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

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Abstract

Scope: Ascorbic acid (ascorbate) is required to recycle tetrahydrobiopterin, which is necessary for neurotransmitter synthesis by the rate-limiting enzymes tyrosine and tryptophan hydroxylases. We sought to determine whether ascorbate might regulate embryonic brain cortex monoamine synthesis utilizing transgenic mouse models with varying intracellular ascorbate levels. Methods and Results: In embryos lacking the sodium-dependent vitamin C transporter 2 (SVCT2), very low levels of brain ascorbate decreased cortex levels of norepinephrine and dopamine by approximately 33%, but had no effect on cortex serotonin or its metabolite, 5-hydroxyindole acetic acid. This decrease in ascorbate also led to a decrease in protein levels of tyrosine hydroxylase, but not of tryptophan hydroxylase. Increased cortex ascorbate in embryos carrying extra copies of the SVCT2 resulted in increased levels of dopamine and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), as well as serotonin and 5-hydroxyindole acetic acid. Conclusion: The dependence of embryonic brain cortex neurotransmitter synthesis and tyrosine hydroxylase expression on intracellular ascorbate emphasizes the importance of receiving adequate ascorbate during development.

Keywords

Ascorbic acid; development; neurotransmitter; sodium-dependent vitamin C transporter 2; tyrosine hydroxylase

1. INTRODUCTION

Ascorbic acid (AA) is widely known for its role as an antioxidant in combating oxidative stress. However, the vitamin does have other functions that are often overlooked. One such role involves its regulation of neurotransmitter synthesis in the brain (Figure 1). AA is known to enhance at least two steps of neurotransmitter synthesis. The first is through an indirect mechanism to recycle tetrahydrobiopterin, which is required for the activation of the

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rate-limiting enzyme for catecholamine synthesis, tyrosine hydroxylase. Tetrahydrobiopterin is also required for tryptophan hydroxylase activity in the synthesis of serotonin. In the second mechanism, AA directly donates an electron to dopamine β -hydroxylase for the conversion of dopamine into norepinephrine within the neurosecretory vesicle. Although these functions of AA are well established, their physiologic relevance is unclear, especially during embryonic development.

AA is highly concentrated and regulated in the brain, suggesting an important role for it in this organ (Harrison and May, 2009; Rice, 2000). It is transported into the brain and into neurons primarily through the sodium-dependent vitamin C transporter 2 (SVCT2) (Harrison and May, 2009; Tsukaguchi et al., 1999). In mice lacking this protein, brain AA levels are negligible and they die at birth (Bornstein et al., 2003; Harrison et al., 2010). This is despite the fact that they are able to make their own AA at this stage and that many organs do not have severe AA deficiency. 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 (Bornstein et al., 2003). The latter result was confirmed in adult mice unable to synthesize their own AA that underwent systemic AA deficiency due to dietary AA depletion, although effects in brain were not reported (Amano et al., 2013). In adult mice unable to synthesize their own AA (*gulonolactone oxidase* knockout mice, *gulo*(−/−)), dietary AA depletion decreased cortex and striatum levels of the serotonin metabolite, 5-hydroxyindole acetic acid, as well as dopamine metabolites in the cortex (Ward et al., 2013).

Cell culture models have been used to determine the effect of AA on proteins involved in neurotransmitter synthesis. For example, treating AA-deficient neuroblastoma cells with AA increased tyrosine hydroxylase mRNA levels (Seitz et al., 1998). We have previously shown that AA treatment increases norepinephrine production within six hours in a neuroblastoma SH-SY5Y cell line (May et al., 2012). In addition, we showed this effect was specific for AA, since it was not mimicked by several other antioxidants. Most important, we found that AA increased tyrosine hydroxylase protein expression, in accordance with the increase in tyrosine hydroxylase mRNA observed by Seitz in a related neuroblastoma cell line, SK-N-SH (Seitz et al., 1998). In the current study, we investigated the effects of cellular AA deficiency and excess on brain neurotransmitter synthesis and tyrosine hydroxylase and tryptophan hydroxylase protein expression in embryonic brain cortex. To do this, we utilized the embryonic SVCT2 knockout mouse to model AA deficiency and a transgenic mouse with increased levels of SVCT2 and AA as a model of a modest excess AA (Harrison et al., 2012).

2. RESULTS

2.1 AA Levels in brain cortex

AA was virtually absent in the cortex of embryos lacking the SVCT2 and decreased 41% in SVCT2(+/-) embryos (Fig 2A). SVCT2-TG embryo cortex, on the other hand, contained 8-11 $\mu\text{mol/g}$ AA, approximately 350% more than wild-type (WT) embryos used for controls in the same AA assays (Fig. 2B).

2.2 Catecholamine Levels

The absence of AA in the cortex of SVCT2(−/−) embryos caused significant decreases in cortex norepinephrine and dopamine contents, by 33% and 32% of SVCT2(+/+), respectively (Fig. 3A and 3B). However, there was no change in the dopamine metabolites, DOPAC and homovanillic acid (Fig. 3C and 3D). There was no significant difference

between the SVCT2(+/+) and SVCT2(+/-) groups in norepinephrine, dopamine, DOPAC, or homovanillic acid despite a modest 12% and 13% decrease in dopamine and homovanillic acid, respectively, in the SVCT2(+/-) group. Increasing cortex AA levels above normal by overexpressing SVCT2 (SVCT2-TG) led to significant increases in dopamine and DOPAC, which were 250% and 411%, respectively (Figure 4B and C). However, there was no significant change in norepinephrine or homovanillic acid (Fig. 4A and 4D).

2.3 Serotonin Levels

The loss of cortex AA, in either the SVCT2(+/-) or SVCT2(-/-) groups, did not affect serotonin levels or its metabolite, 5-hydroxyindole acetic acid (Fig. 5A and 5B). However, increases in cortex AA levels in SVCT2-TG embryos led to significant 200-300% increases in serotonin and 5-hydroxyindole acetic acid compared to wild-type embryos (Fig. 5C and 5D).

2.4 Enzyme Protein Expression Levels

Severe depletion of cortex AA in the SVCT2(-/-) embryos led to a significant 24% decrease in the tyrosine hydroxylase protein level (Fig. 6A). However, the 41% decrease in AA in the SVCT2(+/-) was not enough to alter tyrosine hydroxylase protein level in this group. There were no changes in tryptophan hydroxylase protein (Fig. 6B), in agreement with lack of changes in the serotonin system in these embryos (Fig. 5A and 5B). However, in contrast to increased neurotransmitter levels in SVCT2-TG embryos, increased cortex AA levels in the SVCT2-TG did not alter the levels of tyrosine hydroxylase or tryptophan hydroxylase protein expression (Fig. 6C and 6D).

3. Discussion

The results of this study show that AA levels determine certain neurotransmitter levels in embryonic brain cortex. Severe AA depletion in SVCT2(-/-) mouse embryos caused significant decreases in levels of norepinephrine and dopamine. This significant difference was still observed, despite the variability in the SVCT2(-/-) group. Due to this experimental group having negligible cortex AA levels and thus, receiving the most stress on the system, there could be differences in compensation amongst the embryos to cause the variability in neurotransmitter level. This is in contrast to the control, SVCT2(+/+) group, in which neurotransmitter levels are much less variable. Bornstein *et al* previously reported only trends for decreased levels of norepinephrine and dopamine in the whole brains of such SVCT2(-/-) embryos (Bornstein et al., 2003). Our finding of significant decreases in these two neurotransmitters in the same mouse model embryos could be 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. Further, AA is known to be differentially sequestered in the various cell types within the brain (Rice and Russo-Menna, 1998). In addition, it should be noted that modest 41% AA decreases in SVCT2(+/-) embryos did not affect neurotransmitter levels significantly, indicating that severe AA deficiency is required.

The decreased AA levels in the SVCT2(-/-) embryos also caused modest (24%) but significant decreases in tyrosine hydroxylase protein levels, which could have contributed to the decreases in both dopamine and norepinephrine observed. This finding agrees with previous results in cultured neuronal cells showing that AA supplements to cells lacking AA increased tyrosine hydroxylase mRNA (Seitz et al., 1998) and protein (May et al., 2012). The mechanism by which AA might support tyrosine hydroxylase protein expression remains to be elucidated in cultured cells. On the other hand, since tyrosine hydroxylase protein was not increased in the SVCT2-TG embryos, excess AA does not appear to regulate

tyrosine hydroxylase protein expression. The finding that increased cortex AA in SVCT2-TG embryos did increase dopamine and DOPAC levels supports the notion that most of the AA 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. Failure to see an increase in norepinephrine could reflect saturation of dopamine β -hydroxylase by normal amounts of intracellular AA. The apparent K_m of the enzyme for AA is 0.5-0.6 mM when dopamine conversion to norepinephrine is measured (Levin et al., 1960). Intracellular dopamine concentrations are known to be in the millimolar range (Anden et al., 1966), so the enzyme would be saturated at even normal AA concentrations of 2-10 mM (Harrison and May, 2009). This could in turn cause the observed selective increases in dopamine and DOPAC in the SVCT2-TG embryos without changes in norepinephrine.

Regarding effects of AA on the serotonergic system, Ward *et al* found in the adult AA-depleted *gulo*(-/-) mouse model, serotonin and 5-hydroxyindole acetic acid were significantly decreased in the striatum, but only 5-hydroxyindole acetic acid was modestly decreased in cortex (Ward et al., 2013). In the present study, the spread of the data was too variable to detect changes in serotonin and 5-hydroxyindole acetic acid in the SVCT2(-/-) embryos. In addition, it is important to take into consideration differences in litter variation and compensation that could account for the variability of the SVCT2(-/-) embryos. Therefore, it is difficult to compare raw values of neurotransmitter levels between the SVCT2(-/-) embryos and the TG 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 AA during development. This could be due to tetrahydrobiopterin recycling by another reducing agent such as glutathione in the embryos. On the other hand, increased brain cortex AA in the SVCT2-TG mice also increased levels of both serotonin and 5-hydroxyindole acetic acid. If AA 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.

It is possible the effects on neurotransmitter level and protein synthesis observed in this embryonic model of AA deficiency and excess could be due to the role of AA in cell differentiation and proliferation as described previously (Lee et al., 2003; Lee et al., 2000; Pastor et al., 2013; Yan et al., 2001). AA, along with other important factors, has been shown to aid in differentiation of dopaminergic and serotonergic cells from embryonic stem cells (Lee et al., 2000). Yan *et al* showed AA acts in a non-antioxidant manner to induce dopaminergic cell differentiation and that AA is able to increase dopaminergic cell number by greater than 10-fold compared to cAMP or forskolin (Yan et al., 2001). These findings on the role of AA on cell differentiation suggest the loss of AA in the SVCT2(-/-) embryos could simply reduce the number of dopaminergic and serotonergic neurons, leading to 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 cell number. To be confident cell number is not varying amongst the genotypes, future studies need to be performed to measure the number of proliferating cells and more specifically, the number of dopaminergic and serotonergic cells in this AA-depleted or -excess developmental model.

Still, the AA-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. The results gained from this study prompt further investigation of the role for AA during development.

Although severe AA deficiency is required to impair the dopaminergic and serotonergic systems during development, the increased levels of dopamine and serotonin with increases in intracellular AA do support the notion that AA can regulate neurotransmitter synthesis. Careful monitoring of AA 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 AA levels are decreased in some patients with depression and AA has recently been shown to improve mood and depression in humans (Brody, 2002; Khanzode et al., 2003; Zhang et al., 2011). These studies highlight the significant relationship between AA and mood disorders.

4. EXPERIMENTAL PROCEDURE

4.1 SVCT2 mouse models

Mice containing the heterozygous deletion of the SVCT2 (SVCT2^{+/-}) were originally obtained from Dr. Robert Nussbaum and housed in breeding pairs in a temperature- and humidity-controlled vivarium. They were placed on the C57BL6 background through 9 backcrosses. Homozygous SVCT2^{-/-} mice survive the gestation period but do not survive past birth (Harrison et al., 2010; Sotiriou et al., 2002). On the other hand, SVCT2^{+/-} mice, which have about half the normal levels of AA in the brain cortex, are healthy and fertile. Mice had free access to food and water for the duration of the experiment.

SVCT2-transgenic (TG) mice expressing extra copies of the SVCT2 were generated and also placed on the C57BL6 background, as described previously (Harrison et al., 2012). Homozygous mice for the SVCT2 transgene were housed in breeding pairs in a temperature- and humidity-controlled vivarium. Wild-type C57BL6 mice were used for comparison.

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.

4.2 Sacrifice and tissue collection

Because SVCT2^{-/-} pups are not viable and die shortly after the birth (Sotiriou et al., 2002), we mated SVCT2^{+/-} female and male mice to generate all three genotypes, including SVCT2^{-/-} fetuses. SVCT2^{+/-} dams were provided with 0.33 g/L AA in drinking water with 10 μ M EDTA. Although SVCT2^{+/-} mice can synthesize their own AA, this supplement was given to insure that circulating AA was as high as possible throughout pregnancy. To obtain fetal tissues, pregnant dams were sacrificed by decapitation under isoflurane anesthesia. Male and female fetuses (embryonic days 18.5–19.5) were delivered by Caesarian section and placed on a Petri dish on ice to induce hypothermia. Fetuses were decapitated in which the right and left halves of the cortex were removed from each animal and immediately frozen and stored at -80°C until needed. The right half of the cortex was used for neurotransmitter analysis while one quarter of the left half was used for either protein analysis or AA measurement. The tail was also removed from each embryo and frozen at -20°C for 24 hours before extraction of DNA and determination of genotype by PCR (Harrison et al., 2012). Three to four separate litters were used for AA and neurotransmitter analysis. Eight separate litters were used for protein analysis by Western blotting. C57BL6 wild-type and SVCT2-TG embryonic tissue were collected in a similar manner. Two to three separate litters were used per group for AA and neurotransmitter analysis while three to four litters were used for protein analysis.

4.3 AA Measurement

To measure intracellular AA, tissues were homogenized in a lysis buffer containing 0.1 ml of 25% (w/v) metaphosphoric acid and 0.35 ml of 0.1 M Na₂HPO₄ and 0.05 mM EDTA, pH 8.0. The tissue homogenate was centrifuged at 4°C for 5 min at 13,000×g, and the supernatant was taken for assay of AA. Assay of AA was performed in duplicate by high performance liquid chromatography (HPLC) as previously described (Meredith et al., 2011; Pachla and Kissinger, 1979). Data were expressed per gram wet tissue weight.

4.4 Neurotransmitter Analysis

HPLC coupled with electrochemical detection was used to measure monoamine content as described previously (Ward et al., 2013). 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. Measurements were made using the right hemisphere of the cortex by the Neurochemistry Core Laboratory at Vanderbilt University's Center for Molecular Neuroscience Research (Nashville, TN).

4.5 Western immunoblotting

Immunoblotting was performed as previously described (Meredith et al., 2011). Solubilized protein (30 µg) was subjected to SDS–polyacrylamide gel electrophoresis (7.5% gel) according to the method of Laemmli (Laemmli, 1970). Tyrosine hydroxylase was probed with an affinity-purified rabbit polyclonal antibody (1:400) (sc-14007, Santa Cruz Biotechnology, Santa Cruz, CA). Tryptophan hydroxylase was probed with a monoclonal mouse antibody (1:1000) (T0678, Sigma-Aldrich, St. Louis, MO). Protein levels were expressed relative to β-Actin (1:400) to account for protein loading (sc-1616, Santa Cruz Biotechnology). Anti-rabbit and anti-goat secondary antibodies conjugated to horseradish peroxidase (A6154 and A8919, Sigma-Aldrich, St. Louis, MO) were used with the tyrosine hydroxylase and β-actin primary antibodies, respectively. An anti-mouse secondary antibody conjugated to horseradish peroxidase (sc-2003, Santa Cruz Biotechnology, Santa Cruz, CA) was used in conjunction with tryptophan hydroxylase. Bands were illuminated using ECL Plus Western blotting reagents (RPN 2132, Amersham Biosciences, Piscataway, NJ). Saturation of signal was determined by a film exposure time course. Densitometry was determined using ImageJ, normalized to expression of β-actin, and expressed relative to wild-type control embryos.

4.6 Statistics

Data were analyzed using GraphPad Prism 5.0 version for Windows (GraphPad Software, San Diego, CA). A one-way ANOVA was conducted for each analysis between the SVCT2(+/-) groups, with a Tukey's post-hoc test. A student's "t" test was conducted between the wild-type and SVCT2-TG groups. All results are shown as mean +/- standard error.

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ABBREVIATIONS

AA	ascorbic acid
DOPAC	3,4-dihydroxyphenylacetic acid

gulo	gulonolactone oxidase
SVCT2	sodium-dependent vitamin C transporter 2
WT	wild-type

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HIGHLIGHTS

- Cortex ascorbic acid (AA) concentrations are dependent on the level of the SVCT2.
- Decreased AA levels decrease dopamine and norepinephrine levels.
- Decreased AA levels decrease tyrosine hydroxylase protein levels.
- Increased AA levels increase dopamine, DOPAC, serotonin, and 5-HIAA.

Figure 1.

The role of AA in neurotransmitter synthesis. AA helps to recycle tetrahydrobiopterin which activates the enzymes tyrosine hydroxylase and tryptophan hydroxylase. In addition, AA interacts with the dopamine β-hydroxylase to convert dopamine to norepinephrine.

Figure 2.
Cortex AA levels in embryonic day 18.5-19.5 old mice. A) SVCT2(+/+), (+/-), and (-/-) embryos and B) wild-type and SVCT-TG embryos. #p<0.05 versus other groups.

Figure 3. 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. Cortex A) norepinephrine, B) dopamine, C) DOPAC, and D) homovanillic acid in SVCT2-TG embryos compared to wild-type. # $p < 0.05$ versus wild-type controls.

Figure 5. Cortex serotonin and 5-hydroxyindole acetic acid levels in SVCT2(+/+), (+/-), and (-/-) embryos (A and B) and in wild-type versus SVCT2-TG embryos (C and D). [#]p<0.05 versus other group.

Figure 6. Cortex tyrosine hydroxylase and tryptophan hydroxylase protein levels in SVCT2(+/+), (+/-), and (-/-) embryos (A and B) and wild-type versus SVCT2-TG embryos (C and D). Representative immunoblots shown. [#]p<0.05 versus other groups.