Multiple roles for Ascorbic Acid in the Brain: Transporter Regulation, Neurotransmitter Synthesis, and Brain Endothelial Cell Stability

By

Martha Elizabeth Meredith

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR of PHILOSOPHY

in

Molecular Physiology and Biophysics

May, 2014

Nashville, TN

Approved:

Roger Colbran, PhD

Eric Delpire, PhD

Kate Ellacott, PhD

Owen McGuinness, PhD

Michael Aschner, PhD

ABSTRACT

The roles for ascorbic acid (ASC) in regulation of its transporter, in neurotransmitter synthesis, and in brain endothelial cell barrier permeability were investigated in the brain. First, during mouse development, levels of the main transporter for ASC, the sodium-dependent vitamin C transporter 2 (SVCT2), were low throughout gestation and increased substantially postnatally. This was inversely associated with ASC levels in brain, in which ASC content increased just before birth and decreased postnatally. In adults, low cortex ASC did not affect the SVCT2 level. Second, neurotransmitter levels were increased or decreased in conjunction with altered ASC concentrations in embryos. Dopamine and norepinephrine were decreased in embryos with low levels of cortex ASC, while protein levels of the primary enzyme responsible for synthesis, tyrosine hydroxylase, were decreased. Dopamine and serotonin were increased in embryos with excess ASC. Third, intracellular ASC significantly decreased brain endothelial cell permeability. Culture of endothelial cells in high glucose (25 mM) and activation of the receptor for advanced glycation end-products increased endothelial permeability, an effect that was prevented by intracellular ASC. ASC decreased endothelial permeability under basal glucose conditions by acting, at least in part, through Epac1 and cytoskeleton rearrangement. In all, the data presented here shows the importance of ASC in the brain, especially in the context of development, neurotransmitter synthesis, and brain endothelial cell stability.

Approved by: James M. May, MD

For my family, who have supported me in everything I have pursued.

For my parents, who have always encouraged me to do my best

and take advantage of every opportunity.

For my brothers, Taylor and Patrick, who have always shown me respect

and thus, taught me how to respect others.

For my grandmothers, Nana and Memaw, who have shown me how to be

a grounded, confident woman.

ACKNOWLEDGEMENTS

I am very lucky to have been a part of the nationally-ranked MPB department. This department is truly unlike others. The collaboration and willingness to help in any form or fashion is above and beyond. From the faculty, to the staff, to the students, to the post-docs, everyone has been nothing but generous with reagents, equipment, or advice. There are too many people to thank individually. However, Angie Pernell, Alyssa Hasty, and Chuck Cobb were instrumental in my time as a graduate student.

I would be remiss if I did not mention my boyfriend, Jon, while writing this dissertation. He was always willing to hear about my experiments or pop into lab with me on a quick weekend visit to treat cells. He enjoyed putting on gloves and "playing" science. Mostly though, he provided a distraction from the all-encompassing work to be done. He provided me the opportunities to clear my mind and get out in the woods, allowing me to come back Monday morning with a fresh outlook on the research. Without him, the work weeks would not have gone by as quickly.

My family has been very supportive while I have been in graduate school. I have worked hard (in many aspects of my life), to make them proud. It may have taken them a while to learn what my degree would be in, but after five and a half years, I think they have it. My parents have always been interested in how I have developed as a scientist, whether it was from the publications, or attendance at scientific conferences, or experimental designs. My brothers are happy to know I am finally finished with school after prolonging my life as a student for another 6 years. My aunts, uncles, cousins, and grandmothers have always been interested in my research and I look forward to sharing the different phases with them for years to come.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

5-HIAA: 5-Hydroxyindoleacetic acid

AFR: ascorbate free radical

AGE-BSA: advanced glycation endproduct- bovine serum albumin

AJ: adherens junction

ASC: ascorbic acid, ascorbate

BBB: blood-brain barrier

BEC: brain endothelial cell

BH_4: tetrahydrobiopterin

CDC: Center for Disease Control

DHA: dehydroascorbate

DOPAC: 3,4-Dihydroxyphenylacetic acid

E: embryonic day

eNOS: endothelial nitric oxide synthase

Epac: exchange protein directly activated by cAMP

FITC: Fluorescein isothiocyanate

GLUT: glucose transporter

Gulo: L-gulonolactone oxidase

HMGB1: high-mobility group protein B1

MAP: microtubule associating protein

MDA: malondialdehyde

MMP: matrix metalloproteinase

NE: norepinephrine

ODS: Osteogenic Disorder Shionogi

P: postnatal day

PKA: protein kinase A

PKC: protein kinase C

RAGE: receptor for advanced glycation endproducts

SCOP MBR: scopolamine methyl bromide

SMP30/GNL: senescence marker protein 30/gluconolactonase

SNP: single nucleotide polymorphism

STD: standard

STZ: streptozotocin

SVCT: sodium-dependent vitamin C transporter

SVCT2-TG: sodium-dependent vitamin C transporter 2- transgenic

TH: tyrosine hydroxylase

TJ: tight junction

TPH: tryptophan hydroxylase

VEH: vehicle

WT: wild-type

WTR: water

ZO-1: zona-occludin 1

CHAPTER I

INTRODUCTION

History of Vitamin C

The discovery of vitamin C, is a fairly recent one in comparison to other vital nutrients; it having only been isolated in the 1920s by Albert Szent-Györgyi [1]. However, foodstuffs containing the vitamin had long been used before to "cure" sailors of a disease that reduced them to their beds, and caused loose teeth, bloody gums, depression, and general weakness. Captains such as Magellan and Vasco da Gama lost 70-90% of their crews on long voyages due to the malady [2]. This disease was later separated from other vitamin deficiencies and given the name scurvy. The word scurvy originates from the Latin word *scorbutus*, derived from the Slavonic word *scorb*, which simply means "disease" [3]. Jacques Cartier, in 1535, was the first explorer to note that the sailors who ingested citrus fruits did not get this disease which was noted to have "killed more sailors than storms, shipwrecks, and combat combined during the 18th century" [4]. Cartier had also learned from the local Indians on his travels that the leaves and buds of the Thuja tree, also known as arborvitae, contained a "magical substance" that would bring his sickly sailors back to the strength the ships needed to sail and fight in combat [5]. Unfortunately, word did not travel as easily back then.

Prevention of scurvy

Nearly 200 years (and many lives) later, James Lind, a British Naval surgeon, performed what has often been viewed as one of the first controlled clinical trials, and was able to establish a link between scurvy and the diet, rather than a miasma due to foul air, food toxins, or disease. Providing sailors that had symptoms of scurvy with various available substances such as cider, vinegar, sulfuric acid, or seawater, he noted only the sailors that received oranges and lemons improved to the point that they could nurse their still suffering shipmates. Similar to Cartier's observation, Lind concluded

there must be a specific nutrient in the citrus fruits that was responsible for preventing scurvy and later published this work in 1753 in his *Treatise on the Scurvy* [6].

Isolation of ascorbic acid

Another 200 years later, the Hungarian physiologist, Albert Szent-Györgyi, first isolated the vitamin from the cattle adrenal gland in 1928 and named it hexuronic acid based on the composition [1]. The recognition of who actually discovered vitamin C was a controversy. An American post-doc, Svirbely, had been working on isolating vitamin C in Dr. Charles Glenn King's lab when he joined Szent-Györgyi's lab. Szent-Györgyi allowed him to compare hexuronic acid to the compound Svirbely had been previously investigating. Svirbely wrote to his former mentor to tell him the compounds were the same and that they were planning to submit the results to *Nature.* However, delighted by this finding, King wrote up his work and submitted it to *Science*, which credited King as the discoverer of vitamin C. Szent-Györgyi still submitted his report to *Nature* challenging King's priority in the discovery of vitamin C. The Americans sided with King and his 5-years' worth of work on the compound, while the Europeans sided with Szent-Györgyi, as they knew more about his long-time work with this compound. In the end, recognition was given to Szent-Györgyi, and he received the Nobel Prize in Physiology or Medicine in 1937 for his contribution in the isolation and discovery of vitamin C. Vitamin C was later given the chemical name ascorbic acid (ASC), based on the Latin meaning "without scurvy." When discussing this nutrient in clinical terms, it is referred to as a vitamin, due to the external requirement for life. However, when discussing the roles for the nutrient within the body, it is typically described in by its chemical nature, ASC. These nomenclatures will be used throughout the dissertation.

ASC Metabolism and Roles

Unlike most other mammals, humans have lost the ability to synthesize their own ASC. This is due to a mutation in the gene responsible for the last step in converting *D*-glucose to ASC, L-*gulono-1,4-lactone oxidase* [7]. Therefore, humans, primates, and guinea pigs depend on the diet to obtain enough of the vitamin to prevent scurvy and maintain general health. The recommended daily allowance for healthy adults is 75-90

mg/day [8], but this amount is currently debated amongst the ASC community as to whether or not it is adequate, as will be discussed later. Guinea pigs have long been the animal of choice for the study of vitamin C *in vivo*, since, like humans, they are unable to synthesize the vitamin. Although rats and mice are more useful in laboratory studies due to genetic manipulation, they are able to synthesize their own ASC in the liver. There have, however, been genetic models created or discovered to study the roles of ASC throughout the body in rodents. These models include the Osteogenic Disorder Shionogi (ODS) rat which has a spontaneous mutation preventing the synthesis of ASC [9], the SMP30/GNL mouse that lacks another key enzyme in the ASC biosynthetic and other pathways (gulonolactonase) [10], the ASC transporter deficient mouse (SVCT1(-/-) and SVCT2(-/-)) [11, 12], and a mouse that overexpresses the ASC transporter (SVCT2-transgenic mice) [13]. However, the most common model used that best mimics the human situation involves genetic mutation of *gulonolactone oxidase* in mice (Gulo(-/-) mice), rendering them dependent on dietary ASC [14]. The synthesis of ASC in the liver of most mammals is depicted in Figure 1.1. Also, the targeted proteins responsible for creating the various ASC-models are shown.
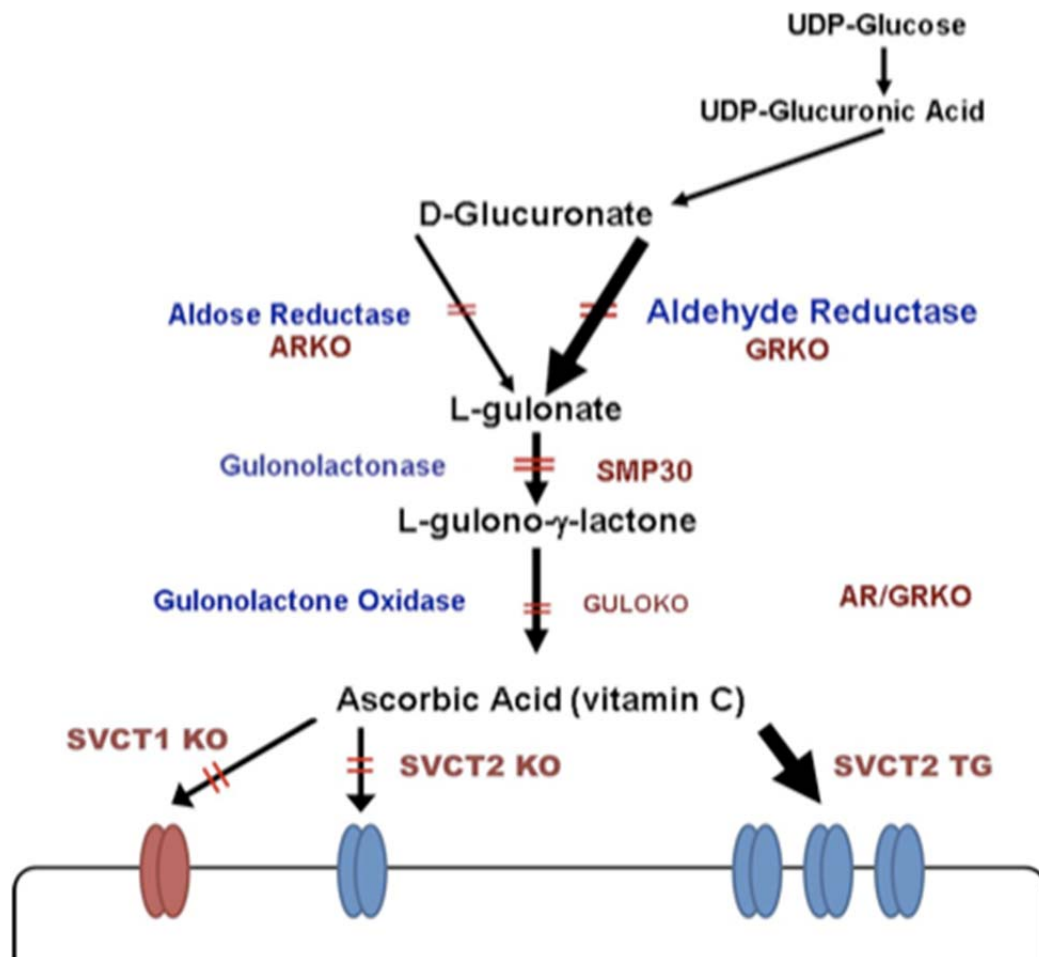
Figure 1.1. Biosynthesis of ASC and genetic models used to study effects of ASC *in vivo*. Modified from Gabbay *et al*. J Biol Chem. 2010 (285) 19510-19520 [15]. Synthesis of ASC occurs in the liver of most mammals. Common genetic models are shown in red while the targeted enzyme is in blue.

Chemistry of ASC

ASC is most well-known for its role as a water-soluble antioxidant. It has an available electron to donate to free radicals and reactive oxygen species, sequestering them and maintaining the redox state of the cell, concomitantly converting ASC into the ASC free radical [16] (Figure 1.2). The ASC free radical can be reduced back to ASC by NAD(P)H-dependent reductases, which have an affinity for the ASC free radical in the low millimolar range [17]. If NAD(P)H reductase activity is inadequate to reduce the ASC free radical back to ASC, the free radical dismutates to form one molecule of ASC and one molecule of the fully oxidized version of ASC, dehydroascorbate (DHA) [18] (Figure 1.2). Unlike the ASC free radical, DHA can be recycled back to ASC by a variety of reductases including a putative DHA reductase, NADPH-dependent reductases, glutathione-dependent thiol transferases, or glutathione [17]. The half-life of DHA is very short, approximately 6 min under physiologic conditions [19, 20]. Therefore, it is either quickly reduced back to ASC or degraded to 2,3-diketo-1-gulonic acid. Due to this rapid degradation, multiple reduction systems are present to prevent this from happening. The wide range of reduction mechanisms for ASC in preventing oxidative stress permits intracellular levels of ASC to be considered a marker of the redox state of a cell.
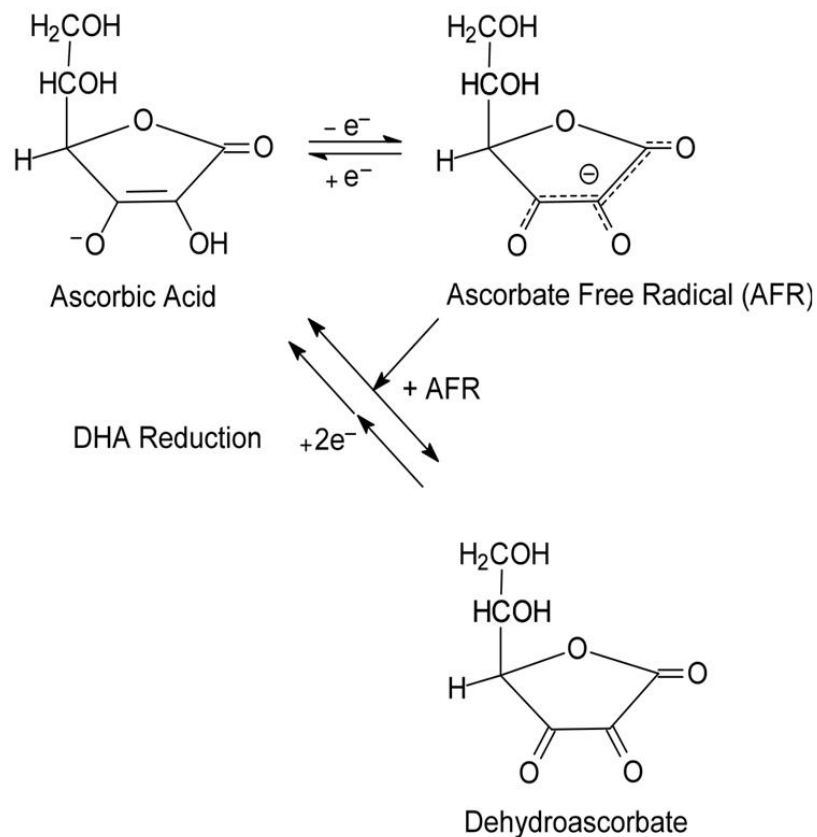
Figure 1.2. Chemistry of ASC. Modified from Harrison and May. Free Radic Biol Med. 45 (2009) 719-730 [21]. ASC donates one electron to produce the ascorbate free radical (AFR). Most commonly, the AFR is reduced back to ASC through various reductases. However, two AFRs can dismutate to form one molecule of ASC and one molecule of the fully oxidized form, dehydroascorbate (DHA). DHA can be recycled back to ASC only if enough reductases are present.

Co-factor role of ASC

In addition to its role as an antioxidant, ASC is a co-factor in multiple enzymatic reactions, where it maintains catalytic transition metals such as iron in their reduced state. Examples include procollagen hydroxylation, nucleic acid demethylation (ten-eleven translocation dioxygenase) [22], histone demethylation (Jumonji demethylase) [23], peptide hormone amidation, and carnitine synthesis [24, 25]. It also aids in the hydroxylation of HIF-1α, which targets it for destruction, altering the response of a cell to hypoxia [26]. Because oxidative stress triggers multiple signaling pathways in cells and because of the role ASC plays in other enzymatic processes throughout the cell, ASC can be considered an important regulator of intracellular signaling processes, many of which have yet to be fully investigated.

Another important co-factor role for ASC that is part of the focus of this dissertation is its role in neurotransmitter synthesis. In the brain and adrenals, ASC is known to enhance two steps of catecholamine synthesis (Figure 1.3). The first is through an indirect mechanism to recycle tetrahydrobiopterin [27-29], which is required for the activation of the rate-limiting enzyme in catecholamine synthesis, tyrosine hydroxylase. Similarly, tetrahydrobiopterin is required for tryptophan hydroxylase activity in the synthesis of serotonin. In the second mechanism, ASC directly donates an electron to dopamine β-hydroxylase [29] for the conversion of dopamine into norepinephrine.

Regulation of these specific neurotransmitters by ASC could have important implications in the functions affected by release of dopamine, norepinephrine, and serotonin. Neurotransmitters aid in relaying a signal from cell-to-cell within the brain, allowing for transmission to the rest of the body. Catecholamines in particular are derived from the essential amino acid tyrosine. Dopamine is produced in the neuronal cell bodies primarily located in the substantia nigra and ventral tegmental area. Dopamine can then be converted into norepinephrine by dopamine β-hydroxylase or degraded by monoamine oxidases and catechol-*O*-methyltransferases. Dopamine is involved in regulating motor functions, mood, and plays a critical role in the reward pathway. Norepinephrine is critical for alertness and also plays a part in reward.

7

Serotonin, on the other hand, is also a monoamine but is derived from tryptophan instead of tyrosine. Serotonin is most well-known for its roles in regulating mood, sleep, and appetite. It is also potentially involved in cognitive functions including learning and memory. Although serotonin is primarily produced in the periphery, the raphe nucleus is the principal source of serotonin in the brain. Serotonin is also degraded by monoamine oxidases.

The ability of ASC to act as a co-factor for tyrosine and tryptophan hydroxylase, as well as dopamine β-hydroxylase, in the synthesis of neurotransmitters, could indicate that ASC regulates these enzymes. In fact, treatment of ASC-deficient neuroblastoma cells with ASC increased tyrosine hydroxylase mRNA [30] and protein [31] levels. The latter is the rate-limiting step in catecholamine synthesis. The May laboratory has previously shown that ASC treatment increases norepinephrine production within six hours in a neuroblastoma SH-SY5Y cell line [31]. In addition, this effect was specific for ASC, since it was not mimicked by several other antioxidants.
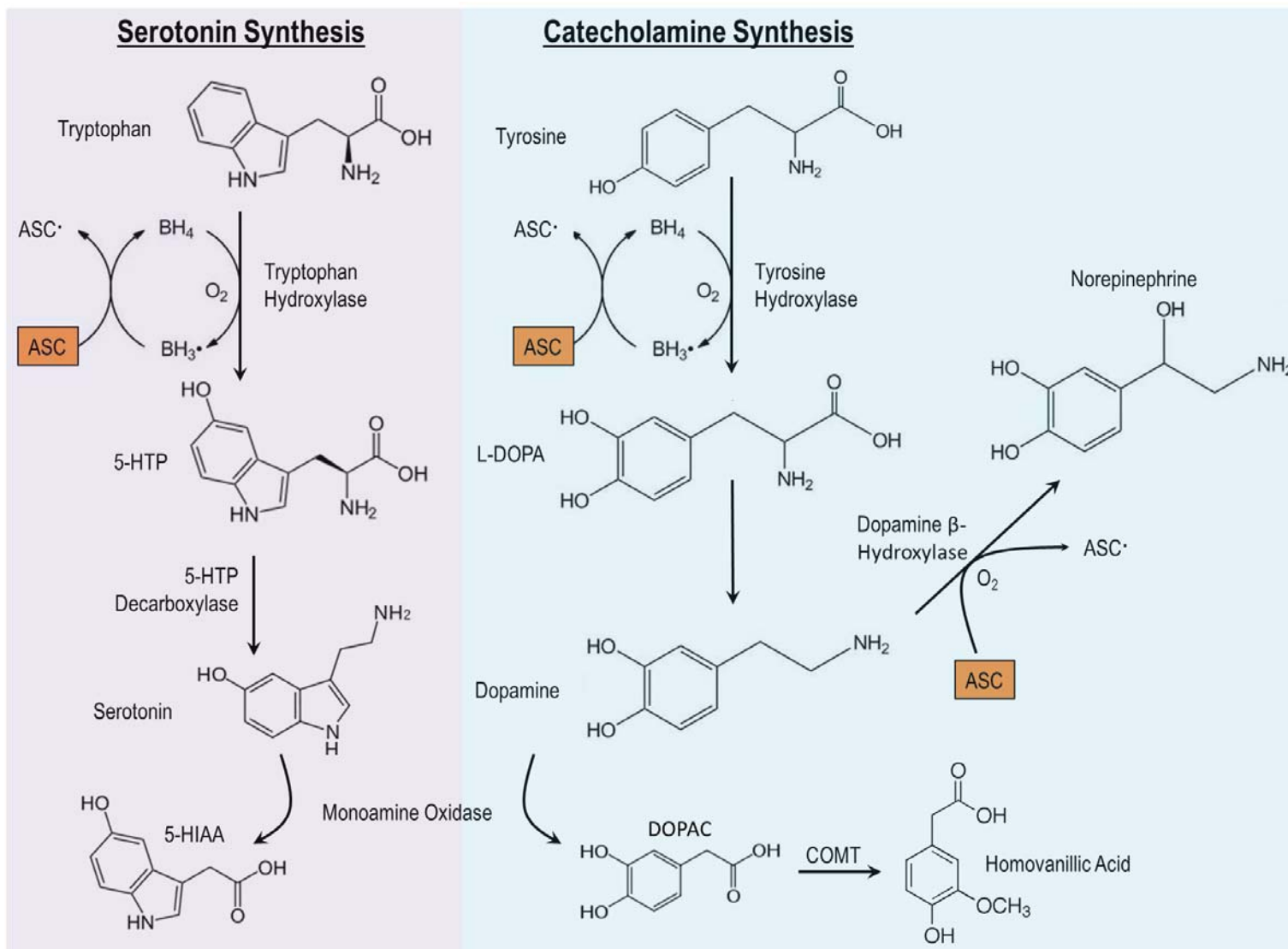
Figure 1.3. ASC regulation of neurotransmitter synthesis. Modified from Meredith and May. Brain Res. 1539 (2013) 7-14 [32]. ASC helps recycle tetrahydrobiopterin (BH_4), a co-factor for tryptophan and tyrosine hydroxylase. ASC also directly acts as a cofactor for dopamine β-hydroxylase to convert dopamine to norepinephrine.

Tissue Concentrations of ASC

The importance of the vitamin throughout the body is highlighted by the differential concentrations in various tissues. The highest ASC concentrations are found in neural-derived tissues and in the brain [21]. Peripheral cells and tissues contain varying amounts of the vitamin: from 40-50 µM in erythrocytes, to 0.4 mM in the muscle to 1 mM in the liver [21]. The adrenal and brain contain the highest levels of ASC, present in concentrations up to 2-10 mM. In addition, the brain is able to sequester and store ASC the longest during deficiency, unlike most other tissues, which show decreases in ASC levels shortly after whole body ASC depletion [33]. Indeed, Lind noted during his voyages that the brains from sailors that had been ravaged by scurvy were still fully intact [6]. The body is an efficient machine and functions in a certain way for a reason. The evidence that the brain has the ability to retain ASC even after the rest of the body is sorely depleted suggests a great need for it in this vital organ.

Low millimolar concentrations of intracellular ASC, much greater than those in the plasma, are critical for its role as an antioxidant [34] as well as a co-factor for dioxygenase enzymes [35]. An efficient recycling system, as mentioned already, helps to maintain high levels of intracellular ASC. In addition, the main transporter for vitamin C, the sodium dependent vitamin C transporter (SVCT), transports ASC in a high affinity sodium- and energy-dependent mechanism to transport ASC against a concentration gradient.

ASC Transport

Transport of DHA through glucose transporters

The oxidized form of ASC, DHA, is transported by glucose transporters. Because DHA levels are typically low in blood, and DHA must compete with glucose for transport, this route of entry into the brain is not considered a primary form of transport. In the brain it is thought that neurons release DHA, which is then taken up by the neighboring astrocytes via glucose transporter 1 (GLUT1) [36]. Astrocytes contain several reductases that are able to recycle DHA back to ASC. These may retain it or possibly release it back into the cerebrospinal fluid allowing for continual uptake by the neurons

as needed. Neurons contain nearly ten times greater ASC than their neighboring astrocytes [37].

Transport of ASC through sodium-dependent transporters

In order to build up and maintain concentrated stores of ASC throughout the body, there must be an energy-dependent transport system in place. ASC is actively transported via two isoforms of the SVCT [38]. The SVCT was first discovered and cloned in 1999 [39] and is part of the solute carrier family, specifically SLC23A. Mouse SVCT1 is a 605 amino acid transporter with 65% amino acid identity to SVCT2, which is 647 amino acids [39-41]. Human SVCT2 shares 95% homology to the rat SVCT2 [42]. Both transporters have similar hydropathy profiles with 12 transmembrane domains each and the N- and C-termini are located on the cytoplasmic side. The transporters are pH sensitive, working in an optimal pH of 5.5 compared to 7.5.

The SVCT1 is primarily responsible for whole body homeostasis ASC. It accomplishes this by mediating ASC absorption in the intestines and resorption in the kidney [39]. The SVCT2 is primarily responsible for wide-spread uptake by the tissues and is present in metabolically active and specialized cells [43]. SVCT2 is the only isoform present in the brain and is the primary transport system for ASC into this organ.

There is evidence that the SVCT2 may undergo regulation via conformational changes and post-translational modifications. The extracellular loop between the 7th and 8th transmembrane domains contains a series of proline residues, which are required for structure stability and transport efficiency [44]. There are glycosylation sites on the 2nd and 3rd extracellular loops and several protein kinase C consensus sites throughout the protein [39]. Changes in glycosylation could also affect transporter function [45]. Further, it has been suggested the phosphorylation of SVCT2 alters the transport activity by altering the protein conformation [46].

ASC Storage in the Brain

Although not basally expressed in the endothelial cells lining the blood vessels throughout the brain, SVCT2 is highly concentrated in the epithelial cells lining the choroid plexus. This organ provides the first of a two-step mechanism of brain ASC

retention. First, the SVCT2 in the choroid plexus concentrates ASC in the cerebrospinal fluid nearly 10-fold by bringing ASC in from the blood (40-60 μM) to the cerebrospinal fluid (200-400 μM) [47]. Second, ASC can be even further concentrated in neurons by nearly 20-fold (2-10 mM). SVCT2 has primarily been found to be expressed in neurons throughout the brain under basal conditions [37, 48, 49], while endothelial cells only express it under stressed conditions [50]. The 10 mM levels in neurons can be compared to glia, which only transport DHA on glucose transporters, and store approximately 1 mM ASC [37].

Expression of SVCT2

Regulation: Transcriptional regulation of SVCT2 has primarily been studied in cell cultures. ASC is the only known substrate for SVCT2 [46]. Previous studies have shown that SVCT2 expression is determined by ASC level in C2C12 myotubes [51], osteoblasts [52], astrocytes [53], and brain endothelial cells (BECs) [54]. Indeed, it has been suggested SVCT2 is up-regulated during differentiation in myotubes [55]. The potential regulation of SVCT2 by differentiation of different cell types could be important during development. However, studies investigating the regulation of SVCT2 *in vivo* are limited.

Localization: The expression of SVCT2 throughout the body relates to the function of ASC in those various cell types or tissues. SVCT2 is primarily expressed in the brain, eye and adrenal [39, 42]. For example, ASC is thought to be critical in protecting the eye from radiation-induced damage [56]. Therefore, SVCT2 is abundantly expressed in the pigmented epithelium of the ciliary body and corneal epithelium. This distribution is reflective of the levels of ASC in these specific areas within the eye [57]. Another example includes the role for ASC in catecholamine synthesis. SVCT2 is abundantly expressed in the adrenal, where in the medulla ASC acts as a co-factor for dopamine β-hydroxylase and is highly concentrated.

Mun *et al* has described the distribution of SVCT2 throughout the brain [45]. Although the levels do not completely agree with the reports of ASC distribution throughout the brain, SVCT2 levels measured by immunohistochemistry are greatest in the hippocampus and frontal cortex with lowest levels in the pons and thalamus [45]. *In*

situ hybridization and immunohistochemical studies also show SVCT2 is localized to neurons, likely causing the great abundance of ASC in this cell type [39, 45]. In addition, the hippocampus and cerebral cortex have been shown to produce high levels of free radicals even under physiological conditions [58]. This supports the increased need for ASC in these specific brain regions to keep oxidative stress levels low. Consistent with levels of ASC in neurons versus glia, *in situ* hybridization of SVCT2 in the rat brain shows SVCT2 is localized to neurons in high concentrations but not in glia [39].

Brain region differences in ASC

Within the brain, there are regional differences in ASC levels, reflecting the results of uptake studies of radiolabeled ASC [59-61]. The cerebral cortex, hippocampus, hypothalamus, and amygdala have consistently shown higher concentrations of ASC [33, 62]. Even when the level of ASC is expressed per cell as opposed to per gram wet tissue, the cortex has approximately 7-times more ASC than cerebellum [60], providing a more accurate representation to the level of ASC.

The regional differences in the brain with ASC levels could also be due to the neuron-rich regions [37]. Neurons contain the greatest amount of ASC and are concentrated in the gray matter, which includes the cerebral cortex and hippocampus. The brain regions with a greater amount of white matter include the posterior regions of the brain including the brain stem and spinal cord. These regions generally have lower ASC contents. The concentration of ASC in neurons also agrees with previous literature in which animals with a greater neuronal density, such as rats and mice, have higher brain ASC concentrations [37].

Regulation of Intracellular ASC

Intracellular ASC concentrations can be regulated by three different mechanisms. The first two have already been discussed and include ASC transport into the cell via the SVCT2 and recycling within the cell by various antioxidants and reductases. The last is by ASC transport out of the cell, or efflux. It is not known how ASC is transported out of the cell. However, it has been proposed ASC efflux occurs through volume-

sensitive anion channels [63], exocytosis by ASC-containing vesicles [64], or by glutamate-ASC hetero-exchange within the brain [65-67].

ASC turnover is low in the brain, with 2% being replaced every hour [68]. Even after days of depletion, ASC is retained in the brain. Harrison *et al* has shown after 4 weeks ASC deficiency in Gulo(-/-) mice, ASC is retained the greatest in cerebellum, thalamus, and hippocampus while the pons and spinal cord contains the least amount of ASC [33]. Although efflux of ASC from neurons and other cells occurs, it is clear that the system is geared to retain ASC within cells.

Oxidative Stress

Oxidative stress is present when the production of reactive oxygen species overwhelms the endogenous antioxidant defense mechanisms. An imbalance in the redox state of the cell can go on to elicit major downstream signaling cascades. One major intracellular source of reactive oxygen species occurs in the mitochondria, in which oxidative phosphorylation generates energy [69]. The transport of ASC into mitochondria to prevent an overwhelming amount of oxidative stress has been controversial. However, recent data suggests there is a mitochondrial ASC transporter with similar kinetics to that described for the SVCT2 [70].

Oxidative stress can damage multiple components of the cell including the DNA, in which 8-oxo-2′-deoxyguanosine is produced, causing single or double stranded DNA breaks when left unrepaired [71]. Protein oxidation can also occur due to increased levels of oxidative stress. Oxidation can occur on all residues but sulfur- and aromatic ring-containing amino acids are most susceptible to oxidation, including cysteine, methionine, and tryptophan [72]. Accumulation of oxidized proteins is associated with many diseases including Alzheimer's disease [73, 74], atherosclerosis, and diabetes [75]. The last major consequence of increased reactive oxygen species is lipid peroxidation. This occurs when species extract hydrogen atoms from lipids, typically polyunsaturated fatty acids, at the cell membrane, creating a fatty acid radical [76]. This radical can then react with a neighboring fatty acid, triggering a chain reaction. Lipid-soluble antioxidants such as vitamin E and ubiquinone are the first line of defense in preventing this lipid-specific propagation.

These three types of modification due to oxidative stress can be reversed but are most detrimental when the amounts are overwhelming within the cell. In conjunction with those three primary consequences, oxidative stress can cause other problems. Oxidative stress can shorten telomeres, a structure that is shortened naturally with age [77]. This increased amount of shortening can increase factors associated with aging and could be a cause of the increased risk in cognitive decline earlier in life, as occurs in diabetes. Reactive oxygen species also regulate several key enzymes involved in the regulation of the cytoskeleton and modification of adhesion molecules. These effects are triggered by modifications to protein kinase C, mitogen-activated protein kinase, and tyrosine phosphatases, to name just a few [78].

The brain has a high rate of oxygen consumption, accounting for nearly 20% of the total body's basal consumption, despite the small percent of total body weight [79]. Therefore, the amount of oxygen to process per gram tissue must require great defense and processing mechanisms of the oxygen. In addition, there are several other causes of oxidative stress in the brain, including neurotransmitter toxicity [80] and excitotoxic amino acids [81].

Importance of ASC in the brain

A whole body knockout of the SVCT2 in the mouse is embryonic or postnatal day 1 lethal. The phenotype of these mice further supports the role for ASC in the brain, especially during this time [11, 46, 82, 83]. The sudden death of SVCT2 mice shortly after birth suggests an importance in ASC in processing the immediate exposure to much greater levels of oxygen in the brain, known as 'birth hyperoxia'. Therefore, during development, it is likely ASC levels should be greater just before birth.

The SVCT2 knockout mice have negligible ASC concentrations in the brain associated with severe capillary hemorrhage. This weakening of the blood vessels in the brain leading to hemorrhaging could be due to the role of ASC in maintaining stability of the BECs at the blood-brain barrier (BBB) as will be introduced next. The SVCT2(+/-) mouse has approximately 30-40% less ASC in the cortex compared to its wild-type littermates [32, 82]. This mouse is viable and fertile and is frequently used in developmental studies and moderately depleted models of cellular ASC deficiency. In

Chapter III, we show that brain ASC content of normal mouse embryos is highest at birth and decreases during postnatal development [37, 83], highlighting the important role for ASC throughout development.

The Blood-Brain Barrier

As mentioned before, the loss of ASC in the brain causes severe hemorrhaging of the blood vessels, suggesting a role for ASC at this structure known as the BBB. The BBB is an intricate neurovascular unit that comprises tightly connected endothelial cells, neurons, astrocytes, pericytes, and extracellular matrix (Figure 1.4). Nearly 130 years ago, before ASC had been isolated, Paul Ehrlich first discovered evidence of a barrier that protected the brain from neurotoxic molecules present in the circulation. When Ehrlich injected a dye into a mammal's circulatory system, every organ in the body was stained except the brain [84]. In addition, Ehrlich's student, Edwin Goldmann, performed the converse experiment in which he injected trypan blue into the cerebrospinal fluid and observed staining of the central nervous system but no staining in peripheral tissues [85]. Although evidence for a BBB has been around for many years, understanding how it functions has proven much more difficult to discern.

Function of the BBB

There are many functions of the BBB but it is primarily responsible for the prevention and regulation of the entrance of certain molecules into and out of the brain. Transport across the BBB is primarily by transcellular transport, through the endothelial cells via active transporters, passive diffusion of lipid soluble molecules, or endocytosis. Paracellular transport of molecules between the endothelial cells is more limited, unlike peripheral blood vessels where molecules can more easily diffuse between the cells [86]. BECs are held together by tight and adherens junctions. Tight junctions are only present in endothelial cells in the brain, not in peripheral endothelial cells, and proteins present here, such as occludin, claudins, and zona-occludin 1, are responsible for keeping neighboring cells tightly connected [87] (Figure 1.4). Zona occludin-1 is responsible for linking occludin and claudin to the intracellular framework of the cell, the cytoskeleton. Adherens junctions are present between all endothelial cells and also help

to hold the cells together but more importantly help to maintain barrier stability. Proteins involved in adherens junctions include VE-cadherin and the catenins. Intracellular signaling pathways that disrupt either the proteins located at the junctions or the cytoskeleton itself, lead to disruptions of the BBB [88].
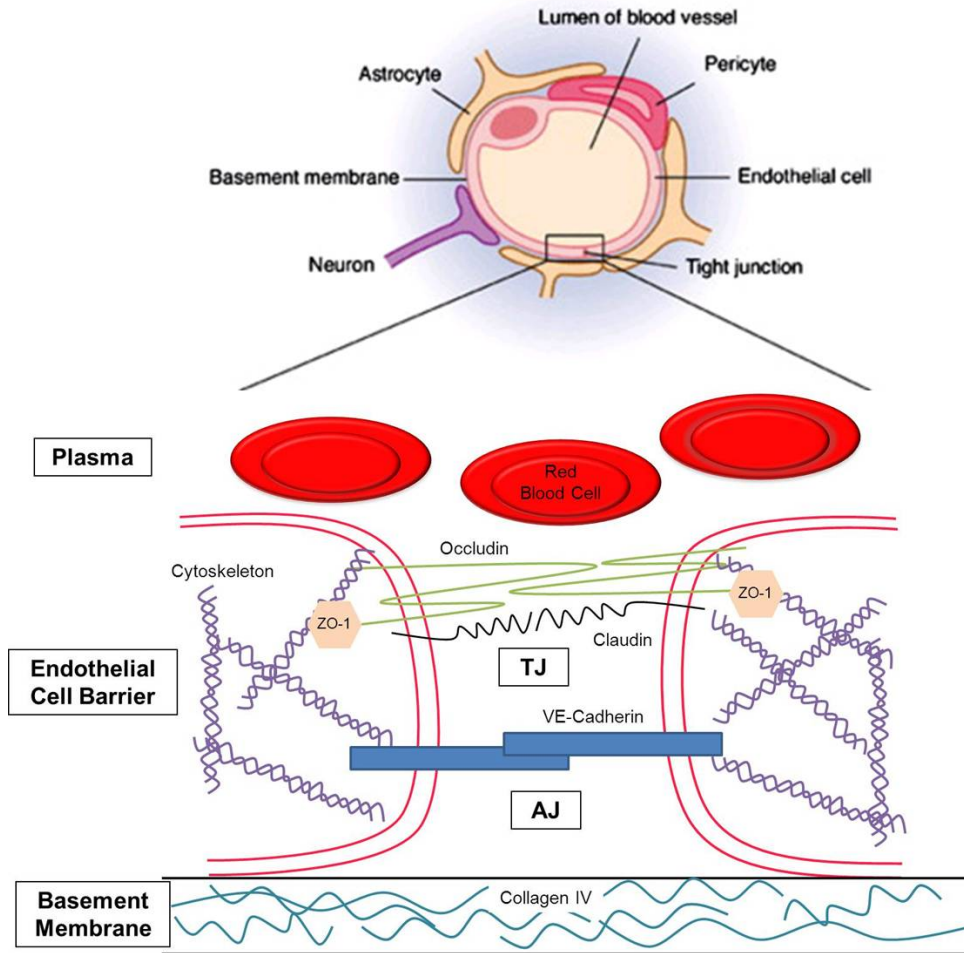
Figure 1.4. Neurovascular unit. Modified from Francis *et al.* Expert Rev Mol Med. 5:15 (2003) 1-19 [89]. Cross section of a brain capillary, depicting the BBB with a close up of the tight junction (TJ) and adherens junction (AJ) resting on the basement membrane primarily composed of type IV collagen.

Maintaining Barrier Stability

Post-translational Modification: BEC barriers are maintained by cell-to-cell interactions via tight and adherens junctions. Modifications of proteins located at these junctions, such as occludin or VE-cadherin, can affect the permeability of the barriers [90-94]. Phosphorylation of proteins located at the junctions has been implicated in altered permeability of the BBB. For example, when occludin is phosphorylated on key serine and threonine residues, it is translocated to the cell membrane or alters the interactions with linking proteins [93]. Han *et al* showed that ASC was able to inhibit protein phosphatase type 2A, likely by decreasing levels of superoxide, to allow for increased phosphorylation of occludin and thus increased expression at the cell membrane [94]. On the other hand, tyrosine phosphorylation of VE-cadherin has been linked with internalization of the protein and increased BEC permeability [91, 95, 96]. In fact, tyrosine phosphorylation in general has been strongly linked to disruption of the endothelial cell barrier and increased permeability, especially in the tissue barriers of tumors [97, 98]. Since superoxide can activate protein tyrosine kinases [99], and phosphatases can be regulated by redox cycling, it may be possible for ASC to prevent this type of modification due to its ability to scavenge superoxide. In addition, these junctional proteins attach to the intracellular cytoskeleton, which includes microtubules and actin filaments. Rearrangement of the cytoskeleton, especially by oxidative stress, has been shown to alter the proteins located at the junctions, leading to a weakened barrier [88, 100, 101].

Cyclic AMP Signaling: Cyclic AMP (cAMP) is an important second messenger in acutely regulating barrier permeability [102, 103]. Activating cAMP downstream signaling cascades affects cytoskeleton rearrangement, which in turn decreases permeability [88, 100]. Increases in intracellular cAMP levels diminish stress fiber formation, allowing for relaxation of the cells, and generate tighter cell-to-cell contacts [104, 105]. Downstream mediators of cAMP, including the canonical protein kinase A (PKA) and the non-canonical exchange protein for activated cAMP (Epac) are implicated in regulating barrier resistance [106, 107]. PKA targets small GTPases including Ras or Raf which ultimately affect actin-myosin contractility [102]. Epac is a more recently discovered protein so the downstream effectors have not been as

19

extensively described. However, activation of the small GTPase Rap1 by Epac has been shown to decrease BEC permeability [88]. In addition, it has been shown Epac can affect the cytoskeleton directly in peripheral endothelial cells, primarily by binding and stabilizing microtubules [88, 108].

Activation of PKA and Epac can trigger these signaling cascades to alter the phosphorylation status of proteins located at the junctions, as well as affect cytoskeleton rearrangement. In addition, it has previously been shown in peripheral endothelial cells that oxidative stress can disrupt cAMP signaling [109, 110], suggesting ASC might be able to prevent the increases in BEC permeability due to the altered cAMP signaling [111].

ASC regulation of BEC stability: The May laboratory has shown a role for ASC in regulating peripheral endothelial cell permeability [112-114]. However, whether or not this remained true in BECs was still to be determined. The mechanism by which ASC is able to improve the endothelial barrier has not been well defined. There is some evidence that nitric oxide may be involved [113]. However, for the studies presented here, previous reports have shown that increased oxidative stress can dysregulate cAMP-dependent mechanisms [109, 110, 115]. ASC has been implicated in regulation of cAMP levels, although the mechanism is not clear [111, 116]. ASC could affect activity of adenylate cyclase or prevent the breakdown of cAMP by inhibiting phosphodieseterases. In addition, ASC can return cAMP to its normal ability to bind downstream effectors by decreasing oxidative stress [111].

Significance of BBB maintenance

Numerous central nervous system pathologies can contribute to breakdown or disruption of the BBB. Multiple sclerosis, stroke, and Alzheimer's disease are common diseases that either arise due to BBB breakdown or exacerbate a weakened BBB [117]. Most research towards investigating the regulation of the BBB is in the context of ischemia or traumatic brain injury. However, these studies have revealed the consequences of BBB disruption can be long-lasting and life threatening. For example, BBB disruption can lead to neuroinflammation and cell death. The goal of Chapters V

and VI is to investigate BBB breakdown in the context of a fairly recent association, namely diabetes.

Diabetes and the BBB

The majority of diabetes-related morbidity and mortality is due to pathologies of the vascular system, including cardiovascular and cerebrovascular disease. A key feature of diabetic complications is disruption of the endothelial barrier and plasma extravasation into various organs [118]. Hyperglycemia in diabetes results in impaired nitric oxide production and activity, which is important in maintaining endothelial function.

Endothelial dysfunction

Endothelial dysfunction is characterized by a blunted vasodilatory response to the release of a variety of factors including growth factors and nitric oxide. Nitric oxide deficiency is a hallmark of endothelial dysfunction and has been implicated in many cardiovascular diseases [119]. Endothelial nitric oxide synthase (eNOS) is the primary enzyme responsible for production of nitric oxide. Nitric oxide regulates vascular tone by free diffusion and relaxation of blood vessels. Tetrahydrobiopterin is an essential co-factor for eNOS in the synthesis of nitric oxide [120]. Diabetes is known to uncouple eNOS in peripheral endothelial cells [121]. When tetrahydrobiopterin levels are inadequate, eNOS becomes unstable or uncoupled, leading to decreases in nitric oxide production and increased superoxide. Decreased production of nitric oxide in the brain due to unstable eNOS worsens the outcome of stroke, as eNOS-derived nitric oxide regulates cerebral blood flow [122]. Indeed, ASC does recycle tetrahydrobiopterin [113] and thus, could help to maintain nitric oxide levels under diabetic conditions.

Endothelial cell regeneration

In addition, hyperglycemic damage to endothelial cells stimulates endothelial cell regeneration. This is especially common in retinal endothelial cells in which high glucose causes an excessive release of angiogenic factors that results in proliferative retinopathy [123]. Vascular injury triggers endothelial progenitor cells to target the

21

ischemic sites, stimulating angiogenesis. This is most common under ischemic conditions in which HIF-1α is activated, leading to transcription of angiogenic factors including vascular endothelial growth factor [124]. The release of this factor mobilizes endothelial progenitor cells from the bone marrow to the location of injury. Type 2 diabetics have decreased levels of circulating endothelial progenitor cells [125]. In addition, not only does hyperglycemia cause endothelial cell loss, but inflammation in and around these cells can also occur during high glucose conditions, causing a multitude of signaling cascades which can perpetuate endothelial dysfunction.

BBB disruption due to diabetes

Although previous studies have focused on peripheral endothelium in organs such as the liver, kidney, and even skeletal muscle, the effect of diabetes on BBB endothelium is gaining interest. Contrary to earlier research, recent studies have shown that the BBB is disrupted with high, chronic circulating levels of glucose [126-130]. People with poorly controlled diabetes have chronically elevated blood glucose levels, which causes increased oxidative stress that, as mentioned before, can alter BBB integrity [131]. Permeability of the blood-retinal barrier is increased dramatically in diabetic retinopathy which is a leading cause of blindness [132]. Risk for developing diabetic retinopathy is associated with duration of diabetes [133]. The blood-retinal barrier has similar features as the BBB, and both are regulated by similar mechanisms. Changes in BBB permeability in diabetic humans have also been reported using MRI [134]. Longitudinal studies involving people with type 1 or 2 diabetes have shown at least a two-fold increased risk of developing impaired cognition compared to people with normal fasting blood glucose levels [135-138]. It is likely this increased risk is due to BBB disruption as vascular dysfunction is critical in neurodegeneration and neuroinflammation, especially in the context of Alzheimer's disease [139].

BBB disruption signaling mechanisms

Despite the range of data implicating diabetes in disruption of the BBB, the molecular mechanisms involved are still under investigation. High glucose levels affect endothelial cells directly, leading to endothelial dysfunction. There are reports that

suggest the increased oxidative stress present in diabetes contributes to barrier instability [140, 141]. Cellular studies using *in vitro* models of the BBB have investigated the molecular mechanisms that occur due to high glucose and show links between PKC/PKA activation, production of reactive oxygen species, and activation of the receptor for advanced glycation end-products (RAGE) during glucose-induced barrier disruption [142-145]. These studies show that glucose ultimately increases barrier permeability by cytoskeleton and junction rearrangement. However, there is little data on what occurs in between the trigger and the ultimate loss of barrier function. Indeed, even the exact trigger of disruption is unclear, but studies suggest it is due to increased oxidative stress in diabetes [146].

RAGE: RAGE is activated by high glucose exposures, enabling intracellular signaling pathways of the endothelial cells that loosen the barrier [147]. Hyperglycemia increases intracellular reactive oxygen species due to the increased flux of glucose metabolism from the glycolysis pathway to the polyol pathway [148]. The increased amount of reactive oxygen species can then increase levels of oxidative stress to affect intracellular processes. In addition, glucotoxicity can activate NFκB, a transcription factor important in mediating inflammation and altering many downstream pathways [148].

Glycation of proteins disturbs their normal function within the cell and allows them to bind RAGE. AGEs are formed when aldehyde or ketone groups of glucose or fructose are covalently bound to free amino groups on proteins. Many of these AGE then bind to and activate RAGE, activation of which leads to increased permeability of endothelial cells [149] and triggering of multiple downstream pathways, including PKC [150], NADPH oxidase [151], or NFκB activation [152] (Figure 1.5). These mediators elicit various downstream effects, but important for the studies presented here, they increase the amount of intracellular reactive oxygen species. These species can then cause multiple potentially detrimental effects including matrix metalloproteinase activation [153], tight junction modification [154], and cytoskeletal rearrangement [101]. All of these events can lead to disruptions of the BBB.

Although the identity of the reactive oxygen species responsible for BBB disruption is unknown, much evidence points to involvement of superoxide. This is

perhaps best illustrated in the mice lacking one copy of the superoxide dismutase, which have increased BBB disruption [155]. However, the effects on BBB permeability could also be due to downstream metabolites of superoxide, such as hydrogen peroxide or peroxynitrite, which have both been shown to lead to BEC disruption [154, 156].

In vivo investigations: Many *in vivo* studies have further explored the effects of glucose on the BBB primarily using streptozotocin diabetic models. The Davis laboratory has shown levels of proteins important in the tight junctions, specifically zona occludins-1 and occludin, are significantly decreased within two weeks of diabetic induction, reflected by increased extravasation of [14C]sucrose into the brain [157]. In addition, matrix metalloproteinase activity, an action involved in degradation of collagen and the extracellular matrix, is significantly increased in the plasma of diabetic rats [157].
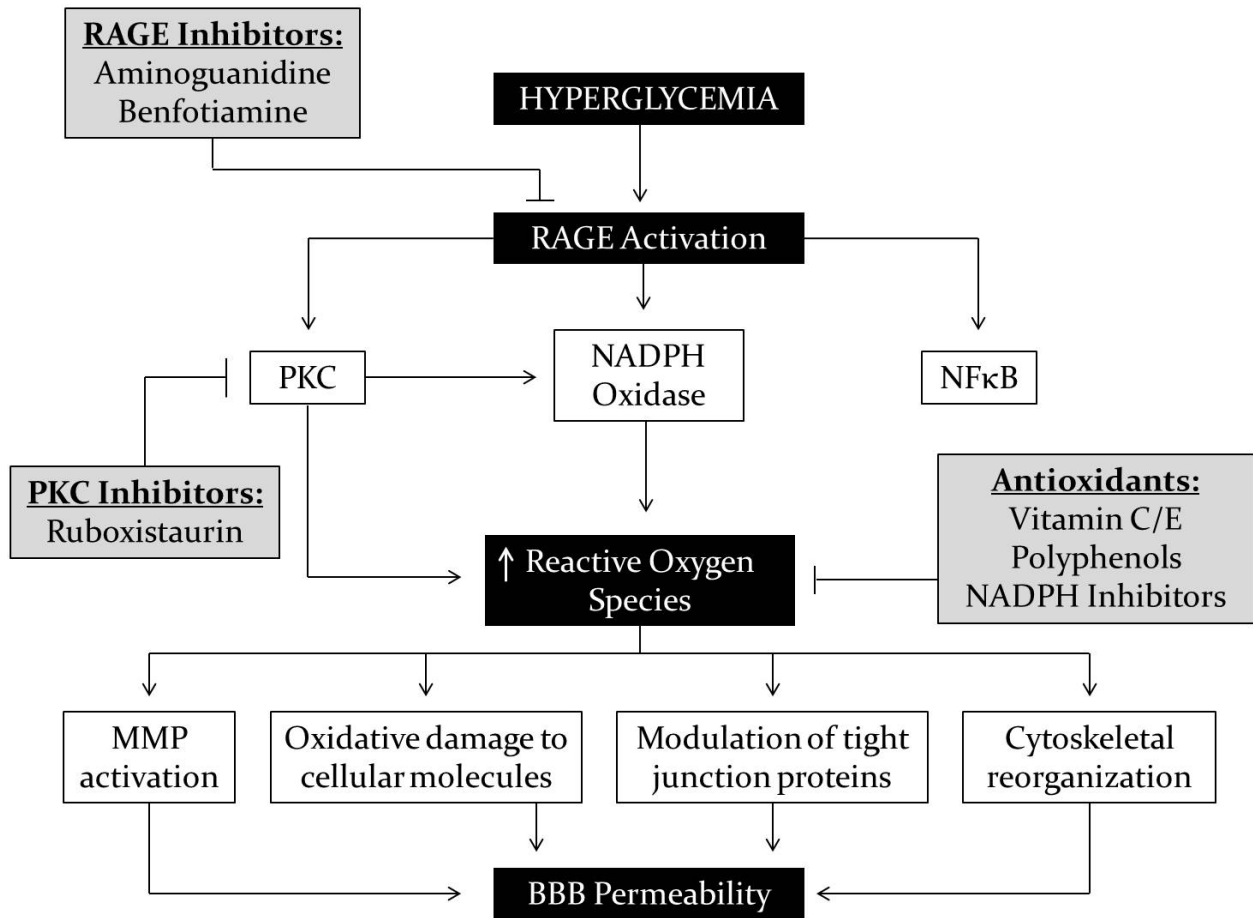
Figure 1.5 RAGE signaling events triggered by hyperglycemia. Modified from Pun *et al.*, Free Radic Res, 43:4 *(*2009) 348-364 and Potenza *et al.*, Curr Med Chem, 16:1 (2009) 94-112 [142, 144]. RAGE activation by high glucose can increase levels of reactive oxygen species. This increase in reactive oxygen species has been shown in BECs to alter many downstream signaling events that ultimately result in BBB permeability. Therapeutic targets (grey boxes) have been suggested as potential mediators of this signaling pathway.

Significance of diabetes, BBB, and ASC interaction

According to the CDC, 8% of the United States population has diabetes and of that 8%, as many as 20% have poor glycemic control, a hemoglobin A1c level greater than 9% [158]. Diabetics have lower circulating levels of ASC despite adequate daily intake [159], which could contribute to increased BBB permeability through endothelial dysfunction due to increased oxidative stress [142]. In addition, leukocyte ASC, one of the best indices for ASC stores in man [160], is decreased by more than 30% in diabetics consuming the recommended daily allowance of vitamin C [161]. Because of the potential consequences due to low ASC, it is important to begin to understand the signaling mechanisms in BECs under high glucose conditions and the role for ASC in this context. Beyond its importance for diabetes, understanding the role ASC plays in endothelial cells could have great significance for other diseases that involve breakdown of any endothelial cell barrier, including stroke, multiple sclerosis, and Alzheimer's disease.

Significance: ASC Deficiency Today

Despite the previously noted controversy regarding the current recommended daily allowance for vitamin C in adults, there is little doubt that associated conditions such as smoking, pregnancy, and diabetes increase the need for the vitamin. Indeed, smokers have a 2.5 fold lower plasma vitamin C level than those of non-smokers [162] and more than one study has shown that diabetics have a 30% lower level than non-diabetics [163]. Dr. Harrison has recently reviewed the literature on plasma vitamin C level in clinical trials, paying close attention to sample preparation and methodologies [164]. Of five separate clinical trials performed in the last 15 years to determine the level of vitamin C deficiency in the general healthy human population, an average of 8% of the population is "deficient" (plasma vitamin C level <11.4 μmol/L) and 21% is "depleted" (plasma vitamin C level 11.4-28 μmol/L) suggesting a need for increased intake, as well as more research on the effects of this deficient supply of vitamin C [163, 165-168]. The recommended daily allowance is calculated based on avoiding deficiency rather than obtaining an optimal amount. Therefore, in the case of vitamin C, the

recommended daily allowance does not target optimal intake, but rather how much of the vitamin is needed to simply prevent scurvy [169].

Single nucleotide polymorphisms associated with SVCTs

From a genetic perspective, there are reported single nucleotide polymorphisms (SNPs) present within the *SCL23A1* and *SLC23A2* genes that encode SVCT1 and SVCT2, respectively, which have been shown to be predictors of plasma ASC levels. As mentioned before, and highlighted by the lethality of the SVCT2(-/-) mouse model, these proteins are the primary form of transport for ASC [170-172]. Many of these studies have linked mutations in these transporters with increased risk for developing various cancers due to the changes in antioxidant capacity [170, 173, 174]. One SNP in *SLC23A1* (SVCT1, rs33972313) lies in exon 8 and causes a missense mutation correlating with decreased levels of circulating ASC. This suggests a conformational change that impairs the activity of the transporter [171]. However, the decreased levels of ASC could also be due to problems in trafficking SVCT1 to the membrane to allow for transport. In addition, SNPs located in introns of *SLC23A2* (SVCT2, rs6053005 and rs6133175) are strong predictors of plasma ASC levels [170]. The consequence of these SNPs on the regulation or activity of SVCT2 has not been investigated.

In addition, multiple groups have found that a SNP within *SLC23A2*, specifically rs6139591, is associated with increased risk for spontaneous preterm birth [174] as well as an acute coronary syndrome in women in which the association is more pronounced with low dietary intake of ASC [175]. This SNP is located in intron 2 and lies 5' upstream of the large gene in a region flanked by conserved sequence, suggesting a functional consequence. Although the functional outcome is not known, it is likely this area regulates gene expression since it lies fairly upstream of the gene itself.

ASC is necessary for collagen formation and membrane tensile strength. Therefore, it is not surprising that potentially altering transport of ASC and thus intracellular ASC levels, could affect the strength of the membrane responsible for holding the developing fetus. In addition to plasma ASC levels and spontaneous preterm birth, another SNP located in *SLC23A2* has been associated with a higher risk for developing the most common form of glaucoma, again linked to the role of ASC as

an antioxidant [172]. Functional studies of these SNPs are still needed to fully corroborate these findings.

Contribution to the field

The purpose of this dissertation research was to better understand the various roles for ASC in the brain. We hypothesized loss of ASC in the brain would impair the known functions of ASC while excess or physiological levels of ASC would rescue these effects. In the studies of Chapter III, we wanted to investigate the regulation of SVCT2 during development as well as by its own substrate, ASC. We hypothesized SVCT2 expression levels would follow a similar pattern to ASC concentration during development. We expected ASC levels would increase throughout development, especially just before birth, to compensate for the increased amount of oxidative stress immediately after birth. In addition, we expected low levels of ASC in adults would cause an up-regulation of SVCT2, dependent on the need for intracellular ASC.

In Chapter IV, we wanted to investigate a separate, yet vital role for ASC specifically in neurons: regulation of neurotransmitter synthesis. Since ASC is a co-factor for neurotransmitter synthesis, we hypothesized low levels of ASC would decrease dopamine, norepinephrine, and serotonin levels and their metabolites by altering the co-factors responsible for neurotransmitter synthesis including tyrosine and tryptophan hydroxylase and dopamine β-hydroxylase. In addition, we expected increased levels of ASC would enhance synthesis by increasing co-factor expression level.

Lastly, Chapters V and VI focus on the regulation of the BBB by ASC under basal and high glucose conditions. We hypothesized ASC would decrease BEC permeability and would prevent the increase in permeability due to high glucose. Since RAGE is involved in endothelial cell permeability, we expected inhibition of this receptor would decrease permeability. In addition, we expected ASC would decrease permeability by affecting phosphorylation of junction proteins and/or alter the cytoskeleton in a cAMP-stimulatory mechanism.

The results gained from this work contribute to the studies on the role for ASC in the brain. Although small concentrations of this vitamin easily prevent the most severe

form of deficiency, scurvy, the mechanisms and impact of what occurs when brain levels are depleted still needs to be investigated. It is hoped that these discoveries will not take another 200 years before being accepted.
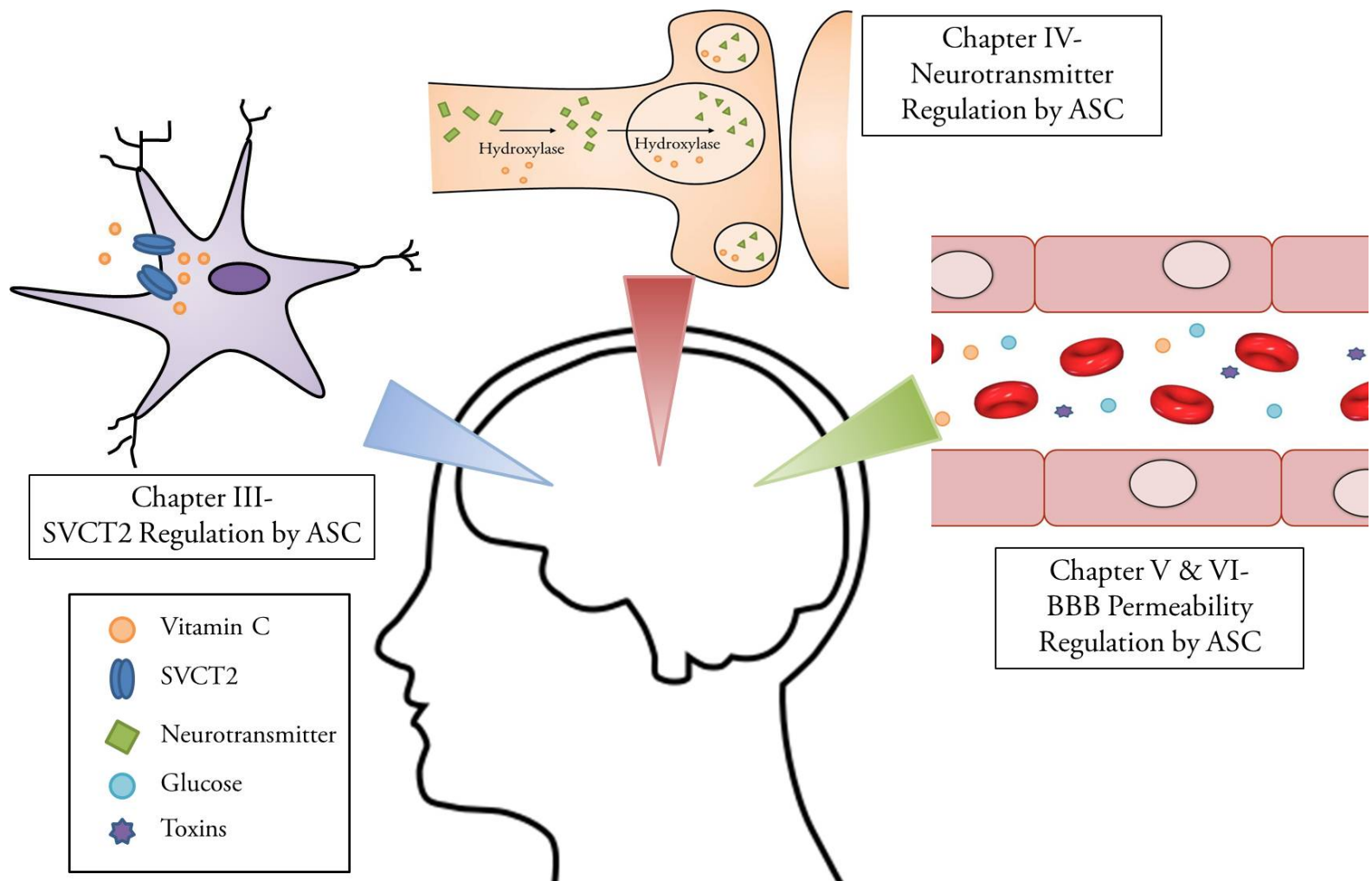
Figure 1.6. Model of the roles for ASC in the brain addressed in this dissertation. The roles for ASC range from developmental regulation of ASC transport (Chapter III) to aiding in synthesis of neurotransmitters (Chapter IV) to regulating the brain endothelial cell barrier (Chapters V and VI).

CHAPTER II

MATERIALS AND METHODS

Animal Care and Usage

Ethics statement: All procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals.*

Animals: Mice were housed in tub cages in a temperature- and humidity-controlled vivarium. Mice were kept on a 12:12-h light:dark cycle, with lights on at 6 AM. They had free access to food and water for the duration of the experiment.

Mice used in Chapter III

Experiment 1: Wild-type (WT) C57BL/6J mice were originally obtained from Jackson Laboratories (stock #000664; Bar Harbor, ME, USA) and maintained as a colony by in-house breeding. WT pregnancies were determined by the presence of a vaginal plug. Mice were sacrificed at embryonic day 15.5 and 18.5, and postnatal day 1, 10, 18, and 32.

Experiment 2: Gulo(-/-) mice were acquired by breeding heterozygous Gulo(+/-) mice, obtained from Mutant Mouse Regional Resource Centers (stock #000015-UCD) and maintained on a C57BL/6J background. All Gulo(-/-) mice were provided deionized water containing 0.33 g/L ASC (standard, STD) (Sigma, USA) with 10 µM EDTA to increase stability of ASC in solution. This is the standard supplement level that provides adult Gulo(-/-) mice with approximately WT levels of ASC in tissues [14, 176]. Similar numbers of males and females were used. At 4-weeks of age, Gulo(-/-) mice were placed on their respective ASC supplements (HIGH 3.33g/L, STD 0.33g/L, LOW 0.033g/L, or WATER (WTR) 0.00g/L) for 4 weeks. Four weeks is enough time for tissue ASC levels to adjust to a steady-state level [82]. More than 4 weeks without ASC supplementation can cause scurvy in Gulo(-/-) mice. Signs of scurvy were monitored in these mice, including weight loss, loss of hair, and changes in gait and posture.

Mice used in Chapter IV

Mice containing the heterozygous deletion of the SVCT2, SVCT2(+/-), were originally obtained from Dr. Robert Nussbaum (UCSF). They were placed on the C57BL/6J background through 9 backcrosses. Homozygous SVCT2(-/-) mice survive the gestation period but do not survive past birth [11, 82]. On the other hand, SVCT(+/-) mice, which have about half the normal levels of ASC in the brain cortex, are healthy and fertile. Because SVCT2(-/-) pups are not viable, we mated SVCT2(+/-) female and male mice to generate all three genotypes, including SVCT2(-/-) embryos. SVCT2(+/-) dams were provided with 0.33 g/L ASC in drinking water with 10 µM EDTA. Although SVCT2(+/-) mice can synthesize their own ASC, this supplement was given to insure that circulating ASC was as high as possible throughout pregnancy.

SVCT2-transgenic (TG) mice expressing extra copies of the SVCT2 were generated and also placed on the C57BL/6J background, as described previously [13]. SVCT2 TG embryos were compared to WT C57BL/6J embryos.

Mice used in Chapter VI

At weaning (3 weeks), Gulo(-/-) mice were placed on standard (0.33 g/L, STD) ASC supplementation supplied with 10 µM EDTA. Similar numbers of males and females were used. After 3 weeks, Gulo(-/-) mice either remained on the STD ASC supplementation or were placed on a low (0.033 g/L, LOW) ASC supplementation. After 2 weeks, mice were treated with either vehicle or streptozotocin (STZ) (200 mg/kg, Sigma). Blood glucose was tested 3 days later by a tail knick using a glucometer (Aviva). Mice with blood glucose levels >250 mg/dL were considered diabetic. Those that had been treated with STZ but did not have blood glucose levels >250 mg/dL were retreated.

A preliminary study was performed to determine water intake in Gulo(-/-) mice. It was found that diabetic mice drink 4-times more water than non-diabetic mice (Figure 2.1). Therefore, diabetic mice on LOW ASC supplementation received 4-fold less ASC in their drinking water (0.00825 g/L).
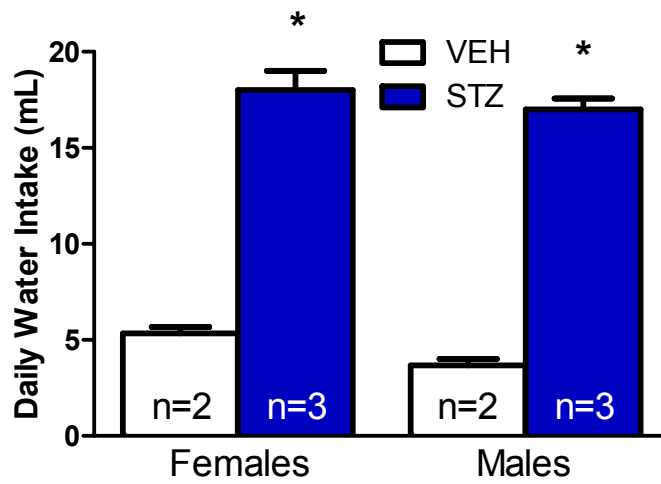
Figure 2.1. Daily water intake for diabetic mice. STZ-treated mice drink approximately 4-times more water after one-week diabetes. *p<0.05 versus vehicle control.

Weight was monitored on a weekly basis to ensure the LOW STZ group did not lose more than 20% body weight (data not shown). Glucose levels were measured again just before sacrifice (either after 6-weeks or 12-weeks diabetes). Only those mice that had received STZ with glucose levels >250 mg/dL at the end of the study were included in the analysis.

Mouse sacrifice and tissue collection

Chapters III and IV: To obtain fetal tissues, pregnant dams were sacrificed by decapitation under isoflurane anesthesia. Male and female fetuses were delivered by Caesarian section and placed on a Petri dish on ice to induce hypothermia. Fetuses were decapitated before tissue collection to ensure death. Postnatal day pups and adult mice were decapitated under isoflurane anesthesia after which the cortex, cerebellum, and liver were removed from each animal and immediately frozen and stored at −80°C until needed.

Chapter VI: Before sacrifice, mice were injected with 100 μL ketamine/xylazine (100 mg/10 mg/kg) intraperitoneally to anesthetize. Once anesthetized, mice received a single tail vein injection of a fixable 10 kDa FITC-dextran (6 week study- 8.5 mg/kg at 1.75 mg/mL; 12 week study- 15 mg/kg at 2.5 mg/mL) (Life Technologies, NY, USA, catalog #D-22913), which circulated for 90 min. Mice were sacrificed by cervical dislocation. Blood was collected via cardiac puncture in EDTA coated tubes. Mice were perfused with 500 mL/kg PBS over 5 min through the left atrium of the heart. After perfusion, tissues (cortex, cerebellum, liver, lung, heart, kidney, spleen) were dissected and immediately frozen on dry ice. The retinas were collected and either stored for dextran quantification or flat-mounted to visualize dextran extravasation. Tissues were stored at -80°C until further analyzed.

ASC Quantification

To measure intracellular ASC, tissues were homogenized in a lysis buffer containing 100 μL of 25% (w/v) metaphosphoric acid and 350 μL of 0.1 M Na_2HPO_4 and 0.05 mM EDTA, pH 8.0. A total of 10 μL buffer was used for each mg of tissue. Cells were washed 2x in KRH before lysis with the buffer. The homogenate was centrifuged

at 4°C for 5 min at 13,000×*g*, and the supernatant was taken for assay of ASC. Assay of ASC was performed in duplicate by high performance liquid chromatography as previously described [82, 177]. Data were expressed per gram wet tissue weight for tissues. For cells, intracellular ASC concentrations were calculated based on the measured intracellular distribution space of 3-*O*-methylglucose relative to protein [178], which was 2.9 µL/mg protein in bEnd.3 cells.

Oxidative stress measurements

Malondialdehyde: Malondialdehyde (MDA), a product of lipid peroxidation, was measured to detect any differences in oxidative stress among the groups. MDA was measured as thiobarbituric acid reactive substances as previously described [82]. Briefly, tissue samples were homogenized in ice cold 5% trichloroacetic acid, centrifuged at 4°C for 5 min at 13,000×*g*, and supernatants were collected. An equal volume of 20 mM thiobarbituric acid was added to the supernatant. Samples were incubated in a 95°C water bath for 35 min, followed by 10 min at 4°C. Fluorescence was read on a Spectramax M5 microplate reader (Vanderbilt Molecular Biology Core Facility, Molecular Devices, USA) with an excitation wavelength of 515 nm and emission wavelength of 553 nm. Samples were run against a linear standard curve. Data were determined per gram wet tissue weight.

Protein Carbonyls: Protein carbonyls, a product of protein oxidation, were measured to further detect differences in oxidative stress among the groups. Weighed samples were homogenized in a volume of 20 mM phosphate buffer (pH 7.4) containing 140 mM KCl and a protease inhibitor cocktail tablet (Roche, USA) (1 mg tissue/10 µL buffer). The homogenates were centrifuged at 750×*g* for 10 min at 4°C to pellet nuclei and cell debris. A BCA assay (Pierce, Thermo Scientific) was performed on the supernatant to measure protein concentration, and samples were diluted to a 1 µg/µL stock solution. A 250 µL aliquot of the diluted sample was transferred to a new 1.5 mL tube and 250 µL of 10 mM 2,4-dinitrophenylhydrazine in 2.5 M HCl was added. Samples were incubated in the dark for 30 min at room temperature, vortexing every 5 min. After incubation, 175 µL of 50% trichloroacetic acid was added, vortexed, and

incubated at -20°C for 30 min before centrifuging the protein pellet at 4°C for 15 min at 9000×g. The supernatant was removed and the pellet was washed three times in 500 μL of ice cold ethanol:ethyl acetate (1:1), centrifuging for 2 min at 9000×g between washes. After the last wash, the pellet was redissolved in 1 mL of 6 M guanidine-HCl. The sample was mixed well and warmed for 15 min at 60°C. Samples were vortexed and 250 μL were loaded in a clear 96-well microtiter plate. The absorbance was measured at 370 nm on a Spectramax M5 microplate reader (Vanderbilt Molecular Biology Core Facility, Molecular Devices, USA).

Eicosanoid Measurements: F_2-Isoprostanes and F_4-Neuroprostanes were measured in the Vanderbilt Eicosanoid core by gas chromatography/negative ion chemical ionization mass spectrometry employing stable isotope dilution as previously described [179, 180].

Dihydrofluorescein: Reactive oxygen species, primarily hydrogen peroxide, were measured as oxidation of dihydrofluorescein diacetate [178]. This non-fluorescent compound diffuses through the cell membrane and once in the cell is de-esterified, which traps it within the cell. Reactive oxygen species within the cell oxidize the compound to the fluorescent dihydrofluorescein. Although dichlorodihydrofluorescein diacetate is commonly used in this assay [181], others have shown dihydrofluorescein is more sensitive [182].

bEnd.3 cells were grown to confluence in black 96-well plates with a clear bottom (Costar 3603, Corning, NY, USA). The cells were rinsed twice with warm KRH to remove culture media and incubated at 37°C in KRH containing 5 mM D-glucose and the indicated concentration of ASC for 60 min. After the 60 min incubation, 20 μM dihydrofluorescein diacetate was added. Dihydrofluorescein was prepared to a 10 mM concentration in DMSO and stored at -20°C in the dark until diluted for use in KRH. The cells were then incubated at 37°C for another 30 min to allow entry of dihydrofluorescein into the cells. After the 30 min, cells were rinsed twice with KRH to remove residual extracellular dihydrofluorescein diacetate before addition of 200 μL KRH containing 5 mM D-glucose. The plate was loaded into a pre-warmed 37°C fluorescence microtiter

plate reader (Synergy H$_4$, Biotek, Vermont, USA). Readings were taken initially and at 36 min at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Data was analyzed as previously described [181]. Briefly, the percentage increase in fluorescence per well over time was calculated by using the formula [(T$_f$ -T$_i$)/ T$_i$ * 100], where T$_f$ = fluorescence at time 36 min and T$_i$ = fluorescence at time 0 min. This method of analysis calculates data that reflects the percentage change of fluorescence over time from the cells in the same well, while also controlling for variability among wells and background fluorescence. Seven separate experiments were performed in triplicate.

Extraction of total RNA and cDNA synthesis

Total RNA was extracted by using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). Tissue samples were homogenized with an electric homogenizer under RNAse-free conditions, and total RNA was extracted according to the manufacturer's protocol. RNA concentrations were determined and confirmed as free from protein contamination by measuring absorbance at 260 and 280 nm. Equal amounts of RNA were used to synthesize cDNA using TaqMan$^®$ Reverse Transcriptase (Sigma-Aldrich) following the manufacturer's protocol. The cDNA was stored at −80 °C until used.

Quantitative RT-PCR

RT-PCR reactions were performed in duplicate by using the iQ SYBR$^®$ Green Supermix (Bio-Rad, Hercules, CA) following the manufacturer's protocol. The forward and reverse primers for SVCT2 were AAGGATGGACGGCATACAAG and TCTGTGCGTGCATAGTAGCC, respectively, and for β-actin, GTTTGAGACCTTCAACACCCC and GTGGCCATCTCCTGCTCGAAGTC. Reactions were performed on an iCycler iQ Multicolor real-time PCR machine (Bio-Rad). SVCT2 mRNA levels were normalized to β-actin and expressed relative to controls. Samples were analyzed using the Pfaffl method [183].

Western Immunoblotting

Immunoblotting was performed by subjecting solubilized protein to SDS-polyacrylamide gel electrophoresis, according to the method of Laemmli [184]. Cells and tissues were lysed in RIPA buffer containing 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and a cocktail of protease inhibitors (Roche Diagnostics, USA, catalog #04693159001) and phosphatase inhibitors (when measuring phosphorylated protein levels) (Roche Diagnostics, USA, catalog #04906845001). Cells were rinsed twice with KRH before lysis. Protein from cell/tissue lysates was measured using the BCA assay (Thermo Scientific). Protein lysates were solubilized by adding equal volumes 5% β-merceptoethanol in Laemmli sample buffer (Bio-Rad, USA). Samples other than those used to probe for the SVCT2 were warmed at 60°C for 10 min prior to gel loading. Membranes were blocked with 5% bovine serum albumin (BSA) in 0.5% tris-buffered saline (TBS)-Tween 20 for 1 hour at room temperature or overnight at 4°C. Proteins were probed with appropriate antibodies (Table 2.1) in 5% BSA in 0.5% TBS-Tween 20. Membranes were washed 3x10 min in 0.5% TBS-Tween 20. A 1 hour secondary incubation in 5% BSA in 0.5% TBS-Tween 20 was performed before washing of the membranes for 3x10 min in 0.5% TBS-Tween 20. Bands were illuminated using ECL Plus Western blotting reagents (Perkin Elmer, Waltham, MA, catalog #ORT2655) and developed using Carestream® Kodak® BioMax® MR film (Sigma-Aldrich, catalog #Z350370).

For detection of phosphorylated occludin (Chapter V), membranes were blocked in 2% ECL Prime blocking reagent (GE Healthcare, UK, catalog #RPN48V) and incubated overnight at 4°C in primary antibody (Rabbit Occludin Ser490, D. Antonetti, U of Michigan). Samples were washed in 0.5% TBS-Tween 20 3x10 min before adding the appropriate secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich, MO, USA) for 1 hour at room temperature. Membranes were again washed 3x10 min with 0.5% TBS-Tween 20 and illuminated with Lumigen TMA-6 (Lumigen, MI, USA, catalog #TMA-100) before developing.

Densitometry was determined using ImageJ (NIH) and target protein was normalized to expression of β-actin or total non-phosphorylated protein and expressed relative to controls. Saturation of signal was determined by a film exposure time course.

Protein	Source	Catalog #	Species	Dilution	Chapter
SVCT2	Santa Cruz	sc-30114	Anti-Rabbit	1:200	III
β-actin	Santa Cruz	sc-1616	Anti-Goat	1:400	III
Tyrosine Hydroxylase	Santa Cruz	sc-14007	Anti-Rabbit	1:400	IV
Tryptophan Hydroxylase	Sigma-Aldrich	T0678	Anti-Mouse	1:1000	IV
pVE-Cadherin	Abcam	AB27776	Anti-Rabbit	1:750	V
VE-Cadherin	Santa Cruz	sc-6458	Anti-Goat	1:1000	V
pOccludin	Univ. Michigan	Gift from D. Antonetti	Anti-Rabbit	1:250	V
Occludin	Invitrogen	33-1500	Anti-Mouse	1:1000	V, VI
Rap1	Cell Signaling	8825	Anti-Rabbit	1:1000	V
Acetylated Alpha Tubulin	Santa Cruz	sc-23950	Anti-Mouse	1:500	V
Alpha Tubulin	Santa Cruz	sc-53646	Anti-Mouse	1:1000	V
Claudin-5	Invitrogen	34-1600	Anti-Mouse	1:800	VI

Table 2.1. List of primary antibodies used for Western immunoblots.

Neurotransmitter Analysis

High performance liquid chromatography coupled with electrochemical detection was used to measure monoamine content as described previously [185]. This included norepinephrine, dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid, as well as serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). Measurements were made using the right hemisphere of the embryonic cortex by the Neurochemistry Core Laboratory at Vanderbilt University's Center for Molecular Neuroscience Research (Nashville, TN). Three to four separate litters were used for ASC and neurotransmitter analysis.

Cell Culture

Murine brain endothelial cells (bEnd.3 cells) were obtained from ATTC (VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. For the assays involving ASC uptake and permeability under basal (5 mM) and high (25 mM) glucose conditions, a 1:1 ratio of media and astrocyte conditioned media was used. Astrocyte-conditioned media from primary rat brain astrocytes is known to tighten the endothelial cell barrier [186]. Astrocyte conditioned media was kindly provided by the laboratory of Michael Aschner. It was noted that although astrocyte-conditioned media helped to tighten the barrier, the within experimental effect of different treatments were unchanged without using this media. Therefore, the experiments probing the basal effects of ASC were performed without the addition of astrocyte conditioned media. bEnd.3 cells have previously been documented as an appropriate *in vitro* model for the BBB [187-189].

The human cerebral microvascular endothelial cell line hCMEC/D3 [190] was a kind gift from Dr. Ashwath Jayagopal and were acquired after the majority of the bEnd.3 cell studies were performed. Therefore, the hCMEC/D3 cells were used intermittently to complement the studies using the bEnd.3 cells. Cells were cultured in Medium 131 (Gibco/Invitrogen, Carlsbad, CA, catalog #M-131-500) containing microvascular growth supplement (Gibco/Invitrogen, catalog #S-005-25) after coating of the plate or filter with 0.1% gelatin (Gibco/Invitrogen, catalog #S-006-100) according to the manufacturer's instructions.

Cells were cultured at 37 °C in humidified air containing 5% CO_2.

Reagents used in Cell Culture

Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) supplied most of the reagent chemicals, including N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (Hepes), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (4-hydroxy-TEMPO, Tempol), and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox). N-benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1) was purchased from EMD Millipore (Billerca, MA, catalog #553030). FPS-ZM1 was initially dissolved in a small amount of dimethylsulfoxide, and then diluted with culture medium such that the final dimethylsulfoxide concentration was 0.06% or less. HMGB1 was purchased from ProSpec (Ness Ziona, Israel, catalog #pro-610). AGE-conjugated bovine serum albumin (AGE-BSA) was purchased from BioVision, Inc. (Milpitas, CA, catalog #2221-10). Perkin-Elmer Life and Analytical Sciences, Inc. (Boston, MA) supplied the [carboxyl-^{14}C]inulin (molecular weight range 5000-5500, 2 mCi/g). H89 dihydrochloride was purchased from Abcam (MD, USA, catalog #120341). ESI-09 (catalog #B133) and HJC0197 (catalog #C-136) were purchased from BioLog (Bremen, Germany). Dihydrofluorescein diacetate (catalog #292648), colchicine (catalog #C9754), cytochalasin A (catalog #C6637), and nocodazole (catalog #M1404) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Assay of Trans-Endothelial Inulin Transfer

Endothelial cells were cultured to confluence in 6-well plates on polyethylene terephthalate cell culture inserts (0.4 micron pores at a density of 2 ± 0.2 x 10^6 pores/cm^2, Falcon BD Biosciences, Franklin Lakes, NJ). After reaching confluence, cells were cultured for another 5-6 days with 1.7 mL of medium in the upper well and 2.8 mL of medium in the lower well. Agents were added above the cells/filter, followed by incubation at 37°C for the times indicated. 20 μL stock [carboxyl-^{14}C]inulin was diluted in 500 μL KRH and stored at -20°C until needed. 10 μL diluted [carboxyl-^{14}C]inulin was added above the filter during the last hour of the transfer experiment. Aliquots (500 μL) of medium above and below the cells/filter were added to 5 mL

Ecolume scintillation fluid (MP Biomedicals, Santa Ana, CA, USA) and sampled for liquid scintillation on a Tri-Carb 2910 TR scintillation counter (Perkin-Elmer, MA, USA). Zhi-Chao Qu performed most of the permeability assays (except the effects of ASC under Basal and High glucose conditions) after the plates and experimental set-up was provided by me. Data analysis and interpretation was performed by myself and Dr. May.

The permeability coefficient for [carboxyl-^{14}C]inulin was corrected for [carboxyl-^{14}C]inulin transfer across filters after removal of cells by treatment with 2 mL of 0.02 M ammonium hydroxide for 10 min [191]. KRH was then added above (1.7 mL) and below (2.8 mL) the cell insert. 10 µL of the diluted [carboxyl-^{14}C]inulin was added above the insert and incubated for 1 h at 37 °C. Aliquots (500 µL) of medium above and below the cells/filter were again taken and diluted in scintillation buffer before being counted for radioactivity. This step adjusts for any changes in permeability due to deposition of matrix material by the cells during culture.

Calculation of permeability coefficients

The permeability coefficient for [carboxyl-^{14}C]inulin was calculated as described [192, 193]. The volume (V), or space, occupied by the agent that was cleared from the luminal to the abluminal side of the cells/filter was calculated as

$$V = \frac{[A]_A - V_A}{[A]_L}$$

(1)

where $[A]_A$ is the abluminal inulin concentration, V_A is the volume of the abluminal chamber, and $[A]_L$ is the final luminal concentration of the inulin. The permeability (P) of each molecule across the cells and filter was calculated as

$$P = \frac{dV/dt}{S}$$

(2)

where "S" is the surface area of the filter on which the cells were cultured. Equation 3 was used to calculate the measured permeability of the cells and filter (P_{F+EC}) as well as

42

that of the filter plus any matrix laid down by the cells (P_F). As mentioned before, this analysis takes into account any effect due to deposition on the filter during culture and allows us to calculate the permeability solely of the BEC monolayer (P_{EC}).

$$\frac{1}{P_{EC}} = \frac{1}{P_{EC+F}} - \frac{1}{P_F}$$

(3)

Rap1 Assay

An active Rap1 detection kit was used as described by the manufacturer's instructions (Cell Signaling, catalog #8818). Cells were grown to confluence in 10 cm dishes for approximately one week in 5 mM glucose media. Briefly, 750 µg protein of the cell lysate was incubated for 60 min with glutathione resin and GST-RalGDS-RBD at 4°C. After elution of the active form of Rap1 with SDS sample buffer containing 200 mM dithiothreitol, samples were incubated at 95°C for 5 min. Samples were subjected to SDS–polyacrylamide gel electrophoresis (10% gel) and transferred overnight at 4°C. Membranes were treated as described in the Western Immunoblot section. GTP-bound levels of Rap1 were expressed relative to total Rap1 protein levels. Positive and negative controls were performed to ensure accuracy of kit. Excess GTPγS and GDP was added to separate control cell lysates in which the intensity of the active Rap1 band at 21 kDa was increased with GTPγS.

Microtubule Stabilization Assay

Microtubule stability was assayed by measuring protein levels of acetylated alpha tubulin relative to total tubulin. bEnd.3 cells were grown to confluence on 10 cm Petri dishes for 6-7 days in 5 mM glucose media. Cells were treated with 100 µM ASC for 90 min. Cells were rinsed once with PBS before lysis in 500 µL RIPA buffer containing protease inhibitors. Cells were scraped off the plate, placed in Eppendorf tubes, sonicated, and placed on ice for 30 min to allow for tubulin depolymerization. After a 1 min spin at 1000xg at 4°C, an equal volume lysate was added to Laemmli SDS buffer with 5% β-merceptoethanol, boiled at 95°C for 5 min and loaded onto the SDS-PAGE

gel. The remaining steps were carried out as described in the Western Immunoblot section.

FITC-Dextran Extravasation Visualization

Weighed cortex samples were homogenized in 400 µL PBS. Homogenates were spun at 7000 x rpm for 10 min. Samples (150 µL) were loaded on a black, 96 well microtiter plate. Fluorescence was measured on a Spectramax M5 microplate reader (Vanderbilt Molecular Biology Core Facility, Molecular Devices, USA) with an excitation wavelength of 590 nm and emission of 617 nm. Standards of the fluorescent dextran were run from 0.0 µg/mL-1.0 mg/mL.

Retinal Flat Mount Visualization

Members of the Ashwath Jayagopal lab, mainly Chauca Moore, dissected retinas from the mice studied in Chapter VI. The eye was dissected in which the cornea and lens were removed. After taking the retina from the eyecup and discarding it, radial cuts were made in all four quadrants to flatten the remaining tissue and mounted in medium. Flat mounts were visualized using the 2x and 4x objective of an inverted microscope fitted with the appropriate excitation and emission filters (Nikon Eclipse Ti microscope, Nikon, Melville, NY) and captured with a Nikon DS-Fi1 camera (Nikon, Melville, NY).

FITC-Dextran Quantification in Retinas

Using the plug-in for the SWIFT program in ImageJ (designed by a group at Harvard, provided by Corey Duprey in the Jayagopal lab), each retina was divided into 4 quadrants, in which the number of fluorescent pixels (in clusters set at a certain threshold) was calculated. The background was removed as intensity not reaching the clustered threshold. The purpose of this quantification was to highlight the disruption of the blood-retinal barrier in the LOW ASC diabetic group, instead of showing only the representative images. Due to the lack of control testing for this analysis, the data is more suggestive, rather than concrete. Therefore, statistical analysis was not performed on these data.

Statistics

      Data were analyzed using SPSS 18.0 for Windows (Chapter III) (IBM, USA) or GraphPad Prism 5.0 version for Windows (Chapters IV-VI) (San Diego, CA). A student's T test was conducted when only two groups were being compared. A one-way ANOVA was conducted when comparing more than two groups. A two-way ANOVA was used when determining an interaction between two variables for two or more groups and is noted in the figure legend (Chapter VI). Data are expressed as ±SEM. After significant omnibus ANOVA, follow-up comparisons were conducted using Fisher's least significant difference (LSD) post-hoc test (Chapter III) or Bonferonni's/Dunnett's post-hoc test (Chapters IV-VI, as listed).

CHAPTER III

Differential regulation of the ascorbic acid transporter SVCT2 during development and in response to ascorbic acid depletion

(Adapted from Meredith, *et al.* (2011). *BBRC.* Nov: 414 (4): 737-742.)

INTRODUCTION

In mammals that can make their own ASC, synthesis can be up-regulated when ASC requirements are increased, such as during pregnancy, due to the drain on maternal supply by the fetus [12, 82]. Because humans and primates have lost the ability to synthesize ASC, as mentioned in Chapter I, adequate dietary intake must occur to ensure optimal levels. However, many studies have shown populations including pregnant women, smokers, and people with diabetes have lower levels of circulating ASC [159, 163, 194] indicating that dietary insufficiency is still a concern in many cases. Protective mechanisms exist to preserve ASC in critical organs, including preferential retention in brain and ASC recycling [33, 195], but the regulation of these is not yet understood.

The experiments presented here were performed to better understand SVCT2 regulation. The regulation of SVCT2 across a range of ASC levels has not been studied *in vivo.* In Experiment 1, we investigated the expression pattern of SVCT2 through a critical period of brain development from embryonic day 15 to postnatal day 32. We expected ASC levels would increase significantly just before birth and return to baseline post-natally. SVCT2 expression levels were expected to follow this similar pattern. In Experiment 2, using adult Gulo(-/-) mice that are unable to synthesize ASC [14], we determined whether changes in tissue ASC level can regulate SVCT2 mRNA and protein *in vivo.* We expected low levels of tissue ASC would cause an increase in SVCT2 expression level. Table 3.1 lays out the experimental plan and the ages or supplementations at which the samples were collected. As a comparison for the brain tissue, liver samples were also collected and analyzed, since liver is the site of synthesis for wild-type mice.

Experiment 1 (Wild-Type)	Experiment 2 (Gulo-/-)
Age (day)	ASC Supplementation
E15.5	Wild-Type
E18.5	HIGH- 3.33 g/L
P1	STD- 0.33 g/L
P10	LOW- 0.033 g/L
P18	WATER- 0 g/L
P32	

Table 3.1. Chapter III experimental design. Experiment 1: Wild-type mice that can synthesize their own ASC were collected at embryonic days 15.5 and 18.5, and postnatal days 1, 10, 18, and 32. Experiment 2: Gulo(-/-) mice were placed on ASC supplements for 4 weeks beginning at four weeks of age as follows: High (HIGH- 3.33 g/L), Standard (STD- 0.33 g/L), Low (LOW- 0.033 g/L), and none (WATER, WTR- 0 g/L).

RESULTS

Experiment 1

Ascorbate content in brain during development

      Cortex ASC levels varied significantly depending on age ($p<0.001$, Figure 3.1A). ASC content was highest at postnatal day 1 (P1) and then decreased significantly, reaching average adult cortex ASC levels by P32. ASC also increased between embryonic day 15.5 (E15.5) and P1. The cerebellum had similarly high levels of ASC throughout development and ASC content was significantly decreased by P32 ($p<0.001$, Figure 3.1B). In contrast, the liver exhibited an opposite pattern of ASC content, which was to increase with age ($p<0.001$, Figure 3.1C). ASC levels were significantly lower in liver during embryogenesis than at older ages and maintained the higher level during postnatal development (P1-P32).

SVCT2 expression during development

      Brain SVCT2 mRNA levels were low and varied inversely with brain ASC content throughout embryogenesis and postnatal development. In the cortex, SVCT2 mRNA increased 5-fold by P32 ($p<0.01$, Figure 3.2A). A similar pattern in SVCT2 mRNA expression was seen in the cerebellum except that increased expression began earlier by P18, and a further increase at P32 ($p<0.001$, Figure 3.2B). The same developmental pattern of SVCT2 expression occurred in liver, where SVCT2 mRNA was low during embryogenesis and then dramatically increased postnatally ($p<0.001$, Figure 3.2C). In contrast to the brain, SVCT2 expression in liver mirrored ASC content instead of being inversely proportional.

      SVCT2 protein levels changed similarly to mRNA levels in the cortex, cerebellum, and liver (Figure 3.2D-F). SVCT2 protein increased with age by about 30-fold in cortex ($p<0.001$, Figure 3.2D) and cerebellum ($p<0.001$, Figure 3.2E) and almost 400-fold in liver ($p<0.001$, Figure 3.2F). Representative blots are shown for each tissue type.
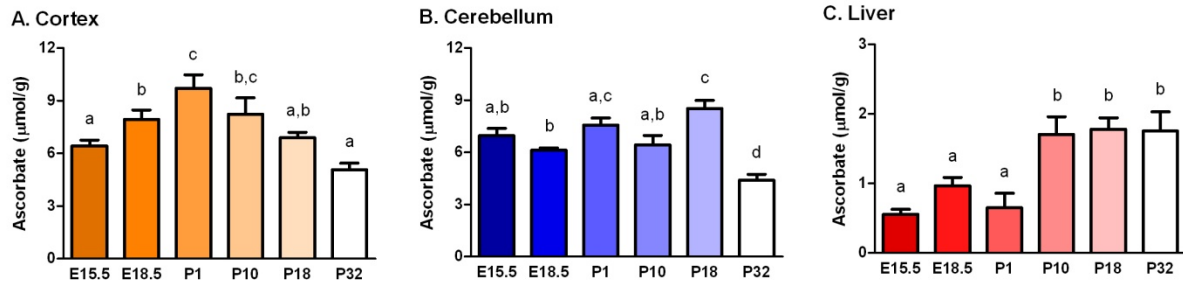
Figure 3.1. ASC content in WT brain and liver during development. ASC concentration in A) Cortex, B) Cerebellum, and C) Liver. Groups that do not share the same letter are different. p<0.05, n=4-6 samples per group
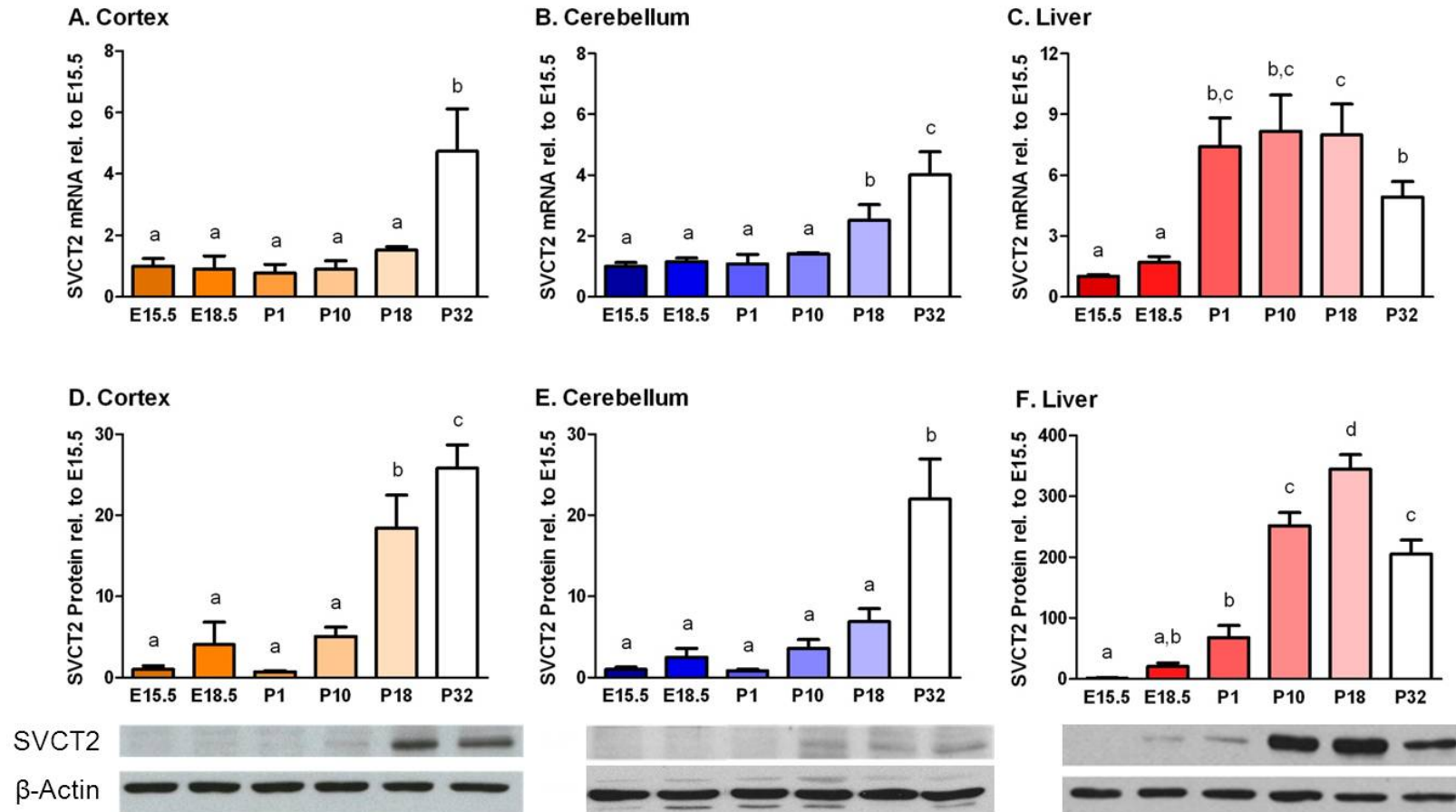
Figure 3.2: SVCT2 expression levels throughout WT development. SVCT2 mRNA in A) Cortex, B) Cerebellum, and C) Liver, n=3-6 samples per group. SVCT2 protein in D) Cortex, E) Cerebellum, and F) Liver, n=6 samples per group. Representative immunoblots are shown. Groups that do not share the same letter are different, p<0.05.

Experiment 2

In Experiment 1, SVCT2 expression followed an inverse relationship with ASC content in the brain but not liver. To further explore the *in vivo* regulation of SVCT2 by its own substrate, separate from any specific developmental processes, adult Gulo(-/-) mice were maintained on different levels of ASC supplementation (HIGH- 3.33 g/L, STD- 0.33 g/L, LOW- 0.033 g/L, WTR- 0.0 g/L) for 4 weeks prior to tissue acquisition.

ASC content in different ASC supplementation groups

As expected, tissue ASC content was directly related to the level of ASC supplementation in the drinking water. In cortex, ASC levels in each supplementation group were significantly different from each other (p<0.001, Figure 3.3A). In cerebellum, ASC levels were similar in the WT, HIGH, and STD groups, with a 5-fold decrease in the LOW group and negligible levels in the WTR group (p<0.001, Figure 3.3B). In the liver, the STD, LOW, and WTR groups had significantly lower ASC than the WT and HIGH groups (p<0.001, Figure 3.3C).

Oxidative stress in different ASC supplementation groups

There was a small increase in oxidative stress in the cortex in the LOW and WTR groups as measured by MDA (Figure 3.4A), although only the WTR group had significantly greater MDA than the WT, HIGH, and STD groups (p<0.05). There was no difference in lipid peroxidation among supplementation groups in the cerebellum (p=0.37) or liver (p=0.29).
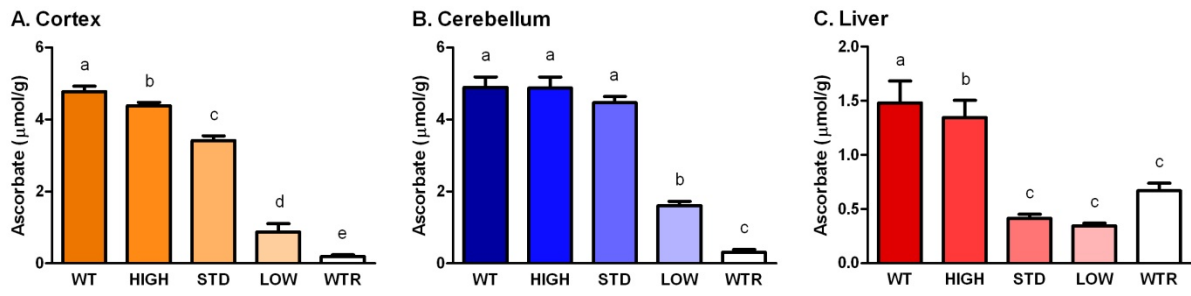
Figure 3.3: ASC content in adult Gulo(-/-) mice on different ASC supplements. ASC concentration in A) Cortex, B) Cerebellum, and C) Liver. Groups that do not share the same letter are different. $p < 0.05$, n=4-7 samples per group
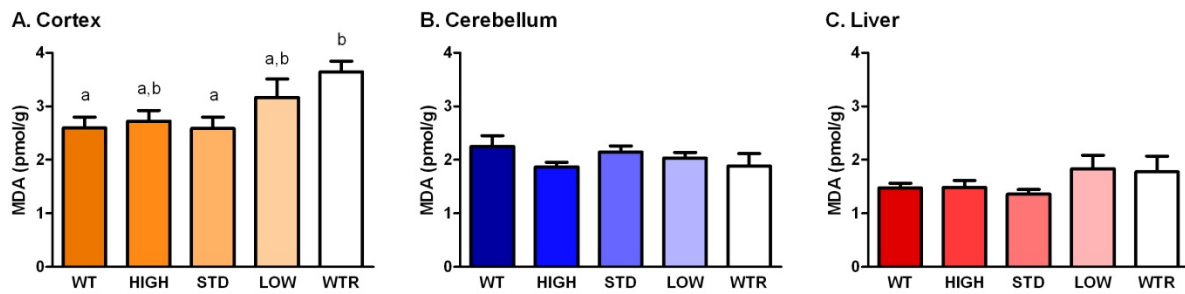
Figure 3.4: MDA content in adult Gulo(-/-) mice on different ASC supplements. MDA levels in A) Cortex, B) Cerebellum, and C) Liver. Groups that do not share the same letter are different, $p < 0.05$, n=5-7 samples per group

SVCT2 expression in different ASC supplementation groups

There were no significant differences among the groups in either of the brain areas in SVCT2 mRNA levels (p's>0.34 Figure 3.5A-B). In the liver, SVCT2 mRNA expression was greater in the lower ASC groups (Figure 3.5C) with the greatest increase in the WTR group compared to WT ($p<0.05$).

There were no significant differences in SVCT2 protein in cortex (p=0.243, Figure 3.5D). Although, there was a strong trend toward elevated SVCT2 protein levels in the cerebellum of the WTR group compared to the WT, HIGH, and STD groups (p=0.06, Figure 3.5E), the omnibus ANOVA was not significant. Similarly, although the liver exhibited a 3-fold increase in the amount of SVCT2 protein in the WTR group compared to WT, the difference did not reach statistical significance (p=0.056, Figure 3.5F). Representative blots are also shown.
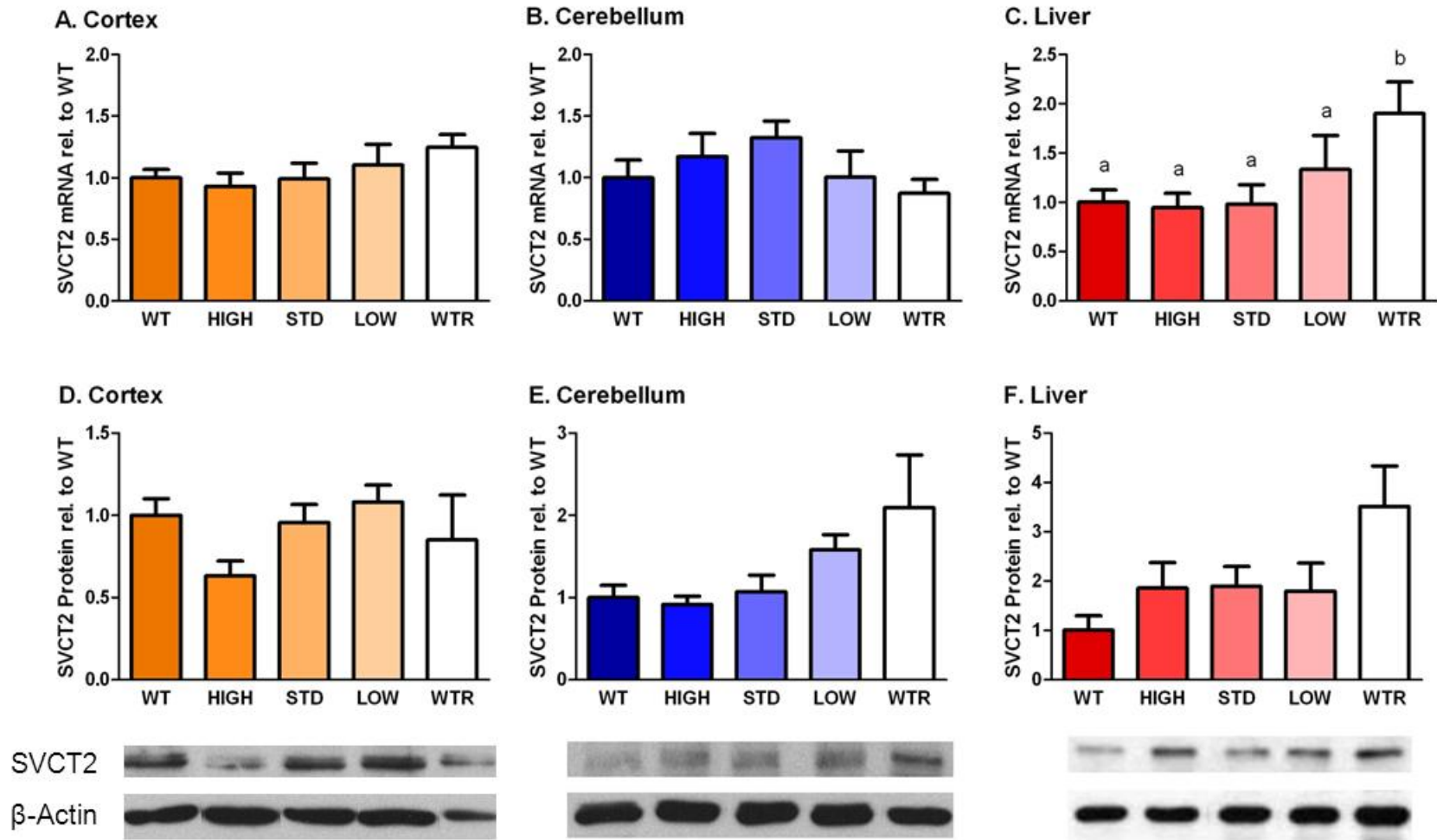
Figure 3.5: SVCT2 expression levels in adult Gulo(-/-) mice on different ASC supplements. SVCT2 mRNA in A) Cortex, B) Cerebellum, and C) Liver. SVCT2 protein in D) Cortex, E) Cerebellum, and F) Liver. Representative immunoblots are shown. Groups that do not share the same letter are different, $p < 0.05$, n=5-7 samples per group

DISCUSSION

The findings presented here are summarized in Figure 3.6. The SVCT2 is thought to determine ASC content in brain and therefore the finding of an inverse relationship between ASC content in the cortex and cerebellum and the pattern of SVCT2 expression in developing mice was unexpected. ASC levels were high throughout embryogenesis and decreased postnatally, similar to previous reports (Figure 3.6A) [37, 196]. However, the relative SVCT2 mRNA and protein expressions were lower than expected in brain at early stages in development, raising the question of how ASC becomes concentrated in embryonic brain tissues. ASC accumulation is unlikely to be via SVCT1, which is not substantially expressed in brain [38]. It is also unlikely that an unknown ASC transporter is present or that uptake of ASC occurs as its oxidized form DHA on glucose transporters, since mice that lack SVCT2 have negligible levels of ASC in the brain [11, 82]. ASC levels may reflect changing cellular distribution in the brain with relatively higher ASC during periods of neurogenesis in the embryo compared to early postnatal life when gliogenesis occurs at a greater rate [37]. Glial cells have been shown to lack the SVCT2 *in vivo* [197] and thus have lower ASC levels than SVCT2-expressing neurons [198]. Although this distinction does not help determine the mechanism behind disproportionate ASC accumulation, it is apparent that even the relatively low levels of SVCT2 are adequate to maintain the high ASC levels in cortex and cerebellum during embryogenesis.

The increase in postnatal SVCT2 expression mirrors that seen with the brain glucose transporters 1 & 3, which are low throughout embryogenesis and increase during postnatal development, reaching adult levels by postnatal day 30 [199, 200]. This coincides with neuronal maturation, suggesting regulation of the SVCT2 during development. The increase in SVCT2 mRNA and protein during postnatal development could thus be due to the increase in neurogenesis. However, the increase in SVCT2 is exponential after postnatal day 18, after the majority of neurogenesis has occurred [201]. There is also substantial growth of the brain vasculature after birth, but increased expression of the SVCT2 in endothelial cells cannot explain its increased postnatal expression, since endothelial cells of the BBB do not express the SVCT2 *in vivo* under basal conditions [202]. The kinetics of SVCT2 could also be variable at the different

developmental stages in the three tissues to allow for increased ASC transport without increased expression, as this has been shown before [46]. This would explain the difference in ASC levels in the cortex versus the cerebellum. Lastly, it is also possible that increased oxidative stress present after birth may decrease ASC content with age and also up-regulate the SVCT2. This possibility formed the basis for Experiment 2, in which we tested directly the likelihood that ASC depletion and oxidative stress levels in brain regulate SVCT2 expression in adult Gulo(-/-) mice with varying dietary intakes of ASC.

As expected, dietary ASC depletion in the adult LOW and WTR groups decreased ASC content in both brain regions and liver (Figure 3.6C-D). These two dietary groups also showed only very minimal increases in lipid peroxidation in cortex, and none in cerebellum or liver. This suggests that ASC depletion to this degree does not cause substantial oxidative stress in these tissues, at least following the short period of deprivation studied here. There must be extensive antioxidant defense mechanisms in place that compensate for the decreased ASC levels. Previously we showed that even the SVCT2(-/-) embryo with very low brain ASC had only modest and localized increases in oxidative stress markers of lipid peroxidation, which supports this notion [82].

Our finding that dietary ASC depletion in Gulo(-/-) mice did not affect SVCT2 mRNA expression in brain cortex or cerebellum agrees with the results of a recent study of SVCT2 regulation in whole brain by dietary ASC depletion in another mouse model that is similarly unable to synthesize ASC (SMP30/GNL) [203]. This group found that removal of ASC from the diet did not affect SVCT2 mRNA levels in total brain, although SVCT2 protein expression was not measured. In the present study, however, SVCT2 protein (but not mRNA) expression was up-regulated in the most extreme two ASC-depleted groups in cerebellum. In addition, it is possible the amount of transporter located at the cell membrane could account for differences in ASC level in the brain. These data suggest that the SVCT2 is differentially regulated in the two tissues, although the mechanisms underlying this difference are still not clear. Again, it is likely there is a difference in antioxidant capacity in these two tissues, as there were differences in oxidative stress in the cortex compared to the cerebellum.

In contrast to brain, ASC content and SVCT2 expression increased with age in developing liver in normal mice (Figure 3.6B). The increase in liver ASC may thus be explained in part by increased SVCT2 expression, but probably more so by the fact that WT fetuses can synthesize their own ASC by embryonic day 15 [204]. In the adult Gulo(-/-) mouse, ASC levels were significantly decreased in the STD supplementation group (Figure 3.6D). This supplementation typically provides WT tissue ASC levels [14]. It is interesting the liver was not able to store ASC in this group at this STD level. Despite the fact that ASC depletion increased both SVCT2 mRNA and protein levels in liver, similar to results observed Amano and colleagues for SVCT2 mRNA [203], ASC levels were not increased. Perhaps the ASC was shunted to the brain in order to provide as much ASC as possible. In the liver, there are additional considerations that do not apply to brain. The liver also expresses the SVCT1 and the exact contribution of each transporter to liver ASC homeostasis has not yet been delineated. SVCT1 mRNA was also measured in our study, with increases in the adult Gulo(-/-) on water supplementation compared to WT and LOW mice (Appendix A1). SVCT2 is thought to transfer ASC from the blood stream into cells. Accordingly, the increase in SVCT2 in response to decreasing liver ASC levels in adult Gulo(-/-) mice seems logical. In contrast, if the same relationship observed in the adult Gulo(-/-) mice held true for developing pups it would predict that SVCT2 would be higher during the embryonic stages of development when all ASC for the liver must be obtained from maternal supply across the placenta. Once pups begin to synthesize their own ASC at the later embryonic stages, the contribution of the SVCT2 might become less significant in the liver. The fact that this is not the case, as reported here, is further indication that additional or alternative mechanisms are in place to supply fetal tissues with ASC, presumably reflecting the importance of maintaining high ASC levels during development.

The results of this study highlight the regulation of ASC and SVCT2 expression in both brain and liver (Figure 3.6). ASC levels are high throughout embryogenesis in the brain and decrease with age, while in the liver they are lower *in utero* and increase postnatally. The high level of ASC in the brain during development reinforces the critical importance of the vitamin at this time. SVCT2 mRNA and protein in the cortex and

cerebellum are low throughout the late gestational developmental stage and increase with age, while liver expression follows a similar pattern, with declines in SVCT2 mRNA and protein by postnatal day 32. In the adult Gulo(-/-) mice, varying tissue ASC content did not alter SVCT2 mRNA or protein levels in the cortex but SVCT2 protein was increased by low tissue ASC levels in the cerebellum and liver. Liver SVCT2 mRNA was also increased with low ASC. These data suggest an alternate regulation mechanism of SVCT2 mRNA and/or protein expression in different tissues to allow for enhanced ASC transport and prevention of oxidative stress.
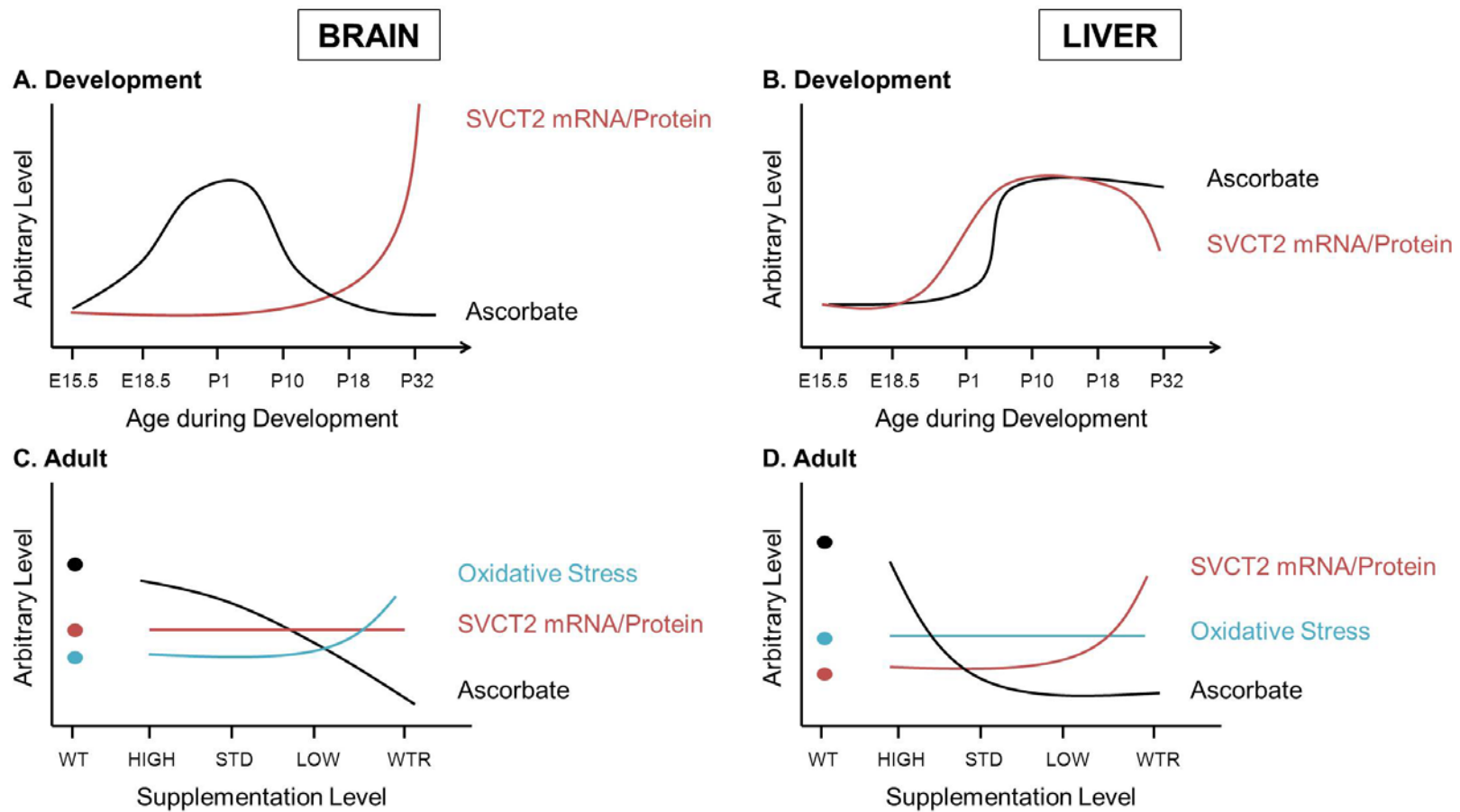
Figure 3.6. Summary model of Chapter III. During brain development, A) ASC levels increase just before birth, returning to baseline postnatally. SVCT2 expression levels increase significantly by P32. B) Liver ASC levels increase by P10 whereas SVCT2 expression precedes this by increasing at P1. C) In adults, ASC level does not affect SVCT2 expression level in brain, despite the increase in oxidative stress. D) Liver SVCT2 is increased due to the decrease in ASC.

CHAPTER IV

**Regulation of embryonic neurotransmitter and tyrosine hydroxylase protein
levels by ascorbic acid**

(Adapted from Meredith, *et al.* (2013). *Brain Research*. Nov: 1539: 7-14.)

INTRODUCTION

As discussed in Chapter III, ASC is highly concentrated and regulated in the
brain, especially during development, suggesting an important role for it in this organ at
this time [21, 37, 83]. This chapter describes in more detail the role for ASC in
neurotransmitter regulation in the developing brain. As mentioned in the introduction,
ASC plays a role in neurotransmitter synthesis by recycling tetrahydrobiopterin, BH_4,
required for tyrosine and tryptophan hydroxylases, and acts as a co-factor for dopamine
β-hydroxylase (Figure 1.3).

Mice lacking the main ASC transporter in the brain, SVCT2(-/-), survive gestation
but do not survive past birth [11]. In attempting to determine whether decreases in brain
catecholamines caused the death of SVCT2(-/-) mice, Bornstein *et al* failed to show
significant decreases in whole brain dopamine and norepinephrine levels in late stage
SVCT2(-/-) embryos, although they did find significant decreases in the adrenal medulla
[205]. The latter result was confirmed in adult mice unable to synthesize their own ASC
that underwent systemic ASC deficiency due to dietary ASC depletion, although effects
in brain were not reported [206]. In adult mice unable to synthesize their own ASC,
Gulo(-/-) mice, dietary ASC depletion decreased cortex and striatum levels of the
serotonin metabolite, 5-hydroxyindole acetic acid, as well as dopamine metabolites in
the cortex [185]. Thus there is evidence, albeit incomplete, that ASC is required for
neurotransmitter maintenance in some tissues.

In the current study, we investigated the effects of cellular ASC deficiency and
excess on brain neurotransmitter synthesis and tyrosine and tryptophan hydroxylase
protein expression in embryonic brain cortex. To do this, we compared embryonic
SVCT2(-/-) mice to their (+/+) and (+/-) littermates as a model of ASC deficiency and a
transgenic mouse with increased levels of SVCT2 and ASC compared to wild-type (WT)

embryos as a model of a modest excess ASC [13]. We hypothesized loss of ASC in the SVCT2(-/-) embryos would decrease levels of dopamine, norepinephrine, and serotonin. This loss of ASC would also decrease tyrosine and tryptophan hydroxylase levels. On the other hand, it was expected excess ASC would increase levels of neurotransmitters and tyrosine and tryptophan hydroxylase enzyme levels.

RESULTS

ASC levels in brain cortex

ASC was virtually absent in the cortex of embryos lacking the SVCT2 and approximately 40% lower in SVCT2(+/-) embryos (p<0.0001, Figure 4.1A). SVCT2-TG embryo cortex, on the other hand, have approximately 350% more ASC than WT embryos (p<0.0001, Figure 4.1B).

Catecholamine Levels

The complete absence of ASC in the cortex of SVCT2(-/-) embryos caused a modest 30% decrease in cortex norepinephrine and dopamine contents (p<0.05, Figure 4.2A-B). However, there was no change in the dopamine metabolites, DOPAC and homovanillic acid (p's>0.169, Figure 4.2C-D). There was no significant difference between the SVCT2(+/+) and SVCT(+/-) groups in norepinephrine, dopamine, DOPAC, or homovanillic acid despite a modest 10% decrease in dopamine and homovanillic acid, in the SVCT(+/-) group.

Increasing cortex ASC levels above normal by overexpressing SVCT2 (SVCT2-TG) led to a 250% increase in dopamine and 400% increase in DOPAC (p<0.001, Figure 4.3B-C). However, there was no significant change in norepinephrine or homovanillic acid (p's>0.18, Figure 4.3A,D).
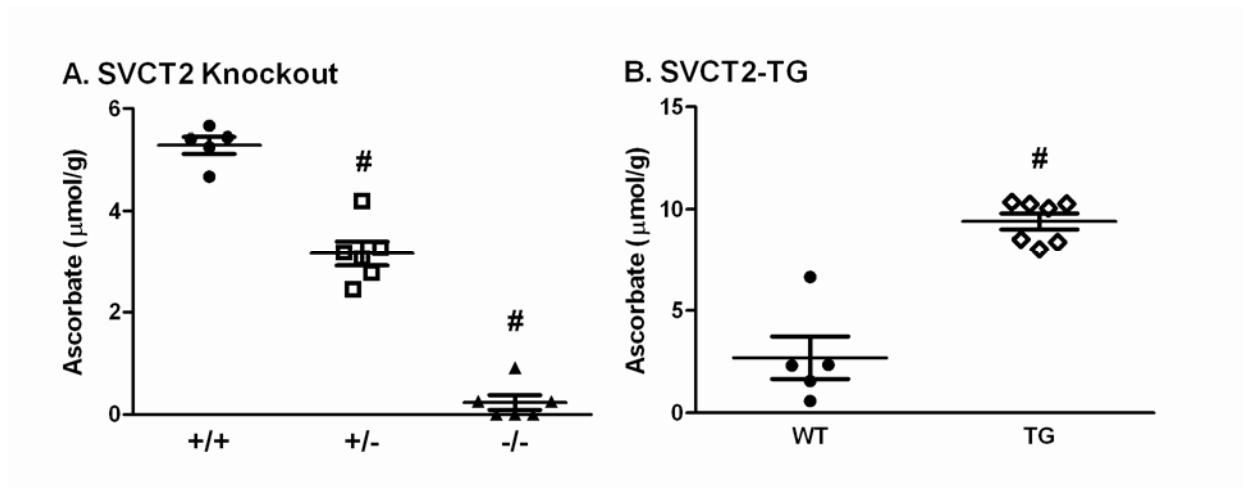
Figure 4.1. Cortex ASC levels in late gestation embryos. Cortex ASC levels in A) SVCT2(+/+), (+/-), and (-/-) embryos and B) WT and SVCT-TG embryos. [#]$p<0.0001$ versus other groups
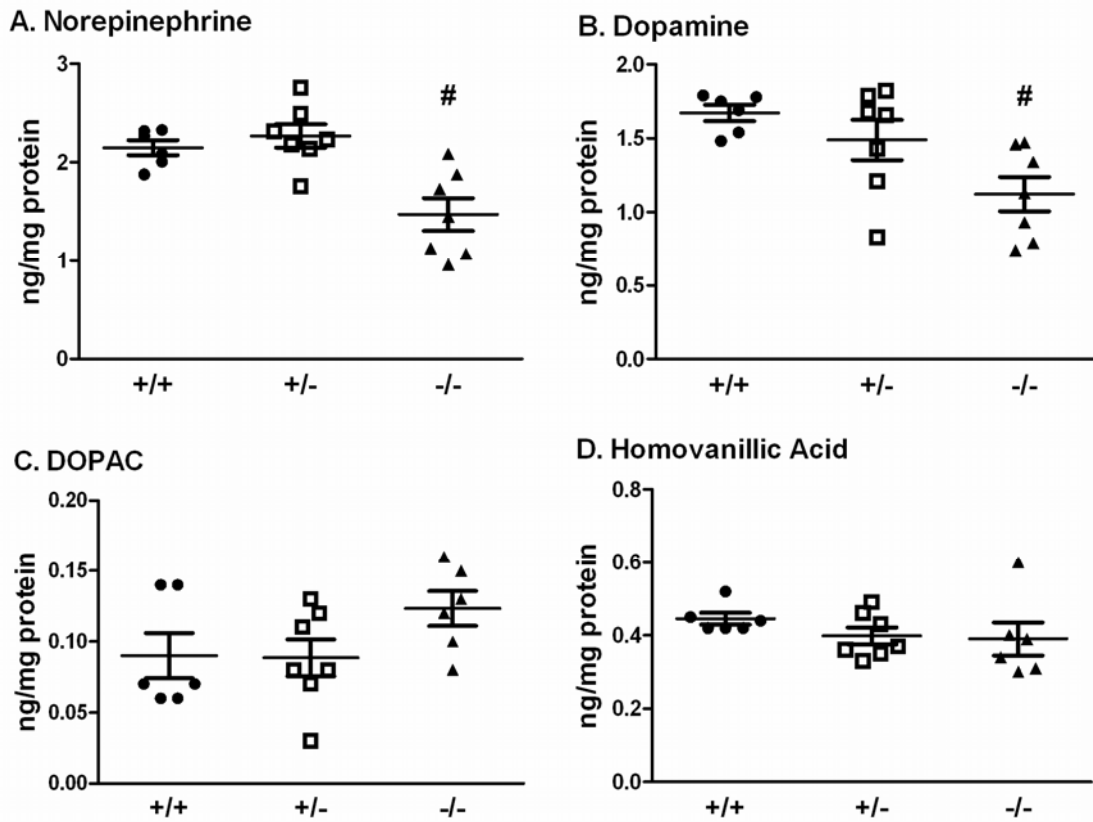
Figure 4.2. Catecholamine levels in ASC deficient embryos. Cortex A) Norepinephrine, B) Dopamine, C) DOPAC, and D) Homovanillic acid in SVCT2(+/+), SVCT2(+/-), and SVCT2(-/-) embryos. [#]p<0.05 versus other groups
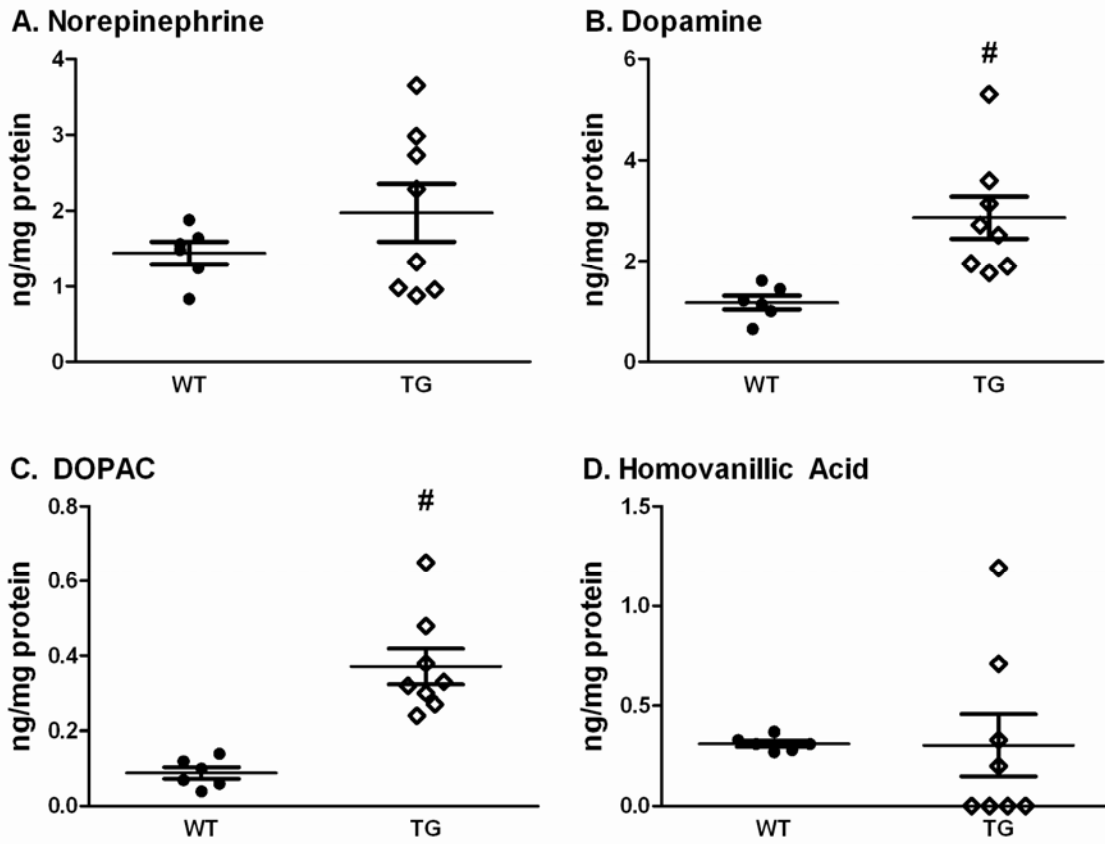
Figure 4.3. Catecholamine levels in embryos with excess ASC. Cortex A) Norepinephrine, B) Dopamine, C) DOPAC, and D) Homovanillic acid in SVCT2-TG embryos compared to WT. [#]p<0.01 versus WT controls

Serotonin Levels

The loss of cortex ASC, in either the SVCT2(+/-) or SVCT2(-/-) groups, did not affect serotonin levels or its metabolite, 5-HIAA ($p's > 0.17$, Figure 4.4A-B). However, increases in cortex ASC levels in SVCT2-TG embryos led to significant 200-300% increases in serotonin and 5-HIAA compared to WT embryos ($p < 0.0001$, Figure 4.4C-D).

Enzyme Protein Expression Levels

Severe depletion of cortex ASC in the SVCT2(-/-) embryos led to an approximate 25% decrease in tyrosine hydroxylase protein level ($p < 0.05$, Figure 4.5A). However, the 40% decrease in ASC in the SVCT2(+/-) did not alter tyrosine hydroxylase protein level in this group. There were no changes in tryptophan hydroxylase protein ($p = 0.83$, Figure 4.5B), in agreement with lack of changes in the serotonin system in these embryos (Figure 4.4A-B).

However, in contrast to increased neurotransmitter levels in SVCT2-TG embryos, increased cortex ASC levels in the SVCT2-TG did not alter the levels of tyrosine hydroxylase or tryptophan hydroxylase protein expression ($p's > 0.17$, Figure 4.5C-D).
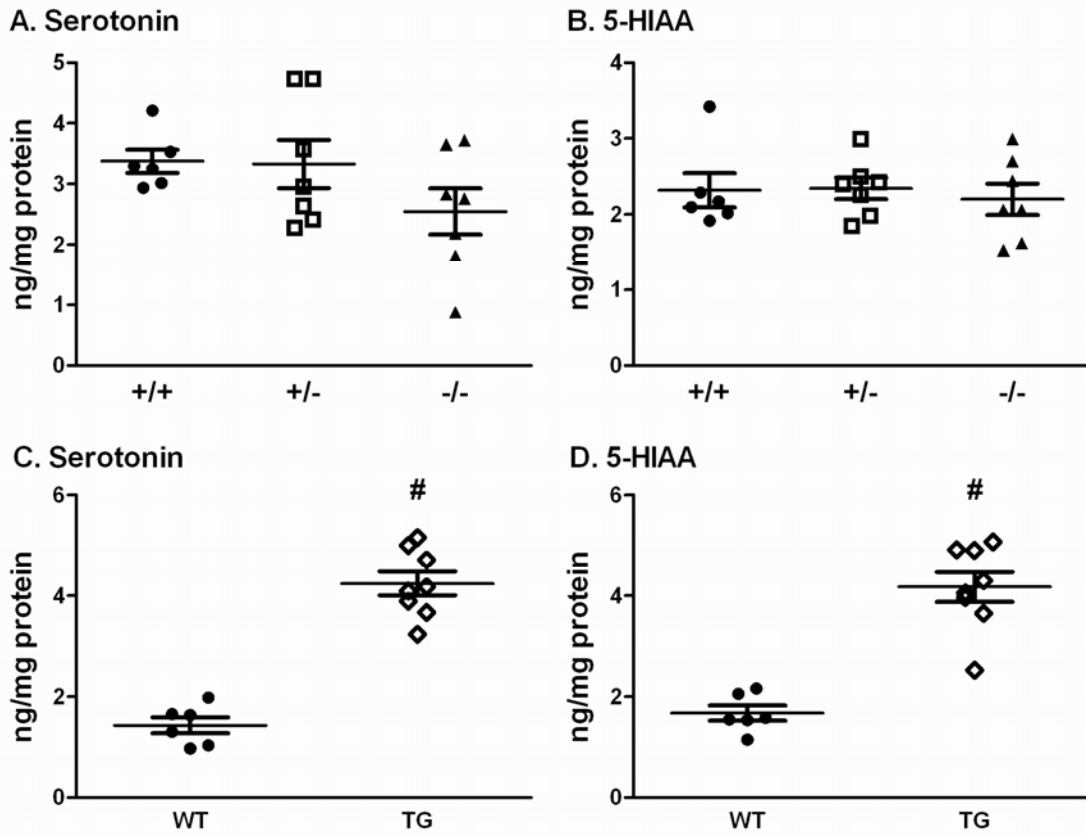
Figure 4.4. Serotonin and metabolite levels. Cortex A,C) Serotonin and B,D) 5-HIAA levels in A-B) SVCT2(+/+), (+/-), and (-/-) embryos and in C-D) WT versus SVCT2-TG embryos. [#]p<0.0001 versus WT controls
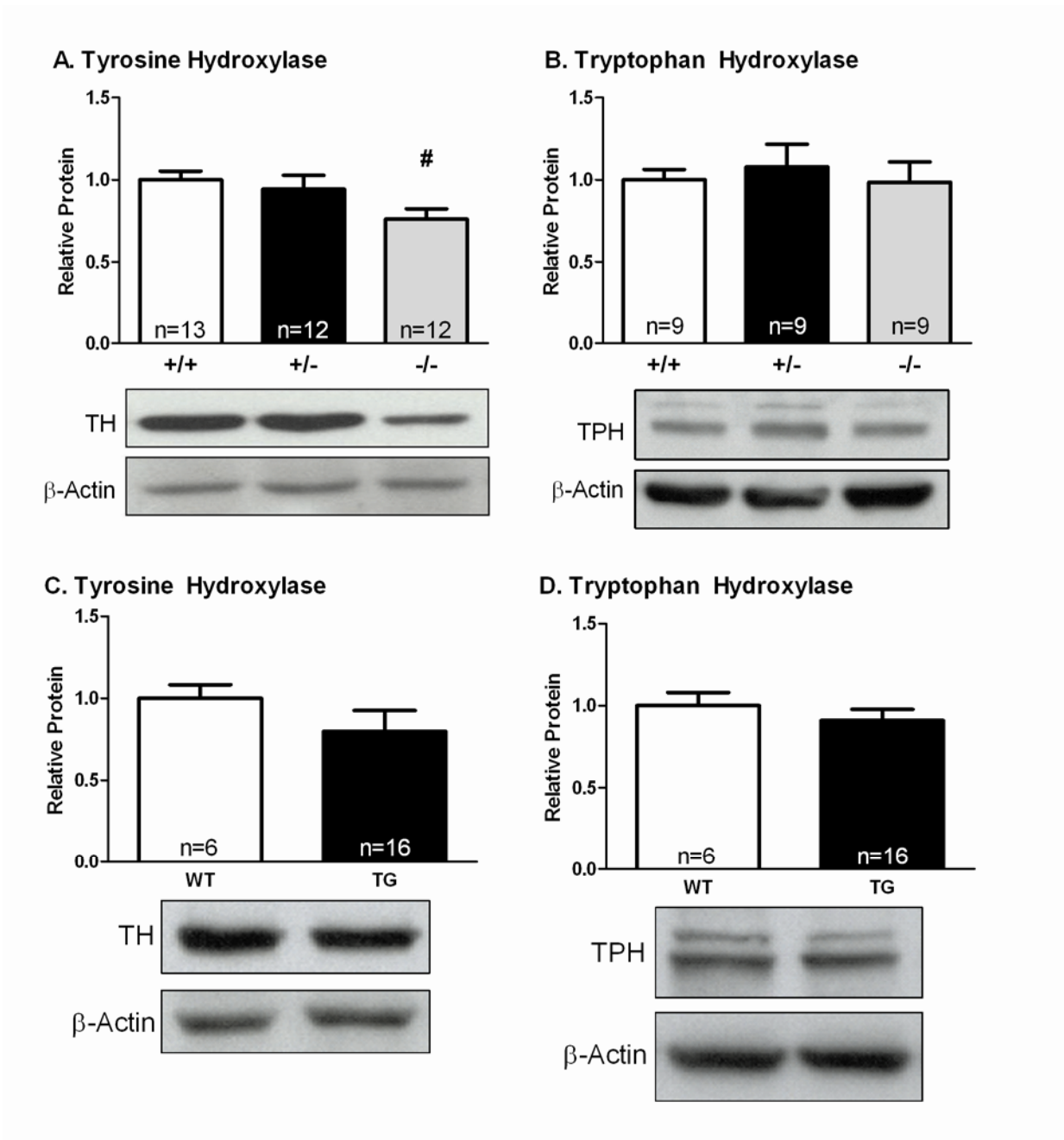
Figure 4.5. Tyrosine and tryptophan hydroxylase protein levels. Cortex A,C) tyrosine hydroxylase (TH) and B,D) tryptophan hydroxylase (TPH) protein levels in A-B) SVCT2(+/+), (+/-), and (-/-) embryos and C-D) WT versus SVCT2-TG embryos. Representative immunoblots are shown. [#]p<0.05 versus other groups

DISCUSSION

The results of this study show that ASC can regulate neurotransmitter levels in embryonic brain cortex as summarized in Figure 4.6. Severe ASC depletion in SVCT2(-/-) mouse embryos caused modest but significant decreases in levels of norepinephrine and dopamine (Figure 4.6, top panel, left). Due to this experimental group having negligible cortex ASC levels and thus, receiving the most stress on the system, there could be differences in compensation amongst the embryos with a minimal change in ASC level, such as in the SVCT2(+/-) embryos. Bornstein *et al* previously reported only trends for decreased levels of norepinephrine and dopamine in the whole brains of SVCT2(-/-) embryos [205]. Our finding of significant decreases in these two neurotransmitters in the same embryonic mouse model is most likely due to the measurement of levels specifically in cortex, rather than whole brain. If so, then even greater differences might be observed in studies focused on areas rich in specific neurotransmitters such as the striatum, which is abundant in dopamine. In addition, ASC is known to be differentially sequestered in the various cell types within the brain [198]. It should be noted that a 40% decrease in ASC level in SVCT2(+/-) embryos did not affect neurotransmitter levels significantly, indicating that a more severe ASC deficiency is required. This data also suggests there are other mechanisms regulating for neurotransmitter synthesis, such as factors present that can also recycle tetrahydrobiopterin.

The decreased ASC levels in the SVCT2(-/-) embryos also caused a modest (25%) but significant decrease in tyrosine hydroxylase protein levels, which could have contributed to the decreases in both dopamine and norepinephrine observed (Figure 4.6, top panel, left). This finding agrees with previous results in cultured neuronal cells which showed the opposite, in that ASC supplements to cells lacking ASC increased tyrosine hydroxylase mRNA [30] and protein [31]. The mechanism by which ASC regulates tyrosine hydroxylase protein expression remains to be elucidated. If tetrahydrobiopterin levels are decreased due to loss of recycling by ASC, its co-factor, tyrosine hydroxylase expression might be down-regulated. On the other hand, since tyrosine hydroxylase protein was not increased in the SVCT2-TG embryos, excess ASC does not appear to regulate tyrosine hydroxylase protein expression in this context

(Figure 4.6, bottom panel, left). The finding that increased cortex ASC in SVCT2-TG embryos did increase dopamine and DOPAC levels supports the notion that most of the ASC effect is related to its known ability to acutely activate tyrosine hydroxylase by preserving tetrahydrobiopterin.

Despite significant increases in dopamine and its metabolite, DOPAC, in the brain cortex of SVCT2-TG embryos, norepinephrine levels were not affected (Figure 4.6, bottom panel, left). Dopamine β-hydroxylase protein levels were measured in the SVCT2(+/-) embryos and were unaltered (Appendix A2). However, they were not measured in the SVCT-TG embryos. Failure to see an increase in norepinephrine could reflect saturation of the dopamine β-hydroxylase by normal amounts of intracellular ASC. The apparent K_m of the enzyme for ASC is 0.5-0.6 mM when dopamine conversion to norepinephrine is measured [207]. Intracellular dopamine concentrations are known to be in the millimolar range [208], so the enzyme would be saturated even at normal ASC concentrations of 2-10 mM [21]. This could in turn cause the observed selective increases in dopamine and DOPAC in the SVCT2-TG embryos without changes in norepinephrine.

Regarding the effects of ASC on the serotonergic system, Ward *et al* found in the adult ASC-depleted Gulo(-/-) mouse model, serotonin and 5-HIAA were significantly decreased in the striatum, but only 5-HIAA was modestly decreased in cortex [185]. In the present study, the spread of the data was too variable to detect changes in serotonin and 5-HIAA in the SVCT2(-/-) embryos (Figure 4.6, top panel, right). It is important to take into consideration differences in litter variation and compensation that could account for the variability of the SVCT2(-/-) embryos. Due to the lack of differences in the serotonergic system in the SVCT2(-/-) embryos, there appear to be adequate amounts of tetrahydrobiopterin to maintain activity of tryptophan hydroxylase in the absence of ASC during development. This is in contrast to the catecholamine system and could be due to tetrahydrobiopterin recycling by another reducing agent such as glutathione in the serotonergic neurons. On the other hand, increased brain cortex ASC in the SVCT2-TG mice also increased levels of both serotonin and 5-HIAA (Figure 4.6, bottom panel, right). If ASC increases tryptophan hydroxylase activity only by recycling tetrahydrobiopterin, then this leads to the conclusion that presumably

normal cortex tetrahydrobiopterin levels are sub-optimal for tryptophan hydroxylase activation. Measuring tetrahydrobiopterin levels using the different ASC models would help to explain this rationale.

It is possible the effects on neurotransmitter level and protein synthesis observed in this embryonic model of ASC deficiency and excess could be due to the role of ASC in cell differentiation and proliferation as described previously [209-212]. ASC, along with other important factors, has been shown to aid in differentiation of dopaminergic and serotonergic cells from embryonic stem cells [209]. Yan *et al* showed ASC acts in a non-antioxidant manner to induce dopaminergic cell differentiation and that ASC is able to increase dopaminergic cell number by greater than 10-fold compared to cAMP or forskolin [210]. These findings on the role of ASC in cell differentiation suggest the loss of ASC in the SVCT2(-/-) embryos could simply reduce the number of dopaminergic and serotonergic neurons, leading to the observed decreases in neurotransmitter and enzyme levels. However, the values of neurotransmitters and enzyme protein levels reported here are expressed per milligram protein, which would take into consideration total cell number. To be confident cell number is not varying amongst the genotypes, future studies should be performed to measure the number of proliferating cells and more specifically, the number of dopaminergic and serotonergic cells in the ASC-depleted and -excess developmental models.

Despite the corollaries described above, the ASC-dependent increases in dopamine and serotonin systems could be of physiologic relevance during development of these neurotransmitter systems in the human fetus both before and after birth. Changes in these systems during development could affect neuronal interactions later in life, and into adulthood. The results gained from this study prompt further investigation of the role for ASC during development.

Although severe ASC deficiency is required to impair the dopaminergic and serotonergic systems during development, the increased levels of dopamine and serotonin due to increases in intracellular ASC support the notion that ASC can regulate neurotransmitter synthesis. Careful monitoring of ASC plasma levels could be beneficial when treating diseases in which there is an imbalance of neurotransmitters, such as in persons with altered mood or depression. Indeed, plasma ASC levels are decreased in

some patients with depression and ASC has recently been shown to improve mood and depression in humans [213-215]. These studies highlight the significant relationship between ASC and neurotransmitter imbalances.
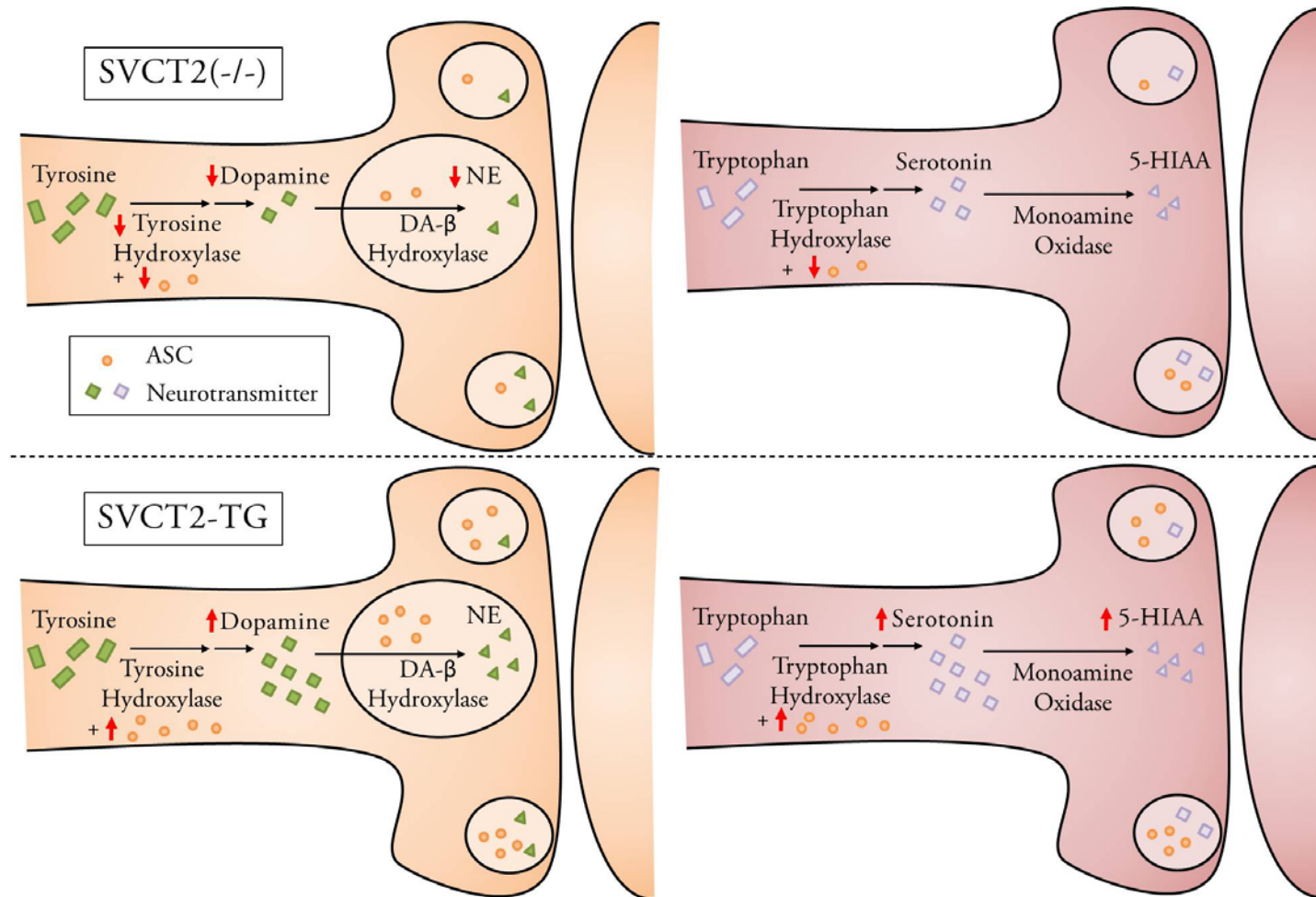
Figure 4.6. Summary model of Chapter IV. Decreased ASC levels concomitantly decrease dopamine, norepinephrine and tyrosine hydroxylase protein levels (top panel). There is no effect on dopamine (DA) β-hydroxylase protein levels or an effect on the serotonin system. On the other hand, increased ASC levels increase dopamine, serotonin, and 5-HIAA levels (bottom panel) without an effect on tyrosine hydroxylase protein levels. Changes in levels due to ASC are highlighted with a red arrow.

CHAPTER V

ASC reverses the high glucose- and RAGE-induced permeability, while signaling through Epac1 and the cytoskeleton under basal glucose conditions to decrease BEC permeability

(Partly adapted from Meredith, *et al.* (2014). *BBRC.* Feb: 445(1):30-5.)

INTRODUCTION

High glucose has been shown to disrupt the brain endothelial cell (BEC) barrier in cell models, rodents, and humans [130, 134, 157, 216-218]. An emerging area in the field is to investigate potential mechanisms as to how diabetes is able to disrupt the BBB, including activation of RAGE, increased oxidative stress, and PKC/PKA activation [143-145]. RAGE activation in particular triggers downstream signaling pathways to increase retinal and peripheral endothelial cell barrier permeability through cytoskeleton rearrangement [219-221]. In addition, our lab has previously shown ASC decreases permeability in peripheral endothelial cells under basal conditions [113, 114, 193].

In this study, I show ASC supplementation decreases permeability of BECs under basal conditions, as well as under high glucose conditions. RAGE activation increases BEC permeability, and both ASC and other antioxidants are able to prevent this increase. In addition, to further investigate the molecular mechanism behind the ability of ASC to tighten the endothelial barrier under basal conditions, I examined both intracellular oxidative stress and phosphorylation of occludin and VE-cadherin. As expected, ASC decreases intracellular oxidative stress. However, through the use of antibodies specific for phosphorylated residues on occludin and VE-cadherin, there was no effect of ASC on phosphorylation. Rather, through the use of pharmacological inhibitors, we found ASC signals through Epac, not PKA, and the cytoskeleton, to tighten the monolayer of BECs. This occurred even though ASC did not affect the activity of the downstream target for Epac, Rap1. In all, these studies show novel signaling mechanisms for the regulation of BBB permeability by increased intracellular ASC.

RESULTS

Effect of high glucose on ASC transport and BEC permeability

ASC accumulation in bEnd.3 cells

Although the ASC content of bEnd.3 cells in culture was very low initially, transport and retention of both DHA and ASC was observed in a dose-dependent manner after 60 min of incubation (Figure 5.1). DHA is normally used for loading cells with ASC as it allows for a more rapid increase in intracellular ASC and insures that extracellular ASC concentrations remain very low. This avoids the risk of redox cycling of ASC with free iron or copper in the culture medium. Intracellular ASC increased at 100 μM DHA and above, but the increase was not as great under high glucose conditions ($p < 0.05$, Figure 5.1A). In addition, intracellular ASC concentrations were significantly greater at 5 mM glucose and 25 mM glucose when incubated with 200 μM ASC ($p < 0.05$, Figure 5.1B).

ASC and glucose effect on permeability

BEnd.3 cell monolayer permeability to [carboxyl-^{14}C] inulin was increased by 50% following culture of the cells under high glucose conditions (Figure 5.2). When treated with 0-200 μM DHA ($p < 0.05$, Figure 5.2A) or 200 μM ASC ($p < 0.05$, Figure 5.2B), permeability was significantly decreased, independent of the glucose concentration. Although intracellular ASC levels differ depending on the form of vitamin C used to load the cells (Figure 5.1), decreases in permeability were similar under basal glucose conditions with either form (0.0024 cm/h for DHA and 0.0026 cm/h for ASC). Since BECs would normally be exposed to much greater ASC than DHA concentrations *in vivo*, subsequent experiments were carried out using ASC to load the cells.
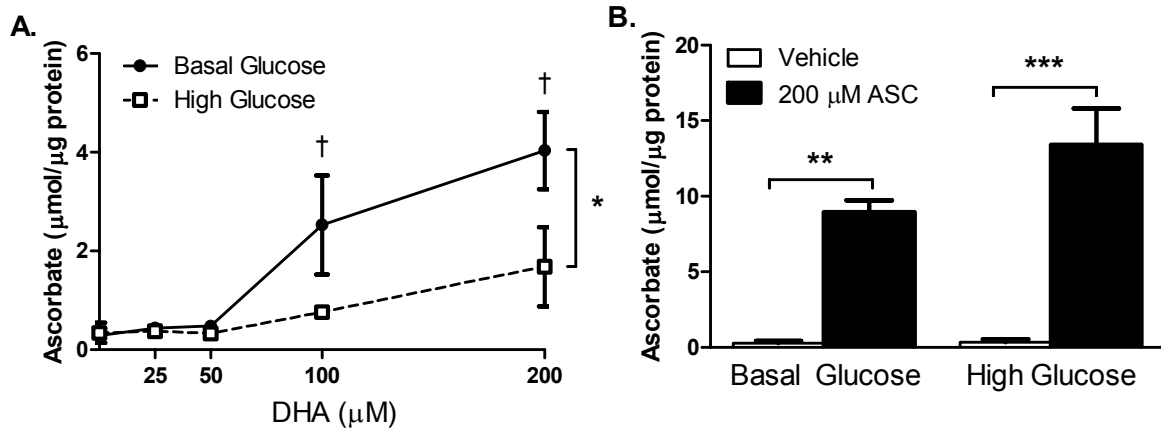
Figure 5.1. BECs transport and store ASC after DHA or ASC incubation. bEnd.3 cells were incubated with increasing concentrations of A) DHA (0-200 µM) or B) ASC (200 µM) for 60 min. Cells were cultured chronically in basal (5 mM) or high (25 mM) glucose with equal parts astrocyte-conditioned medium. A) Two-way ANOVA; interaction, $F=2.686$, $p=0.061$; effect of DHA, $F=10.66$, $p<0.0001$; effect of glucose, $F=7.882$, $p=0.0109$. Bonferonni post-*hoc* analysis: Basal versus High glucose *$p<0.05$, vehicle versus 100 µM or 200 µM DHA [†]$p<0.05$. B) Two-way ANOVA; interaction, $F=3.014$, $p=0.121$; effect of ASC, $F=73.26$, $p<0.0001$; effect of glucose, $F=3.147$, $p=0.114$. Bonferonni post-*hoc* analysis: vehicle versus ASC **$p<0.01$, ***$p<0.0001$. n=3 for each panel.
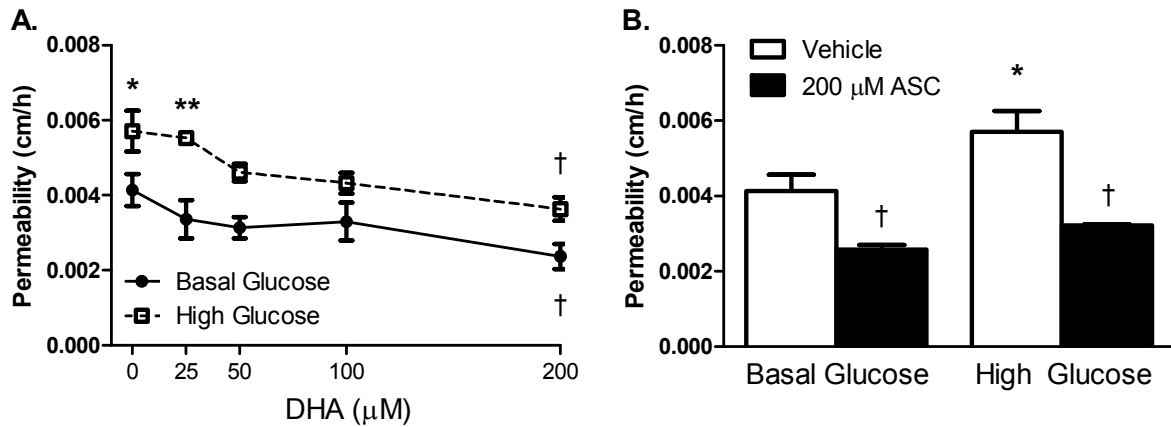
Figure 5.2. Permeability decreases with DHA or ASC incubation. bEnd.3 cells were plated on semi-porous inserts and grown to confluence for 6-7 days. Cells were incubated with A) DHA (0-200 µM) or B) ASC (200 µM) for 30 min before the radiolabeled inulin transfer assay. Cells were cultured chronically in basal (5 mM) or high (25 mM) glucose with equal parts astrocyte-conditioned medium. A) Two-way ANOVA; interaction, $F=0.6344$, $p=0.6438$; effect of DHA, $F=7.324$, $p=0.0008$; effect of glucose, $F=39.17$, $p<0.0001$. Bonferonni post-*hoc* analysis: Basal versus High glucose *$p<0.05$, **$p<0.01$; vehicle versus 200 µM DHA [†]$p<0.05$. B) Two-way ANOVA; interaction, $F=1.734$, $p=0.224$; effect of ASC, $F=32.79$, $p=0.0004$; effect of glucose, $F=9.780$, $p=0.0141$. Bonferonni post-*hoc* analysis: Basal versus High glucose *$p<0.05$, vehicle versus ASC [†]$p<0.05$. n=3 for each panel.

RAGE activation increases BEC permeability

To investigate the effects of RAGE activation on BEC permeability, we utilized human BECs (hCMEC/D3 cells). These cells are the immortalized human version of the bEnd.3 cells. We first confirmed the bEnd.3 cell results on the generality of ASC uptake and effect on permeability in endothelial cells [222]. HCMEC/D3 cells cultured at 25 mM glucose were treated for 60 min with increasing concentrations of the specific RAGE inhibitor FPS-ZM1 [223] before the inulin transfer assay. FPS-ZM1 binds the V domain of RAGE and prevents binding of other substrates, thus preventing activation of RAGE [223]. As indicated in Figure 5.3A, FPS-ZM1 decreased endothelial permeability with a half-maximal effect at a concentration of 0.6 µM (p<0.05). These results suggest RAGE activation mediates most, if not all, of the high glucose-induced increase in endothelial barrier permeability. Previous data shows FPS-ZM1 over this concentration range did not affect basal permeability in peripheral endothelial cells (HUVECs) cultured at 5 mM glucose (results not shown).

If culture of hCMEC/D3 cells at high glucose concentrations increases endothelial barrier permeability by activating RAGE in our model, then known RAGE ligands should also increase permeability at basal glucose concentrations. This hypothesis was tested with optimized concentrations of two RAGE ligands (p<0.05, Figure 5.3B). In hCMEC/D3 cells, a 24 h incubation of either 0.6 µg/ml HMGB1 or 60 µg/ml AGE-BSA increased inulin transfer compared to control. Addition of 200 µM ASC for 30 min before the transfer assay both decreased basal inulin transfer and blunted the increases due to HMGB1, similar to unstimulated conditions (Figure 5.3B). However, we did not observe an ASC effect when cells were treated with AGE-BSA. This could be due to the concentration of AGE-BSA used and further experiments would need to be performed to verify the effect of ASC on permeability after AGE-BSA treatment.
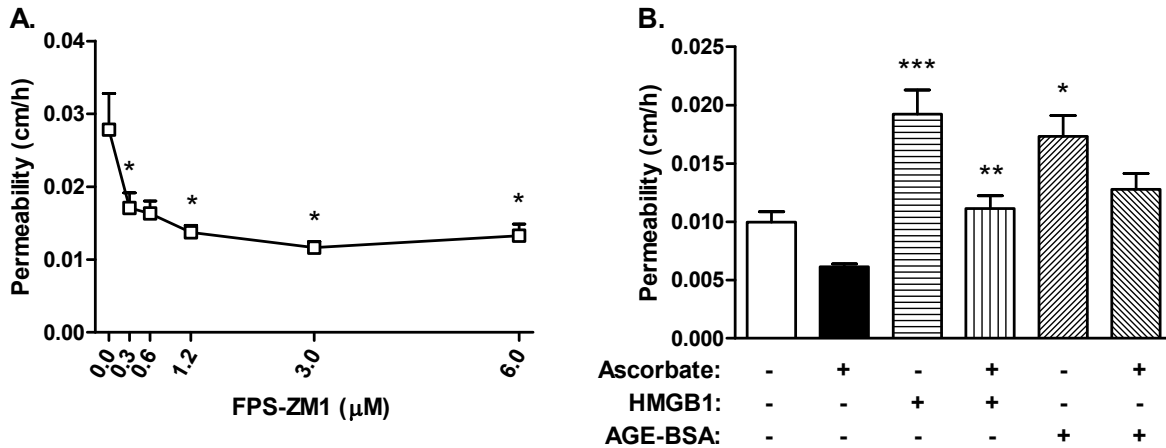
Figure 5.3. ASC prevents BEC leakage when RAGE is activated. HCMEC/D3 cells were plated on semi-porous inserts and grown to confluence for 6-7 days. A) hCMEC/D3 cells cultured at 25 mM glucose were treated with the indicated concentration of FPS-ZM1 for 60 min, followed by the radiolabeled inulin transfer assay. One-way ANOVA, F=5.708, p<0.0008, Dunnett's post-*hoc* analysis: control versus treatment *p<0.05. B) hCMEC/D3 cells cultured at 5 mM glucose were treated for 24 h with 0.6 µg/ml HMGB1 or 60 µg/ml AGE-BSA. On the next day, cells were treated for 30 min with 200 µM ASC before the inulin transfer assay. Results were published with one-way ANOVA analysis, F=12.4, p<0.0001, Bonferonni post-*hoc* analysis: control versus AGE-BSA *p<0.05, HMGB1 versus HMGB1+ASC **p<0.01, control versus HMGB1 ***p<0.001. Upon reflection, a two-way ANOVA was performed: interaction, F=1.383, p=0.266; effect of ASC, F=24.09, p<0.0001; effect of RAGE ligand, F=17.58, p<0.0001; Bonferonni post-*hoc* analysis: control versus HMGB1 (p<0.001), HMGB1 versus HMGB1+ASC (p<0.001), control versus AGE-BSA (p<0.01), control versus AGE-BSA+ASC (p<0.05), n=6 for each panel.

Other antioxidants decrease permeability under high glucose conditions

To assess whether other antioxidants might also acutely reverse the increase in endothelial permeability due to high glucose, hCMEC/D3 cells cultured on filters in 25 mM glucose were treated for 60 min with several antioxidants with different mechanisms of action, followed by the endothelial permeability assay (p<0.05, Figure 5.4). All of the antioxidants at the concentrations used except Tempol tightened the endothelial barrier. However, ASC is able to most effectively decrease permeability. These results suggest that the effect of intracellular ASC to decrease permeability could relate to its function as an antioxidant.

Effect of ASC on BECs under basal glucose conditions

The previous experiments showed BEC permeability decreases with ASC incubation independent of basal or high glucose conditions (Figure 5.2). In addition, RAGE activation increased BEC permeability. However, it appeared as though ASC decreased permeability in an independent fashion, no matter what the glucose concentration was or whether RAGE was activated. Because we still were unsure how ASC was decreasing permeability under basal conditions, we simplified our experiments to focus our efforts on investigating this effect with basal glucose only. The remaining studies were performed in bEnd.3 cells.

Oxidative stress

After determining the actions of ASC could be due to its role as an antioxidant, we wanted to determine whether ASC could decrease intracellular oxidative stress under basal, unstimulated conditions. Reactive oxygen species generated were measured as oxidation of dihydrofluorescein to fluorescein at two ASC concentrations (50 and 200 µM). There was a significant decrease in the basal amount of reactive oxygen species generated following incubation for 90 min with ASC compared to vehicle treated cells (p<0.05, Figure 5.5).
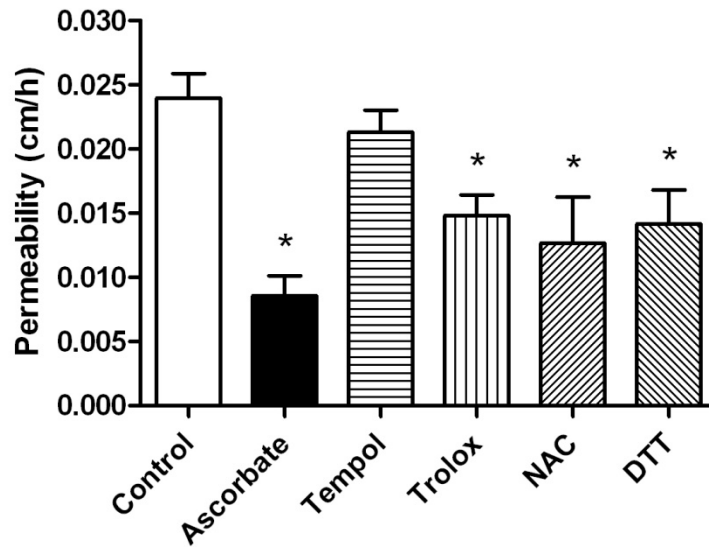
Figure 5.4. Antioxidant reversal of high glucose-induced permeability in BECs. HCMEC/D3 cells cultured for 6-7 days on semi-porous inserts in 25 mM glucose were incubated without any additions (Control) or with additions 60 min prior to the inulin transfer assay as follows: Ascorbate, 125 µM; Tempol, 300 µM; Trolox, 300 µM; *N*-Acetyl Cysteine (NAC), 600 µM; or Dithiothreitol (DTT), 300 µM. One-way ANOVA: F=6.179, $p < 0.0005$; Dunnett's post-*hoc* analysis: control versus treatment *$p < 0.05$, n=6.
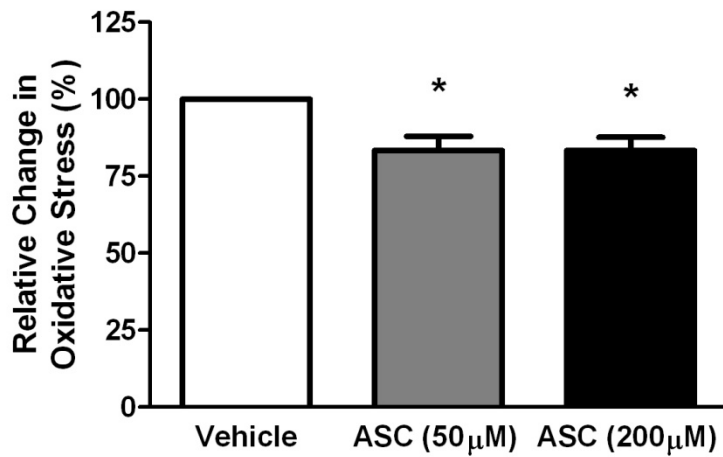
Figure 5.5. ASC decreases intracellular oxidative stress. bEnd.3 cells grown to confluence in 5 mM glucose were incubated with ASC for 60 min prior to addition of 20 μM dihydrofluorescein diacetate, followed by incubation for 30 min before assay of fluorescein fluorescence. One-way ANOVA: $F=7.031$, $p<0.005$, Tukey's post-*hoc* analysis, vehicle versus ASC treatment *$p<0.05$, n=7.

Phosphorylation of junctional proteins

VE-cadherin phosphorylation on Tyr 658 results in dissociation from the p120-catenin complex, causing internalization of VE-cadherin [91]. The exact mechanism for the role in occludin phosphorylation on Ser490 in BEC stability is not known but it has been shown mutation of this residue inhibits trafficking of occludin to the cell membrane [224]. Treating bEnd.3 cells with 100 µM ASC for 90 min did not alter VE-cadherin phosphorylation at residue Tyr658 (Figure 5.6A). Also, 200 µM ASC incubation for 90 min was unable to alter occludin phosphorylation at residue Ser490 (Figure 5.6B).

Involvement of PKA or Epac on permeability

To examine the effect of ASC on activation of cAMP-dependent pathways, known inhibitors of these steps were used in bEnd.3 cells. Due to the variability of basal BEC tightness in these experiments, data was expressed as raw values (Figure 5.7A) for comparison across the literature, as well as normalized values within each experiment (Figure 5.7B) to determine the true relationship between treatments. Statistical analysis was performed on the normalized data. As expected, incubating cells with 100 µM ASC for 90 min led to a significant decrease in permeability compared to vehicle treated cells ($p < 0.05$, Figure 5.7B). The pharmacological agent, H89, is known to selectively inhibit PKA in endothelial cells at a concentration of 10 µM [95]. Indeed, there was an effect on permeability due to a 20 min pre-treatment with H89. However, H89 did not prevent the decrease in permeability due to ASC ($p < 0.05$, Figure 5.7B). ASC still decreased permeability in the presence of PKA inhibition by approximately 40%. Similar results were obtained under the same experimental conditions using a peripheral endothelial cell line, HUVECs (data not shown).

The pharmacological inhibitors, ESI-09 and HJC0197, are known to inhibit Epac [225]. A 20 min pre-treatment with HJC0197 increased basal permeability and both agents blocked the decrease in permeability due to ASC treatment (Figure 5.7B). Lower doses of ESI-09 (6 µM) and HJC0197 (12 µM) provided comparable results, and thus was not included in the data analysis. Similar results were confirmed in HUVECs (data not shown). These results suggest the actions of ASC to alter permeability are mediated through Epac.
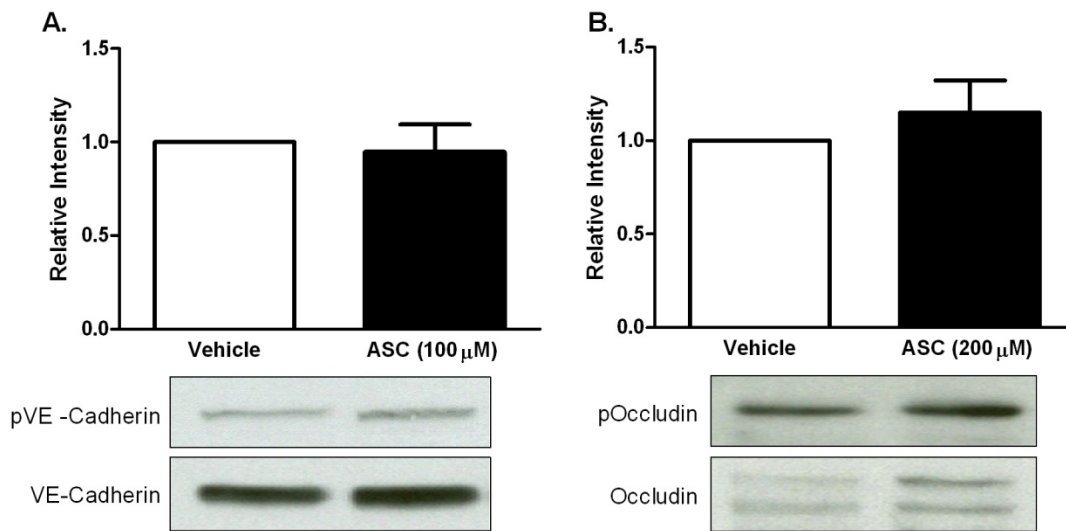
Figure 5.6. Phosphorylation of VE-cadherin and occludin is not affected by ASC. bEnd.3 cells grown to confluence in 5 mM glucose were incubated with ASC (100 or 200 µM) for 90 min. ASC did not affect phosphorylation of either residue A) Tyr658 on VE-cadherin or B) Ser490 on occludin. n=4-6 per group.

A. Raw Permeability Data

B. Relative Permeability Data

ASC (100 μM)	-	+	-	+	-	+	-	+
H89 (10 μM)			+	+				
ESI-09 (18 μM)					+	+		
HJC0197 (30 μM)							+	+
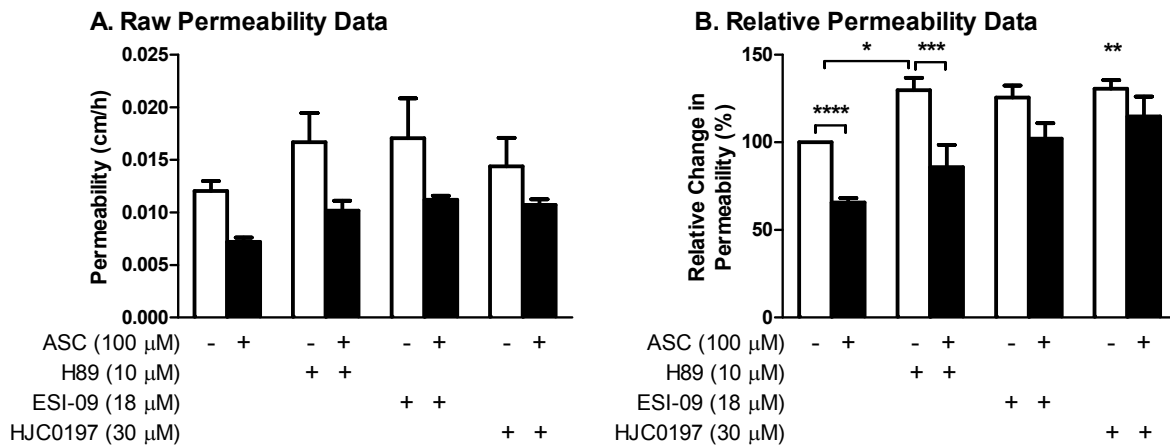
Figure 5.7. Involvement of PKA and Epac on mediating the decrease in permeability due to ASC treatment. bEnd.3 cells were grown to confluence in 5 mM glucose on semi-porous inserts for 6-7 days. Cells were pre-incubated for 20 min with either 10 μM H89 (PKA), 18 μM ESI-09 (Epac), or 30 μM HJC0197 (Epac). Cells were then treated with 100 μM ASC for 30 min before the inulin transfer assay. Data are expressed as A) raw values or B) normalized to control values in each experiment. Two-way ANOVA; interaction, $F=1.672$, $p=0.1835$; effect of ASC, $F=42.6$, $p<0.0001$; effect of inhibitor, $F=20.16$, $p<0.0001$. Bonferonni post-*hoc* analysis: control versus H89 *$p<0.05$, control versus HJC0197 **$p<0.01$, H89 versus H89+ASC ***$p<0.001$, control versus ASC ****$p<0.0001$. n=5-6.

Rap1 activity

Because ASC was found to potentially act through Epac to decrease permeability, we investigated the active role of the downstream mediator of Epac, Rap1. After a 60 min incubation with 100 μM ASC, the GTP-bound form of Rap1 was decreased in bEnd.3 cells, but not significantly (p=0.08, Figure 5.8).

Cytoskeleton contribution

Although we did not see a significant change in Rap1 activity, we wanted to investigate whether the cytoskeleton played a part due to its role in maintaining barrier integrity and because of the possibility that Epac can act independent of Rap1 [88]. Again, 90 min incubation with 100 μM ASC was able to decrease permeability under basal conditions (p<0.05, Figure 5.9B). Inhibiting microtubule or actin polymerization using 10 μM colchicine and 15 μM cytochalasin A, respectively, for 90 min blocked the effect of ASC on decreasing permeability. This suggests the cytoskeleton is important in mediating the effects of ASC on BEC permeability.

Microtubule Stability

To determine whether the effects of ASC were due to microtubule stabilization, we measured acetylated alpha tubulin, a readout of stable microtubules [226]. The levels of acetylated alpha tubulin relative to total alpha tubulin did not change with a 90 min 100 μM ASC incubation (p=0.57, Figure 5.10).
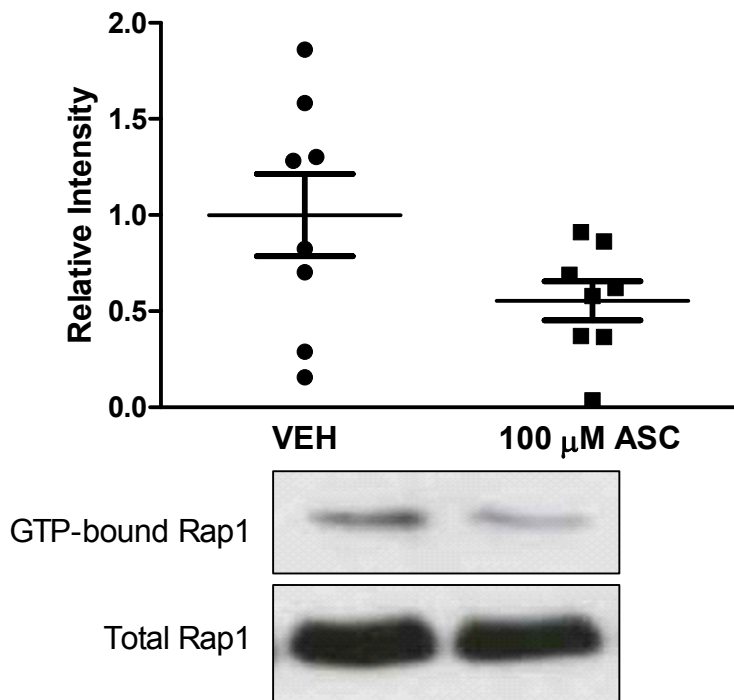
Figure 5.8. Rap1 activity was not affected by ASC. BEnd.3 cells were grown to confluence in 5 mM glucose. Incubating the cells with 100 µM ASC for 60 min appeared to decrease Rap1 activity, but not significantly. n=8.

A. Raw Permeability Data

B. Relative Permeability Data
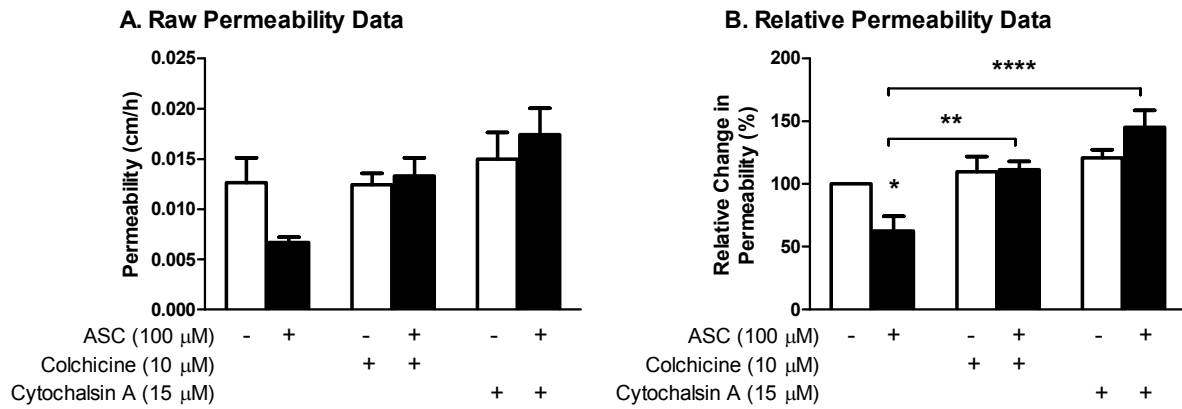
Figure 5.9. Cytoskeleton disruption blocks the effect of ASC on permeability. bEnd.3 cells were grown to confluence in 5 mM glucose on semi-porous inserts for 6-7 days. Cells were incubated with 100 µM ASC concomitantly with 10 µM colchicine or 15 µM cytochalasin A 30 min before the inulin transfer assay. Data are expressed as A) raw values or B) normalized to control values in each experiment. Two-way ANOVA; interaction, $F=5.266$, $p=0.011$; effect of ASC, $F=0.2535$, $p=0.6183$; effect of inhibitor, $F=14.60$, $p<0.0001$. Bonferonni post-*hoc* analysis: control versus ASC *$p<0.05$, ASC versus colchicine+ASC **$p<0.01$, ASC versus cytochalasin A ****$p<0.0001$. n=6.
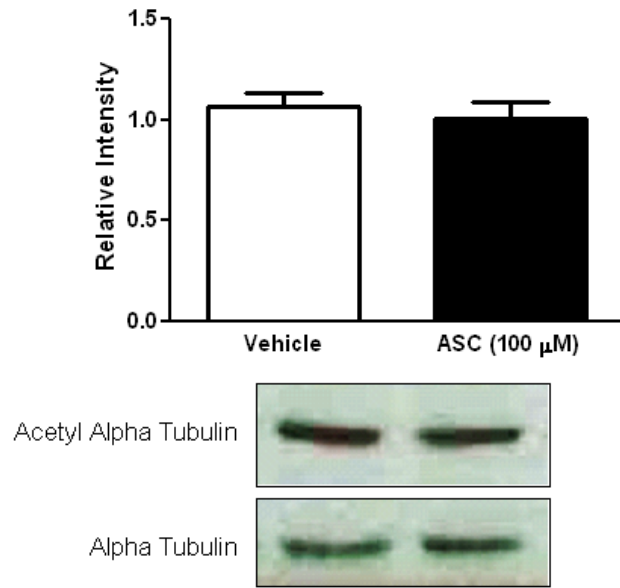
Figure 5.10. ASC had no effect on acetylated alpha tubulin. BEnd.3 cells grown to confluence in 5 mM glucose and incubated with 100 µM ASC for 90 min had no effect on acetylated alpha tubulin compared to untreated controls. n=9.

DISCUSSION

The current study sought to investigate whether and how ASC could prevent the increase in permeability that has been observed in endothelial cells under high glucose (25 mM) conditions [216, 227]. Indeed, we found ASC decreased permeability under these conditions and that high glucose and RAGE activation increased BEC permeability. We further explored the intracellular signaling mechanism behind the ability of ASC to decrease permeability in cells treated at normal glucose (5 mM) and were able to attribute it to alterations in Epac1 signaling and the cytoskeleton. These findings are summarized in Figure 5.11.

BECs take up both the reduced and oxidized forms of ASC within 60 min, under basal and high glucose conditions. It is likely that DHA uptake is not as rapid under high glucose, due to competition with glucose for transport on the glucose transporters. The ability of bEnd.3 cells to increase intracellular ASC levels after DHA incubation also sheds light on the reducing capacity of these cells. One explanation for the trending increase in intracellular ASC under 25 mM glucose conditions could be that the cells with higher glucose have higher ATP levels and thus, are able to increase transport of ASC via the energy-dependent SVCT2 [54].

The increase in intracellular ASC within this time frame decreases permeability significantly whether in basal or high glucose. Our lab has repeatedly observed a decrease in permeability with ASC in different types of peripheral endothelial cells cultured at 5 mM glucose [114, 193]. Another group has shown constant incubation with ASC for 5 days decreases endothelial cell permeability [191]. The authors attributed this change in permeability to collagen deposition by increased ASC levels. However, our studies reveal a much shorter length of incubation required for changes in permeability, suggesting ASC can act acutely to trigger downstream signaling pathways that modulate barrier permeability besides deposition of collagen.

The ability of ASC to decrease high glucose-induced endothelial permeability could involve several independent mechanisms. ASC might decrease endothelial permeability due to its function as an antioxidant, since both thiol and other antioxidants also partially or completely reverse high glucose-induced endothelial barrier leakage. Culture of endothelial cells in high glucose increases superoxide generation [228, 229].

91

Subsequent increases in cellular hydrogen peroxide after the action of superoxide dismutase could then increase endothelial barrier leak [154, 230, 231]. In this scenario, scavenging of superoxide or its downstream products by low millimolar concentrations of ASC [34] could well decrease oxidant-induced increases in endothelial barrier permeability. However, tempol is a superoxide dismutase mimetic, targeting superoxide as its main mechanism in decreasing oxidative stress. In our study, tempol was unable to decrease hCMEC/D3 permeability under high glucose conditions, suggesting superoxide is not responsible for the high glucose-induced permeability. It is possible the amount of tempol used was not concentrated enough to elicit an effect. However, a dose response with tempol or a superoxide dismutase knockdown would need to be performed to verify this hypothesis.

High glucose concentrations in culture also generate AGEs, which bind to and activate RAGE [232]. That RAGE ligands can contribute to high glucose-induced peripheral endothelial barrier leakage is clear from the results of previous studies [149, 233]. Indeed, in our study, a RAGE inhibitor returned high glucose-induced increases in BEC permeability to baseline (Figure 5.11A). However, the ability of ASC to acutely counteract RAGE ligand-mediated endothelial barrier leakage is likely independent, as ASC decreased permeability after RAGE activation similar to unstimulated conditions. The levels of RAGE ligands in the media under high glucose conditions in our model are unknown. Measuring these levels under basal and high glucose conditions would help verify the activation of RAGE by these ligands under high glucose conditions. RAGE activation leads to multiple different signaling pathways, including activation of NADPH oxidase due to increases in intracellular reactive oxygen species [147] or activation of PKC [121]. Antioxidants and reactive oxygen species have been shown to have opposite effects on cell permeability by rearranging the cytoskeleton [234, 235]. Due to the independent nature of the glucose and ASC effects, we wanted to focus on the effects solely due to ASC on barrier permeability. Therefore, the remaining discussion will focus on ASC in the context of basal glucose levels.

As ASC is most known for its role as an antioxidant, we wanted to determine if ASC could decrease intracellular oxidative stress under basal, unstimulated conditions. A 90 min treatment with ASC modestly, but significantly, decreased intracellular reactive

oxygen species production. Therefore, it is possible ASC is able to decrease permeability by improving the redox state of the cell. We wanted to probe this concept further to determine which specific downstream mechanisms might be affected by the changes in the redox state of the cell.

Phosphorylation of proteins located at the cell junctions is known to alter permeability [236, 237]. Indeed, overall tyrosine phosphorylation can be an indicator of barrier strength [97, 98]. Superoxide can activate protein tyrosine kinases [99]. Therefore, we hypothesized that phosphorylation status of proteins located at the junctions were likely to be altered in our model.

VE-cadherin and occludin are two of many important regulators of junctional stability [91, 92, 95]. Previous studies have shown that monolayer permeability is affected by changes in the phosphorylation state of VE-cadherin or occludin, specifically at the residues investigated in the present study [90, 91, 94, 238-240]. The lack of change in phosphorylation in response to ASC in our cellular model (Figure 5.11B) could be explained by the possibility that other important sites of phosphorylation on these proteins are altered. More likely, though, these proteins are shifted due to rearrangement of the cytoskeleton, weakening the cell-to-cell contacts, as has been previously reported [96, 241]. In addition, the mere rearrangement of the cytoskeleton could lead to gaps within the cell barrier. The results using the cytoskeleton inhibitors support these possibilities.

Cyclic AMP has been shown to decrease permeability in endothelial cells [242-245]. There are contradictions in the literature concerning the effects of ASC on cAMP levels. Various groups have shown ASC decreases cAMP levels [110, 246, 247], while other groups show ASC augments cAMP levels under stimulatory conditions [248, 249]. Indeed, Huang *et al* suggest ASC might act in a PKA-independent mechanism [249].

We investigated downstream mediators of the cAMP pathway by using pharmacological inhibitors of PKA and Epac (Figure 5.11C). H89 is a selective inhibitor of cAMP-dependent PKA and has been used throughout the literature at concentrations of 5-10 µM [95]. H89 acts by competing out ATP on the catalytic site for PKA [250]. H89 treatment alone is able to increase permeability (Figure 5.7B) suggesting the agent is able to inhibit PKA to increase BEC permeability. In our study, ASC supplementation

93

decreased permeability when PKA was inhibited, similar to the response observed in vehicle-treated cells. This suggests PKA is not responsible for the decrease in permeability due to ASC. Therefore, we tested whether Epac inhibition affected permeability and if ASC could reverse it, using the Epac-specific inhibitors ESI-09 and HJC0197. These pharmacological agents are membrane-permeant inhibitors of Epac1 and Epac2 [225] but the molecular basis of inhibitory action is not clear. Epac1 is the primary isoform expressed in endothelial cells and is more involved in cell adhesion [88, 95, 107, 251]. HJC0197 treatment alone was able to increase permeability suggesting Epac is also important in mediating BEC permeability. Also, Epac inhibition in our model blunted the effect of ASC on permeability, suggesting ASC signals through Epac1 to decrease permeability.

Epac1 is known to bind and activate Rap1 at the cell membrane, which in turn can decrease barrier permeability [88, 100, 101]. We expected that signaling through Epac1 due to ASC would activate Rap1 and tighten the endothelial barrier. However, ASC incubation did not significantly decrease levels of GTP-bound Rap1, which reflects its activation. Our finding that, if anything, there was a strong trend for ASC to decrease Rap1 activation is puzzling and unexplained. However, Epac1 can bind microtubule associating proteins (MAPs) [101]. Therefore, it is possible ASC enhances Epac1 binding to the polymerizing microtubules which prevents it from activating Rap1. Future studies would need to be performed to investigate the binding of Epac1 to these MAPs under ASC stimulated conditions.

To investigate the effect of the cytoskeleton in our model, we used pharmacological destabilizers of microtubules and actin filaments to determine if these could block the effect of ASC on permeability. Colchicine prevents microtubule polymerization by binding to tubulin while cytochalasin A inhibits actin polymerization by binding to the barbed, fast-growing plus ends of microfilaments. Indeed, the decrease in permeability due to ASC was blocked when cells were treated with either of the inhibitors, suggesting ASC acts through the cytoskeleton. In addition, flavonoids, which are powerful antioxidants, have been shown to stabilize microtubules [234], prompting us to investigate the role for ASC on microtubule acetylation and stabilization.

Treating BECs with 100 μM ASC for 90 min did not affect levels of acetylated alpha tubulin compared to vehicle treated cells. There are other ways to measure microtubule stabilization to verify ASC is not affecting this structure. One way is by staining cells for alpha tubulin and measuring microtubule length after a nocodazole- or cold-induced resistance treatment [234, 252]. Another potential effect ASC might have on the cytoskeleton is by rearranging actin, an effect we have previously shown in peripheral endothelial cells [193]. Indeed, Epac1 can act through Rac1 to alter F-actin arrangement [253]. More studies are needed to confirm the effect of ASC on actin rearrangement in the BEC model.

The results presented here reveal multiple effects for ASC in regulating endothelial cell permeability. First, BEC barrier permeability is decreased when incubated acutely with ASC. Second, high glucose and RAGE activation increased permeability in which ASC and other antioxidants were able to ameliorate this increase. Third, ASC signals through Epac1 to alter the cytoskeleton independent of Rap1. These findings have relevance to microvascular disease caused by the hyperglycemia of diabetes, since replenishment of ASC could well tighten the endothelial barrier and decrease capillary leak of plasma constituents. In addition, the role of ASC in BECs can be applied to other cell types involving permeability of any barrier, whether in the lung, heart, or even tumors.
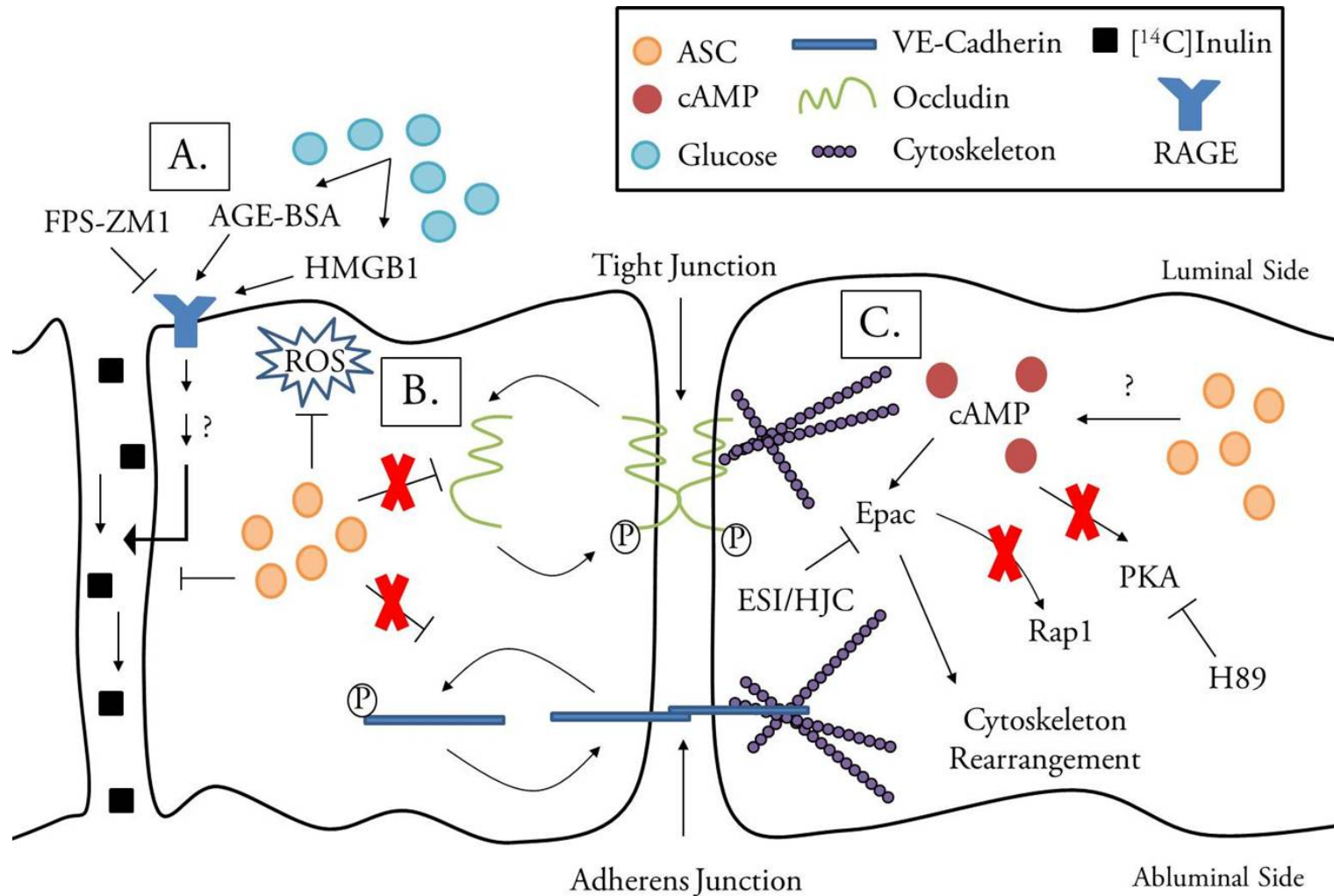
Figure 5.11. Summary model for Chapter V. A) BECs take up and store ASC, which leads to decreases in barrier permeability. High glucose and RAGE activation increases BEC permeability. B) Decreases in permeability due to ASC occur even though phosphorylation of VE-cadherin and occludin is not affected. C) ASC supplementation signals through Epac1, not PKA, which leads to alterations in the cytoskeleton, independent of Rap1, which results in overall improved barrier stability.

CHAPTER VI

Effect of diabetes on blood-brain and -retinal barriers in mice
with decreased ASC levels

INTRODUCTION

We have shown that ASC decreases BEC permeability under both basal and high glucose conditions (Chapter V) [222]. To establish direct clinical relevance, we wanted to determine whether or not we could see the same effects of ASC *in vivo* using the Gulo(-/-) mouse model. Similar to the human condition, ASC levels in these mice are controlled directly through dietary supplementation.

Oral administration of 1000 mg/day ASC for one week augmented endothelial function as measured by brachial artery diameter response in women with type 2 diabetes [254]. Although the mechanism of this improvement was not studied, it strongly suggests a clinical relevance between the links of ASC, endothelial function (in this case at the BBB), and diabetes. As mentioned in Chapter I, multiple studies have shown diabetes disrupts the BBB in rodents, as well as in humans [130, 134, 138, 157]. These findings suggest diabetes may have a cumulative effect on the central nervous system, which takes time to manifest in increased BBB permeability and alterations in the cerebrovascular system. Indeed, such changes in the BBB could account for the finding that people with either type 1 or 2 diabetes have two-fold higher incidence of developing cognitive deficits when compared to healthy subjects, especially as they age [138].

The breakdown of the BBB due to diabetes is thought to be partly due to alterations in the levels of tight junction proteins, claudin and occludin, due to changes in signaling pathways triggered by oxidative stress, as previously mentioned [128, 129]. Huber *et al* showed the changes in BBB structure in rats due to high glucose first occur in the midbrain, a brain region with lower capillary density and blood flow [130]. It is important to note that barrier dysfunction is dynamic and that periods of stress or pain can lead to an acute opening of the BBB, allowing circulating proteins to enter the brain.

Understanding the role ASC plays *in vivo* at the BBB could have great significance for any disease that involves breakdown of the BBB, including multiple

sclerosis and Alzheimer's disease. In addition, ASC supplementation after burn trauma reduces capillary leakage and prevent edema [255]. The ability of ASC to tighten the barrier generated by peripheral and BECs can also be important in cancer metastasis, a vital area of interest for cancer researchers.

It is suggested that because previous reports used albumin extravasation, a very large protein, as an indicator for BBB breakdown, slight disruptions were not detected. Instead, more subtle changes in the structure of the BBB may be taking place and would be better observed using smaller tracers such as fluorescein-labeled dextrans. Due to this dispute among experts in the field concerning the best way to determine BBB disruption *in vivo*, we made use of multiple techniques including tracers such as Evan's blue albumin and FITC-dextran, behavioral testing, and MRI, to determine the optimal way to visualize loss of integrity.

Preliminary studies revealed early on that the behavioral techniques we utilized were not adequate to observe changes in BBB integrity. Scopolamine methylbromide (SCOP MBR) is a drug that cannot cross the BBB unless disruption has occurred, in which treatments should increase ambulatory activity similar to the freely diffusible derivative scopolamine [256]. However, there was no change in activity in diabetic mice with low ASC levels compared to controls when injected with SCOP MBR. It is possible any disruption at the BBB that had occurred was insufficient to allow enough of the drug to enter the brain, and thus increase activity. It was unclear how much SCOP MBR would need to enter the brain to increase activity, and whether our experimental manipulations would permit such an influx, and thus we moved forward without pursuing these behavioral assays.

Our second approach was to attempt to visualize BBB disruption via MRI. To achieve this end we collaborated with Dan Colvin and Tom Yankeelov in the Imaging Institute at Vanderbilt. We used the small molecular weight tracer, gadolinium DPTA, but we were unable to observe differences in a diabetic mouse with low ASC levels compared to a control mouse. Due to the expense of the imaging, and the lack of promising preliminary data, we chose to move forward with a more common method to measure disruption.

In an initial small cohort of mice, we saw promising increases in extravasated Evan's blue albumin in the cortex of mice that had been on a low ASC supplementation for 18 weeks and diabetic for the last 8 of those weeks. However, after measuring cortex ASC levels, we realized something was amiss. Diabetic mice drink approximately 4-fold more of the ASC-supplemented water than control mice, due to dehydration from the high blood glucose (Figure 2.1). The low ASC diabetic mice thus consumed enough water to replete their ASC levels, even at the lowest supplementation level. Therefore, in the remaining studies, ASC supplementation in the water of the low ASC diabetic mice was adjusted to account for the increased intake. Furthermore, to minimize the risk of missing small disruptions in the BBB, we switched to a lower molecular weight fluorescent molecule, a 10 kDa FITC-dextran, instead of the larger sized Evan's blue albumin. This new experimental model is depicted in Figure 6.1.

To supplement our work in the brain, we collaborated with a member of the Vanderbilt Eye Institute, Ashwath Jayagopal, to look at changes in the blood-retinal barrier. As mentioned before in Chapter I, the blood-retinal barrier has similar features to the BBB, including the presence of tight junctions. There is also important clinical significance as the blood-retinal barrier is implicated in diabetic retinopathy, a prevalent problem in diabetics. Much more work in humans has been performed on the molecular mechanisms behind the permeability of this barrier [132]

Here, we show glucose and ASC levels are altered accordingly in the four treatment groups at both 6- and 12-weeks of diabetes. Oxidative stress analysis performed by measuring malondialdehyde (MDA) levels, protein carbonyls, F_2-isoprostanes, and F_4-neuroprostanes, reveals little change between the groups. Additionally, levels of tight junction proteins were analyzed, as we hypothesized levels would be decreased in the low ASC diabetic group. Disruptions in the blood-brain and -retinal barriers were measured by FITC-dextran extravasation quantitatively in the frontal cortex and qualitatively in the retina.

99
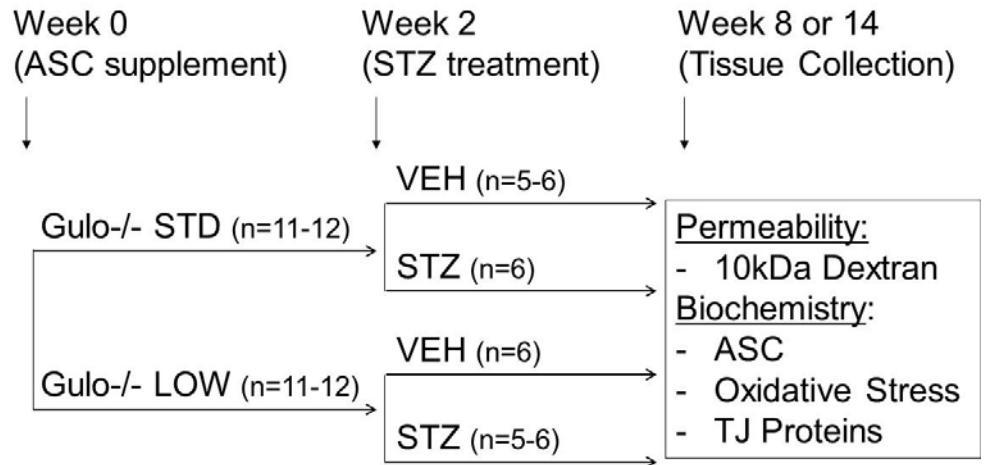
Figure 6.1. Diabetic ASC model. Mice were placed on different ASC supplementations at 6 weeks of age (Week 0) (STD- 0.33 g/L, LOW- 0.033 g/L). Mice were injected two weeks later with vehicle or a 200 mg/kg dose of streptozotocin (STZ). LOW STZ mice were then placed on their new, decreased ASC supplementation level (0.00825 g/L) to account for their increased water intake. Diabetic state was maintained for either 6- or 12-weeks until sacrifice.

RESULTS

Blood Glucose

Blood glucose level did not vary between the randomized groups before STZ injection (Figure 6.2). As expected, blood glucose levels were significantly increased in the STZ mice at the end of 6- or 12-weeks (Figure 6.2B, D). Interestingly, after 12-weeks of diabetes, the LOW STZ group had decreased blood glucose levels compared to the STD STZ group (Figure 6.2D).

ASC Level

LOW ASC supplementation significantly decreased cortex ASC level independent of STZ treatment at 6- and 12-weeks (Figure 6.3).

Oxidative Stress Levels

MDA was used as a marker for lipid peroxidation. There was a significant increase in MDA in the cortex of LOW STZ mice at 6-weeks compared to STD VEH mice (Figure 6.4A). However, there was no change in MDA level in the 12-week diabetic mice (Figure 6.4B). We did not see any difference in the cortex in protein carbonyl levels (Figure 6.4C-D) at either 6- or 12-weeks. Lastly, there was an increased trend for levels of F_2-isoprostanes in the cortex of LOW ASC mice at 6-weeks with significantly increased levels at 12-weeks in the LOW ASC supplemented mice (Figure 6.4E-F). There was no change in F_4-neuroprostanes at either time point (Figure 6.4G-H).
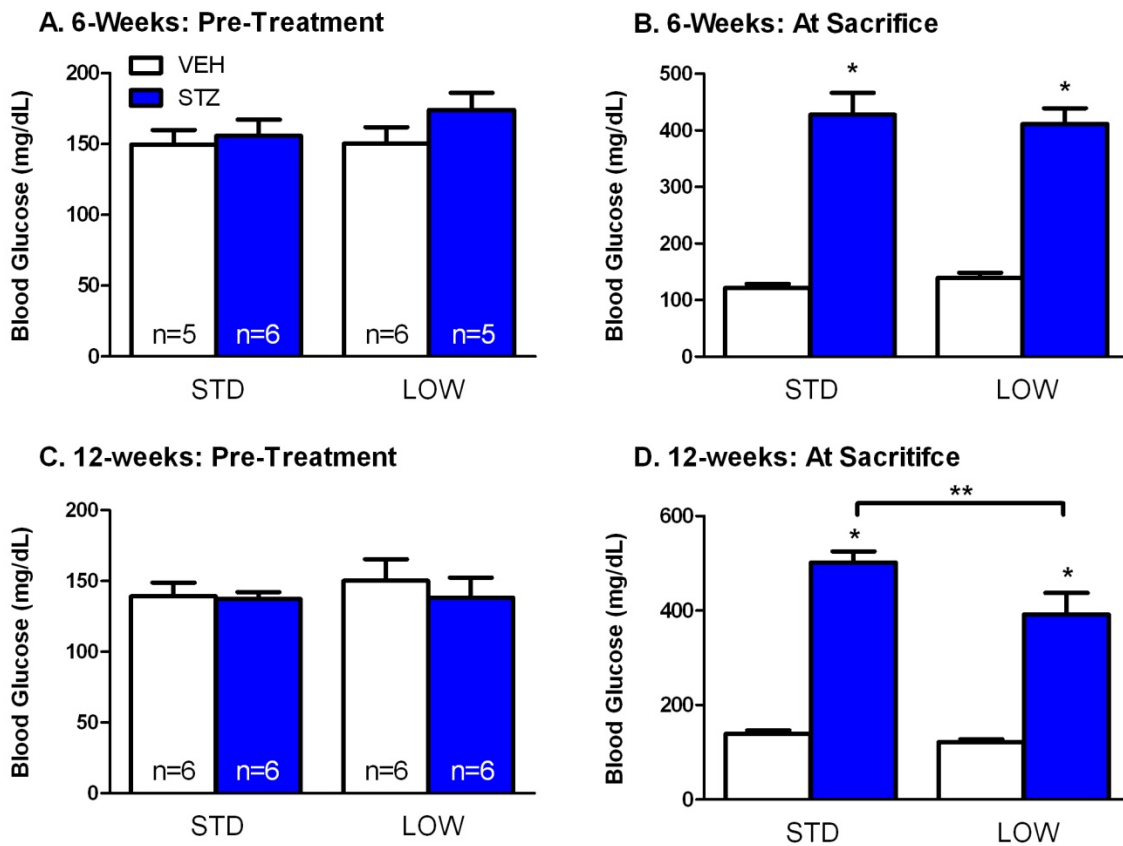
Figure 6.2. Blood glucose levels before and after 6- or 12-weeks of diabetes in Gulo(-/-) mice with STD or LOW ASC supplementation. Blood glucose level did not vary before STZ treatment (A,C) and was significantly increased in mice treated with STZ at sacrifice after 6- or 12-weeks (B,D). *p<0.05 versus VEH, **p<0.05 as marked.
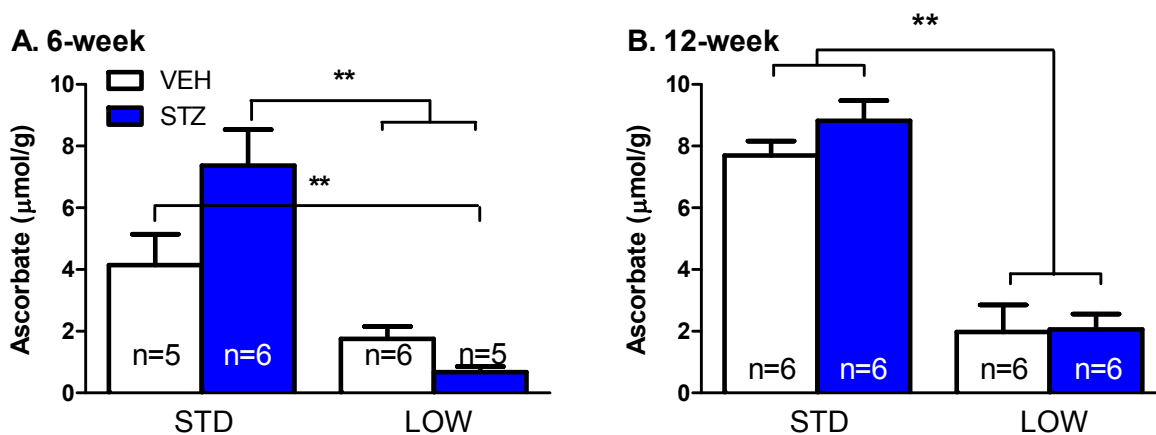
Figure 6.3. ASC levels in cortex of diabetic mice with STD or LOW ASC supplementation. ASC levels were significantly decreased in mice with LOW ASC supplements after either A) 6-weeks or B) 12-weeks of diabetes. **p<0.05 as marked.
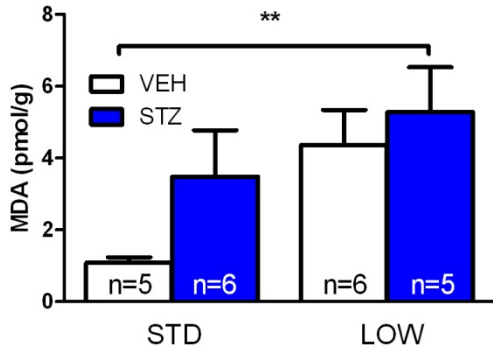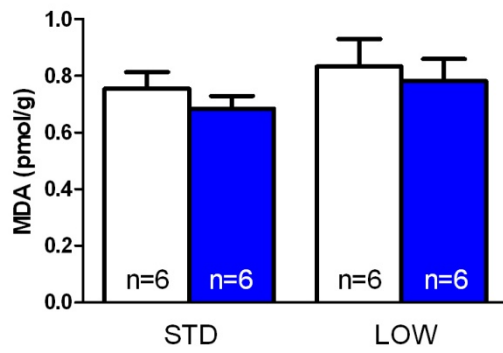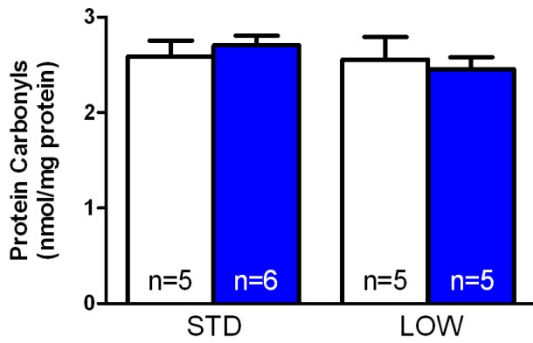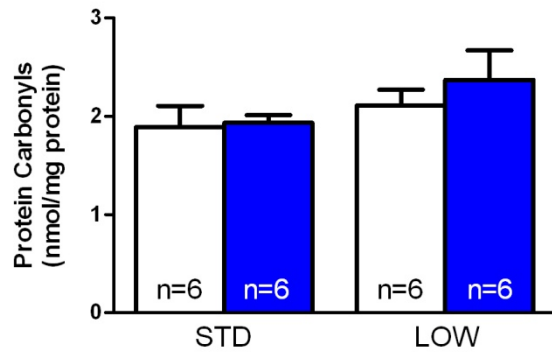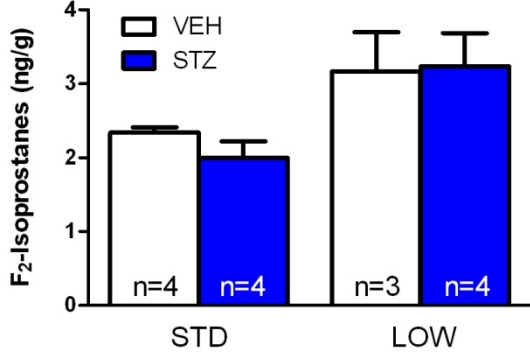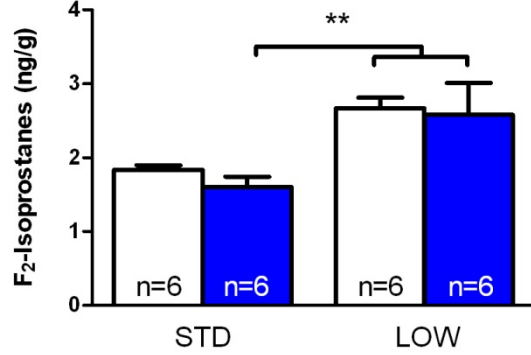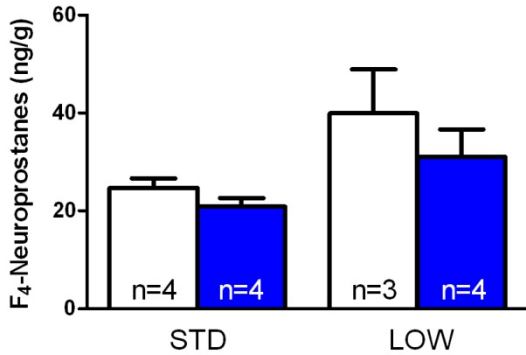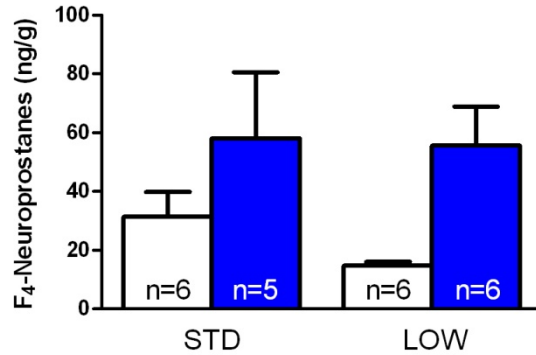
Figure 6.4. Oxidative stress levels in diabetic mice with STD or LOW ASC supplementation. MDA levels were significantly greater in diabetic mice on LOW ASC supplement compared to STD controls at A) 6-weeks but not different at B) 12-weeks. C,D) There was no difference in levels of protein carbonyls at either time point. F_2-isoprostanes were not different at E) 6-weeks but F) did increase significantly in the LOW ASC groups at 12-weeks. G-H) There was no difference in F_4-neuroprostanes at either time point. **$p < 0.05$ as marked.

Tight Junction Protein Levels

Total levels of occludin and claudin-5 in the cortex did not differ between the groups at either time point (Figure 6.5).

FITC-Dextran Extravasation

There was no difference between the groups in levels of extravasated FITC-dextran (Figure 6.6). Levels in the cortex were barely over background fluorescence (as noted by dotted line), suggesting a more sensitive test is needed to detect changes in our model.

Retinal-Barrier Breakdown

Our collaborator collected the retinas from the mice in the experiments above for visualization of dextran leakage. One retina was used for a quantifiable determination of extravasated dextran. Unfortunately, due to the small sample size of the retina, fluorescent dextran levels were not detected. The other retina was flat-mounted for visualization by microscopy (Figure 6.7A-H). As determined by observation of the images by an experienced researcher, as well as the quantification of fluorescent pixels (Figure 6.7I-J), the LOW STZ group trended towards a more severe disruption of the barrier.
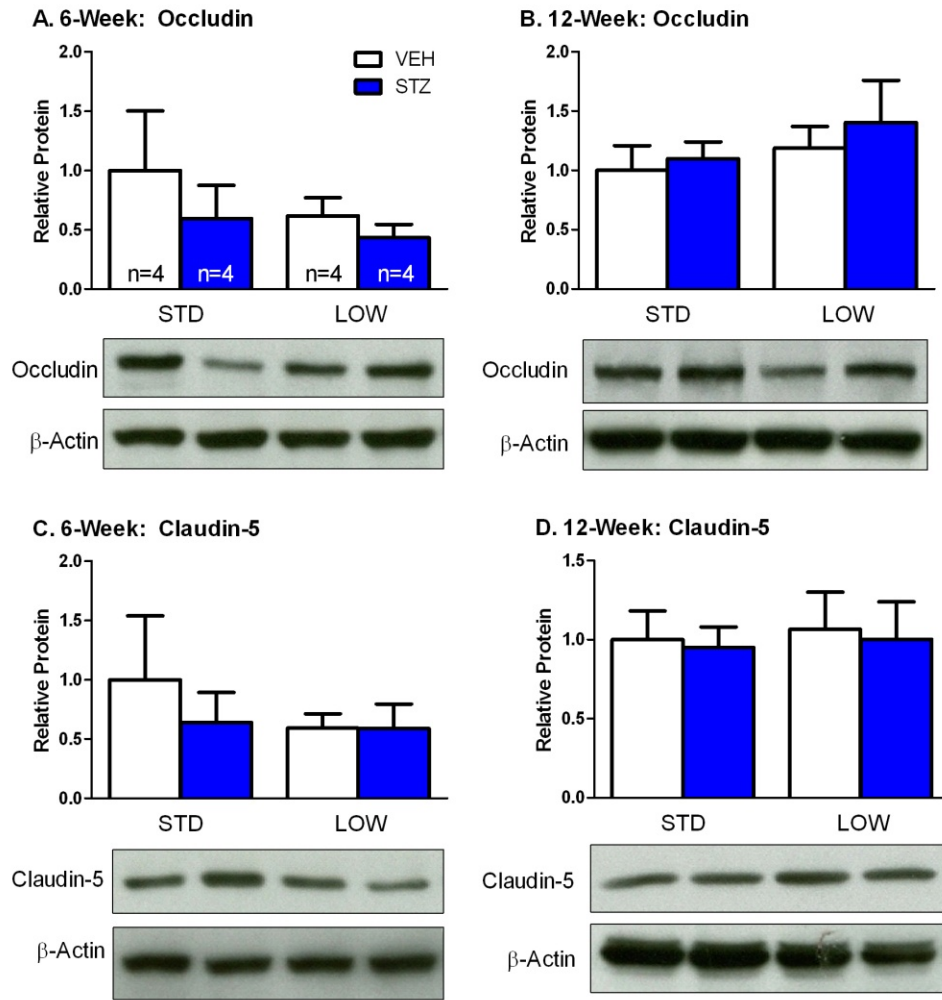
Figure 6.5. Total cortex tight junction protein expression in diabetic mice with STD or LOW ASC supplementation. A-B) Occludin and C-D) Claudin-5 protein levels were unaltered in the cortex of mice at either time point. Representative blots are shown.
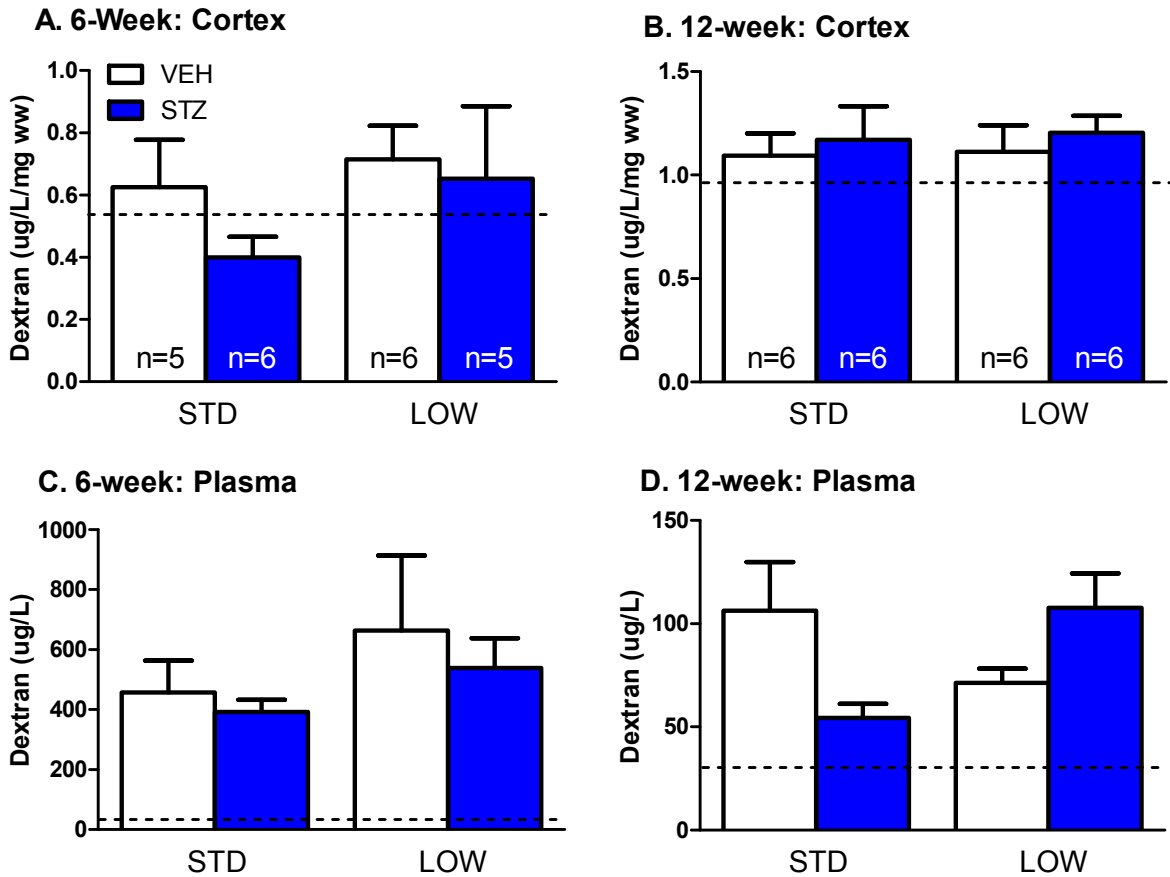
Figure 6.6. Extravasated FITC-dextran in diabetic mice with STD or LOW ASC supplementation. A-B) Levels of FITC-dextran in the frontal cortex did not differ at either 6- or 12-weeks diabetes. C-D) Levels of circulating FITC-dextran did not differ between the groups. Dotted line represents background fluorescence from two mice that did not receive the tracer.
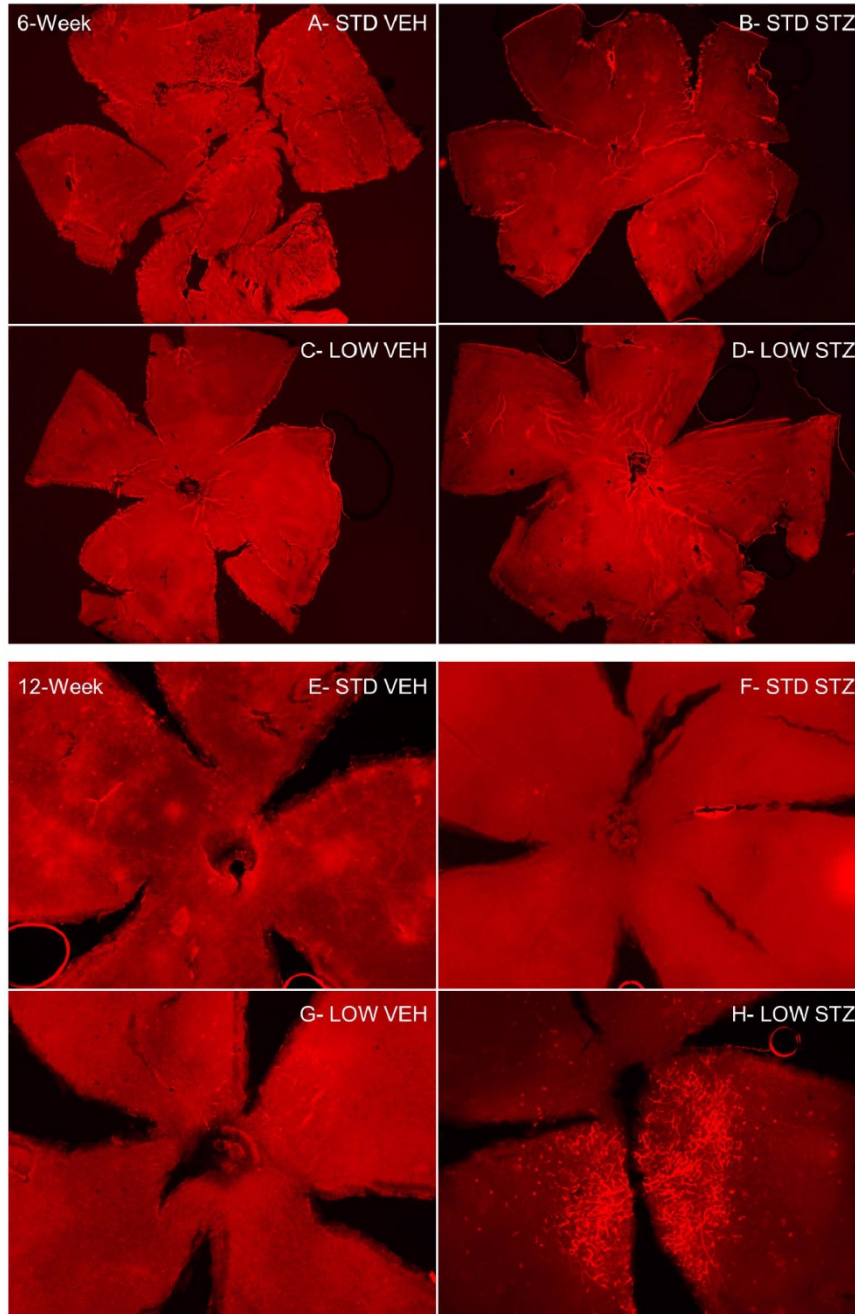
I. 6-week
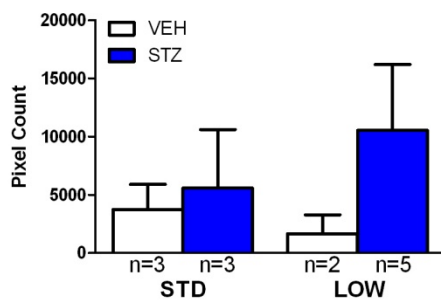
J. 12-week
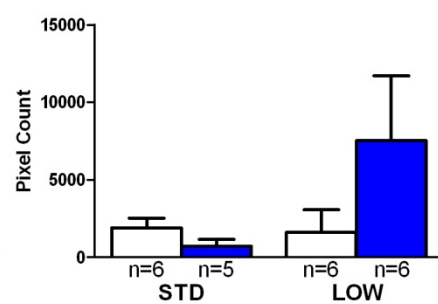
Figure 6.7. FITC-Dextran leakage in the retina after 6- or 12-weeks diabetes with STD or LOW ASC supplementation. Representative FITC-dextran extravasation in flat mounts of retinas from Gulo(-/-) mice on either A,B,E,F) STD or C,D,G,H) LOW ASC supplementation after A-D) 6-weeks diabetes (2x) or E-H) 12-weeks (4x) diabetes. I-J) Quantified pixel intensity of FITC-dextran extravasation in different groups shows increases in the LOW STZ group at both time points.

DISCUSSION

We were able to create a diabetic model with increased blood glucose levels and decreased cortex ASC content determined by external ASC supplementation. All mice gained and maintained weight appropriately over the testing period indicating that STZ and ASC treatments did not adversely affect general health. Tissues were collected at the end of the study for biochemical measurements including ASC content, oxidative stress (as measured by MDA, protein carbonyls, F_2-isoprostanes, and F_4-neuroprostanes), tight junction protein levels, and extravasated FITC-dextran. The results from these studies are summarized in Figure 6.8.

It is unclear why the glucose levels at the 12-week time point in the LOW STZ mice were significantly less than the STD STZ mice. It is unlikely the LOW ASC supplementation directly affected the blood glucose level, as this interaction has not been shown before, and was not observed in the 6-week treatment group. Although the difference is significant, it is important to note that the blood glucose levels in both STZ groups were above 400 mg/dL, a level that is considered severely diabetic.

Differences in ASC levels among the groups in the 6-week diabetic group were more complicated than expected. Cortex ASC in non-diabetic LOW mice was approximately 50% lower than control mice, however, this difference was not significant. In contrast, diabetic LOW mice showed the expected very low ASC level that was significantly different from controls. STD diabetic mice had unusually high cortex ASC, although this was not different from non-diabetic controls. The STD diabetic mice did not have decreased ASC supplementation due to the dehydration because preliminary data showed these mice were not able to increase cortex ASC level above STD control mice level. Future studies should alter ASC supplementation in all diabetic mice in a similar manner due to the increased water intake.

To get a broad idea of the level of oxidative stress that might be going on in our experimental groups, multiple assays were performed. MDA levels are a marker of lipid peroxidation. Although we usually observe changes in MDA levels in mice on a LOW ASC supplement [33, 83] (Chapter III- Figure 3.4), we did not see the same effect in the 12-week diabetic groups. In addition, we did not see any changes in F_4-neuroprostanes and only detected increases at the 12-week time point for the F_2-isoprostanes.

Isoprostanes are a measurement of lipid peroxidation of primarily arachidonic acid while neuroprostanes are mainly derived from docosahexaenoic acid. The difference in the isoprostanes suggests arachidonic acid is more protected by ASC, while docosahexaenoic acid is protected by molecules other than ASC. There was no effect of the experimental paradigm on protein adduction due to increased oxidative stress, as measured by protein carbonyl levels. It is unclear why we were not able to observe many differences in oxidative stress in our model. Multiple other labs have reported STZ-induced diabetes increases oxidative stress in the brain [257, 258]. Therefore, we expected to see even greater oxidative stress due to the combination of diabetes and decreased brain ASC levels. The length of time that the mice were diabetic might have allowed for adaptations to control the oxidative stress. Other antioxidant systems, such as catalase, superoxide dismutase, and glutathione, could be evaluated to determine if these systems were up-regulated.

To determine if the diabetic treatment with different ASC supplements affected levels of two primary tight junction proteins, total cortex occludin and claudin-5 protein levels were measured. Although it would have been better to measure levels in a more homogeneous sample (only in isolated BECs), these proteins are predominantly expressed in endothelial cells in the brain and thus, the total levels most likely represent what is occurring in the BECs. There was no change in total cortex levels. However, *in vitro* studies show the localization of these proteins at the membrane is altered under high glucose and oxidative stress conditions, rather than changes in total levels [245, 259]. Looking at protein levels in different cell fractions would shed light on changes in the location of these proteins. In addition, phosphorylation status of junctional proteins can affect BBB permeability [237]. Therefore, measuring the levels of phosphorylated protein to total protein might shed light on the disruptions due to low ASC and diabetes.

Failure to detect changes in dextran extravasation warrants further technical experimental considerations. First, we looked only at levels of dextran in a homogenate of the frontal cortex. This area has been previously reported to have BBB breakdown under diabetic conditions [260] and a previous report has observed changes in homogenates of brain tissue [130]. There are many other brain regions that could be more susceptible to BBB breakdown in our model, as studies have shown localized

112

changes in the hippocampus, thalamus, striatum, midbrain, and cortex [130, 260]. Additional studies would need to be performed to better understand the regional differences in BBB breakdown. Either micropunches of brain regions could be taken for quantitative measurement of the dextran or immunofluorescence could be performed on tissue slices.

Another reason for a lack of change involves the use of mice in general. Other labs have reported no change in BBB disruption after STZ-diabetes in mice [261, 262] and most of the rodent work on BBB disruption in diabetes has been performed in rats [130, 157]. There are more studies using mice that investigate blood-retinal barrier disruption [140, 263, 264]. Therefore, our results are in agreement with the literature, in that we observe leakage of the tracer into the retina, but not in the brain. The differences in visualizing disruptions are most likely due to size of the sample, and thus a larger model, such as the rat, might help to distinguish differences.

The last few potential technical issues implicate the tracer. One consideration to keep in mind is the length of time the tracer is allowed to circulate. Studies have shown changes in extravasated tracer after a circulation time of anywhere from 1 to 16 hours [130, 265]. We also thought the amount of tracer might have been a limiting factor. For this reason, the 12-week diabetic mice received a greater concentration of the FITC-dextran. However, a 1.5 hour circulation time and increased concentration of tracer had no effect. Additionally, background fluorescence of brain tissue is quite intense, so in order to detect small changes, a tracer that does not compete with the background signal would be best.

Despite several technical difficulties, we were able to successfully create a low ASC, diabetic mouse model (Figure 6.8). Preparative work identified which assays were best-suited to this project and data emphasized the resistance of the C57BL/6 mouse model to oxidative stress. In moving forward to determine the *in vivo* role for ASC in regulating BBB integrity in diabetic conditions, technical parameters need to be further optimized including model used, concentration of tracer, length of time for circulation of tracer, and brain regions investigated. Additionally, instead of measuring flux of a tracer through the BBB, isolating the brain capillaries for biochemical measurements could help to elucidate whether there are alterations present at the BBB.
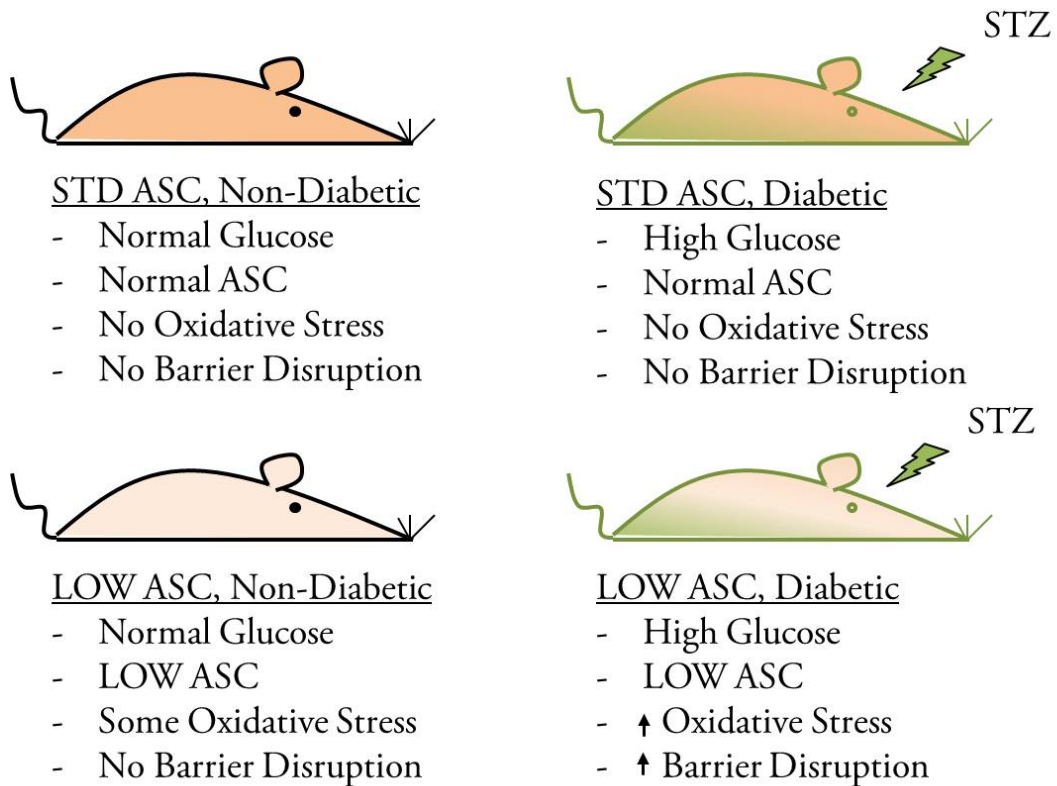
Figure 6.8. Summary model of Chapter VI. Glucose levels were increased in the mice treated with STZ. ASC levels were decreased in mice on LOW ASC supplementation. Oxidative stress was detected in the LOW ASC groups but more pronounced in the LOW ASC diabetic group. Blood-retinal barrier disruption was observed in the LOW ASC diabetic group.

CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The role for ASC in the brain during development, neurotransmitter synthesis, and at the BBB was investigated in the work presented here. Preliminary research suggested a great need for ASC during development [11] and in regulation of SVCT2 [54]. In addition, ASC had been shown to regulate norepinephrine synthesis and tyrosine hydroxylase expression [266]. Lastly, there was support that ASC could stabilize a peripheral endothelial cell barrier [193], suggesting a role for ASC in BECs. Therefore, the following conclusions can be made from the studies investigating these three separate areas in this dissertation.

ASC concentration during development and transporter regulation

To better understand the control of vitamin C transport in the brain, I began these studies by investigating the regulation of the main transporter responsible for this, SVCT2 (Chapter III). During gestation, expression levels of this transporter are low but expression greatly increases by postnatal day 32. This expression pattern did not parallel the levels of ASC during this time period. Instead, ASC levels increased significantly just before birth in the cortex, while declining in the postnatal period. These findings support the increased need for an antioxidant defense, in this case ASC, against the high levels of oxygen right after birth.

Also, in adult mice with varying levels of cortex ASC, SVCT2 expression was unaltered, despite changes in oxidative stress in contrast to data obtained earlier in development. These results suggest that the SVCT2 is differentially regulated depending on the tissue and age. Therefore, it would be interesting to create cell-specific or temporal knockouts of SVCT2 to determine the effects due to loss of ASC in a specific cell type or at a specific time during development. As ASC has been shown to

play a role in differentiation [55], loss of ASC early in development could have lasting detrimental effects that delay certain processes.

In addition, we did not determine how SVCT2 expression in the brain is regulated. Our results suggest that ASC level itself is most unlikely to regulate SVCT2 at this time period. Determining how SVCT2 is regulated at the levels of transcription and translation would help reveal the mechanism behind altered SVCT2 expression. A former post-doc in the May lab investigated the promoter region of SVCT2. Dr. Qiao found transcription factors such as NF-Y and YY1 regulate transcription of SVCT2 [267]. The expression pattern of these factors could help elucidate SVCT2 regulation in the cortex during development.

It would also be important to determine if the transporter activity is regulated during development to increase brain ASC level, independent of transporter level. A former graduate student in the lab, Marquicia Pierce, explored a model to study the kinetics of SVCT2 in synaptosomes, pinched off nerve terminals. Utilizing this model during development would help decipher the transporter kinetics in greater detail. Multiple studies have been performed in peripheral cells suggesting SVCT2 is regulated by an oxidative stress mechanism [268-270], but these were performed in cell cultures, a model with inherent oxidative stress. It would be better to investigate the regulation *in vivo* as cell cultures are under great stress and the expression of SVCT2 appears to be different under culture conditions [271-273]. One *in vivo* example of cells developing expression of SVCT2 is in BECs following ischemic stroke [50].

ASC and neurotransmitter synthesis

In the second part of the study, we investigated the role for ASC in regulating neurotransmitter synthesis (Chapter IV). There was considerable evidence to support our hypothesis the ASC could alter neurotransmitter levels and the enzymes responsible for synthesis because ASC had been shown *in vitro* to regulate tyrosine hydroxylase synthesis, as well as norepinephrine levels [29, 30]. In mice completely depleted of ASC in the cortex, dopamine and norepinephrine levels were significantly decreased, as was tyrosine hydroxylase protein expression. Increasing cortical ASC levels increased dopamine and its metabolite DOPAC significantly. There was no effect

on norepinephrine or tyrosine hydroxylase but there was a significant increase in serotonin and its metabolite 5-HIAA. In all, these results suggest ASC can alter neurotransmitter synthesis, partially by affecting tyrosine hydroxylase protein levels.

Acute intracerebroventricular injections of ASC would help to better define molecular changes *in vivo* in neurotransmitter regulation. A voltammetric microprobe could be stereotaxically placed in the brain region of interest to measure dopamine, serotonin, and ASC levels simultaneously [274]. This would provide a more detailed assessment of the interaction with ASC and neurotransmitter level. In addition, it would investigate the release patterns of these neurotransmitters under different ASC concentrations. Hypothetically, if there is more ASC present in certain brain regions, there could be a greater amount of release of the neurotransmitters due to the increased synthesis.

Although we observed that very low ASC levels were associated with decreased tyrosine hydroxylase protein levels in late-stage embryos, it is still unclear how the two are related; that is, if it is through a positive feedback loop. If the co-factor responsible for tyrosine hydroxylase activation, tetrahydrobiopterin, is not present, the hydroxylase might down-regulate its synthesis. As ASC recycles tetrahydrobiopterin, loss of ASC could lead to a decrease in the level of this co-factor. Tetrahydrobiopterin levels could be measured by taking advantage of Vanderbilt's new Free Radical in Medicine Core that has the ability to measure these levels in biological samples. On the other hand, oxidative stress can trigger multiple downstream signaling pathways and elicit changes in protein synthesis. The neuronal cell line that has been used before to investigate the role of ASC on tyrosine hydroxylase expression, SH-SY5Y cell line, could be utilized to determine the molecular changes taking place due to ASC depletion or supplementation on hydroxylase expression. Since tyrosine hydroxylase protein levels have been shown to be down-regulated under oxidative stress [275], it is likely this could be the reason for decreased expression levels with low ASC.

Our studies involved investigating neurotransmitter synthesis during development. Because this is a critical period for how species are shaped, it would be interesting to determine the long-lasting effects of ASC-deficiency during gestation. Gulo(-/-) mice could also be used to study the effects of varying ASC level during

117

development into adulthood. Decreasing the maternal ASC supplementation also decreases the supply to the pups [276]. Allowing these pups to age with and without ASC repletion would show the effects of ASC deficiency during gestation.

In addition, previous studies have linked low ASC levels with increased depressive-like symptoms, as mentioned in Chapter IV and VIII. Depleting Gulo(-/-) mice of ASC and then testing for behaviors thought to be related to depression, such as the forced swim or tail suspension test, could show a behavior link for ASC in adults. Multiple rodent studies have shown that ASC injections can decrease depressive-like symptoms [277, 278]. However, these studies look at excess ASC injections in rats already able to synthesize their own ASC. Using the Gulo(-/-) model would help relate the findings to humans. Ward *et al* attempted to investigate behavioral changes in Gulo(-/-) mice near scurvy [185]. However, these mice were not very active, skewing the results of the tail suspension test. Interpreting data from tasks indicative of "depressive-like" behaviors that do not involve much activity or strength would be best to try first, such as test tube aggression.

ASC and the BBB

In the last part of this work, the role for ASC at the BBB was investigated (Chapters V, VI). I first explored the effects of ASC in a cell culture model under basal conditions, as well as high glucose conditions. I then looked *in vivo* to begin to determine the effects of ASC in a mouse model with low ASC and diabetes.

First, increased intracellular ASC decreased the monolayer permeability of BECs by about 40% on average. High glucose increased permeability and ASC prevented this during a 60 min incubation. It was also shown RAGE activation increased permeability and ASC as well as other anti-oxidants, countered this effect on permeability. Second, under basal glucose conditions, ASC decreased permeability through signaling via Epac and the cytoskeleton. These studies further investigate the molecular mechanisms behind the regulation of barrier integrity, especially in the context of diabetes.

Although we believe the effects of ASC on permeability are mediated through a cAMP-mediated signaling mechanism, we did not measure cAMP directly due to the great abundance of bound cAMP present in cells and difficulty in observing small

differences over the background signal in a spatial and temporal manner [279, 280]. Additionally, there are other signals that can prime Epac to make it more sensitive to cAMP [281]. Therefore, total levels of cAMP may not change but rather what changes is the sensitivity of Epac to cAMP. However, it still needs to be determined whether ASC increases cAMP and how; whether it is through an oxidative stress mechanism or by affecting cAMP enhancing and degrading enzymes. These studies should begin by using more sensitive analyses, taking into consideration temporal resolution, such as utilizing Fluorescence Resonance Energy Transfer (FRET) to monitor the binding of cAMP to Epac by excitation of a fluorophore.

We did not observe changes in activity of the canonical signaling small GTPase for Epac1, Rap1. Most likely there is some other mediator that is activated by Epac1 to alter cytoskeleton rearrangement. Due to the recent discovery of Epac1, very few substrates have been investigated, other than Rap1. Therefore, other small GTPases could be activated by Epac, as has been suggested for Rac1 [253]. In addition, Epac1 has recently been shown to directly interact with the microtubule associating protein 1b light chain 1 (MAP1B LC1) [281]. This interaction is thought to affect the cytoskeleton via microtubule arrangement independent of a GTPase mediator. Measuring the association of Epac1 and MAP1B LC1 after ASC incubation would help elucidate whether this protein is involved in our model. In addition, utilizing siRNA targeting Epac1 will help confirm the extent of the involvement in Epac's role in mediating a decrease in permeability due to ASC. Transfection of altered or shortened Epac1 sequences would show the sites on this protein responsible for mediating the effects due to ASC. It has been shown previously the catalytic site on Epac1 responsible for facilitating the activation of guanine nucleotide exchange factors, not the cAMP binding site, is also responsible for the interaction of Epac1 with MAP1B LC1 [281]. Therefore, disruption of this site might prevent the ability of ASC to decrease permeability.

Although changes in phosphorylation were not observed on occludin or VE-cadherin, it is still possible these proteins are involved in an ASC effect. Performing immunofluorescence on the cells before and after ASC treatment would show if the proteins are being localized to the cell membrane. Also, cell fractionation and Western immunoblot experiments could be performed. Lastly, tagging the junction proteins with a

fluorescent molecule such as GFP or mCherry would allow for live visualization of the trafficking due to ASC treatment.

The *in vivo* investigations proved to be more difficult than originally hypothesized. Although streptozotocin caused substantial and prolonged diabetes in C57BL/6 mice, we did not observe many of the effects that have been previously reported in diabetic mice, especially in the context of BBB disruption and oxidative stress [130, 157, 282]. Further, although we were able to successfully deplete diabetic Gulo(-/-) mice of ASC, this caused only trends in BBB dysfunction compared to controls. Due to the resistance of the C57BL/6 mouse in our model and small size, it would be best to move forward in studies using a larger rodent model, such as the rat or guinea pig, to better detect disruptions in the BBB. Because small disruptions in the barrier can have major, clinical implications, a more sensitive assay, or a larger sample size is needed. There is a spontaneous mutant ASC deficient rat model, known as the Osteogenic Disorder Shionogi (ODS) rat, that is unable to synthesize its own ASC [9], similar to the Gulo(-/-) mouse. In addition, the guinea pig is not able to synthesize its own ASC and relies on dietary supplementation, similar to humans.

Another potential problem in the observed lack of change in BBB disruption could be due to the tracer used. The current study utilized a 10 kDa FITC-dextran tracer. However, radiolabeled sucrose should be considered as a tracer for future BBB breakdown studies. This molecule is small and can easily be detected in brain samples due to the low background signal [130]. The FITC-dextran signal was not much greater than the background signal fluorescence in the cortex (Figure 6.6).

As mentioned in Chapter I, matrix metalloproteinases are an important mediator of BBB breakdown by regulating the stability of the extracellular matrix. They are activated by increased levels of oxidative stress and thus, are increased in diabetes [283]. These proteinases are primarily responsible for degrading basement membrane. Therefore, it is likely matrix metalloproteinase activity levels could be affected by ASC level in the brain as ASC supplementation decreased the amount of matrix metalloproteinase 9 in a breast cancer model [284]. This information would explore the role of another structure involved in the integrity of the BBB besides the BECs, the extracellular matrix.

As also previously mentioned, SVCT2 is not normally expressed in BECs *in vivo.* Nonetheless, these cells are still able to take up DHA through the glucose transporters and store intracellular ASC. In addition, under stressed conditions, Gess *et al* has shown SVCT2 is up-regulated in BECs, specifically after transient ischemia [50]. Therefore, these cells possess the ability to induce expression of this transporter. It is hypothesized that under hyperglycemia, BECs increase expression of SVCT2 allowing for increased ASC uptake. Collecting or staining BECs for SVCT2 expression measurement from diabetic mice with low ASC would show whether or not SVCT2 can be induced under diabetic conditions.

Conclusion

In all, the results presented here provide two important pieces of information. First, it is clear ASC is required for multiple processes throughout the brain, including development, neurotransmitter synthesis, and BEC stability. Second, there is much more work to be done on fully investigating the molecular mechanisms due to depletion or excess ASC concentration in brain. This continual collection of evidence will help to understand the implications of ASC in normal human health as described next.

CHAPTER VIII

IMPLICATIONS

Vitamin C and Clinical Trials

The reported beneficial effects of vitamin C, especially as an antioxidant, have been propounded to the lay public and may appear to some as being studied extensively. Due to heavy clinical investigation by Linus Pauling in the 1970s in the prevention of diseases from the common cold to cancer, treatment of diseases with this vitamin lost its credibility due to Pauling's reputation as a "quack". Researchers challenged his work on the basis of inappropriate use of control groups, as well as his own personal experimentation in high dose vitamin C regimens [285]. Pauling stood by his work and defended it against other studies trying to repeat the effects, showing no difference [286]. Hopefully with the recent study on the beneficial effects of vitamin C in cancer [287], some of Pauling's clinical studies of vitamin C will be vindicated. This would add another chapter to his diverse accomplishments, which include the Nobel Prize in chemistry for discovering nature of the chemical bond and his Nobel Peace Prize for working to end the possibility of war in the nuclear age.

One major oversight that has plagued clinical trials investigating treatment effects of vitamin C is the method of delivery. It is now clear, the transporter of vitamin C, SVCT1, is limited in its ability to take up large amounts of orally ingested vitamin C. The saturable transport system limits vitamin C uptake in the intestines after oral ingestion [288]. Therefore, plasma vitamin C levels are typically not greater than 100 µM unless injected intravenously. Riboflavin, or vitamin B_2, has similar saturation kinetics [289]. This saturable limit for SVCT2 also holds true for vitamin C transport into the brain. Therefore, it is important to investigate what the maximal concentration is in brain and whether or not subtle regional differences in ASC has a functional effect. Persons with impaired transport of substrates at the choroid plexus as well as those deficient in ASC levels are the key targets in investigating these effects clinically.

In addition, oral ingestion takes time, nearly 3-5 hours, before plasma levels reach a maximal plateau [288]. If the oral dose of ASC is divided into smaller doses throughout the day, a greater percentage of the vitamin can be absorbed compared to if a single, large dose was ingested [288]. Greater attention needs to be paid to the mode and limitations of oral ASC administration. The studies over the past couple of decades showing anecdotal evidence for the effectiveness of vitamin C have been greatly scrutinized and doubted because of the equally abundant negative results, especially when vitamin C is provided as an oral low-dose supplement rather than as repletion of a deficiency [286, 290].

The early scrutiny of this vitamin required future studies to come out cleaner and with better controls. This is exactly the case for a recent report just published in February 2014 in which reconsideration was given to the therapeutic ability of vitamin C in cancer. A well-controlled clinical trial published in *Science Translational Medicine*, in which vitamin C was administered along with standard chemotherapy, suggests vitamin C can improve the side effects due to chemotherapy in ovarian cancer [287]. The researchers also investigated the molecular mechanisms in a cancer cell line and found vitamin C targeted the cancerous cells due to a difference in the metabolism of energy compared to non-cancerous cells.

Vitamin C Safety

Although there is a common concern for safety amongst the general population with mega-doses of vitamin C, the toxic effects of vitamin C have mostly been observed *in vitro*, in people with susceptibility to kidney stones, and in persons with glycolytic enzyme deficiencies [291]. Kidney stone formation and an osmotic diarrhea are the most commonly reported side effects due to high doses. Generally, doses of vitamin C under 10 g orally are not toxic in individuals with normal renal function and those that are not susceptible oxalate stone formation [292].

Vitamin C and Depression

Multiple neurological diseases arise due to neurotransmitter imbalance. Depression is one which is associated with many co-morbidities including diabetes

mellitus, cancer, cardiovascular disease, and anxiety [293, 294]. People with depression have lower circulating levels of anti-oxidants. Indeed, plasma vitamin C levels are decreased in patients with depression [215]. It is unclear whether this is due to the increased oxidative stress that occurs in depression or a decreased vitamin intake due to lack of proper diet [295, 296]. Recent studies suggest the former.

Vitamin C has been shown to improve mood and depression [213, 214]. In one study, blinded, healthy subjects received 3000 mg/day vitamin C supplementation for 14 days and were measured for depression before and after the supplementation period [213]. Even though this dosing regimen was provided orally, the vitamin group still had decreases in the Beck Depression Inventory score, while no change was observed in the placebo group. In another blinded study, acutely hospitalized patients with deficient levels of plasma vitamin C received either vitamin C (500 mg by mouth twice daily) or an identical tablet containing vitamin D. In those receiving vitamin C, plasma levels tripled and mood disturbance score was decreased by nearly 35%, indicating an improvement in mood [214]. The supplementation of two doses of vitamin C throughout the day is more sensible than one large dose to optimally boost levels throughout the day. One could test for neurotransmitter imbalance after a vitamin C regimen to determine more specifically if these are linked. However, for now, these results correlate plasma vitamin C level with improved mood, suggesting that molecular changes take place in the brain after vitamin C supplementation. Most important in subgroups with vitamin C deficiency, emphasis should be directed to replacing a deficit of the vitamin, rather than supplementing already normal levels.

Vitamin C and Diabetes

Diabetes, more specifically hyperglycemia, is primarily associated with increased oxidative stress. As vitamin C is a well-known antioxidant, many researchers have investigated the relationship between vitamin C level and diabetic complications. Indeed, two separate longitudinal clinical trials showed an inverse relationship between plasma vitamin C and hemoglobin A1c level [297, 298]. Specifically, microvascular complications arise from diabetes, in which the increased level of oxidative stress causes endothelial cell dysfunction. Another study has reported an inverse association

in a complication due to diabetes, diabetic retinopathy, and plasma vitamin C level [299]. Nearly 30% of people with diabetes over the age of 40 develop diabetic retinopathy [300].

Diabetes mellitus increases the risk not only for developing cognitive decline later in life but also Alzheimer's disease [301-303]. One recent study shows that this risk is associated with duration of diabetes [303]. Due to the oxidative stress nature of this disease, studies have reported an inverse association between anti-oxidant supplementation, vitamin C included, and development of Alzheimer's disease [304, 305]. In fact, vitamin C levels are greatly decreased by over 50% in people with mild cognitive impairment, as well as Alzheimer's disease [306]. This association was independent of malnutrition as a nutritional assessment was performed before the study.

It is important to keep in mind food consumption when interpreting data from studies evaluating the relationship between nutrients and diseases. For example, diabetics could have decreased levels of a certain vitamin due to poor diet, a common problem in diabetics. However, clinical trials, as seen in the one mentioned above, have begun to document dietary intake through extensive questionnaires. These small details and extra information could add tremendously to the outcome of the study.

Recommended Level of Vitamin C

Irwin Stone, the man responsible for the introduction of Pauling to vitamin C, along with Pauling, have recommended 2-3 grams for daily vitamin C intake [307]. This recommendation is nearly 30 times greater than the standard recommended intake of 75 mg/day for adult females in the United States [8]. Pauling's values are derived from previous studies performed on vitamin C intake and synthesis by other mammals. For example, an average 450-pound gorilla ingests approximately 4.5 g of the vitamin per day based on its diet [308, 309] and it has been reported that unstressed rats, who can synthesize their own ASC, produce the equivalent of nearly 5 g/day for a 155-pound man [310]. Indeed, humans can survive with no clinical symptoms of deficiency on much lower levels of vitamin C, suggesting that human physiology has been able to adapt to this low consumption. However, the level ingested and synthesized by other

mammals under unstressed conditions supports the potential for what we consider mega-doses of vitamin C (in the gram range), especially as a therapeutic agent.

The medical profession has taken a black and white approach to the recommended daily allowance for vitamin C, considering only its ability to prevent the disease scurvy. Recommendations suggest that if there is no scurvy, there is likely no lack of vitamin C. However, scurvy is the ultimate result of vitamin C deficiency. There is a large gap between scurvy and full health in terms of vitamin C that needs to be continually investigated. Indeed, every individual is different in the context of oxidative stressors such as disease, environment, and smoking as well as genetic differences, which could be a reason why clinical trials report contradicting results in the involvement of vitamin C affecting disease outcomes. As personalized medicine becomes more prevalent, it will be interesting to investigate the circumstances that cause some individuals to require more vitamin C than others. The same organization that sets the recommended daily intake for the United States, also sets a less well-known tolerable upper limit level at 2000 mg/day [311]. This agrees with the same daily dose recommended by Pauling.

Recommended daily allowances are just that, recommendations. Some people choose to ingest more while others ingest less, as mentioned in Chapter I with a marked percent of the population that is vitamin C deficient. In fact, although the recommended daily allowance is derived from the ability to prevent scurvy, it is not standard across multiple countries. Keeping in mind that the United States recommends 75-90 mg/day for adult women and men [8], Australia only recommends 45 mg/day for adult men and women [312], while the United Kingdom recommends even less, 40 mg/day [313]. The difference between 40 and 90 mg of vitamin C is rather large when the size of the scale is taken into consideration in supplementing a deficiency or preventing a disease. The problem remains though, in the trust of individuals on the authorities setting the recommendation levels. The greater population might not see a need in acquiring more if it is not recommended. In addition, and potentially more problematic, school lunches are built around the recommended daily allowances. The National School Lunch Program requires one-third of a child's daily recommended intake of vitamin C to be

provided [314]. Therefore, only one-third of the amount recommended to prevent scurvy is going to be provided to school-aged children.

It has been suggested primates lost the ability to synthesize vitamin C by chance during a genetic bottleneck and that this was not selected against due to the freely available nutrient in the foods at the time [308]. The amount of vitamin C ingested by our ancestors, living amongst foods that had plentiful levels, were likely much greater than the amount ingested today, in our Western diets and given the current recommendations. It is thus plausible to think, that many of the chronic illnesses exhibited today could benefit from increased intake of this and other vitamins. The molecular mechanisms required by vitamin C throughout the body is why more studies similar to the ones presented in this dissertation (Fig. 8.1) need to be performed in order to better investigate the effects of ASC in a clinical setting.
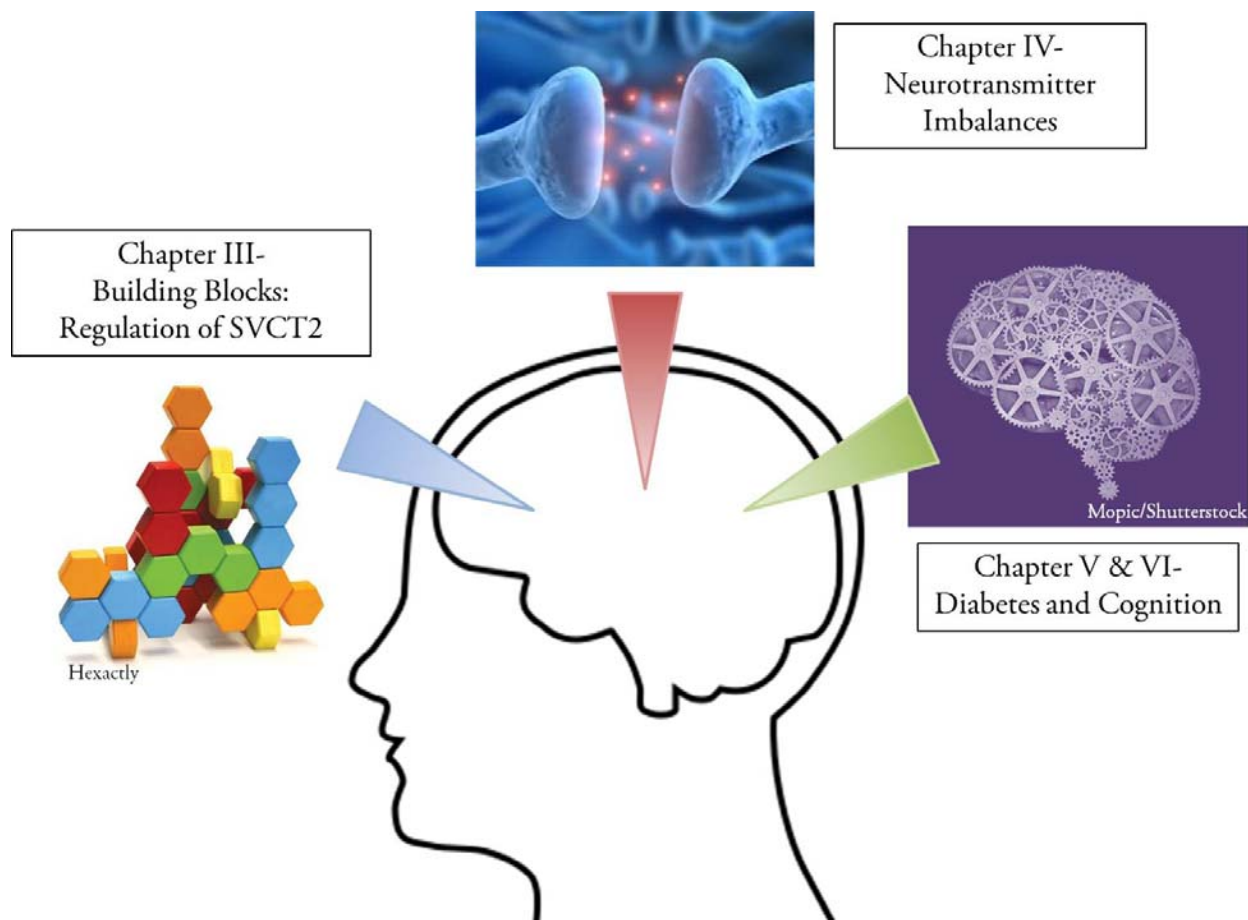
Figure 8.1. Dissertation Model of Translational Implications. Chapter III focused on the basic regulation of the SVCT2 and building blocks of ASC transport. Chapter IV investigated changes in neurotransmitter levels with manipulations of ASC levels, relating these findings to diseases with neurotransmitter imbalances. Chapter V and VI investigated the role for ASC in endothelial cell permeability, an area that is problematic in diabetes and cognition.
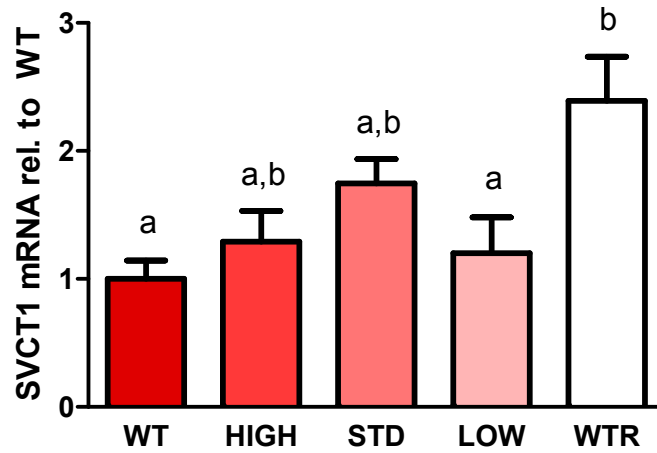
Figure A1. Liver SVCT1 mRNA levels in adult Gulo(-/-) mice. SVCT1 mRNA was increased in the WTR supplemented mice compared to WT and LOW groups. Groups that do not share the same letter are different, p<0.05, n=5-7 samples per group
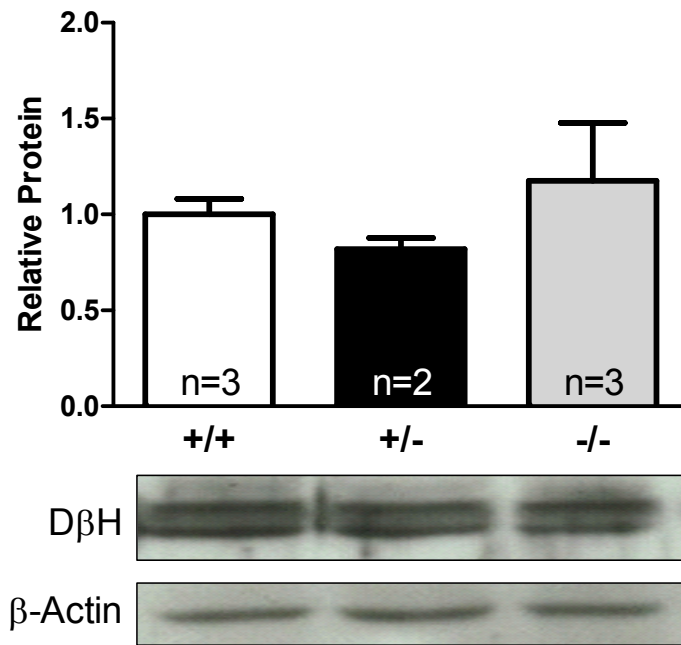
Figure A2. Dopamine β-hydroxylase protein levels in SVCT2(+/-) embryos. Dopamine β-hydroxylase (DβH) protein levels did not differ amongst the SVCT2(+/-) littermates. A representative immunoblot is shown.

REFERENCES

1. Szent-Gyorgyi A: Observations on the function of peroxidase systems and the chemistry of the adrenal cortex: Description of a new carbohydrate derivative. *Biochem J* 1928, 22(6):1387-1409.
2. Lamb J: Preserving the Self in the South Seas, 1680-1840: University of Chicago Press; 2001.
3. Jousset P, Arschagouni J: Practice of medicine: containing the Homeopathic treatment of diseases: A. L. Chatterton & Co.; 1901.
4. Brown SR: Scurvy. Toronto, Ontario, Canada: Thomas Allen Publishers; 2003.
5. Martini E: Jacques Cartier witnesses a treatment for scurvy. *Vesalius : acta internationales historiae medicinae* 2002, 8(1):2-6.
6. Lind J: A Treatise of the Scurvy. Edinburgh: Sands, Murray and Cochran; 1753.
7. Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K: Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. *J Biol Chem* 1994, 269(18):13685-13688.
8. Services UDoAaUDoHaH: Dietary Guidelines for Americans, 2010, 7 edn. Washington, DC: U.S. Government Printing Office; 2010.
9. Mizushima Y, Harauchi T, Yoshizaki T, Makino S: A rat mutant unable to synthesize vitamin C. *Experientia* 1984, 40(4):359-361.
10. Kondo Y, Inai Y, Sato Y, Handa S, Kubo S, Shimokado K, Goto S, Nishikimi M, Maruyama N, Ishigami A: Senescence marker protein 30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy. *Proc Natl Acad Sci U S A* 2006, 103(15):5723-5728.
11. Sotiriou S, Gispert S, Cheng J, Wang YH, Chen A, Hoogstraten-Miller S, Miller GF, Kwon O, Levine M, Guttentag SH *et al*: Ascorbic-acid transporter Slc23a1 is essential for vitamin C transport into the brain and for perinatal survival. *Nature Medicine* 2002, 8(5):514-517.
12. Corpe CP, Tu H, Eck P, Wang J, Faulhaber-Walter R, Schnermann J, Margolis S, Padayatty S, Sun H, Wang Y *et al*: Vitamin C transporter Slc23a1 links renal reabsorption, vitamin C tissue accumulation, and perinatal survival in mice. *JClinInvest* 2010, 120:1069-1083.
13. Harrison FE, Best JL, Meredith ME, Gamlin CR, Borza DB, May JM: Increased expression of SVCT2 in a new mouse model raises ascorbic acid in tissues and protects against paraquat-induced oxidative damage in lung. *PLoSOne* 2012, 7(4):e35623.
14. Maeda N, Hagihara H, Nakata Y, Hiller S, Wilder J, Reddick R: Aortic wall damage in mice unable to synthesize ascorbic acid. *Proceedings of the National Academy of Sciences of the United States of America* 2000, 97(2):841-846.
15. Gabbay KH, Bohren KM, Morello R, Bertin T, Liu J, Vogel P: Ascorbate synthesis pathway: dual role of ascorbate in bone homeostasis. *Journal of Biological Chemistry* 2010, 285:19510-19520.
16. Davies MB, Austin J, Partridge DA: Vitamin C: Its Chemistry and Biochemistry: Royal Society of Chemistry; 1991.
17. May JM: Assessing the reductive capacity of cells by measuring the recycling of ascorbic and lipoic acids. *Methods in molecular biology* 2010, 610:229-243.
18. Bielski BH, Allen AO, Schwarz HA: Mechanism of disproportionation of ascorbate radicals. *Journal of the American Chemical Society* 1981, 103:3516-3518.
19. Koshiishi I, Mamura Y, Liu J, Imanari T: Degradation of dehydroascorbate to 2,3-diketogulonate in blood circulation. *Biochim Biophys Acta* 1998, 1425(1):209-214.

20. Winkler BS: In vitro oxidation of ascorbic acid and its prevention by GSH. *Biochim Biophys Acta* 1987, 925(3):258-264.

21. Harrison FE, May JM: Vitamin C function in the brain: Vital role of the ascorbate transporter (SVCT2). *Free RadicBiol Med* 2009, 45:719-730.

22. Minor EA, Court BL, Young JI, Wang G: Ascorbate induces ten-eleven translocation (Tet) methylcytosine dioxygenase-mediated generation of 5-hydroxymethylcytosine. *J Biol Chem* 2013, 288(19):13669-13674.

23. Guo X, Lu J, Wang Y, Gui Y, Duan X, Cai Z: Ascorbate antagonizes nickel ion to regulate JMJD1A expression in kidney cancer cells. *Acta biochimica et biophysica Sinica* 2012, 44(4):330-338.

24. Burzle M, Hediger MA: Functional and physiological role of vitamin C transporters. *Curr Top Membr* 2012, 70:357-375.

25. Chatterjee IB, Majumder AK, Nandi BK, Subramanian N: Synthesis and some major functions of vitamin C in animals. *Ann N Y Acad Sci* 1975, 258:24-47.

26. Bruegge K, Jelkmann W, Metzen E: Hydroxylation of hypoxia-inducible transcription factors and chemical compounds targeting the HIF-alpha hydroxylases. *Curr Med Chem* 2007, 14(17):1853-1862.

27. Mortensen A, Hasselholt S, Tveden-Nyborg P, Lykkesfeldt J: Guinea pig ascorbate status predicts tetrahydrobiopterin plasma concentration and oxidation ratio in vivo. *Nutr Res* 2013, 33(10):859-867.

28. Wilson JX: Mechanism of action of vitamin C in sepsis: ascorbate modulates redox signaling in endothelium. *Biofactors* 2009, 35(1):5-13.

29. May JM, Qu ZC, Nazarewicz R, Dikalov S: Ascorbic Acid Efficiently Enhances Neuronal Synthesis of Norepinephrine from Dopamine. *Brain Research Bulletin* 2013, 90:35-42.

30. Seitz G, Gebhardt S, Beck JF, Bohm W, Lode HN, Niethammer D, Bruchelt G: Ascorbic acid stimulates DOPA synthesis and tyrosine hydroxylase gene expression in the human neuroblastoma cell line SK-N-SH. *Neuroscience Letters* 1998, 244(1):33-36.

31. May JM, Qu ZC, Meredith ME: Mechanisms of ascorbic acid stimulation of norepinephrine synthesis in neuronal cells. *Biochem Biophys Res Commun* 2012, 426(1):148-152.

32. Meredith ME, May JM: Regulation of embryonic neurotransmitter and tyrosine hydroxylase protein levels by ascorbic acid. *Brain Res* 2013, 1539:7-14.

33. Harrison FE, Green RJ, Dawes SM, May JM: Vitamin C distribution and retention in the mouse brain. *Brain Research* 2010, 1348:181-186.

34. Jackson TS, Xu AM, Vita JA, Keaney JF, Jr.: Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circulation Research* 1998, 83(9):916-922.

35. Arrigoni O, De Tullio MC: Ascorbic acid: much more than just an antioxidant. *Biochimica et Biophysica Acta: General Subjects* 2002, 1569(1-3):1-9.

36. Astuya A, Caprile T, Castro M, Salazar K, Garcia Mde L, Reinicke K, Rodriguez F, Vera JC, Millan C, Ulloa V *et al*: Vitamin C uptake and recycling among normal and tumor cells from the central nervous system. *J Neurosci Res* 2005, 79(1-2):146-156.

37. Rice ME: Ascorbate regulation and its neuroprotective role in the brain. *Trends Neurosci* 2000, 23(5):209-216.

38. Tsukaguchi H, Tokui T, Mackenzie B, Berger UV, Chen XZ, Wang Y, Brubaker RF, Hediger MA: A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature* 1999, 399(6731):70-75.

39. Tsukaguchi H, Tokui T, Mackenzie B, Berger UV, Chen XZ, Wang YX, Brubaker RF, Hediger MA: A family of mammalian Na + -dependent L-ascorbic acid transporters. *Nature* 1999, 399(6731):70-75.

40. Gispert S, Dutra A, Lieberman A, Friedlich D, Nussbaum RL: Cloning and genomic organization of the mouse gene slc23a1 encoding a vitamin C transporter. *DNA Res* 2000, 7(6):339-345.

41. Faaland CA, Race JE, Ricken G, Warner FJ, Williams WJ, Holtzman EJ: Molecular characterization of two novel transporters from human and mouse kidney and from LLC-PK1 cells reveals a novel conserved family that is homologous to bacterial and Aspergillus nucleobase transporters. *Biochim Biophys Acta* 1998, 1442(2-3):353-360.

42. Rajan DP, Huang W, Dutta B, Devoe LD, Leibach FH, Ganapathy V, Prasad PD: Human placental sodium-dependent vitamin C transporter (SVCT2): Molecular cloning and transport function. *Biochemical and Biophysical Research Communications* 1999, 262(3):762-768.

43. Mandl J, Szarka A, Banhegyi G: Vitamin C: update on physiology and pharmacology. *Br J Pharmacol* 2009, 157(7):1097-1110.

44. Liang WJ, Johnson D, Jarvis SM: Vitamin C transport systems of mammalian cells. *MolMembrBiol* 2001, 18(1):87-95.

45. Mun GH, Kim MJ, Lee JH, Kim HJ, Chung YH, Chung YB, Kang JS, Hwang YI, Oh SH, Kim JG *et al*: Immunohistochemical study of the distribution of sodium-dependent vitamin C transporters in adult rat brain. *Journal of Neuroscience Research* 2006, 83(5):919-928.

46. Savini I, Rossi A, Pierro C, Avigliano L, Catani MV: SVCT1 and SVCT2: key proteins for vitamin C uptake. *AminoAcids* 2008, 34:347-355.

47. Stamford JA, Kruk ZL, Millar J: Regional differences in extracellular ascorbic acid levels in the rat brain determined by high speed cyclic voltammetry. *Brain Res* 1984, 299(2):289-295.

48. Castro M, Caprile T, Astuya A, Millan C, Reinicke K, Vera JC, Vasquez O, Aguayo LG, Nualart F: High-affinity sodium-vitamin C co-transporters (SVCT) expression in embryonic mouse neurons. *J Neurochem* 2001, 78(4):815-823.

49. Berger UV, Lu XC, Liu W, Tang Z, Slusher BS, Hediger MA: Effect of middle cerebral artery occlusion on mRNA expression for the sodium-coupled vitamin C transporter SVCT2 in rat brain. *J Neurochem* 2003, 86(4):896-906.

50. Gess B, Sevimli S, Strecker JK, Young P, Schabitz WR: Sodium-Dependent Vitamin C Transporter 2 (SVCT2) Expression and Activity in Brain Capillary Endothelial Cells after Transient Ischemia in Mice. *PLoSOne* 2011, 6(2):e17139.

51. Savini I, Rossi A, Catani MV, Ceci R, Avigliano L: Redox regulation of vitamin C transporter SVCT2 in C2C12 myotubes. *Biochem Biophys Res Commun* 2007, 361(2):385-390.

52. Dixon SJ, Wilson JX: Adaptive regulation of ascorbate transport in osteoblastic cells. *J Bone Miner Res* 1992, 7(6):675-681.

53. Wilson JX, Jaworski EM, Kulaga A, Dixon SJ: Substrate regulation of ascorbate transport activity in astrocytes. *Neurochem Res* 1990, 15(10):1037-1043.

54. Qiao H, May JM: Development of ascorbate transport in brain capillary endothelial cells in culture. *Brain Research* 2008, 1208:79-86.

55. Low M, Sandoval D, Morales B, Nualart F, Henriquez JP: Up-regulation of the vitamin C transporter SVCT2 upon differentiation and depolarization of myotubes. *FEBS Letters* 2011, 585(2):390-396.

56. Rose RC, Bode AM: Ocular ascorbate transport and metabolism. *Comparative biochemistry and physiology A, Comparative physiology* 1991, 100(2):273-285.

57. Kodama T, Kabasawa I, Tamura O, Reddy VN: Dynamics of ascorbate in the aqueous humor and tissues surrounding ocular chambers. *Ophthalmic research* 1985, 17(6):331-337.

58. Halliwell B, Gutteridge JM: Free radicals, lipid peroxidation, and cell damage. *Lancet* 1984, 2(8411):1095.

59. Milby K, Oke A, Adams RN: Detailed mapping of ascorbate distribution in rat brain. *Neuroscience Letters* 1982, 28(1):15-20.

60. Kratzing CC, Kelly JD: Tissue levels of ascorbic acid during rat gestation. *IntJVitamNutrRes* 1982, 52(3):326-332.

61. Hornig D: Distribution of ascorbic acid, metabolites and analogues in man and animals. *Annals of the New York Academy of Sciences* 1975, 258:103-118.

62. Oke AF, May L, Adams RN: Ascorbic acid distribution patterns in human brain. A comparison with nonhuman mammalian species. *AnnNYAcadSci* 1987, 498:1-12.

63. Siushansian R, Tao L, Dixon SJ, Wilson JX: Cerebral astrocytes transport ascorbic acid and dehydroascorbic acid through distinct mechanisms regulated by cyclic AMP. *Journal of Neurochemistry* 1997, 68(6):2378-2385.

64. Daniels AJ, Dean G, Viveros OH, Diliberto EJ, Jr.: Secretion of newly taken-up ascorbic acid by adrenomedullary chromaffin cells. *Science* 1982, 216(4547):737-739.

65. Wilson JX, Peters CE, Sitar SM, Daoust P, Gelb AW: Glutamate stimulates ascorbate transport by astrocytes. *Brain Research* 2000, 858(1):61-66.

66. Grunewald RA, Fillenz M: Release of ascorbate from a synaptosomal fraction of rat brain. *NeurochemInt* 1984, 6(4):491-500.

67. Corti A, Casini AF, Pompella A: Cellular pathways for transport and efflux of ascorbate and dehydroascorbate. *Archives of Biochemistry and Biophysics* 2010, 500(2):107-115.

68. Spector R: Vitamin homeostasis in the central nervous system. *New England Journal of Medicine* 1977, 296:1393-1398.

69. Ballard JW: Drosophila simulans as a novel model for studying mitochondrial metabolism and aging. *Experimental gerontology* 2005, 40(10):763-773.

70. Munoz-Montesino C, Roa FJ, Pena E, Gonzalez M, Sotomayor K, Inostrosa E, Munoz C, Gonzalez I, Maldonado M, Soliz C *et al*: Mitochondrial ascorbic acid transport is mediated by a low-affinity form of the sodium-coupled ascorbic acid transporter-2 (SVCT2). *Free Radic Biol Med* 2014.

71. Murray JM, Stiff T, Jeggo PA: DNA double-strand break repair within heterochromatic regions. *Biochem Soc Trans* 2012, 40(1):173-178.

72. Berlett BS, Stadtman ER: Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 1997, 272(33):20313-20316.

73. Aksenov MY, Aksenova MV, Butterfield DA, Geddes JW, Markesbery WR: Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* 2001, 103(2):373-383.

74. Halliwell B: Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs & aging* 2001, 18(9):685-716.

75. May JM, Harrison FE: Role of Vitamin C in the Function of the Vascular Endothelium. *AntioxidRedoxSignal* 2013.

76. Mylonas C, Kouretas D: Lipid peroxidation and tissue damage. *In Vivo* 1999, 13(3):295-309.

77. Saretzki G, Von Zglinicki T: Replicative aging, telomeres, and oxidative stress. *Ann N Y Acad Sci* 2002, 959:24-29.

78. Kitagishi Y, Matsuda S: Redox regulation of tumor suppressor PTEN in cancer and aging (Review). *International journal of molecular medicine* 2013, 31(3):511-515.

79. Halliwell B: Oxidative stress and neurodegeneration: where are we now? *Journal of Neurochemistry* 2006, 97(6):1634-1658.

80. Wrona MZ, Dryhurst G: Oxidation of serotonin by superoxide radical: implications to neurodegenerative brain disorders. *Chemical research in toxicology* 1998, 11(6):639-650.

81. Mailly F, Marin P, Israel M, Glowinski J, Premont J: Increase in external glutamate and NMDA receptor activation contribute to H2O2-induced neuronal apoptosis. *J Neurochem* 1999, 73(3):1181-1188.

82. Harrison FE, Dawes SM, Meredith ME, Babaev VR, Li L, May JM: Low vitamin C and increased oxidative stress cell death in mice that lack the sodium-dependent vitamin C transporter SVCT2. *Free Radical Biology and Medicine* 2010, 49:821-829.

83. Meredith ME, Harrison FE, May JM: Differential regulation of the ascorbic acid transporter SVCT2 during development and in response to ascorbic acid depletion. *Biochemical and Biophysical Research Communications* 2011, 414:737-742.

84. Ehrlich P: Das sauerstufbudurfnis des organismus. Eine Farbenanalytische, Hirschwald, Berlin; 1885.

85. Goldmann EE: Vitalfarbung am zentralnervensystem. *Abhandl Konigl preuss Akad Wiss* 1913, 1:1-60.

86. Pardridge WM: Blood-brain barrier biology and methodology. *J Neurovirol* 1999, 5(6):556-569.

87. Carvey PM, Hendey B, Monahan AJ: The blood-brain barrier in neurodegenerative disease: a rhetorical perspective. *J Neurochem* 2009, 111(2):291-314.

88. Sehrawat S, Cullere X, Patel S, Italiano J, Jr., Mayadas TN: Role of Epac1, an exchange factor for Rap GTPases, in endothelial microtubule dynamics and barrier function. *MolBiolCell* 2008, 19(3):1261-1270.

89. Francis K, van Beek J, Canova C, Neal JW, Gasque P: Innate immunity and brain inflammation: the key role of complement. *Expert reviews in molecular medicine* 2003, 5(15):1-19.

90. Mangialardi G, Katare R, Oikawa A, Meloni M, Reni C, Emanueli C, Madeddu P: Diabetes causes bone marrow endothelial barrier dysfunction by activation of the RhoA-Rho-associated kinase signaling pathway. *Arterioscler Thromb Vasc Biol* 2013, 33(3):555-564.

91. Potter MD, Barbero S, Cheresh DA: Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state. *J Biol Chem* 2005, 280(36):31906-31912.

92. Xiao K, Allison DF, Buckley KM, Kottke MD, Vincent PA, Faundez V, Kowalczyk AP: Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. *J Cell Biol* 2003, 163(3):535-545.

93. Blasig IE, Bellmann C, Cording J, Del Vecchio G, Zwanziger D, Huber O, Haseloff RF: Occludin protein family: oxidative stress and reducing conditions. *Antioxid Redox Signal* 2011, 15(5):1195-1219.

94. Han M, Pendem S, Teh SL, Sukumaran DK, Wu F, Wilson JX: Ascorbate protects endothelial barrier function during septic insult: Role of protein phosphatase type 2A. *Free Radical Biology and Medicine* 2010, 48(1):128-135.

95. Fukuhara S, Sakurai A, Sano H, Yamagishi A, Somekawa S, Takakura N, Saito Y, Kangawa K, Mochizuki N: Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. *MolCell Biol* 2005, 25(1):136-146.

96. Cullere X, Shaw SK, Andersson L, Hirahashi J, Luscinskas FW, Mayadas TN: Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. *Blood* 2005, 105(5):1950-1955.

97. Matsuyoshi N, Hamaguchi M, Taniguchi S, Nagafuchi A, Tsukita S, Takeichi M: Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J Cell Biol* 1992, 118(3):703-714.

98. Behrens J, Vakaet L, Friis R, Winterhager E, Van Roy F, Mareel MM, Birchmeier W: Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine

phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J Cell Biol* 1993, 120(3):757-766.

99.　Rao RK, Basuroy S, Rao VU, Karnaky Jr KJ, Gupta A: Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. *Biochem J* 2002, 368(Pt 2):471-481.

100.　Zucchetti AE, Barosso IR, Boaglio AC, Luquita MG, Roma MG, Crocenzi FA, Sanchez Pozzi EJ: Hormonal modulation of hepatic cAMP prevents estradiol 17beta-D-glucuronide-induced cholestasis in perfused rat liver. *Dig Dis Sci* 2013, 58(6):1602-1614.

101.　Mei FC, Cheng X: Interplay between exchange protein directly activated by cAMP (Epac) and microtubule cytoskeleton. *MolBiosyst* 2005, 1(4):325-331.

102.　Parnell E, Smith BO, Palmer TM, Terrin A, Zaccolo M, Yarwood SJ: Regulation of the inflammatory response of vascular endothelial cells by EPAC1. *Br J Pharmacol* 2012, 166(2):434-446.

103.　Spindler V, Schlegel N, Waschke J: Role of GTPases in control of microvascular permeability. *Cardiovasc Res* 2010, 87(2):243-253.

104.　Maurice DH: Subcellular signaling in the endothelium: cyclic nucleotides take their place. *Curr Opin Pharmacol* 2011, 11(6):656-664.

105.　Curry FR, Adamson RH: Tonic regulation of vascular permeability. *Acta Physiol (Oxf)* 2013, 207(4):628-649.

106.　Sayner SL: Emerging themes of cAMP regulation of the pulmonary endothelial barrier. *Am J Physiol Lung Cell Mol Physiol* 2011, 300(5):L667-678.

107.　Gloerich M, Bos JL: Epac: defining a new mechanism for cAMP action. *Annu Rev Pharmacol Toxicol* 2010, 50:355-375.

108.　Metrich M, Berthouze M, Morel E, Crozatier B, Gomez AM, Lezoualc'h F: Role of the cAMP-binding protein Epac in cardiovascular physiology and pathophysiology. *Pflugers Archiv : European journal of physiology* 2010, 459(4):535-546.

109.　Zhou L, Beattie MC, Lin CY, Liu J, Traore K, Papadopoulos V, Zirkin BR, Chen H: Oxidative stress and phthalate-induced down-regulation of steroidogenesis in MA-10 Leydig cells. *Reproductive toxicology* 2013, 42:95-101.

110.　Kim JE, Song SE, Kim YW, Kim JY, Park SC, Park YK, Baek SH, Lee IK, Park SY: Adiponectin inhibits palmitate-induced apoptosis through suppression of reactive oxygen species in endothelial cells: involvement of cAMP/protein kinase A and AMP-activated protein kinase. *The Journal of endocrinology* 2010, 207(1):35-44.

111.　Raynaud F, Evain-Brion D, Gerbaud P, Marciano D, Gorin I, Liapi C, Anderson WB: Oxidative modulation of cyclic AMP-dependent protein kinase in human fibroblasts: possible role in psoriasis. *Free Radic Biol Med* 1997, 22(4):623-632.

112.　May JM, Qu ZC: Ascorbic acid prevents increased endothelial permeability caused by oxidized low density lipoprotein. *Free RadicRes* 2010, 44(11):1359-1368.

113.　May JM, Qu ZC: Nitric oxide mediates tightening of the endothelial barrier by ascorbic acid. *Biochemical and Biophysical Research Communications* 2011, 404(2):701-705.

114.　May JM, Qu ZC: Ascorbic acid prevents oxidant-induced increases in endothelial permeability. *Biofactors* 2011, 37(1):46-50.

115.　Itani H, Liu X, Sarsour EH, Goswami PC, Born E, Keen HL, Sigmund CD: Regulation of renin gene expression by oxidative stress. *Hypertension* 2009, 53(6):1070-1076.

116.　Lopez BE, Rodriguez CE, Pribadi M, Cook NM, Shinyashiki M, Fukuto JM: Inhibition of yeast glycolysis by nitroxyl (HNO): mechanism of HNO toxicity and implications to HNO biology. *Arch Biochem Biophys* 2005, 442(1):140-148.

117.　Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ: Structure and function of the blood-brain barrier. *Neurobiol Dis* 2010, 37(1):13-25.

118.　Plante GE, Alfred J, Chakir M: The blood vessel, linchpin of diabetic lesions. *Metabolism* 1999, 48(3):406-409.

119. Ignarro LJ, Cirino G, Casini A, Napoli C: Nitric oxide as a signaling molecule in the vascular system: an overview. *J Cardiovasc Pharmacol* 1999, 34(6):879-886.
120. Alp NJ, Channon KM: Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arterioscler Thromb Vasc Biol* 2004, 24(3):413-420.
121. Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M, Skatchkov M, Thaiss F, Stahl RA, Warnholtz A *et al*: Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circulation Research* 2001, 88(2):E14-E22.
122. Endres M, Laufs U, Liao JK, Moskowitz MA: Targeting eNOS for stroke protection. *Trends in neurosciences* 2004, 27(5):283-289.
123. Avogaro A, Albiero M, Menegazzo L, de Kreutzenberg S, Fadini GP: Endothelial dysfunction in diabetes: the role of reparatory mechanisms. *Diabetes Care* 2011, 34 Suppl 2:S285-290.
124. Besler C, Doerries C, Giannotti G, Luscher TF, Landmesser U: Pharmacological approaches to improve endothelial repair mechanisms. *Expert review of cardiovascular therapy* 2008, 6(8):1071-1082.
125. Fadini GP, Sartore S, Agostini C, Avogaro A: Significance of endothelial progenitor cells in subjects with diabetes. *Diabetes Care* 2007, 30(5):1305-1313.
126. Rechthand E, Smith QR, Latker CH, Rapoport SI: Altered blood-nerve barrier permeability to small molecules in experimental diabetes mellitus. *J Neuropathol Exp Neurol* 1987, 46(3):302-314.
127. Ennis SR, Betz AL: Sucrose permeability of the blood-retinal and blood-brain barriers. Effects of diabetes, hypertonicity, and iodate. *Invest Ophthalmol Vis Sci* 1986, 27(7):1095-1102.
128. Chehade JM, Haas MJ, Mooradian AD: Diabetes-related changes in rat cerebral occludin and zonula occludens-1 (ZO-1) expression. *Neurochem Res* 2002, 27(3):249-252.
129. Hawkins BT, Davis TP: The blood-brain barrier/neurovascular unit in health and disease. *Pharmacol Rev* 2005, 57(2):173-185.
130. Huber JD, VanGilder RL, Houser KA: Streptozotocin-induced diabetes progressively increases blood-brain barrier permeability in specific brain regions in rats. *AmJPhysiol Heart CircPhysiol* 2006, 291:H2660-H2668.
131. Sheikh-Ali M, Chehade JM, Mooradian AD: The Antioxidant Paradox in Diabetes Mellitus. *Am J Ther* 2009.
132. Klaassen I, Van Noorden CJ, Schlingemann RO: Molecular basis of the inner blood-retinal barrier and its breakdown in diabetic macular edema and other pathological conditions. *Progress in retinal and eye research* 2013, 34:19-48.
133. Tapp RJ, Shaw JE, Harper CA, de Courten MP, Balkau B, McCarty DJ, Taylor HR, Welborn TA, Zimmet PZ, AusDiab Study G: The prevalence of and factors associated with diabetic retinopathy in the Australian population. *Diabetes Care* 2003, 26(6):1731-1737.
134. Starr JM, Wardlaw J, Ferguson K, MacLullich A, Deary IJ, Marshall I: Increased blood-brain barrier permeability in type II diabetes demonstrated by gadolinium magnetic resonance imaging. *J Neurol Neurosurg Psychiatry* 2003, 74(1):70-76.
135. Fontbonne A, Berr C, Ducimetiere P, Alperovitch A: Changes in cognitive abilities over a 4-year period are unfavorably affected in elderly diabetic subjects: results of the Epidemiology of Vascular Aging Study. *Diabetes Care* 2001, 24(2):366-370.
136. Allen KV, Frier BM, Strachan MW: The relationship between type 2 diabetes and cognitive dysfunction: longitudinal studies and their methodological limitations. *Eur J Pharmacol* 2004, 490(1-3):169-175.

137. Yaffe K, Blackwell T, Kanaya AM, Davidowitz N, Barrett-Connor E, Krueger K: Diabetes, impaired fasting glucose, and development of cognitive impairment in older women. *Neurology* 2004, 63(4):658-663.

138. Huber JD: Diabetes, cognitive function, and the blood-brain barrier. *Current pharmaceutical design* 2008, 14(16):1594-1600.

139. Nicolakakis N, Hamel E: Neurovascular function in Alzheimer's disease patients and experimental models. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 2011, 31(6):1354-1370.

140. Feng Y, Wang R, Xu J, Sun J, Xu T, Gu Q, Wu X: Hydrogen-rich saline prevents early neurovascular dysfunction resulting from inhibition of oxidative stress in STZ-diabetic rats. *Curr Eye Res* 2013, 38(3):396-404.

141. Liu C, Wu J, Zou MH: Activation of AMP-activated protein kinase alleviates high-glucose-induced dysfunction of brain microvascular endothelial cell tight-junction dynamics. *Free Radic Biol Med* 2012, 53(6):1213-1221.

142. Pun PB, Lu J, Moochhala S: Involvement of ROS in BBB dysfunction. *Free radical research* 2009, 43(4):348-364.

143. Taguchi A: Vascular factors in diabetes and Alzheimer's disease. *J Alzheimers Dis* 2009, 16(4):859-864.

144. Potenza MA, Gagliardi S, Nacci C, Carratu MR, Montagnani M: Endothelial dysfunction in diabetes: from mechanisms to therapeutic targets. *Current medicinal chemistry* 2009, 16(1):94-112.

145. Wang D, Luo P, Wang Y, Li W, Wang C, Sun D, Zhang R, Su T, Ma X, Zeng C *et al*: Glucagon-like peptide-1 protects against cardiac microvascular injury in diabetes via a cAMP/PKA/Rho-dependent mechanism. *Diabetes* 2013, 62(5):1697-1708.

146. Price TO, Eranki V, Banks WA, Ercal N, Shah GN: Topiramate treatment protects blood-brain barrier pericytes from hyperglycemia-induced oxidative damage in diabetic mice. *Endocrinology* 2012, 153(1):362-372.

147. Warboys CM, Toh HB, Fraser PA: Role of NADPH oxidase in retinal microvascular permeability increase by RAGE activation. *Invest OphthalmolVisSci* 2009, 50(3):1319-1328.

148. Sena CM, Pereira AM, Seica R: Endothelial dysfunction - A major mediator of diabetic vascular disease. *Biochimica et Biophysica Acta* 2013.

149. Esposito C, Gerlach H, Brett J, Stern D, Vlassara H: Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties. *Journal of Experimental Medicine* 1989, 170:1387-1407.

150. Yuan SY: Protein kinase signaling in the modulation of microvascular permeability. *VasculPharmacol* 2002, 39(4-5):213-223.

151. Kahles T, Luedike P, Endres M, Galla HJ, Steinmetz H, Busse R, Neumann-Haefelin T, Brandes RP: NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. *Stroke* 2007, 38(11):3000-3006.

152. Ramasamy R, Vannucci SJ, Yan SS, Herold K, Yan SF, Schmidt AM: Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology* 2005, 15(7):16R-28R.

153. Haorah J, Ramirez SH, Schall K, Smith D, Pandya R, Persidsky Y: Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *J Neurochem* 2007, 101(2):566-576.

154. Lee HS, Namkoong K, Kim DH, Kim KJ, Cheong YH, Kim SS, Lee WB, Kim KY: Hydrogen peroxide-induced alterations of tight junction proteins in bovine brain microvascular endothelial cells. *Microvascular Research* 2004, 68(3):231-238.

155. Maier CM, Hsieh L, Crandall T, Narasimhan P, Chan PH: A new approach for the investigation of reperfusion-related brain injury. *Biochem Soc Trans* 2006, 34(Pt 6):1366-1369.
156. Mayhan WG, Mayhan JF, Sun H, Patel KP: In vivo properties of potassium channels in cerebral blood vessels during diabetes mellitus. *Microcirculation* 2004, 11(7):605-613.
157. Hawkins BT, Lundeen TF, Norwood KM, Brooks HL, Egleton RD: Increased blood-brain barrier permeability and altered tight junctions in experimental diabetes in the rat: contribution of hyperglycaemia and matrix metalloproteinases. *Diabetologia* 2007, 50(1):202-211.
158. CDC: http://www.cdc.gov/chronicdisease/resources/publications/AAG/ddt.htm. Accessed Jan 2011.
159. Stankova L, Riddle M, Larned J, Burry K, Menashe D, Hart J, Bigley R: Plasma ascorbate concentrations and blood cell dehydroascorbate transport in patients with diabetes mellitus. *Metabolism* 1984, 33(4):347-353.
160. Jacob RA, Skala JH, Omaye ST: Biochemical indices of human vitamin C status. *American Journal of Clinical Nutrition* 1987, 46:818-826.
161. Cunningham JJ, Ellis SL, McVeigh KL, Levine RE, Calles-Escandon J: Reduced mononuclear leukocyte ascorbic acid content in adults with insulin-dependent diabetes mellitus consuming adequate dietary vitamin C. *Metabolism: Clinical and Experimental* 1991, 40:146-149.
162. Wrieden WL, Hannah MK, Bolton-Smith C, Tavendale R, Morrison C, Tunstall-Pedoe H: Plasma vitamin C and food choice in the third Glasgow MONICA population survey. *J Epidemiol Community Health* 2000, 54(5):355-360.
163. Johnston CS, Thompson LL: Vitamin C status of an outpatient population. *J Am Coll Nutr* 1998, 17(4):366-370.
164. Harrison FE: A critical review of vitamin C for the prevention of age-related cognitive decline and Alzheimer's disease. *J Alzheimers Dis* 2012, 29(4):711-726.
165. Johnston CS, Solomon RE, Corte C: Vitamin C status of a campus population: college students get a C minus. *J Am Coll Health* 1998, 46(5):209-213.
166. Cahill L, Corey PN, El-Sohemy A: Vitamin C deficiency in a population of young Canadian adults. *Am J Epidemiol* 2009, 170(4):464-471.
167. Gan R, Eintracht S, Hoffer LJ: Vitamin C deficiency in a university teaching hospital. *J Am Coll Nutr* 2008, 27(3):428-433.
168. Hampl JS, Taylor CA, Johnston CS: Vitamin C deficiency and depletion in the United States: the Third National Health and Nutrition Examination Survey, 1988 to 1994. *Am J Public Health* 2004, 94(5):870-875.
169. Levine M, Dhariwal KR, Welch RW, Wang Y, Park JB: Determination of optimal vitamin C requirements in humans. *Am J Clin Nutr* 1995, 62(6 Suppl):1347S-1356S.
170. Duell EJ, Lujan-Barroso L, Llivina C, Munoz X, Jenab M, Boutron-Ruault MC, Clavel-Chapelon F, Racine A, Boeing H, Buijsse B *et al*: Vitamin C transporter gene (SLC23A1 and SLC23A2) polymorphisms, plasma vitamin C levels, and gastric cancer risk in the EPIC cohort. *Genes & nutrition* 2013, 8(6):549-560.
171. Timpson NJ, Forouhi NG, Brion MJ, Harbord RM, Cook DG, Johnson P, McConnachie A, Morris RW, Rodriguez S, Luan J *et al*: Genetic variation at the SLC23A1 locus is associated with circulating concentrations of L-ascorbic acid (vitamin C): evidence from 5 independent studies with >15,000 participants. *The American journal of clinical nutrition* 2010, 92(2):375-382.
172. Zanon-Moreno V, Ciancotti-Olivares L, Asencio J, Sanz P, Ortega-Azorin C, Pinazo-Duran MD, Corella D: Association between a SLC23A2 gene variation, plasma vitamin C levels, and risk of glaucoma in a Mediterranean population. *Molecular vision* 2011, 17:2997-3004.

173.   Chen AA, Marsit CJ, Christensen BC, Houseman EA, McClean MD, Smith JF, Bryan JT, Posner MR, Nelson HH, Kelsey KT: Genetic variation in the vitamin C transporter, SLC23A2, modifies the risk of HPV16-associated head and neck cancer. *Carcinogenesis* 2009, 30(6):977-981.

174.   Erichsen HC, Engel SA, Eck PK, Welch R, Yeager M, Levine M, Siega-Riz AM, Olshan AF, Chanock SJ: Genetic variation in the sodium-dependent vitamin C transporters, SLC23A1, and SLC23A2 and risk for preterm delivery. *American Journal of Epidemiology* 2006, 163(3):245-254.

175.   Dalgard C, Christiansen L, Vogel U, Dethlefsen C, Tjonneland A, Overvad K: Variation in the sodium-dependent vitamin C transporter 2 gene is associated with risk of acute coronary syndrome among women. *PloS one* 2013, 8(8):e70421.

176.   Harrison FE, Yu SS, Van Den Bossche KL, Li L, May JM, McDonald MP: Elevated oxidative stress and sensorimotor deficits but normal cognition in mice that cannot synthesize ascorbic acid. *J Neurochem* 2008, 106(3):1198-1208.

177.   Pachla LA, Kissinger PT: Analysis of ascorbic acid by liquid chromatography with amperometric detection. *Methods Enzymol* 1979, 62:15-24.

178.   Jones W, Li X, Qu ZC, Perriott L, Whitesell RR, May JM: Uptake, recycling, and antioxidant actions of alpha-lipoic acid in endothelial cells. *Free Radic Biol Med* 2002, 33(1):83-93.

179.   Roberts LJ, 2nd, Montine TJ, Markesbery WR, Tapper AR, Hardy P, Chemtob S, Dettbarn WD, Morrow JD: Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. *J Biol Chem* 1998, 273(22):13605-13612.

180.   Milne GL, Sanchez SC, Musiek ES, Morrow JD: Quantification of F2-isoprostanes as a biomarker of oxidative stress. *Nat Protoc* 2007, 2(1):221-226.

181.   Wang H, Joseph JA: Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic Biol Med* 1999, 27(5-6):612-616.

182.   Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM: Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2',7'-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. *Free Radic Biol Med* 1999, 27(1-2):146-159.

183.   Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research* 2001, 29(9):e45.

184.   Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227(5259):680-685.

185.   Ward MS, Lamb J, May JM, Harrison FE: Behavioral and monoamine changes following severe vitamin C deficiency. *J Neurochem* 2013, 124(3):363-375.

186.   Arthur FE, Shivers RR, Bowman PD: Astrocyte-mediated induction of tight junctions in brain capillary endothelium: an efficient in vitro model. *Brain Research* 1987, 433(1):155-159.

187.   Brown RC, Morris AP, O'Neil RG: Tight junction protein expression and barrier properties of immortalized mouse brain microvessel endothelial cells. *Brain Res* 2007, 1130(1):17-30.

188.   Omidi Y, Campbell L, Barar J, Connell D, Akhtar S, Gumbleton M: Evaluation of the immortalised mouse brain capillary endothelial cell line, b.End3, as an in vitro blood-brain barrier model for drug uptake and transport studies. *Brain Res* 2003, 990(1-2):95-112.

189.   Watanabe T, Dohgu S, Takata F, Nishioku T, Nakashima A, Futagami K, Yamauchi A, Kataoka Y: Paracellular barrier and tight junction protein expression in the immortalized brain endothelial cell lines bEND.3, bEND.5 and mouse brain endothelial cell 4. *Biol Pharm Bull* 2013, 36(3):492-495.

190. Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P *et al*: Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J* 2005, 19(13):1872-1874.

191. Utoguchi N, Ikeda K, Saeki K, Oka N, Mizuguchi H, Kubo K, Nakagawa S, Mayumi T: Ascorbic acid stimulates barrier function of cultured endothelial cell monolayer. *Journal of Cellular Physiology* 1995, 163:393-399.

192. Siflinger-Birnboim A, del Vecchio PJ, Cooper JA, Blumenstock FA, Shepard JM, Malik AB: Molecular sieving characteristics of the cultured endothelial monolayer. *JCell Physiol* 1987, 132(1):111-117.

193. May JM, Qu ZC, Qiao H: Transfer of ascorbic acid across the vascular endothelium: mechanism and self-regulation. *Am J Physiol Cell Physiol* 2009, 297(1):C169-178.

194. Madruga de Oliveira A, Rondo PH, Barros SB: Concentrations of ascorbic acid in the plasma of pregnant smokers and nonsmokers and their newborns. *Int J Vitam Nutr Res* 2004, 74(3):193-198.

195. Linster CL, Van Schaftingen E: Vitamin C. Biosynthesis, recycling and degradation in mammals. *FEBS J* 2007, 274(1):1-22.

196. Kratzing CC, Kelly JD, Kratzing JE: Ascorbic acid in fetal rat brain. *J Neurochem* 1985, 44(5):1623-1624.

197. Berger UV, Hediger MA: The vitamin C transporter SVCT2 is expressed by astrocytes in culture but not in situ. *Neuroreport* 2000, 11(7):1395-1399.

198. Rice ME, Russo-Menna I: Differential compartmentalization of brain ascorbate and glutathione between neurons and glia. *Neuroscience* 1998, 82(4):1213-1223.

199. Vannucci SJ, Clark RR, Koehler-Stec E, Li K, Smith CB, Davies P, Maher F, Simpson IA: Glucose transporter expression in brain: relationship to cerebral glucose utilization. *Dev Neurosci* 1998, 20(4-5):369-379.

200. Nagamatsu S, Sawa H, Nakamichi Y, Katahira H, Inoue N: Developmental expression of GLUT3 glucose transporter in the rat brain. *FEBS Lett* 1994, 346(2-3):161-164.

201. Rice FL, Gomez C, Barstow C, Burnet A, Sands P: A comparative analysis of the development of the primary somatosensory cortex: interspecies similarities during barrel and laminar development. *The Journal of comparative neurology* 1985, 236(4):477-495.

202. Mun GH, Kim MJ, Lee JH, Kim HJ, Chung YH, Chung YB, Kang JS, Hwang YI, Oh SH, Kim JG *et al*: Immunohistochemical study of the distribution of sodium-dependent vitamin C transporters in adult rat brain. *J Neurosci Res* 2006, 83(5):919-928.

203. Amano A, Aigaki T, Maruyama N, Ishigami A: Ascorbic acid depletion enhances expression of the sodium-dependent vitamin C transporters, SVCT1 and SVCT2, and uptake of ascorbic acid in livers of SMP30/GNL knockout mice. *Arch Biochem Biophys* 2010, 496(1):38-44.

204. Kratzing CC, Kelly JD: Tissue levels of ascorbic acid during rat gestation. *Int J Vitam Nutr Res* 1982, 52(3):326-332.

205. Bornstein SR, Yoshida-Hiroi M, Sotiriou S, Levine M, Hartwig HG, Nussbaum RL, Eisenhofer G: Impaired adrenal catecholamine system function in mice with deficiency of the ascorbic acid transporter (SVCT2). *FASEB J* 2003, 17(13):1928-1930.

206. Amano A, Tsunoda M, Aigaki T, Maruyama N, Ishigami A: Effect of ascorbic acid deficiency on catecholamine synthesis in adrenal glands of SMP30/GNL knockout mice. *Eur J Nutr* 2013.

207. Levin EY, Levenberg B, Kaufman S: The enzymatic conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. *J Biol Chem* 1960, 235:2080-2086.

208. Anden NE, Hfuxe K, Hamberger B, Hokfelt T: A quantitative study on the nigro-neostriatal dopamine neuron system in the rat. *Acta Physiol Scand* 1966, 67(3):306-312.

209. Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD: Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* 2000, 18(6):675-679.
210. Yan J, Studer L, McKay RD: Ascorbic acid increases the yield of dopaminergic neurons derived from basic fibroblast growth factor expanded mesencephalic precursors. *J Neurochem* 2001, 76(1):307-311.
211. Lee JY, Chang MY, Park CH, Kim HY, Kim JH, Son H, Lee YS, Lee SH: Ascorbate-induced differentiation of embryonic cortical precursors into neurons and astrocytes. *Journal of Neuroscience Research* 2003, 73(2):156-165.
212. Pastor P, Cisternas P, Salazar K, Silva-Alvarez C, Oyarce K, Jara N, Espinoza F, Martinez AD, Nualart F: SVCT2 vitamin C transporter expression in progenitor cells of the postnatal neurogenic niche. *Front Cell Neurosci* 2013, 7:119.
213. Brody S: High-dose ascorbic acid increases intercourse frequency and improves mood: a randomized controlled clinical trial. *Biol Psychiatry* 2002, 52(4):371-374.
214. Zhang M, Robitaille L, Eintracht S, Hoffer LJ: Vitamin C provision improves mood in acutely hospitalized patients. *Nutrition* 2011, 27(5):530-533.
215. Khanzode SD, Dakhale GN, Khanzode SS, Saoji A, Palasodkar R: Oxidative damage and major depression: the potential antioxidant action of selective serotonin re-uptake inhibitors. *Redox Rep* 2003, 8(6):365-370.
216. Allen CL, Bayraktutan U: Antioxidants attenuate hyperglycaemia-mediated brain endothelial cell dysfunction and blood-brain barrier hyperpermeability. *Diabetes Obes Metab* 2009, 11(5):480-490.
217. Bondan EF, Martins Mde F: Blood-brain barrier breakdown and repair following gliotoxic drug injection in the brainstem of streptozotocin-diabetic rats. *Arq Neuropsiquiatr* 2012, 70(3):221-225.
218. Lorenzi M, Healy DP, Hawkins R, Printz JM, Printz MP: Studies on the permeability of the blood-brain barrier in experimental diabetes. *Diabetologia* 1986, 29(1):58-62.
219. Mohammad G, Siddiquei MM, Othman A, Al-Shabrawey M, Abu El-Asrar AM: High-mobility group box-1 protein activates inflammatory signaling pathway components and disrupts retinal vascular-barrier in the diabetic retina. *Exp Eye Res* 2013, 107:101-109.
220. Wolfson RK, Chiang ET, Garcia JG: HMGB1 induces human lung endothelial cell cytoskeletal rearrangement and barrier disruption. *Microvasc Res* 2011, 81(2):189-197.
221. Guo XH, Huang QB, Chen B, Wang SY, Li Q, Zhu YJ, Hou FF, Fu N, Brunk UT, Zhao M: Advanced glycation end products induce actin rearrangement and subsequent hyperpermeability of endothelial cells. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* 2006, 114(12):874-883.
222. Meredith ME, Qu ZC, May JM: Ascorbate reverses high glucose- and RAGE-induced leak of the endothelial permeability barrier. *Biochem Biophys Res Commun* 2014.
223. Deane R, Singh I, Sagare AP, Bell RD, Ross NT, LaRue B, Love R, Perry S, Paquette N, Deane RJ *et al*: A multimodal RAGE-specific inhibitor reduces amyloid beta-mediated brain disorder in a mouse model of Alzheimer disease. *J ClinInvest* 2012, 122(4):1377-1392.
224. Murakami T, Felinski EA, Antonetti DA: Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. *J Biol Chem* 2009, 284(31):21036-21046.
225. Almahariq M, Tsalkova T, Mei FC, Chen H, Zhou J, Sastry SK, Schwede F, Cheng X: A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion. *Molecular Pharmacology* 2013, 83(1):122-128.
226. Maruta H, Greer K, Rosenbaum JL: The acetylation of alpha-tubulin and its relationship to the assembly and disassembly of microtubules. *J Cell Biol* 1986, 103(2):571-579.

227. Leal EC, Martins J, Voabil P, Liberal J, Chiavaroli C, Bauer J, Cunha-Vaz J, Ambrosio AF: Calcium dobesilate inhibits the alterations in tight junction proteins and leukocyte adhesion to retinal endothelial cells induced by diabetes. *Diabetes* 2010, 59(10):2637-2645.

228. Cosentino F, Hishikawa K, Katusic ZS, Luscher TF: High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation* 1997, 96(1):25-28.

229. Cai S, Khoo J, Channon KM: Augmented BH4 by gene transfer restores nitric oxide synthase function in hyperglycemic human endothelial cells. *CardiovascRes* 2005, 65(4):823-831.

230. Cai H: Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences. *CardiovascRes* 2005, 68(1):26-36.

231. Siflinger-Birnboim A, Lum H, del Vecchio PJ, Malik AB: Involvement of Ca 2+ in the H2O2-induced increase in endothelial permeability. *AmJPhysiol* 1996, 270:L973-L978.

232. Hudson BI, Schmidt AM: RAGE: a novel target for drug intervention in diabetic vascular disease. *PharmRes* 2004, 21(7):1079-1086.

233. Hirose A, Tanikawa T, Mori H, Okada Y, Tanaka Y: Advanced glycation end products increase endothelial permeability through the RAGE/Rho signaling pathway. *FEBS Letters* 2010, 584:61-66.

234. Touil YS, Fellous A, Scherman D, Chabot GG: Flavonoid-induced morphological modifications of endothelial cells through microtubule stabilization. *NutrCancer* 2009, 61(3):310-321.

235. Kratzer E, Tian Y, Sarich N, Wu T, Meliton A, Leff A, Birukova AA: Oxidative stress contributes to lung injury and barrier dysfunction via microtubule destabilization. *AmJRespirCell MolBiol* 2012, 47(5):688-697.

236. Nico B, Ribatti D: Morphofunctional aspects of the blood-brain barrier. *Curr Drug Metab* 2012, 13(1):50-60.

237. Dejana E, Orsenigo F, Lampugnani MG: The role of adherens junctions and VE-cadherin in the control of vascular permeability. *Journal of cell science* 2008, 121(Pt 13):2115-2122.

238. Stamatovic SM, Keep RF, Andjelkovic AV: Brain endothelial cell-cell junctions: how to "open" the blood brain barrier. *Curr Neuropharmacol* 2008, 6(3):179-192.

239. Murakami T, Frey T, Lin C, Antonetti DA: Protein kinase cbeta phosphorylates occludin regulating tight junction trafficking in vascular endothelial growth factor-induced permeability in vivo. *Diabetes* 2012, 61(6):1573-1583.

240. Takenaga Y, Takagi N, Murotomi K, Tanonaka K, Takeo S: Inhibition of Src activity decreases tyrosine phosphorylation of occludin in brain capillaries and attenuates increase in permeability of the blood-brain barrier after transient focal cerebral ischemia. *J Cereb Blood Flow Metab* 2009, 29(6):1099-1108.

241. Lin JL, Huang YH, Shen YC, Huang HC, Liu PH: Ascorbic acid prevents blood-brain barrier disruption and sensory deficit caused by sustained compression of primary somatosensory cortex. *J Cereb Blood Flow Metab* 2010, 30(6):1121-1136.

242. Birukova AA, Burdette D, Moldobaeva N, Xing J, Fu P, Birukov KG: Rac GTPase is a hub for protein kinase A and Epac signaling in endothelial barrier protection by cAMP. *Microvasc Res* 2010, 79(2):128-138.

243. Patterson CE, Lum H, Schaphorst KL, Verin AD, Garcia JG: Regulation of endothelial barrier function by the cAMP-dependent protein kinase. *Endothelium* 2000, 7(4):287-308.

244. Deli MA, Dehouck MP, Abraham CS, Cecchelli R, Joo F: Penetration of small molecular weight substances through cultured bovine brain capillary endothelial cell monolayers:

the early effects of cyclic adenosine 3',5'-monophosphate. *Exp Physiol* 1995, 80(4):675-678.

245. Horai S, Nakagawa S, Tanaka K, Morofuji Y, Couraud PO, Deli MA, Ozawa M, Niwa M: Cilostazol strengthens barrier integrity in brain endothelial cells. *Cellular and molecular neurobiology* 2013, 33(2):291-307.

246. Kaya F, Belin S, Diamantidis G, Fontes M: Ascorbic acid is a regulator of the intracellular cAMP concentration: old molecule, new functions? *FEBS Lett* 2008, 582(25-26):3614-3618.

247. Bordignon B, Mones S, Rahman F, Chiron J, Peiretti F, Vidal N, Fontes M: A derivative of ascorbic acid modulates cAMP production. *Biochem Biophys Res Commun* 2013, 439(1):137-141.

248. Lopez-Lluch G, Buron MI, Alcain FJ, Quesada JM, Navas P: Redox regulation of cAMP levels by ascorbate in 1,25-dihydroxy- vitamin D3-induced differentiation of HL-60 cells. *Biochem J* 1998, 331 (Pt 1):21-27.

249. Huang W, Yang Z, Lee D, Copolov DL, Lim AT: Ascorbic acid enhances forskolin-induced cyclic AMP production and pro-ANF mRNA expression of hypothalamic neurons in culture. *Endocrinology* 1993, 132:2271-2273.

250. Engh RA, Girod A, Kinzel V, Huber R, Bossemeyer D: Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl protein kinase inhibitors H7, H8, and H89. Structural implications for selectivity. *J Biol Chem* 1996, 271(42):26157-26164.

251. Kooistra MR, Corada M, Dejana E, Bos JL: Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. *FEBS Lett* 2005, 579(22):4966-4972.

252. Nguyen HL, Chari S, Gruber D, Lue CM, Chapin SJ, Bulinski JC: Overexpression of full- or partial-length MAP4 stabilizes microtubules and alters cell growth. *Journal of cell science* 1997, 110 (Pt 2):281-294.

253. Umapathy NS, Zemskov EA, Gonzales J, Gorshkov BA, Sridhar S, Chakraborty T, Lucas R, Verin AD: Extracellular beta-nicotinamide adenine dinucleotide (beta-NAD) promotes the endothelial cell barrier integrity via PKA- and EPAC1/Rac1-dependent actin cytoskeleton rearrangement. *J Cell Physiol* 2010, 223(1):215-223.

254. Regensteiner JG, Popylisen S, Bauer TA, Lindenfeld J, Gill E, Smith S, Oliver-Pickett CK, Reusch JE, Weil JV: Oral L-arginine and vitamins E and C improve endothelial function in women with type 2 diabetes. *Vasc Med* 2003, 8(3):169-175.

255. Kremer T, Harenberg P, Hernekamp F, Riedel K, Gebhardt MM, Germann G, Heitmann C, Walther A: High-dose vitamin C treatment reduces capillary leakage after burn plasma transfer in rats. *J Burn Care Res* 2010, 31(3):470-479.

256. Cosquer B, Vasconcelos AP, Frohlich J, Cassel JC: Blood-brain barrier and electromagnetic fields: effects of scopolamine methylbromide on working memory after whole-body exposure to 2.45 GHz microwaves in rats. *Behav Brain Res* 2005, 161(2):229-237.

257. Moghaddam H, Baluchnejadmojarad T, Roghani M, Khaksari M, Norouzi P, Ahooie M, Mahboobi F: Berberine Ameliorate Oxidative Stress and Astrogliosis in the Hippocampus of STZ-Induced Diabetic Rats. *Mol Neurobiol* 2013:1-7.

258. Yang H, Fan S, Song D, Wang Z, Ma S, Li S, Li X, Xu M, Xu M, Wang X: Long-term streptozotocin-induced diabetes in rats leads to severe damage of brain blood vessels and neurons via enhanced oxidative stress. *Molecular medicine reports* 2013, 7(2):431-440.

259. Lochhead JJ, McCaffrey G, Quigley CE, Finch J, DeMarco KM, Nametz N, Davis TP: Oxidative stress increases blood-brain barrier permeability and induces alterations in occludin during hypoxia-reoxygenation. *Journal of cerebral blood flow and metabolism :*

official journal of the International Society of Cerebral Blood Flow and Metabolism 2010, 30(9):1625-1636.

260. Karolczak K, Rozalska S, Wieczorek M, Labieniec-Watala M, Watala C: Poly(amido)amine dendrimers generation 4.0 (PAMAM G4) reduce blood hyperglycaemia and restore impaired blood-brain barrier permeability in streptozotocin diabetes in rats. *International journal of pharmaceutics* 2012, 436(1-2):508-518.

261. Liu H, Zhang D, Xu X, Liu X, Wang G, Xie L, Pang X, Liu L: Attenuated function and expression of P-glycoprotein at blood-brain barrier and increased brain distribution of phenobarbital in streptozotocin-induced diabetic mice. *Eur J Pharmacol* 2007, 561(1-3):226-232.

262. Kamei J, Hirano S, Miyata S, Saitoh A, Onodera K: Effects of first- and second-generation histamine-H1-receptor antagonists on the pentobarbital-induced loss of the righting reflex in streptozotocin-induced diabetic mice. *Journal of pharmacological sciences* 2005, 97(2):266-272.

263. Muranaka K, Yanagi Y, Tamaki Y, Usui T, Kubota N, Iriyama A, Terauchi Y, Kadowaki T, Araie M: Effects of peroxisome proliferator-activated receptor gamma and its ligand on blood-retinal barrier in a streptozotocin-induced diabetic model. *Invest OphthalmolVisSci* 2006, 47(10):4547-4552.

264. Sugimoto M, Cutler A, Shen B, Moss SE, Iyengar SK, Klein R, Folkman J, Anand-Apte B: Inhibition of EGF signaling protects the diabetic retina from insulin-induced vascular leakage. *The American journal of pathology* 2013, 183(3):987-995.

265. Min LJ, Mogi M, Shudou M, Jing F, Tsukuda K, Ohshima K, Iwanami J, Horiuchi M: Peroxisome proliferator-activated receptor-gamma activation with angiotensin II type 1 receptor blockade is pivotal for the prevention of blood-brain barrier impairment and cognitive decline in type 2 diabetic mice. *Hypertension* 2012, 59(5):1079-1088.

266. May JM, Qu ZC, Meredith ME: Mechanisms of ascorbic acid stimulation of norepinephrine synthesis in neuronal cells. *Biochemical and Biophysical Research Communications* 2012, 426(1):148-152.

267. Qiao H, May JM: Interaction of the transcription start site core region and transcription factor YY1 determine ascorbate transporter SVCT2 exon 1a promoter activity. *PLoSOne* 2012, 7(4):e35746.

268. Chothe PP, Chutkan N, Sangani R, Wenger KH, Prasad PD, Thangaraju M, Hamrick MW, Isales CM, Ganapathy V, Fulzele S: Sodium-coupled vitamin C transporter (SVCT2): expression, function, and regulation in intervertebral disc cells. *Spine J* 2013, 13(5):549-557.

269. Hierro C, Monte MJ, Lozano E, Gonzalez-Sanchez E, Marin JJ, Macias RI: Liver metabolic/oxidative stress induces hepatic and extrahepatic changes in the expression of the vitamin C transporters SVCT1 and SVCT2. *European journal of nutrition* 2013.

270. Sangani R, Naime M, Zakhary I, Ahmad S, Chutkan N, Zhu A, Ha Y, Hamrick M, Isales C, Elsalanty M *et al*: Regulation of vitamin C transporter in the type 1 diabetic mouse bone and bone marrow. *ExpMolPathol* 2013.

271. Wu X, Itoh N, Taniguchi T, Nakanishi T, Tatsu Y, Yumoto N, Tanaka K: Zinc-induced sodium-dependent vitamin C transporter 2 expression: potent roles in osteoblast differentiation. *Archives of Biochemistry and Biophysics* 2003, 420(1):114-120.

272. Qiao H, May JM: Macrophage differentiation increases expression of the ascorbate transporter (SVCT2) *Free RadicBiol Med* 2009, 46 1221-1232.

273. Fujita I, Hirano J, Itoh N, Nakanishi T, Tanaka K: Dexamethasone induces sodium-dependent vitamin C transporter in a mouse osteoblastic cell line MC3T3-E1. *BrJ Nutr* 2001, 86(2):145-149.

274. Hocevar SB, Zivin M, Milutinovic A, Hawlina M, Hutton E, Ogorevc B: Simultaneous in vivo measurement of dopamine, serotonin and ascorbate in the striatum of experimental

rats using voltammetric microprobe. *Frontiers in bioscience : a journal and virtual library* 2006, 11:2782-2789.

275.   Sai Y, Wu Q, Le W, Ye F, Li Y, Dong Z: Rotenone-induced PC12 cell toxicity is caused by oxidative stress resulting from altered dopamine metabolism. *Toxicology in vitro : an international journal published in association with BIBRA* 2008, 22(6):1461-1468.

276.   Harrison FE, Meredith ME, Dawes SM, Saskowski JL, May JM: Low ascorbic acid and increased oxidative stress in gulo(-/-) mice during development. *Brain Research* 2010, 1349:143-152.

277.   Moretti M, Budni J, Dos Santos DB, Antunes A, Daufenbach JF, Manosso LM, Farina M, Rodrigues AL: Protective effects of ascorbic acid on behavior and oxidative status of restraint-stressed mice. *J Mol Neurosci* 2013, 49(1):68-79.

278.   Binfare RW, Rosa AO, Lobato KR, Santos AR, Rodrigues AL: Ascorbic acid administration produces an antidepressant-like effect: evidence for the involvement of monoaminergic neurotransmission. *ProgNeuropsychopharmacolBiol Psychiatry* 2009, 33(3):530-540.

279.   Ponimaskin EG, Heine M, Zeug A, Voyno-Yasenetskaya T, Salonikidis PS: Monitoring Receptor-Mediated Changes of Intracellular cAMP Level by Using Ion Channels and Fluorescent Proteins as Biosensors. In: *Serotonin Receptors in Neurobiology.* Edited by Chattopadhyay A. Boca Raton (FL); 2007.

280.   Matin A, Matin MK: Cellular levels, excretion, and synthesis rates of cyclic AMP in Escherichia coli grown in continuous culture. *J Bacteriol* 1982, 149(3):801-807.

281.   Borland G, Gupta M, Magiera MM, Rundell CJ, Fuld S, Yarwood SJ: Microtubule-associated protein 1B-light chain 1 enhances activation of Rap1 by exchange protein activated by cyclic AMP but not intracellular targeting. *Mol Pharmacol* 2006, 69(1):374-384.

282.   Chatuphonprasert W, Lao-Ong T, Jarukamjorn K: Improvement of superoxide dismutase and catalase in streptozotocin-nicotinamide-induced type 2-diabetes in mice by berberine and glibenclamide. *Pharmaceutical biology* 2013.

283.   Zlokovic BV: The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 2008, 57(2):178-201.

284.   Cha J, Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M: Ascorbate supplementation inhibits growth and metastasis of B16FO melanoma and 4T1 breast cancer cells in vitamin C-deficient mice. *International journal of oncology* 2013, 42(1):55-64.

285.   Cameron E, Pauling L: Supplemental ascorbate in the supportive treatment of cancer: reevaluation of prolongation of survival times in terminal human cancer. *ProcNatlAcadSciUSA* 1978, 75(9):4538-4542.

286.   Creagan ET, Moertel CG, O'Fallon JR, Schutt AJ, O'Connell MJ, Rubin J, Frytak S: Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. A controlled trial. *The New England journal of medicine* 1979, 301(13):687-690.

287.   Ma Y, Chapman J, Levine M, Polireddy K, Drisko J, Chen Q: High-dose parenteral ascorbate enhanced chemosensitivity of ovarian cancer and reduced toxicity of chemotherapy. *Science translational medicine* 2014, 6(222):222ra218.

288.   Mayersohn M: Ascorbic acid absorption in man--pharmacokinetic implications. *Eur J Pharmacol* 1972, 19(1):140-142.

289.   Mayersohn M, Feldman S, Gibaldi M: Bile salt enhancement of riboflavin and flavin mononucleotide absorption in man. *The Journal of nutrition* 1969, 98(3):288-296.

290.   Moertel CG, Fleming TR, Creagan ET, Rubin J, O'Connell MJ, Ames MM: High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy. A randomized double-blind comparison. *The New England journal of medicine* 1985, 312(3):137-141.

291. Vitamin C [http://lpi.oregonstate.edu/infocenter/vitamins/vitaminC/]
292. Pauling L: How to live longer and feel better: Avon; 1987.
293. Beydoun MA, Beydoun HA, Boueiz A, Shroff MR, Zonderman AB: Antioxidant status and its association with elevated depressive symptoms among US adults: National Health and Nutrition Examination Surveys 2005-6. *Br J Nutr* 2012:1-16.
294. Kupfer DJ, Frank E, Phillips ML: Major depressive disorder: new clinical, neurobiological, and treatment perspectives. *Lancet* 2012, 379(9820):1045-1055.
295. Scapagnini G, Davinelli S, Drago F, De Lorenzo A, Oriani G: Antioxidants as antidepressants: fact or fiction? *CNS Drugs* 2012, 26(6):477-490.
296. Payne ME, Steck SE, George RR, Steffens DC: Fruit, vegetable, and antioxidant intakes are lower in older adults with depression. *J Acad Nutr Diet* 2012, 112(12):2022-2027.
297. Kositsawat J, Freeman VL: Vitamin C and A1c Relationship in the National Health and Nutrition Examination Survey (NHANES) 2003-2006. *J AmCollNutr* 2011, 30(6):477-483.
298. Harding AH, Wareham NJ, Bingham SA, Khaw K, Luben R, Welch A, Forouhi NG: Plasma vitamin C level, fruit and vegetable consumption, and the risk of new-onset type 2 diabetes mellitus: the European prospective investigation of cancer--Norfolk prospective study. *Archives of Internal Medicine* 2008, 168(14):1493-1499.
299. Tanaka S, Yoshimura Y, Kawasaki R, Kamada C, Tanaka S, Horikawa C, Ohashi Y, Araki A, Ito H, Akanuma Y *et al*: Fruit intake and incident diabetic retinopathy with type 2 diabetes. *Epidemiology* 2013, 24(2):204-211.
300. CDC: National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. US Depart of Health and Human Services CDC; 2011.
301. Xu WL, Qiu CX, Wahlin A, Winblad B, Fratiglioni L: Diabetes mellitus and risk of dementia in the Kungsholmen project: a 6-year follow-up study. *Neurology* 2004, 63(7):1181-1186.
302. Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM: Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology* 1999, 53(9):1937-1942.
303. Huang CC, Chung CM, Leu HB, Lin LY, Chiu CC, Hsu CY, Chiang CH, Huang PH, Chen TJ, Lin SJ *et al*: Diabetes mellitus and the risk of Alzheimer's disease: a nationwide population-based study. *PloS one* 2014, 9(1):e87095.
304. Zandi PP, Anthony JC, Khachaturian AS, Stone SV, Gustafson D, Tschanz JT, Norton MC, Welsh-Bohmer KA, Breitner JC: Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements: the Cache County Study. *Archives of Neurology* 2004, 61(1):82-88.
305. Fotuhi M, Zandi PP, Hayden KM, Khachaturian AS, Szekely CA, Wengreen H, Munger RG, Norton MC, Tschanz JT, Lyketsos CG *et al*: Better cognitive performance in elderly taking antioxidant vitamins E and C supplements in combination with nonsteroidal anti-inflammatory drugs: the Cache County Study. *Alzheimer's & dementia : the journal of the Alzheimer's Association* 2008, 4(3):223-227.
306. Rinaldi P, Polidori MC, Metastasio A, Mariani E, Mattioli P, Cherubini A, Catani M, Cecchetti R, Senin U, Mecocci P: Plasma antioxidants are similarly depleted in mild cognitive impairment and in Alzheimer's disease. *Neurobiology of aging* 2003, 24(7):915-919.
307. Pauling L: Evolution and the need for ascorbic acid. *Proc Natl Acad Sci U S A* 1970, 67(4):1643-1648.
308. Hemila H: Vitamin C supplementation and common cold symptoms: Problems with inaccurate reviews. *Nutrition* 1996, 12(11-12):804-809.
309. Bourne GH: Vitamin C and Immunity. *British Journal of Nutrition* 1949, 2(04):341-347.
310. Stone I: On the genetic etiology of scurvy. *Acta geneticae medicae et gemellologiae* 1966, 15(4):345-350.

311. Hathcock JN, Azzi A, Blumberg J, Bray T, Dickinson A, Frei B, Jialal I, Johnston CS, Kelly FJ, Kraemer K *et al*: Vitamins E and C are safe across a broad range of intakes. *American Journal of Clinical Nutrition* 2005, 81(4):736-745.
312. Health ANHaMRCatNZMo: Nutrient Reference Values for Australia and New Zealand Including Recommended Dietary Intakes: Vitamin C. In. Australia; 2006.
313. Health GBDo: Dietary Reference Values for Food Energy and Nutrients for the United Kingdom: Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy: Stationery Office; 1991.
314. National School Lunch Program [http://frac.org/federal-foodnutrition-programs/national-school-lunch-program/]