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Calsyntenin-3 interacts with the sodium-dependent vitamin C transporter-2 to regulate vitamin C uptake

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Abstract

Ascorbic acid (AA) uptake in neurons occurs via a Na^+-dependent carrier-mediated process mediated by the sodium-dependent vitamin C transporter-2 (SVCT2). Relatively little information is available concerning the network of interacting proteins that support human (h)SVCT2 trafficking and cell surface expression in neuronal cells. Here we identified the synaptogenic adhesion protein, calsyntenin-3 (CLSTN3) as an hSVCT2 interacting protein from yeast two-hybrid (Y2H) screening of a human adult brain cDNA library. This interaction was confirmed by co-immunoprecipitation, mammalian two-hybrid (M2H), and co-localization in human cell lines. Co-expression of hCLSTN3 with hSVCT2 in SH-SY5Y cells led to a marked increase in AA uptake. Reciprocally, siRNA targeting hCLSTN3 inhibited AA uptake. In the J20 mouse model of Alzheimer's disease (AD), mouse (m) SVCT2 and mCLSTN3 expression levels in hippocampus were decreased. Similarly, expression levels of hSVCT2 and hCLSTN3 were markedly decreased in hippocampal samples from AD patients. These findings establish CLSTN3 as a novel hSVCT2 interactor in neuronal cells with potential pathophysiological significance.

Keywords

Vitamin C; Transport; Interactor; Alzheimer's disease; SVCT2; CLSTN3

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CRediT authorship contribution statement

Veedamali S. Subramanian: Conceptualization, Validation, Formal analysis, Methodology, Investigation, Visualization, Writing – original draft, Writing – review and editing, Project administration, Supervision, Resources, Funding acquisition. **Trevor Teafatiller:** Validation, Formal analysis, Methodology, Investigation, Visualization, Writing – original draft, Writing – review and editing. **Janielle Vidal:** Investigation, Visualization. **Gihan S. Gunaratne:** Investigation, Visualization. **Carlos J. Rodriguez-Ortiz:** Investigation, Visualization. **Masashi Kitazawa:** Supervision, Resources, Methodology, Writing – review and editing. **Jonathan S. Merchant:** Conceptualization, Supervision, Writing – original draft, Writing – review and editing.

Declaration of competing interest

The authors declare that there are no competing interests associated with the manuscript.

1. Introduction

Vitamin C (ascorbic acid: AA) is an essential micronutrient that is required for normal cellular health and development in humans. This vitamin is involved in an array of vital functions including acting as an enzymatic cofactor and antioxidant [1,2]. AA is able to neutralize reactive oxygen species (ROS) by enacting defensive pathways that promote renewal of endogenous antioxidants [3]. Insufficient vitamin C levels negatively impact processes that accelerate cognitive decline, β-amyloid accumulation and deposition in Alzheimer's disease (AD) [3–7], the sixth leading cause of death in the United States [8]. The high metabolic activity of the brain makes this organ particularly susceptible to oxidative stress and free radical damage. Brain tissue has the highest concentration of AA in the body and its levels in AD patients plasma are significantly lower [3,9–11]. Supplementation of AA lead to reduced oxidative stress, decreased secretion of inflammatory factors and lower β-amyloid plaque burden in an AD experimental model [3]. Therefore, optimization of vitamin C body homeostasis may defend against age-related cognitive decline and AD [4]. These observations underscore the importance of understanding the molecular mechanisms that optimize homeostasis of this micronutrient in the brain.

Human cells do not produce vitamin C; they obtain the vitamin C from their surroundings following intestinal absorption and blood circulation. Mice are able to synthesize vitamin C in the liver which is then distributed to all tissues via circulation, but endogenous synthesis does not fully meet the metabolic need of brain cells, so there is still a dependence on intestinal vitamin C absorption and its circulation. This is evidenced by studies utilizing knockout mouse models [12,13]. AA uptake into cells is achieved by means of a Na^+-dependent carrier-mediated process. In neurons, AA uptake is reliant on the human sodium-dependent vitamin C transporter-2 (hSVCT2), the predominantly expressed vitamin C transporter isoform in the brain [10,14,15]. hSVCT2 is a member of the major facilitator superfamily of membrane transport proteins, which exhibits a twelve transmembrane (TM)-spanning structural arrangement with NH_2- and COOH-terminals, as well as glycosylation and phosphorylation sites [16–18]. The NH_2- and COOH-terminal amino acid sequences of hSVCT2 are important for cell surface expression [16,19]. These cytoplasmic regions may associate with accessory proteins that regulate hSVCT2 trafficking, targeting and/or function. Prior studies have established the significance of such interactions for other water-soluble vitamin transporters in various cellular systems [20–25]. However, little is known about the vitamin C transporter interactome, and how this is modulated to customize transport properties in different cell types including cells with a high requirement for vitamin C such as neurons. In this study, we identified and validated calsyntenin-3 as an hSVCT2 accessory protein and regulator of cellular vitamin C uptake.

2. Materials and methods

2.1. Reagents

[^{14}C]-AA (specific activity: 2.8 mCi/mmol, radiochemical purity: >97%) was purchased from PerkinElmer (Boston, MA). The human-derived neuroblastoma SH-SY5Y cells and human-derived embryonic kidney HEK-293 cells were obtained from American Type

Culture Collection (ATCC) (Manassas, VA). The services provided by Integrated DNA Technologies (San Diego, CA) were used to synthesize the DNA oligonucleotide primers. TissueScan, a human brain cDNA array, human brain tissue qPCR array (HBRT301) was obtained from OriGene (OriGene Biotechnology, Rockville, MD). All chemicals and molecular biological reagents were bought from commercial vendors and of analytical/molecular biology grade.

2.2. Cell culture and transfection

The SH-SY5Y cell line was cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F-12) medium or the HEK-293 cell line was cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC), each with added fetal bovine serum (FBS; Gemini Bio Products, West Sacramento, CA) [10–20% (*v/v*)], glutamine (0.29 g/L), sodium bicarbonate (2.2 g/L), penicillin (100,000 U/L), and streptomycin (10 mg/L) in T75-cm^2 flasks. The cell culture incubation conditions were maintained at 37 °C (5% CO_2-95% O_2) and the cell culture media was changed every 2–3 days. Cells destined for uptake and molecular biological analysis or live cell imaging were cultured on 12-well plates (Corning Life Sciences, Tewksbury, MA) or glass-bottomed petri dishes (MatTek, Ashland, MA). Upon growth reaching 90% confluence, transient transfection was performed using 2 μg of plasmid DNA and 2 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) followed by continued culture for 48 h before analysis.

2.3. Human and mouse hippocampus tissue preparation

After deep anaesthesia with sodium pentobarbital (60 mg/kg), J20 mice (a model which overexpresses human APP with two mutations linked to familial AD – the Swedish (KM670/671NL) and Indiana (V717F) mutations – with transgene expression driven by the PDGF-β promoter in a C57BL/6 genetic background; [26]) or WT littermate control (C57BL/6 J) were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4). Hippocampi were then dissected and flash frozen in dry ice. Human hippocampal tissue from nondemented and AD patients were provided by the University of California, Irvine (UCI), Alzheimer's Disease Research Centre (ADRC). Brain autopsy consent was obtained under protocol approved by the Institutional Review Board (IRB) at UCI and de-identified tissue was used for the current study. T-Per extraction buffer (150 mg/mL, Pierce Biotechnology, Rockford, IL) was used in combination with protease and phosphatase inhibitors (Fisher Scientific) to homogenize human and mouse hippocampal frozen tissue. Lysates were then subjected to centrifugation (20,000 ×*g* for 30 min at 4 °C) and used for Western blotting. Total RNA was prepared from frozen human and mice hippocampal tissue using Direct-zol RNA miniprep following the manufacturer's protocol (Zymo Research Corporation, Irvine, CA). The University of California, Irvine (AUP-18-180) Institutional Animal Care and Use Committee (IACUC) approved the protocols in this study.

2.4. Yeast two-hybrid (Y2H) and 1-by-1 Y2H assays

The commercial services at Hybrigenics Services SAS (Evry, France) were utilized to perform yeast two-hybrid (Y2H) and "1-by-1" Y2H interaction assays [27]. For Y2H assay, a pP6 prey plasmid containing random-primed human adult brain cDNA library was screened with a pB27 bait plasmid comprising a LexA DNA binding domain (DBD) fused

with the PCR-amplified hSVCT2 C-terminal (567–650 amino acids) (N-LexA-hSVCT2-C). The bait and prey constructs were derived from pBTM116 [28] and pGADGH [29] vector backbones, respectively. YHGX13 (Y187 ade2-101::loxP-kanMX-loxP, matα) and L40ΔGal4 (mata) yeast strains were used to screen one hundred and fifty four million colons (15-fold the complexity of the library) via a mating approach [27]. Selective medium without histidine, leucine, and tryptophan identified two hundred and fifty-three His+ colonies. The PCR products of positive clone prey fragments were sequenced and subsequently those sequences identified the corresponding interacting proteins with a confidence score (Predicted Biological Score, PBS) assigned to each potential interaction found within the GenBank database (National Center for Biotechnology Information) [30]. For "1-by-1" Y2H assay, the hSVCT2-C terminal fragment was cloned with the LexA DBD into pB27 and the hCLSTN3 protein fragment (507–639 amino acid) was cloned into the activation domain (AD) containing plasmid pP6. Sequencing of the DBD and AD constructs confirmed their identity. The reporter gene, HIS3 activity was used to determine the level of hSVCT2 and hCLSTN3 interaction following a growth assay without histidine [27].

2.5. Co-immunoprecipitation assay and Western blotting

HEK-293 cells transiently co-transfected with hSVCT2-YFP and hCLSTN3-HA (together with non-transfected controls) were used in parallel co-immunoprecipitation studies. Cells were lysed in phosphate buffered saline (PBS) supplemented with 1% Triton X-100, complete EDTA-free protease inhibitor cocktail (Roche Applied Sciences, NJ), and Halt phosphatase inhibitor cocktail (Thermo Fisher, Waltham, MA). Cleared supernatant was collected from protein lysates after centrifugation (16,000xRCF for 10 min at 4 °C). 500 μg aliquots of lysate from transfected cells was incubated with 2 μg/mL of either antihemagglutinin (HA) antibody raised in mouse (Santa Cruz Biotechnology, Dallas, TX), anti-SVCT2 antibody raised in rabbit (previously characterized [31]), or corresponding isotyped control antibodies (Santa Cruz Biotechnology) for 1 h at 4 °C. Samples were then supplemented with protein-G agarose beads (Roche Applied Sciences, NJ) and incubated on a tube rotator overnight at 4 °C. The beads were then briefly centrifuged to form a pellet and the supernatant was aspired. The beads were then washed with PBS + 1% Triton X-100 three times. Elution of the immunoprecipitated proteins was done by incubating beads with 2× Laemmli sample buffer in a 95 °C water bath for 10 min. Samples were then separated on Any kD™ Criterion™ TGX gels (Bio-Rad Laboratories Inc., Hercules, CA) and transferred to nitrocellulose membranes for Western blot analysis using an iBlot 2 transfer system (Thermo Fisher). Nitrocellulose membranes were blocked at room temperature in Tris buffered saline containing 5% milk and 0.1% Tween 20 for 1 h. Incubation with 1 μg/mL anti-hSVCT2 antibodies [31], 0.4 μg/mL anti-HA antibody (Santa Cruz Biotechnology, sc-7392), and 0.4 μg/mL anti-GAPDH antibody (Santa Cruz Biotechnology, sc-47724) followed. Protein samples isolated from human/mouse normal or AD hippocampus were separated on NuPAGE 4–12% Bis-Tris mini gels. The blots were probed with human/mouse SVCT2 (1:1000 dilutions) or human/mouse CLSTN3 (1:1000 dilution) (Proteintech, Rosemont, IL, 13302-1-AP) with β-actin antibodies (Thermo Fisher, MA5-15739). Subsequently, the blots were washed three times with PBS-0.1% Tween 20, then probed with secondary antibodies [anti-rabbit and/or anti-mouse IRDye 680 (P/N: 926-68020) or IRDye 800 (P/N: 926-32211); LI-COR Biosciences, Lincoln, NE] in 1:30000

dilutions. Individual protein band fluorescence was resolved by a LI-COR Odyssey infrared imaging system and the intensities were analyzed with LI-COR software.

2.6. Mammalian two-hybrid (M2H) assay

The CheckMate™ mammalian two-hybrid (M2H) system (Promega, Madison, WI) was utilized to assess protein-protein interaction in mammalian cells. The hSVCT2 and hCLSTN3 full-length sequences were cloned into pBIND and pACT vectors, respectively. The resulting inframe pACT-hCLSTN3 and pBIND-hSVCT2 constructs (2 μg each) were co-transfected into SH-SY5Y cells to induce fusion of hSVCT2 with the DBD of GAL4 and fusion of hCLSTN3 with the AD of VP16, respectively. The firefly luciferase gene within pG5*luc* vector served as a reporter gene. After 48 h of post-transfection, Promega's Dual-luciferase Reporter Assay System reagents and a GloMax 20/20 luminometer were used to measure firefly luciferase activity which was normalized to Renilla luciferase activity [20–22].

2.7. Fluorescence microscopy

HEK-293 cells were cultured to 90% confluence on MatTek glass-bottomed petri dishes and co-transfected with hSVCT2-YFP and hCLSTN3-mCherry plasmid DNA (2 μg each) and 2 μL of Lipofectamine 2000 (Invitrogen). Live cell images were taken at 60× with a Nikon CFI Plan APO λ 60xH oil lens on the Keyence BZ-X810 All-in One Inverted Fluorescence Phase Contrast Microscope (Keyence Corporation, Itasca, IL). hSVCT2-YFP was imaged at 488 nm while hCLSTN3-mCherry was imaged at 555 nm. The system uses 80 W Halide lamp as the fluorescence light source and images were analyzed using the Keyence Analyzer software.

2.8. Small interfering RNA (siRNA) analysis

hCLSTN3 siRNA was purchased from Ambion (Austin, CA). Lipofectamine RNAiMAX reagent (Invitrogen) was used to transiently transfect SH-SY5Y cells (~90% confluence) with hCLSTN3 siRNA (Sense: GGAUCGAAUAUGCACCAGGtt, antisense: CCUGGUGCAUAUUCGAUCCtt) and negative control siRNA (scrambled). After 48 h, hSVCT2 and hCLSTN3 mRNA expression levels were quantified using total RNA isolated with TRIzol reagent (Ambion) and the effect of hCLSTN3 knockdown on ^{14}C-AA uptake was determined.

2.9. Real-time PCR (RT-qPCR) analysis

SH-SY5Y cells or human/mouse normal/AD hippocampus total RNA samples were reverse transcribed using a Superscript II kit (Invitrogen). The resultant cDNA template was then amplified by RT-qPCR with hSVCT2, hCLSTN3, mSVCT2, mCLSTN3, hβ-actin and mβ-actin primers (Table 1). The different regions of the normal human brain cDNA obtained from OriGene Biotechnology was directly subjected to RT-qPCR using hSVCT2 or hCLSTN3 specific primers (Table 1). β-Actin was used as a reference target to normalize relative expression values and C_t data were calculated using the relative relationship method [32].

2.10. Uptake studies

Confluent SH-SY5Y cells in 12-well plates were used for ^{14}C-AA uptake analysis after 48 h of transient transfection. The labeled ^{14}C-AA (0.1 μCi) was added to the Krebs-Ringer (K-R) buffer and incubated for 30 min at 37 °C. The incubation was stopped by washing the cells with ice-cold K-R buffer, then the radioactivity and protein contents were measured as described [22,31].

Table 1 shows the primer sequence and combination of primers used to generate each hSVCT2 and hCLSTN3 construct by PCR. Restriction sites for *Hind III* (boldface text), *Sac II* (italics underlined text), *Xba1* (boldface italics) and *Sal1* (underlined text) were added to the hSVCT2 and hCLSTN3 primers to allow subsequent sub-cloning into the YFP-N1, mCherry, pBIND and pACT vectors.

2.11. Statistical analyses

Uptake values represent mean ± SEM of multiple experimental preparations ($n > 3$) using different passages of SH-SY5Y cells and are presented as a percentage relative to controls. Statistical significance was assessed using Student's *t*-test (*P*-value of <0.05). Data from Western blot analysis, RT-qPCR and live cell imaging are representative outcomes from independent experimental replicates.

3. Results

3.1. hCLSTN3 interacts with the hSVCT2 C-terminus

To identify candidate hSVCT2 interactors, we used a yeast two-hybrid (Y2H) screening approach. The hSVCT2 COOH-domain (567–650 amino acids) was used as a bait (Fig. 1A) in a LexA fusion construct for a Y2H screen against preys in a human adult brain cDNA library. This cytoplasmic region of hSVCT2 has previously been shown to regulate hSVCT2 function [16]. The prey fragments of two hundred and fifty-three His+ colonies (from a total of 154 million screened clones) were selected for PCR amplification and their 5′ and 3′ ends were sequenced. Bioinformatic analyses prioritized calsyntenin-3 (CLSTN3: also known as Alcβ, CDHR14 and CSTN3) as a good confidence interactor. The calsyntenin family consists of three (CLSTN1-3) synaptic adhesion proteins belonging to the cadherin superfamily [33,34]. CLSTN3 was identified as a synaptogenic adhesion molecule that is widely expressed in human brain and directly validated in neuronal cells [34,35].

To interrogate the validity of hSVCT2 and hCLSTN3 interaction, a direct ("1-by-1") Y2H analysis was performed. The DBD, N-LexA-hSVCT2-C and a N-GAL4 AD-hCLSTN3 construct (507–639 amino acids) were used as 'bait' and 'prey', respectively. The higher yeast dilution and restrictive growth medium [−Leucine, −Histidine, −Tryptophan, +3 aminotriazole (3AT; 10 mM, an inhibitor of the HIS gene product)] culture conditions supported colony growth for the hSVCT2-hCLSTN3 interaction compared to appropriate positive and negative controls (Fig. 1B). The combined results from the discovery Y2H screening analysis and the direct ("1-by-1") Y2H assays establish that hCLSTN3 is a candidate accessory protein partner for the hSVCT2.

3.2. hCLSTN3 interacts with hSVCT2 in mammalian cells

Next, interaction between hCLSTN3 and hSVCT2 was assessed by co-immunoprecipitation. Epitope-tagged constructs, hCLSTN3-HA and hSVCT2-YFP, were transiently co-transfected into HEK-293 cells and isolated lysates were immunoprecipitated using either anti-HA or anti-hSVCT2 antibodies. Immunoblotting with both anti-hSVCT2 and anti-HA antibody revealed that the hCLSTN3-HA immunoprecipitate contains hSVCT2 and the hSVCT2 immunoprecipitate contains hCLSTN3 (Fig. 1A). Conversely, lysates immunoprecipitated using isotyped control IgG displayed absence of both hSVCT2 and hCLSTN3 in HEK-293 cells (Fig. 2A).

Next, we performed a firefly luciferase assay to further confirm the identified interaction between hSVCT2 and hCLSTN3 in SH-SY5Y cells. pBIND-hSVCT2 and pACT-hCLSTN3 plasmids were co-expressed in SH-SY5Y cells in the presence of pG5*luc* reporter plasmid and Renilla-normalized firefly luciferase activity assay was determined following 48 h of post-transfection. The SH-SY5Y cells co-expressing pBIND-hSVCT2 and pACT-hCLSTN3 plasmids showed a markedly increased luciferase activity when compared to cells transfected with the pBIND and pACT control vectors alone (Fig. 2B).

Finally, we examined hSVCT2 and hCLSTN3 co-localization in HEK-293 cells. hSVCT2-YFP and hCLSTN3-mCherry plasmids were co-transfected into HEK-293 cells and construct localization imaged 48 h later. As shown before, hSVCT2 expression was present at the cell surface and within a variety of intracellular vesicles [16]. HEK-293 cells expressing hCLSTN3-mCherry displayed colocalization with hSVCT2 within intracellular vesicles (Fig. 2C).

3.3. Effect of hCLSTN3 on hSVCT2 function

Next, we examined the effect of hCLSTN3 on hSVCT2 transporter function in SH-SY5Y cells. SH-SY5Y cells were transiently transfected with either hSVCT2 alone, or hCLSTN3 and hSVCT2 together, and ^{14}C-AA uptake was examined. Cells co-expressing hSVCT2 and hCLSTN3 displayed enhanced ^{14}C-AA uptake compared to cells expressing hSVCT2 alone (Fig. 3A). Next, we determined the effect of knockdown of endogenous hCLSTN3 on ^{14}C-AA uptake. In cells transfected with hCLSTN3 siRNA, ^{14}C-AA uptake was markedly lower compared to SH-SY5Y cells transfected with scrambled control siRNA (Fig. 3B). To verify the efficiency of the siRNA knockdown, RT-qPCR was performed and a marked reduction in hCLSTN3 mRNA levels were observed in hCLSTN3 siRNA transfected SH-SY5Y cells compared with scrambled siRNA transfected cells (Fig. 3C). The expression level of hSVCT2 mRNA was not altered in hCLSTN3 siRNA transfected cells compared with controls (Fig. 3D). Collectively, these data demonstrate that hCLSTN3 co-expression enhanced cellular AA uptake, while silencing of endogenous hCLSTN3 impaired AA accumulation in SH-SY5Y cells.

3.4. Expression of SVCT2 and CLSTN3 in normal and AD hippocampus

Previous studies have shown that the plasma vitamin C levels in AD patients are lower than those of healthy subjects [9,11,36]. Reciprocally, maintenance of normal ascorbate levels appears to counteract age-related cognitive impairment and AD [4]. To assess whether

expression of CLSTN3 and SVCT2 is perturbed in AD models, we first utilized the J20 mouse model. This model overexpresses human APP, resulting in higher Aβ levels than mice expressing equivalent amounts of wild-type human APP. In the J20 model, expression levels of mSVCT2 (Fig. 4A) and mCLSTN3 mRNA (Fig. 4C) were significantly decreased in hippocampal samples compared to WT littermates. A similar decrease was observed for protein expression by Western blotting (Fig. 4B & D).

Is down regulation also observed in human samples? First, we profiled expression of hSVCT2 and hCLSTN3 in 24 different regions of normal human brain. Results showed that both hSVCT2 (Fig. 5A) and hCLSTN3 (Fig. 5D) were present in hippocampus. Next, we examined the hSVCT2 and hCLSTN3 mRNA and protein levels in both normal and AD hippocampus samples, which also revealed that hSVCT2 and hCLSTN3 mRNA levels were lower in the human AD hippocampus compared with control samples (Fig. 5B & E). Similarly, protein expression levels for both hSVCT2 (Fig. 5C) and hCLSTN3 (Fig. 5F) were decreased in AD hippocampus compared to control samples. These data demonstrate lower levels of CLSTN3 and SVCT2 expression in both an AD mouse model as well as clinical samples.

4. Discussion

Accessory proteins can impact the expression and function of their membrane transporter partners by regulating subcellular trafficking, cell surface retention and protein perdurance within the cell [20–22,37]. For both vitamin C transporters (hSVCT1 and hSVCT2), comparatively little is known about their interactome. Identification of hSVCT1 and hSVCT2 accessory proteins will help decode the physiological roles of these transporters and advance our understanding of how vitamin C uptake changes in different disease states. Knowledge of hSVCT interactions is also important beyond basic cell biology as strategies that target accessory proteins and their binding interfaces are receiving increasing attention as a therapeutic approach [38–42]. Here we have focused on hSVCT2, the key pathway for vitamin C uptake in neurons, and have identified a new accessory protein regulator, hCLSTN3. This interaction was confirmed by Y2H, “1-by-1” Y2H, M2H, co-immunoprecipitation as well as co-localization in human-derived cell lines. AA uptake was up-regulated following overexpression of hCLSTN3 in SH-SY5Y cells. Reciprocally, knockdown of hCLSTN3 inhibited AA uptake.

CLSTN3 is a single-pass transmembrane protein [956 amino acids (~130 kDa)] of the cadherin superfamily localized to the synaptic membrane, where it interacts with α-neurexin to promote excitatory and inhibitory synapse development in neurons [43,44] and is highly expressed in many cellular systems including neuronal, renal, intestinal, liver and eye [35]. These cellular systems also highly express the SVCT2. CLSTN3 has also shown to be linked to AD pathology [45]. Intriguingly, CLSTN3 also interacts with β-amyloid protein precursor (APP) by binding through their cytoplasmic domains to a shared adaptor protein, X11L. These three proteins (APP-X11L-CLSTN3) form a tripartite complex in the brain that stabilizes the intracellular APP metabolism [46]. Calsyntenins have received attention as biomarkers for AD [47]. hSVCT2 also likely plays a role in AD pathogenesis. Hippocampal neurons, the locus of cognitive impairment/decline in the AD brain, have

high concentrations of vitamin C [6,48–50]. The potential protective function that vitamin C exerts against age-related cognitive impairment/decline and AD may prove beneficial if considered for therapeutic applications given the fact that AD patients exhibit low plasma vitamin C levels [4]. hSVCT2 as well as hCLSTN3 are expressed in human hippocampus (Fig. 5A & D). However, both proteins are markedly decreased in both human AD hippocampal samples, and J20 mice. This is consistent with other data showing that hCLSTN3 is decreased in AD hippocampus [51]. This down regulation would be expected to impair neuronal vitamin C homeostasis. Any correlation in these changes in expression levels and disease state would require considerable further investigation.

Together, our findings demonstrate hCLSTN3 interacts with hSVCT2, with hCLSTN3 expression being positively correlated with vitamin C uptake. Down regulation of both proteins in AD disease states suggests impaired vitamin C homeostasis in the AD brain.

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Data availability

All supporting data are included within the main article and are available from the corresponding author upon request.

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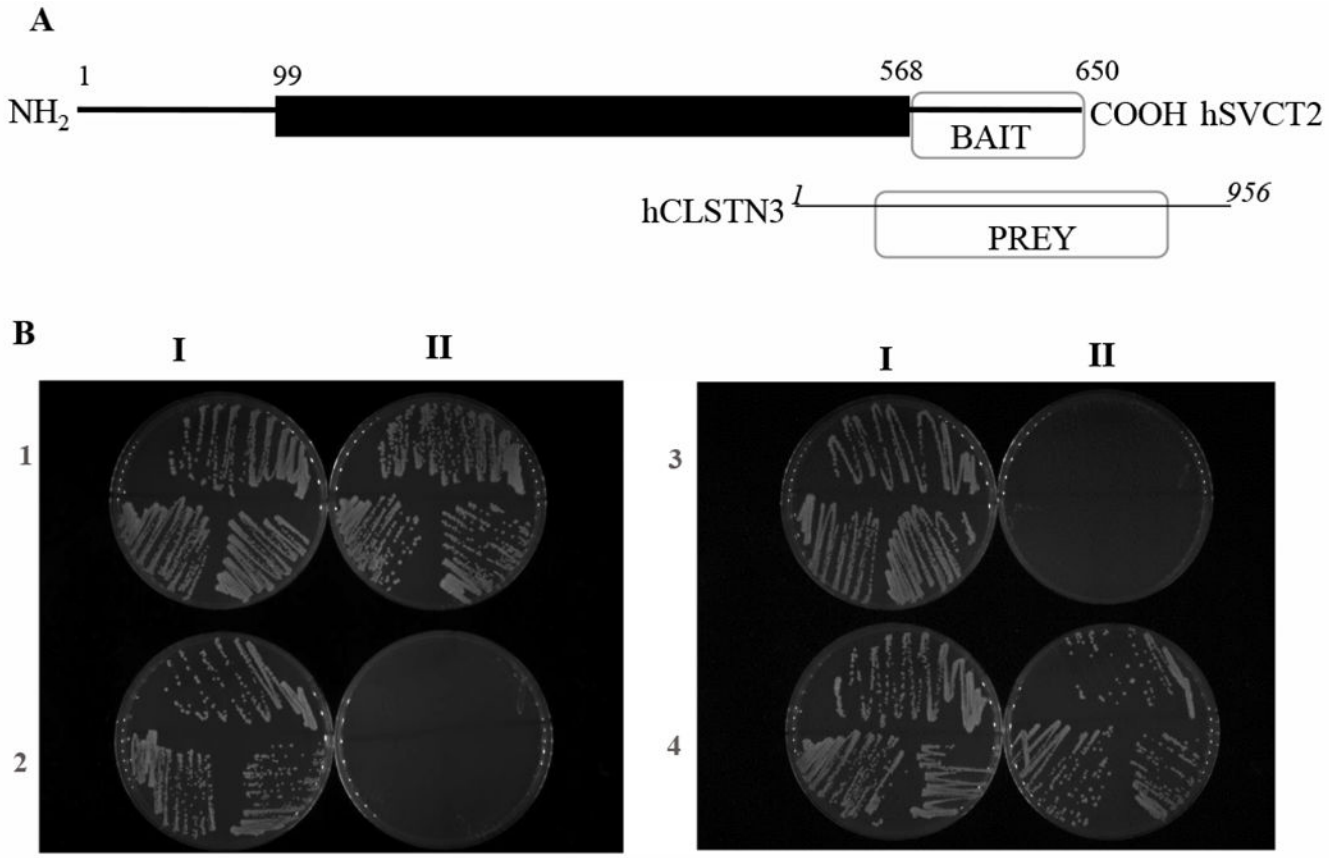

Fig. 1.

Interaction of hSVCT2 and hCLSTN3. (A) Schematic depiction of the coding regions of hSVCT2 and hCLSTN3 with carboxyl domain of hSVCT2 used for Y2H analysis boxed. (B) Images of '1-by-1' Y2H interactivity growth assay on -histidine petri dishes. I: selective medium without tryptophan and leucine; II: selective medium without tryptophan, leucine and histidine. 1. Interaction positive control (SMAD and SMURF), 2. Negative control (empty bait plasmid and hCLSTN3 prey), 3. Negative control (hSVCT2 + empty prey plasmid) and 4. Interaction (hSVCT2 and hCLSTN3).

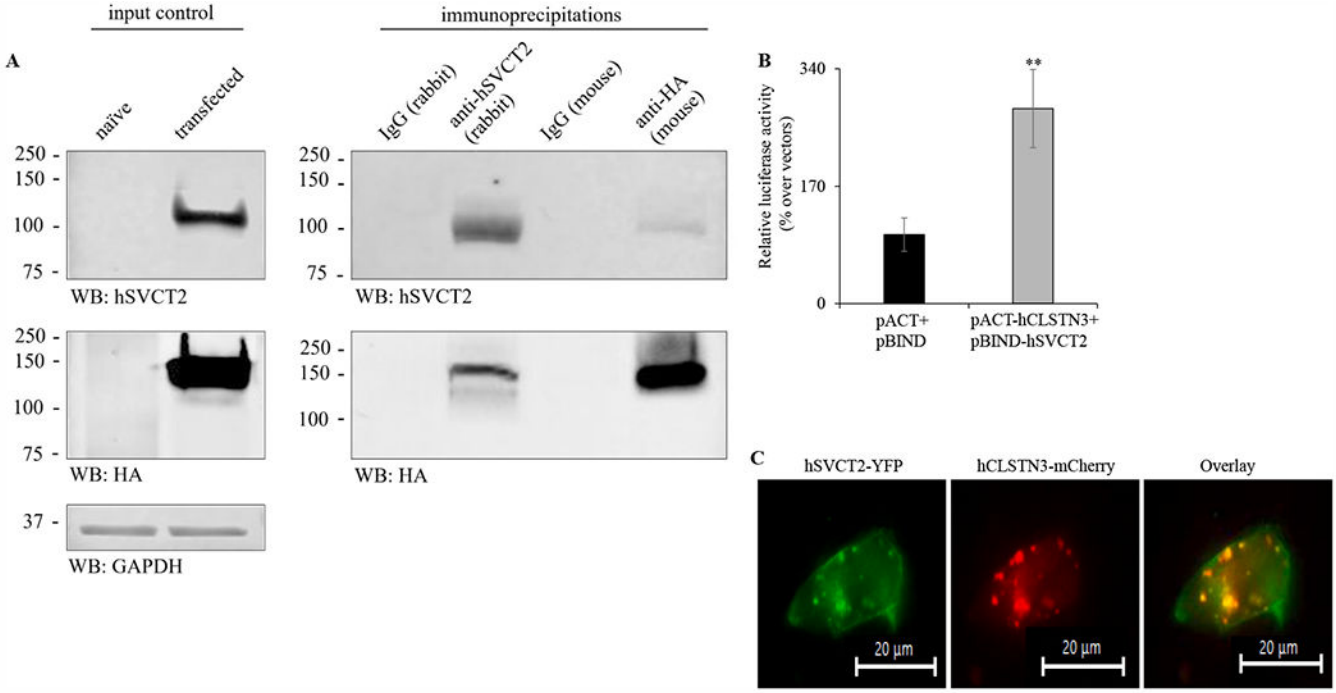

Fig. 2.

Conformational aspects of hCLSTN3 interaction with hSVCT2. (A) HEK-293 cells transiently expressing hSVCT2-YFP and hCLSTN3-HA were lysed and subsequently used for immunoprecipitation with anti-hSVCT2 and anti-HA antibodies as described in the "Methods". Western blotting with the anti-hSVCT2 and anti-HA antibodies was then used to analyze immunoprecipitates separated on Any kD™ Criterion™ TGX gels. Control (ctrl), immunocomplex obtained from isotyped control IgG immunoprecipitated lysates. (B) Plasmids (pBIND-hSVCT2 and pACT-hCLSTN3) were co-transfected into SH-SY5Y cells in the presence of pG5*luc* vector. The dual luciferase assay system was able to quantify the Renilla-normalized firefly luciferase activity in transfected SH-SY5Y cells after 48 h. Values are the mean ± SEM of at least 5 different experiments and firefly luciferase activity was presented in folds over the vectors set at 1. $^{**}P < 0.01$. (C) HEK-293 cells co-expressing hSVCT2-YFP and hCLSTN3-mCherry constructs following transient transfection. Live cell imaging was then done following 48 h of post-transfection. Fluorescence of YFP (left), mCherry (middle) and an overlay of images (right) are shown for cells expressing both constructs.

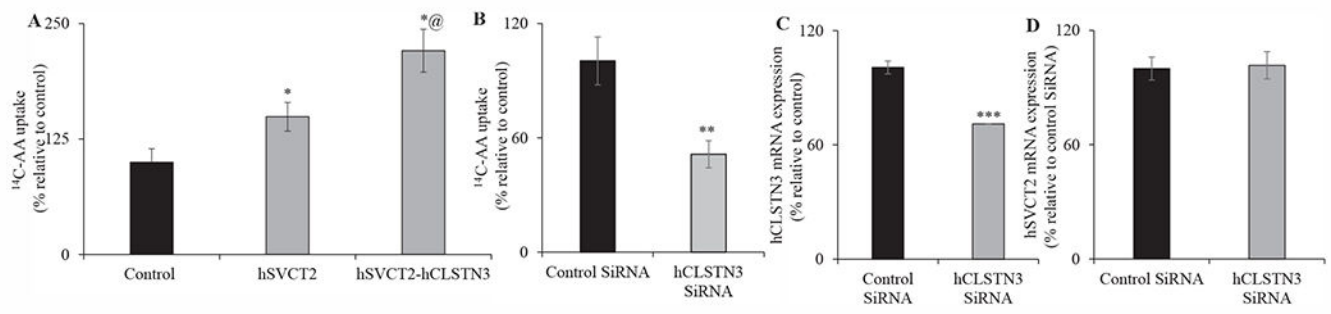

Fig. 3.

Functional effects of the hSVCT2 and hCLSTN3 protein-protein interaction. (A) SH-SY5Y cells were transiently transfected with hSVCT2-YFP alone or co-transfected with hSVCT2-YFP and hCLSTN3-HA and ^{14}C-AA uptake (0.1 μCi; pH 7.4; 30 min) was performed after 48 h of transfection. (B) The hCLSTN3 gene specific siRNA or control siRNA (scrambled) transfected SH-SY5Y cells were utilized for ^{14}C-AA uptake assay after 48 h of siRNA transfection. (C & D) RT-qPCR was done using hCLSTN3 as well as hSVCT2 gene-specific primers and cDNA reverse transcribed from RNA that was prepared from SH-SY5Y cells transfected with either the hCLSTN3 specific siRNA or the control siRNA (scrambled). Values are mean ± SEM of at least 3–5 independent uptake analyses with multiple batches of cells. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; $^{@}$significant compared to hSVCT2.

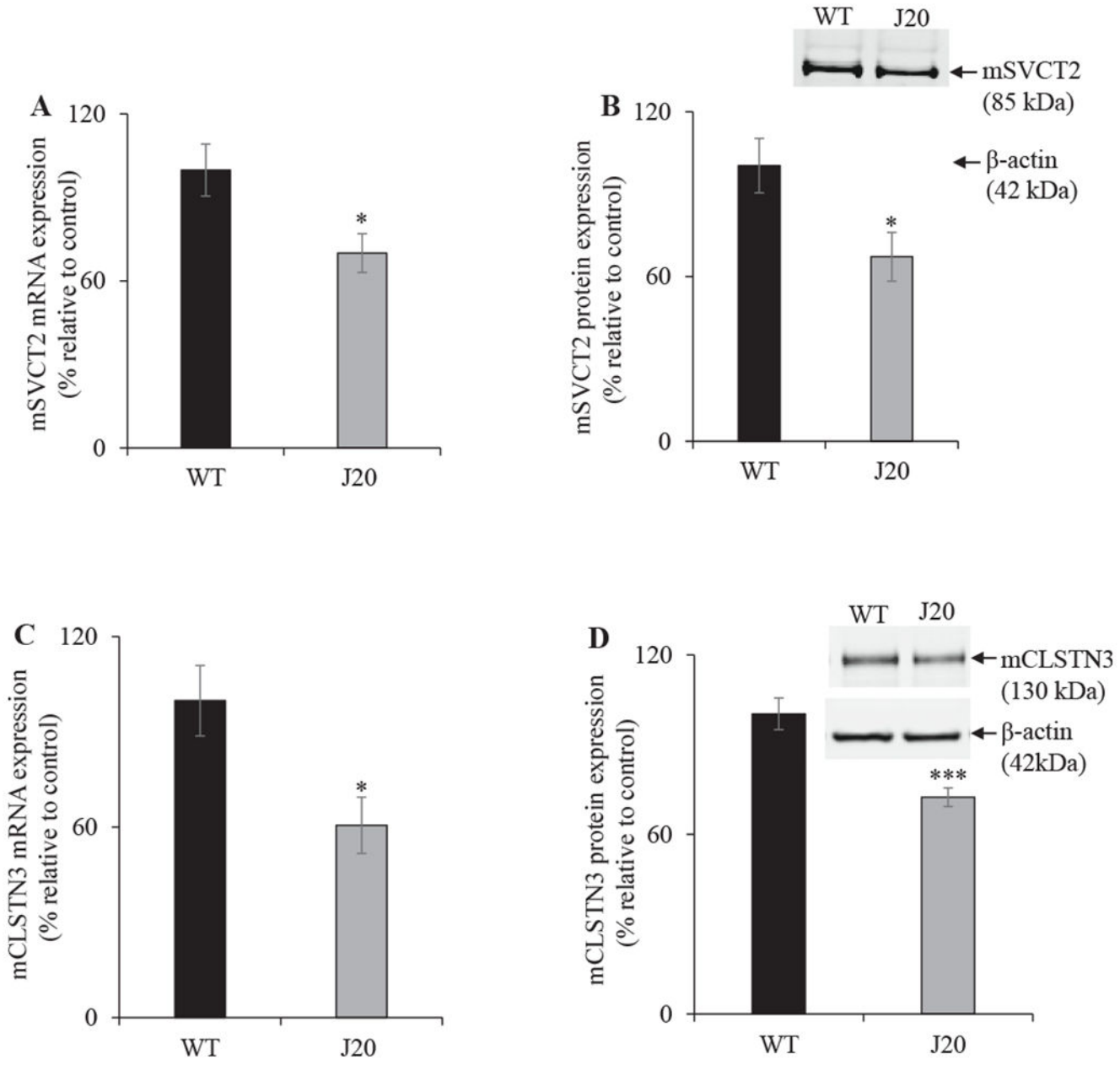

Fig. 4.

Effect of AD on the levels of expression of mSVCT2 and mCLSTN3 protein and mRNA in J20 and WT littermate mice hippocampus samples. cDNA from J20 and WT mice hippocampus were used for RT-qPCR analysis to determine the mRNA expression of mSVCT2 (A) and mCLSTN3 (C) relative to (β-actin. 40 μg of protein lysate from J20 and WT littermate mice hippocampus was used for Western blot to quantify the mSVCT2 (B) and mCLSTN3 (D) protein expression. Values are mean ± SEM of at least 5–7 sets of samples. ***P < 0.001; *P < 0.05.

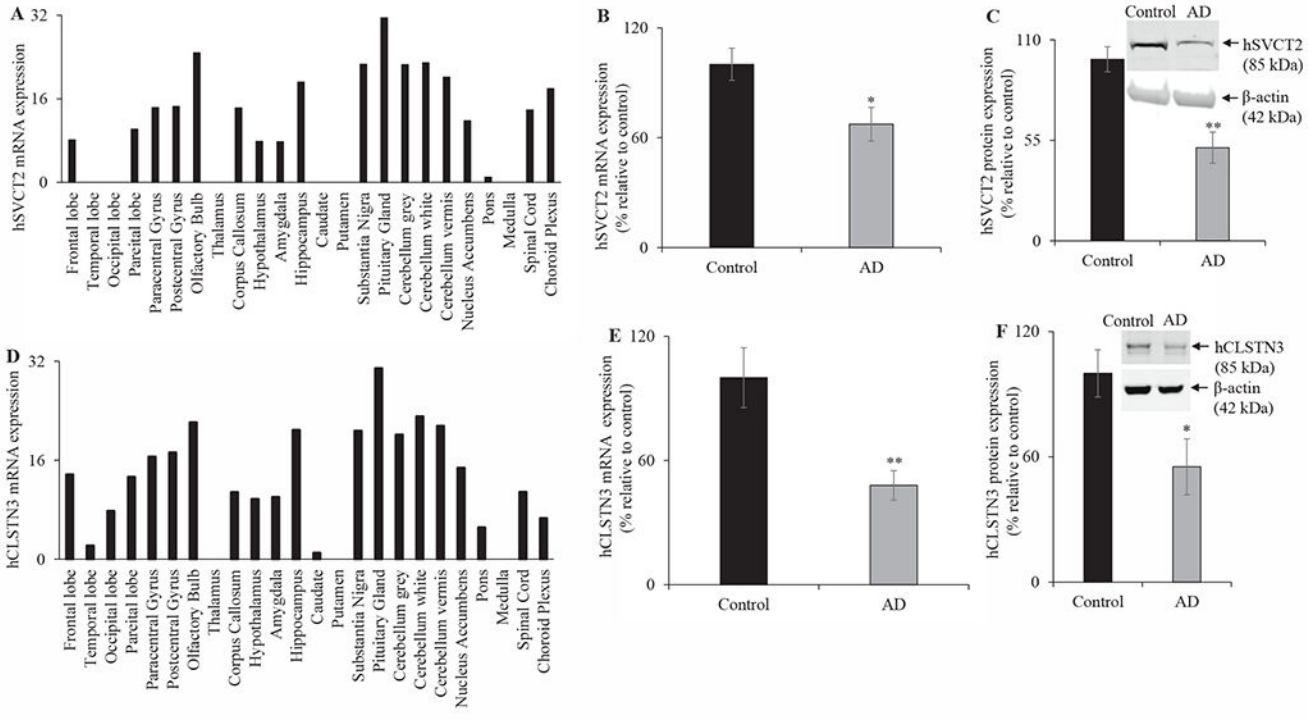

Fig. 5.

Effect of AD on expression level of hSVCT2 and hCLSTN3 protein and mRNA in human normal and AD hippocampus samples. cDNA from 24 different regions of the normal human brain were obtained from a commercial source (“Materials and methods”) and RT-qPCR was performed to quantify the mRNA expression of hSVCT2 (A) and hCLSTN3 (D) relative to β-actin. cDNA from human normal and AD patient samples were analyzed by RT-qPCR to quantify the mRNA expression of hSVCT2 (B) and hCLSTN3 (E) relative to β-actin. Protein isolated from human normal and AD patients hippocampus was used for Western blot to determine the hSVCT2 (C) and hCLSTN3 (F) protein expression levels. Values are mean ± SEM of at least 4–10 sets of samples. $^{**}P < 0.01$, $^{*}P < 0.05$.

Table 1

Combination of primers used to prepare constructs and RT-PCR analysis.

Gene name	Forward & reverse primers (5′-3′)
Mammalian two-hybrid assay	
hSVCT2	GCGTCGACTTATGATGGGTATTGGTAAGAAT; GC***TCTAGA***CTATCCCGTGGCCTGGGAGTC
hCLSTN3	GCGTCGACTTATGACCCTCCTGCTGCTG; GC***TCTAGA***TTAGTAGCGGTGTGGGG
Imaging studies	
hSVCT2	CCC**AAGCTT**ATGATGGGTATTGGTAAGAATAC; TCC*CCGCGG*TCCCGTGGCCTGGGAGT
hCLSTN3	CCC**AAGCTT**ATGACCCTCCTGCTGCTG; TCC*CCGCGG*GTAGCGGTGTGGGG
Real-time PCR studies	
hSVCT2	TCTTTGTGCTTGGATTTTCGAT; ACGTTCAACACTTGATCGATTC
hCLSTN3	CAACACGGTCCTACTGAATCC; CTCTCCTGTCGCCTTGTCA
hβ-actin	CATCCTGCGTCTGGACCT; TAATGTCACGCACGATTTCC
mSVCT2	AACGGCAGAGCTGTTGGA; GAAAATCGTCAGCATGGCAA
mCLSTN3	GGACAAGGCAACGGGTGAA; GCCACAGTCATAAGCCTGAATG
mβ-actin	ATCCTCTTCCTCCCTGGA; TTCATGGATGCCACAGGA