REGULATION OF VITAMIN C TRANSPORT IN BRAIN

By

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DEDICATION

To God and Family:

Especially my parents, Mark & Regina,
for being such a beautiful example of faith, love and strength

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AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	5' adenosine monophosphate-activated protein kinase
BCA	bicinchoninic acid
BH4	tetrahydrobiopterin
BSA	bovine serum albumin
CNS	central nervous system
CSF	cerebral spinal fluid
DDF	dideoxforskolin
DHA	dehydroascorbic acid
DHPG	(S)-3,5-dihydroxyphenylglycine
DIDS	4,4′- diisothiocyanatostilbene-2,2′-disulfonic acid
DMSO	dimethyl sulfoxide
DOPA	3,4-hydroxyphenyl alanine
EAAC1	excitatory amino-acid carrier 1
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLAST	glutamate aspartate transporter
GLT-1	glutamate transporter-1
GLU	glutamate
GLUT	glucose transporter
GPβ	G-protein β subunit
GSH	reduced glutathione
Gulo	gulonolactone oxidase enzyme
HEK	human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
iGluR	inotropic glutamate receptors
KRH	Krebs-ringer HEPES buffer
MDA	malondialdehyde
mGluR	metabotropic glutamate receptor
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NET	norepinephrine Transporter
NMDAR-1	N-methyl-D-aspartate Receptor
NPPB	5-nitro-2-(3-phenylpropylamino) benzoic acid
PLN	phloretin
PSD-95	postsynaptic density protein 95
RDA	recommended dietary allowance
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCG	superior cervical ganglion

SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SITS	4-aceta- mido-4′-isothiocyanostilbene-2,2′-disulfonic acid
SLC23A	solute carrier family 23 member 1, SVCT2
SPZ	sulfinpyrazone
SVCT2	sodium - dependent vitamin C transporter type-2
TCA	trichloroacetic acid
TH	tyrosine hydroxylase
VC	vitamin C
VE	vitamin E
VMAT2	vesicular monoamine transporter–type 2,
VRAC	volume-regulated anion channel
VSOAC	volume-sensitive osmolyte and anion channel

CHAPTER I

INTRODUCTION

OVERVIEW OF VITAMIN C IN HEALTH

History of Vitamin C

As with most medical breakthrough discoveries, the beginning of vitamin C (VC) research was initiated by necessity and urgency. VC is an essential micronutrient for humans as its deficiency leads to scurvy, a life-threating condition characterized by fatigue, hemorrhages, bleeding gums, and eventually death. Early accounts of the disease may date as far back as the ancient Egyptians (1-3). Since then, the cure for scurvy seems to have been discovered, forgotten and rediscovered numerous times (4). For decades, sailors suffered from the disease after long voyages at sea. In 1753, James Lind, a Scottish physician aboard the H.M.S. Salisbury, performed the first recorded clinical trial that investigated whether dietary intervention could offer his disease-stricken crewmates some reprieve (5). At the time, it was postulated that acid or malt wort contained the cure (5). He assessed the effects of six different remedies in two seaman each that spanned six days: [1] a quart of cider, [2] twenty-five drops of elixir vitriol (mixture of sulfuric acid, alcohol, and aromatics - usually ginger and cinnamon), [3] two spoonful's of vinegar, [4] a course of sea-water, [5] two oranges and a lemon, and [6] a nutmeg, garlic, and mustard seed concoction. He documented that citrus fruits, particularly lemons and oranges, were the only food that relieved the sailors' symptoms and afforded them full recovery. He concluded that an "anti-scorbutic factor" in these food items was the key to preventing scurvy (6). After decades of the British Navy disregarding Lind's findings, lemon or lime juices were finally incorporated into sailors' rations in the mid-1800's.

It was not until the first half of the 20th century when various conditions began to be described as diseases due to deficiency of an essential nutrient. In 1912, Casmir Funk proposed a list of "vitamins"; compounds not synthesized within the body whose deficiency lead to severe diseases. In this list he used the letter "C" to denote the still unidentified factor known to cure scurvy (7). In 1929, the factor was isolated and identified. A research team led by the Hungarian chemist Albert Szent-Gyorgyi described the molecule's ability to inhibit biological oxidation reactions (8). It was originally called *L*-hexuronic acid, but when it was found to have anti-scurvy activity in animals, the suggestion was made to rename the compound to ascorbic acid, derived from a- (meaning "no") and scorbutus (scurvy). Szent-Gyorgyi later shared the Nobel Prize for his discovery of the structure of ascorbic acid.

Both animals and plants need ascorbic acid to live. All plants and most mammals can synthesize ascorbic acid from glucose in liver. In 1957, Albert Lehninger determined that unlike most species, humans are not able to perform the crucial last step in the synthesis pathway: conversion of l-gulono-γ-lactone into ascorbic acid via the gulonolactone oxidase enzyme. Nishikimi & Yagi (9) discovered that the gene responsible for coding the enzyme is present but non-functional due to several mutations. In fact, gorillas, chimps, orangutans, some monkeys, guinea pigs and select bats have this phenotype. Therefore, ascorbic acid is only a true "vitamin" to a select few species and must simply be considered an essential nutrient in others.

Chemistry of Vitamin C

Ascorbic acid, the reduced form of VC, is the species that contains all known biological activity. It is water-soluble and at neutral pH it is ionized at the C-3 hydroxyl group (Figure 1.1) (4). At the heart of its versatility is the capacity to donate up to two electrons to other compounds. The one-electron oxidation of ascorbic acid results in the relatively inactive ascorbate free radical

being formed. NADH- and NADPH-dependent reductases within the cell are able to reduce this

radical back to ascorbic acid. However, when the radical accumulates, two of these molecules

can dismutate, resulting in one molecule of ascorbic acid and one molecule of dehydroascorbic

acid (DHA). Due to the structural stress on the molecule caused by three ketone groups, the

half-life of DHA is very short in physiologic buffers (~6 mins) (10;11). The cell can readily reduce

DHA back to ascorbic acid by glutathione (GSH), thiol transferases or NADPH-dependent

reductases (12). The name 'vitamin C' refers to the combination of all three molecules (ascorbic

acid, ascorbate free radical and dehydroascorbic acid) in biological systems since all of the

oxidized states of ascorbic acid are eventually reduced to active form. Over 98% of VC in

plasma exists as ascorbic acid, with dehydroascorbate concentrations on the order of 1-2 μM

(13). Therefore, for simplicity I will refer to ascorbate and ascorbic acid as VC whenever the

distinction between the two is irrelevant.
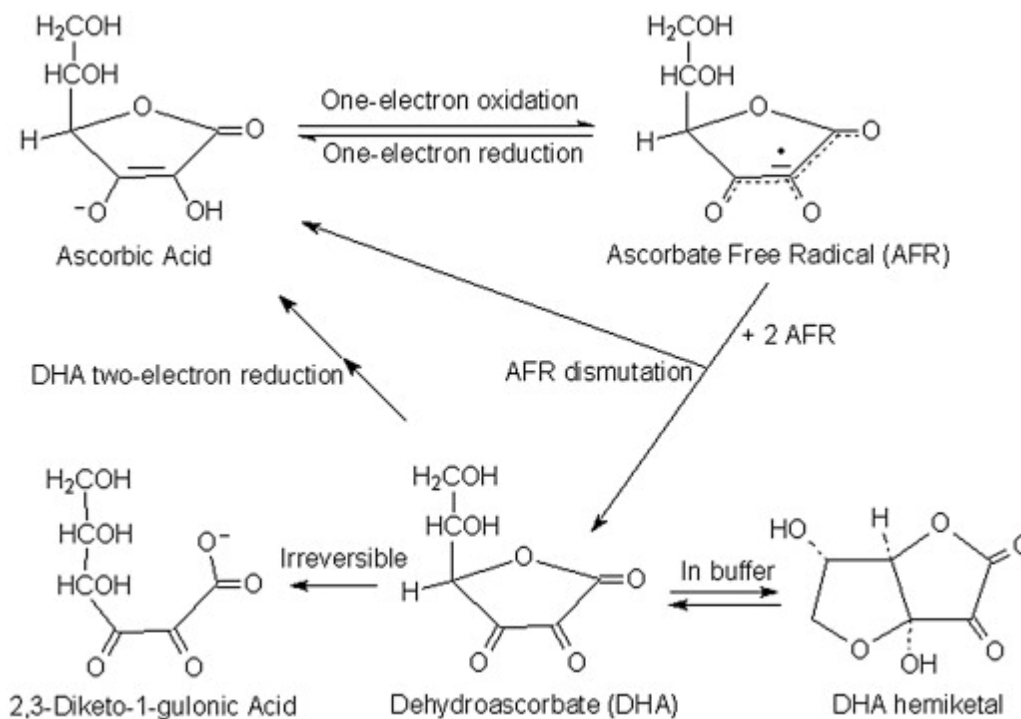
Figure 1.1: VC Metabolism. Adapted from (4)

Vitamin C Functions

Antioxidant functions

Oxidative stress arises from the imbalance of oxidants and antioxidants in favor of the former. Accumulation of these molecules can lead to damage to lipids, proteins or DNA and can eventually cause cellular death. VC is one of the most important low molecular weight antioxidants. Chain-breaking donor antioxidants, like VC, provide an electron, often with an accompanying proton, to reduce radical species (Figure 1.1). This slows or eliminates potentially damaging oxidative chain reactions after they begin. VC also acts as a direct antioxidant to scavenge end-phase oxidants, such as peroxynitrite, which would otherwise damage cellular proteins. It reacts both with endogenous (14) and exogenous reactive oxygen species (ROS) and to a certain extent, reactive nitrogen species (15-17).

Recycling: Vitamins C and E function as co-antioxidants in lipid peroxidation

A synergistic relationship exists between water-soluble VC and lipid-soluble vitamin E (VE), particularly α-tocopherol. While the vitamins partition to different parts of the cell, the phenol group of tocopherol, the site of antioxidant action, is at the water-membrane interface and easily accessible to VC (18). VC donates an electron to the α-tocopheroxyl radical and thus restores its ability to intercept carbon- or oxygen-based radicals in the lipid phase. The physiologic implications have been demonstrated in numerous *in vitro* and *in vivo* studies in which VC has been shown to preserve or at least spare α-tocopherol in lipid micelles, cell membranes, and in human erythrocytes. It has been more difficult to show sparing *in vivo*, but two more recent studies have strongly suggested this synergism between the two vitamins.

In guinea pigs, which cannot make either vitamin, Hill, et al. (19) found that after 3 weeks of a VE-deficient diet, as little as 5 days of VC deprivation led to an ascending paralysis and a

respiratory death in most animals. This phenotype may reflect the neuronal damage associated with VE deficiency, not scurvy. No changes were observed in guinea pigs deficient in only a single vitamin. Surprisingly, although F_2-isoprostanes in liver and brain were increased in the doubly deficient animals, this increase was modest. Nonetheless, subsequent studies (20) confirmed the neurologic damage due to inflammation by showing widespread destruction of the long-track neurons in the pons and spinal cord in the doubly deficient animals, but no sign of damage in either single deficiency. Indeed, VC prevents loss of α-tocopherol and decreases lipid peroxidation in cultured neuron cells subjected to oxygenated medium (21). Together, these studies suggested that a modest decrease in CNS VC significantly accelerated VE deficiency syndrome.

More recently, evidence for a sparing effect of VC on α-tocopherol has been found in human smokers (22). In this study, smokers were found to have decreased plasma α- and γ-tocopherol levels that were completely restored to normal after 3 days of supplementation with 500 mg of VC daily. These results could reflect scavenging of smoke-derived radicals to spare α-tocopherol, but also support the notion derived from *in vitro* and animal studies that VC recycles VE.

Vitamin C and Tetrahydrobiopterin Recycling

VC also recycles an important enzyme cofactor, tetrahydrobiopterin (BH_4), thus keeping it in its reduced state to allow for full catalytic activity (23;24). BH_4 reduces Fe^{3+} back to Fe^{2+} to maintain activation and by so doing preserves the catalytic iron of several dioxygenase enzymes in the active ferrous form (25). Table 1.1 lists known enzymes that require BH_4 to function properly.

Vitamin C serves as a co-factor for monoxygenases and dioxygenases.

Oxygenases incorporate one or two atoms of dioxygen in a variety of substrates in metabolic pathways. Monooxygenases are enzymes that incorporate one hydroxyl group as two atoms of dioxygen are reduced to one hydroxyl group and one H_2O molecule by the concomitant oxidation of NADPH. Dioxygenases are enzymes that incorporate two dioxygen atoms into substrates (26). Oxygenases are involved in many pathways including catecholamine, neuropeptide, and collagen synthesis. VC serves as a co-factor for both monooxygenases (Table 1.3) and dioxygenases (Table 1.3). Indeed, scurvy is a disease caused by the malfunction of several key dioxygenases. (Table 1.4).

Table 1.1: Enzymes that require Tetrahydrobiopterin function.

Enzyme	Function
Tyrosine hydroxylase (TH)	Responsible of the conversion of tyrosine to 3,4-hydroxyphenyl alanine (DOPA), the first and rate-limiting step of norepinephrine synthesis
Phenylalanine hydroxylase	Catalyzes L-tyrosine from *L*-phenylalanine. Its deficiency causes phenylketonuria.
Tryptophan hydroxylase	*Fi*rst and rate-limiting step in serotonin synthesis, catalyzing the production of 5-hydroxy-tryptophan from *L*-tryptophan
Alklglycerol monooxygenase	Catalyzes the formation of 1-hydroxyalkyl-*sn*-glycerol from 1-alkyl-*sn*-glycerol
Nitric oxide synthase	Catalyzes production of *L*-citrulline and nitric oxide from *L*-arginine and is key for nitric oxide generation

Table 1.2: Monoxygenases that require Vitamin C as a co-factor.

Enzyme	Function	Reference
Dopamine-β-hydroxylase	Catalyzes the conversion of dopamine to norepinephrine. It is soluble within vesicles and also exists as a constituent of the membranes of secretory vesicles of norepinephrine-producing neurons in the CNS, sympathetic ganglia and adrenal medulla cells. It requires copper as a cofactor and oxygen and VC as co-substrates.	(27;28) (29)
Peptidylglycine α-amidating monoxygenase	Catalyzes the production of α-amidated peptides from neuroendocrine peptides and is crucial for neurotransmitter synthesis as well	(30)

Table 1.3: Dioxygenases that require Vitamin C as a co-factor.

Enzyme	Function	Reference
6-*N*-trimethyl-*L*-lysine hydroxylase and γ-butyrobetaine hydroxylase	Two VC dependent dioxygenases that are required in carnitine synthesis.	(31)
Aspartyl (asparaginyl) β-hydroxylase	Responsible for the post-translational modification of Asp residues present in EGF-like domains.	(32)
4-hydroxyphenylpyruvate dioxygenase	An Fe(II) containing non-heme oxygenase, which catalyzes the oxidation of 4-hydroxyphenylpyruvate to homogentisate. This is one of the many steps in tyrosine catabolism and oxidation.	(33)
Proyl and lysyl hydroxylases	Catalyzes hydroxylation of proline or lysine to hydroxyproline and hydroxylysine, respectively. These selective modifications are essential for proper collagen folding. Lack of VC leads to the formation of dysfunctional collagen in blood vessels and bones. Furthermore, a novel class of prolyl hydoxylases has been shown to regulate gene expression via the transcription factor HIF1α. VC supplies electrons reguired for the hydroxylation of HIF1α, which then targets the transcription factor for proteosomal degradation, thus decreasing its activity.	(7;34) (7;35) (36;37)
Jumonji histone demethylase 1a/1b (Jhdm1a/1b)	Catalyzes the formation of mono-methylated histone H3 form α-ketoglutarate and trimethyated histone H3. This may account for the recent findings that VC is required for progression of stem cells through differentiation and development.	(38-40)
Nucleic acid demethylase	Catalyzes the production of thymine in DNA from α-ketoglutarate and 3-methyl- thymine found in DNA. This also may contribute to epigenetic regulation by VC.	(39)

Table 1.4: Correlation between scurvy symptoms and loss in the activity of specific dioxygenases due to vitamin C deficiency. Adapted from (31)

Symptom	Putative Target affected	Dioxygenase(s) Involved
Hemorrhages/ Spots	Blood vessels Various collagen types (III,IV)	Prolyl/lysyl hydroxylase
Rotten or loose teeth	Dentin Various collagen types (I, II, IV)	Prolyl/lysyl hydroxylase
Rigid tendons	Various collagen types (I, XII, XIV)	Prolyl/lysyl hydroxylase
Bone/ Cartilage fragility	Various collagen types (I, II, IX, X, XI)	Prolyl/lysyl hydroxylase
Lassitude	Carnitine biosynthesis	Trimethyl-lysine dioxygenase butyrobetaine dioxygenase
Vision problems	Synthesis of vitamin A	β-carotene 15-15' dioxygenase
Neurological disorders	Noradrenaline/ dopamine biosynthesis	Tyrosine hydroxylase dopamine hydroxylase

Adequate amounts of vitamin C

Due to the critical roles played by VC, obtaining and sustaining adequate cellular concentrations is essential. In humans, a physiologic deficiency of VC will occur in about 90 days when normal body stores, usually ~1500 mg, decrease below 350 mg or serum levels reach <11.4 µM (2).

As little as 10 mg/day of VC is needed to maintain stores > 11.4 µM and prevent scurvy, however saturation of plasma and tissue levels occurs at 200 mg/day (70 µM) (41). Excess amounts will be excreted as unmetabolized VC in the urine (42). Fruits and vegetables are the best sources of VC. Citrus fruits, tomatoes and tomato juice, and potatoes are major contributors of VC to the American diet (43). Currently in the U.S., the Recommended Dietary

Allowance (RDA) for VC is 120% of the estimated average requirement and therefore is 90 mg/day for adult men and 75 mg/day for adult women. This concentration was set based on levels needed to maintain near-maximal neutrophil concentration with minimal urinary excretion of VC (42). There is growing evidence that the amount needed for optimal health is far more than that. In scenarios where there is an increase of oxidative stress and the subsequent metabolic turnover of VC, such as smoking, lactation, pregnancy, the requirement is increased by 35 mg/day (43).

Table 1.5: Recommended Dietary Allowances (RDAs) for Vitamin C. Taken from (41)

Age	Male	Female	Pregnancy	Lactation
0–6 months	40 mg*	40 mg*		
7–12 months	50 mg*	50 mg*		
1–3 years	15 mg	15 mg		
4–8 years	25 mg	25 mg		
9–13 years	45 mg	45 mg		
14–18 years	75 mg	65 mg	80 mg	115 mg
19+ years	90 mg	75 mg	85 mg	120 mg
Smokers	Individuals who smoke require 35 mg/day more VC than nonsmokers.			

* Adequate Intake (AI)

State of Vitamin C status in the US and effects on health

In the 2012 National Report of Biochemical Indicators of Diet and Nutrition in U.S Population, (http://www.cdc.gov/nutritionreport/pdf/Nutrition) serum VC concentrations were reported to be deficient (< 11.4 µM) in 6% of the population 6 years and older. This is estimated to be 15.8 million people. At least the same amount of people had low (11.4 - 23 µM) VC levels. Although overt scurvy manifestation is rare, it is likely that this population may develop a form of latent scurvy characterized by fatigue, dull aches and irritability (2;44). Furthermore, since scurvy is relativity uncommon in the US, occasionally it is misdiagnosed based on symptoms. Low levels

of VC can lead to loss of bone mass, inadequate collagen formation, hemorrhaging, decline in cellular energy status, impaired neurotransmitter production and eventually death (45). This is especially relevant for individuals with low intake due to psychiatric disorders or alcoholism that present to clinicians with rheumatologic or dermatologic complaints that mask the underlying signs of early scurvy (45-47). Therefore, awareness of the disease is essential to make the proper diagnosis.

Importance of Vitamin C in the brain

The brain is perhaps one of the most sheltered organs during progression of VC deficiency, having both some of the highest levels of VC in any organ, achieved by a unique two-step concentrative mechanism (discussed below). For example, whereas cells within most organs have VC levels of 1-2 mM, those within brain can be as high as 4-6 mM (48). Further, neurons have rates of oxidative metabolism as high at ten-fold those of other tissues (49) and thus are likely to generate more ROS than other cell types. The burden of oxidative stress warrants the high antioxidant VC levels in neural tissues. This may be especially important in neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's, in which there is oxidative stress due to secondary inflammation (50;51). Another factor in both health and disease is likely the role of VC as co-factor for several crucial dioxygenase enzymes in the CNS, as described above. Finally VC has been implicated in neurotransmitter regulation (52), as will be discussed below.

However, there is a huge gap in the understanding the role of VC in brain health and disease due to the lack of information on the mechanisms involved in tissue distribution and homeostasis. It is this lack that prompted the selection of this thesis project, first to determine whether vitamins C and E interact to support behavior *in vivo*, then to dissect potential mechanisms at the sub-cellular level by studying VC uptake, efflux, and metabolism in the

neuronal synapse. Key to both of these topics is what is known of brain and VC homeostasis. Although most information relates to VC uptake by the brain and CNS, our work also suggests a role for efflux in of the vitamin, at least in synapses. Accordingly the next sections will discuss VC transport regulation, with emphasis on the brain and neurons.

VITAMIN C TRANSPORT REGULATION

Plasma VC levels depend on dietary absorbance and/or hepatic synthesis and ultimately determine the concentration available for all tissues (41;42). Therefore all species depend on transport of VC across cell membranes for redistribution into other tissues. VC transport between tissues is a balance of uptake and efflux. While VC uptake mechanisms are well established, identification of the proteins mediating efflux still remains elusive. The brain, as noted above, is unique among organs in having a two-step uptake mechanism that derives from its transporter, the Sodium-dependent VC Transporter type 2 (SVCT2) (53). Although the SVCT2 is likely to be the primary mechanism by which the brain acquires VC, there is another mechanism, as described next.

Regulation of Brain Vitamin C Uptake

Glucose Transporters

DHA represents less than 5% of total VC in plasma and can be transported across the membrane via glucose transporters (54). A number of GLUTs: GLUT1, GLUT3, and GLUT4 (55;56) recognize DHA because of structural similarity to glucose. However, these proteins do not recognize ascorbic acid. Facilitative transport of DHA is similar to glucose in that it is transported down a concentration gradient into cells. The affinity of glucose transporters for DHA is similar to that for glucose (57;58), thus, there is competition between the two substrates.

At normal physiologic glucose concentrations (5 mM), DHA uptake is likely very low, considering that its extracellular concentrations are very low micromolar, as noted previously. Although DHA can be transported across the blood-brain barrier when concentrations are high (59), competition with glucose deters this from being is not a major mechanism for VC entry into the brain (53). However, once DHA is in the cells, reduction mechanisms convert DHA to ascorbic acid very efficiently (17;60) and this mechanism could become important in cases of severe oxidative stress, such as in the periumbral area of stroke, where there is reperfusion after ischemia. Nonetheless, most brain VC under normal circumstances is acquired on the SVCT2, as described next.

Sodium Dependent Vitamin C Transporters

Ascorbic acid represents ~95% of total VC in plasma and is transported into tissues solely by SVCT's. The Hediger group cloned two SVCT type transporters (61) from the solute carrier (*Slc)* transporter family. The *Slc23a1* gene encodes the SVCT1 protein, and *Slc23a2* encodes the SVCT2 protein. The two transporters share 65% amino acid identity with minor differences between species (61). They both have 12 trans-membrane spanning motifs with the N- and C-termini located on the cytoplasmic side of the membrane and several putative sites for post-translational modification. These transporters are stereospecific for *L*-ascorbic acid and are driven by the Na^+ electrochemical gradient. In a two- step manner, SVCT1 and SVCT2 work in concert to maintain proper levels of VC within the body. These isoforms have different tissue distribution, and functional properties depending on the cell, tissue and species.

SVCT1 (*slc23A1*) mRNA is found in epithelial tissues involved in VC absorption (intestine) and re-absorption (kidney). SVCT1 has a low affinity for VC (apparent K_m 66-237 μM) and higher capacity (V_{max}) compared to SVCT2 (62). SVCT1-deficient mice have VC levels about half normal, but appear to develop normally and have sufficient VC tissue levels to prevent scurvy.

Once VC is in the plasma, SVCT2 helps distribute the vitamin to all of the other cell types. Two other SVCT orphan transporters (SVCT3 and SVCT4) have been cloned, but are most likely nucleobase transporters and are not known to transport VC (63).

SVCT2 Background

SVCT2 depends on Na^+ ions as replacement with K^+, Li^+ or choline abolishes uptake (64). The transport cycle for SVCT2 is characterized by the transport of 2 molecules of Na^+ for every one molecule of VC. Na^+ increases the transport rate of VC in a cooperative manner by decreasing the transport K_m without affecting the V_{max}. Therefore, SVCT2 (in most cells) has a relatively high affinity for VC (K_m = 20-40 µM), reflective of plasma concentrations (30-50 µM). Godoy, et al. showed that Ca2+ and Mg2+ ions are also required for SVCT2 function (65).

SVCT2 *(slc23a2)* mRNA and protein expression is evident in liver, lung, placenta, eye, and spleen. However, the highest expression is in brain in areas of high neuronal density like the hippocampus and cerebellum (66). The heterogeneous distribution of SVCT2 in the brain can be appreciated in the visualization of SVCT2 gene expression provided by the *in situ* hybridization staining depicted in the Allan Brain Atlas (Figure 1.2).
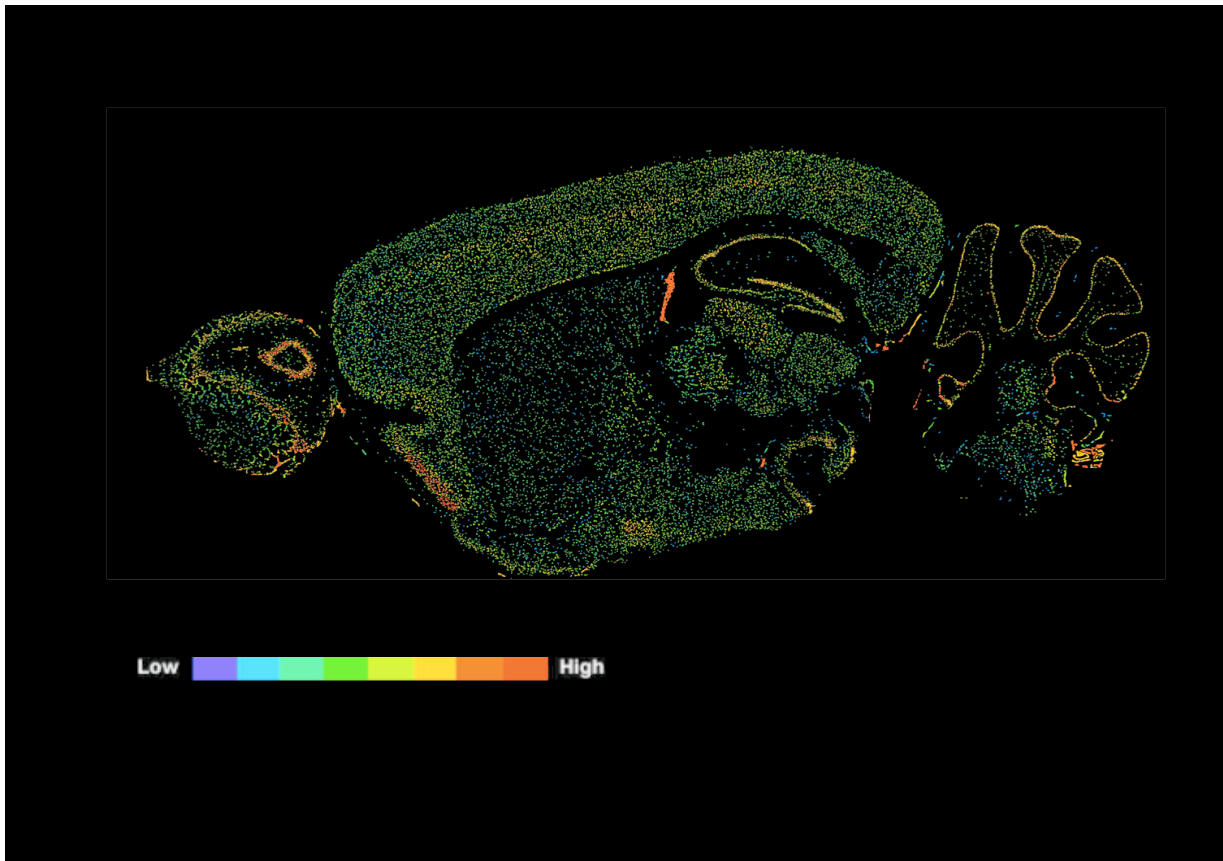
Figure 1.2: Visualization of *In situ* hybridization staining for SVCT2 gene expression in mouse brain. (Image credit: Allen Institute for Brain Science, mouse.brain-map.org)

The mechanisms that regulate SVCT2 expression are yet to be defined. Mice that are homozygous knock-out mutants for SVCT2 die at birth with low levels of VC in organs that only express SVCT2 and undetectable levels in the brain (67), attesting to the importance of this transporter in brain health. VC is transported to the brain in a two-step mechanism because it cannot cross the blood-brain barrier. Indeed, the SVCT2 is not present in blood-brain barrier endothelial cells *in vivo* (68), perhaps accounting for failure of VC to enter the brain where the blood-brain barrier is very tight. In the first step, VC is transported from the blood to the CSF in the third ventricle by SVCT2 expressed in the epithelial cells that make up the choroid plexus. This generates CSF concentrations of 200-400 µM in humans (69;70). In the second step, VC is transported from the CSF into neurons via SVCT2 expressed in the neuronal plasma

membrane. This two-step mechanism contributes to neuronal VC concentration of ~3-10 mM, depending on neuronal density (71). Indeed, although neurons express the SVCT2 in abundance, glial cells do not express the protein *in vivo*, and thus likely have much lower VC contents (48).

Currently, there are no biochemical or structural data for SVCT2 due to the fact that purified proteins have not been obtained. Primary amino acid sequence and Western blot analysis is the basis of all of the information available for SVCT2. Based on these analyses, SVCT2 has potential *N*-glycosylation sites on an exofacial loop between trans-membrane segments 3 and 4 (61;72). Also, there are putative sites for protein kinase-A and protein kinase-C regulation on the cytoplasmic portions as shown in Figure 1.3 (61).

There have been few studies correlating SVCT2 structure with function. Cell culture studies suggest that these sites are functional in acute regulation given that VC transport is decreased by both dibutyryl cyclic AMP (73;74) and by protein kinase-C agonists (72;75). Our lab has shown in endothelial and SH-SY5Y cells that transport rates are inhibited by extracellular sulfhydryl reagents, indicating the four exofacial cysteines may be involved in the transport mechanism (76). Our lab has also shown that SVCT2 is regulated by VC levels (77). There is also evidence that intracellular VC and GSH concentrations affect VC transport (76). Even under times of severe VC deficiency, the brain retains ~25% of original levels while the rest of the body has been depleted (78) suggesting that the brain may have efficient mechanisms for regulating VC transport. It is remains unclear if VC concentration can affect the transport of VC by altering SVCT2 affinity for VC. Despite the potential importance of the SVCT2 to brain VC levels, function, and antioxidant defense, as is evident from the foregoing, we know very little about how changes in the expression and function of the SVCT2 regulate VC homeostasis in the CNS.
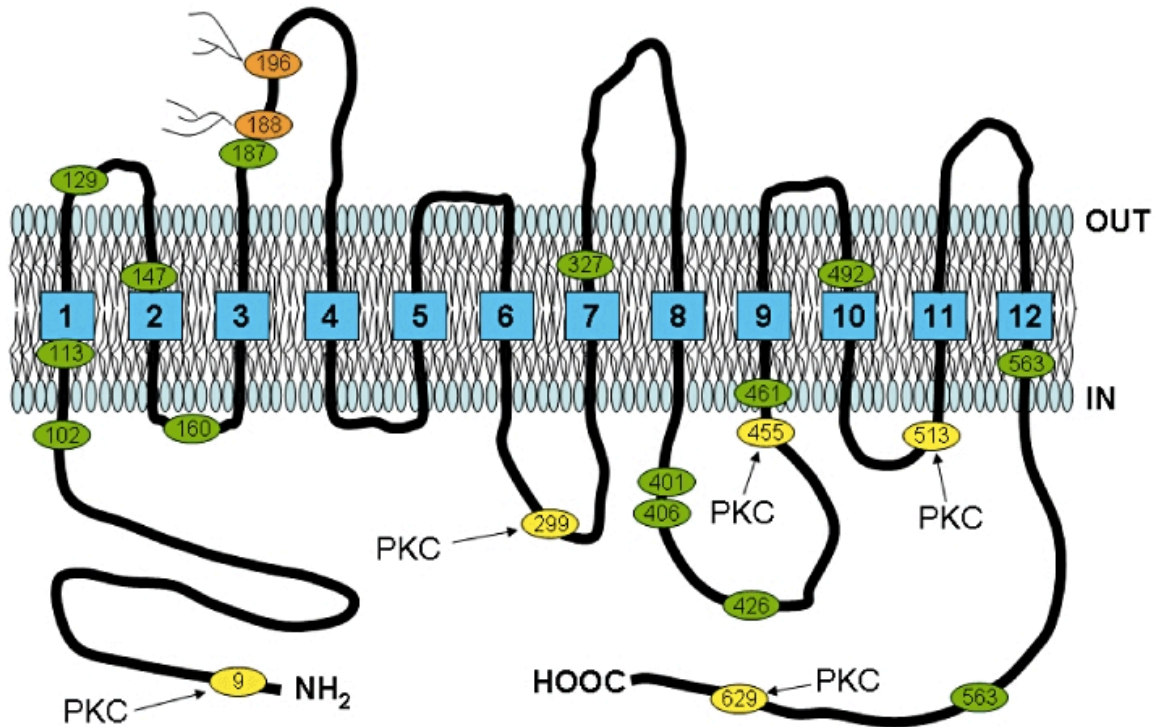
Figure 1.3: Structural model of SVCT2 based on its hydropathy profile. Taken from (79) Schematic diagram based on the proposed sequence and hydropathy analysis of the human SVCT2 (64). The human SVCT2 is predicted to have 12 membrane-spanning regions with intracellular amino (NH2-) and carboxy (HOOC-) termini. Locations of potential extracellular glycosylation sites are noted in brown, cysteine residues are noted in green and sites for protein kinase C phosphorylation are noted in yellow.

Regulation of Vitamin C Efflux in Brain

The extracellular concentration of VC in the CSF has been shown to have significant impact on physiologic functions as discussed later in this chapter. Simple diffusion of VC out of cells across the plasma membrane is not thermodynamically probable due its size and negative charge. Furthermore, although DHA is more hydrophobic than VC and could exit on glucose transporters, DHA intracellular concentrations are low (12) and not likely to be the source of extracellular VC. As aforementioned, VC uptake is homeostatically regulated and there is increasing evidence that VC efflux mechanisms are regulated as well. Even though there has

been very little research devoted to elucidating these efflux mechanisms, a few possible pathways have arisen including: [1] volume-sensitive anion channels, [2] ascorbate-ascorbate homeoexchange, and [3] glutamate-ascorbate heteroexchange.

Volume-Regulated Anion Channels

A number of physiologic scenarios can cause cells to swell, during which the intracellular volume increases. Volume-Sensitive Osmolyte and Anion Channels (VSOACs) and Volume-Regulated Anion Channels (VRACs) are anion channels located in the plasma membrane that mediate the compensatory decrease of intracellular volume and prevent the cell from bursting. While the molecular identities of the channels are uncertain (80), electrophysiological studies have shown that VC may be transported through these types of channels (81). This type of VC efflux mechanism has been mostly studied in neurons and astrocytes and has received the most experimental support out of the three mechanisms mentioned (82;83). A variety cell types including hepatocyte-like HepG2 cells (84), coronary artery endothelial cells (85), K562 cells (86), astrocytes (87), and SH-SY5Y neuroblastoma cells (83) have shown VC efflux that is sensitive to anion channel blockers, suggesting that such channels facilitate this pathway. These studies use generic anion channel inhibitors such as 4,4′- diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-aceta- mido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), and the VSOAC-inhibitors dideoxforskolin (DDF) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB). More relevant to the likely function of these channels in cells, it has also been shown in astrocytes that hypotonic solutions can stimulate VC release in astrocytes by cell swelling (88).

Ascorbate-ascorbate homeoexchange

The ascorbate-ascorbate homeoexchange theory is based on the observation that the addition of extracellular VC prompts the release of preloaded intracellular VC from adrenal cortical cells (89). Our lab has published a similar study using EA.hy926 endothelial cells, in which dose-dependent release of preloaded ^{14}C-VC was induced by the addition of unlabeled VC (90). A single transporter capable of this moving VC both in and out of cells has not been identified. An alternative explanation for this phenomenon is that SVCT2 is bidirectional, depending on local conditions. While this activity is characteristic of facilitative diffusion transporters, such as the GLUTs, it is less likely to be a property of an energy and Na^+-dependent transporter, such as the SVCT2. Further research is necessary to determine which, if any, physiologic conditions can allow this type of function.

Glutamate-ascorbate heteroexchange

The excitatory neurotransmitter *L*-glutamate activates glutamate receptors on the cell surface and synaptic cleft. Proper clearance of glutamate via uptake is essential as over-activation of these receptors causes oxidative stress and excitotoxicity. It has been shown that the uptake of extracellular glutamate stimulates VC efflux in astrocytes (52). Glutamate uptake is also associated with ischemia-induced efflux of VC from neurons and glia (71;91;92). This data suggests that these two processes, glutamate uptake and VC efflux, may be linked in a single mechanism. The glutamate-ascorbate heteroexchange theory postulates that glutamate uptake is dependent on VC release and that there is a heteroexchange of the two molecules within the intracellular and extracellular space via one or more transporters (52;93;94). However, while Wilson and colleagues confirmed that glutamate increases VC efflux from cerebral astrocytes, intracellular VC did not have an effect on glutamate uptake, suggesting that heteroexchange may not be the correct mechanism (95). Later, Wilson concluded that the release of VC was attributed to glutamate-induced cell swelling followed by opening of the aforementioned anion

channels (88).   Our lab found similar results in SH-SY5Y neuroblastoma cells: glutamate

caused release of intracellular VC, but neither intra- nor extracellular VC affected glutamate

uptake, and does not support a heteroexchange mechanism (83).

Investigating vitamin C regulation and its effects on behavior

Rebec noted that extracellular VC had an effect on the behavior in rats. In electrophysiological

studies he observed that increased extracellular VC stimulated behavior and also that

conversely, a decrease in extracellular VC inhibited behavior (96;97). It has long been

suggested that depressive behavior and despair is one of the earliest signs of scurvy (2). There

is now emerging evidence in rodents and humans that VC supplements can alter behavior with

specific reference to mood. In several studies in stressed mice (despite their ability to make VC),

supplements of the vitamin improved behavior and synergized with the effect of fluoxetine in the

tail suspension test, considered am appropriate test of behavioral despair (98-100). Two human

studies also support a role for replenishing VC to improve mood and depression. In the first,

college students known to be very low in VC levels due to poor dietary intake had improved

mood following VC supplements in a randomized controlled clinical trial (101). Further,

hospitalized patients supplemented with VC at a dose of 1 gram daily had significantly improved

mood and decreased depression compared to a matched control group of patients who received

an identical tablet containing vitamin D. The physiologic implications of VC transport and efflux

mechanisms are that not only VC concentrations are carefully regulated but are also important

for molecular neuronal function, cognition and behavior. Dysregulation of VC may lead to an

impaired neurobehavioral phenotype. One way to gain insight on VC's role in behavior is to

elucidate its regulation in the highly specialized region of the neuron, the synaptic terminal,

where the biochemical signal propagation occurs. VC regulation in this region has received little

attention. Thus, I have conducted the following studies for my thesis and investigated whether

dysregulation of VC leads to an altered neurobehavioral phenotype and also whether and how VC is regulated at the nerve terminal.

SPECIFIC AIMS

Specific Aim 1: To determine if and by what mechanism increased oxidative stress by combined vitamin C and E effects neuronal SVCT2 function and/or expression.

Specific Aim 2: To determine if and by what mechanism increased oxidative stress effects vitamin C regulation at the nerve terminal.

CHAPTER II

MATERIALS AND METHODS

ANIMAL MODELS

Wild type (Gulo$^{+/+}$; SVCT2 $^{+/+}$)

Mice denoted as "wild-type mice" are from C57BL/6J background. These mice were originally obtained from Jackson laboratories (stock #000664) and a colony was maintained in-house. 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 6AM. All procedures conformed to Institutional IACUC guidelines. Mice had free access to food and water for the duration of experiment.

L-gulonolactone oxidase deficient mice (Gulo$^{-/-}$)

Mice heterozygous for the *gulonolactone oxidase* gene were originally obtained from Mutant Mouse Regional Resource Centers (http://www.mmrrc.org, #000015-UCD) and maintained on a C57BL/6J background. Gulo$^{-/-}$ mice are unable to synthesize VC and were supplied with 0.33g/L VC in their drinking water except during experimental manipulations. This supplementation level provides adult (non-pregnant) gulo$^{-/-}$ mice with approximately wild-type levels of VC in tissues (102;103). To minimize oxidation of VC, deionized water was supplemented with 0.01 mmol/L EDTA.

Sodium-dependent Vitamin C Transport Type 2 heterozygous mice (SVCT2$^{+/-}$)

Mice that were heterozygous for *slc23a2* gene that encodes for SVCT2 mice were originally provided by Dr. Robert Nussbaum on the 129/SvEvTac background and were backcrossed more than 10 generations to the C57BL/6J background as described (104).

L-gulonolactone oxidase and SVCT2 deficient mice (Gulo$^{-/-}$; SVCT2$^{+/-}$)

To enable both systemic and intracellular VC depletion, gulo$^{-/-}$ mice were crossed with mice lacking one allele of the SVCT2 (SVCT2$^{+/-}$) (67) to generate gulo$^{-/-}$/SVCT2$^{+/+}$; gulo$^{-/-}$/SVCT2$^{+/-}$ and gulo$^{-/-}$/SVCT2$^{/-}$ littermates. These were bred separately from both the Gulo$^{+/+}$ and SVCT2$^{+/+}$ groups.

Genotypes of each mouse were confirmed using PCR as outlined in *genotyping.*

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 6AM. All procedures conformed to Institutional IACUC guidelines. Mice had free access to food and water for the duration of experiment.

EXPERIMENTAL DIETS

Table 2.1: Macronutrient compositions of experimental diets utilized in Specific Aims I-II.

	Normal Chow	**Western Diet* (VE+)**	**Western Diet* (VE-)**
Supplier	Southern Agriculture	Harlan Teklad	Harlan Teklad
Catalog No.	5001	TD.07310	TD.07310
Macronutrient (% Calories Provided)			
Fat	12.1	33.6	33.6
Protein	28.0	17.1	17.1
Carbohydrate	59.8	49.3	49.3
Lard	--	16	16**
Sucrose	3.7	34	34
Cholesterol		0.2	0.2
Vitamin E, IU/kg	49	50-150	0
Vitamin C, mg/gm	0	0	0

*An atherogenic diet

**"Vitamin-Free" Test Casein (alcohol-extracted) and tocopherol-stripped lard are used to limit endogenous vitamin E

Normal rodent chow that contained essentially no VC and 42 IU/kg of VE (total tocopherols) (Lab Diet, #5001). VE-deficient and supplemented diets were custom made by Harlan Teklad (TD.07310 and TD.07311 respectively). Western diet (16% lard, 34% sucrose, 0.2 cholesterol), (TD.07310)

BEHAVIORAL ASSESSMENTS

Weight

Scurvy in mice typically manifests after 5-6 weeks of VC deprivation (102;103) and early signs include weight loss. Mice were weighed on a standard gram scale twice per week and observed for signs of overt scurvy.

Motor & 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. Prior to the session, non-toxic ink was applied to the hind paws of the mice. The mice were then allowed to walk freely along a narrow 5-inch corridor lined with paper. Average stride length was calculated as the distance between two consecutive footprints, averaged from 3 strides as described (103;104).

Locomotor Activity

In each 10 min session, the mice were allowed to individually explore an open 27.9 X 27.9 cm chamber (Med Associates, OFA-510). Locomotor activity (distance traveled and velocity) was automatically recorded by the breaking of infra-red beams as described (103). After each mouse the chamber was cleaned with 10% alcohol solution. Mice were tested in the same chamber for their repeated trials.

Inverted Screen

Mice were assessed for general muscle grip strength in the inverted screen test. In each 5 min trial, mice were placed on a one-centimeter 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 min trial, mice were placed on a ridged, rotating beam that slowly accelerated from 4 rpm to 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 sec 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 sec; 3.5 = stabilized itself within 60 sec; 3 = attempts to stabilize itself but fails to do so within 60 sec; 2 = remains hanging from the wire for 60 sec; 0 = falls from wire within 60 sec.

Horizontal Beam

Similar to the wire hang, in each 60 sec trial, mice were placed to hang by their front legs in the middle of a plastic beam suspended horizontally between two platforms. A score indicating the activity of the mouse is displayed as the following: 4 = stabilized itself to a four-paw grip position within 30 sec; 3.5 = stabilized itself to a four-paw grip position within 60 sec; 3 = attempts to stabilize itself but fails to do so with 60 sec; 2 = remains hanging from the beam for 60 sec; 0 = falls from wire within 60 sec as described (103).

Zero Maze

Anxiety was assessed by exploration patterns in the elevated zero maze. In each 5 min 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 min 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.

Behavioral Assessments and data analysis were performed in part through the use of the Murine Neurobehavior Core Laboratory at the Vanderbilt University Medical Center.

For all of the assessments or assays, mice were deeply anesthetized with isoflurane and killed quickly by decapitation.

Synaptosome Preparations and Fractionation Steps (Chapter IV)
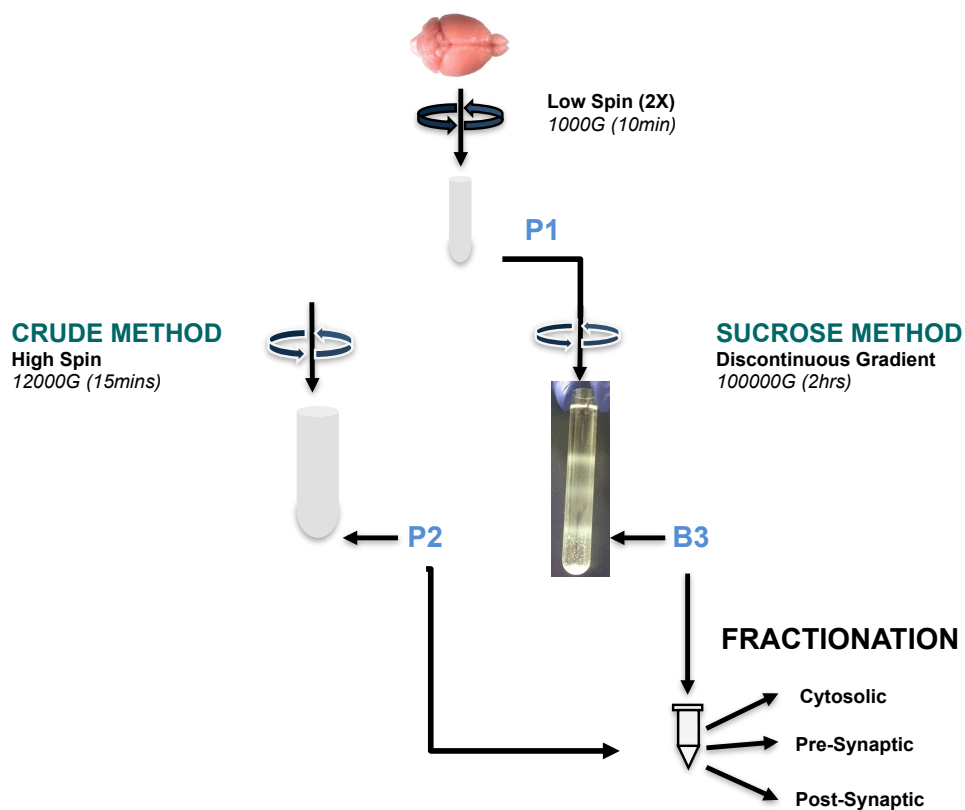
Figure 2.1: Flowchart of Overall Synaptosome Preparation.

Crude Synaptosome Preparation

The whole cortex region of a mouse brain was dissected out on a cold block and added to 9 ml of homogenization buffer (0.32 M sucrose/ 4.3 mM HEPES solution, 9 µl of 1000X protease inhibitor cocktail (1.54 µM aprotinin, 10.7 µM leupeptin, 0.948 µM pepstatin, 200 µM PMSF)) in

a 10 ml glass homogenizer tube. The mixture was homogenized with a hand-held Teflon pestle (approximately 7 strokes). The homogenate was then transferred to a 15 ml plastic tube for centrifugation at 1000X G for 5 min at 4°C. The supernatant (S1) was then transferred to a 15 ml plastic tube and then centrifuged at 12,000X G for 15 min at 4°C. The pellet was suspended in assay buffer (1300 μl of Krebs-Ringer Hepes buffer (10 mM HEPES, 128 mM, 5.2 mM KCl, 1 mM NaH_2PO_4, 10 mM Glucose, 2.2 mM $MgSO_4$ 1.2 mM $CaCl_2$)) and then transferred to an Eppendorf tube for assays.

Enriched Synaptosome Preparation

Prefrontal cortex brain tissue (half or the cortex) was homogenized as above in 10 ml of 0.32 M homogenization buffer (0.32 M sucrose in 4.2 mM Hepes (pH 7.4), 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.54 μM aprotinin, 10.7 μM leupeptin, 0.948 μM pepstatin, 200 μM PMSF) in a 15 ml conical tube. The homogenate was removed and centrifuged at 1000 g (2250 rpm) on a tabletop centrifuge at 4° C for 10 min to pellet nuclei and membrane debris (P1a). Supernatant was transferred to clean 50 ml conical tube on ice (S1a). P1a pellets were gently resuspended in 10 ml of 0.32 M sucrose solution the centrifugation step was repeated (P1b). Supernatant (S1b) was transferred to 50 ml conical tube containing S1a. The supernatant was brought 20 ml with 0.32 M sucrose solution and centrifuged at 10,000XG at 4° C for 20 minutes to produce P2 pellet (contains crude synaptosomes, mitochondria, and microsomes). P2 pellets were gently suspended in 3.4 ml of 0.32 M sucrose solution and aliquot was taken for Western blotting.

The remaining suspension of P2 pellet was applied to a discontinuous sucrose gradient. This was prepared in Beckman 14 ml polycarbonate tubes in which 3 ml of 0.8 M, 1.0 M and 1.2 M sucrose solutions were slowly layered in that order. Three milliliters of crude synaptosome sample were layered on top of gradient and balanced with 0.32 M sucrose solution. Samples were then centrifuge in Beckman ultracentrifuge (Optima LE-80K) using an SW 40 Ti rotor at

100,000XG (23712 rpm) at 4° C for 2 hours. Enriched synaptosomes were collected from interface of the 1.0 M and 1.2 M solutions and transferred to clean 15 ml conical tube. Fractions were diluted with 3 ml of the 0.32 M sucrose solution and centrifuged in same rotor at 100,000XG for 30 min at 4° C.

Synaptosome Fractionation

Pre- and post-synaptic fractions in both enriched and crude synaptosome preparations were obtained by the following protocol. The P2 pellet from an enriched or crude synaptosome preparation was gently suspended in 4 ml hypotonic lysis buffer (Hypotonic lysis buffer: 20 mM Tris (pH 6.0), 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Triton, 1.54 µM aprotinin, 10.7 µM leupeptin, 0.948 µM pepstatin, and 200 µM phenylmethyl sulfonyl fluoride (PMSF)). This was incubated on ice for 20 minutes to lyse membranes and transferred to 5 ml ultracentrifuge tubes (Beckman Ultra Clear, catalog #344057). Samples were centrifuged at 100,000 XG (28,700 rpm) in a SW 55 Ti rotor in a Beckman centrifuge (Optima LE-80K) for 120 minutes at 4ºC. The supernatant (S3), which contains cytosolic proteins from both pre- and post- synaptic terminals, was transferred to another tube and placed on ice. Pellet P3 was suspended in 1 ml of Tris buffer, pH 8.0 (Tris Buffer: 20 mM Tris (pH 8.0), 1% Triton X-100, 1.54 µM aprotinin, 10.7 µM leupeptin, 0.948 µM pepstatin, and 200 µM PMSF). This was transferred to plastic 2 ml Eppendorf tubes and incubated on ice for 20 minutes. The samples were centrifuged at 10,000XG for 30 minutes at 4ºC, then the supernatant (S4) containing the presynaptic fraction was transferred to Eppendorf tubes. The pellet (P4) was suspended in 200 µl of 1x PBS/1% SDS and centrifuged at 10,000XG for 30 minutes to obtain the supernatant (S5) containing the post-synaptic fraction, as described in Figure 2.2.

Concentration of cytosolic/perisynaptic and presynaptic fractions

To ensure adequate amounts of protein were studied, samples were concentrated using 3K concentrator tubes (Amincon Ultra, Millipore) that were centrifuged at 3400 rpm at 4°C until volumes reach ~250 µl. Once the correct volume was reached, membranes were washed with homogenization buffer, and transferred to new Ependorff tubes for assays.
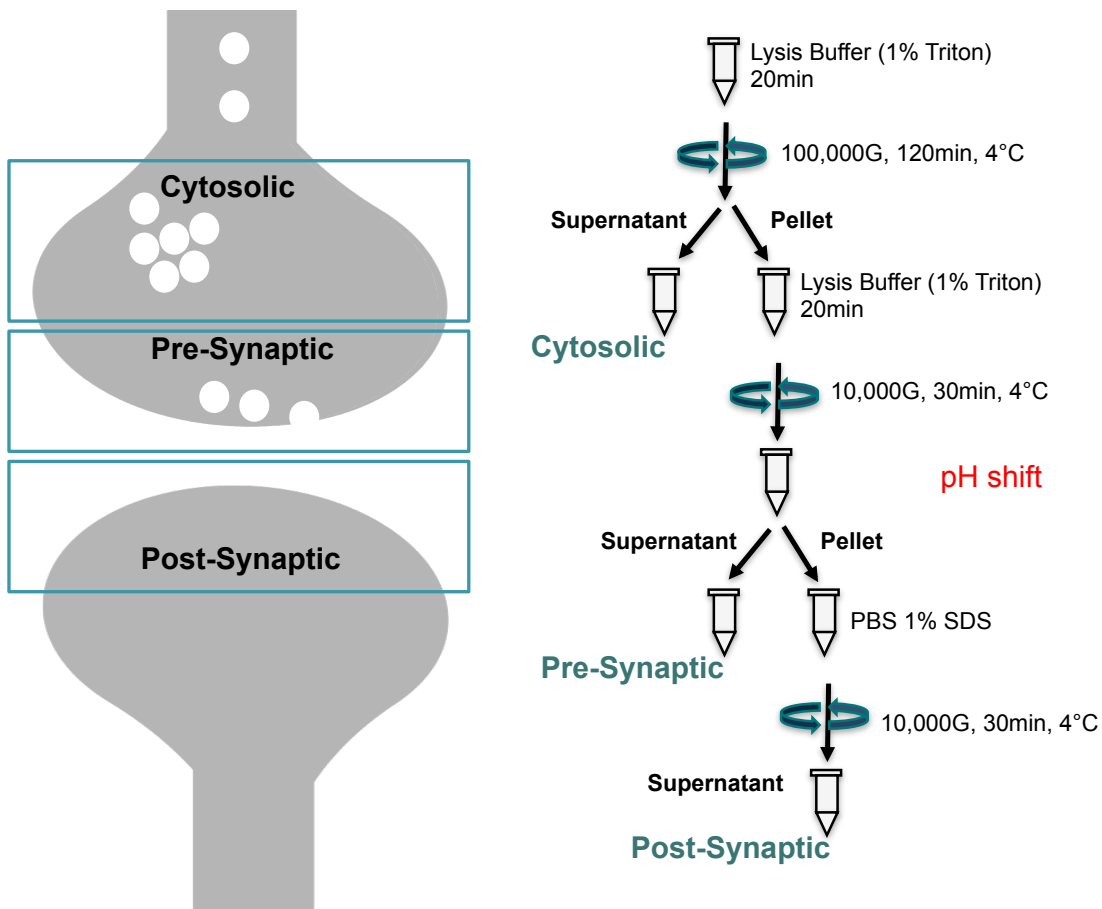
Figure 2.2: Flowchart of Synaptosome Fractionation.

Fura Red-AM Assay

Crude synaptosome preparations were centrifuged at 13,000 rpm for 2 min at 4°C to pellet synaptosomes. The pellet was then suspended in 1300 µL of the Fura Red-AM (Invitrogen,

F3021) solution (1300 μL of assay buffer with glucose and 5.3 μL of 1 mM Fura Red-AM dye). The mixture was then incubated in a 37°C bath for 30 minutes and centrifuged again at 13,000 rpm for 2 min at 4°C. The supernatant was removed and the pellet was suspended in 1300 μL of the assay buffer with glucose. Following this, 50 μL of the preparation treated as noted in text at 37°C. At the end of each incubation period samples were treated with 1 mL of ice-cold assay buffer (-glucose), centrifuged at 13,000 rpm for 2 min at 4°C, and the supernatant discarded. This was followed by addition of 250 μL of 60% methanol in water to lyse the synaptosomes. After a brief centrifugation to clear undissolved material, 100 μL aliquots were transferred to a clear plate in a microtiter plate reader and fluorescence was read at excitation 420/emission 610 nm.

BIOCHEMICAL ASSESSMENTS

Tissue Preparations and Assays

If fresh tissues were not needed, cortex, cerebellum and liver were removed and frozen in dry-ice and stored at -80 °C until needed.

Protein determination

The Pierce BCA Protein Assay Kit *(Thermoscientific Cat No. 23225)* is based on bicinchoninic acid (BCA) and allows for the colorimetric detection and quantitation of total protein within a tissue sample. Prior to determination assay, tissues were homogenized with a rubber policeman in RIPA buffer (10 mM Tris-Cl (pH 8.0),1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 140 mM NaCl). According to the manufacturer's instructions, 2.5 μL of the unknown protein sample was added to a 96-well microplate. The assay working reagents (Reagent A and Reagent B) were mixed in a 1:50 ratio and 200 μL was added to each well. A bovine serum albumin (BSA) standard curve (0, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0

µg/ml) was also added to the plate. The plate was covered in Parafilm and incubated in a 37°C bath for 10min. After cooling to room temperature the plate was then read with a spectrophotometer at an absorbance of 562 nm.

Western blot analysis

Appropriate amounts of protein samples were mixed with 4X Sample Buffer and loaded into a well on a gel (5-20 µg total protein per lane) along with a Kaleidoscope Precision Plus Protein Standard (Bio- Rad). Samples were subjected to denaturing electrophoresis on a 7.5% acrylamide gel. Protein samples were transferred Immobilon PVDF membrane (pore size 0.45), according to the manufacturer's instructions (Millipore). Membranes were blocked in blocking buffer (5% BSA or dry milk, 0.5% Tween 20) for 1-hour at room temperature or overnight at 4°C. Membranes were then incubated with primary antibodies (diluted 1:200-1000) in blocking buffer at 4 °C with gentle rocking overnight. Blots were washed (3 times for 10 min each) at room temperature in TBS-T (Tris-Buffered Saline; 150 mM NaCl, 20 mM Tris pH 7.5 with 0.5% (v/v) Tween 20; Sigma Aldrich; St. Louis, MO). Then, blots were incubated with species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:10000) in blocking buffer for 1-hour at room temperature and subsequently, washed in TBS-T (3 times for 10 min). Antibody detection was performed with Western Lightening Plus-ECL Enhanced Chemiluminescence Substrate Kit (Perkin Elmer) and Carestream Kodak BioMax MR Film (Kodak, Rochester, NY). Band intensity from X-ray film detection was analyzed by densitometry using ImageJ software from the National Institutes of Health.

Primary antibodies used for immunoblotting are the following: SVCT2 (S-19), VMAT2 (H-90), Actin (I-19), (all from Santa Cruz Biotechnology). GAPDH (Milllipore, MAB-374), NMDAR1 (BD Pharmingen, 556308), PSD-95 (Neuromab, 75-028), GβP (Santa Cruz, SC-378) for the synaptosome fractionation studies were kindly provided by Katherine Betke in Dr. Heidi Hamm's

laboratory (Vanderbilt University). Secondary antibodies used at a 1:10000 dilution for immunodetection are the following: anti-rabbit IgG HRP and anti-goat IgG HRP conjugate purchased from Santa Cruz Biotechnology.

Genotyping

Tail clippings were added to 100 μL extraction buffer and 25 μL tissue buffer. Samples were incubated 20 minutes at room temperature and then 3 minutes at 95°. The reaction was stopped with the addition of 100 μL neutralization buffer. Each PCR reaction contained 10 uL PCR Master Mix, 2 μL RNA/ DNAase free water, 1 μL of each primer according to gene probe (SVCT 2 or Gulo). Samples were processed in DNA Engine® Peltier Thermal Cycler (Bio-Rad). 10 μL of sample was loaded in each well and run on 1.5% agarose gel (1.5 g agarose, 100 ml 1X TAE, and 10 μL Ethidium Bromide) at 95V for 30mins.

Primers were purchased from Integrated DNA Technologies (Coralville, IA)

Gulo Primer 1: 5'- CGC GCC TTA ATT AAG GAT CC -3'

Gulo Primer 2: 5'- GTC GTG ACA GAA TGT CTT GC -3'

Gulo Primer 3: 5'- GCA TCG CAG TGA CTA AGG AT -3'

SVCT2 Primer (50): 5'- CAT CTG TGC GTG CAT AGT AGC -3'

SVCT2 Primer (51): 5'- CAC CGT GGC CCT CAT TG -3'

SVCT2 Primer (54): 5'- TCT GAG CCC AGA AAG CGA AG -3'

SVCT2 Primer (55): 5'- GAT GGA CGG CAT ACA AGT TC -3'

Antioxidant Analysis

Vitamin C

In both chapters, VC was measured as ascorbic acid in metaphosphoric acid extracts of tissues as described (103) using ion-pair HPLC with electrochemical detection.

Vitamin E

In both chapters, VE was measured as α-tocopherol in extraction buffer containing reagent alcohol, 10 mg/ml butylated hydroxytoluene, 3% SDS and hexane and subjected to HPLC analysis as described (105).

Reduced glutathione

Vitamin C tissue extracts were used to also measure reduced glutathione by an adaptation of the *o*-phthalaldehyde method as described (106). Data for all assays were expressed per gram tissue weight (wet weight).

Oxidative Stress Analysis

Malondialdehyde

To assess lipid peroxidation, malondialdehyde was measured by homogenizing samples in 5% TCA solution and reacted with thiobarbituric acid as described (106).

F_4-neuroprostanes and F_2-isoprostanes

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 (107). These analyses were performed in the Vanderbilt University Eicosanoid Core Laboratory.

STATISTICAL ANALYSIS

Behavioral Data: (Chapter III)

In Experiment 1, for all assessments that were conducted at both time points, repeated measures ANOVA was conducted with Time as the repeated variable (Test 1, Test 2) and vitamin C status (VC+, VC-) and vitamin E status (VE+, VE-) as the between groups 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 groups variables. In Experiment 2, Repeated measures ANOVA were conducted with Time as the repeated variable (Test 1, Test 2, Test 3) and genotype of the *gulonolactone oxidase* gene (gulo$^{+/+}$, gulo$^{-/-}$) and vitamin C transporter (SVCT2$^{+/+}$, SVCT2$^{+/-}$) as the between groups variables.

Biochemical and oxidative stress markers: (Chapter III, 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 groups variables. In Experiment 2, univariate ANOVA was conducted with genotype of the *gulonolactone oxidase* gene (gulo$^{+/+}$, gulo$^{-/-}$) and transporter (SVCT2$^{+/+}$, SVCT2$^{+/-}$) as the between groups 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 X 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

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respective figure legend. All statistical analyses were performed by IBM SPSS Statistics Software (Version 19).

CHAPTER III

COMBINED VITAMIN C AND E DEFICIENCY INDUCES MOTOR DEFECTS IN GULO$^{-/-}$/SVCT2$^{+/-}$

Adapted from Pierce, M. R., DiAsio, D. L., Rodrigues, L. M., Harrison, F. E., & May, J. M. (2013). Combined vitamin C and E deficiency induces motor defects in gulo$^{-/-}$/SVCT2$^{+/-}$ mice. Nutritional Neuroscience, 16(4), 160-173.

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 (49). Chief among the central nervous system's defenses against oxidative stress are low molecular weight antioxidants such as vitamins E and C. VE (mostly α-tocopherol) is lipid-soluble and provides protection in cellular membranes. Vitamin C (VC) 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) (61). This transporter is responsible for generating VC concentrations up to 2-6 mM in neurons (53). Mice that are homozygous for the SVCT2 gene deletion die shortly after birth with undetectable VC levels in the brain (67). This demonstrates the importance both of VC content and regulation by SVCT2 in brain.

In vitro (108) and *in* vivo (19) studies have demonstrated a synergistic relationship between VC and VE that helps to preserve neuronal function and survival (21). Guinea pigs, like humans, are unable to synthesize VC and have long served as a model for study of both VE and VC deficiencies. Using this model, it was demonstrated that first, high dietary fat and cholesterol exacerbated chronic VC deficiency by both decreasing VC levels and increasing its oxidation ratio (109). Second, a diet deficient in both vitamins had greater oxidative stress than animals deficient in either vitamin alone (19). 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 (20). 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) (102), which is required for VC synthesis. VC tissue levels in these mice can be readily manipulated by dietary means (102;103). VE can also be removed from their diets, thus yielding a combined VC and VE 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 (110;111). This model is physiologically relevant because humans deficient in one vitamin are often marginal in the other. Furthermore, those most likely to have VC deficiency tend to eat a diet low in fruits and vegetables (112;113) and high in triglycerides and cholesterol.

The purpose of this study was to determine the effect of oxidative stress due to combined VC and VE 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 VC, induced double vitamin deficiency in gulo mice that also lacked one allele for the SVCT2 and included further behavioral testing.

PILOT STUDY

Our laboratory initially developed the double-deficient model of neurological damage in guinea pigs, as noted above. The double-deficient diet was adapted to mice with the goal of developing a sustainable oxidative stress to assess responsiveness of the SVCT2 expression and distribution. Since the SVCT2 determines VC localization, the goal was to determine how the brain responds to combined VC deficiency and oxidative stress due to lower dietary antioxidants in terms of VC distribution and function. A pilot study was conducted to determine the optimal time for the vitamin deficiencies since the combined VE and VC deficient diet should produce more oxidative stress than the VE deficient diet alone.

In contrast to guinea pigs, mice can synthesize VC. Therefore, in the mouse double-deficient model, all mice have targeted deletion of gulonolactone oxidase (Gulo$^{-/-}$). This enzyme catalyzes the last step in the synthesis of VC and is missing in humans and guinea pigs. When mice were not on an experimental diet they were supplemented with VC (0.33 g/L) in their drinking water (~2.5 mg/mouse), which provided levels equal to wild-type control. Treatments were made in de-ionized water with 0.01 M EDTA to increase VC stability.

To establish the mouse double-deficient model, five week-old Gulo$^{-/-}$ mice were placed on a VE and VC deficient diet (Harlan Teklad, diet #TD88163). Gulo$^{-/-}$ mice that are not provided VC supplementation develop scurvy in about 4-5 weeks (102). Therefore, 3 weeks of VC deficiency was chosen to ensure low VC levels but to avoid scurvy. Mice are relatively resistant to VE

deficiency, requiring more than 10 months on a solely VE-deficient diet before they develop symptoms including shaking of the head and ataxia. Therefore, 2, 4, and 6 months of VE deficiency was the time-points chosen as assessment points which were then followed by the complete removal of VC.

Table 3.1 shows that both VC and VE were decreased to levels about 10% of normal in brain cortex and liver by the diets. The formation of malondialdehyde (MDA) was measured to assess lipid peroxidative stress in these mice. Cortex MDA levels were increased at both 2 and 4 months of VE deficiency. Liver MDA levels were increased at all time points. These results confirm increased oxidative stress in these mice. Liver F_2-isoprostane levels showed a similar trend and further indicate increased oxidant stress generation. Because there was no significant increase in MDA at 6 months and since there was weight loss observed in the 6 month group before VC was removed, we concluded 6 months was not optimal and focused on the 2 and 4 month groups, which both had increased MDA levels in cortex and liver. Between these two groups there were more motor defects in the 4-month group. In wild-type C57/B6 adult mice (20 g female; 25 g male) stride length is ~58 mm (103). However, in my study, gait was adversely affected in the combined VC and VE group. In the 2 month group the average gait length was ~46 mm in the control group and ~36 mm in the 4 month group. Locomotor activity was normal for age and strain; there was no difference between 2 and 4 months indicating that they were not grossly affected by scurvy. Therefore, I concluded that 4 months was the optimal time for VE-deficiency.

These data demonstrate that combined dietary depletion of the two vitamins in mice unable to make VC causes detectable behavioral changes associated with increased oxidant stress in brain cortex. These results also demonstrate the feasibility of using this *in vivo* model of oxidant

stress in the planned studies to assess the contribution of each vitamin to the induced oxidative

stress as described in the next section.

Table 3.1: Pilot Study - Summary of the effects of combined Vitamin C and E deficiency on experimental mice groups.

All four experimental groups were subjected to 0,2,4 or 6 months of vitamin E deprivation followed by 3 weeks of vitamin C deprivation. Assessment values shown for groups were normalized to the $Gulo^{+/+}$ (wild-type) had normal amounts of vitamin E in their diets. Normal $Gulo^{+/+}$ 0 MON values are represented as "++++" while other groups are shown with variable numbers of pluses relative to normal. Relative biochemical assessments of the groups, including vitamin C, vitamin E, MDA, F_2- Isoprostanes and F_4- Neuroprostanes concentration, are shown for cortex and liver tissues. Also shown, are relative group performance values for several motor coordination tests. A comprehensive display of the data can be found in Appendix 1.

		Vitamin E Deprivation Time			
		0 MON	2 MON	4 MON	6 MON
BIOCHEMICAL ASSESSMENTS	**Cortex Concentration**				
	Vitamin C	++++	+	+	+
	Malondialdehyde	++++	++++++	++++++	++++++
	F_2- Isoprostanes	++++	++++++	++++++	++++++
	F_4- Neuroprostanes	++++	++++	++	++
	Liver Concentration				
	Vitamin E	++++	+	+	+
	Malondialdehyde	++++	+++++++	+++++++	+++++++
	F_2- Isoprostanes	++++	++++++++	++++++++	++++++++
BEHAVIORAL ASSESSMENTS	**Motor-Coordination Performance**				
	Ambulatory Distance	++++	++++	++++	+++++
	Ambulatory Velocity	++++	++++	++++	++++
	Inverted Screen	++++	++++++	++++++	+++++

EXPERIMENTAL DESIGN AND BEHAVIOR ASSESSMENT SCHEDULE

Experiment 1 Design:

Male and female gulo$^{-/-}$ mice were maintained on a normal rodent chow that contained essentially no VC and 42 IU/kg of VE (total tocopherols). At 4-5 weeks of age, the mice were randomly divided into four groups on a Western diet (16% lard, 34% sucrose, 0.2 cholesterol). This was to increase oxidative stress and to provide a physiologic condition relevant to humans, as those deficient in these vitamins are likely to also consume a diet high in saturated fats and sucrose.

The four treatment groups each consisted of 16 weeks of VE treatment via diet followed by 3 weeks of VC treatment via water supplementation: (1) control group - VE treatment as 150 IU/kg with continued supplementation of VC at 0.33 g/L; VE+VC+ (N=9), (2) VC deficient group - VE treatment as 150 IU/kg and a final VC treatment of 0 g/L; VE+VC- (N=8), (3) VE deficient group - VE treatment as 0 IU/kg with continued VC supplementation of 0.33 g/L; VE-VC+ (N=6), and (4) doubly deficient group - VE treatment as 0 IU/kg with a final VC treatment 0 g/L VE-VC- (N=8). The four groups, containing approximately equal males and females, are shown in Experimental Scheme 1 (Figure 3.1).
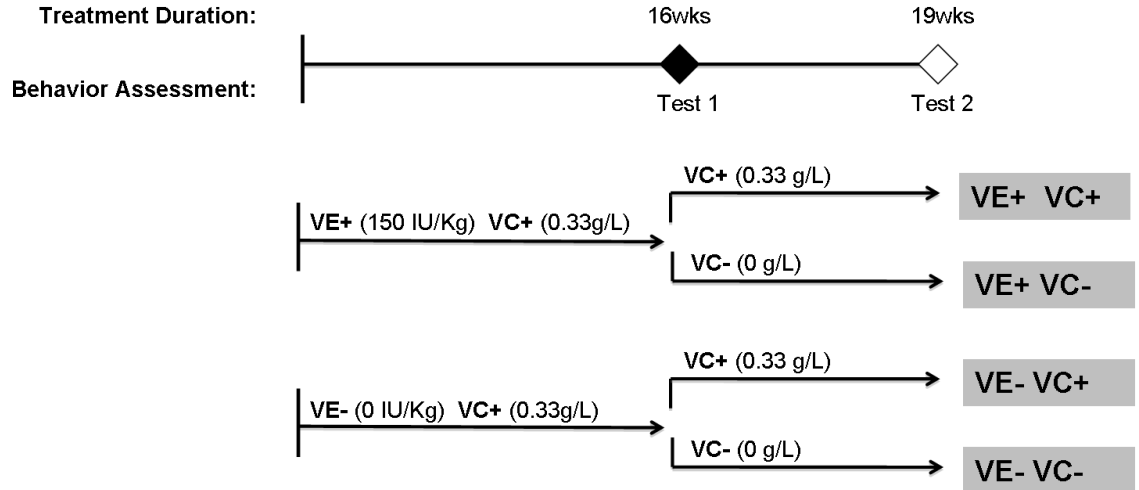
Figure 3.1: Experimental Scheme 1, Combined vitamin C & E deficient diets.
Mice started VE treatments at 4-5 weeks of age with continued VC supplementation. After 16 weeks of VE treatments mice were tested (Test 1) to observe changes in behavior. VC treatment lasted 3 weeks, after which another set of behavior tests were performed (Test 2). Mice were then sacrificed for biochemical analysis.

Experiment 1 Behavior Assessment Schedule:

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

Experiment 2 Design:

To enable both systemic and intracellular VC depletion, gulo$^{-/-}$ mice were crossed with mice lacking one allele of the SVCT2 (SVCT2$^{+/-}$) to generate gulo$^{-/-}$/SVCT2$^{+/-}$ mice. 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 VE-deficient, Western diet (TD.07310) and were provided with 0.33 g/L VC in their drinking water for 16 weeks. The mice were then all deprived of VC 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 (Figure 3.2).
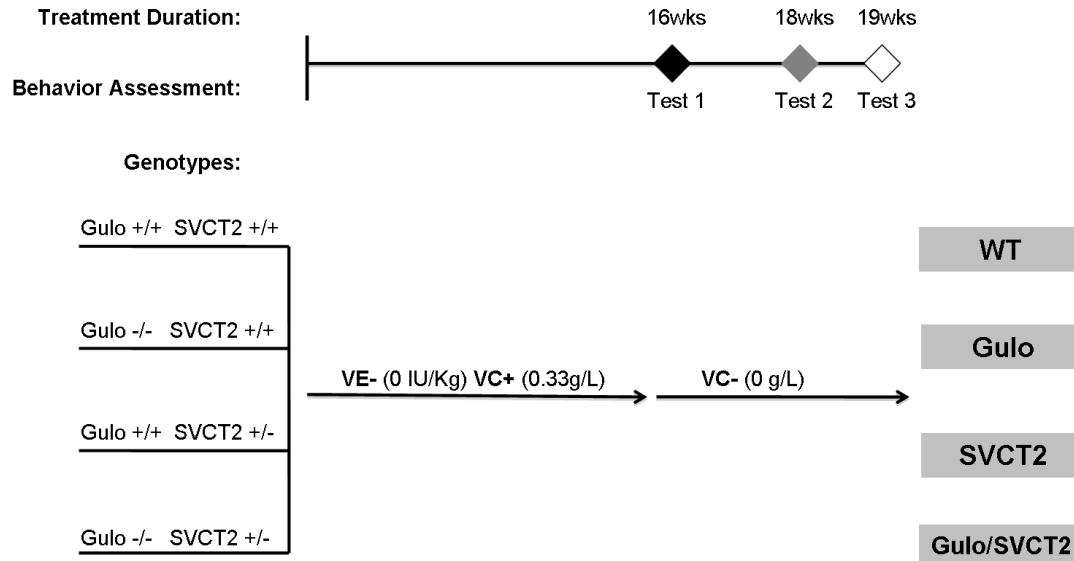
Figure 3.2: Experimental Scheme 2, Combined vitamin deficiency effects on gulo$^{-/-}$ SVCT2$^{+/-}$ mice.

Mice started VE deprivation at 4-5 weeks of age with continued VC supplementation. After 16 weeks mice were tested (Test1) to observe changes in behavior. VC treatment lasted 3 weeks, after which another set of behavior tests were performed after 2 weeks and followed-up with another at 3 weeks (Test 2 and 3 respectively). Mice were then sacrificed for biochemical analysis.

Experiment 2 Behavior Assessment Schedule:

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 at 16 weeks after VE deficient diet followed by two separate assessments at weeks 2 and 3 of the VC treatment phase which corresponds to 18 and 19 weeks of VE deficiency, (Test 2 and Test 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 two included one session of gait, Y-maze, horizontal beam, wire-hang, and Rota-rod test, sequentially.

All behavioral tests were performed under similar lighting. Repeated testing of cohorts was performed at the same time of day (morning or afternoon).

RESULTS

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

Body Weight and general appearance

As expected, the weights of all mice increased with age. During VC 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 ($P= 0.56$) (Figure 3.3A). There were no physical signs of early scurvy in any of the mice following VC 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 X VC, or Time X VE interactions (P's>0.062). There were, however, significant interaction effects of Time X VC and of Time X VC X VE (P's<0.038) (Figure 3.3B). 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 test 1 and test 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) (Figure 3.3C). Both VC deficient groups (indicated by the dashed lines) explored less than the VC-supplemented groups at test 2 (P<0.01) (Figure 3.3C). 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 X VC X VE (P<0.01) (Figure 3.3D). 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's> 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) (Figure 3.3E). Results in Figure 3.3F are from the Roto-rod probe trial only (day 2 of each 2-day test session). VE 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 VC 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) (Figure 3.3F).
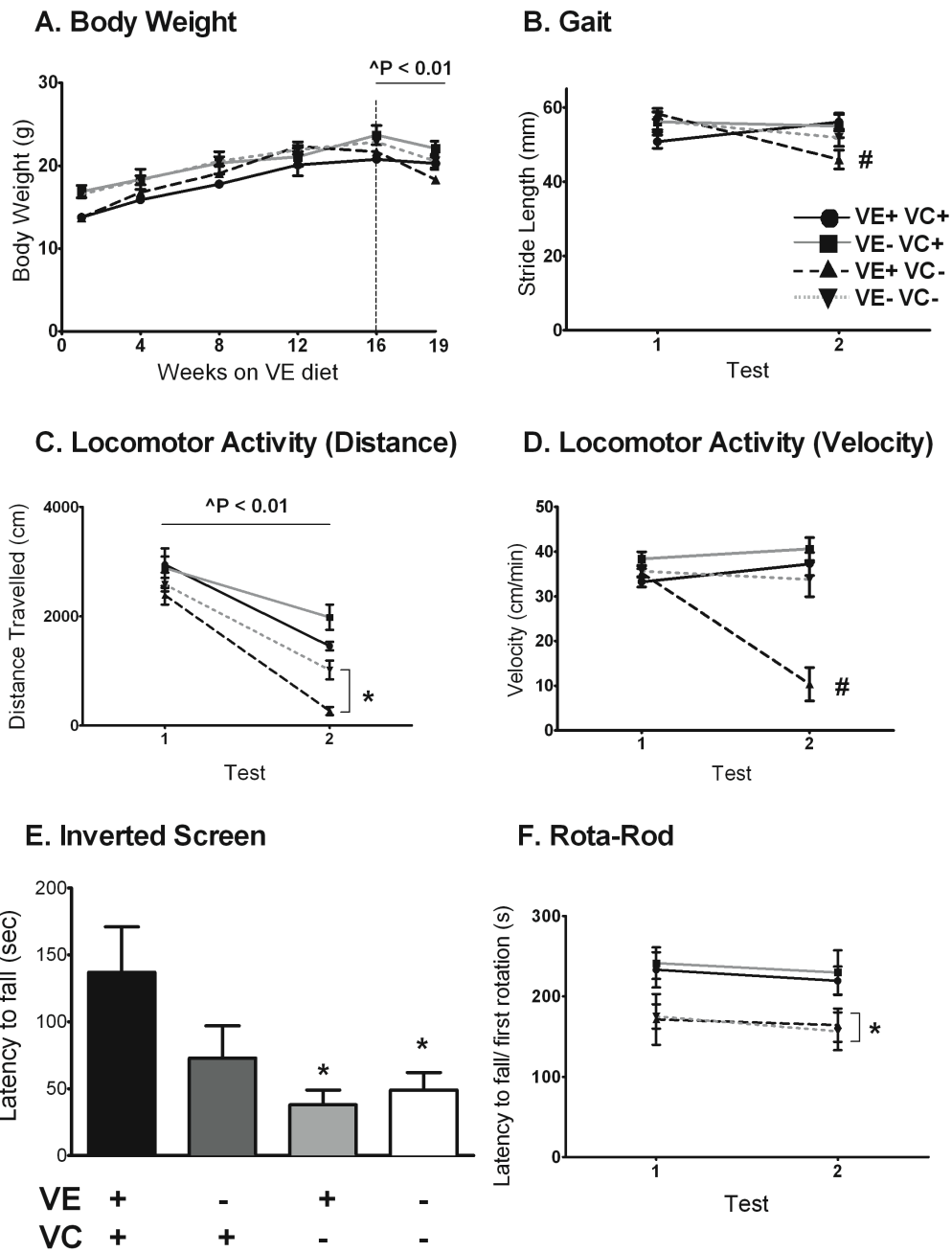
Figure 3.3: Behavioral phenotype of combined vitamin C & E deficiency.
Behavioral assessments included changes in body weight (A) stride length (B) locomotor activity
(C) and motor coordination on the Rota-rod (D) Displayed are group means ± standard error of
means.
^ Denotes a significant difference between Test 1 and Test 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

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VC and α-Tocopherol measurements of brain cortex, and liver

VC 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 VC status, since it receives dietary VC from the portal system and does not synthesize the vitamin in gulo$^{-/-}$ mice. As expected, VC deprivation for 3 weeks regardless of VE treatment lowered the VC contents of cortex and liver. In cortex, VC levels decreased 86% in VE+VC- and VE-VC- groups compared to the VE+VC+ group (P<0.01) (Figure 3.4A). Liver VC decreased 73% and 78% in the VE+VC- and VE-VC- groups, respectively (Figure 3.4B). 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's<0.05), VE deprivation without VC deprivation lowered VC in VE-VC+ compared to VE+VC+ (P<0.05).

As expected, VE deprivation for 16 weeks lowered α-tocopherol in cortex (Figure 3.4C) and liver (Figure 3.4D). In cortex, there were main effects of both VC (P<0.05) and VE (P<0.01) status, with no significant VC X VE status interaction (P=0.356). In liver, there were main effects of VE and VC deprivation (P's<0.01). A significant VC X VE status interaction in liver (P=0.01) indicated that although deprivation of either vitamin led to decreases in liver α-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's>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 VC by at least 80% (P<0.01). VE-VC- decreased 88% and 97% in cortex and liver tissue, respectively.

Measures of oxidative stress

Diets did not affect GSH contents in cortex or liver (P's>0.448) (Figure 3.4E and F). MDA levels in cortex and liver were increased in the VE-VC- group compared to the groups with normal VE intakes (Figure 3.5A and B). VC 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 increased almost 3-fold in cortex in groups deprived of VE compared to groups on adequate VE (P<0.01; Figure 3.5C). F_2-isoprostanes, derived from non-enzymatic peroxidation of arachidonic acid, were also increased in cortex to a lesser extent with VC 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 levels 7-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 (Figure 3.5D). F_4-neuroprostanes, derived from peroxidation of docosahexaenoic acid, were about 30-fold higher than those of F_2-isoprostanes (compare Figure 3.5C to 5E). Similar to F_2-isoprostanes, VE deprivation increased F_4-neuroprostanes in cortex (P<0.05) (Figure 3.5E).
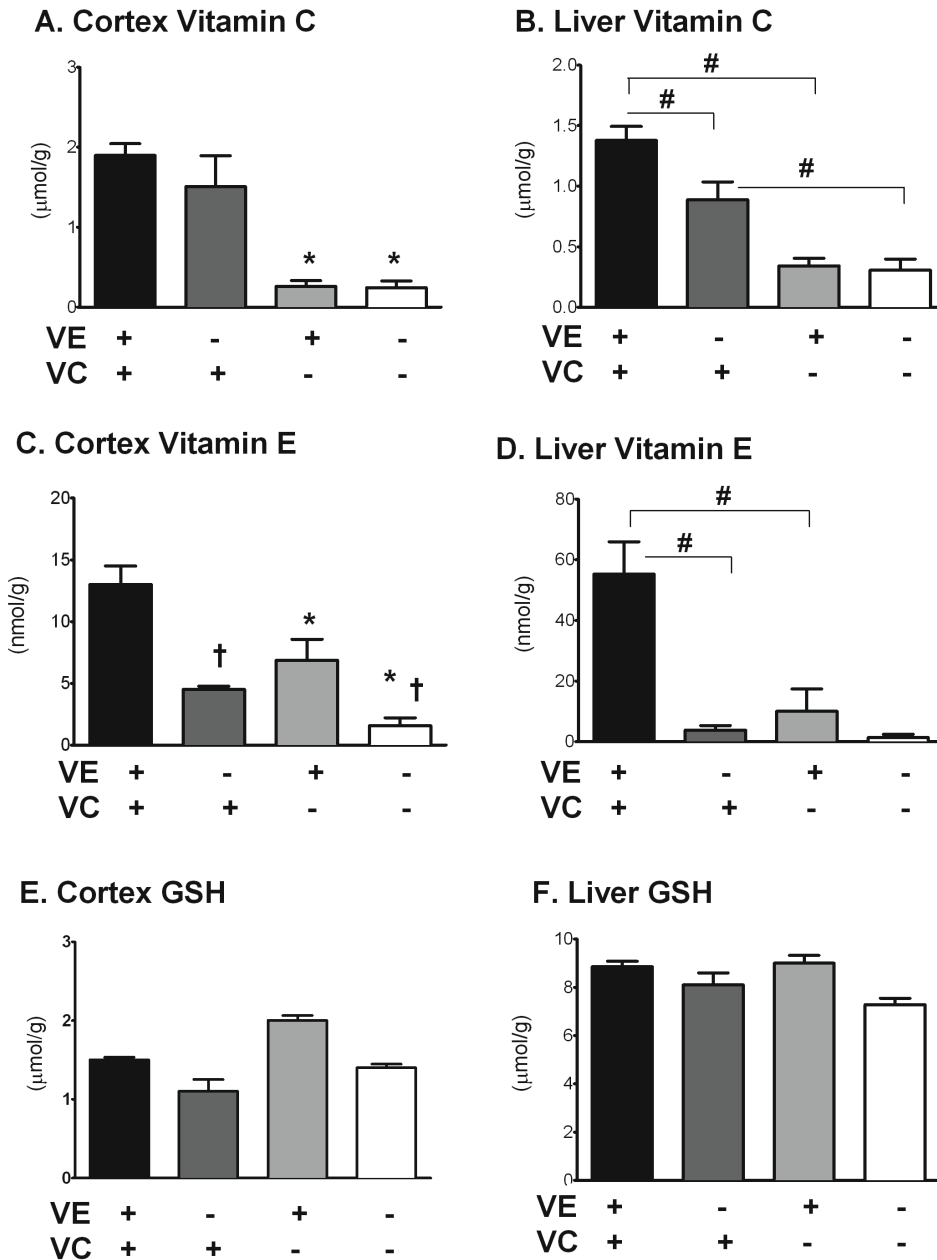
Figure 3.4: Combined vitamin C & E deficiency decreases antioxidant levels in cortex and liver.

Biochemical assessment included measurements of VC in cortex (A) and liver (B) and glutathione (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)
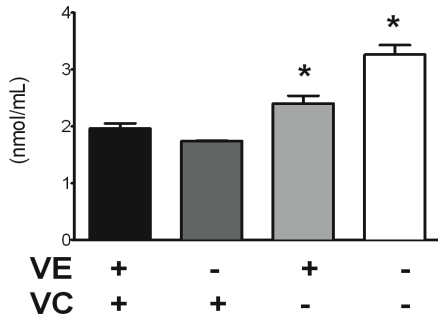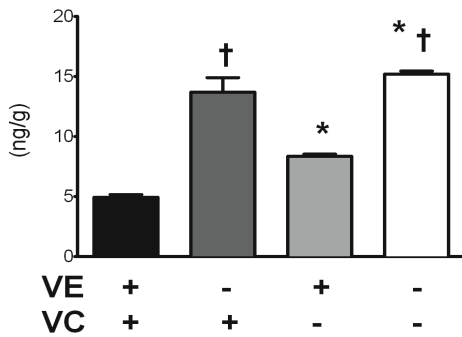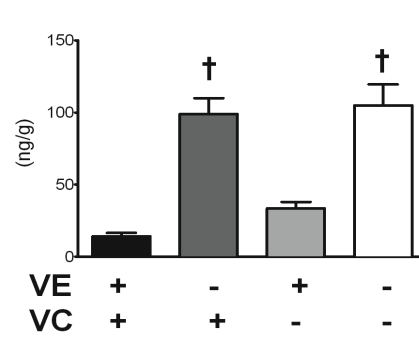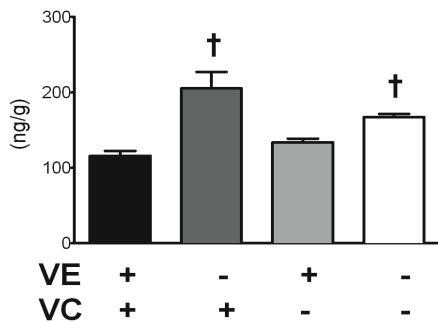
Figure 3.5: Vitamin E deficiency induces oxidative stress.
Biochemical assessment included measurements of malondialdehyde in cortex (A) and liver (B);
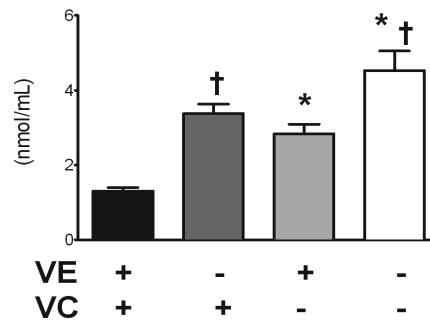F_2- isoprostanes in cortex (C) and liver (D); and F_4- neuroprostanes in cortex (E).
* Denotes a significant difference between VC+ and VC- groups ($p < 0.05$)
† Denotes a significant difference between VE+ and VE- groups ($p < 0.05$)

Experiment 2 - Combined vitamin C & E deficiency in gulo$^{-/-}$/SVCT2$^{+/-}$ mice

In Experiment 2, a cellular VC deficiency was added to the systemic VC deficiency, using gulo$^{-/-}$ mice that had been crossed with mice heterozygous for the VC transporter SVCT2 (SVCT2$^{+/-}$). All mice were on the same Western diet and were deprived of VE for 16 weeks, followed by VC-deficient intakes as described for Experiment 1. This experiment was designed to confirm the effects observed in the VC- and E-depleted gulo$^{-/-}$ mice in Experiment 1 and to expand motor testing by adding further 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 differences between the groups (Figure 3.6A). None of the mice had overt signs of severe scurvy, although gulo$^{-/-}$/SVCT2$^{+/-}$ mice lost weight following 3 weeks of VC deficiency (P<0.01).

Behavioral Assessment

Changes in behavioral phenotype were observed during the last 3 weeks of the diet treatments. Mice were assessed both under normal VC supplementation (Test 1) and after 2 (Test 2) and 3 (Test 3) weeks of VC deprivation (Experimental Scheme 2, Figure 3.2).

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) (Figure 3.6B). There was a main effect of SVCT2 genotype (P<0.05), but not of gulo genotype (P=0.17), and also a Gulo X 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's>0.38).

Analysis of locomotor activity revealed significant main effects of time, both gulo and SVCT2 genotype and a Time X Gulo interaction (P's<0.05) (Figure 3.6C). However, these effects were all subsumed by a significant 3-way interaction among the factors (Time x Gulo X SVCT2, P<0.01). All mice explored less across test sessions (P's<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's<0.05). The Time X Gulo X 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 travelled at this time point). There were no other velocity differences among the groups.

On the inverted screen test, there was a Time X Gulo X 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's>0.1) (Figure 3.6D). 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 test 1 to test 2 despite VC deprivation in the gulo$^{-/-}$ group (Figure 3.6E). During this period a decrease in fall latency was observed in the gulo$^{-/-}$/SVCT2$^{+/-}$ group. There was interaction of Time X Gulo X SVCT2 (P<0.05). The gulo$^{-/-}$/SVCT2$^{+/-}$ group was the only group that performed more poorly on the Rota-rod across 3 weeks of VC

deprivation (P<0.01). This group also demonstrated shorter fall latencies compared to other test groups at the final session (P's<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 (Figure 3.6F), with a Time X Gulo X 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's<0.01). Although a similar trend was observed in the wire hang test (Figure 3.6G), the differences did not reach statistical significance. There were main effects of SVCT2 genotype (P<0.01) because the SVCT2$^{+/-}$ mice performed more poorly (higher scores) 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's>0.062) (Figure 3.6G). 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 in the percent of time mice in each group spent in the open arm of the zero maze indicating no evidence of increased anxiety (Figure 3.6H). 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 gulo$^{-/-}$/SVCT2$^{+/-}$ group, consistent with their decreased tendency to explore (Figure 3.6C).
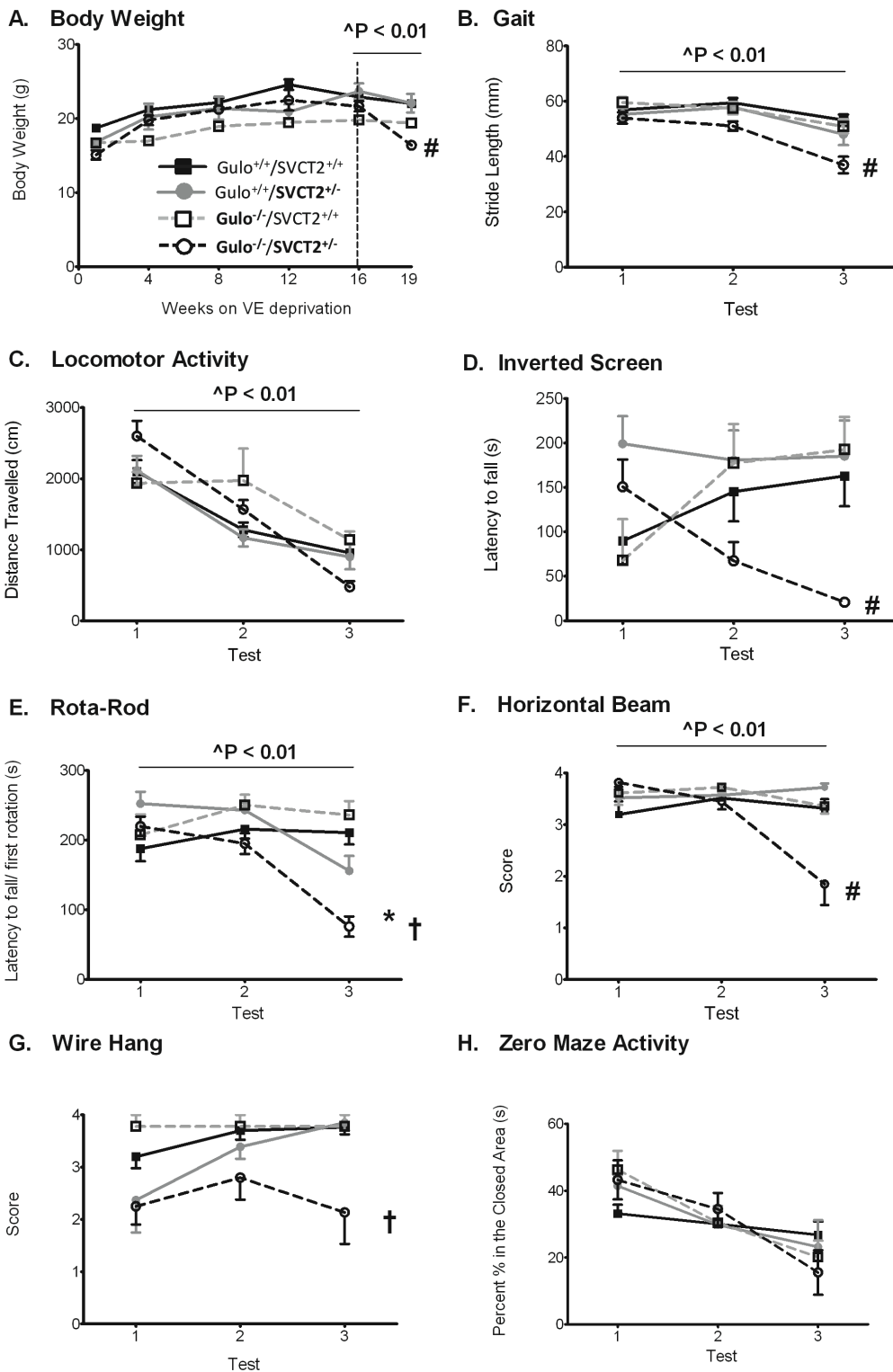
Figure 3.6: Genetic modification of gulo$^{-/-}$/SVCT2$^{+/-}$ alters behavioral phenotype.
Behavioral assessments included changes in body weight (A) stride length (B) locomotor activity (C) and motor coordination including inverted screen (D) Rota-rod (E) horizontal beam (F) wire hang (G) and zero-maze (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 X SVCT2 interaction effect (p<0.05)

Vitamin C & E content of brain cortex, cerebellum and liver

The VC 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 VC. As in Experiment 1, placing $gulo^{-/-}$ mice on a VC-free diet for 3 weeks decreased VC in each tissue type by 95%, 85% and 87%, respectively. In the cortex, overall VC 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 X SVCT2 ($P<0.05$) because VC was decreased in all $SVCT2^{+/-}$ mice. Similarly, in $gulo^{-/-}$ mice, there was no further effect of SVCT2 genotype on VC level ($P=0.93$), but being heterozygous for SVCT2 did lead to lower VC in $gulo^{+/+}$ cortex ($P<0.05$) (Figure 3.7A). 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 ($P=0.073$) (Figure 3.7B). In liver, lower VC was seen in $gulo^{-/-}$ mice than in $gulo^{+/+}$ controls ($P<0.01$) (Figure 3.7C).

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 VE-deficient diet (compare Figure 3.4D and E to Figure 3.7D and F). In addition to this lowering due to VE-deficient diets alone, α-tocopherol levels were significantly decreased in cortex (but not cerebellum and liver) in the 3 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 ($P<0.05$). VE 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 (Figure 3.7D-F), however, overall levels were lower in the cerebellum than the cortex. Liver VE levels were often undetectable and no different between the groups (Figure 3.7F).

Measures of oxidative stress

Cortex GSH levels in were increased in gulo$^{-/-}$/SVCT2$^{+/-}$ mice deprived of VC compared to wild type and to SVCT2$^{+/-}$ mice (Figure 3.8A). There was a main effect of both gulo and SVCT2 genotypes (P's<0.01). A similar trend was observed in cerebellum with the highest levels in the gulo$^{-/-}$/SVCT2$^{+/-}$ mice; however the interaction between Gulo X SVCT2 was not significant (P=0.070) (Figure 3.8B). The same effect was not observed in liver (Figure 3.8C). MDA levels were increased in cortex in all of the gulo$^{-/-}$ mice (P<0.01) mice after VC deprivation compared to the other two gulo$^{+/+}$ groups (Figure 3.8D). There were no additional effects of SVCT2 genotype. MDA levels were not different in cerebellum among the genotypes, consistent with the slight changes in VE content. In liver, MDA was increased compared to wild type in the two VC-deprived groups (Figure 3.8F). There was a main effect of both gulo and SVCT2 (P's<0.01). Together, these results show that VC deprivation, especially in the gulo$^{-/-}$/SVCT2$^{+/-}$ mice, causes a modest increase in oxidative stress in cortex and liver.
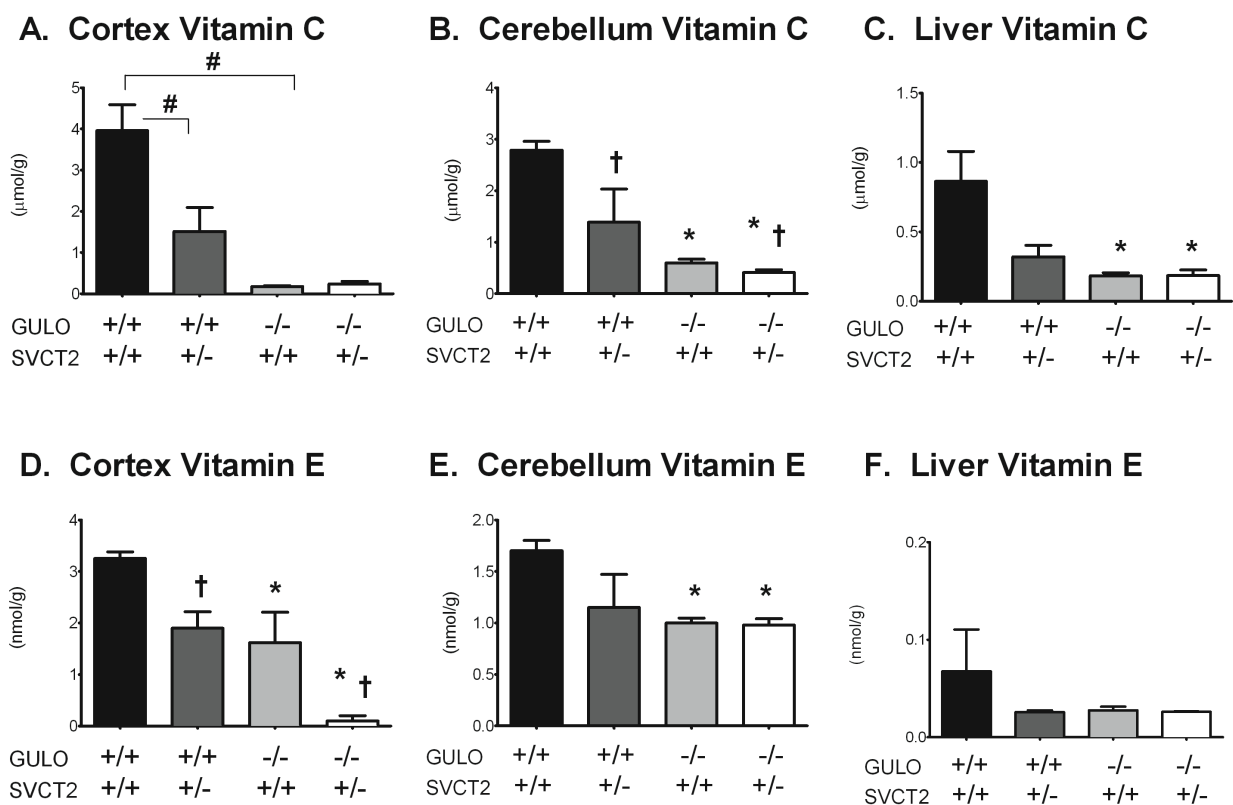
Figure 3.7: Combined vitamin C & E deficiency decreases antioxidants in cortex, cerebellum and liver in gulo$^{-/-}$/SVCT2$^{+/-}$.
Biochemical assessment included measurements of VC in cortex (A) cerebellum (B) and liver (C); VE in cortex (D) cerebellum (E) and liver (F).
* Denotes a significant difference 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 two groups (p<0.05)
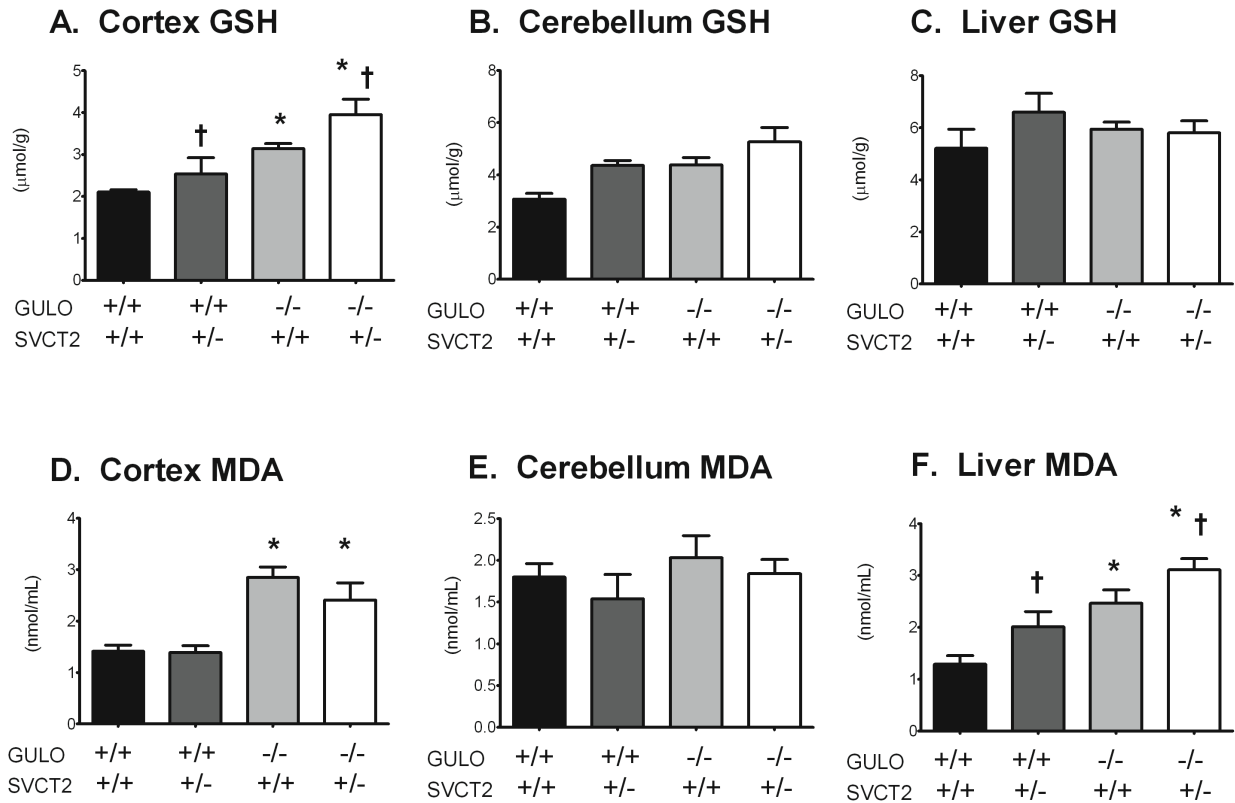
Figure 3.8: Combined vitamin C & E deficiency increases oxidative stress in cortex and liver in gulo^{-/-}/SVCT2^{+/-}.
Oxidative stress assessment included measurements of glutathione in cortex (A) cerebellum (B) and liver (C); malondialdehyde in cortex (D) cerebellum (E) and liver (F). Displayed are 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 ($p < 0.05$)

DISCUSSION

In Experiment 1, we investigated the effects of a combined VE & VC deficient diet on the neurobehavioral phenotype and oxidative stress measures in mice unable to synthesize their own VC. In contrast to guinea pigs, mice were more tolerant of combined VE and VC deprivation in terms of neurological deficits and survival. VE deficiency takes approximately 10 months to cause motor symptoms in mice compared to just 4-5 weeks in guinea pigs (114). In guinea pigs deprived of VE for only 2 weeks and VC for only 5-6 days, an ascending paralysis leading to death was observed (19), whereas as long as 10 months of VE deprivation in normal mice does not shorten stride or impair Rota-rod performance, despite an 80% decrease in VE levels in cortex and liver (115). Thus, it was not surprising that in the present study a VE-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.

VC 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 VC 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 VC-deprived groups showed poor performance on the Rota-rod. However, this difference was already apparent following the VE deprivation period, suggesting that it occurred by chance and was unrelated to the VC 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, VE deprivation alone did increase both cortex and liver F_2-isoprostanes, an established and sensitive marker of lipid peroxidation due to oxidative stress (116). Cortex F_4-neuroprostanes were also modestly increased by VE deprivation independently of VC status. For both markers, this could relate to the location of VE 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 VE deprivation in this manner causes a modest increase in brain lipid peroxidation, motor skills defects induced by short-term VC deprivation were not worsened by VE deficiency. VC deprivation consistently decreased VE content in both the cortex and liver. These results support those of *in vitro* studies showing that VC can recycle oxidized VE as α-tocopherol in lipid bilayers (108). Despite this role, VC deficiency in mice did not increase F_2-iso- or F_4-neuroprostanes, the most specific markers of lipid peroxidation (116), irrespective of VE depletion.

Since deficiency of VC rather than VE was related to defects in motor performance in Experiment 1, to accentuate this, a cellular deficit in VC 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. Only stride length and locomotor velocity remained unaffected. Decreased performance following VC 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 (117), 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 VE-supplemented groups in Experiment 1, so it is possible that this exacerbated

the motor skills defects. However, since VE deprivation did not affect motor performance independently of VC in Experiment 1, the contribution of VE deficiency to the observed motor skills defects would seem to be minimal.

VC 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 VC uptake and content in both brain and liver. This is surprising (and unexplained) for liver, since these mice can synthesize their own VC in this organ. VC 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 VC. However, VC contents of cortex and liver, although low, were no different than in gulo$^{-/-}$ mice deprived of VC. 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 VC, the cerebellum had better retention of VC and fewer changes in F$_2$-isoprostane levels compared to the cortex (103). Data in the present study support the idea that the cerebellum is better protected against VC depletion and subsequent increased oxidative stress.

The present results suggest that although VE 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 VC deficiency. Motor skills deficits became more extensive when cellular VC deficiency due to a decrease in cellular VC uptake was superimposed on VE deprivation and then followed by an acute deprivation of VC. Even though the mice in this study had no overt physical signs of scurvy beyond weight loss, it is likely that cellular deficiency of VC 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 VC deficiency in zebrafish showed altered cellular energy metabolism homeostasis by activation of the purine nucleotide cycle (118). This suggests that VC deficiency may lead to inadequate energy production, which could also contribute 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 VC 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 skill defects as young adults, with increases in oxidative stress markers (103). 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 VC deficiency, our results show that a 16-week deficiency of VE modestly increased oxidative stress markers, but did not affect mouse motor skills or coordination. VC deficiency in addition to VE deficiency only minimally increased oxidative stress markers compared to the single deficiency model. However, VC deficiency did begin to impair motor and coordination skills, deficits in which become more evident when the cellular uptake of VC is decreased. A summary of the observed phenotypes respective of the genotypes is shown in Table 3.2. 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.

Table 3.2: Summary of the effects of combined Vitamin C and E deficiency on experimental mice groups.
All four experimental groups were subjected to 4 months of vitamin E deprivation followed by 3 weeks of vitamin C deprivation. Assessment values shown for groups were normalized to the Gulo$^{+/+}$/SVCT2$^{+/+}$ (wild-type) group. Normal Gulo$^{+/+}$/SVCT2$^{+/+}$ values are represented as "++++" while other groups are shown with variable numbers of pluses relative to normal. Relative biochemical assessments of the groups, including vitamin C, vitamin E, GSH, and MDA concentration, are shown for cortex, cerebellum and liver tissues. Also shown, are relative group performance values for several motor coordination and anxiety tests.

		GULO +/+ SVCT2 +/+	GULO +/+ SVCT2 +/-	GULO -/- SVCT2 +/+	GULO -/- SVCT2 +/-
BIOCHEMICAL ASSESSMENTS	**Cortex Concentration**				
	Vitamin C	++++	++	+	+
	Vitamin E	++++	+++	+++	+
	Glutathione	++++	++++	+++++	+++++++
	Malondialdehyde	++++	++++	++++++	++++++
	Cerebellum Concentration				
	Vitamin C	++++	+++	++	+
	Vitamin E	++++	+++	++	++
	Glutathione	++++	++++	++++	++++
	Malondialdehyde	++++	++++	+++	++++
	Liver Concentration				
	Vitamin C	++++	++	+	+
	Vitamin E	++++	++	++	++
	Glutathione	++++	++++++	++++	++++
	Malondialdehyde	++++	++++++	++++++	++++++++
BEHAVIORAL ASSESSMENTS	**Motor-Coordination Performance**				
	Gait Length	++++	++++	++++	+++
	Locomotor Activity	++++	++++	++++	++
	Inverted Screen	++++	++++	++++	+
	Rota-Rod	++++	+++	++++	+
	Horizontal Beam	++++	++++	++++	++
	Wire Hang	++++	++++	++++	++
	Anxiety Test Performance				
	Y-Maze Alterations	++++	++++	++++	++++
	Zero-Maze Open/Close Ratio	++++	++++	++++	++++

CHAPTER IV

VITAMIN C TRANSPORT AT THE NERVE TERMINAL

Manuscript in Preparation

INTRODUCTION

Vitamin C (VC), an antioxidant not biosynthesized in humans, is an essential nutrient in the central nervous system (CNS) and neurons (49;119). Facilitated transport of VC across the plasma membrane of the neuron is a balance of two transport systems: uptake of VC inside the cell into the cytoplasm and efflux of VC outside the cell into the extracellular CSF. Dysregulation of VC concentrations on either side of the plasma membrane can lead to increased oxidative stress and abnormal neuronal development and function (53). Many cell models have been used to characterize the mechanisms for each transport system including neuron primary culture, neuroblastoma cell lines, human embryonic kidney (HEK) cells, and endothelial cells. However, how VC is transported in specialized regions within cells has received less attention. The most active region of the neuron is arguably the synapse, or nerve terminal, yet there are very few studies that investigate VC transport in this area. Neuronal pre-synaptic release sites are highly metabolically active and undergo a considerable amount of oxidative stress. Understanding the contribution for each transport system, uptake and efflux, at the nerve terminal is essential for fully appreciating the role of VC in neuronal health.

It is well established that the primary mechanism of VC uptake in the brain is mediated by the Sodium-dependent Vitamin C Transporter-type 2 (SVCT2) (53). Driven by the Na^+ electrochemical gradient, the SVCT2 transports one molecule of VC along with 2 molecules of Na^+ into the intercellular space (65). Mice that are homozygous for the SVCT2 knockout gene mutation have very low levels of VC in brain tissue despite the ability to synthesize the vitamin on their own (67). Equally as important as the function of the SVCT2 is its localization within the cell. *In situ* hybridization studies have shown that the SVCT2 mRNA is most neuronal cell types however, not glia (71). Studies with primary culture of mouse hippocampal neurons suggested that the SVCT2 was localized to the plasma membrane of the cell body and punctate areas in axons (120). Although these results suggest that the SVCT2 may reside in vesicular structures in neurons, they do not shed light on if the SVCT2 is present or functional specifically at the nerve terminals.

Cellular models for VC efflux in brain include astrocytes (87;88) and SH-SY5Y neuroblastoma cells (83). The mechanisms for VC efflux in the brain are unclear but some appear to be mediated by channel proteins (82). Protein-mediated VC efflux from cells may occur spontaneously (temperature-dependent), within a heteroexchange system with other molecules or be glutamate-induced, which is observed when cells are stimulated with the excitatory neurotransmitter, glutamate. Concerning the latter, it is postulated that glutamate uptake causes cell swelling that in turn triggers volume-regulated anion channels (VRACs) to release osmolytes to prevent swelling-induced cell lysis. VC appears to be one of the substances released by VRACs in astrocytes (87) and neurons (83). This type of glutamate-induced VC efflux may be of physiologic relevance as glutamate is the most common excitatory neurotransmitter and comprises ~95% of the neuronal projections in cortex tissue (121). Furthermore, electrophysiologic studies in rats showed that glutamate-induced VC efflux was associated with neurobehavioral changes (96;122). Although most VC efflux mechanism studies

have been performed in astrocytes, it is unclear if similar mechanisms exist within neurons as well. This is especially relevant considering that most of the brain's VC is concentrated in neurons.

The physiologic link between how neuronal VC transport is regulated in culture and it is regulated *in vivo* may lie in the VC uptake and efflux systems maintained at the synapse. Synaptic sites are specialized regions that may have distinct mechanisms to regulate VC uptake and efflux to ensure proper neuronal function. Synaptosome preparations are an established model for studying synaptic mechanisms as these pinched off nerve terminals contain all the required protein machinery for synaptic function. VC uptake and efflux has been partially described in synaptosomes (123). These studies observed that VC could be taken up by synaptosomes when incubated with an excess of VC concentration. Furthermore, VC efflux from synaptosomes could occur spontaneously or following incubation with physiologic glutamate concentrations. However, the mechanisms in each of the systems have not yet been characterized. Therefore, the studies described in this paper investigate the SVCT2 expression and function in VC uptake in synaptosomes. Also, we investigated the potential role of VRACs in glutamate-induced VC efflux from synaptosomes.

RESULTS

Neuronal SVCT2 localization

To localize the SVCT2 in neurons with regard to known neuronal transporters, cultured mouse superior cervical ganglia (SCG) were immunostained with antibodies to the norepinephrine transporter (NET) (Figure 4.1A), the SVCT2 (Figure 4.1B), and to the vesicular monoamine transporter-2 (VMAT2) (Figure 1C). The rationale was that co-localization of the SVCT2 with either of these two transporters would help to identify the vesicles containing the SVCT2. The SVCT2 antibody stained fibers and particulate structures that corresponded in many areas with nerve fiber staining of the NET (Figure 4.1D). On the other hand, the SVCT2 and the VMAT2 co-localized to particulate structures within nerve fibers (Figure 4.1E).

The superior cervical ganglion has relatively large presynaptic boutons (~5 µm). Using confocal fluorescence imaging, it is possible to take a section through the base of the bouton that forms the presynaptic side of the synaptic cleft (graphic representation, Figure 4.1F). Since it shows proteins located in the presynaptic bouton membrane and in vesicles just below it, this approach can be used to determine whether proteins or transporter co-localize in this important region. When such a sectioned bouton was examined, all three transporters co-localized (inset to Figure 4.1D and E), suggesting that the SVCT2 co-localizes with both the norepinephrine and VMAT2 transporters in the presynaptic bouton membrane. This shows that the vesicles containing both transporters, as well as the VMAT2 transporter, have fused with the presynaptic membrane.

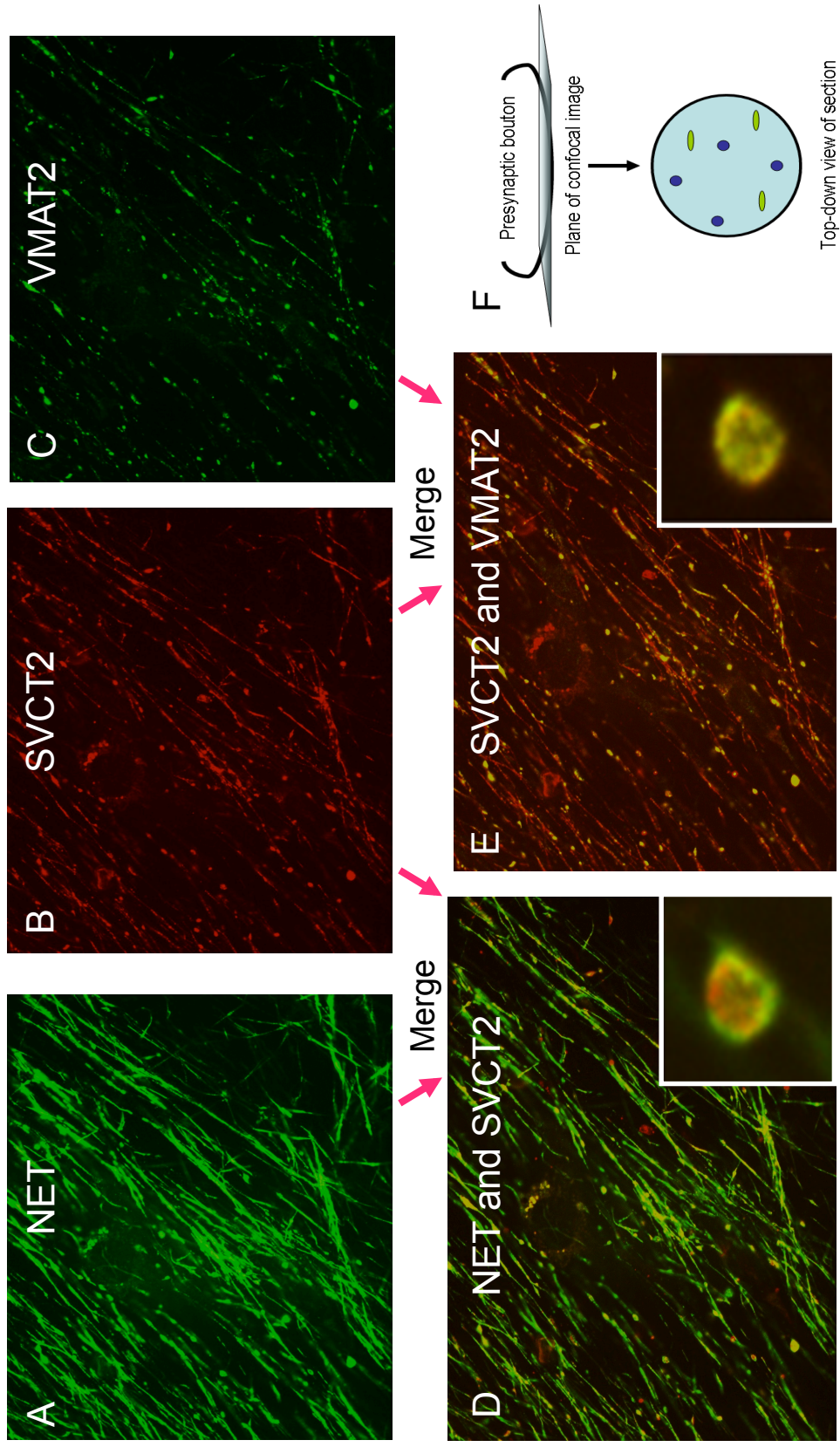**Figure 4.1: Immunostaining of the SCG presynaptic bouton.**
Top panel: Immunostaining for [A] Norepinephrine Transporter (NET), [B] SVCT2 and [C] vesicular monoamine Transporter Type 2 (VMAT2). Bottom panel: Co-localization of transporters; merging of immunostaining images of [D] NET and SVCT2 and [E] SVCT2 and VMAT2. [F] Graphic representation of bouton structures in insets for panels D and E.

SVCT2 expression at the cortical pre-synaptic terminal

To assess the location of SVCT2 protein within the nerve terminal we prepared synaptosomes from cortical tissue of male wild-type mice 4-6 months of age. Enriched synaptosome preparations were separated into three fractions: [1] cytosolic, which contains mostly detergent-soluble cytosolic proteins including synaptic vesicles and peri-synaptic membrane fragments; [2] pre-synaptic, and [3] post-synaptic. The latter two fractions contain detergent-resistant membrane fragments of the active zone that are soluble at a pH of 6.0 and 8.0, respectively. The relative enrichment of synaptic proteins was compared to whole synaptosomes (unfractionated preparation) and assessed by Western immunoblots (Figure 4.2A and B). The cytosolic marker Glyceraldehyde 3-Phosphate Dehydrogenase, (GAPDH, Figure 4.2C), was enriched in the cytosolic fraction. VMAT2 (Figure 4.2D), was also found in the cytosolic fraction, but was more prominent in the pre-synaptic fraction. Two proteins located in the post-synaptic terminal, *N*-methyl-*D*-aspartate Receptor, (NMDAR1, Figure 4.2E) and Postsynaptic Density Protein 95 (PSD-95, Figure 4.2F) were enriched in the post-synaptic fraction. These markers confirmed that the fractionation procedure enriches sub-cellular proteins found in these parts of the synapse. Under these conditions, SVCT2 was predominantly enriched in the pre-synaptic fraction (Figure 4.2G), suggesting that SVCT2 is indeed localized at the synapse, at the pre-synaptic terminal specifically.
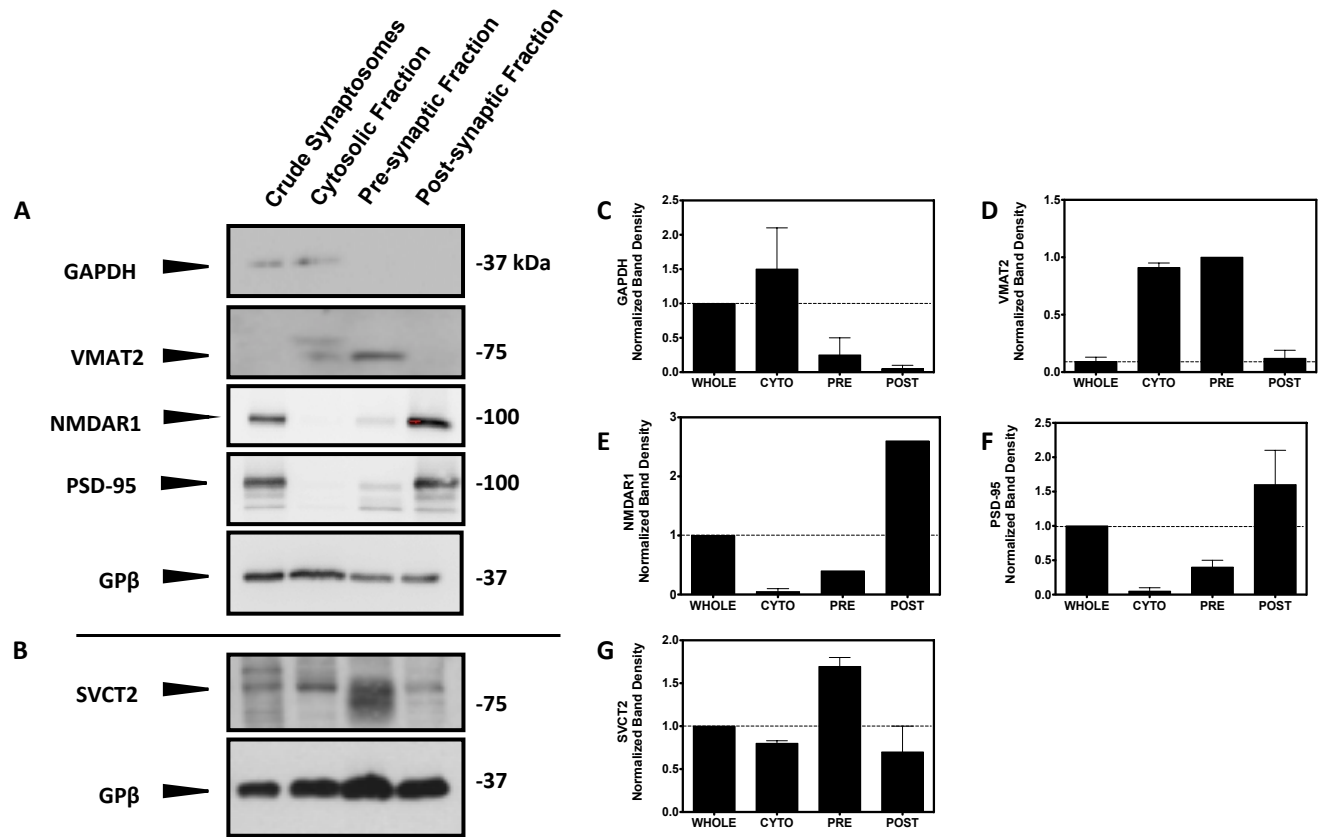
Figure 4.2: SVCT2 is localized in pre-synaptic fractions.
[A] Western immunoblots of cortical synaptosome preparations and fractionated samples.
Samples containing 5µg of protein were subjected to SDS-PAGE. Samples were probed for
cytosolic protein, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH); pre-synaptic protein,
Vesicular Monoamine Transporter (VMAT2); and two post-synaptic proteins, N-methyl-D-
aspartate Receptor (NMDAR1) and Postsynaptic Density Protein 95 (PSD-95).
[B] Samples containing 30µg of protein were subjected to SDS-PAGE under same fractionation
conditions and probed for SVCT2. Bands in each lane were normalized by G-protein β subunit
expression and compared to the initial crude synaptosome preparation (first lane).
Quantification of [C] GAPDH, [D] VMAT2, [E] NMDAR1, [F] PSD-95 and [G] SVCT2 are
represented as the mean ± SEM of two different experiments.

SVCT2 function in crude cortical synaptosomes

Net VC uptake

To determine if SVCT2 functions at the nerve terminal, we performed experiments to characterize net VC uptake. We found that crude synaptosomes contained endogenous VC at ~80 nmol/g (Figure 4.3A). Upon incubation for 30 min with 250 µM unlabeled VC, a two-fold increase in VC content was observed (Figure 4.3A). Two known SVCT2 inhibitors, sulfinpyrazone and phloretin prevented this uptake (Figure 4.3A). Similar results were observed in studies using radiolabeled ^{14}C-VC, which also showed net uptake of ^{14}C-VC uptake at ~80-100 nmol/g when incubated for 30 min (Figure 4.3B). Radiolabeled VC uptake was also inhibited by sulfinpyrazone and phloretin, as well as by replacement of sodium with choline. It has been shown that the oxidized form of VC, dehydroascorbic acid (DHA) can be transported on the GLUTs in neurons and astrocytes (54). However, the GLUT transporter competitive inhibitor, 3-*0*-methylglucose at a concentration that will substantially compete for dehydroascorbic acid, did not affect ^{14}C-VC uptake. This indicated that ^{14}C-VC uptake was independent of GLUT function.
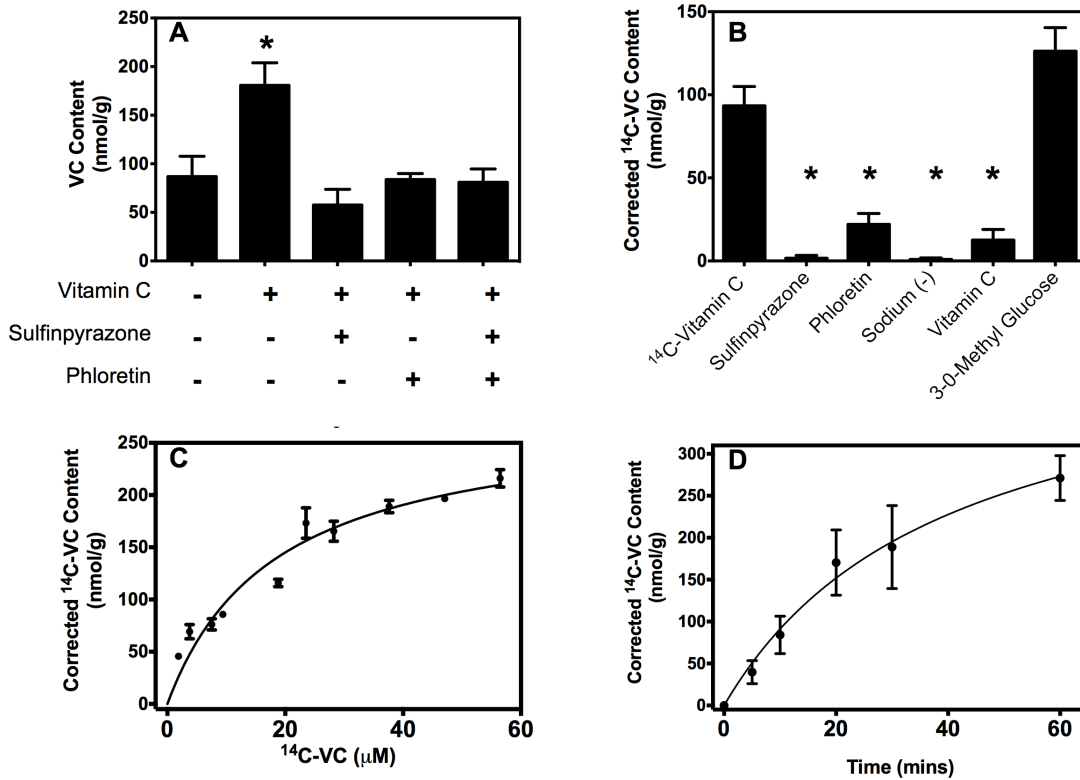
Figure 4.3: Net Vitamin C uptake in cortical crude synaptosomes.
[A] Crude synaptosomes were incubated for 30 min without (control) and with 250 μM VC only or 250 μM VC and 10 min pre-incubation with 1 mM sulfinpyrazone and/or 0.1 mM phloretin.
[B] Crude synaptosomes were pre-incubated for 10 minutes with 1 mM sulfinpyrazone, 0.1 mM phloretin, sodium-free KRH buffer, 1mM unlabeled VC, or 40 mM 3-o-methylglucose before the addition of ^{14}C-VC (0.05 μCi, 7 μM) and further incubated for 30 minutes. Background ^{14}C-VC signal was determined by assessing the signal detected after lysing synaptosomes. This amount was then subtracted from all of the readings.
[C] Crude synaptosomes were incubated with ^{14}C-VC (0.05μCi, 7μM) for different periods of time or [D] concentration for 10 minutes. The calculated K_m was ~12-15 μM. The results represent the mean ± SEM of a representative experiment performed in duplicate. *P<0.05

Kinetic characterization of VC uptake

To determine if the kinetic properties of VC uptake in synaptosomes were similar to those expected if transport occurred via SVCT2, time and concentration curves were performed using radiolabeled VC. The uptake of ^{14}C-VC was linear for about 20 min (Figure 3C). Using an incubation time in the linear phase of uptake (10 min), uptake was saturable the and apparent K_m and V_{max} of 18.31 ± 4.7 µM and 277 ± 28.15 nmol/g/30 min, respectively. These data suggest that the VC uptake in synaptosomes is consistent with SVCT2 function.

Net VC efflux out of cortical synaptosomes

VC transport is a balance between uptake and efflux. In order to assess the contribution of VC movement out of synaptosomes, a series of efflux studies were performed to observe the release of endogenous VC from non-loaded synaptosomes over time. Two types of VC efflux mechanisms were studied: spontaneous and glutamate-induced. Spontaneous endogenous VC efflux was measured since net uptake would depend on efflux occurring at 37°C. We also assessed glutamate-induced VC efflux because of its physiologic relevance.

Temperature dependence of *spontaneous VC efflux*

No VC efflux was observed at 4°C, whereas at 37°C, the endogenous VC concentration steadily decreased over time and after 30 min had decreased to ~80 – 100 nmol/g, comparable to the uptake experiments over the similar time frame (Figure 4.4A). To determine the contribution of SVCT2 to VC efflux, synaptosomes were incubated with the SVCT2 inhibitors, phloretin and sulfinpyrazone, for 30 min at 37°C. The presence of these inhibitors did not inhibit efflux but rather enhanced efflux by 25% and 50%, respectively (Figure 4.4C and E). This is likely due to the decrease in net VC uptake caused by the inhibition of the SVCT2, resulting in an apparent increase of net efflux. This suggests that the SVCT2 is not responsible for VC efflux, but that

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influx does play a role in VC homeostasis in the synaptosome. To ensure that this efflux was not due to a compromise in the integrity of the membrane, synaptosomes were pre-loaded with Fura Red[TM] dye for 30 min at 37°C. The synaptosomes were centrifuged and re-suspended in buffer. A subsequent time course experiment assessed efflux of the water-soluble intracellular dye over 30 min at 37°C. There was minimal leakage of the dye, suggesting that synaptosome integrity is maintained during the course of this type of experiment (Figure 4.5).
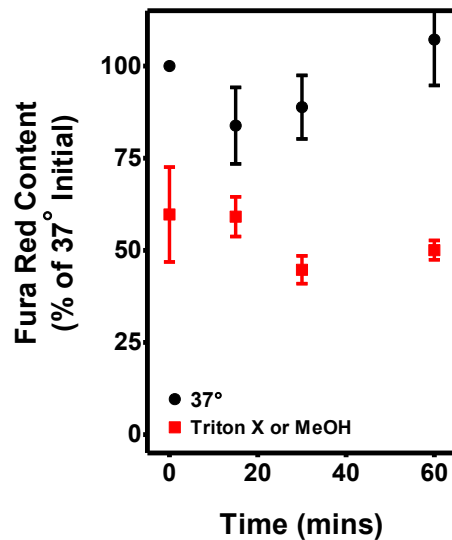
Figure 4.4: Synaptosome membrane integrity is maintained during the course of experiment.
Synaptosomes were pre-loaded with Fura Red dye for 30 minutes at 37°C and then incubated at 37°C with or without 5% Triton X-100 (Sigma T-9284) or 60% MeOH. The results represent the mean ± SEM, N=4.
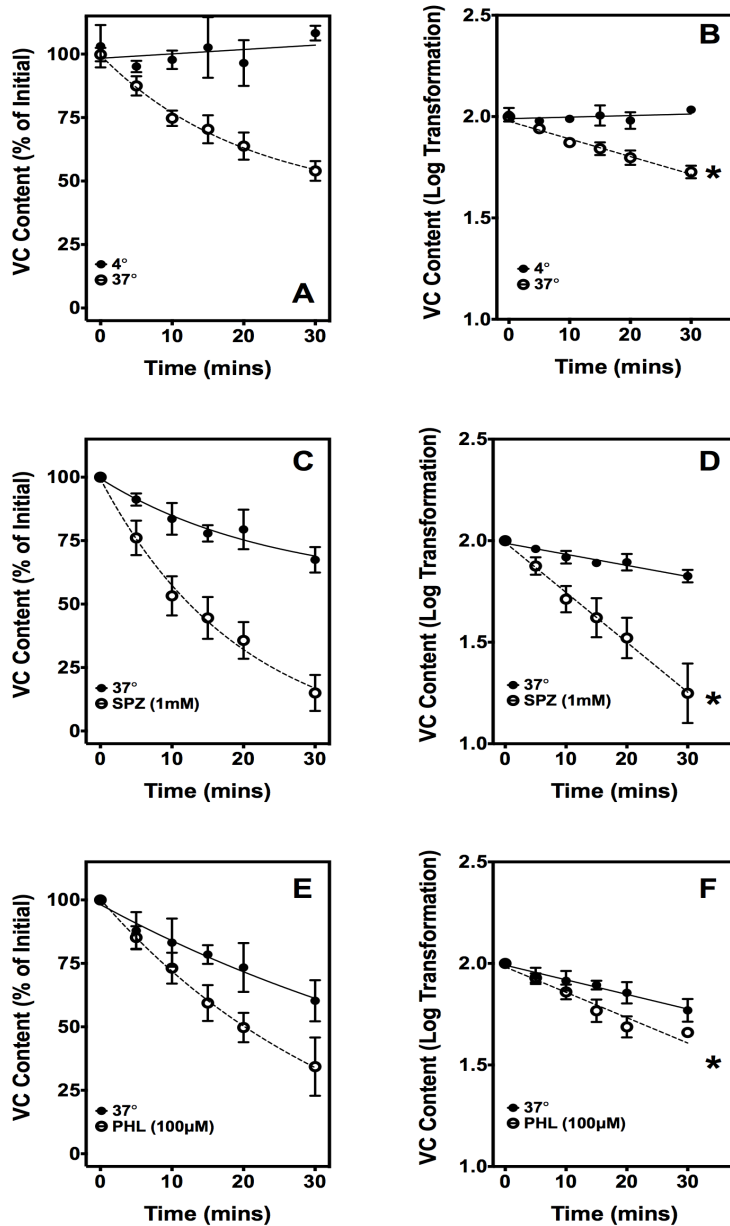
Figure 4.4: Spontaneous Vitamin C Efflux.
Time course of spontaneous VC efflux. [A] Relative VC content over 30 min at 4°C (closed circles) and 37°C (open circles). The initial synaptosomal VC concentration was ~250-300 nmol/g. [B] Logarithmic transformation of data in panel A where (*) denotes a significant difference between the slopes of the two lines. [C] Relative VC content over 30mins of efflux at 37°C with (open circles) and without (closed circles) 1 mM sulfinpyrazone [D] Logarithmic transformation of data in panel C where (*) denotes a significant difference between the slopes of the two lines. [E] Relative VC content over 30mins of efflux at 37°C with (open circles) and without (closed circles) 100 µM phloretin [F] Logarithmic transformation of data in panel C where (*) denotes a significant difference between the slopes of the two lines.

Hypotonic-Induced VC Efflux

To determine the effect of osmolality on VC efflux in synaptosomes, preparations were incubated in hypotonic conditions and VC content was assessed after 5 min. Figure 4.5 shows VC content of synaptosomes in isotonic buffer (300 mOsm), hypotonic Na+ control buffer (300 mOsm, with choline replacement), and hypotonic buffer (200 mOsm). VC content decreased ~50% after incubation with hypotonic buffer alone. This decrease was completely eliminated in the presence of 1 mM DIDS, an ion channel inhibitor known to inhibit VRAC (124). These data suggest that VC efflux from synaptosomes is enhanced by hypotonicity independent of the sodium concentration and mediated by an anion channel.
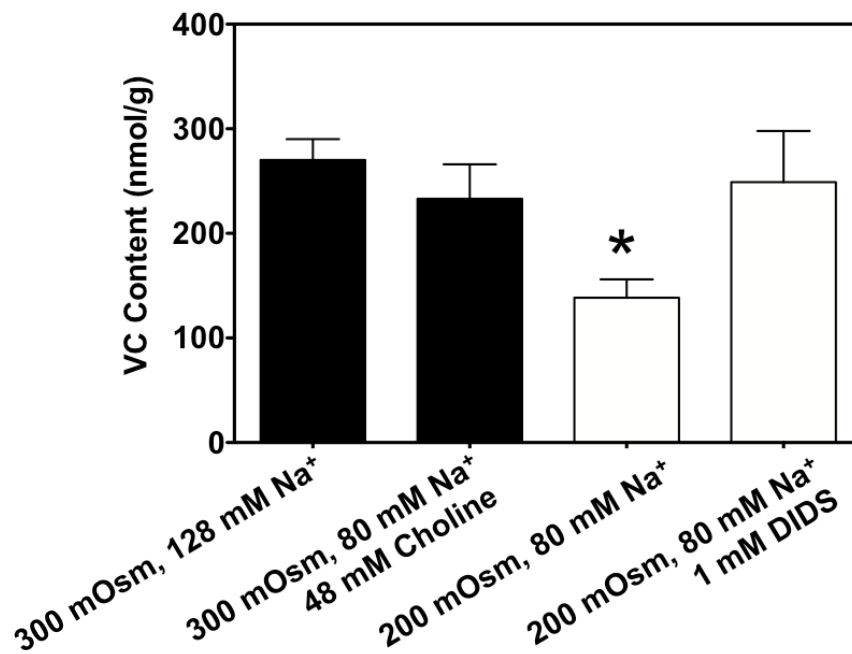

Figure 4.6: Hypotonic solution-induced vitamin C efflux.
Crude synaptosomes were incubated in buffers of various tonicities and sodium content for 5 min and residual synaptosomal VC content was measured after 5 min at 37°C. Samples shown by the last bar were pre-incubated with 1 mM DIDS for 5 min at 37°C before the efflux experiment. N = 2, with $p < 0.05$ compared to other bars.

Glutamate-Induced VC Efflux

To determine the mechanism for glutamate-induced VC efflux in synaptosomes, preparations were incubated with increasing concentrations of glutamate (Figure 4.6). A decrease of about 50% was observed when 200 µM glutamate was incubated with the synaptosomes for 30 min. Therefore, this concentration was chosen for subsequent experiments. Time course experiments confirmed that there was a significant increase in VC efflux at 37°C in the presence of glutamate (200 µM) resulting in an additional 15% decrease in VC content by the end of 30 min compared to 37° at 30min (Figure 4.7A and B). Pre-treatment of synaptosomes with 1 mM DIDS before the addition of 200 µM glutamate significantly decreased VC-efflux compared to 200 µM glutamate alone (Figure 4.7C and D). These data suggest that glutamate-induced VC efflux in cortical synaptosomes may occur on volume-regulated anion channels, as it is sensitive to the VRAC inhibitor, DIDS. As a control for the DIDS effect on basal (spontaneous) VC efflux, synaptosomes were incubated with DIDS (1mM) for 30 min at 37°C. The presence of DIDS failed to either inhibit or enhance efflux.
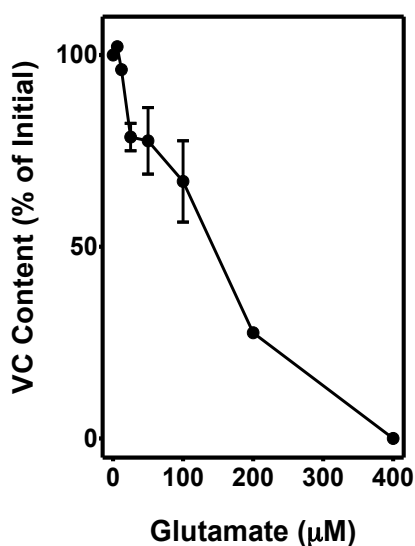
Figure 4.7: Glutamate concentration curve.
Vitamin C content of synaptosomes incubated with increasing concentrations of glutamate (0-400 µM), expressed as a fraction of control. N=3.
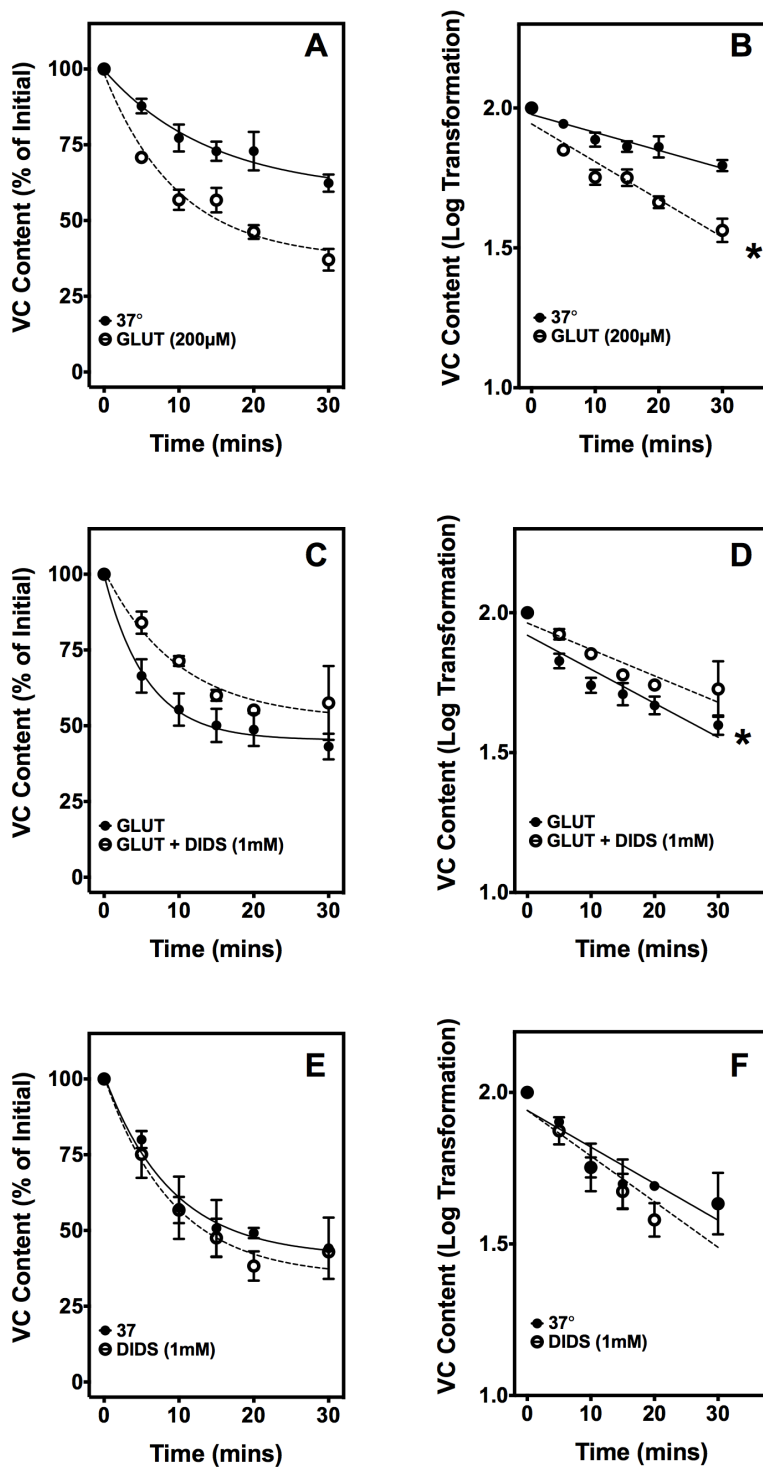
Figure 4.8: Glutamate Induced Vitamin C Efflux.
Time course of glutamate-induced VC efflux. [A] Relative VC content over 30 minutes 37°C with (open circles) and without (closed circles) 200 µM glutamate. [B] Logarithmic transformation of data in panel A where (*) denotes a significant difference between the slopes of the two lines. [C] Relative VC content over 30 minutes 37°C with (open circles) and without (closed circles) 1mM DIDS. [D] Logarithmic transformation of data in panel C where (*) denotes a significant difference between the slopes of the two lines. [E] Relative VC content over 30 minutes 37°C with (open circles) and without (closed circles) 1mM DIDS. [F] Logarithmic transformation of data in panel C

DISCUSSION

Neuronal VC concentration depends on VC transport across the plasma membrane is critical for proper neuronal function. VC is highly concentrated in neurons compared to surrounding glia cells that lack SVCT2 *in vivo* (71). VC transport has been extensively studied in several cell lines however, the physiologic link between how neuronal VC transport is regulated in culture and it is regulated *in vivo* may lie in the VC uptake and efflux systems maintained at the synapse. Using synaptosomes as a model to investigate VC regulation at the nerve terminal, this paper describes the SVCT2 expression and function in VC uptake and also the potential role of VRACs in glutamate-induced VC efflux.

First, we determined that the SVCT2 is expressed at the nerve terminal of superior cervical ganglia cells in the peripheral nervous system. Immunostaining of these cells showed colocalization of the SVCT2 with both the NET and VMAT2 at the presynaptic bouton. This data is not the first to show localization of the SVCT2 in catecholamine excreting cells. SVCT2 is also present in adrenal chromaffin cells, and likely accounts for their high intra-vesicular VC concentrations (125). Furthermore, Bornstein et al. (126) showed that adrenal chromaffin cell function is severely impaired and had morphological abnormalities in SVCT2 null mice. Based what is known about VMAT2 localization (127), these results may support the notion that the SVCT2 is carried in regulated dense core vesicles that are stored at presynaptic release sites.

In order to determine if the SVCT2 was expressed in nerve terminals in the CNS, enriched cortical synaptosome preparations were used. Uptake of VC in primary culture neurons is dependent on SVCT2 expression (120). Thus it was not surprising that SVCT2 was present in cortical synaptosome preparations. We determined the SVCT2 expression at the nerve terminal to be localized primarily in the presynaptic fraction some expression in the cytosolic fraction. This may indeed imply SVCT2 localization in several distinct compartments, as the cytosolic

fraction contains proteins that are localized peri-synaptically and in membrane-bound organelles including synaptic vesicles and mitochondria. It has recently been shown that HEK-293 mitochondria express SVCT2 (128).

In order to determine if the SVCT2 was functional at nerve terminals in the CNS, crude cortical synaptosome preparations were used. While the role of SVCT2 has been established in whole neurons, this study is the first to characterize its function at the nerve terminal. SVCT2 may function to reuptake VC after depolarization at the pre-synaptic terminal and actively transport VC inside the secretory vesicles in the cytosolic compartment. To determine if VC uptake in synaptosomes was consistent with SVCT2 function we performed uptake studies. Consistent with previous studies, VC uptake plateaued and saturated over time and concentration, characteristic of facilitated transport on a protein transporter rather than a channel. Kinetic analysis of VC uptake in synaptosomes showed that the apparent K_m was similar to that of the SVCT2 in neuronal cell systems. Furthermore, known SVCT2 inhibitors, sulfinpyrazone and phloretin inhibited VC uptake in synaptosomes. Therefore, SVCT2 likely mediates VC transport at the nerve terminal.

Extracellular VC concentration in the CNS is maintained homeostatically (129). Meile and Fillenz reported that extracellular VC is regulated homeostatically *in vivo*, in awake, behaving animals (130). In these studies, 100 –1000 µM VC was perfused through a microdialysis probe, with voltammetric detection of extracellular VC in the tissue adjacent to the probe. Concentrations that were lower than 400 µM caused extracellular VC to fall, whereas higher concentrations caused an increase in extracellular VC. After each perturbation, extracellular VC recovered within a few minutes, which demonstrates homeostasis. Homeostatic regulation of extracellular VC suggests that the extracellular compartment of brain tissue might be an important site of action for VC (71). To determine role of the SVCT2 in VC efflux, we

investigated spontaneous efflux from synaptosomes. Spontaneous VC efflux occurred at 37°C but not at 4°C. This is consistent with results of previous studies of VC efflux from rat synaptosomes (123). While SVCT2 appeared to mediate VC uptake into synaptosomes, it opposed efflux, since the latter was increased by its inhibition. Spontaneous VC efflux most likely does not involve SVCT2. Additional studies will have to be performed to determine the mechanism of spontaneous VC efflux.

There have been several papers published recently that have emphasized VC release in conjunction with a number of neurotransmitters including glutamate (131-133). Glutamate-induced VC efflux has been studied in astrocytes but the proteins mediating this process in neurons and synaptosome model are unknown. However, there is growing evidence that suggests that VRAC might be involved in glutamate-induced VC efflux particularly after cell swelling in astrocytes (88). We hypothesize that a similar process is happening at the nerve terminal and that VC efflux was occurring on VRACs in synaptosomes as well.

Our data shows glutamate indeed stimulates VC efflux and that co-incubation with VRAC inhibitor, DIDS, attenuates glutamate-induced VC efflux. These data suggest DIDS-sensitive VRACs could mediate glutamate-induced VC efflux at the nerve terminal. Recent studies by Lane and Lawen (133) demonstrated that the effects of glutamate on VC efflux in astrocytes are not due to the isolated activation of iGluRs, or mGluRs, since the release of VC from astrocytes cannot be emulated by agonists that are selective for NMDA and AMPA iGluRs or group 1 mGluRs. It is unclear if group II or III mGluRs are involved. This supports the concept that glutamate uptake is essential for VC efflux.

One caveat to this study is that DIDS in a non-specific anion channel inhibitor that could inhibit glutamate uptake and thus subsequently VC efflux (134;135). Therefore we investigated the

effect of osmolality on VC efflux. To determine if the change in osmolality associated with the adding glutamate could be responsible for VC efflux, we assessed VC efflux in a hypotonic buffer. Hypotonicity-induced VC efflux indeed occurred, and was attenuated with DIDS suggesting that DIDS-sensitive VRACs could mediate VC efflux due to swelling. This also indicates a glutamate-independent inhibition of VC efflux by DIDS, and DIDS had no effect on spontaneous VC efflux. This data also indicates that DIDS influence on VC efflux is independent of glutamate uptake.

In conclusion, we were able to provide evidence that SVCT2 protein is expressed predominately at the pre-synaptic terminal and functions to mediate VC uptake at the synapse. VC efflux from synaptosomes occurs in several conditions including spontaneous induction and glutamate-induction. While the mechanisms for VC efflux are not completely clear, our studies suggest that VRACs are likely to have a contribution in glutamate-induced VC efflux. Additional studies will need to be performed to determine if other types of VC efflux, including depolarization-induced VC efflux occurs via similar processes (Figure 4.8).

Understanding transport mechanisms for VC are crucial to understanding VC's role at the synapse. Currently, lack of molecular identification of VRACs is a significant drawback in the assessment of their role in VC efflux. Furthermore, as putative VRACs contribution accounts for less than 50% of the VC efflux by many of the cells tested, including hepatocyte-like Hep-G2 cells (84), SH-SY5Y neuroblastoma cells (83), and K562 cells (86), other release pathways are probably significant contributors. Further studies will have to be performed in order to characterize proteins involved in VC efflux.
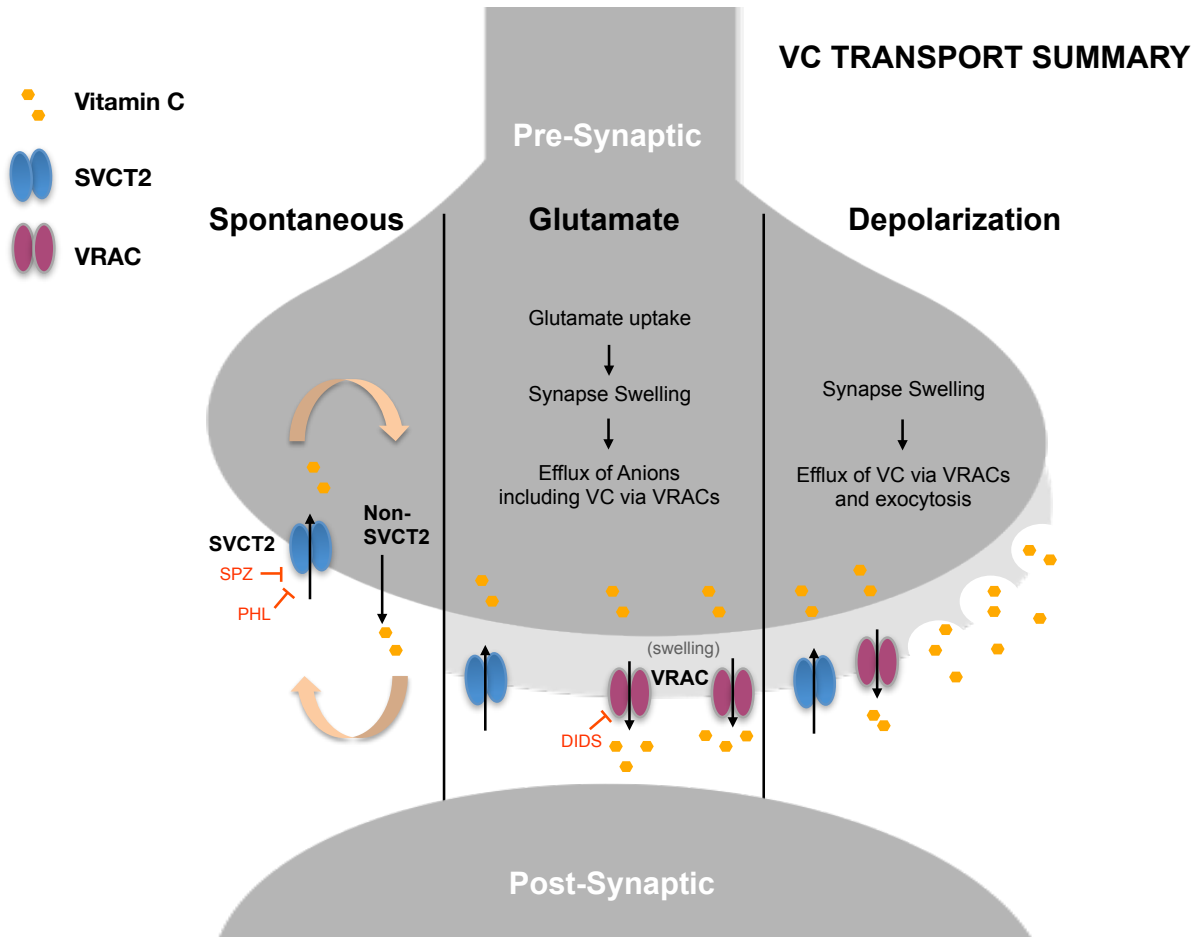
Figure 4.9: Model of Vitamin C Uptake and Efflux at a Representative Nerve Terminal.
VC transport is a balance between uptake and efflux. SVCT2 protein is expressed predominately at the pre-synaptic terminal and functions to mediate VC uptake at the synapse. VC efflux occurs in several conditions including (1) spontaneous induction, (2) glutamate-induction and (3) depolarization-induction. There are several proteins that could mediate glutamate uptake in neurons including EAAC1 and GLAST (136). glutamate-induced VC efflux is associated with synaptosome swelling which trigger VC permeable DIDS-sensitive VRACs. Depolarization induces VC efflux by exocytosis of VC contain secretory vesicles.

CHATPER V

SUMMARY AND CONCLUSIONS

BACKGROUND

We know a great deal about VC, but its function is as elusive as it is ubiquitous. The full picture of VC's contribution to human health has yet to be seen. There has always been a sense of urgency to understand this molecule and with its discovery, the dogma was born: the body needs just enough VC to prevent deficiency or scurvy to be healthy. Albert Szent-Gyorgyi, however, championed the notion that there was a wide gap between scurvy and full health. Linus Pauling, a renowned leader in the study of VC function, postulated that animals capable of synthesizing VC made more than the amount required for optimum health because of the burden of metabolic processes. Furthermore, these amounts could be used to calculate the ideal amount for human consumption, and suggested the RDA for VC should be 250 – 4000 mg per day based on his observation that many population groups were not ingesting enough VC for full saturation of tissues (137). Mark Levine, the leading expert on optimal VC concentration in tissues, may not support VC intakes as high as Pauling, but nonetheless has reinforced the idea of tissue saturation by assessing the pharmacokinetics of VC absorption. Currently, the paradigm is beginning to shift to appreciate the importance of adequate VC concentrations for optimal health as well as the necessity for a more comprehensive understanding of how VC impacts normal physiology (138). To date, we have determined VC's antioxidant and non-antioxidant roles in processes that vary widely from the integrity of tissue structure (collagen), to regulation of DNA function and transcription, and to the essential defense from ROS and RNS in virtually every cell

type. In this document I have initiated an investigation of a mechanism that couples the regulation of VC transport with motor coordination and proper neuronal function.

COMBINED VITAMIN C AND E DEFICIENCY ALONG WITH DECREASED SVCT2 EXPRESSION CAUSES MOTOR COORDINATION DEFECTS

First, it is debatable if the RDA of VC is sufficient for optimal health. Although fully developed scurvy is not as prevalent in the US, VC deficiency and "marginal status," defined as less than (20%) and (10%) of RDA value, respectively, is a condition that effects up to 30% of the US population (113;139). To further complicate the problem, populations that are deficient in VC are also likely to have a diet that is deficient in other vitamins, low on fruits vegetables and high in fat and sucrose. These caveats are known to influence the bioavailability and subsequently the regulation of VC. In order to elucidate how these conditions of low VC levels affect neuronal health it is important to understand how VC is regulated in the brain.

The role of VC as an antioxidant has received more attention than the other biological functions of VC. Oxidative stress has been implicated in many neurodegenerative diseases. However, the mechanisms of how dietary deficiencies of antioxidant contribute to pathology of these diseases are not fully understood. Therefore, in specific aim 1, we initially evaluated if oxidative stress due to combined VC and VE deficiency had a neurobehavioral phenotype. A pilot study was conducted to determine the optimal parameters for adapting the well-known guinea pig model to mice. We employed the use the $Gulo^{-/-}$ mouse model because of its physiologic relevance, as these mice, like humans, were dependent on dietary VC. Results from the pilot study indicated the optimal time to assess behavior was after 4 months of VE deprivation followed by 3 weeks of VC deprivation. At this time, mice are asymptomatic because scurvy has not fully developed

despite the 80% and 75% decrease in VE and VC respectively. These conditions were used in subsequent studies to determine the individual contribution of each vitamin deficiency and also the effect of the combined vitamin deficiencies to oxidative stress.

In a second study, we expanded the biochemical and behavioral assessment of the experimental mice to include VC, VE, GSH and MDA in cortex, cerebellum and liver tissues as well and anxiety test. We concluded that the combined vitamin deficiency resulted in only a modest increase of oxidative stress, particularly lipid-peroxidation. However, as observed in our pilot study, there were no major alterations in behavior. It was not until SVCT2 protein expression was decreased that a more extensive motor skills deficit was observed. The model using Gulo$^{-/-}$/SVTC2$^{+/-}$ was intended to further decrease cellular VC by limiting uptake of the vitamin. However, this was not observed, as overall VC concentrations in the cortex and cerebellum were not drastically different. Furthermore, oxidative stress, as measured by MDA, was not markedly increased. Nonetheless, the combined vitamin deficiency produced a profound effect on the neurologic phenotype in these mice that included motor-coordination defects.

Therefore, in regard to our original question of whether there is a neurological phenotype that is associated with oxidative stress and/ or VC deficiency, our results show that a 16-week deficiency of VE modestly increased oxidative stress markers, but did not affect mouse motor skills or coordination. VC deficiency in addition to VE deficiency only minimally increased oxidative stress markers compared to the single deficiency model. However, VC deficiency alone did begin to impair motor and coordination skills, deficits in which become more evident when the cellular uptake of VC is decreased. The mechanism does not appear to be due to an increase in lipid peroxidation and remains to be determined.

Possible causes of neurobehavioral changes in cellular vitamin C deficiency.

Acute generalized (whole body) scurvy

Although the mice in this study had no overt physical signs of scurvy beyond weight loss, it is clear that cellular deficiency of VC due to partial loss of the SVCT2 in the gulo$^{-/-}$/SVCT2$^{+/-}$ mice hastened deficits in motor and coordination skills. The latter could be early manifestations of acute or latent scurvy characterized by fatigue, dull aches and irritability. This may explain poor performance on behavioral tasks. Therefore, even if the deficits are due to latent scurvy, this provides evidence that VC deficiencies associated with the earliest stages of scurvy could have cognitive or neurobehavioral effects. In the human population, symptoms of acute scurvy are similar, but there are very few studies that look at the effect of low VC levels on motor-coordination (2;140). Indeed scurvy does cause severe lassitude (lack of physical or mental energy) and asthenia (weakness) in humans, however the terminal symptomatology of scurvy is mostly due to vessel and organ rupture because of collagen defects rather than neurological deficits. This is likely because VC is retained in the brain even under severe VC depletion (78;141). As noted earlier, such retention in the brain may suggest important neural function. Although the disease has been associated with paraparesis (partial paralysis of the lower limbs) in humans, death appears to be due more to complications of systemic collagen dysfunction and not to a clear neurologic syndrome (44).

Imbalance in energy homeostasis

Kirkwood, et al. (118) used the zebrafish model to investigate the role of VC in energy homeostasis. This model is useful, as it does not require genetic manipulation because zebrafish, like humans, cannot make VC. They concluded that whole-body VC deficiency in zebrafish altered cellular energy-metabolism homeostasis by activation of the purine nucleotide cycle. Their studies showed that VC decreased adenosine monophosphate (AMP) levels by elevating

AMP deaminase (AMPD) activity, however it is unclear if recruitment of the AMP receptor is altered. This suggests that VC deficiency may lead to inadequate energy production, which could also contribute to the behavioral deficits observed in our studies in specific aim 1. In a follow-up (142) study by the same group, it was shown that VE deficiency caused an increase in oxidative stress that led to depletion of VC. Subsequent damage to the muscle fibers resulted in impaired muscle function. While we do not think that the oxidative stress in the combined VC and VE deficient Gulo$^{-/-}$ mouse model was severe enough to cause extensive muscle damage in the short time-span of our experiments, further studies will have to be performed to determine the role of VC in energy homeostasis, Furthermore, any effects of VC on the activity of AMPK should be investigated, as it may have a broader impact on cellular energy metabolism.

DYSREGULATION OF VITAMIN C UPTAKE VIA ALTERED NEURONAL SVCT2 EXPRESSION IMPAIRS NEURONAL FUNCTION.

In the studies for specific aim 1, it appeared that regulation of VC uptake, not necessarily VC concentration alone, had a substantial contribution to behavior. This was evidenced by the fact that the Gulo$^{-/-}$/SVCT2$^{+/+}$ group and the Gulo$^{-/-}$/SVCT2$^{+/-}$ group on the same combined VC- and VE-deficient diet had decidedly different behavioral phenotypes, despite the fact that VC concentrations in cortex or cerebellum tissue were not significantly different. There is a growing body of evidence that suggests that not only VC concentration matters, but also local, acute transport of VC in and out of neurons affects their function (82). Limitations of the studies performed in specific aim 1 include not being able to determine how VC is compartmentalized within the brain tissues. Thus, localized severe VC deficiencies in crucial brain areas or even cells (e.g., Purkinje cerebellar neurons) could have caused the motor and behavioral changes observed. This caveat should also be considered at the sub-cellular level for what is arguably the

most important region for neural signal propagation, the nerve terminal. Decreases in synaptic VC transport by the SVCT2 protein could lead to acute impaired neuronal function. Evidence for this possibility derives from functional SVCT2 and electrophysiology studies in hippocampal neuronal culture. Qiu et al. in the May lab (120) showed that neurons from SVCT2$^{-/-}$ mice displayed miniature excitatory post-synaptic currents (mEPSC) that were smaller in amplitude and occurred less frequently compared to wild-type neurons, suggesting that SVCT2 activity is required for normal glutamatergic function. This paralleled a decrease in the number of dendritic branches. Furthermore, it was observed that SVCT2$^{-/-}$ neurons expressed fewer GluR1 clusters, indicating that the transporter is required for normal development of glutamatergic function of cultured hippocampal neurons. These changes, coupled with the aforementioned localization of the SVCT2 in the presynaptic bouton in superior cervical ganglion neurons of the peripheral nervous system, led me to investigate whether SVCT2 was expressed and functional at nerve terminals in the central nervous system (CNS) in specific aim 2.

TRANSPORT OF VITAMIN C IS TIGHTLY REGULATED AT THE NERVE TERMINAL IN THE CNS

There are no studies to date that have investigated the mechanisms of VC transport at the neuronal synapse in the CNS. Therefore, the goal of specific aim 2 was to determine how VC transport was regulated at the cortical nerve terminal. We employed the use of synaptosomes, which have long served as a model of the nerve terminal (143;144). These pinched off nerve endings are fully functional and can be used to investigate transport of molecules at the nerve terminal. It has been established that VC is transported in and out of synaptosomes (123), but how this occurred has not been fully characterized.

Although it is well established that the SVCT2 was the primary mediator of VC uptake in neurons (53), the studies described in this document were the first study to demonstrated that SVCT2 was indeed present in pre-synaptic fraction of synaptosomal preparations. With a series of uptake studies with inhibitors we were able to characterize VC uptake and conclude that it is likely mediated by SVCT2. The inhibitors sulfinpyrazone and phloretin, while not specific for SVCT2, are well-documented inhibitors of VC uptake (82). The fact that the transporter is present and functional in this specialized region of the neuron is supporting evidence for compartmentalized non-antioxidant roles of VC within the neuron.

VC efflux has been characterized in many cell types, as noted previously. However, the proteins that mediate VC transport out of most cells are still unknown. With a series of inhibitor studies in hepatocytes, Upston, et al. (84) were able to distinguish proteins that mediated DHA uptake from those that mediated VC release, but the latter were not identified. In the brain, it has long been established by the studies of Rebec and others that VC uptake and efflux is highly correlated with changes in behavior (52), For example, the level of behavioral activation in rodents is correlated with the level of striatal VC release (145). These changes were thought to be mediated by the striatal glutamatergic system (146). *In vivo* studies using voltammetric electrode recordings showed that extracellular VC signals were directly related to the amounts of microinjected glutamate. This signal was dependent on glutamate reuptake as it was eliminated in the presence of inhibitors of glutamate uptake, homocysteic acid and *d*,*l*-threo-β-hydroxy-aspartic acid (147).

At the cellular level, studies to elucidate the mechanisms of VC efflux have been carried out mainly in primary cultures of rat astrocytes. Wilson, et al. (87) hypothesized that glutamate uptake triggered the release of VC. Using primary cultures of rat cerebral astrocytes they observed that extracellular glutamate increased VC efflux. Hypertonic media inhibited this efflux.

Wilson's group did not find evidence that glutamate-ascorbate heteroexchange mechanism was involved, since levels of intracellular VC did not affect glutamate uptake. They proposed rather that the tonicity of the extracellular fluid was one determinant of VC efflux. The anion channel inhibitors 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) inhibited both Na^+-dependent glutamate uptake and VC efflux. These studies were able to distinguish between mediating proteins as two other inhibitors of volume-sensitive organic anion channels (1,9-dideoxyforskolin and 5-nitro-2-(3-phenylpropylamino) benzoic acid) (NPPB) did not slow glutamate uptake but prevented stimulation of VC efflux (87). This prompted them to propose that glutamate causes cell swelling, which then allows VC efflux on volume-sensitive or –regulated anion channels.

Using these experiments as a guide, I hypothesized that a similar mechanism could mediate efflux in synaptosomes. Our studies showed that indeed spontaneous and glutamate-induced VC efflux occurred in synaptosomes. In our spontaneous studies we observed that net VC transport was a combination of uptake and efflux. SVCT2 inhibition had a greater concentration-dependence on apparent VC efflux then we originally expected, as a drastic decrease in VC content was observed. While the mechanism behind this type of efflux was not fully characterized, the inhibitor study showed that SVCT2 largely mediates uptake and does not function bi-directionally. Furthermore, VRACs may not contribute to spontaneous efflux, as incubation with DIDS did not affect VC concentrations during the time course experiments.

My studies of glutamate-induced VC efflux suggest that VRACs may mediate efflux as DIDS did inhibit decreases in VC levels. Furthermore, this effect was recapitulated with hypotonic solutions without glutamate. However, future studies should include the use of additional inhibitors to gain a clearer understanding of the types of channels involved in efflux. For example, the inhibitor IAA-94 targets different chloride ion channels (CLIC) that may contribute to VC efflux. Synaptosomes appeared to be more sensitive to the use of inhibitor solvents such

as DMSO and ethanol as even low concentrations seem to lyse the synaptosomes and preclude uptake studies with NPPB and niflumic acid, which target ClC-1 and ClC-2, respectively. The next steps for the continuation of the work will include inhibitor studies with 1,9-dideoxyforskolin as well as studies leading to identification of VRAC proteins involved. Currently, none of the presumed VRACs have been identified and therefore there is no antibody known to be specific for these channels. Once identified, it will be necessary to determine using siRNA and knockout experiments whether the channel(s) in question in fact mediate VC efflux and under what conditions, including changes in osmolality and glutamate stimulation.

What implications does this research have for in vivo models?

 The fact that spontaneous VC efflux appeared to level off after ~50% decrease in VC suggests that intracellular VC levels may regulate its transport. *In vivo* models have shown changes in SVCT2 protein levels in response to intracellular VC concentration in brain tissue (77). In an attempt to determine whether and how intracellular VC levels may alter SVCT2 protein expression at the nerve terminal, I compared SVCT2 expression in enriched synaptosome fractions prepared from Gulo$^{-/-}$ mice on VC deficient diet with that of Gulo$^{-/-}$ that were supplemented with VC. However, there was no change in protein levels in any of the fractions. Furthermore, I was not able to detect a consist increase in VC uptake in crude synaptosome preparations from these mice. Although, I did not observe an effect of intracellular VC concentration by assessing SVCT2 uptake in Gulo$^{-/-}$ mice with or without VC supplementations, an interesting assessment would be the comparison of VC uptake in synaptosomes prepared from mice with different protein expression levels of SVCT2 (SVCT2$^{+/-}$ and SVCT2-Transgenic).
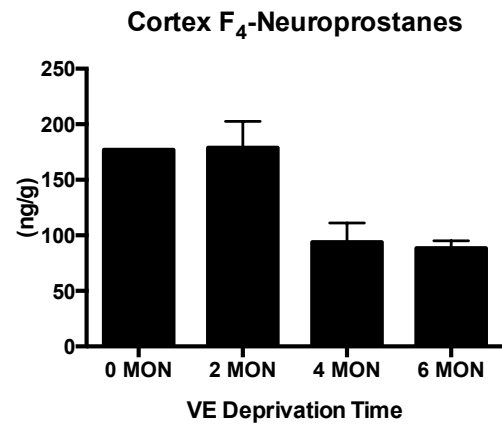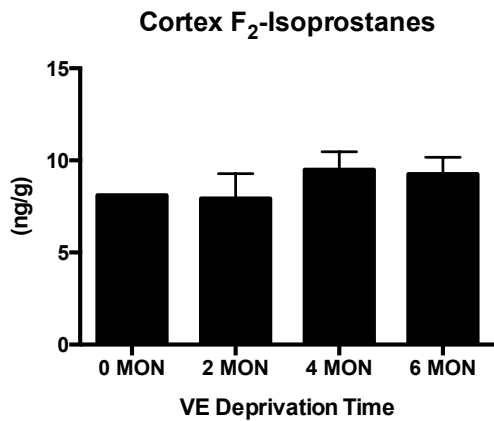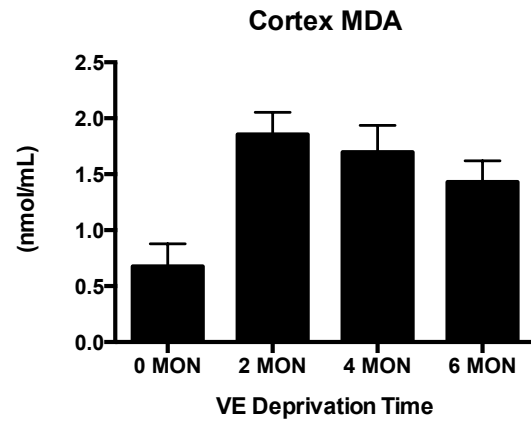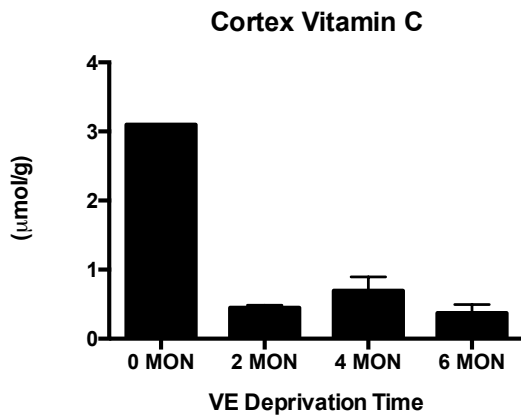
This research can impact human health by providing insight on how VC is regulated in the brain, particularly the cortical neuron. Understanding how this antioxidant's contributes to the bigger

picture of brain health and function is crucial for the advancement of therapeutic options that can combat specific forms of oxidative stress that are implicated in neurodegeneration.

APPENDIX

PILOT STUDY BIOCHEMICAL AND BEHAVIOR ASSESMENT

Wildtype (Gulo$^{+/+}$; SVCT2$^{+/+}$) mice were subject to 0, 2, 4 or 6 months of vitmain E deprivation followed by 3 weeks of vitamin C deprivation.

Cortex Vitamin C

Cortex MDA

Cortex F$_2$-Isoprostanes

Cortex F$_4$-Neuroprostanes

Liver Vitamin E

Liver Isoprostanes

Weight 2 MON

Weight 4 MON

Weight 6 MON

Ambulatory Distance

Ambulatory Velocity

Inverted Screen

Gait
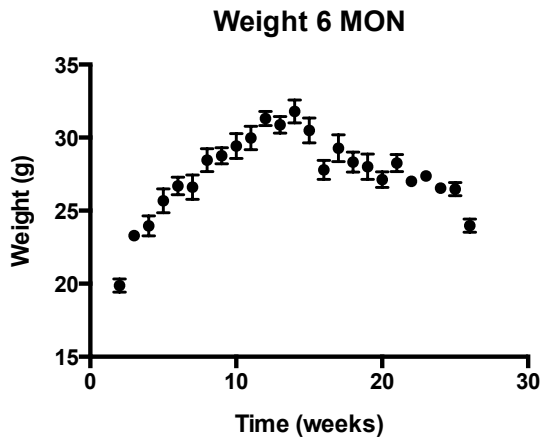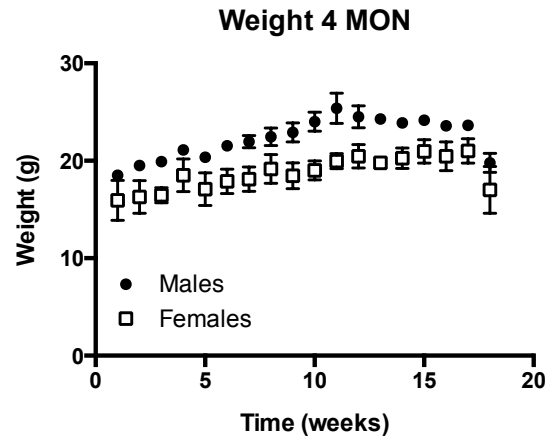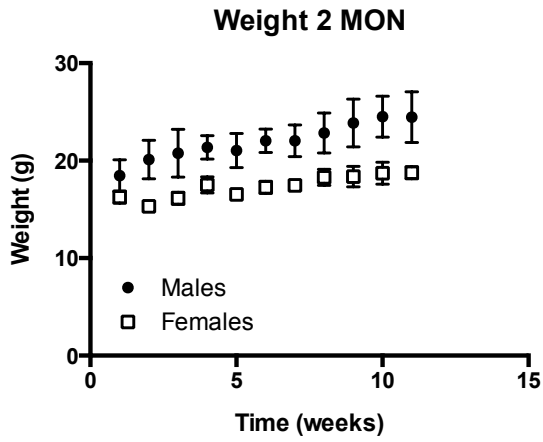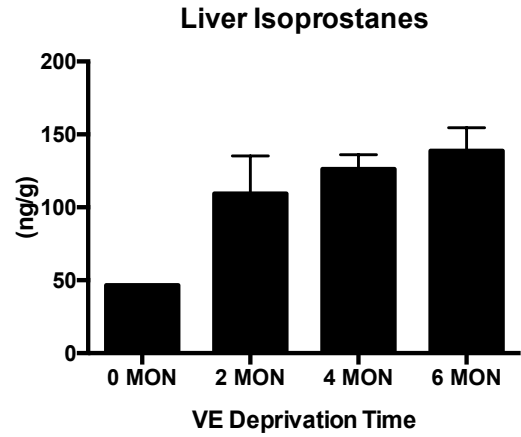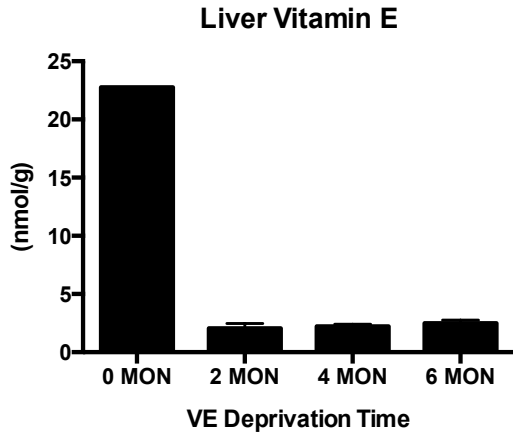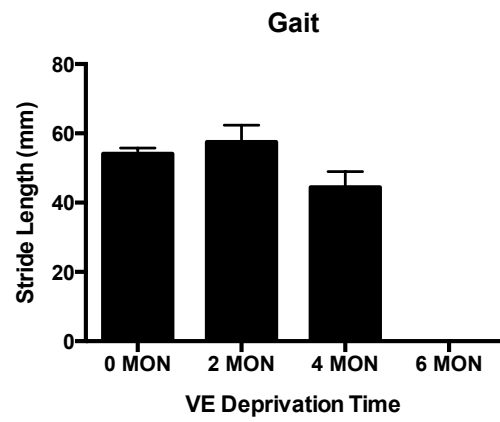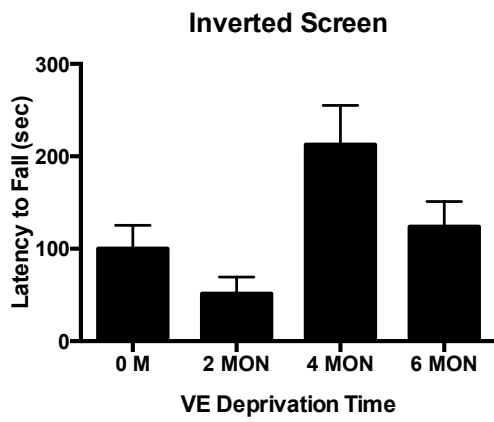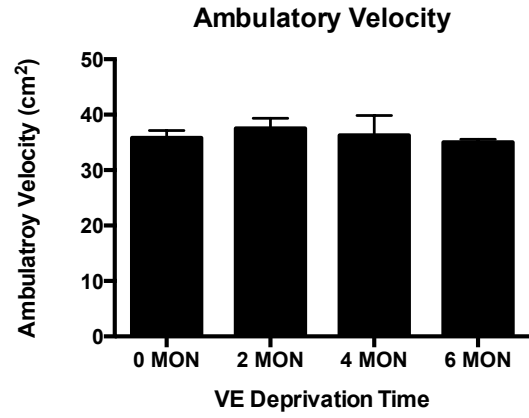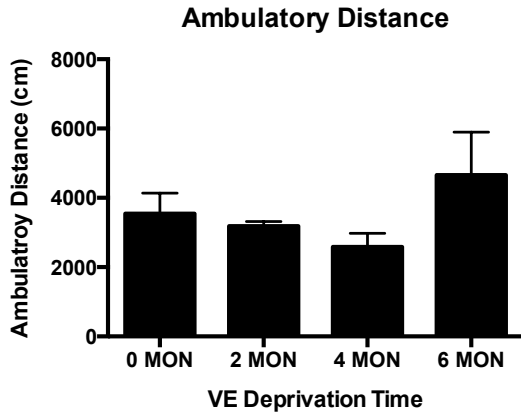
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