Original Article

Electroacupuncture Alleviates Memory Deficits in APP/PS1 Mice by Targeting Serotonergic Neurons in Dorsal Raphe Nucleus*

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[Abstract] Objective: Alzheimer's disease (AD) has become a significant global concern, but effective drugs able to slow down AD progression is still lacked. Electroacupuncture (EA) has been demonstrated to ameliorate cognitive impairment in individuals with AD. However, the underlying mechanisms remains poorly understood. This study aimed at examining the neuroprotective properties of EA and its potential mechanism of action against AD. **Methods:** APP/PS1 transgenic mice were employed to evaluate the protective effects of EA on *Shenshu* (BL 23) and *Baihui* (GV 20). Chemogenetic manipulation was used to activate or inhibit serotonergic neurons within the dorsal raphe nucleus (DRN). Learning and memory abilities were assessed by the novel object recognition and Morris water maze tests. Golgi staining, western blot, and immunostaining were utilized to determine EA-induced neuroprotection. **Results:** EA at *Shenshu* (BL 23) and *Baihui* (GV 20) effectively ameliorated learning and memory impairments in APP/PS1 mice. EA attenuated dendritic spine loss, increased the expression levels of PSD95, synaptophysin, and brain-derived neurotrophic factor in hippocampus. Activation of serotonergic neurons within the DRN can ameliorate cognitive deficits in AD by activating glutamatergic neurons mediated by 5-HT_{1B}. Chemogenetic inhibition of serotonergic neurons in the DRN reversed the effects of EA on synaptic plasticity and memory. **Conclusion:** EA can alleviate cognitive dysfunction in APP/PS1 mice by activating serotonergic neurons in the DRN. Further study is necessary to better understand how the serotonergic neurons-related neural circuits involves in EA-induced memory improvement in AD.

Keywords: Alzheimer's disease; electroacupuncture; dorsal raphe nucleus; hippocampus; serotonergic neurons; glutamatergic neurons; 5-HT1B; cognitive impairment; chemogenetic manipulation; synaptic plasticity

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Alzheimer's disease (AD) is the predominant neurodegenerative condition and the primary cause of dementia[1]. It is distinguished by permanent cognitive impairment, amnesia, and the inability to perform personal daily tasks independently[2]. With the global population aging, it is projected that more than 150 million individuals will be diagnosed with AD by 2025[3], which poses a significant public health issue worldwide. The etiology of AD is characterized by the presence of amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs), which occur due to the aberrant buildup of Aβ and hyperphosphorylated tau proteins[2]. Despite extensive studies, no viable medications have been developed to cure this severe disease due to its complex pathophysiology, making it a top priority for further investigation.

In recent years, accumulating experimental evidence has suggested that the restoration of damaged brain circuits could be a highly promising method for treating AD[4, 5]. The cholinergic[6], noradrenergic[7], and serotonergic neural circuits[8] have a substantial influence on synaptic transmission, neuroinflammation, oxidative stress, and glucose metabolism in AD. These circuits offer new insights into comprehending and treating this disease[9]. Increasing evidence has elucidated the significant function of the serotonergic circuit, which includes serotonin (5-HT) and its receptors, in AD[8, 10]. The dorsal raphe nucleus (DRN) is the primary origin of 5-HT in the brain. It transmits projection fibers to several brain regions and influences a range of processes, such as memory, emotion, sleep and wakefulness, anxiety, and reward[11]. Increasing

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amounts of data indicate that the DRN is among the initial areas in which hyperphosphorylated tau proteins are expressed[12, 13]. Postmortem examinations have additionally shown that the loss of serotonergic neurons in the DRN commences at a very early stage, even in the absence of cognitive deterioration, in patients with AD[14–16]. The presence of early-stage disruption in the serotonergic system and a lack of 5-HT could contribute to the early onset of neuropsychiatric symptoms in AD[17, 18]. In addition, serotonergic neurons are the main target of selective serotonin reuptake inhibitors, which have been linked to a delayed progression from mild cognitive impairment to AD dementia[19]. These inhibitors have also been demonstrated to be neuroprotective in models of AD[20]. Consequently, the restoration of serotonergic dysfunction could offer promising prospects for the treatment of AD.

Acupuncture, a prominent nonpharmacological treatment in Chinese medicine, has been utilized for thousand years to address various neurological conditions, including stroke, pain, mood disorders, and sleeplessness. Recent clinical trials have demonstrated that acupuncture can successfully reduce cognitive deterioration in AD patients. These findings imply that acupuncture has the potential to be used as a complementary therapy for AD[21–23]. However, the underlying mechanisms remain unverified. Recent clinical studies have shown that acupuncture can enhance functional connectivity between the RN and other parts of the brain[24, 25]. In our previous study, we found that electroacupuncture (EA) can improve cognitive dysfunction in rats with AD-like pathology by modifying the glycogen synthase kinase-3β gene in the DRN through epigenetic mechanisms[26]. However, the effects of EA on 5-HTergic neurons in the DRN and synaptic plasticity have not yet been examined. We performed the present study to clarify whether serotonergic neurons in the DRN are involved in cognitive impairment in AD patients and to investigate whether EA influences the activity of serotonergic neurons in the DRN.

1 MATERIALS AND METHODS

1.1 Animals

The current animal study received approval from the Ethics Committee of Hubei University of Chinese Medicine (ethics number: HUCMS202206003) and was performed in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The mice used in the experiment were acquired from Liaoning Changsheng Biotechnology Co., Ltd. (China). The license number is SYXK(E) 2017–0067. The age of all the mice was 4 months, and their weight was 30.0 ± 2 g. The AD model mice used in the study were male APP/PS1 double transgenic mice, while the control group consisted of C57BL/6J mice. The mice were housed individually in cages under optimal conditions, with a 12-h light/dark cycle, a constant temperature of 20–22°C, and an air humidity of 50% ± 10%. Prior to the experiment, all the mice had unrestricted access to food and water and were allowed to adapt for a minimum of one week. For experiment 1, the mice were randomly assigned to 3 groups: the model group (APP/PS1), the control group (C57BL/6J wild-type mice), and the EA group (APP/PS1 + EA) (n=12 per group). The random number approach was employed for the assignment. For experiment 2, APP/PS1 mice were randomly allocated into 6 groups: model group, vehicle group, EA + Gi group, EA + vehicle group, EA group, and Gq group. Each group consisted of 12 mice. The comprehensive data are displayed in table 1.

1.2 Preparation of Viruses

Adeno-associated viruses (AAV2/9) were used for chemogenetic manipulation. The rAAV-Ef1a-DIO-hM3D(Gq)-EGFP-WPREs (PT-0988, BrainVTA, China) combined with rAAV-TPH2-CRE-WPRE-hGH polyA (PT-0396, BrainVTA, China) was designed to activate serotonergic neurons in the DRN. The rAAV-EF1a-DIO-hM4D(Gi)-EGFP-WPREs (PT-0987, BrainVTA, China) combined with rAAV-TPH2-CRE-WPRE-hGH polyA (PT-0396, BrainVTA, China) was designed to inhibit serotonergic neurons in the DRN. rAAV-EF1a-DIO-EGFP-WPRE-hGH polyA (PT-0795, BrainVTA, China) was used as the control vehicle.

1.3 Virus Injection

All procedures were conducted under stereotaxic guidance and aseptic conditions. First, the mice were sedated using 1% pentobarbital sodium and then secured onto the stereotaxic equipment after their heads were shaved. The position of the DRN was determined using Paxinos and Watson's Rat Brain Atlas (6th edition) using the following coordinates: anteroposterior (AP) –4.59 mm, mediolateral (ML) 0 mm, and dorsoventral (DV) –3.25 mm. The CA1 area was located at the following coordinates: AP –2.06 mm, ML ±1.13 mm, and DV –1.37

Table 1 The details of each experimental group

Experiment	Group	n	Descriptions of treatments
Experiment 1	Control group	12	None
	Model group	12	None
	EA group	12	EA treatment
Experiment 2	Model group	12	Model mice with i.p. saline
	Vehicle group	12	Model mice with vehicle virus injection and i.p. saline
	EA + Gi group	12	Model mice with EA treatment, Gi virus injection, and i.p. clozapine N-oxide
	EA + vehicle group	12	Model mice with EA treatment, vehicle virus injection, and i.p. saline
	EA group	12	Model mice with EA treatment
	Gq group	12	Model mice with Gq virus injection, and i.p. clozapine N-oxide

i.p.: intraperitoneal

mm. To activate or inhibit DRN serotonergic neurons, 200 nL of virus [either rAAV-Ef1a-DIO-hM3D(Gq)-EGFP-WPREs, rAAV-EF1a-DIO-hM4D(Gi)-EGFP-WPREs, or rAAV-EF1a-DIO-EGFP-WPRE-hGH polyA] was injected into the DRN area at a rate of 40 nL/min. A viral vector, namely, 200 nL rAAV-TPH2-CRE-WPRE-hGH polyA, was administered by injection into the CA1 region. The mice in the vehicle, EA + vehicle, and Gq groups were injected with rAAV-TPH2-CRE-WPRE-hGH polyA in the left CA1 area. The mice in the EA + Gi group received bilateral injections of rAAV-TPH2-CRE-WPRE-hGH polyA into the CA1 area. This was done since unilateral inhibition could be compensated for by the other side.

1.4 EA Treatment

At 14th day after the mice were injected with the virus, they were treated with EA. The EA intervention followed the methods reported in our earlier investigation[26]. The mice were securely immobilized in a supine position using our custom-made soft fabric materials without the administration of anesthetic. The mice in the EA group, EA + Gi group, and EA + vehicle group were administered EA at acupoint GV20 (Baihui) and alternating unilateral L23 (Shenshu). GV20 is positioned at the midpoint of the parietal bone, whereas BL23 is located next to the second lumbar vertebra, 3 mm away from the median dorsal line, as per the acupoint location standard for the experimental mouse[27]. Stainless steel needles, measuring 13 mm in length and 0.25 mm in diameter, were inserted at a 15° angle to a depth of 3–5 mm at GV20. They were also inserted vertically to a depth of 3–5 mm at BL23. Subsequently, the needle handles were connected to an EA apparatus (HANS-100A, China) with a frequency of 2 Hz and an intensity of 2 mA. This procedure was performed for 30 min every other day for a total of 1 month. During the course of the treatment, all of the animals were in a calm state and showed no indications of discomfort. The mice in the non-EA treatment groups were subjected to the same wrapping procedure without any additional interventions.

1.5 Chemogenetic Manipulation

Chemogenetic modification was conducted two weeks after viral injection. The mice in the EA + Gi group and EA + vehicle group were intraperitoneally administered clozapine N-oxide (1 mg/kg) (CNO, BrainVTA, China) or saline in identical amounts 30 min prior to EA treatment. Simultaneously, mice in the model, vehicle, and Gq groups were intraperitoneally injected with either CNO (1 mg/kg) or an equivalent quantity of saline.

1.6 Novel Object Recognition Test

Following the administration of EA, the novel object recognition (NOR) test was carried out to evaluate the recognition memory capacity of the mice. The experiment was conducted for 3 days. On the first day, the mice were acclimated to an empty space measuring 40 cm × 40 cm × 50 cm for 10 min. On the second day, the mice were reintroduced to the device with two identical objects, labeled A and B. The acquisition session lasting 10 min was performed on the third day. The mice were presented with the same experimental items, A and B. However, during the familiarization session also lasting 10 min, object B was replaced with object C that the mice had not previously become accustomed to. Exploratory activity is the act of using the nose or forepaws to smell or touch objects that are within a distance of less than 2 cm. The act of sitting or strolling about the object was not classified as a form of exploration behavior. The recognition index (RI) was established as the ratio of time spent examining a novel object to the total time spent exploring two objects[28].

1.7 Morris Water Maze Test

Following the completion of the NOR test, the Morris water maze (MWM) test was conducted to evaluate the spatial learning and memory abilities of the mice. The experimental approach was consistent with the methodology described in our prior investigation[26].

A water maze utilizing a circular pool with a diameter of 100 cm and a height of 50 cm was employed. The water temperature was 24 ± 2°C. The pool was partitioned into 4 quadrants of equal size. A white platform with a diameter of 10 cm was positioned 1 cm below the surface of the water. Following a 5-day experiment in which the mice were allotted one min to locate the hidden platform, a spatial probing test was conducted on the 6th day to assess memory retention. The swimming tracks were captured using an overhead video camera positioned above the pool. The tracking and analysis system captured and measured the escape delay and the time spent in the target quadrant.

1.8 Golgi Staining

A Golgi Stain Kit (Servicebio, China) was used to perform Golgi staining. Briefly, after quick washing with distilled water to remove the blood from the surface, a vibratome (Leica VT1000S, Leica Microsystems, Germany) was used to cut the coronal slices (5–10 mm thick) that contained the hippocampal regions. After fixation with Golgi stain fixing solution, the tissues were stained in Golgi staining solution at 26°C for 2 consecutive weeks. Then, the tissue blocks were placed into a fresh Golgi dye solution, which was changed at a frequency of 3 days. After staining, the brain tissues were transferred to tissue-processing solution for 1 h at 4°C without light for 3 days. Next, the brain tissues were cut into 60 μm thick blocks, followed by development using Golgi developer solution. Finally, the number of dendritic spines per 10 μm was determined using ImageJ software.

1.9 Western Blotting

The expression levels of 5-HT_{1B}, PSD95, SYP, BDNF, GluA 1, Glu 2/3, and VGLUT 1 were detected using Western blotting. The hippocampus was immediately separated on ice. Then, equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane via the wet transfer method. After blocking with 5% fat-free milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with the primary antibodies mentioned in table 2. Next, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for

Table 2 Antibody information

Antibodies	Immunofluorescence	Western blotting	Companies	Species	Cat No.
GAPDH	/	1:10000	ABclonal	Rabbit	AC002
5-HT_{1B}	/	1:1000	ABclonal	Rabbit	A18285
PSD95	/	1:500	ABclonal	Rabbit	A0131
SYP	/	1:1000	Santa Cruz	Mouse	Sc-166940
BDNF	/	1:1000	ABclonal	Mouse	A18129
GluA 1	/	1:1000	ABclonal	Rabbit	A11643
Glu 2/3	/	1:500	ABclonal	Rabbit	A2754
VGLUT 1	/	1:1000	Abcam	Rabbit	Ab227805
c-Fos	1:300	/	Servicebio	Mouse	GB12069
TPH2	1:500	/	Abcam	Rabbit	ab184505
Neurograin	1:900	/	Abcam	Rabbit	ab217672

1 h. An enhanced chemiluminescence kit (Cat# abs920, Abisin, China) was used to detect the protein signal. The gray values of the protein bands were quantified using ImageJ software.

1.10 Immunofluorescence Staining

After anesthesia with 1% pentobarbital sodium, the animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde prepared in 0.1 mol/L PBS, pH 7.4. After perfusion, the brains were separated and fixed in 4% paraformaldehyde at 4°C for 24 h. Then, the brain tissues were dehydrated and embedded in paraffin. Coronal slices containing the hippocampus and DRN were cut into 5 μm sections, followed by antigen retrieval as previously described[26]. After blocking with 10% donkey serum (with 0.3% Triton X-100) for 1 h at 37°C, the slides were incubated at 4°C overnight with primary antibodies, as shown in table 2. Subsequently, the sections were incubated with the corresponding fluorophore-conjugated secondary antibodies for 1 h at 37°C. After the slides were coverslipped, a virtual slide microscope (VS-120, Olympus, Japan) was used to obtain fluorescence microscopy images of the neurons. Brain slides from at least 4 mice were randomly selected for the quantification of positive neurons.

1.11 Statistical Analysis

The Kolmogorov-Smirnov test was performed to assess the normality of the distribution of the data. Normally distributed data are presented as mean ± standard deviation (SD). GraphPad Prism 8.0 software (San Diego, USA) was used for performing the statistical analysis. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used. $P<0.05$ was considered to indicate statistical significance.

2 RESULTS

2.1 EA Effectively Improved Learning and Memory in APP/PS1 Mice

To evaluate the therapeutic effects of EA on AD, EA was administered at acupoints GV20 and BL23 for 30 min every other day for one month. The NOR test revealed that the RIs of the mice in both the model and EA groups were considerably greater than those of the control group ($P<0.01$, fig. 1C and 1F), while the RIs of the mice in the EA group were greater than those of the model group ($P<0.01$, fig. 1C and 1F). The MWM test revealed a substantial increase in escape latency in both the model and EA groups compared to that in the control group starting on day 3 ($P<0.01$, fig. 1A and 1D). Compared to those in the model group, mice in the EA group exhibited a significant reduction in escape latency starting on day 4 ($P<0.01$, fig. 1A and 1D). Both the model group and the EA group showed a substantial decrease in the amount of time spent in the target quadrant compared to the control group. The duration of time spent in the target quadrant by the EA group was significantly greater than that spent by the model group ($P<0.01$, fig. 1B and 1E). These results indicated that EA mitigated memory deficits in APP/PS1 mice, which is in line with our prior findings.

2.2 Enhanced Neuronal Activity in DRN Induced by EA Treatment Could Contribute to Rescue of Learning and Memory Ability

Mounting evidence has suggested that 5-HT and its receptors are involved in the regulation of cognition and memory[29, 30]. The DRN serves as the primary origin of 5-HT in the brain. The structure includes widespread neuronal connections with the hippocampus, entorhinal cortex, prefrontal cortex, amygdala, basal forebrain, and hypothalamus. It is involved in functions such as learning and memory, emotion, sensation and movement, and the reward circuit[31]. Hence, we hypothesized that neuronal activity in the DRN could be associated with the enhancement of memory caused by EA. The immunofluorescence results indicated that the proportions of c-Fos-positive neurons in the DRN were considerably lower in both the model and EA groups than in the control group ($P<0.01$, fig. 2A and 2B). As hypothesized, EA treatment significantly increased the proportion of c-Fos-positive neurons in the DRN ($P<0.05$, fig. 2A and 2B). This indicates that EA has the potential to improve cognitive impairments by stimulating neurons in the DRN. The 5-HT_{1B} receptor is a vital subtype of the 5-HT receptor family and has been demonstrated to be involved in the modulation of excitatory synaptic transmission and memory[29, 32]. Subsequently, the presence of 5-HT_{1B} in the hippocampus was investigated. Western blotting analysis revealed a significant decrease in the level of 5-HT_{1B} in the model group compared to that in the control group ($P<0.01$, fig. 2C and 2D). However, treatment with EA significantly increased these levels ($P<0.05$, fig. 2C and

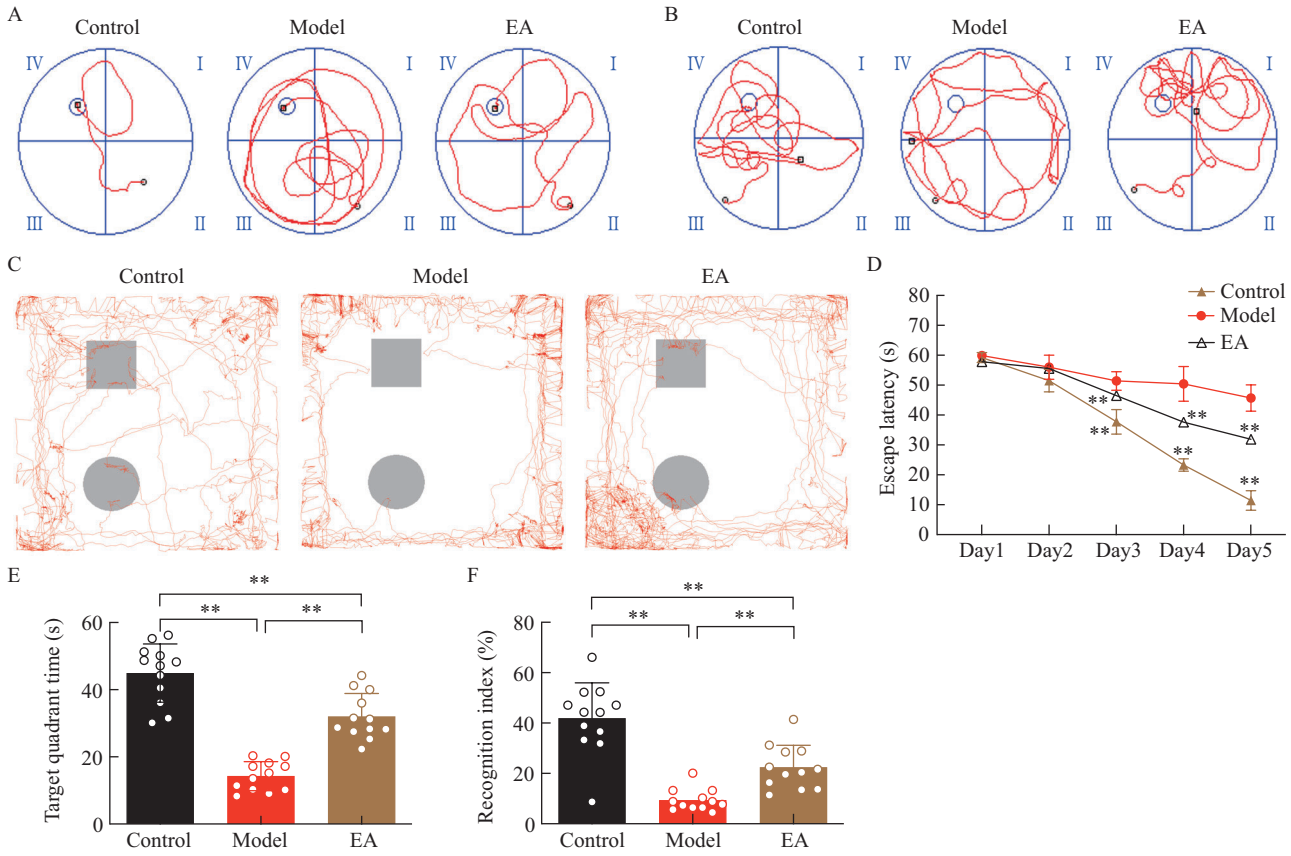

Fig. 1 EA attenuates learning and memory deficits in APP/PS1 mice

The learning and memory abilities of the mice in each group were tested using novel object recognition and Morris water maze tests after EA treatment. A and B: representative movement trajectories of mice to find the hidden platform in the navigation trial and paths in the spatial probe trial. C: representative paths of mice in the novel object recognition test. D: comparisons of escape latency in the navigation trial in each group. E: comparisons of target quadrant time in the spatial probe trials. F: comparisons of the recognition indices of the mice in the novel object recognition test. The data are presented as mean ± SD (n=12). $^{**}P$<0.01 (one-way analysis of variance with post hoc Dunnett's post hoc test)

2D). To observe the impact of EA on dendritic spine density, Golgi staining was performed. As shown in fig. 2, the dendritic spine density in mice in the model and EA groups was substantially lower than that in the control group (P<0.01, fig. 2E and 2F). EA therapy effectively reversed the reduction in the number of dendritic spines (P<0.05, fig. 2E and 2F).

2.3 Inhibition of Serotonergic Neurons in DRN Reversed Beneficial Effects of EA on Memory in APP/PS1 Mice

Subsequently, we employed a chemogenetic manipulation technique to experimentally evaluate our hypothesis. A diagram illustrating the process of virus injection and EA treatment is shown in fig. 3A. To selectively control serotonergic neurons in the DRN region, we administered rAAV-Ef1a-DIO-hM3D(Gq)-EGFP-WPREs or rAAV-EF1a-DIO-hM4D(Gi)-EGFP-WPREs into the DRN area and injected rAAV-TPH2-Cre-WPRE-hGH polyA into the CA1 area on day 1 (fig. 3A).

We observed a substantial increase in the RI in the NOR test of mice in the EA + Gi, EA + vehicle, EA, and Gq groups compared to that of mice in the model group (P<0.01, fig. 3D and 3G). The RIs of the mice in the EA group were significantly greater than those in the EA + Gi group (P<0.01, fig. 3D and 3G). However, there was no significant difference in the RI among the EA + vehicle, EA, and Gq groups (P>0.05, fig. 3D and 3G). Furthermore, beginning on day 4, the time it took for the mice to escape in the EA + Gi, EA + vehicle, EA, and Gq groups was considerably shorter than that in the model group (P<0.01, fig. 3B and 3E). The mice in the EA group exhibited a shorter escape latency than in the EA + Gi group (P<0.01, fig. 3B and 3E). In the spatial trial, the time spent in the target quadrant by mice in the EA + Gi, EA + vehicle, EA, and Gq groups was considerably longer than that in the model group (P<0.01, fig. 3C and 3F). Additionally, mice in the EA group spent more time in the target quadrant than in the EA + Gi group (P<0.01, fig. 3C and 3F). Collectively, our observations suggest that inhibiting serotonergic neurons in the DRN may counteract the beneficial effects of EA on memory impairment in APP/PS1 mice.

Immunofluorescence labeling revealed a significant decrease in the number of c-Fos$^+$ serotonergic neurons in the DRN of mice in the model group compared to the EA + Gi, EA + vehicle, EA, and Gq groups (P<0.01, fig. 4A and 4C). As hypothesized, the number of c-Fos$^+$ serotonergic neurons in the EA + Gi group was significantly lower than that in the EA group (P<0.01, fig. 4A and 4C). This indicates that EA has the ability to activate serotonergic neurons in

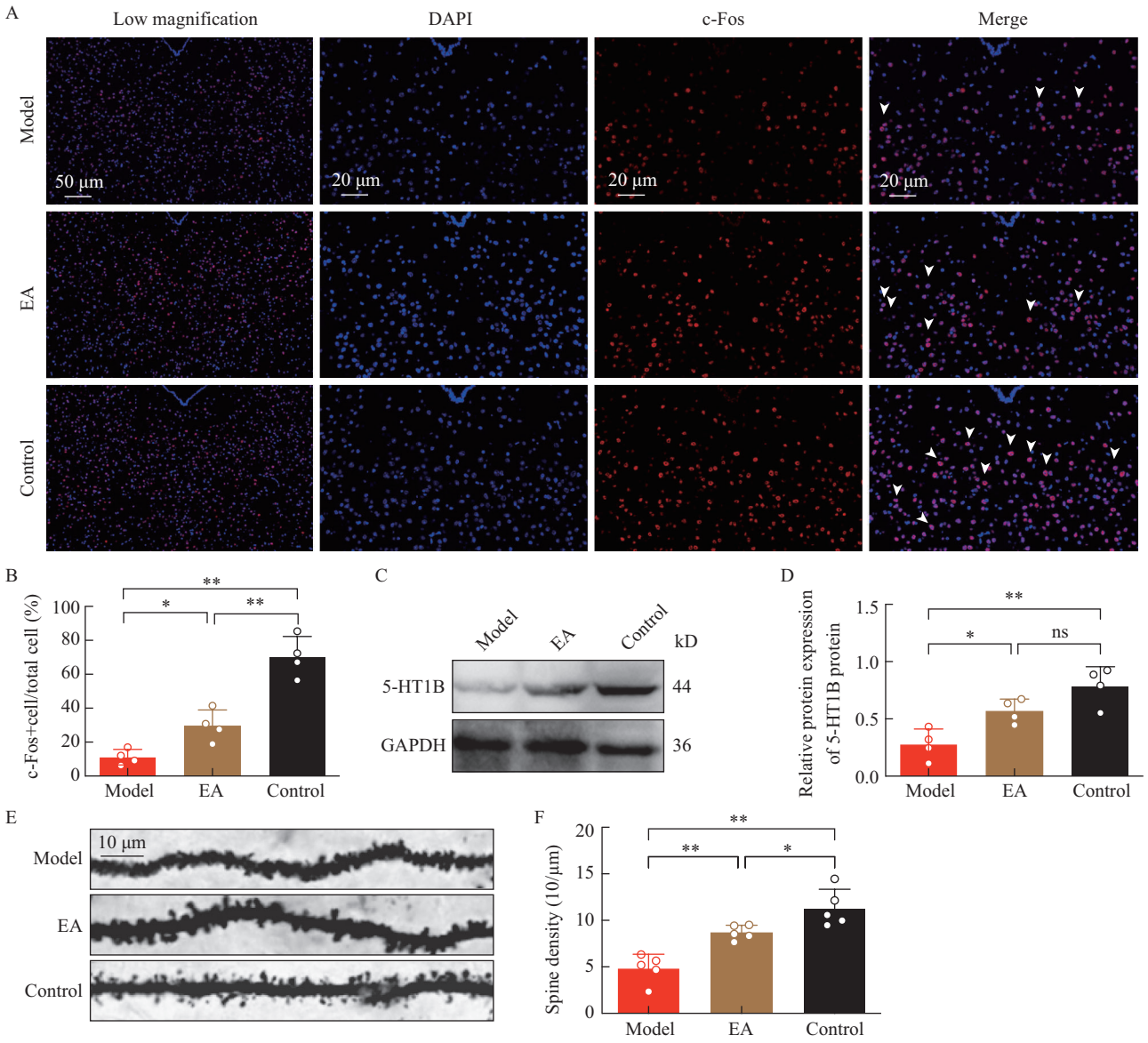

Fig. 2 Effects of EA on neuronal activity in the DRN, 5-HT1B levels and spine density in the hippocampus
A: representative immunofluorescence staining of c-Fos$^+$ (red) in the DRN. Arrows indicate c-Fos$^+$ cells. B: quantification of the number of c-Fos$^+$ cells in the DRN. C: The expression of 5-HT$_{1B}$ in the hippocampus was detected by Western blotting. D: quantification of 5-HT$_{1B}$/GAPDH. n=4 per group. E: representative Golgi staining of dendritic spines in the hippocampus. F: quantification of spine density (10/μm). n=5 per group. The data are shown as mean ± SD. $^*P$<0.05, $^{**}P$<0.01 (one-way analysis of variance with post hoc Dunnett's post hoc test)

APP/PS1 mice. In addition, Western blot analysis revealed a substantial decrease in the expression of 5-HT$_{1B}$ in the hippocampus of the model group compared to the EA + Gi, EA + vehicle, EA, and Gq groups (P<0.01, fig. 4B and 4D). The expression of 5-HT$_{1B}$ in the EA + Gi group was considerably lower than that in the EA group (P<0.01, fig. 4B and 4D). These results suggest that suppressing serotonergic neurons in the DRN can result in reduced expression of 5-HT$_{1B}$ in the hippocampus. Conversely, EA can activate serotonergic neurons in the DRN, leading to the increased expression of 5-HT$_{1B}$ in the hippocampus.

2.4 EA Activated Serotonergic Neurons in DRN and Subsequently Activated Hippocampal Glutamatergic Neurons

The association between memory deficits in AD and disruption of glutamatergic neurotransmission is generally acknowledged[33]. Consequently, we proceeded to examine whether suppressing serotonergic neurons in the DRN could affect the activity of glutamatergic neurons in the hippocampus. Immunofluorescence labeling revealed a substantial decrease in the number of c-Fos$^+$ neurogranin neurons in the hippocampal CA1 region of mice in the model group compared to the EA + Gi, EA + vehicle, EA, and Gq groups (P<0.01, fig. 5A and 5F). The quantity of c-Fos$^+$ neurogranin neurons in the EA group was considerably greater than that in the EA + Gi group (P<0.01, fig. 5A and 5F). Vesicular glutamate transporter 1 (VGLUT1) serves as a distinctive indicator for serotonergic neurons. In addition, the GluA1 receptor and Glu2/3 play a role in hippocampal synaptic plasticity[34]. Next, we employed the Western blot analysis to detect the levels of VGLUT1, GluA1, and Glu2/3 in

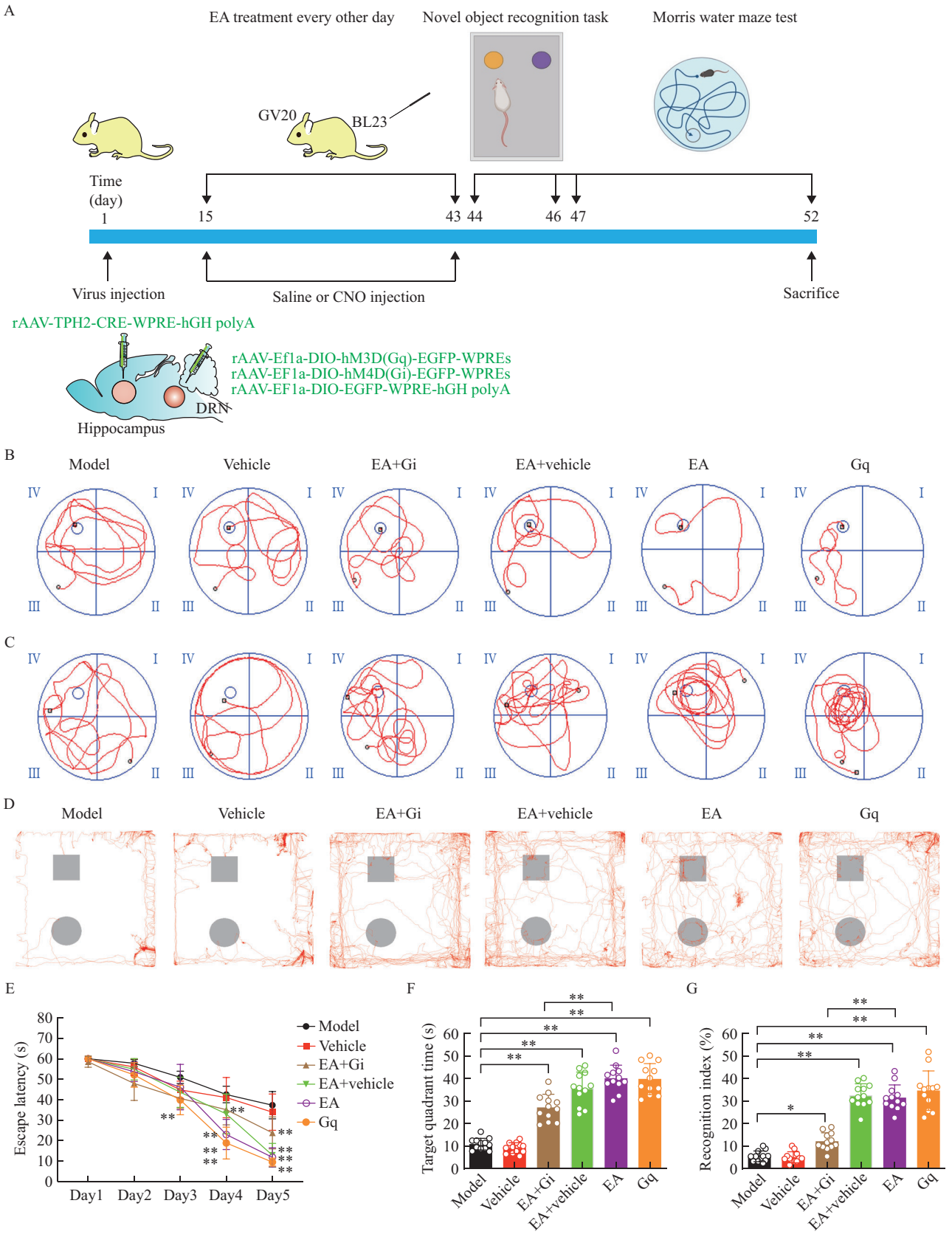

Fig. 3 The inhibition of serotonergic activity in the DRN can block the ameliorative effect of EA on memory deficits

A: flow chart of the experiment and location of virus injection in a sagittal view of the mouse DRN and hippocampal CA1 area. B: representative movement trajectories of mice to find the hidden platform in the navigation trial. C: representative paths in the spatial probe trial. D: representative paths of mice in the novel object recognition test. E: comparisons of escape latency in the navigation trial in each group. F: comparisons of target quadrant time in the spatial probe trials. G: comparisons of the recognition indices of the mice in the novel object recognition test. The data are presented as mean ± SD (n=12). $^{*}P$<0.05, $^{**}P$<0.01 (one-way analysis of variance with post hoc Dunnett's post hoc test)

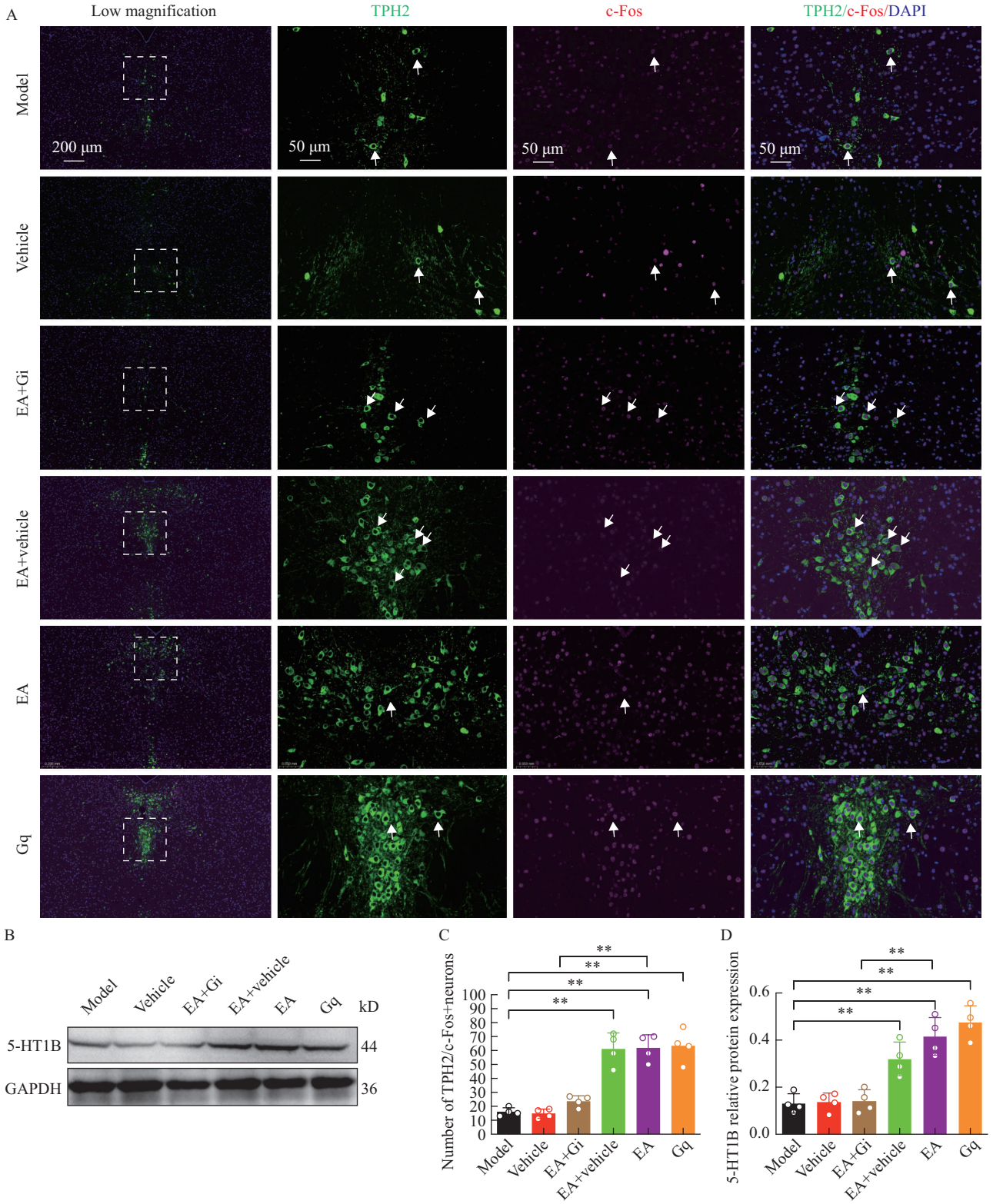

Fig. 4 The inhibition of serotonergic activity in the DRN can reverse the EA-induced increase in 5-HT1B levels in the hippocampus
A: representative immunofluorescence staining of c-Fos$^+$ (red), TPH2 (green), and DAPI (blue) in the DRN. Arrows indicate TPH2/c-Fos$^+$ neurons. B: The expression of 5-HT$_{1B}$ in the hippocampus was detected by Western blotting. C: quantification of the number of TPH2/c-Fos$^+$ neurons in the DRN. D: quantification of 5-HT$_{1B}$/GAPDH. n=4 per group. The data are shown as mean ± SD. $^*P$<0.05, $^{**}P$<0.01 (one-way analysis of variance with post hoc Dunnett's post hoc test)

the hippocampus. In our study, we observed significant decreases in the expression levels of VGLUT1, GluA1, and Glu2/3 in the model group compared to the EA + Gi, EA + vehicle, EA, and Gq groups (P<0.01, fig. 5B–5H). Additionally, the expression levels of VGLUT1, GluA1, and Glu2/3 were significantly lower in the EA + Gi group than in the EA group (P<0.01, fig. 5B–5H). These results indicate that EA can activate glutamatergic neurons in the

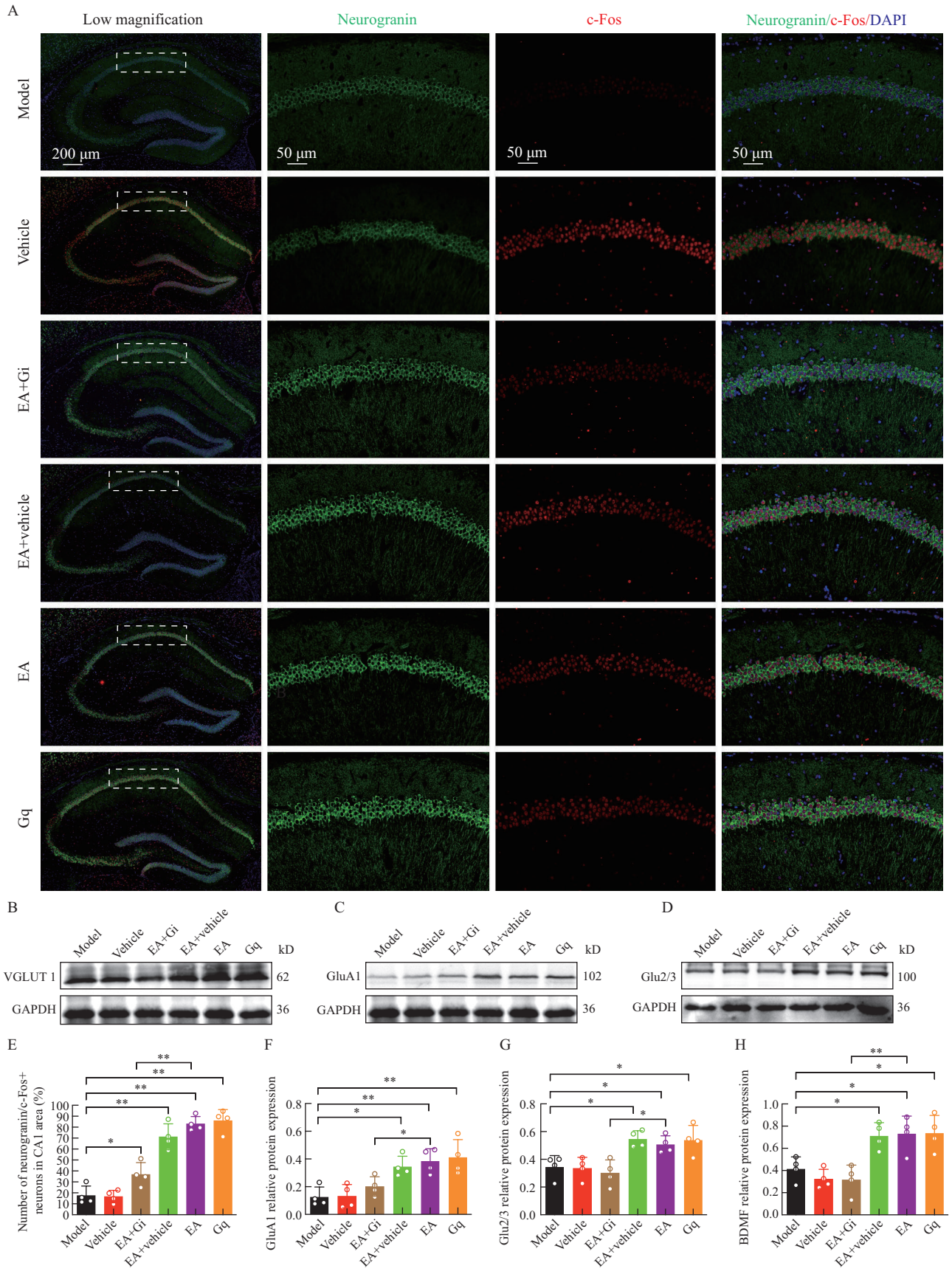

Fig. 5 The inhibition of serotonergic activity in the DRN can block EA-induced activation of hippocampal glutamatergic neurons
A: representative images of c-Fos$^+$ (red), neurogranin (green), and DAPI (blue) immunofluorescence staining in the hippocampus. Arrows indicate neurogranin/c-Fos$^+$ neurons. B–D: The expression of VGLUT1 (B), GluA1 (C), and Glu2/3 (D) in the hippocampus was detected by Western blotting. E: quantification of the number of neurogranin/c-Fos$^+$ neurons in the hippocampal CA1 area. F–H: quantification of VGLUT1/GAPDH (F), GluA1/GAPDH (G), and Glu2/3/GAPDH (H). n=4 per group. The data are shown as mean ± SD. $^*P$<0.05, $^{**}P$<0.01 (one-way analysis of variance with post hoc Dunnett's post hoc test)

hippocampus by activating serotonergic neurons in the DRN.

2.5 Inhibition of Serotonergic Neurons in DRN Blocked Protective Effects of EA on Synaptic Plasticity Injury in APP/PS1 Mice

Glutamatergic neurons closely participate in the modulation of synaptic plasticity[35]. Subsequently, Golgi staining was used to assess the density of dendritic spines in the CA1 region of the hippocampus. Our study revealed a significant decrease in the density of dendritic spines in mice in the model group compared to the EA + Gi, EA + vehicle, EA, and Gq groups (P<0.01, fig. 6A and 6C). The density of dendritic spines in the mice in the EA group was significantly greater than that in the EA + Gi group (P<0.01, fig. 6A and 6C). Mounting evidence has shown that postsynaptic density 95 protein (PSD95), synaptophysin (SYN), and brain-derived neurotrophic factor (BDNF) are strongly linked to the process of synaptic transmission. We employed Western blotting to detect PSD95, SYN, and BDNF expression. EA treatment led to a significant increase in the expression of PSD95, SYN, and BDNF in the hippocampus compared to the model group (P<0.01, fig. 6B–6F). The expression of PSD95, SYN, and BDNF in the EA + Gi group was significantly lower than that in the EA group (P<0.01, fig. 6B–6F), as anticipated. These data indicate that EA can reduce synaptic plasticity injury in APP/PS1 mice.

3 DISCUSSION

Prior research has established that the DRN plays a vital role in modulating learning and memory[36, 37]. Furthermore, our earlier research revealed a strong connection between tau pathology in the DRN and the ability of EA to improve cognitive impairment by regulating DRN pathology[26]. However, the specific mechanisms by which neurons in the DRN contribute to the neuroprotective effects of EA remain unclear. The current study provides evidence that reduced serotonergic neuron activity in the DRN leads to decreased glutamatergic neuron activity in the hippocampal CA1 region and synaptic damage in APP/PS1 mice. Additionally, chemogenetic activation of serotonergic neurons in the DRN ameliorates cognitive impairment. EA had a similar effect on serotonergic neuron activation. The inhibition of serotonergic neurons reversed the effects of EA. These findings indicate that EA can activate glutamatergic neurons in the hippocampal CA1 area by activating serotonergic neurons in the DRN, thus ameliorating synaptic injury and cognitive dysfunction.

It is widely accepted that kidney deficiency and

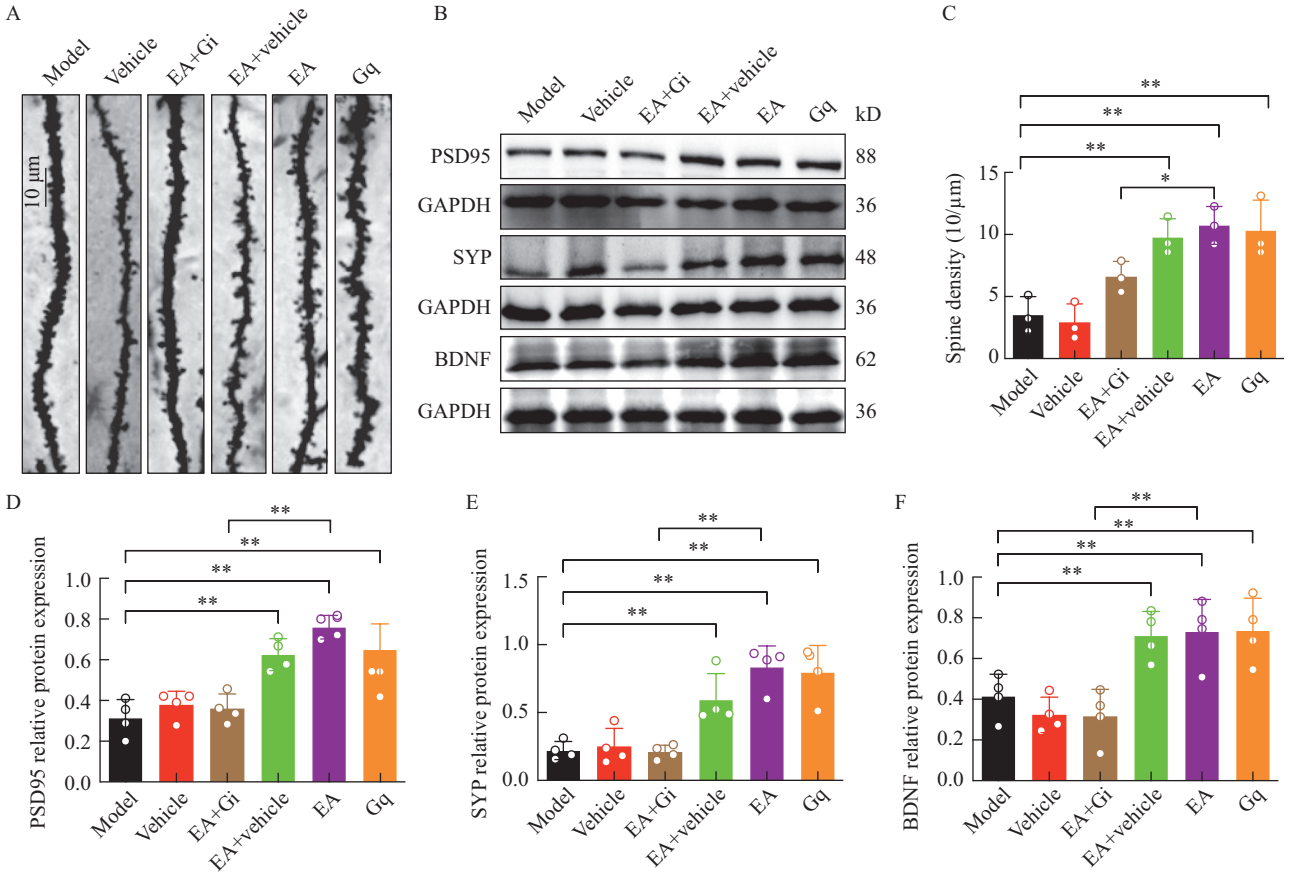

Fig. 6 The inhibition of serotonergic activity in the DRN can reverse the EA-induced enhancement of hippocampal synaptic plasticity
A: representative Golgi staining of dendritic spines in the hippocampus. B: The expression of PSD95, SYP, and BDNF in the hippocampus was detected by Western blotting. C: quantification of spine density (10/μm). n=3 per group. D–F: quantification of PSD95 (D), SYP (E), and BDNF (F) expression. n=4 per group. The data are shown as mean ± SD. $^{**}P$<0.01 (one-way analysis of variance with post hoc Dunnett's post hoc test)

Governor vessel occlusion as a consequence of qi stagnation and blood stasis play a significant role in the occurrence of dementia based on Chinese medicine theory. The results from our previous data mining analysis also showed that *Baihui* (GV 20) was the primary acupoint for treating AD[38]. Therefore, in the present study, the acupoints *Shenshu* (BL 23) and *Baihui* (GV 20), which are able to nourish kidney essence and modify the governor vessel, were selected to treat cognitive dysfunction in APP/PS1 mice. Mounting experimental evidence also supports the beneficial effects of acupuncture on AD[39–41]. In our previous review, we concluded that acupuncture can alleviate cognitive impairment in AD via multiple targets and pathways, including the modulation of Aβ metabolism, tau phosphorylation, neurotransmitters, neurogenesis, synaptic plasticity, autophagy, neuronal apoptosis, neuroinflammation, cerebral glucose metabolism, and brain response[42].

Increasing evidence indicates that the DRN and locus coeruleus in the brainstem are associated with the occurrence of AD[12, 17, 20]. Hyperphosphorylated tau proteins in the DRN can be observed in the presymptomatic phase of AD[13]. In addition, there is a correlation between serotonin disturbance in the DRN and prodromal symptoms of AD, such as depression, irritability, and disruptions in circadian rhythm. Furthermore, this serotonin disturbance is directly linked to the advancement of the clinical symptoms of AD[16, 43]. The AD brain exhibits reduced amounts of 5-HT, 5-HT receptors, and metabolites[44]. Hence, directing interventions toward the serotonergic system could present a new and promising approach for the treatment of AD. It has been recently proven that optogenetic activation of serotonergic neurons in the DRN projecting to the ventral tegmental area preferentially reactivates positive memory ensembles in the dorsal dentate gyrus[45]. In this study, we demonstrated that the chemogenetic activation of serotonergic neurons in the DRN leads to enhanced memory in APP/PS1 mice. These findings shed light on the potential of targeting serotonergic neurons in the DRN as a therapeutic approach for AD. Recent findings indicate that EA can exert antidepressive and analgesic effects by modulating serotoninergic neurons in the DRN[46, 47]. Furthermore, Wang *et al* recently demonstrated that EA was able to reorganize the function of the raphe nucleus-associated serotonergic system to treat chronic neck pain[24]. The current study demonstrated that EA effectively activates serotonergic neurons in the DRN and alleviates memory deficits. DRN serotonergic neurons receive widespread projections from various brain regions[48]. The nucleus tractus solitarius (NTS) is one of the main brain areas affected by acupuncture stimulation[49]. Approximately 80% of vagus nerve fibers are afferent fibers that can send both somatic and general visceral signals to the NTS, which in turn sends fibers to other brainstem nuclei, including the locus coeruleus, DRN, and parabrachial nucleus[50]. Mounting evidence indicates that acupuncture can modulate brain function by modulating vagus nerve activity[51, 52]. Liu *et al* recently reported that vagus nerve stimulation induced the activation of glutamate receptor-mediated TrkB signaling in the NTS, which in turn activated serotonergic neurons in the DRN and norepinephrinergic neurons in the locus coeruleus[53]. We speculate that there might be a potential vagal afferent-NTS-DRN serotonergic circuit involved in the modulation of memory. Acupuncture can activate DRN serotonergic neurons by activating the vagal afferent NTS-DRN circuit. Neural tracing methods and optogenetic approaches in the future are encouraged to validate potential neural circuits.

The DRN sends intense serotonergic inputs to the hippocampus[54]. Therefore, we further investigated the effects of EA on the levels of 5-HT_{1B} and glutamatergic neurons, which are abundant in the hippocampus. 5-HT_{1B} plays a significant role in regulating the release of neurotransmitters such as glutamate, dopamine, acetylcholine, and GABA[32, 55, 56]. Studies have shown that the levels of 5-HT_{1B} receptors in the hippocampus, hypothalamus and frontal cortex are significantly reduced in patients with AD and Parkinson's disease and are also associated with cognitive decline[57, 58]. 5-HT_{1B} receptor antagonists decrease glutamate levels in the hippocampus and prefrontal areas[59], while activation of 5-HT_{1B} receptors promotes glutamate release[60], suggesting that the hippocampal 5-HT_{1B} receptor is able to impact the glutamatergic system. Wang *et al* demonstrated that chemogenetic activation of serotonergic neurons in the median raphe nucleus ameliorated memory deficits via activation of 5-HT_{1A} and 5-HT_{3A} receptors[61]. Similarly, in the present study, we also observed that chemogenetic inhibition of serotonergic neurons in the DRN led to decreased levels of 5-HT_{1B} and inactivation of glutamatergic neurons in the hippocampal CA1 area, while chemogenetic activation of serotonergic neurons reversed cognitive dysfunction in AD mice, indicating that a possible 5-HT^{DRN}-GluCA1 neural circuit may be associated with memory impairment in AD. Consistent with these findings, it has been demonstrated that activation of serotonergic terminals in the hippocampal CA1 region enhances spatial memory[62].

The 5-HT_{1B} receptor in the hippocampal CA1 area not only increased the density of dendritic spines on glutamatergic neurons but also led to an increase in the expression of the Kalirin-7, PSD95, and GluA2/3 subunits of AMPA receptors, thus improving the spatial memory of rats[63]. EA has been reported to ameliorate middle cerebral artery occlusion-induced cognitive deficits by upregulating the 5-HT_{1A} receptor and PKA kinase[64]. Our research revealed that EA ameliorated memory deficits by upregulating 5-HT_{1B} to activate glutamatergic neurons.

Disruption of the glutamatergic system is strongly associated with the clinical symptoms observed in AD[65]. Glutamate, which is released by glutamatergic neurons, interacts with ionotropic glutamate receptors such as AMPA receptors[66] and NMDA receptors[67]. The GluA1-GluA4 subunits of the AMPA receptor have a significant impact on the establishment and maintenance of long-term potentiation[66]. Increasing evidence indicates that AMPA receptors play a crucial role in the regulation of

synaptic plasticity, which in turn affects memory[68–70]. Therefore, the density of dendritic spines and the expression levels of synapse-associated proteins were examined. PSD95, one of the main synapse-associated proteins in the PSD, can increase the number and size of dendritic spines, promote the maturation of presynaptic terminals, coordinate synaptic maturation, and stabilize the postsynaptic membrane[71]. PSD95 has also been demonstrated to be essential for maintaining dendritic spine stability[71, 72]. The abundant presence of SYN on the synaptic vesicle membrane also contributes to the development of synapses and synaptic transmission. BDNF is one of the most abundant neurotrophic factors in the central nervous system and can enhance synaptic plasticity and neurogenesis[73, 74].

The protective effects of EA on neural plasticity have been investigated extensively. EA can rescue spatial memory deficits by activating BDNF/TrkB/Erk signaling to promote synaptic plasticity[75]. Zhang *et al* demonstrated that EA increased dendritic spine density and protein levels of PSD95 and SYN in chronic unpredictable mild stress mice by inhibiting the GluN2B/CaMKII/CREB pathway[76]. In our earlier investigation, we discovered that EA attenuated the hippocampal loss of dendritic spines by inactivating the GSK3β/mTOR pathway[77]. In line with our previous findings[77], we showed that EA effectively prevented the reduction in the number of dendritic spines in the hippocampus. Additionally, it increased the protein levels of PSD95, SYN, and BDNF by activating serotonergic neurons in the DRN, which subsequently activated glutamatergic neurons in the hippocampal CA1 area.

This study has some limitations that should be noted. First, decreased excitability of glutamatergic neurons in the hippocampal CA1 area was observed when serotonergic neurons in the DRN were chemogenetically inhibited, suggesting that a possible 5-HT^{DRN}-GluCA1 neural circuit could be involved in memory modulation. However, the neuronal activity of other neurons involved in the regulation of memory, including dopaminergic and noradrenergic neurons, has not been investigated. Furthermore, it is currently unclear whether the DRN has direct or indirect projections to glutamatergic neurons in the CA1 area. In future studies, neural tracing and optogenetic manipulation in combination with calcium imaging *in vivo* should be utilized to uncover the underlying neuronal networks.

In conclusion, we demonstrated that chemogenetic activation of serotonergic neurons in the DRN can relieve cognitive deficits in APP/PS1 mice, probably by subsequently activating glutamatergic neurons mediated by 5-HT$_{1B}$ and enhancing synaptic plasticity. Furthermore, EA can alleviate learning and memory impairment in APP/PS1 mice by activating serotonergic neurons in the DRN.

Conflict of Interest Statement

All the authors confirm that there are no conflicts of interest.

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