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SVCT2–mediated ascorbic acid uptake buffers stress responses via DNA hydroxymethylation reprogramming of S100 calcium-binding protein A4 gene

Qian-Qian Han [a,1], Peng-Fei Wu [a,b,c,d,1,**], Yi-Heng Li [a], Yu Cao [a], Jian-Guo Chen [a,b,c,d,***], Fang Wang [a,b,c,d,*]

[a] Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China
[b] The Key Laboratory of Neurological Diseases (HUST), Ministry of Education of China, Wuhan, 430030, China
[c] Laboratory of Neuropsychiatric Diseases, The Institute of Brain Research, Huazhong University of Science and Technology, Wuhan, 430030, China
[d] The Key Laboratory for Drug Target Researches and Pharmacodynamic Evaluation of Hubei Province, Wuhan, 430030, China

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ABSTRACT

Vitamin C, a key antioxidant in the central nervous system, cycles between ascorbic acid and dehydroascorbic acid under pathophysiological conditions. Clinical evidence supports that the absence of vitamin C may be linked to depressive symptoms, but much less is known about the mechanism. Herein, we show that chronic stress disrupts the expression of ascorbic acid transporter, sodium-dependent vitamin C transport 2, and induces a deficiency in endogenous ascorbic acid in the medial prefrontal cortex, leading to depressive-like behaviors by disturbing redox-dependent DNA methylation reprogramming. Attractively, ascorbic acid (100 mg/kg-1000 mg/kg, intraperitoneal injection, as bioequivalent of an intravenous drip dose of 0.48 g–4.8 g ascorbic acid per day in humans) produces rapid-acting antidepressant effects via triggering DNA demethylation catalyzed by ten-eleven translocation dioxygenases. In particular, the mechanistic studies by both transcriptome sequencing and methylation sequencing have shown that S100 calcium binding protein A4, a potentially protective factor against oxidative stress and brain injury, mediates the antidepressant activity of ascorbic acid via activating erb-b2 receptor tyrosine kinase 4 (ErbB4)-brain derived neurotrophic factor (BDNF) signaling pathway. Overall, our findings reveal a novel nutritional mechanism that couples stress to aberrant DNA methylation underlying depressive-like behaviors. Therefore, application of vitamin C may be a potential strategy for the treatment of depression.

1. Introduction

Major depressive disorder, a severe mental health disorder that affects 5%–20% of the general population [1], is predicted to be one of the three leading causes of burden of disease by 2030. Although antidepressants such as selective serotonin reuptake inhibitors are commonly used, there are obvious limitations in the clinical treatment. Approximately one-third of patients with major depressive disorder do not respond to current antidepressants and the delayed onset of drug action remains a significant unmet clinical need. In the past twenty years, emerging evidence has shown that specific micronutrients, such as vitamin D, vitamin B12 and folate, may be associated with mental disorders [2,3]. Vitamin C is a water-soluble micronutrient that is essential for humans with high safety profile. Case reports indicate that severe deficiency of vitamin C, including scurvy, is linked to psychiatric symptoms [4,5], including major depressive disorder, anorexia nervosa, weakness, and even suicidal attempts, and a case report has indicated

* Corresponding author. Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China.
** Corresponding author. Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China.
*** Corresponding author. Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China.
E-mail addresses: wupengfeipharm@foxmail.com (P.-F. Wu), chenj@mails.tjmu.edu.cn (J.-G. Chen), wangfangtj0322@163.com (F. Wang).
[1] Contributed equally.

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Abbreviations

AAV	adeno-associated virus
BDNF	brain derived neurotrophic factor
CRS	chronic restraint stress
CSDS	chronic social defeat stress
CUS	chronic unpredictable stress
Ddias	DNA damage induced apoptosis suppressor
ErbB4	erb-b2 receptor tyrosine kinase 4
FST	forced swim test
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLUT	glucose transporter
GSH	reduced glutathione
GSSH	oxidized glutathione
LV	lentivirus
mPFC	medial prefrontal cortex
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
SIT	social interaction test
SPT	sucrose preference test
SVCT	sodium-dependent vitamin C transporter
S100A4	S100 calcium binding protein A4
TET	ten-eleven translocation
TrkB	tropomyosin related kinase B
TST	tail suspension test

that supplementation with vitamin C ameliorates depressive symptoms in scurvy patients, which is caused by cancer diagnosis [6]. Notably, major depressive disorder is known to precede the physical symptoms of scurvy in certain patients [6,7]. However, until now, very little is known about the role and its mechanism of endogenous vitamin C in the pathophysiology of major depressive disorder.

Vitamin C has two biological forms: the reduced form, ascorbic acid, and the oxidized form, dehydroascorbic acid. The brain preferentially maintains a high level of ascorbic acid (2–10 mM) [8], even during a deficiency in peripheral tissues [9]. Ascorbic acid is a key antioxidant in the central nervous system [10], and its deficiency leads to the abnormalities of brain development [11]. In addition to its role as a cellular antioxidant, ascorbic acid works as a co-factor by maintaining the availability of Fe^{2+} via the reduction of Fe^{3+}, which enables the implementation of ten-eleven translocation (TET) enzyme-mediated oxidation of 5-methylcytosine to 5-hydroxymethylcytosine in DNA [12], followed by epigenome reprogramming of DNA methylation patterns. Furthermore, ascorbic acid affects the activity of TET proteins as a cofactor for their correct folding [13]. In the last decade, ascorbic acid has been demonstrated to play a pivotal role in remodeling the epigenome by promoting TETs-dependent DNA oxidation in embryonic stem cells [14,15]. Converging evidence from animal and human studies reveal that psychosocial stressors may facilitate the development of depression by driving epigenetic changes such as altered DNA methylation patterns [16,17]. Similarly, mounting evidence has revealed oxidative stress emerged as a key player in the pathogenesis of major depressive disorder [18,19]. Oxidative stress is likely to increase the extent of ascorbic acid oxidation and turnover following dehydroascorbic acid generation. Considering the possible role of TET enzymes in stress-induced responses [20,21], a disrupted ascorbic acid homeostasis may couple altered DNA methylation state with oxidative stress under depression.

In mammals, endogenous ascorbic acid homeostasis is tightly regulated by sodium-dependent vitamin C transporter (SVCTs), which comprise two isoforms SVCT1 and SVCT2. They are encoded by the solute carrier family 23 member 1 and 2 genes, SLC23A1 and SLC23A2, respectively, and actively cotransport sodium and ascorbic acid. SVCT1 is the predominant transporter of ascorbic acid in the intestine, while ascorbic acid is transported into the brain via SVCT2 [9]. Previous reports have indicated that oral administration of ascorbic acid (500 mg twice daily, 10 d) can improve mood in humans [22] and increase the antidepressant efficacy as an adjunct (1000 mg per day, 3–6 months) to fluoxetine [23]. Several research efforts have been devoted to the antidepressant-like effect of ascorbic acid in mice [24]. Pretreatment with ascorbic acid at a low dosage (1 mg/kg-10 mg/kg) prevents acute or chronic stress-induced depressive-like behaviors [25,26]. Chronic ascorbic acid administration (1 mg/kg, gavage, 21 d) produces an antidepressant-like effect in the mouse [27] and ameliorates behavioral despair induced by tumor necrosis factor [28]. Interestingly, some studies indicated that ascorbic acid may work as a candidate of ketamine-like drugs [29,30]. Oral administration of ascorbic acid reaches a maximum level of about 70 μmol/L in plasma, whereas intravenous administration may reach a maximum level around 50 mmol/L at doses higher than 70 g/m^2 [8]. Thus, treatment with ascorbic acid by oral or intravenous administration may be a potential therapeutic approach to overcome epigenetic dysregulation underlying diseases, especially cancer [31–33]. Although SVCT2 has profound impact on the vitamin C homeostasis in brain, until now, much less is known about its pathological role. Some reports have suggested that impaired SVCT2 function may contribute to the pathogenesis of Huntington's disease and peripheral nerve injury [34,35]. Here, we found that impaired SVCT2-dependent ascorbic acid homeostasis mediated depression-related DNA hypermethylation, and rewriting stress-associated DNA methylation signatures by ascorbic acid (100 mg/kg-1000 mg/kg intraperitoneal injection, bioequivalent of an intravenous drip dose of 0.48 g–4.8 g ascorbic acid per day in humans) produced a rapid-onset antidepressant effect.

2. Materials and methods

2.1. Agents

Ascorbic acid (A92902), phloretin (P7912), niclosamide (N3510), ANA-12 (SML0209), and AG1478 (T4182) were purchased from Sigma-Aldrich (St. Louis, USA). Other agents of the highest reagent grade were obtained from commercial suppliers. The artificial cerebrospinal fluid contained: 119 mM NaCl, 1.3 mM $MgSO_4$, 3.5 mM KCl, 11.0 mM glucose, 26.2 mM $NaHCO_3$, 1.0 mM NaH_2PO_4 and 2.5 mM $CaCl_2$, pH 7.4. Saline was used as vehicle for ascorbic acid. Phloretin, ANA-12 and AG1478 were each dissolved in 50% dimethyl sulfoxide (DMSO) diluted with artificial cerebrospinal fluid. Niclosamide was administered as a suspension in 10% Cremophor® EL (C107105, Aladdin, Shanghai, China) dissolved with saline.

2.2. Animals

Six to eight-weeks old male C57BL/6J mice (SJA laboratory, Hunan, China) were housed four per cage in environment of 22 °C ± 2 °C with a relative humidity of 40%–60% and 12 h light-dark cycle. Mice had ad libitum access to water and a normal rodent chow diet. Experiments involving animals were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Review Committee for the Use of Human or Animal Subjects of the Huazhong University of Science and Technology. All animal studies were reported in compliance with the ARRIVE guidelines. Animals were randomly assigned groups and tested by an individual blind to the genotype and experimental design. The experiments were performed during the light phase except some stressors in the chronic

unpredictable stress (CUS) procedure including: inversion of day/night light cycle, stroboscopic illumination.

2.3. Stress procedures

Three different stress procedures including CUS, chronic social defeat stress (CSDS) and chronic restraint stress (CRS) were used in our study. For CUS procedure, mice were single-housed in plastic cages, and others remained as controls were housed in groups of four or five. CUS procedure was induced as described previously with some slight modifications [36]. In brief, all but the controls were singly housed and subjected to the following stressful stimuli over 35 d: swimming in cold water (4 °C) for 6 min, food and water deprivation for 12 h, cage tilting (45°) for 12 h, stroboscopic illumination (flashing twice per sec with a stroboscope in dark) for 12 h, restrictions in a small tube (50 mL) for 3 h, water bedding for 12 h, freezing in a cylindrical container with ice for 3 h, bedding deprivation for 24 h, foot shock (10 s, 0.5 mA, 5 times, 30 s intervals) and reversed cycle of light-dark. Each animal was exposed to two stressors per day. To prevent the adaptions to stress, mice were not experienced the same stressor for 4 consecutive days. The stress procedure continued for 5 weeks prior to behavioral tests. Unstressed control mice were handled only for weighing, behavioral measurements or injections.

The CSDS procedure was performed similar with methods used in our previous studies [36,37]. Briefly, single housed CD-1 mice (4–5 months of age) were selected according to aggressive behavior. A single invading C57BL/6J mouse was exposed to a novel CD-1 aggressor for 5–10 min daily for 10 consecutive days. After each defeating, the C57BL/6J mice and CD-1 aggressor were separated using plastic divider with holes to allow visual, olfactory, and auditory contact for the next 24 h. The non-defeated control C57BL/6J mice were housed in cages under the same condition.

For CRS procedure, C57BL/6J mice were divided randomly into the control and CRS group after one week of adaptation to the housing conditions. The restraint stress was conducted as previously described with minor modifications [38]. To induce CRS, animals were singly housed and individually placed head-first into well-ventilated 50 mL cylindrical plastic tubes, which were then plugged with a 4.5 cm long middle tube to make animals almost immobile in it. Each experimental mouse was restrained 4–6 h per day without food or water for 14 consecutive days.

2.4. Behavioral tests

Brief descriptions of the behavioral experiments, including the sucrose preference test (SPT), social interaction test (SIT), forced swim test (FST, the temperature of water was 25 °C ± 1 °C), and tail suspension test (TST) were provided below. Open field test and elevated plus maze were performed according to our previous published reports [36–38]. Mice were placed into testing room 60 min prior to starting each behavioral test to adapt to the environment. Behavioral analysis was performed in a double-blind fashion.

SPT was performed as previously described [36]. Mice were individually housed and acclimatized to two identical bottles containing either tap water or 1% sucrose solution, respectively. The positions of two bottles were exchanged every 6 h to eliminate position preference. After habituated for 48 h, mice were deprived of water for 12 h and then exposed to two pre-weighed bottles containing either tap water or 1% sucrose solution. After 2 h, bottles were weighed and the sucrose preference was reported as the percentage calculated as: sucrose solution consumption/total liquid consumption × 100%. The total liquid consumption was calculated as sucrose solution consumption + water consumption.

SIT was conducted as previously described [37]. Briefly, the experimental mice were individually placed into an open field apparatus (45 × 45 cm) with a wire-mesh cage (10 × 6 cm) at one end. Movements were videotaped for 2.5 min in the absence (target absent) and presence of an unfamiliar CD-1 mouse in the cage (target present), respectively. Total time spent in the interaction zone was obtained using Anymaze software (Stoelting Co, Wood Dale, IL, USA). Social interaction ratio was calculated as time spent in an interaction zone at the "target present" phase/time spent in an interaction zone at the "target absent" phase. Mice with interaction ratio <1 were considered as susceptible and interaction ratio ≥1 were considered as resilient to CSDS.

FST was performed as previously published methods [36]. Briefly, mice were individually placed in a clear cylinder (20 cm in diameter, 30 cm in height) containing fresh water (25 °C ± 1 °C, 20 cm depth). A 6 min swim test session was videotaped under bright light and tracked by an Anymaze software (Stoelting Co, Wood Dale, IL, USA). Immobility time was defined as the time spent by the mice floating in the water without struggling or only slightly moving to keep the head above the water. Immobility time was scored during the last 4 min by an investigator unaware of animal grouping.

TST was conducted as previously described [36]. Mice were suspended with tape, fixed on a metal hook (about 2 cm away from tip) and suspended 20 cm above the floor for 6 min. Then, we recorded the duration time of immobility of each mouse with Anymaze software (Stoelting Co, Wood Dale, IL, USA). Immobility time was defined as the time spent by the mice exhibited no body movement and was manually evaluated by an experimenter blinded to testing conditions.

2.5. Ascorbic acid administration

Mice were administrated with ascorbic acid (10, 100, or 1000 mg/kg) by a single intraperitoneal injection. Twenty-four hours after drug administration, animals were used for behavioral studies or biochemical assays. For the behavioral studies, separated mice were used for each time point and measured at different time points.

2.6. Tissue collection

After sacrifice, the coronal slices (300–350 μm) were cut by vibratome (Leica VT 1000S, Wetzlar, Germany) in the ice-cold artificial cerebrospinal fluid, which was oxygenated with 95% O_2 + 5% CO_2. Sections containing hippocampus, medial prefrontal cortex (mPFC), nucleus accumbens and basolateral amygdala were collected and dissected on ice using a dissecting microscope according to the histology atlas of Paxinos and Franklin (2001, The Mouse Brain in Stereotaxic Coordinates 2nd edn, San Diego, CA: Academic). Brain tissue were immediately frozen on dry ice and stored at −80 °C.

2.7. Measurement of oxidative stress parameters

The level of oxidative stress was evaluated in the mPFC and nucleus accumbens of mice subjected to CUS by measurement of reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio using GSH and GSSG assay kit (S0053, Beyotime Biotechnology, Haimen, China) and malondialdehyde using malondialdehyde assay kit (A003-1, Nanjing Jiancheng Bioengineering institute, Nanjing, China) according to the manufacturer's instructions [39]. Amplex Red method was used to measure the concentration of H_2O_2. Supernatants from brain tissue were reacted with horseradish peroxidase (31,490, Thermo Fisher Scientific, Massachusetts, USA) in the presence of Amplex Red (50 μM, A12222, Invitrogen, Carlsbad, USA) to get resorufin in tris-(hydroxymethyl)-aminomethane buffer (pH 8.5, 100 mM). Fluorescence of resorufin (Ex/Em = 530/590) was detected with a SpectraMax Gemini XS fluorimeter (Molecular Devices, California, USA).

2.8. Ascorbic acid and iron assay

Ascorbic acid contents were detected in different brain subregions of mice subjected to CUS and in the mPFC of mice at 24 h after ascorbic

acid administration (ascorbic acid assay kit, MAK075, Sigma-Aldrich, St. Louis, USA) [40]. In this assay, wet tissues were weighed and rapidly homogenized in cold ferric reducing/antioxidant and ascorbic acid buffer in the darkness. After 20 min, lysates were centrifuged at 13,000 g for 10 min at 4 °C, and supernatants were collected into a new tube. To remove protein, supernatants were transferred to 10 kDa molecular weight cut off spin filters (UFC8010, Merck Millipore, Darmstadt, Germany) for centrifuging at 7500 g for 10 min at 25 °C. Fluids were collected to assay the content of ascorbic acid immediately. Antioxidants in the fluids reduced Fe^{3+} into Fe^{2+}, which resulted in a colorimetric (593 nm) reaction product in ferric reducing/antioxidant and ascorbic acid buffer. The parallel sample that added ascorbic acid oxidase was used as a background for the measurement of ascorbic acid. Absorbance of the reaction product was detected with the microplate reader (Infinite® 200 Pro, Tecan, Hombrechtikon, Switzerland).

The intracellular Fe^{2+}, Fe^{3+} and total iron levels were examined using iron assay kit (ab83366, abcam, Cambridge, UK) according to the manufacturer's instructions [41]. Briefly, brain tissues were weighed, washed with cold phosphate-buffered saline, and homogenized in iron assay buffer. After centrifuging at 16,000 g for 10 min at 4 °C, supernatants were collected to react with iron buffer or iron reducer in iron assay buffer for testing Fe^{2+} or total iron concentration, respectively. Then, the iron probe was added intoeach sample before mixing, and then incubated for 1 h. Absorbance of the reaction product (optical density = 593 nm) was immediately measured on a microplate reader (Infinite® 200 Pro, Tecan, Hombrechtikon, Switzerland).

2.9. Western blotting

As we have reported [38], frozen tissue sample were homogenized in radio-immunoprecipitation assay lysis buffer (P0013C, Beyotime Biotechnology, Haimen, China) containing protease and phosphatase inhibitors (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfonate, protease inhibitor mixture, pH 7.4) on ice. After 30 min, the lysates were centrifuged at 12, 000 g for 20 min at 4 °C, and the supernatants were harvested and quantified using Coomassie blue protein-binding assay (P0017F, Beyotime Biotechnology, Haimen, China). The proteins were incubated for 5 min at 95 °C with 3 vol of loading buffer (P0015, Beyotime Biotechnology, Haimen, China). After denaturation, 30 μg of protein samples were separated by 8/10/12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to the nitrocellulose membranes (HATF00010, Merck Millipore, Billerica, USA). For immunodetection, the membranes were blocked with Tris-buffered saline +0.1% Tween 20 containing 5% bovine serum albumin (10711454001, Merck Millipore, Billerica, USA; 2 h, room temperature), and incubated overnight at 4 °C with diluted primary antibodies. The following primary antibodies were used: β-actin (1:2000; sc-47778, Santa Cruz Biotechnology, California, USA); Glucose transporter 1 (GLUT1, 1:1000, PA1-46152, Thermo Fisher Scientific, Massachusetts, USA); Glucose transporter 3 (GLUT3, 1:1000, ab191071, abcam, Cambridge, UK); Glucose transporter 4 (GLUT4, 1:1000, NBP1–49533SS, Novus, Colorado, USA); SVCT1 (1:1000, CSB-PA887038LA01HU, CUSABIO, Houston, USA); SVCT2 (1:1000, A6740, ABclonal, Wuhan, China); TET1 (1:500, 09–872, Merck Millipore, Billerica, USA), TET2 (1:250, ab124297, abcam, Cambridge, UK); S100 calcium binding protein A4 (S100A4, 1:1000, ab197896, abcam, Cambridge, UK); Brain derived neurotrophic factor (BDNF, 1:1000, ab108319, abcam, Cambridge, UK). After washes in tris-buffered saline with tween 20, the bands were incubated with secondary antibody (1:10,000, LI-COR, Lincoln, USA) for 1 h at room temperature. The antibody-reactive bands were visualized using the Odyssey® Infrared Imaging system (LI-COR, Lincoln, USA) and quantified using ImageJ (NIH, Bethesda, USA) normalized to β-actin. Protein relative levels were shown as fold change relative to control group.

2.10. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA of brain tissue was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). RNA quantity was quantified using the ultrafine ultraviolet spectrophotometer (NanoUV-3000, Thermo Fisher Scientific, Massachusetts, USA). Reverse transcription from 1 μg of RNA was performed in a volume of 20 μL using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (6210A, Takara Bio Inc, Kusatsu, Japan). qRT-PCR was conducted using TB Green Premix Ex Tag (RR820A, Takara Bio Inc, Kusatsu, Japan) and measured by the CFX 96 Real-Time PCR System Detector (Bio-Rad, California, USA) with 0.5 μL of template and 0.3 μM of primer. The parameters for qRT-PCR were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as the internal control. The primer sequences used in the qRT-PCR as follows (5′–3′): *Tet1*-forward: AACAAGAGGCCCCAGAG; *Tet1*-reverse: TTCTTCCCCATGACCAC; *Tet2*-forward: CTCCTGGTGAACAAAGTCA GAATGG; *Tet2*-reverse: CTAATAGCTGCCAGATCAGGACC; *Tet3*-forward: CCGGATTGAGAAGGTCATCTAC; *Tet3*-reverse: AAGATAA-CAATCACGGCGTTCT; *S100A4*-forward: AGGAGCTACTGACCAGGGAG; *S100A4*-reverse: ATGGCAATGCAGGACAGGAA; *DNA damage induced apoptosis suppressor (Ddias)*-forward: GTTGCAGGCTTCCACCAAAG; *Ddias*-reverse: TTTCAAGGCTCTCAGCAGCA; *GAPDH*-forward: ATGGT-GAAGGTCGGTGTG; *GAPDH*-reverse: CATTCTCGGCCTTGACTG. Gene expression was calculated by using $2^{-\Delta\Delta CT}$ method and presented as the fold change relative to control.

2.11. DNA extraction and dot blot assay

Genomic DNA was extracted from tissues with the Genomic DNA Extraction Kit (9765, Takara Bio Inc, Kusatsu, Japan) according to the manufacturer's instructions. Dot blot were conducted using the published methods [42]. A proper amount of DNA was heated at 95 °C for 10 min and placed on the ice immediately. Denatured DNA was serially diluted in 2 × Saline Sodium Citrate buffer and then spotted on nitrocellulose membrane (BS-NC-22, Biosharp, Hefei, China) with the Bio-Dot Apparatus (Bio-Rad, California, USA). The membrane was dried at 80 °C for 2 h, and blocked with 5% bovine serum albumin in tris-buffered saline with tween 20 for 1 h at room temperature. Afterwards, blots were incubated with a primary antibody against 5-hydroxymethylcytosine (1:10,000, 39,769, Active motif, California, USA) and 5-methylcytosine (1:1000, 61,479, Active motif, California, USA) overnight at 4 °C. Finally, immunoblots were visualized by MicroChemi (DNR Bio-Imaging Systems, Jerusalem, Israel) with an ECL kit (32,106, Thermo Fisher Scientific, Massachusetts, USA) after washing three times with tris-buffered saline with tween 20. The same blot was stained in 0.02% methylene blue liquid to ensure equal loading.

2.12. Stereotaxic surgery and cannula infusion

For microinjection of drugs into target brain regions, mice were anesthetized with pentobarbital sodium (45 mg/kg, intraperitoneal injection) and fixed to the stereotaxic apparatus (RWD Life Science Co., Shenzhen, Guangdong Province, China). The guide cannulas (RWD Life Science Co., Shenzhen, Guangdong, China) were inserted bilaterally into the mPFC region (AP = −2.0 mm anteroposterior; ± 0.4 mm mediolateral; −2.0 mm dorsoventral relative to Bregma). Dental cement was used on the skull to affix guide cannulas. Animals were placed on a heating pad until recovery from anesthesia. Drugs, including ascorbic acid (0.1 mM, 1 μL per side), phloretin (200 μM, 1 μL per side),

niclosamide (S100A4 transcription inhibitor, 4 μM, 1 μL per side), ANA-12 (tropomyosin related kinase B (TrkB) antagonist, 2.45 mM, 1 μL per side) and AG1478 (erb-b2 receptor tyrosine kinase 4 (ErbB4) blocker, 50 μM, 1 μL per side) were injected into mPFC region at the speed of 0.1 μL/min with an internal cannula one week after recovery.

2.13. Genetic approaches

Lentivirus (LV) containing short hairpin RNA (shRNA) was acquired from Genechem Co., Ltd (Shanghai, China). Cytomegalovirus was chosen to be the vector based on its rapid expression. To knockdown TET1 and TET2, mice were infected with LV expressing U6-MCS-Ubi-enhanced green fluorescent protein (EGFP) shRNA specific for TET1 and TET2. The sequence targeted by LV-*TET1* shRNA was GCAGATGGCCGTGACACAAAT, the sequence targeted by LV-*TET2* shRNA was GAGCGTTCCTCAGTATCATTT. LV-CON (TTCTCCGAACGTGTCACGT) was used as control vector to eliminate the impact of virus alone. The final titers of LV-*TET1* shRNA and LV-*TET2* shRNA were 3.0×10^8 TU·ml^{-1} and 5.0×10^8 TU·ml^{-1}, respectively. For *in vivo* lentiviral microinjections, lentiviral vector (2 μL) was bilaterally targeted to the mPFC region (+2.0 mm anteroposterior; ± 0.4 mm mediolateral; − 2.0 mm dorsoventral relative to Bregma) at a rate of 0.1 μL min^{-1} followed by an additional 10 min to allow optimal virus diffusion. Brain tissues were collected for determination of gene expression 14 d after virus injection.

To overexpress S100A4, adeno-associated virus (AAV) 2/9 carrying S100A4 and Flag (CMV-bGlobin-MCS-EFGP-3FLAG-WPRE-hGH-polyA; AAV-S100A4, titer: 1.38×10^{12} VG·ml^{-1}) or only Flag (AAV-GFP, titer: 1.02×10^{12} VG·ml^{-1}) were constructed by AAV Helper-Free System. The microsyringe was utilized to infuse 0.8 μL of virus at a flow rate of 0.08 μL min^{-1} followed by an additional 10 min rest period to prevent backflow. The efficacy of AAV-S100A4 was verified at least 4 weeks following surgeries. Viruses used in the experiments were obtained from Genechem Co., Ltd (Shanghai, China), kept at −80 °C, and thawed immediately before injection.

2.14. Immunofluorescence

Immunofluorescence was conducted as described previously [38]. To determine the localization of SVCT2 in neuron or microglia, male C57BL/6J mice (8 weeks old) were intracardially perfused with physiological saline followed by 4% paraformaldehyde dissolved in phosphate-buffered saline. Brain was then post-fixed in 4% paraformaldehyde for 24 h at 4 °C before transferring into a 15%–30% sucrose gradient for 2 d. Brain was rapidly frozen and coronally sectioned at a thickness of 30 μm using a freezing microtome (Leica Microsystems, Wetzlar, Germany). Sections containing mPFC region were incubated in blocking solution (phosphate-buffered saline supplemented with 0.3% TritonX-100 and 5% bovine serum albumin) for 1 h at room temperature. After washing in phosphate-buffered saline, brain slices were incubated with primary antibody overnight at 4 °C. The primary antibodies used as follows: anti-Neuron (anti-NeuN, 1:500, MAB377, Merck Millipore, Billerica, MA); anti-ionized calcium binding adapter molecule 1 (anti-Iba1, 1:200, ab5076, abcam, Cambridge, UK); anti-SVCT2 (1:200, A6740, ABclonal, Wuhan, China).

To detect the localization of SVCT2 with astrocyte, AAV containing GfaABC$_1$D promoter and enhanced green fluorescent protein (rAAV-GfaABC1D-EGFP-SV40 polyA, 0.8 μL, titer: 1.27×10^{12} VG·ml^{-1}) was microinjected into the mPFC of male C57BL/6J mice (7 weeks old). Virus was purchased from BrainVTA Co., Ltd (Wuhan, China). Mice were intracardially perfused 28 d following viral injection (11 weeks old). Brain was removed, post-fixed, gradient dehydrated and sliced. Sections containing mPFC region were blocked with blocking solution and incubated with anti-SVCT2 (1:200, A6740, ABclonal, Wuhan, China) overnight at 4 °C and reacted with Alexa dye-tagged secondary antibodies (Invitrogen, Carlsbad, USA) for 2 h at room temperature on the next day. Signals of immunofluorescence were visualized using a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan).

2.15. Confocal image of dendritic spines

Dendritic spines were quantified according to previous reports with some modifications [36]. Mice were microinjected into the mPFC with LV-CON (TTCTCCGAACGTGTCACGT) to visualize the neurons, and then subjected to different treatments after two weeks. At 24 h after systemic injection of ascorbic acid, mice were deep anesthetized with sodium pentobarbital (45 mg/kg, intraperitoneal injection) and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in phosphate-buffered saline (0.1 M, pH 7.4). Brains were post-fixed in 4% paraformaldehyde overnight and dehydrated using graded sucrose solution. Serial coronal slices (50 μm thick) containing the mPFC were acquired using a freezing microtome (Leica Microsystems, Wetzlar, Germany). For analysis of dendritic spines, sections were loaded on slides and then the confocal Z-stack images were observed in a confocal microscope (FV1000, Olympus, Tokyo, Japan) using a 100× oil-immersion objective lens. The images were semiautomatically traced via the Imaris software (Bitplane, Zurich, Switzerland). The lengths and branching patterns in dendrites of neurons were quantified, and the results were shown as density of spines per μm relative to control.

2.16. RNA-sequencing and differentially expressed genes analysis

Mice were killed 24 h after intraperitoneal injection of ascorbic acid. Brains were removed, coronally sliced and mPFC tissue was rapidly dissected and frozen on dry ice. The frozen mPFC tissue from mice with different treatments were sent to OE Biotech Co., Ltd (Shanghai, China) [43] for RNA-sequencing analysis. Total RNA was obtained from mPFC using the mirVana miRNA Isolation Kit (AM1561, Thermo Fisher Scientific, Massachusetts, USA) and RNA integrity was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Cruz Biotechnology, California, USA) with RNA integrity number >8.0. In all, cDNA reverse-transcribed from 50 ng RNA was used for library construction using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, USA). One hundred fifty bp of cDNA fragments were sequenced from both ends by HiSeq TM 2500 (Illumina, San Diego, USA). All differentially expressed genes were generated using the DESeq estimating function and nbinomTest. At least 2.0-fold change and $p < 0.05$ was identified as significant difference among the comparisons across groups. Gene ontology analyses were performed by database for annotation, visualization and integrated discovery bioinformatics platform with highest stringency settings. Gene expression patterns were analyzed by hierarchical cluster analysis.

2.17. DNA methylation analysis by pyrosequencing

Genomic DNA was isolated with All Prep DNA/RNA Mini Kit (80,004, Qiagen, Dusseldorf, Germany) from mPFC tissue according to manufacturer's protocol. DNA was bisulfite converted using EpiTect Bisulfite Kit (59,104, Qiagen, Dusseldorf, Germany). Primer target CpG sites within the promoter and intron region of S100A4 were designed in PyroMark Assay Design 2.0 (Qiagen, Dusseldorf, Germany), and the region of interest was amplified using PyroMark® PCR Kit (978,703, Qiagen, Dusseldorf, Germany). The primers used in PCR were presented in Table S1. PCR were performed in an iQ5 real-time PCR system (Bio-Rad, California, USA) using the following cycling conditions: 95 °C for 3 min, 40 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, 72 °C for 7 min and 4 °C for terminating. The products of PCR were sequenced on the PyroMark Q96 ID machine (Qiagen, Dusseldorf, Germany) using PyroMark Q96 Advanced Reagents (974,022, Qiagen, Dusseldorf, Germany) according to manufacturer's instructions. The methylation status in each sample were quantified using the Pyro Q-CpG software (Qiagen, Dusseldorf, Germany). Pyrosequencing was conducted by Feiming

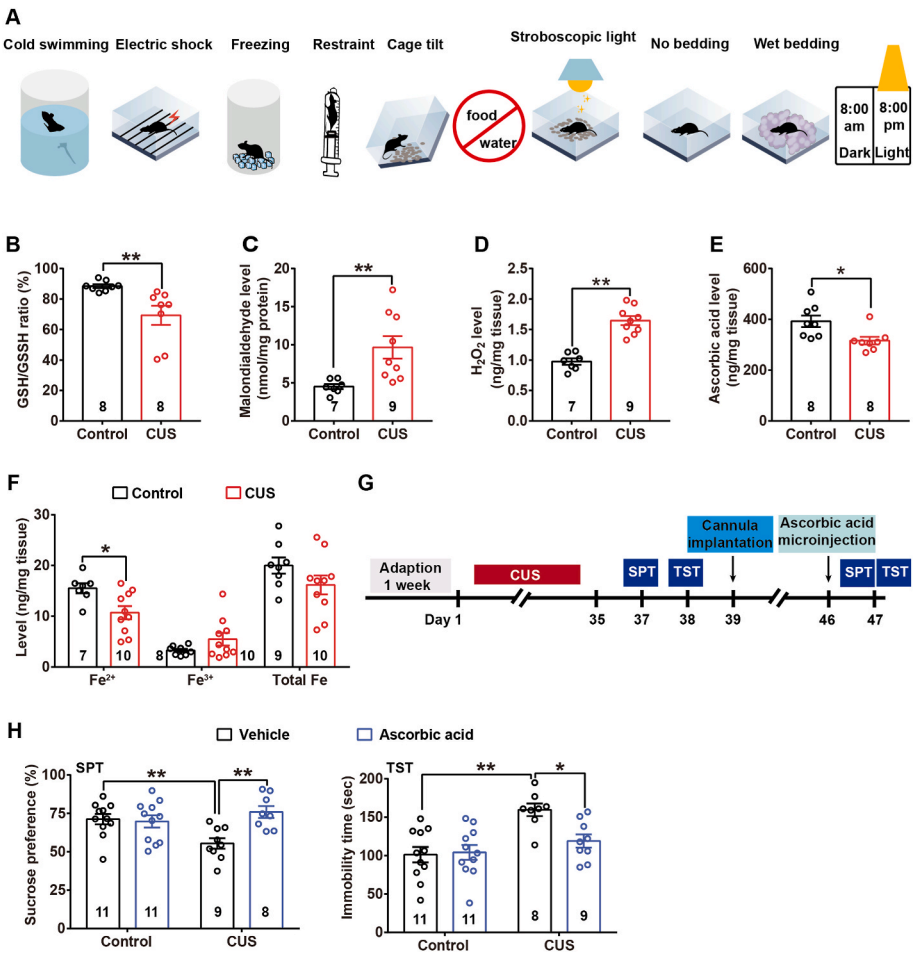

Fig. 1. Impaired ascorbic acid homeostasis in the mPFC contributes to chronic stress-induced depressive-like behaviors of mice. (A) Schematic of stressors utilized in the CUS procedure. **(B–D)** The level of GSH/GSSG + GSH ratio, malondialdehyde and H_2O_2 in the mPFC of control and CUS-treated mice. **(E)** The level of ascorbic acid in the mPFC of control and CUS-treated mice. **(F)** The level of Fe^{2+}, Fe^{3+} and total iron in the mPFC of control and CUS-treated mice. **(G)** Experiment timeline of CUS, stereotaxic surgery, cannula infusion and behavioral tests. **(H)** Results of SPT and immobility time in the TST 24 h after infusion of ascorbic acid (0.1 mM, 1 μL per side) into the mPFC. All data were represented as mean ± SEM and analyzed by Student's t-test (B–F) or two-way ANOVA (H) with Bonferroni's post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001.

Biotech Co., Ltd (Wuhan, China).

2.18. Statistics

Data analyses were carried out using GraphPad Prism 7 (GraphPad Prism, California, USA). For all experiments, difference between two groups was performed using Student's t-test, and difference among three or more groups was performed using one-way or two-way ANOVA followed by Bonferroni's test for the post-hoc analysis. Details on statistical tests used and statistical significance are included in the relevant figure legend. Sample sizes were estimated according to our previous experience performing similar experiments and justified by the power analyses. Mice used for studies were randomly arranged into groups. All data are shown mean ± SEM and statistical significance is identified at $p < 0.05$. The details for all statistical tests are showed in Table S2.

3. Results

3.1. CUS increases oxidative stress and disturbs ascorbic acid homeostasis in mPFC

CUS paradigm is a widely used animal model of major depressive disorder. Following exposure to different stressors for 35 d (Fig. 1A), CUS-treated mice displayed decreased sucrose preference ($t = 4.81$, $p < 0.001$; Fig. S1A). In both TST ($t = 4.44$, $p < 0.001$; Fig. S1B) and FST ($t = 4.80$, $p < 0.001$; Fig. S1C), an increase in immobility time was observed. We found that the levels of oxidative stress were significantly higher in CUS-treated mice, as indicated by depletion of the GSH ($t = 3.01$, $p = 0.009$; Fig. 1B), an elevation in the levels of malondialdehyde ($t = 3.00$, $p = 0.010$; Fig. 1C) and H_2O_2 ($t = 6.97$, $p < 0.001$; Fig. 1D) in the mPFC and nucleus accumbens (GSH/GSSG: $t = 2.70$, $p = 0.017$; malondialdehyde: $t = 2.55$, $p = 0.024$; H_2O_2: $t = 2.52$, $p = 0.025$; Fig. S1D) of CUS-treated mice compared with that of control.

Considering that the oxidation of ascorbic acid and its redox homeostasis are closely associated with a decrease in GSH concentrations, the level of ascorbic acid was measured in CUS-treated mice. It was shown that the level of ascorbic acid was significantly decreased in the mPFC ($t = 2.80$, $p = 0.014$; Fig. 1E), but not in the hippocampus, nucleus accumbens or basolateral amygdala of CUS-treated mice (hippocampus: $t = 0.21$, $p = 0.836$; nucleus accumbens: $t = 1.30$, $p = 0.210$; basolateral amygdala: $t = 0.54$, $p = 0.600$; Fig. S1E). Iron is essential as a redox metal in several cellular functions, such as TETs-dependent DNA oxidation. The availability of Fe^{2+} via the reduction of free transition metal ions by ascorbic acid controls TETs activity [12]. We found that the level of Fe^{2+} was decreased, but Fe^{3+} and total iron remained unchanged in the mPFC after CUS (Fe^{2+}: $t = 2.72$, $p = 0.016$; Fe^{3+}: $t = 1.56$, $p = 0.138$; total iron: $t = 1.51$, $p = 0.152$; Fig. 1F), indicating a disturbed iron redox homeostasis may occur. Then, ascorbic acid (0.1 mM, 1 μL per side) was bilaterally infused into the mPFC to restore the physiological levels of ascorbic acid in CUS-treated mice (Fig. 1G). We found that local administration of ascorbic acid in the mPFC increased sucrose preference and decreased immobility time in the TST in CUS-treated mice (SPT: $F_{(1, 35)} = 8.51$, $p = 0.006$; TST: $F_{(1, 35)} = 5.23$, $p = 0.028$; Fig. 1H). Together, these results indicate that chronic stress increases oxidative stress levels and disrupts ascorbic acid homeostasis in the mPFC.

3.2. Deficits in SVCT2-dependent ascorbic acid uptake in the mPFC mediates depression-like behaviors

Growing evidence suggests that intracellular ascorbic acid levels are maintained by SVCTs and GLUTs. Immunofluorescence analyses showed that SVCT2, the main transporter maintaining ascorbic acid level in the adult brain, was expressed in the neuron, astrocyte and microglia of mPFC (Fig. 2A). To identify the relevance of chronic stress and vitamin C transporters, we analyzed the protein expression in the mPFC of CUS-treated mice by Western blot, and found that CUS significantly decreased the expression of SVCT2, but not SVCT1 in the mPFC (SVCT1: $t = 1.14$, $p = 0.274$; SVCT2: $t = 2.17$, $p = 0.044$, Fig. 2B), which was expressed more highly in intestine (Fig. S2A). No changes in GLUT1, GLUT 3 and GLUT 4 protein level were observed in the mPFC of CUS-treated mice (GLUT 1: $t = 0.14$, $p = 0.894$; GLUT 3: $t = 0.05$, $p = 0.963$; GLUT 4: $t = 0.16$, $p = 0.874$; Fig. 2C). And SVCT2 protein expression was also decreased in the mPFC of CSDS and CRS-treated mice (CSDS: $t = 2.92$, $p = 0.009$; CRS: $t = 2.31$, $p = 0.038$; Fig. 2D). Furthermore, the expression of SVCT2 in the hippocampus remained unchanged ($t = 0.47$, $p = 0.644$; Fig. S2B), whereas its level was elevated in the nucleus accumbens ($t = 2.65$, $p = 0.020$; Fig. S2C) and basolateral amygdala of CUS-treated mice ($t = 2.09$, $p = 0.049$; Fig. S2D). Taken together, the deficits in SVCT2 expression may confer a mechanism underlying CUS-induced ascorbic acid deficiency in the mPFC.

Next, we asked whether inhibition of SVCT2-mediated ascorbic acid uptake in the mPFC was sufficient to induce depression-like behaviors. Phloretin, a nonselective inhibitor that disrupts various vitamin C transporters, such as SVCT2, SVCT1, and glucose transporters [44,45], was used to mimic the deficiency of ascorbic acid in the mPFC. Phloretin (200 μM, 1 μL/per side) was infused into the mPFC for 7 d (Fig. 2E) and a lower ascorbic acid level were observed ($t = 2.66$, $p = 0.017$; Fig. 2F). It was shown that local administration of phloretin into the mPFC was sufficient to induce depressive-like behaviors, such as a decreased sucrose preference and an increased immobility time of mice in the FST (SPT: $t = 2.61$, $p = 0.017$; FST: $t = 2.82$, $p = 0.011$; Fig. 2G). These results indicate that the deficits in SVCT2-dependent ascorbic acid uptake in the mPFC may confer to the pathophysiology of depression.

3.3. Ascorbic acid upregulates SVCT2 expression and produces rapid antidepressant actions in the mice

Previous reports have indicated that a high level of exogenous ascorbic acid may restore SVCT2 expression in a positive feedback-dependent manner [46]. To demonstrate the effect of ascorbic acid on SVCT2 expression, SVCT2 protein levels were measured in the mPFC of mice that received intraperitoneal injection of ascorbic acid ranging from 10 mg/kg to 1000 mg/kg. We found that both the protein expression of SVCT2 (10 mg/kg: $t = 1.76$, $p = 0.100$; 100 mg/kg: $t = 2.28$, $p = 0.037$; 1000 mg/kg: $t = 2.86$, $p = 0.011$; Fig. 3A) and the level of ascorbic acid ($F_{(3, 24)} = 7.65$, $p < 0.001$; Fig. 3B) were significantly increased in the mPFC of mice at 24 h after treatment with ascorbic acid (100 mg/kg and 1000 mg/kg). Next, the effects of ascorbic acid on behavioral despair were examined in the TST and FST (Fig. 3C). It was found that ascorbic acid at dosages of 100 mg/kg, 200 mg/kg and 1000 mg/kg produced an antidepressant action in the TST and FST at 24 h after administration (TST, $F_{(3, 35)} = 7.94$, $p < 0.001$; FST, $F_{(3, 34)} = 6.38$, $p = 0.002$; Fig. 3D and TST: $t = 2.84$, $p = 0.011$; FST: $t = 2.75$, $p = 0.013$; Fig. S3A), without affecting immobility time in FST at 0.5 h, 1 h, 3 h, 6 h, and 12 h after administration with a dosages of 100 mg/kg (0.5 h: $t = 0.92$, $p = 0.368$; 1 h: $t = 0.48$, $p = 0.231$; 3 h: $t = 0.45$, $p = 0.655$; 6 h: $t = 1.74$, $p = 0.095$; 12 h: $t = 0.98$, $p = 0.336$; Fig. S3B). On the other hand, ascorbic acid at dosages of 10 mg/kg, 100 mg/kg exhibited little influence on the open field test (Center%: $F_{(3, 31)} = 1.96$, $p = 0.141$; Total distance: $F_{(3, 29)} = 6.54$, $p = 0.002$; Fig. S3C) and elevated plus maze (open-arm duration: $F_{(3, 28)} = 0.76$, $p = 0.524$; $F_{(3, 31)} = 0.67$, $p = 0.576$; Fig. S3D), indicating that ascorbic acid exerted no effect on anxiety behaviors. However, mice treated with ascorbic acid (1000 mg/kg) exhibited impaired locomotor capacity, as indicated by a significant decrease in total distance moved in the open field test (Fig. S3C).

To further evaluate the antidepressant effect of ascorbic acid, we

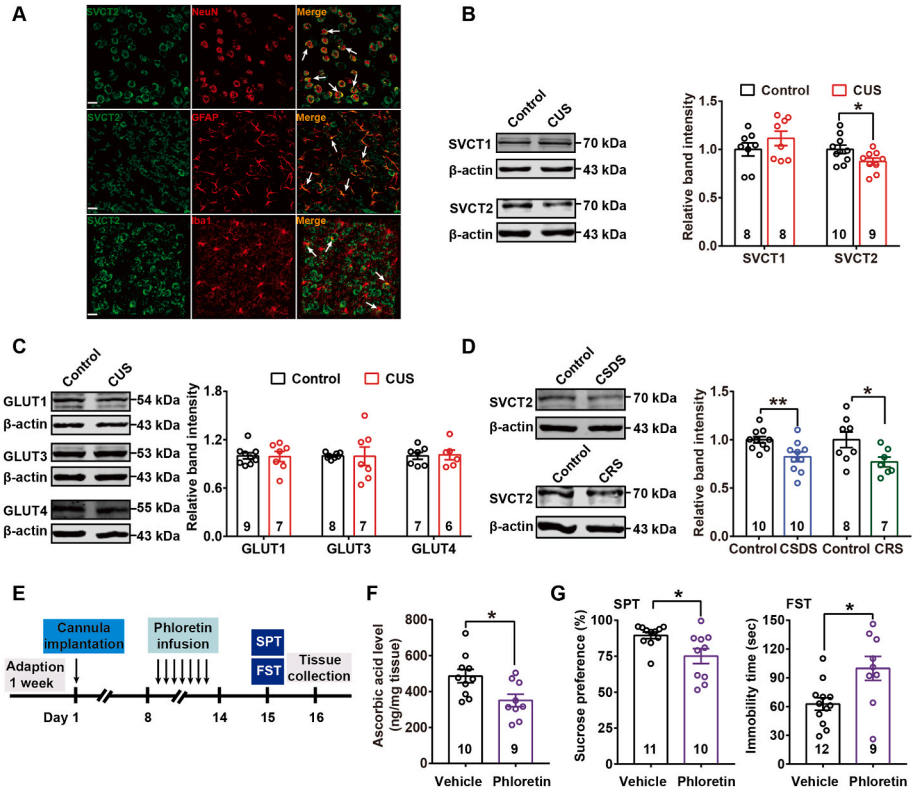

Fig. 2. Deficit in the SVCT2-dependent ascorbic acid uptake in the mPFC mediates depression-like behaviors. **(A)** Localization of SVCT2 in neuron, astrocyte or microglia in the mPFC as visualized by immunofluorescence assay, scale bar = 20 μm. **(B)** Western blotting analysis for SVCT1 and SVCT2 in the mPFC of control and CUS-treated mice. **(C)** Western blotting analysis for GLUT1, GLUT3 and GLUT4 in the mPFC of control and CUS-treated mice. **(D)** Western blotting analysis for SVCT2 in the mPFC of CSDS and CRS-treated mice. **(E)** Experiment timeline of stereotaxic surgery, cannula infusion and behavioral tests. **(F)** The level of ascorbic acid in mPFC after local infusion of phloretin (200 μM, 1 μL per side) into the mPFC for 7 d. **(G)** Results of SPT and immobility time in the FST after local infusion of phloretin into the mPFC for 7 d. All data were represented as mean ± SEM and analyzed by Student's t-test, *$p < 0.05$.

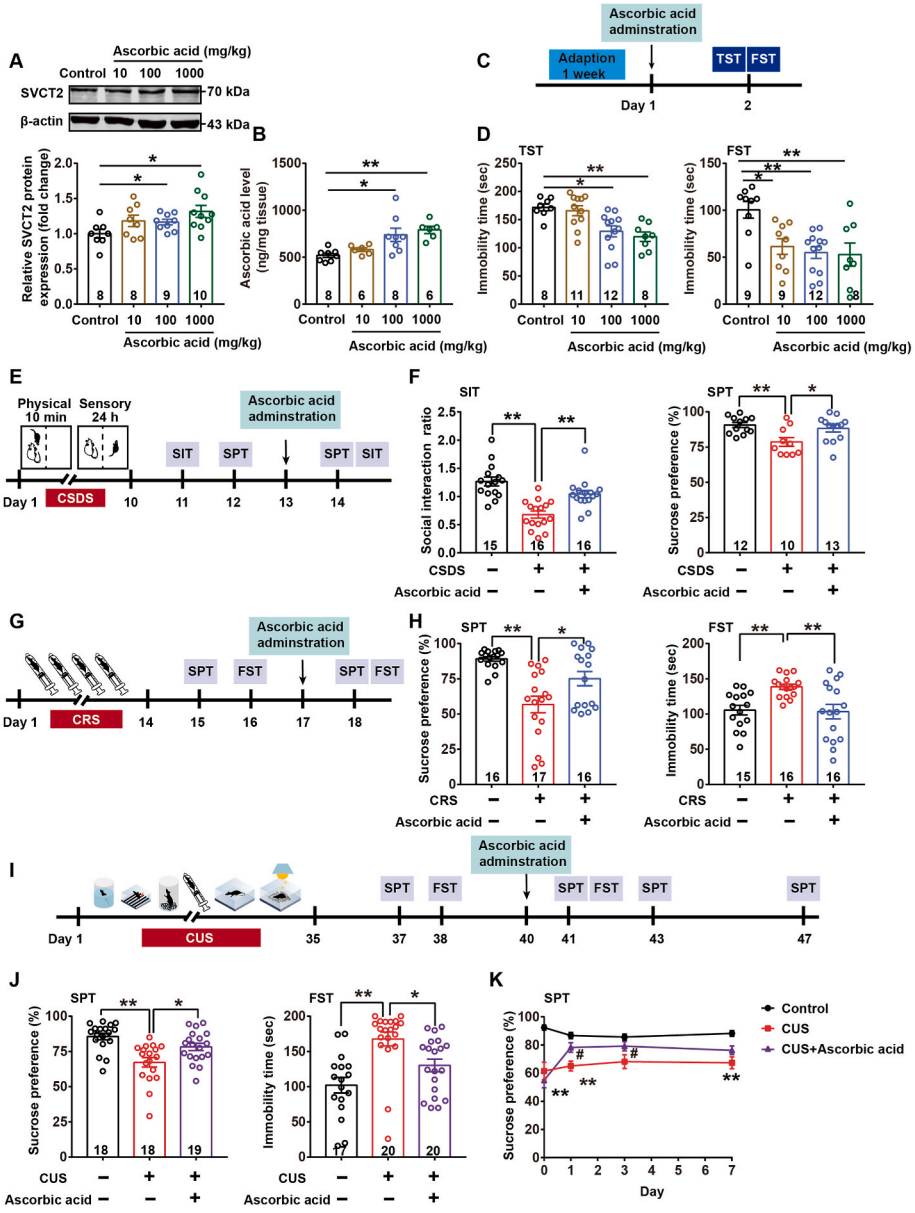

Fig. 3. Ascorbic acid upregulates SVCT2 expression in mPFC and elicits rapid antidepressant effects. (A) SVCT2 protein expression in the mPFC 24 h after ascorbic acid administration. **(B)** The level of ascorbic acid in the mPFC 24 h after ascorbic acid administration. **(C)** Schematic timeline of ascorbic acid treatment and behavioral tests. **(D)** The immobility time in TST and FST 24 h after ascorbic acid treatment. **(E)** Schematic timeline of CSDS, ascorbic acid treatment and behavioral tests. **(F)** Behavioral consequences of ascorbic acid (100 mg/kg) treatment in SIT and SPT for CSDS-treated mice. **(G)** Schematic timeline of CRS, ascorbic acid treatment and behavioral tests. **(H)** Behavioral consequences of ascorbic acid (100 mg/kg) treatment in SPT and FST for CRS-treated mice. **(I)** Schematic timeline of CUS, ascorbic acid treatment and behavioral tests. **(J)** Behavioral consequences of ascorbic acid (200 mg/kg) treatment in SPT and FST for CUS-treated mice. **(K)** Results of SPT at 1 d, 3 d and 7 d after ascorbic acid (200 mg/kg) treatment in CUS-treated mice. All data were represented as mean ± SEM and analyzed by Student's t-test (A) or one-way ANOVA (B, D, F, H, J, K) with Bonferroni's post-hoc test, *p < 0.05, **p < 0.01vs control, #p < 0.05 vs CUS.

employed three mouse models of depression, including CSDS, CRS and CUS. As shown in Fig. 3E and F, intraperitoneal injection of a single dosage of ascorbic acid (100 mg/kg) counteracted the social avoidance ($F_{(2, 44)} = 18.39$, $p < 0.001$) and the decrease in sucrose preference ($F_{(2, 32)} = 6.12$, $p = 0.006$) induced by CSDS at 24 h after administration. However, these behavioral consequences has been reported to be reversed by daily treatment with tricyclic antidepressants or fluoxetine for 2 weeks [43,47]. Consistently, intraperitoneal injection of ascorbic acid (100 mg/kg) caused rapid antidepressant effects in both SPT and FST in the CRS-treated mice (SPT: $F_{(2, 46)} = 12.27$, $p < 0.001$; FST: $F_{(2, 44)} = 7.08$, $p = 0.002$; Fig. 3G and H) at 24 h after administration, but not in CUS-treated mice (SPT: $F_{(2, 38)} = 9.40$, $p < 0.001$; TST: $F_{(2, 36)} = 7.36$, $p = 0.002$; FST, $F_{(2, 42)} = 19.25$, $p < 0.001$; Fig. S4). A higher dosage of ascorbic acid (200 mg/kg) exerted rapid antidepressant effect in CUS-treated mice at 24 h after administration (SPT: $F_{(2, 52)} = 10.91$, $p < 0.001$; FST: $F_{(2, 54)} = 10.86$, $p < 0.001$; Fig. 3I and J). Moreover, increased sucrose preference was observed at 1 and 3 d after intraperitoneal injection of ascorbic acid, indicating that the antidepressant effect of ascorbic acid on anhedonia lasts for at least 3 d (0 d: $F_{(2, 34)} = 17.07$, $p < 0.001$; 1 d: $F_{(2, 34)} = 11.98$, $p < 0.001$; 3 d: $F_{(2, 42)} = 10.83$, $p < 0.001$; 7 d: $F_{(2, 44)} = 10.29$, $p < 0.001$; Fig. 3K). These results consistently demonstrate that intraperitoneal injection of a single high-dosage of ascorbic acid elicits fast and sustained antidepressant effects in stressed mice.

3.4. TETs knockdown in mPFC blocks the rapid antidepressant action of ascorbic acid

Based on that the formation of TET-dependent 5-hydroxymethylcytosine has been reported to mediate pharmacological effects of ascorbic acid [31,48,49], we wondered whether this mechanism could also mediate the antidepressant effects of ascorbic acid. First, we evaluated

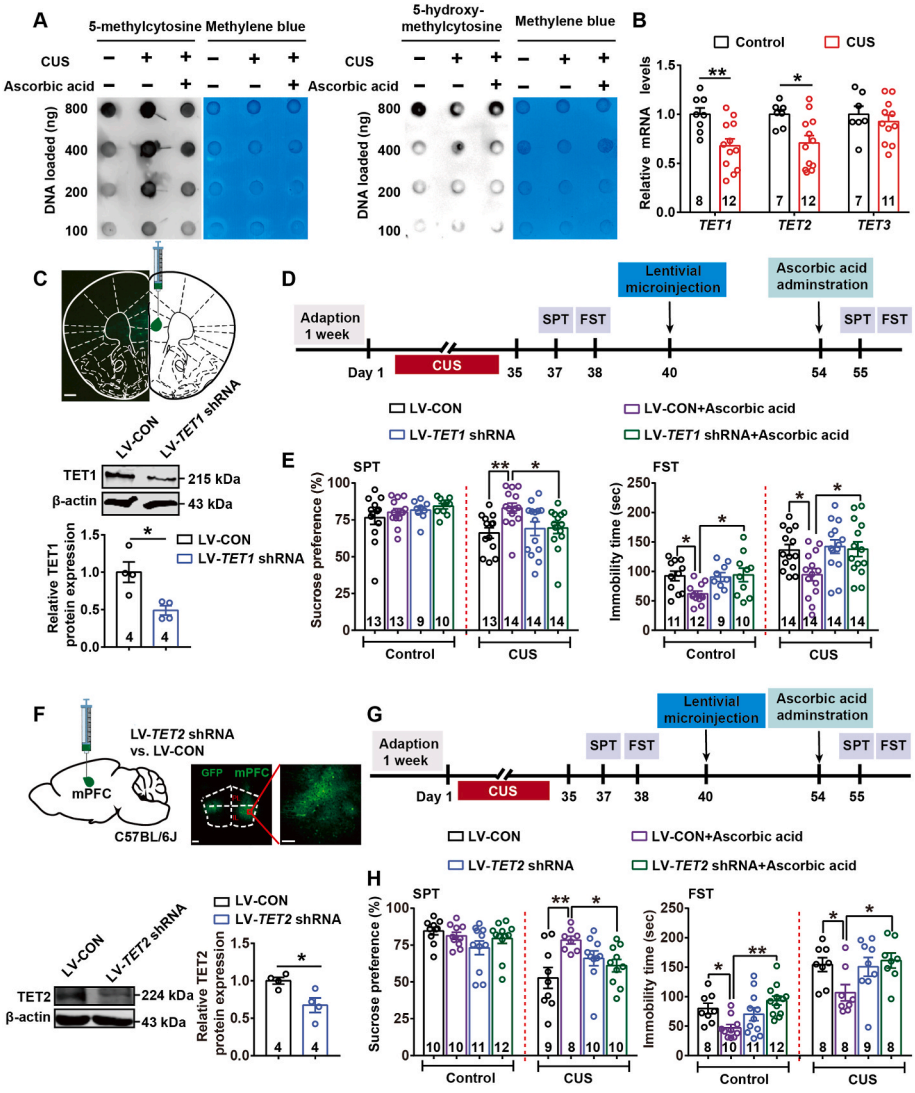

Fig. 4. TET1/2 in the mPFC are required for the rapid antidepressant effects of ascorbic acid. (A) Dot blot assay showing global decrease in 5-methylcytosine and increase in 5-hydroxymethylcytosine in the mPFC after intraperitoneal injection of ascorbic acid (200 mg/kg) in CUS-treated mice. **(B)** *Tets* mRNA expression in the mPFC of control and CUS-treated mice. **(C)** Representative virus-mediated gene transfer and TET1 protein levels in the mPFC two weeks after microinjection of LV-*TET1* shRNA, scale bar = 200 μm. **(D)** Experimental timeline for lentivirus injection, drug administration and behavioral tests. **(E)** Behavioral consequences of TET1 knockdown in the SPT and FST for control and CUS-treated mice. **(F)** Viral strategy, representative EGFP expression and TET2 protein levels in the mPFC two weeks after microinjection of LV-*TET2* shRNA, scale bar = 100 μm. **(G)** Experimental timeline for lentivirus injection, drug administration and behavioral tests. **(H)** Behavioral consequences of TET2 knockdown in the SPT and FST for control and CUS-treated mice. All data were represented as mean ± SEM and analyzed by Student's t-test (B, C, F) or two-way ANOVA (E, H) with Bonferroni's post-hoc test, *p < 0.05, **p < 0.01.

the effect of CUS and ascorbic acid on the levels of 5-methylcytosine and 5-hydroxymethylcytosine, and found that CUS significantly increased 5-methylcytosine levels, but decreased 5-hydroxymethylcytosine levels in mPFC, which could be restored by administration of ascorbic acid (200 mg/kg, intraperitoneal injection, Fig. 4A). Moreover, CUS decreased the mRNA level of *TET1* and *TET2*, but not *TET3* in the mPFC (*TET1*: $t = 3.16$, $p = 0.005$; *TET2*: $t = 2.73$, $p = 0.014$; *TET3*: $t = 0.70$, $p = 0.492$; Fig. 4B), indicating that chronic stress disrupted TET-dependent epigenetic function. To explore the role of TET1 and TET2 in the antidepressant effects of ascorbic acid, LV-containing EGFP and shRNA targeting *TET1* and *TET2* was used to specifically knockdown of TETs in the mPFC of mice. Two weeks after bilateral microinjection of LV into the mPFC, the expression of TET1 ($t = 3.39$, $p = 0.015$; Fig. 4C) and TET2 ($t = 3.02$, $p = 0.023$; Fig. 4F) protein was decreased in EGFP-expressing mice. We found that genetic knockdown of TET1 (Fig. 4D and E) or TET2 (Fig. 4G and H) in the mPFC exerted no effect on depression-related behaviors, including the sucrose preference and immobility time in the FST. However, ascorbic acid failed to exert antidepressant action in the mice microinjected with LV-*TET1* shRNA (Control-SPT: $F_{(1, 41)} = 0.02$, $p = 0.882$; CUS-SPT: $F_{(1, 51)} = 4.49$, $p = 0.182$; Control-FST: $F_{(1, 38)} = 4.33$, $p = 0.044$; CUS-FST: $F_{(1, 52)} = 2.95$, $p = 0.092$; Fig. 4D and E) or LV-*TET2* shRNA (Control-SPT: $F_{(1, 39)} = 1.94$, $p = 0.171$; CUS-SPT: $F_{(1, 33)} = 8.98$, $p = 0.005$; Control-FST: $F_{(1, 37)} = 10.34$, $p = 0.003$; CUS-FST: $F_{(1, 29)} = 4.40$, $p = 0.044$; Fig. 4G and H). Taken together, these results suggest that both TET1 and TET2 in the mPFC are involved in the antidepressant action of ascorbic acid.

3.5. *S100 calcium binding protein A4 (S100A4) works as a key downstream effector underlying the antidepressant effects of ascorbic acid*

TET-dependent epigenetic function can alter the expression of numerous mRNAs. To evaluate whether ascorbic acid has an impact on stress-sensitive gene expression, we performed RNA-sequencing, and the heatmap diagram showed a significant change in the transcriptional signature in mPFC after chronic stress exposure (Fig. 5A top). There were 33 genes down-regulated and 3 genes up-regulated in the mPFC under CUS (Table S3). Subsequently, we conducted hierarchical cluster analysis of gene expression changes in the mPFC of CUS mice treated with ascorbic acid (Fig. 5A bottom). When compared with CUS model mice, there were 17 and 7 genes downregulated and upregulated in ascorbic acid (200 mg/kg) treatment group, respectively (Table S4). Gene ontology analysis revealed that many distinguishable gene patterns changed in CUS-treated mice were related to the extracellular matrix, proteinaceous extracellular matrix and negative regulation of the apoptotic process (Fig. 5B, top). After ascorbic acid treatment, the changed genes were strongly correlated with neuron projection, positive regulation of transcription by RNA polymerase II and extracellular region (Fig. 5B, bottom). Venn diagram was used to study the intersection

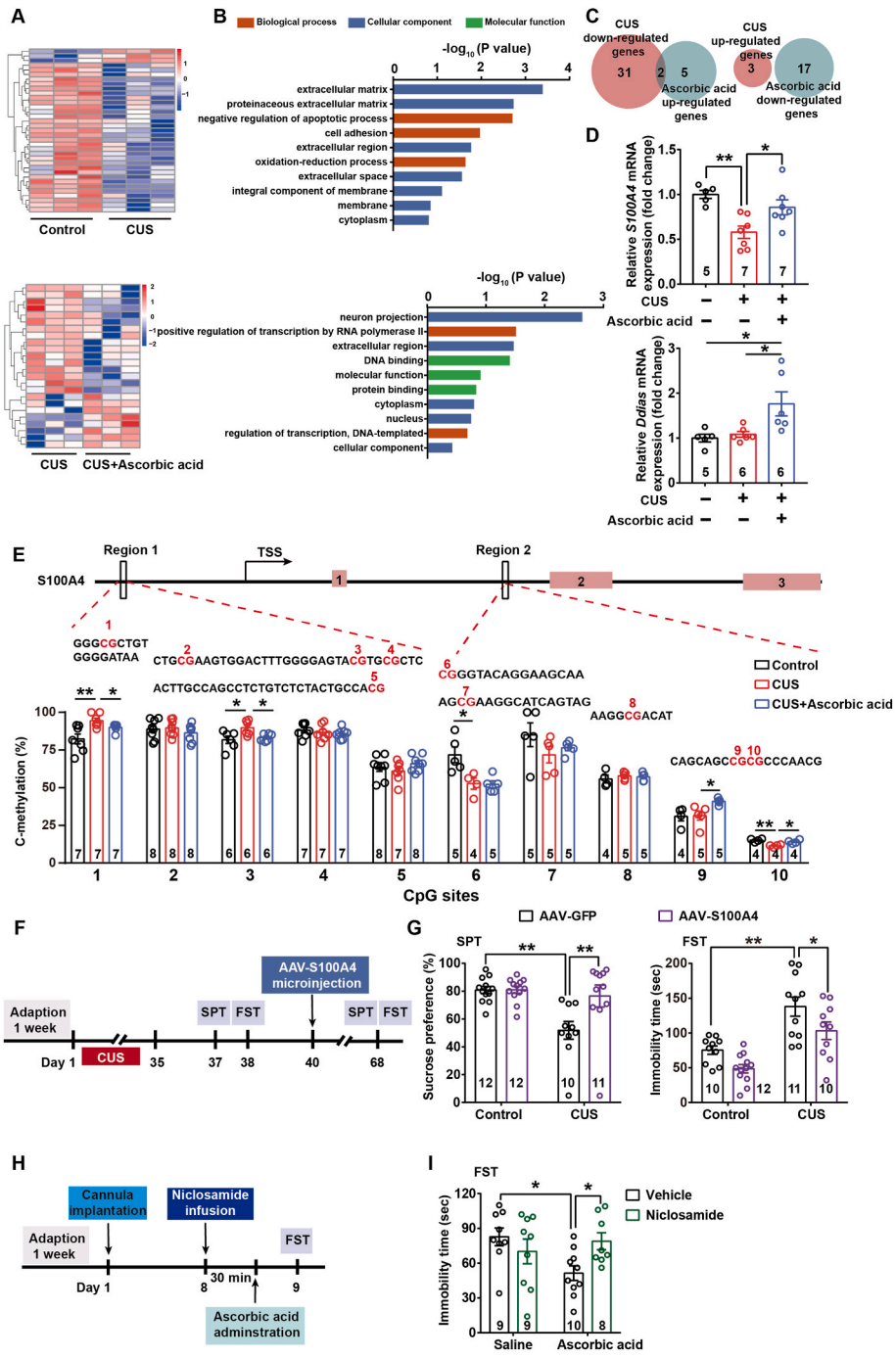

Fig. 5. S100A4 works as a key downstream effector of epigenetic therapy with ascorbic acid. **(A**, Top) Heatmap of gene expression profiles in the mPFC of control and CUS-treated mice. Bottom, heatmap of gene expression profiles in the mPFC of non-treated and ascorbic acid-treated CUS mice. Each group had three biological repeats. **(B**, top**)** Top ten gene ontology enrichment terms in differentially expressed genes in the mPFC between control and CUS-treated mice. Bottom, top ten gene ontology enrichment terms in differentially expressed genes in the mPFC between nontreated and ascorbic acid-treated CUS mice. **(C)** Venn diagram of differentially expressed genes revealed small overlap between genes downregulated induced by CUS and genes upregulated followed by ascorbic acid (200 mg/kg, intraperitoneal injection) treatment. **(D)** S100A4 and *Ddias* mRNA expression in the mPFC of control mice, vehicle-treated CUS mice and ascorbic acid-treated CUS mice. **(E)** Bar graphs indicating the altered methylation levels of S100A4 at each CpG site. Red boxes represent exons. **(F)** Experimental timeline of CUS, virus injection and behavioral tests. **(G)** Behavioral consequences of S100A4 overexpression in the SPT and FST for control and CUS-treated mice. **(H)** Experimental timeline of stereotaxic surgery, cannula infusion, drug treatment and behavioral tests. **(I)** FST immobility time 24 h after ascorbic acid treatment with or without niclosamide (4 μM, 1 μL per side) pretreatment. All data were represented as mean ± SEM and analyzed by one-way ANOVA (C), Student's t-test (E) or two-way ANOVA (G, I) with Bonferroni's post-hoc test, *p < 0.05, **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of differentially expressed genes regulated by CUS and ascorbic acid treatment. We found that two differentially expressed genes (S100A4; Ddias) were downregulated by CUS, but upregulated after ascorbic acid treatment (Fig. 5C left). There was no overlapping between gene upregulation induced by CUS and gene downregulation followed by ascorbic acid treatment (Fig. 5C right). Furthermore, we found that only S100A4 was significantly downregulated by CUS and upregulated after ascorbic acid treatment (*S100A4*: $F_{(2, 16)} = 8.52$, $p = 0.003$; *Ddias*: $F_{(2, 14)} = 5.79$, $p = 0.015$; Fig. 5D). Given that DNA methylation status plays a key role in regulating gene expression, pyrosequencing, a highly accurate technique for measuring the relative methylation level at each CpG island, was conducted to detect the methylation level of S100A4. Five primers for amplification were designed and 10 CpG sites in regions 1 and 2 were quantitatively analyzed. There were significant differences in methylation levels at CpG sites 1 ($t = 3.33$, $p = 0.006$), 3 ($t = 2.51$, $p = 0.031$), 6 ($t = 2.84$, $p = 0.025$), 9 ($t = 0.01$, $p = 0.926$) and 10 ($t = 5.34$, $p = 0.020$) in CUS-exposed mice when compared with that of control mice, and the changes at sites 1 ($t = 2.42$, $p = 0.032$), 3 ($t = 2.89$, $p = 0.016$) and 10 ($t = 3.646$, $p = 0.011$) were reversed by treatment with ascorbic acid (Fig. 5E). These results highlight that ascorbic acid regulates S100A4 expression by altering DNA methylation status.

S100A4, a member of the S100 family, is predominantly expressed in astrocytes [50] and has been reported to exert neuroprotective effects [51]. Therefore, we further examined whether S100A4 in the mPFC functions as an endogenous antidepressant. Mice that received microinjection with AAV-S100A4 to overexpress S100A4 displayed a marked elevation in the S100A4 protein level after four weeks ($t = 4.29$, $p = 0.008$; Fig. S5). The behavioral results showed that S100A4

overexpression in the mPFC increased sucrose preference and decreased immobility time in the FST (SPT: $F_{(1, 41)} = 5.66$, $p = 0.022$; FST: $F_{(1, 39)} = 0.15$, $p = 0.702$; Fig. 5F and G) in CUS-treated mice. To gain further insight into the role of S100A4 in the antidepressant effect of ascorbic acid, niclosamide (4 μM, 1 μL/side), an inhibitor of S100A4 transcription [52], was bilaterally infused into the mPFC 30 min before intraperitoneal injection of ascorbic acid (Fig. 5H). We found that niclosamide prevented the ascorbic acid-induced decrease in immobility time in the FST ($F_{(1, 32)} = 6.06$, $p = 0.019$; Fig. 5I), suggesting that S100A4 mediates the antidepressant effects of ascorbic acid.

3.6. The antidepressant effects of ascorbic acid are mediated by activation of S100A4- Erb-b2 receptor tyrosine kinase 4 (ErbB4)-brain derived neurotrophic factor (BDNF) signaling pathway

Next, we focused on the molecular mechanism underlying the antidepressant activity of S100A4. Recent studies have reported that silencing S100A4 decreases the expression of BDNF in a mouse model of oxygen-induced retinopathy [53]. Thus, we asked whether S100A4 regulated BDNF expression in the mPFC of CUS-treated mice. As shown in Fig. 6A, a higher level of BDNF ($t = 5.04$, $p < 0.001$) was observed in the mPFC of S100A4-overexpressing mice. Given that intraperitoneal injection of ascorbic acid (200 mg/kg) exerted an antidepressant effect via upregulation of S100A4, we next measured the protein expression level of BDNF after ascorbic acid treatment. The levels of BDNF were elevated in the mPFC of mice 24 h after ascorbic acid treatment ($F_{(1, 33)} = 0.24$, $p = 0.629$; Fig. 6B). BDNF is the major causal agent for the changes in spine density under stress, thus, confocal microscopy was used to measure dendritic spine densities in the mPFC of CUS-treated mice 24 h after ascorbic acid treatment. Administration of ascorbic acid significantly reversed the CUS-induced decrease in spine densities in the mPFC ($F_{(1, 42)} = 3.63$, $p = 0.064$; Fig. 6C). Recent studies have reported that S100A4 regulates neuronal survival and plasticity by activating the ErbB4 receptor [54], and that ErbB4 ablation downregulates the expression of BDNF and its receptor TrkB [55]. To ask whether ErbB4 was also involved in the antidepressant effects of ascorbic acid, AG1478 (50 μM, 1 μL per side), a selective ErbB4 receptor blocker [56], was infused into the mPFC 3 h before administration of ascorbic acid (Fig. 6D). Behavioral results revealed that ascorbic acid-induced increase in sucrose preference and decrease in immobility time in the FST were prevented by microinjection of AG1478 into the mPFC (Control-SPT: $F_{(1, 35)} = 1.00$, $p = 0.324$; CUS-SPT: $F_{(1, 38)} = 9.77$, $p = 0.003$; Control-FST: $F_{(1, 33)} = 3.25$, $p = 0.080$; CUS-FST: $F_{(1, 37)} = 16.03$, $p < 0.001$; Fig. 6E). Furthermore, blockade of the BDNF-TrkB pathway by intra-mPFC infusion of ANA-12 (2.45 mM, 1 μL per side), a TrkB non-competitive inhibitor, also abolished the antidepressant activity of ascorbic acid (Control-SPT: $F_{(1, 25)} = 1.56$, $p = 0.224$; CUS-SPT: $F_{(1, 43)} = 9.97$, $p = 0.003$; Control-FST: $F_{(1, 46)} = 1.03$, $p = 0.316$; CUS-FST: $F_{(1, 38)} = 8.48$, $p = 0.006$; Fig. 6F and G). Altogether, these findings suggest that the S100A4-ErbB4-BDNF-TrkB signaling pathway mediates the mechanism underlying the antidepressant-like effects of ascorbic acid (see Fig. 7).

4. Discussion

Converging evidence suggests that psychosocial stressors may trigger the onset of depression by driving gene expression changes via epigenetic reprogramming of DNA methylation patterns [57,58]. However,

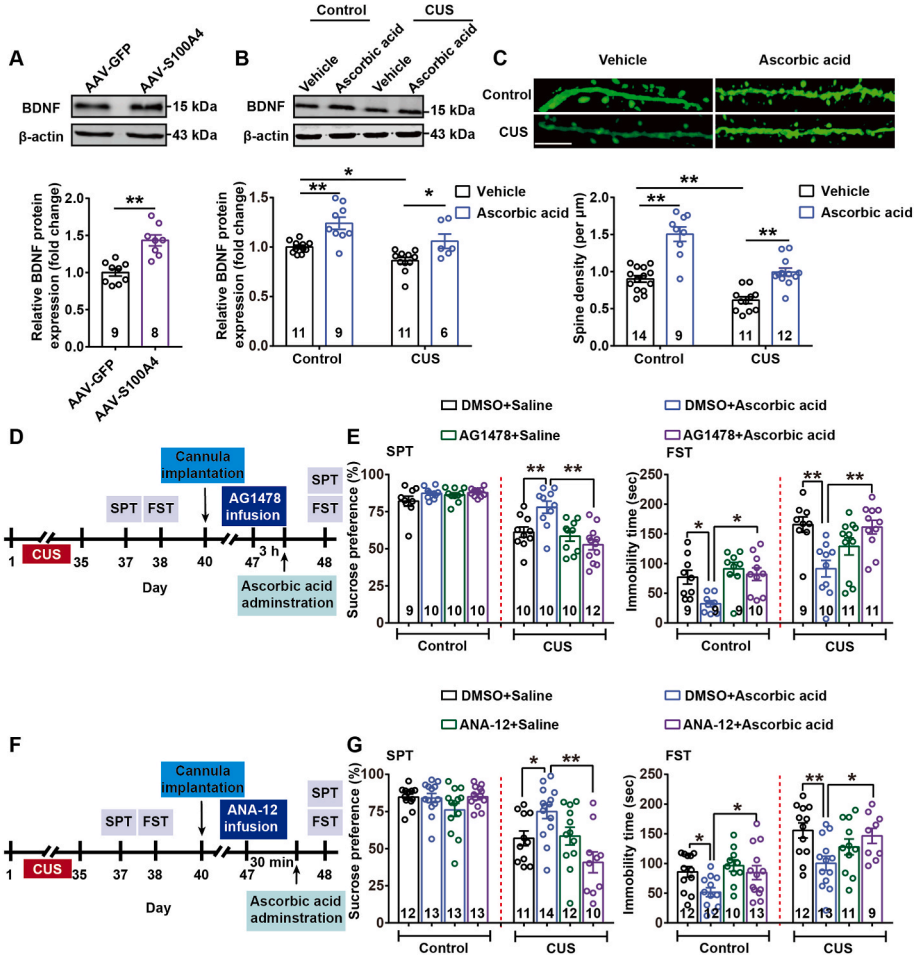

Fig. 6. Ascorbic acid produces rapid antidepressant action via activating S100A4-ErbB4-BDNF-TrkB signaling pathway. (A) The expression of BDNF protein in the mPFC of CUS mice microinjected with AAV-S100A4 or AAV-GFP. **(B)** The expression of BDNF protein in the mPFC 24 h after ascorbic acid (200 mg/kg) administration. **(C)** Representative confocal images and quantification of dendritic spines from neurons in the mPFC 24 h after administration with ascorbic acid, scale bar = 5 μm. **(D)** Experimental timeline of CUS, stereotaxic surgery, cannula infusion, drug treatment and behavioral tests. **(E)** Results of SPT and FST immobility time 24 h after ascorbic acid treatment with or without AG1478 (50 μM, 1 μL per side) pretreatment. **(F)** Experimental timeline of CUS, stereotaxic surgery, cannula infusion, drug treatment and behavioral tests. **(G)** Results of SPT and FST immobility time 24 h after ascorbic acid treatment with, or without, ANA-12 (2.45 mM, 1 μL per side) pretreatment. All data were represented as mean ± SEM and analyzed by Student's t-test (A) or two-way ANOVA (B, C, E, G) with Bonferroni's post-hoc test, *$p < 0.05$, **$p < 0.01$.

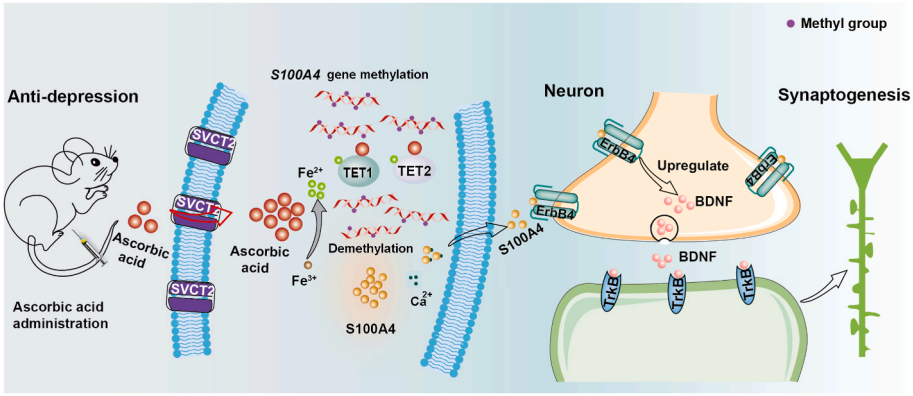

Fig. 7. Schematic model for the rapid antidepressant effect of ascorbic acid. A high dose of ascorbic acid restores ascorbic acid homeostasis in the mPFC and stimulates TETs-catalyzed epigenome reprogramming, resulting in an increase in S100A4 expression, following by an activation of S100A4-ErbB4-BDNF-TrkB-dependent synaptogenesis to elicit rapid antidepressant activity.

whether the 'plastic' characteristic of DNA methylation accounts for a promising possibility of rapid-acting antidepressants remains largely unknown. Ascorbic acid, an endogenous antioxidant with an unusually high safety profile, has been recently discovered as a key cofactor for TET dioxygenases that controls DNA demethylation. Here, we reported that ascorbic acid (200 mg/kg) exerted a rapid-acting antidepressant--like effect in mice by promoting TET-dependent DNA oxidation to reprogram DNA methylation patterns, followed by activation of S100A4-ErbB4-BDNF-TrkB signaling pathway. These results imply that epigenetic therapy with ascorbic acid may act as a potential approach against stress-related disorders.

The pathophysiology of depression is still not clearly understood. A deficiency in specific nutrients, such as vitamin D, has been suggested to play a role in the development of depression [2,3,59]. Antidepressant treatment does not lead to a satisfactory response in approximately 30% of patients [60], and the low medication compliance rates as well as the high recurrence rate suggest nutrients as a promising adjunct to improve drug efficacy. From early studies of scurvy, some studies have indicated that deficiency of vitamin C may be a contributory factor to depressive symptoms [4,6]. Although these clinical studies indicated a possible nutritional psychiatry mechanism underlying vitamin C deficiency, very little is known about the role of endogenous ascorbic acid homeostasis in major depressive disorder development. As the main transporter of ascorbic acid, SVCT2 serves as a key regulator of endogenous ascorbic acid homeostasis in the brain. Here, we observed that chronic stress induced ascorbic acid deficiency in the mPFC by disrupting the expression of SVCT2, leading to increased susceptibility to depression. Over the past three decades, the influence of nutrition and nutrients, such as vitamin D, vitamin B12, folate, and zinc, on mental health has received growing attention through epidemiological and experimental findings [59], but the mechanism through which specific nutrients and depression are correlated is not clear. Our present data provided a novel possibility that couples specific nutrients to depression, that is, stress impairs the absorption or metabolism of nutrients, followed by the disruption of physiological function, which supports the nutrition psychiatry hypothesis [61]. For the limitation of detection sensitivity, the content of plasma ascorbic acid was not tested in this study. Although the decreased expression of SVCT2 in the mPFC highlighted the role of central ascorbic acid depletion in stress-related disorders, the alterations in ascorbic acid in other tissues should be considered. For instance, the requirement for ascorbic acid in brain would lead to an increase in the synthesis of ascorbic acid in the liver, which may increase the circulating plasma level of ascorbic acid. Considering SVCT2 was the main transporter of ascorbic acid in the brain, the possible increase in ascorbic acid synthesis could not compensate its deficits in the central nervous system, which was evidenced by our observation that a decreased ascorbic acid level in the mPFC 24 h after CUS treatment. On the contrary, some other stress-related alterations, such as oxidative stress, may decrease plasma ascorbic acid level. However, the relationship between stress and circulating plasma level of ascorbic acid may be complex and the effect of peripheral compensatory mechanism on brain ascorbic acid level could not be fully excluded. A systematic study for ascorbic acid plasma level in stressed mice at different time points are required in the next study.

Increasing evidence on the molecular underpinnings of major depressive disorder posits a mechanistic role of epigenetic DNA modifications to account for individual variability, especially DNA methylation [57,58,62]. DNA demethylation rewrites DNA methylation signatures and regulates gene expression. As the most important epigenetic regulators of DNA demethylation, TET enzymes promote 5-hydroxymethylcytosine modification of genes to initiate DNA demethylation. Previous reports have indicated that elevated TET activity may be involved in the antidepressant action of sodium butyrate [63] and metformin [64], and loss of TET2 increased the susceptibility of mice to depression [20,21]. Ascorbic acid affects TET activity not only by maintaining the availability of Fe^{2+} but also by affecting the activity of TET proteins as cofactors [13,14]. In our study, ascorbic acid exerted rapid-acting antidepressant responses in a TET1-and TET2-dependent manner, which was different from previous reports [20,21,63,64]. We did not observe other changes in behavioral consequences (such as SPT and FST) after knockdown of TET1 or TET2 gene in the mPFC, which was similar with some previous reports from TET1 knockout mice [65,66]. Considering the vital role of TETs in the epigenetic mechanisms, other effects of TET1 or TET2 knockdown on behaviors may also exist. The stress-induced ascorbic acid deficiency is mainly due to impaired SVCT2 function. Another possible mechanism for stress-related ascorbic acid deficiency may be the elevated oxidative stress level [67], which may oxidize ascorbic acid into dehydroascorbic acid. The antidepressant effect of ascorbic acid on anhedonia lasted for at least 3 d, which may be associated with the sustained alterations in gene expression profile in the mPFC via TETs-dependent epigenetic mechanisms [68]. In addition, the relatively high concentration of ascorbic acid in neuron may contribute to its lasting antidepressant effects. In our experimental settings, we observed an increase of 52% $\pm$ 0.07% in ascorbic acid level in the brain at 24 h after ascorbic acid administration. This increase may result from multiple mechanisms, such as an increased SVCT2 level (132% $\pm$ 0.09% of control group), an increased initial uptake via the choroid plexus into the CSF, and the saturation of periphery excretory pathway by a high-dosage ascorbic acid administration. In the brain, the absorption rate of ascorbic acid was faster than its excretion rate [69–71], and multiple intracellular recycling mechanisms may help to maintain a high level of intracellular ascorbic acid in neurons.

Our findings suggested a beneficial role of S100A4, a member of the large family of S100 proteins that has long been implicated mainly in

tumorigenesis and metastasis [50,72]. Emerging evidence has supported that S100A4 may act as a potentially protective factor against oxidative stress and brain injury [50,73], and exert physiological roles in the nervous system [51,74,75]. Interestingly, both transcriptome sequencing and methylation sequencing data highlight a critical role of S100A4 in the antidepressant activity of ascorbic acid. A previous study revealed that silencing S100A4 downregulated BDNF expression in oxygen-induced ischemic retinopathy [53], whereas ErbB4 receptor, a key receptor for S100A4 protein signals, regulates the expression of BDNF and its receptor, TrkB [54]. Our study revealed that chronic stress inhibited S100A4 expression. Taken together with our results, a dysregulated S100A4-ErbB4-BDNF-TrkB pathway may contribute to the pathogenesis of depression.

In cancer research, epigenetic therapy with high doses of vitamin C is being explored as a promising therapeutic approach [31–33,76]. For cancer therapy, a high-dose vitamin C (10–150 g/d, intravenous administration) [49,77,78] is required to achieve and maintain a high concentration of ascorbic acid in the plasma. In our study, we found that single intraperitoneal injection of ascorbic acid (100–1000 mg/kg) produced epigenetic responses in the mPFC and alleviated depressive symptoms. Using the body surface area normalization method described by FDA draft guidelines (human dosage = mice dosage × km mice/km human, km mice = 3, km human = 37), this dosage was equivalent to 0.48–4.8 g ascorbic acid per day in humans. It should be noted that the administration route significantly impacted the bioavailability of vitamin C [79,80] for its dose-dependency and non-linearity of the pharmacokinetics. Considering the difference in bioavailability between intraperitoneal injection and oral administration, the dosage used in our experiments may be equivalent to an oral dose of 1.5–15 g/d or an intravenous drip dose of 0.48–4.8 g ascorbic acid per day in humans. This dosage of vitamin C seems lower than the epigenetic therapy used in cancer. In previous experimental reports, the intracellular concentrations of ascorbic acid in the brain were many-fold higher than those in the plasma [70]. The ability of the brain to maintain a high ascorbic acid level by SVCT2 may help decrease the effective dose. A translation study is required in the next study. Notably, the differences in basic homeostasis of ascorbic acid between mice and humans should be considered for the translation of our findings. Due to the functional mutation in the L-gulonolactone oxidase gene, unlike mice, humans are incapable of synthesizing vitamin C. Thus, a higher effective dosage than a 15 g/d oral dose or a 4.8 g/d intravenous dose may be required in depression patients. In addition, some other factors such as age, gender and diet may also influence dosage translation between mice and humans [81,82].

It should be noted that there are several limitations of the present study. First, although three classical stress procedures were used to induce depression-type behaviors in mice, phenotype in rodents could not fully mimic the symptoms in depressive patients. Thus, the results of animal model should be verified by a translational study in the future. Second, we only used male mice because female mice were limited in some stress procedures, such as CSDS [83], which was caused by repeated experiences of social defeat stress by male CD-1 mice. In next study, the antidepressant effect of ascorbic acid should also be investigated in female mice using other procedures. Third, the content of ascorbic acid in brain is regulated by several factors [84]. Ascorbic acid is transported from plasma to the cerebrospinal fluid across the epithelium of the choroid plexus and absorbed by brain cells [85,86]. Except for SVCT2, the transport process in the choroid plexus may also be affected by chronic stress. Besides, the main mechanism responsible for the epigenetic action of ascorbic acid is due to its function as a cofactor of alpha-ketoglutarate-dependent dioxygenases (αKGDD). In addition to TETs, other αKGDDs, such as the JmjC domain-containing histone demethylase family and AlkB, are also known to be epigenetic regulators that can be potentially regulated by ascorbic acid [87,88]. Although our data showing that genetic knockdown of TET1 or TET2 ameliorated the effects of ascorbic acid, other mechanisms may also contribute to the epigenetic therapy effect of ascorbic acid. Similar to TETs, hypoxia inducible factor hydroxylases require ascorbic acid as a cofactor to recycle Fe^{2+} [89], and its transcriptional effects may also be involved in the regulation of ascorbic acid. The highest dose of ascorbic acid (1000 mg/kg) decreased the locomotor activity (Fig. S3C), which may be due to the toxic effect of high dose of ascorbic acid. The decreased locomotor activity may impact the TST and FST immobile time in the high dosage group. We found that infusion of phloretin, a nonselective inhibitor of vitamin C transporters, into the mPFC induced depressive-like behaviors in mice (Fig. 2G). Considering that SVCT2 was the main transporter of vitamin C in the brain, the behavioral consequences of phloretin may be closely associated with its action on SVCT2. However, the possible role of SVCT1 and glucose transporters in the effect of phloretin could not be excluded. For the study of mechanism by differentially transcriptome, we identified 36 differentially expressed genes (*Col6a6, Ddias, S100A4, Pnpla1, Rpe65, Shcbp1*, etc) in the mPFC of stressed animals, and 24 differentially expressed genes (*Tbx6, Xlr4a, Mup17, Ddias, Fcer2a, S100A4*, etc) in the mPFC of ascorbic acid-treated stressed animals ($p < 0.05$ and fold change >2 were defined as significant difference). Among them, two differentially expressed genes (*Ddias, S100A4*) that inversely regulated by CUS and ascorbic acid treatment under the higher criterion were identified and validated by qRT-PCR. Notably, when $p < 0.05$ or fold change >1.2 was identified as significant difference, there were 23 genes (*Ddias, S100A4, Cmb1, Mid1, Msantd1*, etc) inversely regulated by CUS and ascorbic acid. Thus, multiple downstream mechanisms may underlie the antidepressant activity of ascorbic acid.

In conclusion, our results suggest that the deficits in endogenous homeostasis of ascorbic acid may bridge oxidative stress with epigenetic mechanisms underlying nutrition psychiatry. Targeting the stress-coupled 'plastic' DNA methylation state by ascorbic acid candidates as a novel rapid-acting antidepressant strategy in future.

Author contributions

Qian-Qian Han performed animal experiments, stereotaxic surgery and molecular experiments. Peng-Fei Wu conceived the study, helped molecular methodology, and analyzed the RNA sequencing data. Yi-Heng Li and Yu Cao contributed to animal experiments and stereotaxic surgeries. Peng-Fei Wu and Qian-Qian Han wrote the paper and draw the figures. Fang Wang, Peng-Fei Wu and Jian-Guo Chen supervised the project, designed the experiments, revised the manuscript, and supported funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.redox.2022.102543.

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