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Low brain ascorbic acid increases susceptibility to seizures in mouse models of decreased brain ascorbic acid transport and Alzheimer's disease

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Abstract

Seizures are a known co-occurring symptom of Alzheimer's disease, and they can accelerate cognitive and neuropathological dysfunction. Sub-optimal vitamin C (ascorbic acid) deficiency, that is low levels that do not lead the sufferer to present with clinical signs of scurvy (e.g. lethargy, hemorrhage, hyperkeratosis), are easily obtainable with insufficient dietary intake, and may contribute to the oxidative stress environment of both Alzheimer's disease and epilepsy. The purpose of this study was to test whether mice that have diminished brain ascorbic acid in addition to carrying human Alzheimer's disease mutations in the amyloid precursor protein (APP) and presenilin 1 (PSEN1) genes, had altered electrical activity in the brain (electroencephalography; EEG), and were more susceptible to pharmacologically-induced seizures. Brain ascorbic acid was decreased in APP/PSEN1 mice by crossing them with sodium vitamin C transporter 2 (SVCT2) heterozygous knockout mice. These mice have an approximately 30% decrease in brain ascorbic acid due to lower levels of SVCT2 that supplies the brain with ASC. SVCT2+/-APP/PSEN1 mice had decreased ascorbic acid and increased oxidative stress in brain, increased mortality, faster seizure onset latency following treatment with kainic acid (10 mg/kg i.p.), and more ictal events following pentylenetetrazol (50 mg/kg i.p.) treatment. Furthermore, we report the entirely novel phenomenon that ascorbic acid deficiency alone increased the severity of kainic acid- and pentylenetetrazol-induced seizures. These data suggest that avoiding ascorbic acid deficiency may be particularly important in populations at increased risk for epilepsy and seizures, such as Alzheimer's disease.

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Keywords

Alzheimer's disease; mouse model; Vitamin C; Electroencephalography; Kainic acid; Pentylenetetrazol

1. Introduction

Seizures are a co-occurring adverse event in Alzheimer's disease (AD), related to amyloid precursor protein (APP) and presenilin 1 (PSEN1) mutations in familial AD, but also affecting many sporadic AD cases, with estimates of prevalence of up to 64 % (Friedman et al., 2012). Non-convulsive seizures (e.g. absence or partial seizures) are harder to distinguish from other abnormal behaviors (Pandis and Scarneas, 2012) and may be under-reported in AD, particularly by non-medical caregivers.

Ascorbic acid (ASC, vitamin C) is a critical antioxidant in the brain. ASC levels are depleted or deficient in up to 30% of Western populations, particularly in the elderly and hospitalized (Harrison, 2012). ASC is carefully controlled in the brain parenchyma via the sodium dependent vitamin C transporter, SVCT2, which transfers ASC at the choroid plexus from blood into cerebral spinal fluid, and also from extracellular fluid into neurons. This two-step transport process allows accumulation in the brain to far exceed that in blood, except under conditions of prolonged insufficient intake.

Pre- and post-seizure treatments with exogenous ASC moderate the severity of seizures and resultant neurological damage in rodent models (Dong et al., 2013; Gonzalez-Ramirez et al., 2010; Naseer et al., 2011; Santos et al., 2009; Xavier et al., 2007), but ASC deficiency, the more common state in humans, has not been investigated in epilepsy. The objective of this study was to demonstrate whether chronic ASC deficiency increased seizure susceptibility and severity in a mouse model of AD by measuring mortality, behavioral response, and electroencephalography (EEG) responses to pharmacologically-induced seizures, targeting two neurotransmitter systems, GABAergic and glutamatergic.

2. Methods

2.1 Animals

Heterozygous SVCT2 knockout mice (SVCT2^{+/-}; Sotiriou et al., 2002) were crossed with a bigenic mouse carrying two mutations known to cause familial (early-onset) AD (APP^{SWE}/PSEN1^{ΔE9}; Jackson Laboratories, stock #005864; Fig. 1A). Mice aged 12 to 18 weeks, were maintained in a temperature and humidity controlled environment with *ad libitum* access to food and water. These mice can synthesize ASC and received no additional supplementation. All procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. All experiments were run, and data analyzed, with the experimenter blinded to genotype.

2.2 Kainic acid (KA) seizure induction

Seizure induction was performed in female mice (n=28) by administration of KA 10 mg/kg, i.p. (Sigma-Aldrich, St. Louis, MO). Seizure-related activity was scored according to a modified Racine scale (Fig. 1B).

2.3 EEG headmount affixation surgery, recording and analysis

Male mice (n=24), were fitted with a prefabricated headmount (Pinnacle Technology Inc.) comprised of three channels; 2 EEG to assess the electrical impulses of the brain, and 1 EMG (electromyography) to measure the muscular activity evoked in the nuchal muscles. Two mice died following surgeries (wild-type and SVCT2+/-APP/PSEN1). Following a 1-week recovery period, synchronized video-EEG/EMG recordings were conducted to assess baseline activity over a 24-h period, quantified in uniform 5-min segments each hour (Arain et al., 2012). Each mouse was then injected with a single dose of the GABA_A receptor antagonist, pentylenetetrazol (PTZ; Sigma-Aldrich, St. Louis, MO) 50 mg/kg, i.p. to induce seizure-related activity and monitored during the first 15 min after administration (Binder et al., 2004; Rauca et al., 1999).

A trained observer assessed the spike-and-wave discharges (SWDs) including specific seizure-related events (absence seizures, myoclonic jerks) following previously determined guidelines (Akman et al., 2010; Chung et al., 2009; Snead et al., 1999). SWDs associated with absence seizures and myoclonic jerks were correlated with the appropriate behavioral manifestations in the accompanying video of the EEG/EMG recordings. Abnormal discharges (absence seizure-like activity) and spike discharges (myoclonic jerk-like activity) were quantified regardless of a detectable associated behavior with specific seizure-related events.

2.4 Ascorbic acid and malondialdehyde (MDA)

ASC was measured by an ion pair HPLC and electrochemical detection as previously described (Harrison et al., 2008). MDA was measured as thiobarbituric reactive substances as previously described (Harrison et al., 2010).

2.5 Statistics

Data were analyzed using GraphPad Prism version 5.0 for Mac. KA-seizure induction data, ASC, MDA and EEG data were analyzed using a 2(SVCT2 genotype) X 2(APP/PSEN1 genotype) Univariate ANOVA with Bonferroni *post hoc* comparisons following significant interactions. Mortality data were analyzed using a Chi² test against expected mortality.

3. Results and Discussion

3.1 Mortality

There was a startling increase in unexplained deaths prior to 6 months of age in the SVCT2+/-APP/PSEN1 mice (32% of mice) compared to the other three genotypes (8–10% of mice; Fig. 1C). SVCT2+/-APP/PSEN1 mice were observed undergoing seizure-like behaviors in the home cage. Seizures are also a known complication of APP and PSEN1 mutations and are the likely cause of death in these mice. Increased mortality was previously

noted in APP/PSEN1 mice that were unable to synthesize ASC due to inactivation of the *gulo* gene for ASC synthesis and which had been maintained on low ASC supplementation (Harrison et al., 2010), supporting the hypothesis that low ASC contributes to increased mortality through sudden, unexplained deaths in this mouse model of AD.

3.2 KA-induced seizures

In order to pursue seizures as a potential cause of death, we treated mice with KA which causes seizures through glutamate excitotoxicity. SVCT2+/-, APP/PSEN1 and SVCT2+/-APP/PSEN1 mice progressed to stage 3 (repetitive movements) much more quickly than wild-type mice (Fig. 1D; APP/PSEN1: $F_{1,24}=27.02$, $p<0.01$; SVCT2+/-: $F_{1,24}=14.31$, $p<0.05$). SVCT2+/-APP/PSEN1 mice were the only genotype observed to progress past stage 3, to die or require euthanization ($n=2$) according to humane endpoints following severe tonic-clonic seizures.

3.3 EEG activity

We next hypothesized that such a severe phenotype would manifest itself as aberrant neural electrical activity, and undertook EEG measurements on 4–6 mice per genotype. Under baseline conditions, there was a trend toward increased incidence of absence seizures and myoclonic jerks in SVCT2+/-APP/PSEN1 mice, although in neither case were differences significant ($F_s<2.12$, $p_s>0.16$; Fig. 2A, B). Abnormal discharges and spike discharges were not appreciably different in any of the genotypes.

Given these suggestive, but inconclusive data, we determined whether increased seizure susceptibility would also be apparent following seizure induction with PTZ. Following PTZ administration, abnormal EEG discharges (SWDs without an associated behavior arrest) were 3 to 6-fold higher in mutant genotypes compared to wild-types although this was not significant ($F_s<2.29$, $p_s>0.15$, Fig. 2C). Significantly more myoclonic jerks were observed in SVCT2+/-APP/PSEN1 mice compared to other genotypes (SVCT2 $F_{1,16}=15.67$, $p<0.05$; interaction $F_{1,16}=31.5$, $p<0.01$; Fig. 2D; I). Spike discharges were also more common in mice heterozygous for SVCT2+/- ($F_{1,16}=30.28$, $p<0.05$, Fig. 2E, J). The greatest number was observed in SVCT2+/- APP/PSEN1 mice, driving a trend toward an additional main effect of APP/PSEN1 genotype ($F_{1,16}=13.56$, $p=0.07$). The latency to first spike discharge was significantly shorter in all three mutant genotypes compared to wild-types ($F_s>10.24$, $p_s<0.01$, Fig. 2F). These data suggest that mice of SVCT2+/- and APP/PSEN1 genotypes have aberrant electrical activity that may lower their seizure threshold.

3.4 ASC and oxidative stress

Additional mice ($n=14$) were treated with PTZ but not assessed with EEG for inclusion in biochemical assays. SVCT2+/- and SVCT2+/-APP/PSEN1 mice had significantly decreased brain ASC, approximately 30% lower than wild-types ($F_{1,30}=63.19$, $p<0.001$, Fig. 2G), which was in line with previous reports of these mice (Sotiriou et al., 2002). SVCT2+/- and SVCT2+/-APP/PSEN1 also had increased MDA, which was used as a marker of lipid peroxidation ($F_{1,30}=13.82$, $p<0.05$, Fig. 2H).

Given these findings, we hypothesize that oxidative stress contributed to the differences reported in these mice and ASC plays a major role as an antioxidant in cells or at the synapse during seizures. Previous data showed that ASC (250–500 mg/kg i.p.) decreased seizure severity and increased latency to onset following administration of KA, pilocarpine and PTZ. ASC also attenuated oxidative stress during and after active seizure phase and attenuated hippocampal neuronal loss and markers of apoptosis (e.g. caspase-3) and autophagy (Dong et al., 2013; Gonzalez-Ramirez et al., 2010; Naseer et al., 2011; Santos et al., 2009; Xavier et al., 2007). Nevertheless, given the clear data with both KA (kainate receptor agonist) and PTZ (GABA_A antagonist) it is likely that ASC also interacts with these neurotransmitter systems in some way. Multiple methods indicate disrupted glutamate transport in human AD postmortem samples (Proper et al., 2002) and mouse models of AD (Minkeviciene et al., 2008).

4. Conclusions

Previous work in this area has been undertaken in wild-type rodents which synthesize their own ASC and have normal ASC transport, and without consideration of the additional sensitivity found in AD. This is the first report where decreased brain ASC, at clinically-relevant levels, impacts seizure susceptibility in both wild-type and APP/PSEN1 mice. We therefore suggest that models of altered brain ASC (such as the SVCT2^{+/-} line used here or the gulo^{-/-} line that, like humans, cannot synthesize ASC) may be particularly useful, and more relevant to animal-based research into seizure-related mechanisms. The most surprising and important finding from this study was that ASC deficiency alone impacted seizure activity. Cognitive decline is accelerated by 5–7 years in AD patients with concomitant epilepsy (Vossel et al., 2013). It is, therefore, possible that preventing deficiency could be a cost-effective and simple way to diminish the occurrence or severity of seizures in some at-risk populations.

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Abbreviations

ASC	ascorbic acid
AD	Alzheimer's disease
APP	amyloid precursor protein
EEG	electroencephalography
EMG	electromyography
KA	kainic acid
MDA	malondialdehyde
PSEN1	presenilin 1

PTZ	pentylentetrazol
SVCT2	sodium dependent vitamin C transporter

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HIGHLIGHTS

Ascorbic acid deficiency shortened latency to onset of kainic acid-induced seizures

APP/PSEN1 mutations shortened latency to onset of kainic acid-induced seizures

SVCT2+/-APP/PSEN1 mice have abnormal EEG response to pentylentetrazol

Figure 1. Unexplained deaths and increased seizure susceptibility in SVCT2^{+/-} APP/PSEN1 mice

(A) Breeding scheme, genotype and phenotype for experimental mice. (B) Modified Racine scale used for behavioral scoring of seizures. Humane endpoint for euthanization was stage 6, tonic clonic seizures, for 2 consecutive minutes. (C) Increased mortality in SVCT2^{+/-} APP/PSEN1 mice reported as percent deaths from unexplained causes in the colony. Data are for all litters bred in the in-house colony over the past 4 years that reached 6-months of age, and were not removed for other experiments. Total numbers of mice considered given above their respective bars. Data analyzed by χ^2 difference from expected value $^{+++}p < 0.001$. (D) SVCT2^{+/-}, APP/PSEN1 and SVCT2^{+/-} APP/PSEN1 mice have shorter latency to onset of seizure stage 3 following treatment with 10 mg/kg KA (i.p.) in n=6–8 females per genotype. Data were analyzed by 2(APP/PSEN1 genotype) X 2(SVCT2 genotype) ANOVA. * $p < 0.05$, different from wild-type following Bonferroni *post hoc* comparisons. Mean $\pm$ Std. Dev. shown.

Figure 2. Seizure-related phenotype expressed in APP/PSEN1 and SVCT2+/-APP/PSEN1 mice in response to PTZ

(A) Occurrence of absence seizures and (B) myoclonic jerks were slightly, but not significantly, increased in SVCT2+/-APP/PSEN1 mice under baseline conditions. EEG recordings were analyzed for the first 15-minutes following administration of 50 mg/kg PTZ (i.p.). (C) Abnormal discharges varied among the groups but not significantly so. (D) Number of myoclonic jerks was greater in mice that were heterozygous for SVCT2. (E) Number of spike discharges, which represent myoclonic jerk-like activity without an associated behavior, was also increased in SVCT2+/- and SVCT2+/-APP/PSEN1 mice. (F) The latency to the first observation of a spike discharge was also significantly shorter in

APP/PSEN1, SVCT2+/- and SVCT2+/-APP/PSEN1 mice. **(G)** Cortex ascorbic acid levels were significantly decreased in SVCT2+/- and SVCT2+/-APP/PSEN1 mice. **(H)** Lipid peroxidation measured by MDA was significantly elevated in SVCT2+/- and SVCT2+/-APP/PSEN1 mice. A sample EEG/EMG readout for **(I)** a myoclonic jerk and **(J)** spike discharge. The EEG recording data are from 4 WT, 5 APP/PSEN1, 6 SVCT2+/-, and 5 SVCT2+/-APP/PSEN1 mice. The ASC and MDA determination data are from 10 WT, 9 APP/PSEN1, 8 SVCT2+/-, and 7 SVCT2+/-APP/PSEN1 mice. All mice were males. *p<0.05, **p<0.01 different from wild-type following Bonferroni *post hoc* comparisons. Bars show mean $\pm$ SEM.