Received: July 14, 2022 Revised: November 1, 2022 Accepted: November 15, 2022

https://doi.org/10.1016/j.neurom.2022.11.014

Electroacupuncture Ameliorates Cognitive Impairment by Regulating γ-Amino Butyric Acidergic Interneurons in the Hippocampus of 5 Familial Alzheimer's Disease Mice

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ABSTRACT

Objectives: γ-amino butyric acid (GABA)–ergic dysfunction in excitatory and inhibitory (E/I) imbalance drives the pathogenesis of Alzheimer's disease (AD). Inhibitory interneurons play an important role in the regulation of E/I balance, synaptic transmission, and network oscillation through manipulation of GABAergic functions, showing positive outcomes in AD animal models. Mice expressing 5 familial AD mutation (5xFAD) exhibited a series of AD-like pathology and learning and memory deficits with age. Because electroacupuncture (EA) treatment has been used for a complementary alternative medicine therapy in patients with AD, we aimed to examine any usefulness of EA therapy in GABA interneuron function and its associated synaptic proteins, to determine whether EA could effectively improve inhibitory transmission and network oscillation and eventually alleviate cognitive impairments in 5xFAD mice, and to further elucidate the GABAergic system function underlying the antidementia response of EA.

Materials and Methods: 5xFAD mice were used to evaluate the potential neuroprotective effect of electroacupuncture at Baihui (DU 20) and Dazhui (DU 14) through behavioral testing, immunofluorescence staining, electrophysiology recording, and molecular biology analysis.

Results: First, we observed that EA improved memory deficits and inhibitory synaptic protein expression. Second, EA treatment alleviated the decrease of somatostatin-positive interneurons in the dorsal hippocampus. Third, EA attenuated E/I imbalance in 5xFAD mice. Last, EA treatment enhanced theta and gamma oscillation in the hippocampus of 5xFAD mice.

Conclusions: EA stimulation at DU20 and DU14 acupoints may be a potential alternative therapy to ameliorate cognitive deficits in AD through the regulation of the function of the GABAergic interneuron.

Keywords: 2 Hz stimulation, Alzheimer's disease, electroacupuncture, interneurons

Conflict of Interest: The authors reported no conflict of interest.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease associated with increasing cognitive impairment leading to dementia[1] and usually occurs in people aged 65 years or older. With the aging of the population, the prevalence of AD increases yearly, and the burden of AD on social medical care and economic life will increase accordingly.[2] The pathological changes in patients with AD are characterized by amyloid plaques, tau protein phosphorylation, and formation of neurofibrillary tangles in nerve cells.[3] However, the precise pathophysiology in all its complexity remains unclear, and there is still no effective treatment for AD.

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Source(s) of financial support: This work was supported by grants from National Natural Science Foundation of China, China (Grant numbers 81704168 to Jiaying Zhao); Natural Science Foundation of Guangdong Province, China (Grant numbers 2017A030310359 to Jiaying Zhao); Guangdong Provincial Key Laboratory of Chinese Medicine and Acupuncture, China (Grant numbers 2017B030314143 to Nenggui Xu).

Reports have shown that cognitive deficits with aberrant network activity can be detected decades before the onset of the clinical symptoms of AD.[4] In studies in animal models, it is reported that the mechanisms of network abnormalities, especially oscillation rhythm degradation by amyloid-β (Aβ) in brain slices, may involve excitatory and inhibitory (E/I) imbalance and cellular excitability.[5,6] E/I balance finely tunes neural network activity within a narrow temporal window by modulating strengths and weights of excitatory and inhibitory neurotransmission associated with external stimuli.[7] According to recent studies, dysfunction of γ-amino butyric acid (GABA) interneurons promotes amyloid plaque deposition and hyperphosphorylated tau sequesters and leads to cognitive impairment, which accounts for the E/I imbalance and related excitotoxicity.[8,9] In turn, these pathological markers exacerbate GABA interneuron dysfunction. Normalization of E/I balance through manipulation of GABAergic functions has shown positive outcomes in preclinical and clinical studies.[10,11] In addition, network synchronization and oscillation of brain rhythm receiving inhibitory tone from GABA interneurons can regulate the activity of memory.[12] The frequency of neural network oscillations ranges widely. Delta (1.5–4 Hz), theta (4–10 Hz), beta (10–30 Hz), and gamma (30–80 Hz) rhythms reflect large periodic changes in neuronal aggregation excitability levels in the brain during encoding, storing, and retrieving information. Theta and gamma oscillations are the most widely studied because they are closely related to higher cognitive function and memory task execution.[13,14] A decrease in gamma and theta rhythms has been observed in multiple brain regions of patients with AD,[15] and manipulation of the GABAergic interneurons could restore gamma oscillation.[16]

Although AD cannot yet be cured, electroacupuncture (EA) has been widely used for a complementary alternative medicine therapy in patients with AD.[17-19] A series of studies has suggested that EA could improve synaptic plasticity, and attenuate amyloidosis and cognitive deficits in animal models of AD.[20-22] However, the potential mechanisms of EA treatment in patients with AD remain elusive. Dazhui (DU14) and Baihui (DU20) are commonly used acupoints for the treatment of neurological and psychiatric disorders such as epilepsy, anxiety, and dementia, and of neuropathic pain through modulation of GABA neurotransmitters and related receptors.[23-25] In this study, five-month-old mice expressing 5 familial AD mutation (5xFAD) were EA stimulated at Baihui (DU20) and Dazhui (DU14), both of which are located on the Du/govern vessel. We examined any usefulness of EA therapy in GABA interneuron function and its associated synaptic proteins to determine whether EA could effectively improve inhibitory transmission and network oscillation, eventually alleviate cognitive impairments of 5xFAD mice, and further elucidate the GABAergic system function underlying the antidementia response of EA.

EXPERIMENTAL PROCEDURE

Animals

The male and female 5xFAD mice used in this study were from Nanjing University (Nanjing, China) and housed in three to five groups, with free access to water and feed. The 5xFAD mice used in this study were inbred with wild-type (WT) (C57/BL6) mice to generate heterozygous offspring, which has been previously described[26]. These mice express gene mutation APP with KM670/671NL (Swedish mutation), I716V (Florida), V717I (London), and PS1 (M146L and L286V). The 5xFAD mice were randomly divided into two groups, including the 5xFAD and electroacupuncture (5xFAD +EA) groups, of 12 mice with six males and six females in each group. Age-matched WT littermates served as the normal control (WT) group. Most of the mice were five months old at the start of tests in this study and were killed at the age of six months for further tests. All mice were housed in a standard environment [temperature (22+2) °C; humidity 55%; 12-hour light-dark cycle]. The study was conducted in strict accordance with the Animals Care and Use Committee of Guangzhou University of Chinese Medicine.

Electroacupuncture Treatment

EA treatment was performed six times per week for four weeks. The stainless-steel needles (0.16 × 7mm, Suzhou Medical Appliance Factory, Suzhou, China) were horizontally inserted at a depth of 2 to 3 mm into the DU14 and DU20 acupoints. DU14 is located at the depression of the seventh cervical spinous, whereas DU20 is located at the intersection of the sagittal midline and the line linking the two ears. Stimulation was generated using the electronic stimulation generator (Master-8, AMPI, Israel), and the stimulation parameters were set as continuous waves of 2 Hz, 1 mA, 30 minutes. No corresponding treatments were performed on the 5xFAD mice group and WT groups, although the mice were restrained once a day to ensure an equivalent trial condition.

Behavioral Analysis

Morris Water Maze Test

The Morris water maze (MWM) test was used to measure the hippocampus-dependent spatial memory, as described before.[27] The pool was filled with water (22+2) °C, and the hidden platform (4.5 cm in diameter) was submerged 1 cm below the opaque water surface in the third quadrant of the pool. All mice were trained in a learning period for four trials per day for five consecutive days. In each trial, mice were placed into water facing the pool wall from quadrant 4 in sequence. After 60 seconds to find the hidden platform, mice that failed to find the hidden platform in 60 seconds were guided to the platform, where they remained for 10 seconds. After five days of training, mice were returned to the pool from the first quadrant, with the hidden platform absent for 60 seconds. The swim path was recorded by the video analysis system from Shanghai Jiliang Software Technology Co, Ltd (Shanghai, China).

Novel Object Recognition Task

The aim of the novel object recognition (NOR) task was to measure episodic hippocampal memory, as previously described.[28] All mice were put into the apparatus 30 minutes before beginning the task to habituate to the situation. In the training session, two identical objects were placed near the two corners at either end of one side of the chamber. Mice were placed individually into the open field facing the center of the opposite wall and allowed to explore the objects for 10 minutes. After 60 minutes, the test session was performed and the mice allowed to explore two dissimilar objects for 5 minutes. Exploration was defined by directing the nose or forepaws to the object at less than 5 cm and/or touching the object. Moving the object or standing on the object was not considered exploratory behavior. The time spent exploring the familiar object and the novel object was calculated by computer. The discrimination index and the preference index were calculated as the time spent with the novel object divided by the total time

spent exploring either object in two sessions. To prevent olfactory cues, objects and arenas were cleaned with 70% ethanol between trials.

Western Blot

Western-blot experiments were performed as previously described in our study.[16] Briefly, hippocampal tissues were isolated and immediately frozen in liquid nitrogen, then homogenized with RIPA lysis buffer (Beyotime) supplemented 1 mM phenylmethanesulfonyl fluoride (PMSF) and inhibitors of protease and phosphatase (10 μg/mL each of aprotinin, leupeptin, and pepstatin). An aliquot of 40 μg of proteins of tissue lysate was electrophoresed on 10% and 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked in 5% nonfat milk for one hour at room temperature and then incubated overnight at 4 °C with the following primary antibodies: rabbit anti–glutamate decarboxylase 65 (GAD65) (1:2000, Abcam), mouse anti–parvalbumin (PV) (1:3000, Abcam), rat anti–somatostatin (SST) (1:2000, ImmunoStar), rabbit anti–calretinin (CR) (1:2000, Abcam), mouse anti–gephyrin (1:1000, Abcam), rabbit anti–vesicular GABA transporter (VGAT) (1:1000, Santa Cruz), rabbit anti–synaptophysin (1:1000, Abcam), rabbit anti–postsynaptic density 95 (PSD95) (1:2000, Santa Cruz), or rabbit anti–β-actin (1:1000, Abcam), respectively. After several washes, the membranes were incubated with Alexa Fluor 488 donkey antirat, Alexa Fluor 488 goat anti-rabbit, or Alexa Fluor 488 goat antimouse (1:3000, Cell Signaling Technology) for one hour at room temperature. Films were scanned with Image Quant software (Tanon, Shanghai, China). ImageJ was used to quantify the intensities of protein bands. The relative protein expression was normalized to β-actin.

Immunostaining

The procedure of immunofluorescence was performed as previously described.[29] For animal experiments, mice were anesthetized with 1% pentobarbital sodium and intracardially perfused with saline, followed by 4% paraformaldehyde (PFA, in 0.1M phosphate buffer, pH = 7.4). Mice brains were removed postfixed in 4% PFA for 24 hours and dehydrated in 15% and 30% sucrose at 4 °C. Brains were embedded in optimum cutting compound and cut into 40 μm sections. Free-floating sections were washed in phosphate-buffered saline (PBS), blocked in a buffer containing 1% bovine serum albumin, 10% goat serum, and 0.3% Triton X-100 (Sigma Aldrich) for one hour at room temperature, and then incubated with primary antibodies (rabbit anti-PV (Abcam), rat anti-SST (Millipore), rabbit anti-CR (Abcam), rabbit anti–beta amyloid 1–42 antibody (Abcam)) at 4 °C for 24 hours (1:500 dilution in NCM universal antibody diluent (New Cell & Molecular Biotech Co, Suzhou, China) for all antibodies). After being washed in PBS, sections were incubated with secondary antibodies (Alexa Fluor 488 goat antirabbit, Alexa Fluor 488 goat antirat [Abcam]) at 37 °C for one hour (1:800 dilution in NCM universal antibody diluent for all antibodies). Finally, sections were then washed in PBS, and 4', 6-diamidino-2-phenylindole counterstaining was performed to label cell nuclei. The fluorescent images were taken using a confocal microscope (Nikon Eclipse Ti, Japan). The total number of PV+, CR+, SST+ neurons or amyloid 1–42 plaques in dCA1 (4–6 sections per mouse) was acquired using ImageJ (NIH).

In Vitro Electrophysiology

Slice Preparation

The slices were prepared as before.[30] In brief, mice (aged six months) were decapitated after being anesthetized; next, the brain was placed in Vibroslice (VT 1200S; Leica) to prepare hippocampus slices (300 μm) in ice-cold ACSF. For whole-cell recording, the slice-cutting solution NMDG-HEPES ACSF (in mM) contained 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 sodium ascorbate, 3 sodium pyruvate, 0.5 $CaCl_2 \cdot 2H_2O$, and 10 $MgSO_4 \cdot 7H_2O$. After being cut, the slices were recovered in 300 mL NMDG-HEPES ACSF for 35 minutes, with the temperature at 33 ± 1 °C. Next, slices were transferred into the HEPES holding ACSF at room temperature for 1 to 1.5 hours. The HEPES holding ACSF was (in mM) 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 sodium ascorbate, 3 sodium pyruvate, 2 $CaCl_2 \cdot 2H_2O$, and 2 $MgSO_4 \cdot 7H_2O$.

Electrophysiological Recording

Brain slices were recovered in holding ACSF and then shifted to the recording chamber, continuously perfused (3 mL/min) with recording ACSF at 32 to 34 °C. The recording ACSF (in mM) contains 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 12.5 glucose, 5 HEPES, 2 $CaCl_2 \cdot 2H_2O$, and 2 $MgSO_4 \cdot 7H_2O$. All solutions were filled with 95% O_2/5% CO_2 (vol/vol) and adjusted to 280 to 310 mOsm. Whole-cell membrane patch-clamp recording at CA1 in the hippocampus was accomplished using an infrared-sensitive CCD camera (ORCA-Flash4.0 LT C11440, HAMAMA-TSU, Japan) with a 40× water-immersion lens (NIR APO DIC N2; Nikon, Japan) and vertical microscope. The patch pipette (4–5 MΩ) solution contained (in mM) 125 CsCH3SO3, 10 HEPES, 5 CsCl, 0.2 EGTA, 4 Mg-ATP, 1 MgCl2, 5 Qx-314, 10phosphocreatine, 0.3 Na-GTP, pH 7.30, and 280~300 mOsm. The spontaneous excitatory postsynaptic currents (sEPSCs) were recorded with a voltage clamp at −60mV, whereas the spontaneous inhibitory postsynaptic currents (sIPSCs) were measured at +10mV; all recordings were used with a 1550B digitizer and Multiclamp 700B amplifier (Molecular Devices, CA) and analyzed with Clampfit 10.7 (Molecular Devices). All data were collected with the series resistance in the initial values (20–30 MΩ), sampled in 10 kHz, and filtered in 1 kHz. All solution formulation was according to the protocol by Jonathan T. Ting et al.[31]

In Vivo Electrophysiological Recordings

After the behavior tests, mice from the three groups were anesthetized with isoflurane (RWD, Shenzhen, China) and placed in a stereotactic apparatus for a long-term implant surgery. The scalp was shaved, and 75% ethanol was used to sterilize the surgical area before the scalp was exposed. The coordinate site for local field potential (LFP) recordings was determined according to the mice brain atlas in stereotaxic coordinates (anterior, 1.5 mm; lateral, 2.0 mm; horizontal, 1.5 mm from bregma). Three self-tapping screws (F000CE094, Morris Precision Screws and Parts) were attached to the skull, and 16-channel microelectrode arrays (1 × 0.15 mm, with 0.1 mm interelectrode spacing, nickel chromium, < 1MΩ) were implanted into mice hippocampus CA1 under aseptic conditions; finally, a custom stainless-steel headplate was affixed using dental cement (Shanghai New Century Dental Material Co, Ltd). The LFP recordings were conducted one week after the microelectrode implant surgery.

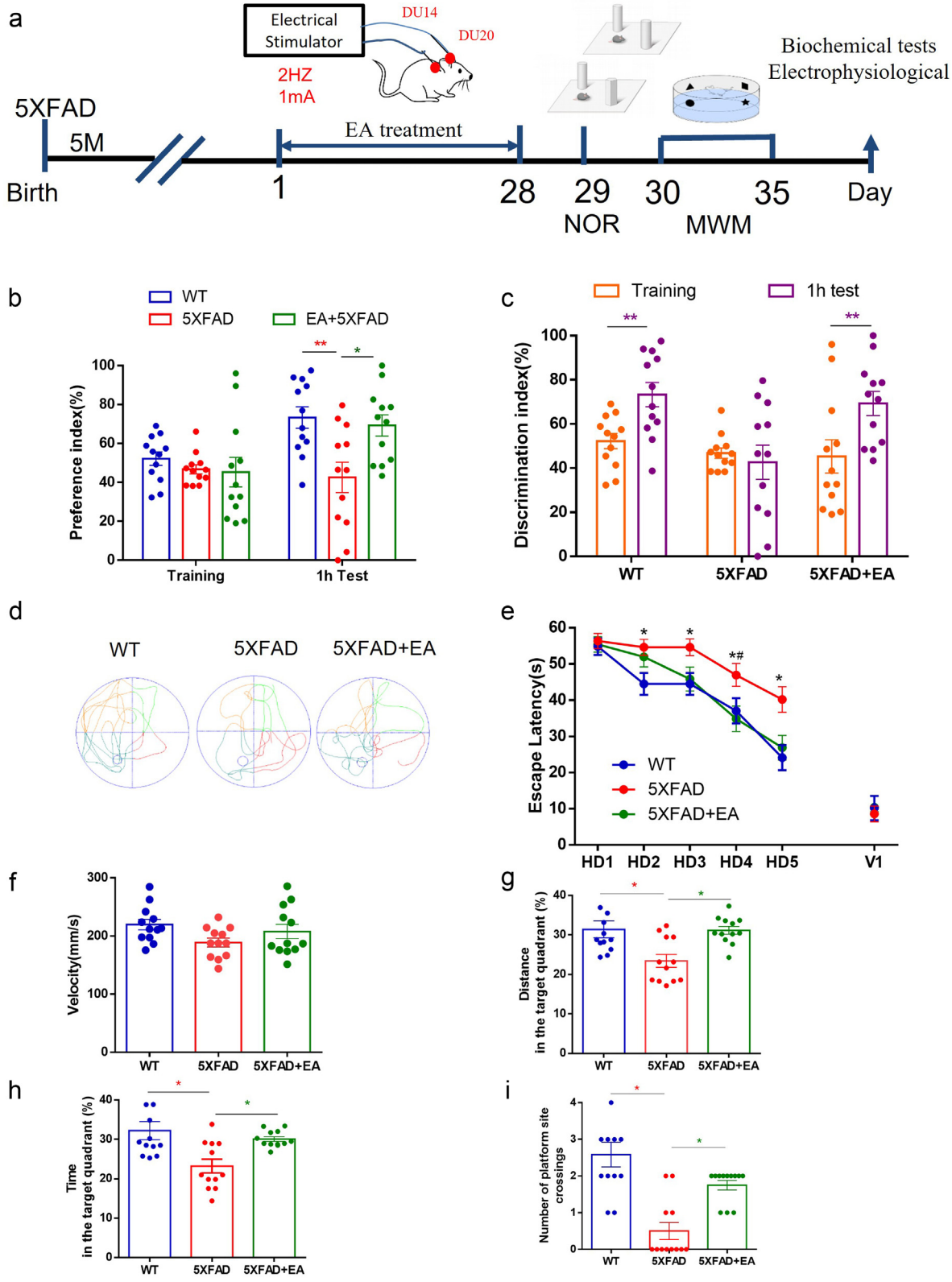

Figure 1. EA ameliorated memory deficit in 5xFAD mice. a. The experimental schedule of the 5xFAD mice, EA treatment, behavior test, biochemical tests, and electrophysiological recording. The preference index (b) and the discrimination index (c) in novel object recognition test among the WT-mice group, the 5xFAD-mice group, and the 5xFAD +EA-mice group. The WT-mice group and the 5xFAD +EA-mice group spent more time exploring the novel object than the familiar one, whereas no difference was found in the 5xFAD-group mice. (n = 12 mice per group. t-test, t = 0.32, p = 0.0037 for WT group; t = 0.61, p = 0.5109 for 5xFAD group; t = 2.568, p = 0.0176 for 5xFAD +EA group). d. Representative swim paths in target quadrant from each group during the probe trials. e. Increased latency in 5xFAD mice to reach the hidden platform in MWM, compared with WT mice, and latency was reduced by EA stimulation. (n = 12 per group. Group: $F_{2,94}$ = 15.098, $p < 0.05$; day:

Nissl Staining

Mice were perfused with 4% paraformaldehyde (PFA, pH 7.4) after anesthesia with 1% pentobarbital sodium, as previously described in detail.[32] Briefly, after postfixation in the 4% PFA for 24 hours at 4 °C and dehydration in 15% and 30% sucrose at 4 °C, the brains were cut into 40 μm-thick coronal sections on a cryostat (Leica CM1900-1-1, Germany). The slices of hippocampus were mounted on slides with polylysine. The slices were dried overnight and rehydrated in distilled water. After 20 minutes submergence in 1% cresyl violet, the slices were rinsed in distilled water and dehydrated in graded series of ethanol. Next, sections were immersed in xylene and mounted in neutral balsam, and the covers lipped. Nissl staining was performed to examine whether the microelectrode was placed in the pyramidal layer of the medial CA1 region.

Statistical Analysis

All data in this study were analyzed through the program of Prism 8.0 (Graphpad Software Inc). The results of automatic or blind measurements were expressed as mean ± SEM and analyzed using one-way or two-way analysis of variance (ANOVA) with least significance difference post hoc tests or using Student's *t* test when applicable. Differences of $p < 0.05$ are considered statistically significant.

RESULTS

EA Ameliorated Cognitive Impairments in 5xFAD Mice

To investigate the effects of EA treatment on spatial reference, recognition learning, and memory in 5xFAD mice, we conducted both the NOR task and MWM test (Fig. 1a). In the NOR test exposure, the control group and the 5xFAD receiving EA treatment (5xFAD +EA) mice spent more time with a novel object than with a familiar one (with increased discrimination index and preference index). However, no significant object-discrimination differences were detected with the 5xFAD group (Fig. 1b,c). In the MWM test (Fig. 1e), the escape latency in all mice groups showed a downward trend over five consecutive days. However, the 5xFAD +EA mice exhibited shorter escape latency than did the 5xFAD mice. On day 6 of the testing session, the navigation paths showed that the times of passing the hidden platform position were increased in the 5xFAD +EA mice compared with the 5xFAD group (Fig. 1d,i). Simultaneously, the 5xFAD +EA-group mice spent more time and longer distance in the target quadrant than in other quadrants, than did the 5xFAD-group mice (Fig. 1g,h). No difference was found between the 5xFAD +EA mice and littermate control mice. No differences in velocity were seen among the three groups (Fig. 1f). Together, these results suggest that EA treatment can ameliorate the cognitive impairments in MWM and NOR tests of 5xFAD mice.

EA Treatment Increases the Expression of Gephyrin, Glutamate Decarboxylase 65, and Somatostatin Proteins in Five-Month-Old 5xFAD Mice

To better understand the molecular mechanism underlying cognitive and memory deficits, we investigated whether the deficiency of synaptic proteins and GABAergic neuron related proteins (which are known to play key roles in inhibitory synaptic transmission) were recovered by using EA treatment in the hippocampus of 5xFAD mice (Fig. 2a). Western-blot analysis revealed a decrease in the level of gephyrin protein in the 5xFAD group mice compared with that in the littermate WT mice, whereas gephyrin protein expression level was significantly enhanced after EA treatment (Fig. 2b). No significant difference was found in levels of synapsin1 and (PSD95) proteins (Fig. 2d,e) or in VGAT levels (Fig. 2c). These results indicate EA treatment reversed the down-regulated levels of inhibitory synaptic proteins in the hippocampus of 5xFAD mice.

According to the results of enhanced inhibitory synaptic protein expression by EA treatment in 5xFAD mice, we further explored whether EA could regulate interneuron-related proteins. Here, we measured the expression of GAD65, PV, SST, and CR proteins in the hippocampus (Fig. 2f). We found EA treatment reversed the reduced GAD65 and SST protein levels in the hippocampus of 5xFAD mice (Fig. 2g,j), whereas the level of PV protein expression level did not change (Fig. 2h). No difference was observed in the CR protein expression level among the three groups (Fig. 2i). These results suggest that EA upregulates the decreased expression of inhibitory synaptic proteins.

EA Treatment Alleviates SST Neuron Reduction in the Dorsal Hippocampus of Five-Month-Old 5xFAD Mice

Interneuron loss in the dorsal hippocampus has been observed in 5xFAD mice,[33] and transplant Nav1.1-overexpressing interneuron, which is predominantly present in PV-expressing interneurons, could alter network activity and cognitive dysfunction in AD.[6] To further explore whether EA treatment plays a role in interneurons in AD process, we have conducted PV, CR, and SST immunostaining in the three subfields CA1 (comprising the CA1 pyramidal cell layer, stratum oriens, stratum radiatum, and stratum lacunosum moleculare), CA3 (comprising stratum oriens and stratum lucidum), and dentate gyrus (DG, comprising molecular layer, granule cell layer, and hilus). The number of PV+ and SST+ cells was significantly reduced in the 5xFAD-group mice in the three subfields compared with the number in the littermate WT mice (Fig. 3a,b), whereas numbers of CR+ cells were unchanged (Fig. 3c). Quantitative analyses of the number of PV+ cells revealed a significant increase in the dorsal part of the CA1 area in the 5xFAD +EA group compared with that of the 5xFAD group (Fig. 3b). We found that EA treatment enhanced the reduced number of SST+ cells in the total dorsal hippocampus (Fig. 3d). It has been reported that SST expression is reduced by 50% in AD and that it is related to the formation of Aβ oligomers[34] (AβO). Therefore, we found that EA treatment alleviated Aβ1–42 deposition in 5xFAD mice (Supplementary Data Fig. 1a,b). These results suggest that EA alleviates SST-neuron reduction in the dorsal hippocampus and PV-neuron reduction in the CA1 area of five-month-old 5xFAD mice.

EA Treatment Mitigates E/I Imbalance in 5xFAD Mice

GABA interneuron dysfunction has previously been associated with network hyperexcitability in AD,[35] and the neuronal network

$F_{4,188} = 30.689$, $p < 0.001$; group × day: $F_{6,282} = 1.423$, $p > 0.05$; two-way ANOVA with repeated measures). f. Swim speeds did not differ significantly among all groups ($n = 12$ mice per group; one-way ANOVA, $p > 0.05$). g, h. Reduced percentage of time, distance spent in target quadrant of mice in 5xFAD group, compared with mice in WT group, and percentage of time and distance spent in platform quadrant was increased after EA treatment ($n = 12$ mice per group; one-way ANOVA, $F = 6.974$, $p = 0.0030$ for distance; $F = 6.997$, $p = 0.0029$ for time). i. Platform crossing times in the testing phase, probe trial on day 6 of the MWM test ($n = 12$ mice per group. $F = 215.9$, $p < 0.001$). Values are the mean ± SEM: $^*p < 0.05$, $^{**}p < 0.01$. [Color figure can be viewed at www.neuromodulationjournal.org]

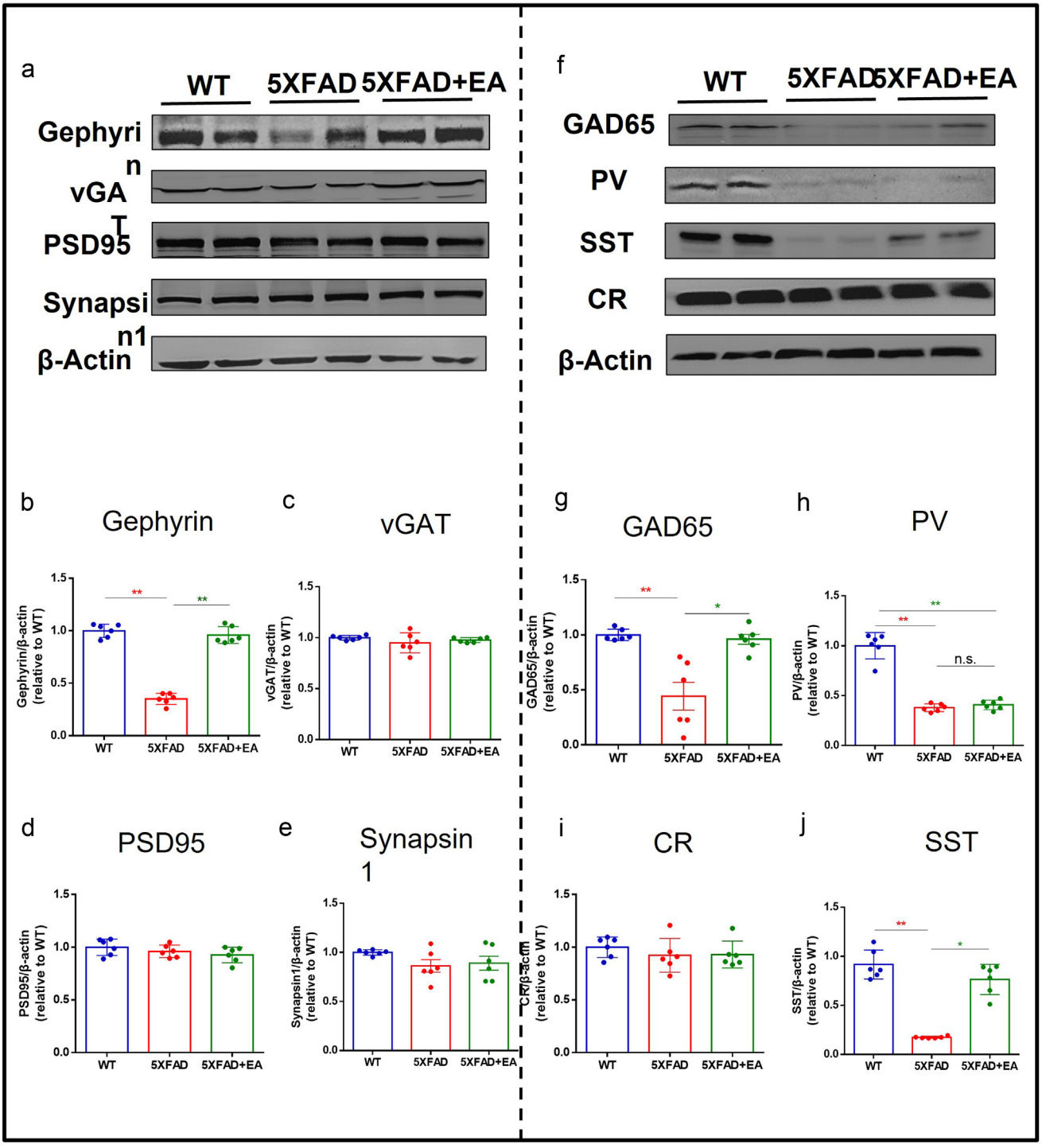

Figure 2. EA treatment improved inhibitory synaptic protein and SST protein expression in the hippocampus of 5xFAD mice. a. Representative Western blot of synapse-associated proteins in the hippocampus. b–e. Quantification of Western blot of the synapse-associated proteins in the hippocampus ($n = 6$ mice per group; one-way ANOVA, $F = 49.43$, $p < 0.001$ for gephyrin protein; $F = 1.498$, $p > 0.05$ for VGAT protein; $F = 0.188$, $p > 0.05$ for PSD95 protein; $F = 0.505$, $p > 0.05$ for synapsin1 protein). f. Representative Western blot of GABA-associated proteins in the hippocampus. g–j. Quantification of Western blot of the GABA-associated proteins in the hippocampus ($n = 6$ mice per group; one-way ANOVA, $F = 49.79$, $p < 0.001$ for GAD65 protein; $F = 116.6$, $p < 0.001$ for PV protein; $F = 1.802$, $p = 0.199$ for CR protein; $F = 12.39$, $p < 0.001$ for SST protein) ($n = 6$ mice for each group). The levels of synapse-associated proteins were standardized on the basis of the respective level of β-actin. The values were expressed as relative changes to the respective WT mice, which were set to 1. Data are the mean ± SEM: *$p < 0.05$, **$p < 0.01$. n.s., not significant. [Color figure can be viewed at www.neuromodulationjournal.org]

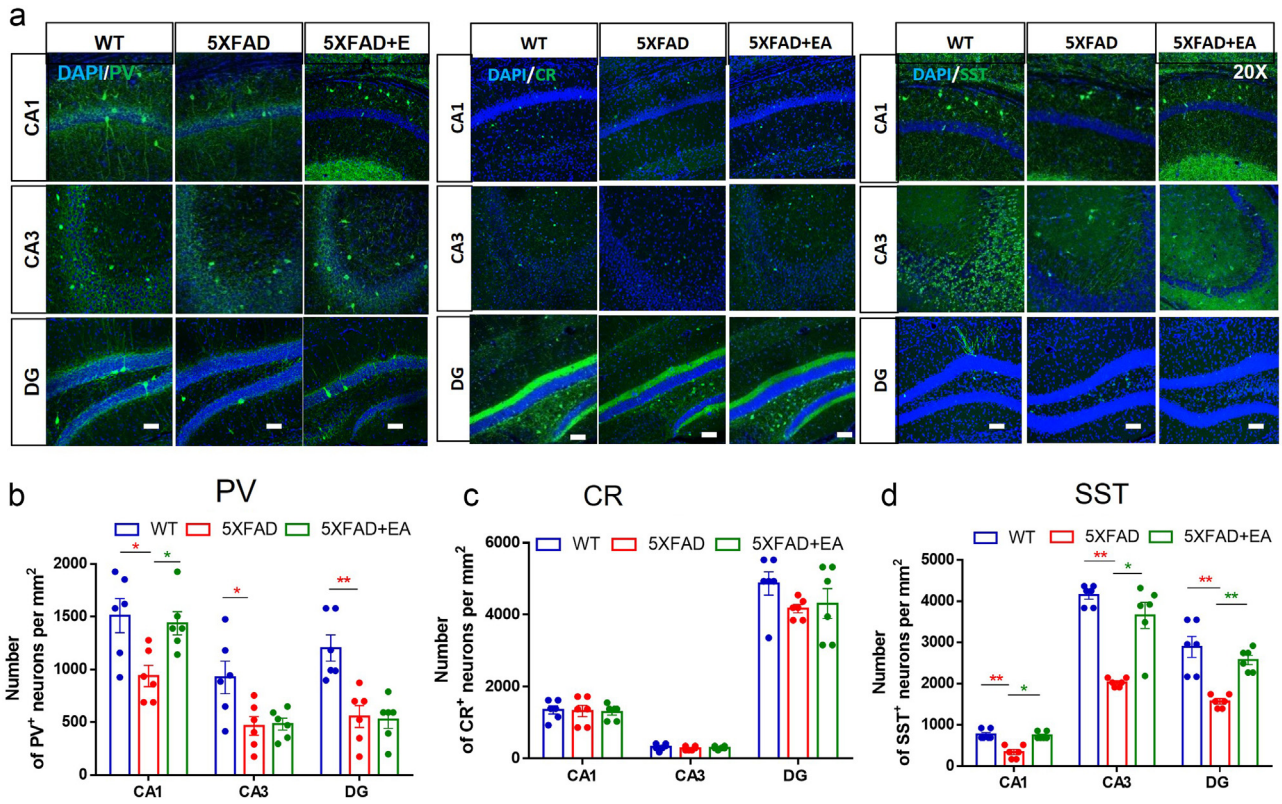

Figure 3. EA treatment alleviates SST-neuron reduction in the dorsal hippocampus of five-month-old 5xFAD mice. a. Representative images showing the distribution of PV neurons or CR neurons or SST neurons in the CA1, CA3, and DG regions in the dorsal hippocampus of 5xFAD and WT mice. Scale bar, 50 μm. b. Number of PV + neurons per mm^2 in the CA1, CA3, and DG regions in the dorsal hippocampus of three groups (one-way ANOVA; for CA1 region, F = 6.065, $p = 0.012$; for CA3 region, F = 5.907, $p = 0.053$; for DG region, F = 3.124, $p = 0.062$). c. Number of CR + neurons per mm^2 for CA1, CA3, and DG regions in the dorsal hippocampus among three groups (one-way ANOVA; for CA1 region, F = 0.057, $p = 0.945$; for CA3 region, F = 0.674, $p = 0.525$; for DG region, F = 1.399, $p = 0.277$). d. number of SST + neurons per mm^2 of CA1, CA3, and DG region in the dorsal hippocampus of WT-, 5xFAD-, and 5xFAD +EA-group mice (one-way ANOVA, for CA1 region, F = 12.870, $p = 0.0474$; for CA3 region, F = 12.192, $p = 0.002$; for DG region, F = 17.710, $p < 0.00$). (WT, $n = 6$; 5xFAD, $n = 6$; 5xFAD +EA, $n = 6$). Data are the mean ± SEM: $^*p < 0.05$, $^{**}p < 0.01$. [Color figure can be viewed at www.neuromodulationjournal.org]

alterations have been identified as impairments of excitatory and inhibitory synaptic transmission.[36] Therefore, we hypothesized that the decreased inhibitory synaptic proteins might accompany E/I imbalance. Accordingly, we recorded sEPSCs and sIPSCs of the same pyramidal neurons in dCA1 (Fig. 4a). The 5xFAD mice exhibited a dramatically lower sIPSCs frequency and area than did the control littermates. In these mice, EA treatment upregulated the sIPSCs frequency. No change in amplitude was seen among the three groups (Fig. 4e–h). We found that the frequency and area of sEPSCs on the dCA1 pyramidal neurons increased significantly in 5xFAD mice compared with those in the WT group. EA treatment significantly reduced the frequency and area of sEPSCs but did not change the amplitude from the level of the 5xFAD-group mice (Fig. 4c,d,g). The charge ratio of sEPSC/sIPSC in 5xFAD mice increased compared with that of the control mice, whereas EA treatment significantly decreased the E/I ratio compared with that of the 5xFAD-group mice (Fig. 4i). These results suggest that EA treatment can rescue the impaired synaptic transmission and normalize the E/I balance in the dorsal hippocampus that may contribute to cognitive deficit in 5xFAD mice.

EA Enhances Theta and Gamma Oscillations in 5xFAD Mice

Network oscillation dysfunction, such as impaired theta and gamma oscillation, is associated with GABAergic microcircuits.[37] To determine whether EA can regulate the decreased inhibitory input from GABA interneurons in the hippocampus of five-month-old 5xFAD mice, we recorded LFPs simultaneously in the CA1 areas of hippocampal in 5xFAD mice, 5xFAD +EA mice, and WT mice (Fig. 5a,b). As indicated by the corresponding spectrograms of the representative LFP traces (Fig. 5e–g), theta and gamma oscillations could be reliably recorded in all animals. Comparing these, we found that the power of theta and gamma oscillation was significantly lower in 5xFAD mice than in WT mice, whereas EA treatment reversed the decreased theta and gamma power of LFPs in 5xFAD mice (Figs. 5c and 5d). This finding suggests that the decreased theta and gamma oscillation present in the 5xFAD mice could be rectified by EA treatment.

DISCUSSION

In our study, we found that four weeks of EA stimulation at DU20 and DU14 acupoints could effectively ameliorate memory deficits, improve the expression of gephyrin and SST proteins, normalize E/I imbalance, and enhance theta and gamma oscillation in the hippocampus of 5xFAD mice.

Our results showed that five-month-old 5xFAD mice exhibited significant memory deficits in NOR and MWM testing, whereas EA treatment at DU20 and DU14 acupoints ameliorated cognitive

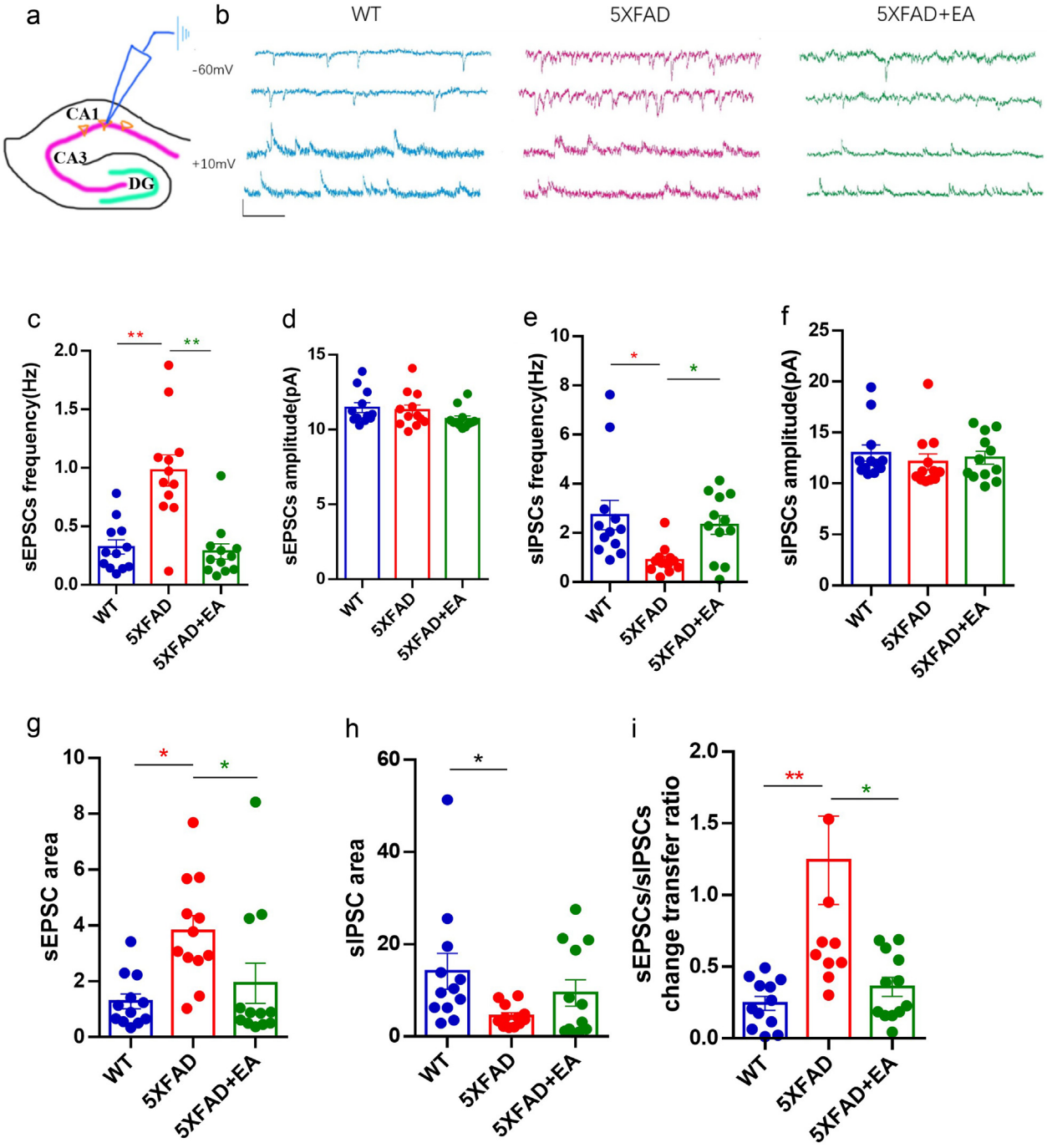

Figure 4. EA normalized E/I imbalance in 5xFAD mice. a. Schematic of the patch-clamp experimental set up: electrode was placed in the dorsal hippocampus CA1. b. Representative recording from WT, 5xFAD, and 5xFAD +EA mice at −60mV and +10mV. Similar recordings from age-matched 5xFAD mice are shown in red. The pyramidal cells recorded in WT mice are shown in blue, and 5xFAD +EA mice are shown in green. Scale bars on the left refer to all four data sets. c–f. sEPSCs frequency was significantly higher in the 5xFAD mice, whereas amplitude did not significantly change in these pyramidal cells and was correlated with a significantly lower frequency of sIPSCs. EA treatment significantly lowered sEPSC frequency and enhanced sIPSC frequency. Amplitude did not differ in the excitatory cells among the three groups. (n = 12 cells from three mice per group, one-way ANOVA, F = 16.76, $p < 0.001$ for sEPSC frequency; f = 1.608, $p > 0.05$ for sEPSC amplitude; F = 4.781, $p = 0.0153$ for sIPSC frequency; F = 0.456, $p > 0.05$ for sIPSC amplitude). g, h. sEPSCs area was significantly higher in 5xFAD mice whereas sIPSCs area was significantly decreased in these pyramidal cells. EA treatment significantly lowered sEPSC area but did not alter sIPSC area. (n = 12 cells from three mice per group, F = 5.159, p = 0.0114 for sEPSC area; F = 2.776, $p > 0.05$ for sIPSC area). i. Quantification of the sEPSCs:sIPSCs ratio in pyramidal neurons among WT-, 5xFAD-, and 5xFAD +EA-group mice. (n = 12 cells from three mice per group, one-way ANOVA, F = 8.799, $p < 0.001$). n = 12 or 13 cells from six mice per group. (Data are the mean ± SEM: *p <0.05, **p <0.01, n = 3 animals per cohort, scale bars: 2 seconds, 50 pA). [Color figure can be viewed at www.neuromodulationjournal.org]

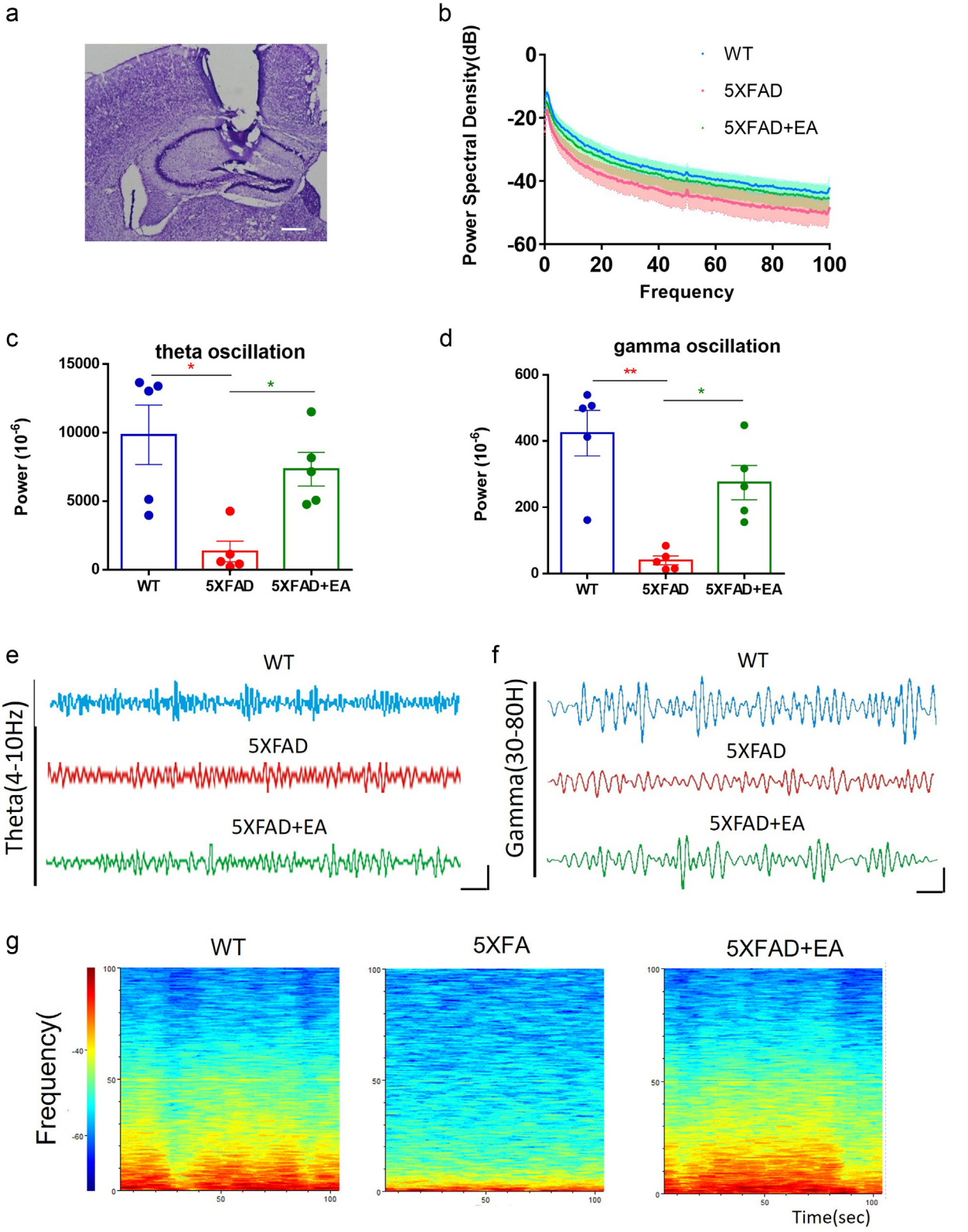

Figure 5. EA treatment increased power in theta and gamma of 5xFAD mice. a. Nissl staining showed the site of microelectrode placed. b. LFP recorded in CA1 among WT-, 5xFAD-, and 5xFAD +EA-group mice. c. Mean and standard deviation of the normalized power spectrum during theta in three groups of mice (n = 5 mice per group, one-way ANOVA, F = 8.480, p = 0.0051). d. Mean and standard deviation of the normalized power spectrum during gamma in three groups of mice (n = 5 mice per group, one-way ANOVA, F = 14.85, $p < 0.001$). e. Representative theta oscillation trace and hot plot of three groups. (Scale bars: 500 milliseconds, 200μV). f. Representative gamma oscillation trace and hot plot of three groups. (scale bars: 200 milliseconds, 200μV). g. Time-resolved power spectra averaged across electrode in dCA1 among three groups, but for a broader band of the frequency spectrum (0–100 Hz) (Data are the mean ± SEM: *$p < 0.05$, **$p < 0.01$). [Color figure can be viewed at www.neuromodulationjournal.org]

impairment, which is consistent with previous studies.[38–40] Another study reported that electroacupuncture at Baihui (GV 20) and Shenting (GV 24) ameliorated neuroinflammation-mediated cognitive deficits in Ps cDKO mice.[41] Clinical research indicated that EA improved the cognitive function of patients with mild impairment.[42] Together, research strongly indicates that EA can provide effective complementary therapy for dementia. Furthermore, our study showed that EA stimulation can improve the expression of SST interneurons, in addition to the inhibitory synaptic proteins gephyrin and GAD65. Gephyrin is a microtubule and dynein light chain-binding protein, transported along microtubule tracks, that was proposed to be anchored to microtubules at postsynaptic membrane specializations.[43] GAD65 is preferentially found in nerve terminals and considered to be the source of the neurotransmitter GABA.[44] In postmortem AD brains and AD model animals, decreased frontal, temporal, and parietal GABA concentrations were observed, in addition to decreased gephyrin and GAD65 protein expression.[45,46] In this study, we found a reduction in hippocampal GAD65 expression of 5xFAD mice; however, some controversial findings in recent studies showed high GABA concentration in reactive astrocytes in the DG of 5xFAD mice,[47,48] so some factors should be considered for these results. First, differences in age and sex in 5xFAD mice were present among these studies, and GABA concentration may be dynamic with age and sex differences. Second, astrocytes contribute to the complex cellular pathology of AD, especially in the glutamate/GABA-glutamine cycle; several astrocyte-specific components of the glutamate/GABA-glutamine cycle are perturbed in AD, whereas restoring astrocyte metabolism may serve as an approach to arrest or even revert the clinical progression of AD.[49] Our study has not investigated the role of astrocytes, and we will conduct more research on reactive astrocytes in relation to GABA activity. Meanwhile, we did not find any change in expression of excitatory synaptic proteins or VGAT protein. VGAT is the vesicular transporter of GABA (mainly in GABAergic and glycinergic presynaptic terminals)[50] and has different functions to those of gephyrin, which forms the postsynaptic scaffold of the inhibitory synapse or GAD65 (GABA-synthesizing enzymes).[51] This suggests that the increased inhibitory postsynaptic proteins and GABAergic neuron proteins may contribute to the effect of EA on memory deficits. Interestingly, this study indicated that EA treatment especially improves SST-protein expression, which specifically acts on associational/commissural synapses.[52] Taken together, these results suggest that EA treatment can improve inhibitory synaptic function, likely through SST+ neurons.

Other recent studies indicate that the disruption of GABA receptors leads to E/I imbalance, which has been regarded as a key mechanism of both epileptogenesis and neurodegeneration disorder.[53,54] At present, all approved clinical drugs for AD are modulators of the cholinergic and glutamatergic systems and have shown limited effect.[55] Our research suggests that EA may target GABA receptors and normalize E/I imbalance. Overexpression of Aβ-induced network hyperexcitability in early AD model mice,[56] in turn, would exacerbate the aggregation of Aβ. Studies showed that Aβ impairs the inhibitory synaptic transmission from the dysfunction of the GABAergic interneurons, leads to hyperexcitability of pyramidal neurons, and often exhibits increased E/I balance.[57] In our study, results indicate that EA treatment may ameliorate synaptic impairment through regulation of GABAergic interneurons. However, SST+ and PV+ interneurons could play different important roles in the hippocampal inhibitory network, owing to their varying abilities to effectively synchronize dendritic inhibition and mediate lateral inhibition, respectively.[58] According to our results, EA may improve synapse-specific dysfunctions by regulating PV and SST interneurons and improving E/I balance. This ameliorating effect on synaptic function may be due to a close relationship with GABA postsynaptic protein gephyrin and an increased expression of SST protein, but the specific mechanism still needs to be further explored.

Gamma and theta oscillations are the most widely studied because of their close relation with cognitive function and memory task. Gamma oscillation is also involved in the process of memory recall, which was observed in the electroencephalogram results of patients with AD.[59] Reports showed that gamma oscillations are produced mainly by a network of inhibitory interneurons, and hence, gamma oscillations synchronize neurons by activating local excitatory neurons and rapidly inhibiting neurons related to synaptic plasticity.[60] Theta rhythm is also involved in the EC–CA1 circuit and is important in memory arousal. It is reported that exogenous Aβ injection would disrupt theta rhythm in the hippocampus and lead to a decrease in learning and memory ability.[61] Interestingly, interneuron subtype-specific dysfunctions of SST- and PV-interneuron input to CA1 pyramidal cells may selectively lead to theta and gamma frequencies, respectively, by AβO-induced impairments in early AD.[62,63] In addition, this study proves that Nav1.1-overexpressing interneuron transplants enhance the behavior-dependent modulation of gamma oscillations.[6] We found decreased gamma and theta oscillations in 5xFAD mice, and EA treatment could attenuate the impaired oscillations. A previous study showed that causal disruption of either PV+- or SST+-expressing interneuron activity impairs the generation of slow γ oscillations in the ventral hippocampus ex vivo, and SST+ interneuron activation, along with general network excitation, is sufficient to generate high-frequency γ oscillations in the same preparation.[64] This, combined with our results above, indicates that EA may improve cognitive function through regulating mainly SST interneurons, which contribute to gamma and theta oscillation improvement.

Together, the results of this study strongly highlight that EA can reverse cognitive dysfunction and regulate SST interneurons in 5xFAD mice, indicating the beneficial role of EA in GABAergic system. Regarding the regulatory effects of EA on E/I imbalance and on oscillations of gamma and theta, we suggest that EA may attenuate cognitive impairment through modulating the inhibitory synaptic protein and different function of PV and SST interneurons. However, how EA regulates the GABAergic neurons needs to be further explored. Moreover, additional research in other brain areas and other ages of 5xFAD mice is needed to reveal the underlying mechanism of neuroprotection of EA and seek better strategies to ameliorate and prevent AD cognitive impairment.

CONCLUSIONS

In conclusion, our findings showed that EA at DU20 and DU14 improved memory deficits and inhibitory synaptic protein expression. We propose that the underlying mechanism of EA for normalization of E/I imbalance in addition to the enhancement of theta and gamma oscillation in the hippocampus of 5xFAD mice may be related to the function of SST+ and PV+ interneurons.

Therefore, EA stimulation at DU20 and DU14 acupoints may be a potential alternative therapy for ameliorating cognitive deficits in AD mice through regulating the function of GABAergic interneurons.

DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding author upon request.

Authorship Statements

Jiaying Zhao and Nenggui Xu designed the experiments. Hongzhu Li conducted the behavioral test, Western blot, and in vivo electrophysiology experiments. Runyi Wang and Xiaoling Fang conducted the electroacupuncture treatment. Lanfeng Lai conducted the in vitro electrophysiology experiments and the data analysis. Hongzhu Li and Jiaying Zhao wrote the manuscript. Nenggui Xu helped revise the manuscript. All authors read and approved the final manuscript.

How to Cite This Article

Li H., Lai L., Li X., Wang R., Fang X., Xu N., Zhao J. 2022. Electroacupuncture Ameliorates Cognitive Impairment by Regulating γ-Amino Butyric Acidergic Interneurons in the Hippocampus of 5 Familial Alzheimer's Disease Mice. Neuromodulation 2022; ■: 1–12.

SUPPLEMENTARY DATA

To access the supplementary material accompanying this article, visit the online version of *Neuromodulation: Technology at the Neural Interface* at www.neuromodulationjournal.org and at https://doi.org/10.1016/j.neurom.2022.11.014.

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COMMENTS

This study showed that EA improved memory deficits and inhibitory synaptic protein expression. It proposes that the underlying mechanism of EA for normalization of E/I imbalance as well as enhancement of theta and gamma oscillation in the hippocampus of 5xFAD mice may be related to SST+ and PV+ interneuron's function. Therefore, EA stimulation may be a potential alternative therapy for ameliorating cognitive deficits in AD mice through regulating GABAergic interneuron's function. This finding is very helpful for the study of the effect and mechanism of EA on AD.

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