

# Electroacupuncture Attenuates Reference Memory Impairment Associated with Astrocytic NDRG2 Suppression in APP/PS1 Transgenic Mice

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**Abstract** Electroacupuncture (EA) has demonstrated therapeutic potential for the treatment of Alzheimer's disease (AD). A previous study reported that N-myc downstream-regulated gene 2 (NDRG2) was upregulated in the brain of patients with AD. In the present study, we investigated the effects of repeated EA administration on reference memory impairment and the role of NDRG2 in an amyloid precursor protein (APP)/presenilin-1 (PS1) double transgenic mouse model. Age-matched wild-type and transgenic mice were treated with EA (once per day for 30 min) for 4 weeks (four courses of 5 days EA administration and 2 days rest) beginning at 10 months of age. At seven and ten postnatal months of age and following a 4-week EA treatment regime, mice received training in the Morris

water maze (MWM) and a probe test. Brain tissue was analyzed via Western blot and double-label immunofluorescence. Beginning at 7 months of age, APP/PS1 mice began to exhibit deficits in reference memory in the MWM test, an impairment associated with upregulation of glial fibrillary acidic protein (GFAP) and NDRG2. Four weeks of EA administration significantly ameliorated cognitive impairments and suppressed GFAP and NDRG2 upregulation. In conclusion, our findings demonstrated that EA administration can alleviate reference memory deficits and suppress NDRG2 upregulation in an AD transgenic mouse model. This study provides supportive evidence for EA as an effective therapeutic intervention for AD, as well as NDRG2 as a novel target for AD treatment.

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## Abbreviations

AD	Alzheimer's disease
APP	β-Amyloid precursor protein
EA	Electroacupuncture
GFAP	Glial fibrillary acidic protein
NDRG2	N-myc downstream-regulated gene 2
LTD	Long-term depression

## Introduction

Alzheimer's disease (AD) is one of the most common progressive neurodegenerative disorders in the aging population

and accounts for approximately 60–80 % of all age-related dementias [1]. AD is the fourth leading cause of death that accelerates the end of life. Due to a lack of effective treatment options, it has become a significant global social burden [2, 3]. Therefore, it is imperative to find new and more effective treatments for AD.

One survey reported that 55 % of patients with AD had tried at least one form of complementary medicine with the hopes that these therapies could improve their overall quality of life and delay further decline in cognitive functioning [4]. Animal models have reported that acupuncture could improve cognitive impairment in vascular dementia [5, 6]. Electroacupuncture (EA), which does not require traditional Chinese medicinal training, is a simple and effective modern acupuncture method used in the treatment of many diseases. Our previous study has reported that EA at the Baihui acupoint (GV 20) shows cerebral protective effects [7] and ameliorates hypergravity-induced impairment of learning and memory [8]. However, the possible beneficial effects of acupuncture or EA on cognition in patients with AD remain uncertain [9, 10].

In the central nervous system, astrocytes are abundant and play an important role in brain homeostasis and neuronal function [11]. Under pathological circumstances, the activation of astrocytes results in abandonment of their typical neurosupportive function for an inflammatory role, which is thought to contribute to neurodegeneration. This astrocyte-related pathomechanism may be a potential therapeutic target for AD [12]. Human N-myc downstream-regulated gene 2 (NDRG2) is expressed in astrocytes throughout different brain regions related to memory, including the cerebral cortex, hippocampus, and the olfactory bulb [13]. Our previous study reported an association between NDRG2 and ischemia reperfusion injury in the MCAO rat model, where post-ischemia was correlated with increased NDRG2 regulation [14]. Another study also reported that NDRG2 participated in diabetic cognitive dysfunction [15]. Furthermore, Mitchelmore found that NDRG2 was upregulated at both the RNA and protein levels in AD patients, suggesting that it may be involved in the pathogenesis of this disease [16]. These findings suggest that NDRG2 may be a novel AD-associated protein.

In an attempt to confirm the therapeutic effects of repeated EA (GV 20) treatment for AD-associated cognitive impairments, experiments were conducted on APP/PS1 transgenic mice that developed an AD-like pathology with amyloid plaques, astrocytosis, and microgliosis. We also investigated the potential mechanisms underlying the repeated EA treatment, focusing on the novel molecular target of NDRG2, which may be associated with AD.

## Methods

### Animals

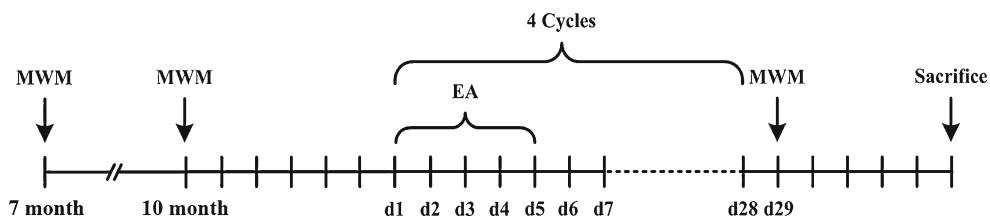
In the current study, we used a double transgenic APPSwe/PS1 (B6C3-Tg (APPswe, PSEN1dE9)85Dbo/J) mouse model. APPSwe is the Swedish mutation of the amyloid precursor protein, whereas PS1 is the mutant form of human presenilin 1. APP/PS1 mice were maintained by crossing the double transgenic mice with C57BL/6J X C3H hybrid wild-type mice. These mice were genotyped by PCR analysis of genomic DNA from tail biopsies. APP/PS1 transgenic and age-matched wild-type mice in our study were obtained from the HFK Bioscience Company. All mice were male, weighing 18–22 g, and housed at 20–25 °C with 60 % relative humidity under controlled conditions (12-h light/dark cycle). Furthermore, the mice had free access to standard rodent diet and tap water. Behavioral testing was conducted by researchers who were blind to the mice's group. The experiments were performed in accordance with the national guidelines for the care and use of laboratory animals and were approved by the Experimental Animal Welfare and Committee of the Fourth Military Medical University.

### Experimental Protocol

Mice were divided into four groups: wild-type (Con), wild-type with repeated EA (Con+EA), APP/PS1 (APP), and APP/PS1 with repeated EA administration (APP+EA). Each group contained 12 mice. The mice in Con+EA and APP+EA groups received the same standardized EA administration, while the mice in the Con group and APP group were anesthetized without EA. It has been demonstrated that APP/PS1 mice encode a mutated allele of the human amyloid precursor protein (APP) and presenilin 1 (PS1) genes, as well as exhibit extensive amyloid plaque deposition in the brain by 6–7 months of age [17]. In addition, Yu Y et al. [18] verified that the memory of 3-month-old transgenic mice was normal, whereas 9-month-old APP/PS1 mice displayed memory deficits and numerous brain A $\beta$  deposits. Ultimately, we chose 7-month-old mice to make sure of the cognitive impairments, and 10-month-old mice to evaluate the curative effect of repeated EA administration. The experimental timeline is shown in Fig. 1. After the completion of the last behavioral test, mice were sacrificed for pathologic analyses.

### EA Administration

After 1 % pentobarbital sodium (40 mg/kg, i.p.) anesthesia, mice were stimulated with EA (G6805-2 EA Instrument, Model No. 227033, Qingdao Xinsheng Ltd., China) at the Baihui acupoint (GV 20), which is located at the intersection of the sagittal midline and the line linking two rat ears. The



**Fig. 1** Schematic representation of the methodology used. Morris water maze (MWM) test was performed 7 and 10 months after birth. After the 10-month MWM test, an individual EA session was administered daily

for 30 min, 5 days/week, and 2 days rest for a period of 4 weeks. After the last EA administration, MWM test was carried out. Mice used for other studies were sacrificed 24 h after the last behavioral observations

intensity of 1 mA and frequency of 2/15 Hz were selected for the EA administration as described in our previous study [19]. A fine needle (0.5 mm in diameter) placed at GV 20 was connected to one electrode of the EA stimulator, as well as two electrodes on the ears. The tremor of the ears during administration was regarded as effective EA stimulation. An individual EA session was administered daily for 30 min, 5 days/week, and 2 days rest for a period of 4 weeks. During anesthesia and EA, the mice's core temperature was maintained at  $37.0 \pm 0.5$  °C by surface heating or cooling (Spacelabs Medical Inc.).

#### Morris Water Maze Test

At postnatal months 7 and 10, spatial learning and reference memory were evaluated using a conventional Morris water maze test (MWM) ( $n=6$ /group) as previously described [20]. The Morris water maze apparatus was placed in a warm and quiet room with ample surrounding visual cues. The apparatus consisted of a white colored pool (122 cm in diameter and 51 cm in height), which was divided into four quadrants and was filled with water to 40 cm in depth at 20–22 °C. Four positions around the edge of the tank were arbitrarily designated as N, S, E, and W, providing four alternative start positions and dividing the tank into four quadrants: NE, SE, SW, and NW. A hidden escape platform (10 cm in diameter) was placed in the center of one of the quadrants (the target quadrant) and was changed semi-randomly every day according to the following pattern (repeated twice): SE, NW, NE, SW, SE, and NW. Mice were released into the water facing the tank wall. Individual trials were 1 min in length, and the intertrial interval was 15 s. Any mouse that failed to locate the platform within 60 s was placed on the platform by hand. During training, mice received four trials per day from four principal start locations and were tested over a 5-day period. Twenty-four hours after the final trial, a probe test was performed to assess spatial memory. The mice were released from novel start positions at 30-s intervals. The trials were recorded and analyzed by an

automated analyzing system (Dig-Behav, Jiliang Co., Ltd., Shanghai, China).

#### Double-Label Immunofluorescence Assays

To remove the blood and fix the brain tissue, three mice in each group were anesthetized and transcardially perfused with physiologic saline followed by 4 % paraformaldehyde (PFA). Fixed brains were cut in 12-μm sagittal sections. Subsequent to sectioning, brain slices were immersed in 0.3 % H<sub>2</sub>O<sub>2</sub> in methanol for 15 min and incubated with 10 % normal goat serum for 30 min. Sections were probed with anti-NDRG2 mouse polyclonal antibody (1:200; Abnova Corporation, Taipei, Taiwan) and anti-GFAP rabbit monoclonal antibody (1:1000; Abcam, USA) in 1 % BSA-PBS overnight at 4 °C in a humidified box. Afterwards, sections were incubated with anti-mouse FITC-tagged secondary antibody (1:200; CWBIO, Beijing, China) and anti-rabbit Cy3-tagged secondary antibody (1:200; CWBIO, Beijing, China) for 1 h at room temperature. Finally, the sections were mounted with 50 % glycerol for examination under a fluorescence microscope. Fluorescent images were observed with a confocal laser microscope (FV1000; Olympus, Tokyo, Japan), and images were captured with Fluoview 1000 (Olympus).

#### Western Blot Analysis

The hippocampus was lysed in modified radioimmuno-precipitation assay buffer for Western blot analysis. Total protein concentrations were determined by using the BCA Protein Assay Kit (Pierce, USA). Firstly, 40-μg protein samples were separated on 10 % SDS-polyacrylamide gel by electrophoresis. Proteins were then transferred onto nitrocellulose membranes. The membranes were incubated with 10 % SDS-polyacrylamide gel (1:2000; Abcam, USA) or anti-GAPDH rabbit monoclonal antibody (1:500; Boster, Wuhan, China) after blocking with 5 % nonfat milk. Subsequently, membranes were incubated for 1 h at room temperature with secondary antibodies conjugated to IRDye800 (1:20000; Rockland Inc., USA). After blotting, immunoreactive signals were visualized with chemiluminescence luminol reagents

(ECL; Amersham Bio-Sciences, UK) and an Odyssey infrared imaging system (LI-COR Inc., USA).

### Statistical Analysis

All data is presented as mean $\pm$ SEM. A multifactorial analysis of variance (ANOVA) for repeated measurement was employed for analyzing escape latencies and percent time spent in quadrants in the Morris water maze test. Tukey's test was further used as a post hoc test to detect between-group differences. One-way ANOVA was employed for analyzing other data obtained in these experiments followed by LSD (equal variances assumed) or Dunnett's T3 (equal variances not assumed) post hoc test.  $P<0.05$  was considered statistically significant.

## Results

### Cognitive Impairment in APP/PS1 Mice

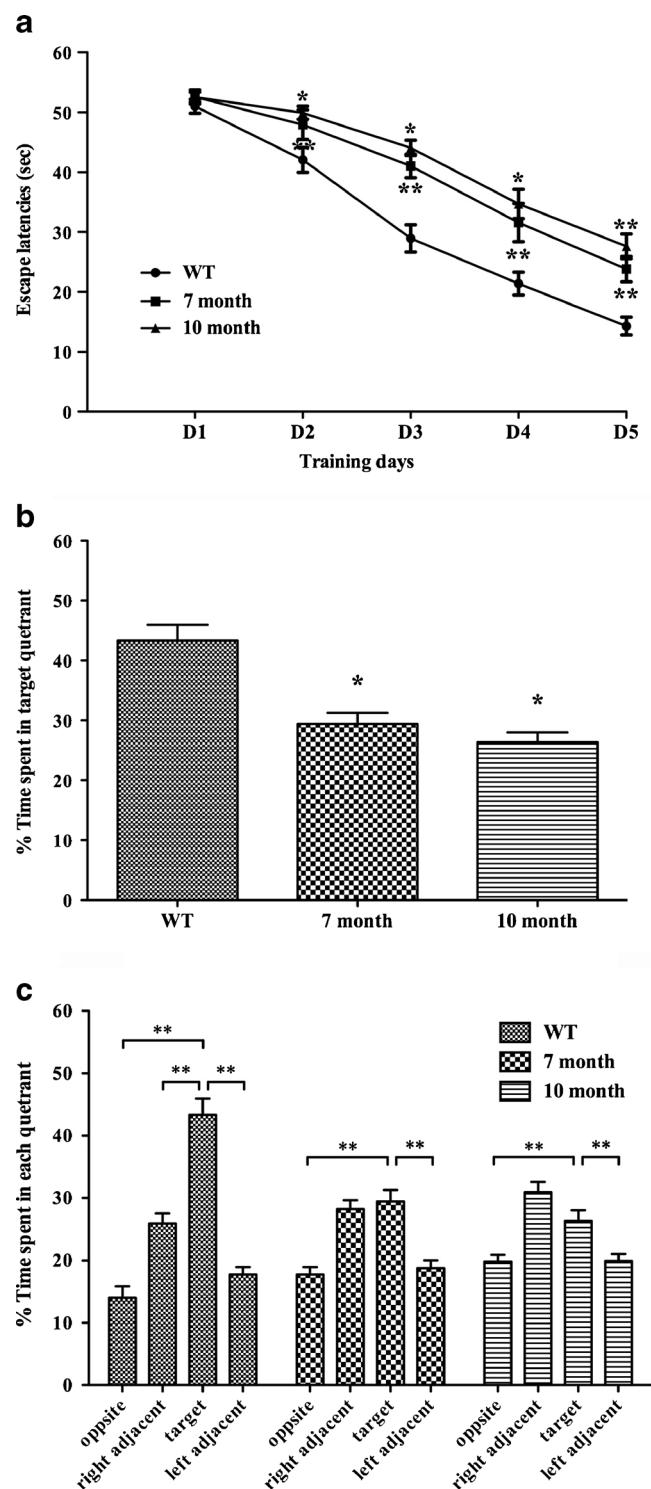
The main effect of the groups showed statistical significance [ $F(4,24)=188.072, P<0.05$ ]; the escape latency of the 7- and 10-month-old groups was significantly decreased compared with the wild-type group ( $P<0.01$ ; Fig. 2a). During the probe trial, the percentage of time spent in the target quadrant was used for statistical analysis. Post hoc test for the groups showed that mice in the 7- and 10-month-old groups spent less time in the target quadrant compared with the wild-type group ( $P<0.01$ ; Fig. 2b). The mice spent more time in the target quadrant compared to other quadrants except the 10-month-old group ( $P<0.01$ , respectively; Fig. 2c). These findings suggest that the APP/PS1 transgenic mice develop spatial reference memory impairment features, beginning at least after the seventh postnatal month.

### GFAP and NDRG2 Were Upregulated in APP/PS1 Mice

The expression of NDRG2 was examined by double-label immunofluorescent staining of GFAP and NDRG2 (green) ( $n=3$ /group; Fig. 3). The data illustrates that almost all the astrocytes expressed NDRG2, which was exclusively restricted to GFAP-immunopositive cells. The APP group showed an increased arborization when compared to the wild-type mice.

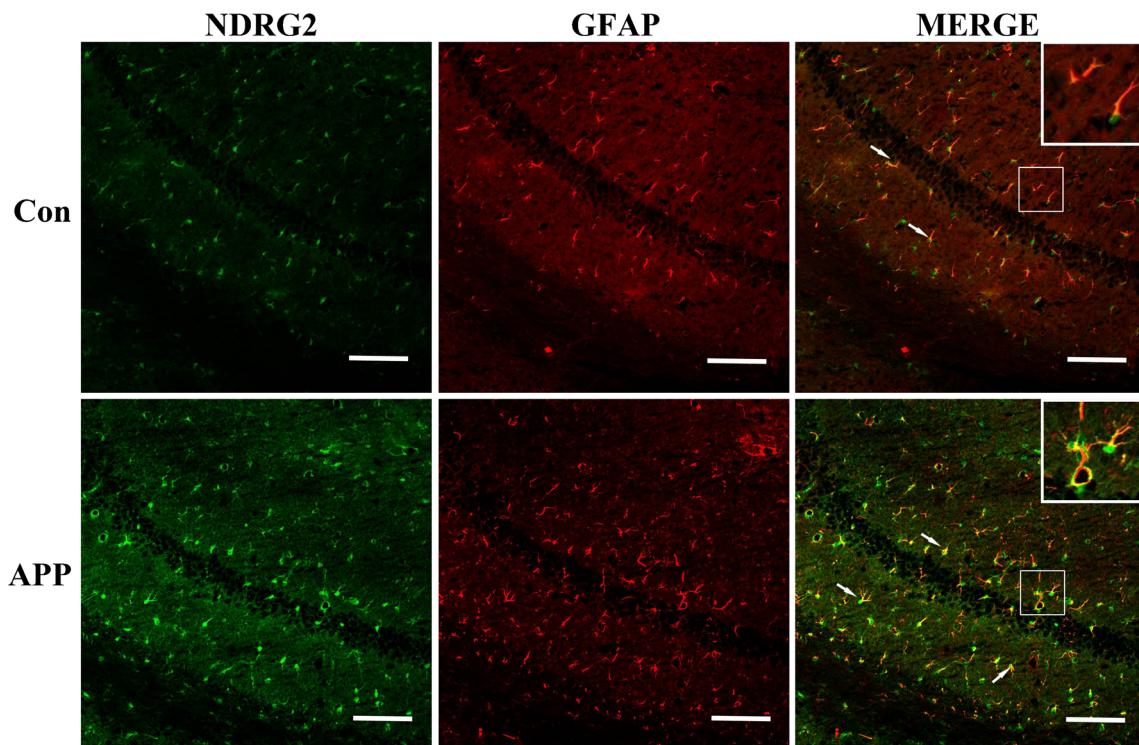
### EA Inhibits Activation of Astrocytes and Upregulation of NDRG2 in APP/PS1 Mice

To explore the underlying cellular and molecular mechanisms of repeated EA treatment of cognitive impairment, the protein levels of GFAP and NDRG2 were investigated. Immunofluorescence double-labeling for GFAP and NDRG2 revealed a reduction in GFAP-positive cells and decreased arborization



**Fig. 2** APP/PS1 mice show cognitive impairment features 7 months after birth. **a** Mice in the 7th and 10th months show longer latencies for reaching the platform compared with the wild-type mice. **b** The probe test indicated that the time spent in the target quadrant was decreased 7 and 10 months after birth compared with the wild-type mice. **c** Percent time spent in the target quadrant in all probe trials is shown (\* $P<0.05$  from the Con group, \*\* $P<0.01$  from the Con group)

in the APP+EA group in comparison to the APP group. In addition, the upregulation of NDRG2 was partially reversed



**Fig. 3** GFAP and NDRG2 were upregulated in APP/PS1 mice. Immunofluorescence stains for NDRG2 (green) and GFAP (red) in brain sections. The expressions of GFAP and NDRG2 were increased (white

arrowheads) in the APP group. The merged yellow images indicate co-localization of NDRG2 and GFAP (white arrowheads). The inset displays a part of the image that has been magnified. Bar=20  $\mu$ m

(Fig. 4a). As shown in Fig. 4b, the semiquantitative analysis of the Western blot indicated that repeated EA significantly decreased the expression of GFAP and NDRG2 in the APP+EA group compared to the APP group (GFAP  $1.22 \pm 0.11$ ,  $P < 0.05$  vs.  $0.89 \pm 0.08$  in the APP group; NDRG2  $1.12 \pm 0.07$ ,  $P < 0.05$  vs.  $0.84 \pm 0.08$  in the APP group), suggesting that repeated EA inhibited astrocytosis and NDRG2 in APP mice. Interestingly, repeated EA also attenuated the expression of NDRG2 in the brain of age-matched wild-type mice.

#### Repeated EA Ameliorates AD-Induced Cognitive Impairment

##### Repeated EA Ameliorates AD-Induced Learning Deficiency

After 4 weeks of repeated EA administration, spatial learning and memory were assessed using the Morris water maze test ( $n=6$ /group). The main effect for all of the groups showed statistical significance [ $F(4,40)=50.655$ ,  $P < 0.05$ ]; post hoc test revealed that the escape latency of the APP group was significantly greater than the Con group ( $P < 0.01$ ). While the poor performance of APP mice was improved by administration of EA, repeated EA reduced the escape latencies of the APP+EA group in comparison to APP mice ( $P < 0.01$ ; Fig. 5a). In other words, the results suggest that acquisition or retention of long-term memory was enhanced in APP/PS1 mice by repeated electroacupuncture.

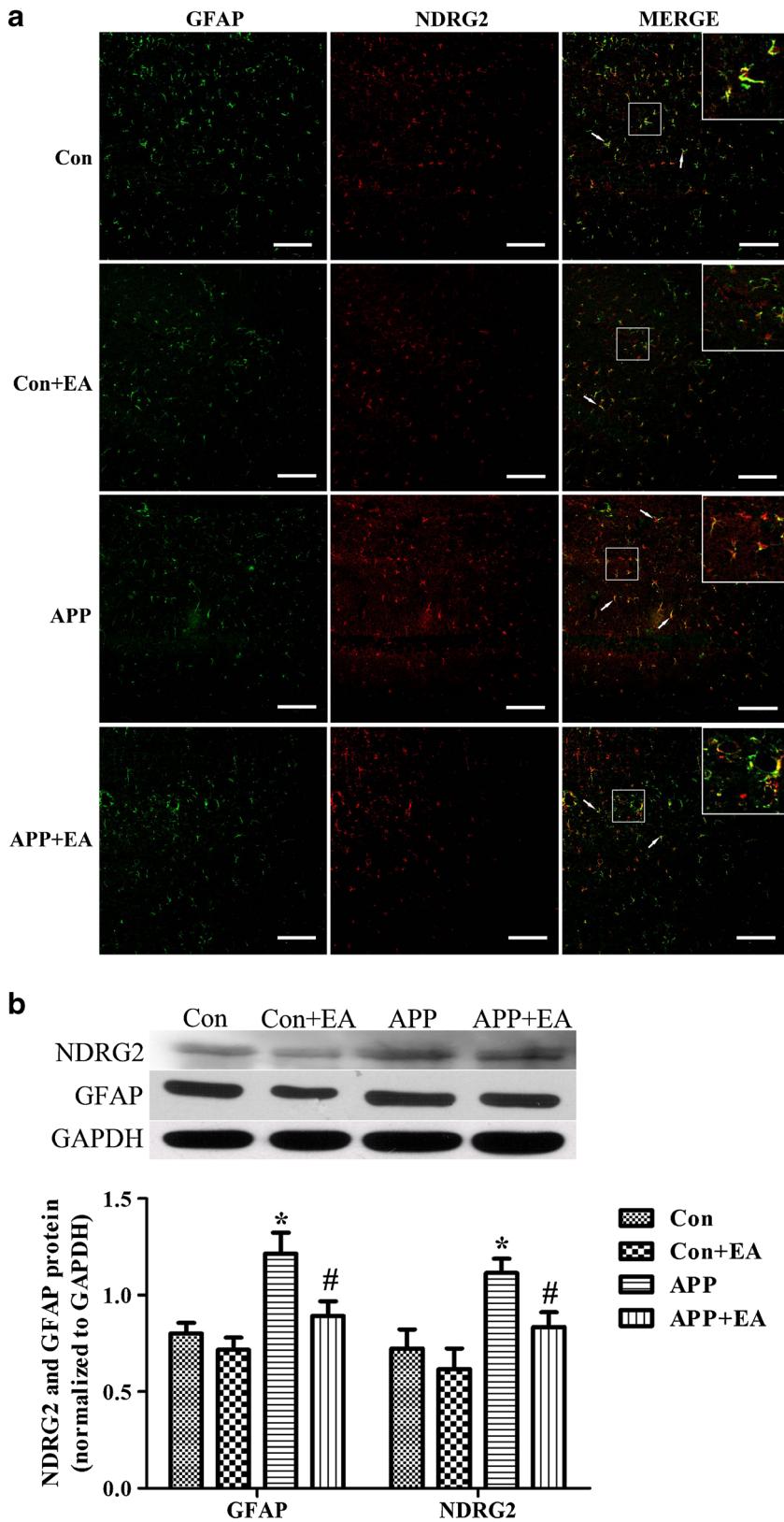
##### Repeated EA Ameliorates AD-Induced Memory Decline

During the probe trial, we evaluated the mice's retrieval of spatial memory by measuring the time spent in each quadrant. Post hoc test for the groups showed that the APP/PS1 transgenic mice with repeated EA spent significantly more time in the target quadrant than APP/PS1 mice ( $P < 0.01$ ; Fig. 5b). Post hoc test for the quadrants showed that the mice spent more time in the target quadrant compared to other quadrants except the APP group ( $P < 0.01$ , respectively; Fig. 5c). During the training period, swimming velocity was not affected by exercise ( $P > 0.05$ , data not shown). Statistical analyses revealed a significant increase in the number of platform crossings in the APP+EA group when compared with APP mice ( $P < 0.01$ ; Fig. 5b). Taken together, these results imply that repeated EA attenuated the spatial reference memory impairment in the APP/PS1 transgenic mice.

#### Discussion

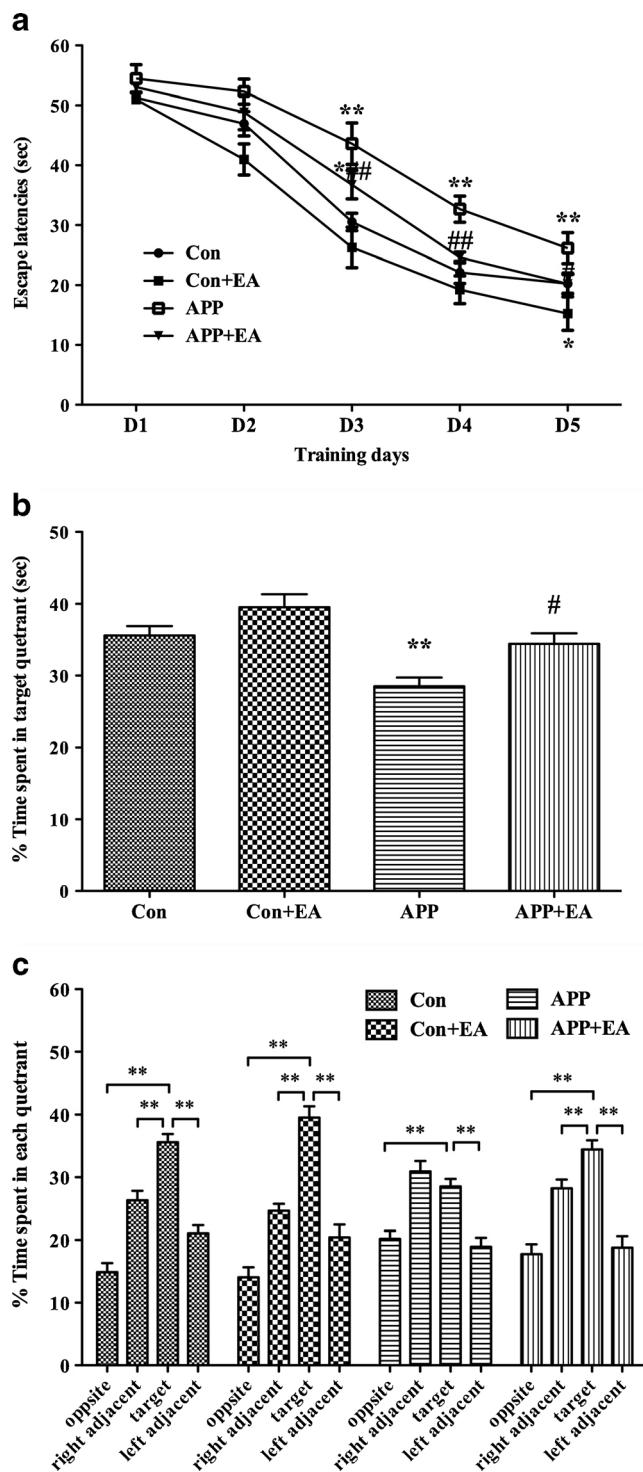
In the present study, we found that repeated EA administration ameliorated reference memory impairment in APP/PS1 double transgenic mice and reduced the upregulation of astrocytic NDRG2. This suggests that EA administration alleviates AD risk by inhibiting reference memory decline, potentially

**Fig. 4** Repeated EA attenuates the AD-associated upregulation of GFAP and NDRG2. **a** Representative double immunofluorescence staining for NDRG2 (green) and GFAP (red) in brain sections. The upregulation of GFAP and NDRG2 are attenuated (white arrowheads) in the APP+EA group. The inset displays a part of the image that has been magnified. Bar=20  $\mu$ m. **b** The protein expression of NDRG2 was evaluated by using Western blot. Repeated EA significantly downregulated the protein expression of NDRG2 in APP/PS1 mice. Data are means $\pm$ SEM ( $n=5$  in each group)



associated with the reduction of NDRG2. Our study provides new insight into a unique, potential AD therapy.

AD is a neurodegenerative disease with major clinical hallmarks of memory loss, dementia, and cognitive



**Fig. 5** Repeated EA ameliorates APP/PS1 mice's cognitive impairments in Morris water maze test. **a** Mice in the APP group show longer latencies for reaching the platform on days 3–5 compared with the Con group. These poor performances of APP/PS1 mice were improved by repeated administration of EA in the APP+EA group. **b** The probe test indicated that the time spent in the target quadrant decreased in the APP group compared with the Con group. These decreases are reversed by repeated

administration of EA in the APP+EA group. Con group: age-matched wild-type mice; Con+EA group: age-matched wild-type mice with repeated administration of EA; APP group: 11-month-old APP/PS1 mice; APP+EA group: 11-month-old APP/PS1 mice administered with repeated EA. **c** Percent time spent in the target quadrant in all probe trials is shown. \*\* $P < 0.01$  from the Con group, # $P < 0.05$  from the APP group

impairment [18]. Memory loss is one of the earliest symptoms of AD. There is no single treatment that can prevent or cure it.

Most current treatments focus on helping people to maintain mental functioning, managing behavioral symptoms, or

slowing down the progression of AD. For example, current FDA-approved medications for the treatment of AD include acetylcholine esterase inhibitors for mild to moderate cases and memantine, an *N*-methyl-D-aspartate receptor antagonist for the treatment of moderate to severe AD. However, neither of these pharmaceutical interventions has been proven to inhibit the progression of AD [21–23]. This could be attributed to the current researcher's narrow focus on symptomatically treating AD, as opposed to addressing the mechanisms underlying this neurological disorder. Therefore, there is a great need for developing therapeutic strategies based on the underlying pathogenetic cascade of events that characterize AD.

Acupuncture has been widely used for a range of neurological disorders [24]. Recently, a study has claimed that the use of acupuncture or EA for the treatment of AD and dementia has shown to be effective in improving intelligence [10]. Despite its popularity, the current literature on EA administration is contradictory [9]. Our previous study has demonstrated that EA pretreatment at the Baihui acupoint (GV 20) ameliorates hypergravity-induced impairment of learning and memory [8]. In the present study, we demonstrated that 4 weeks of EA administration ameliorated AD-induced cognitive impairments.

The hippocampus is the structural foundation of learning and memory. Long-term depression (LTD) is a long-lasting modification of synapses, particularly well studied in the CA1 region of the hippocampus. It has been reported that an acute exposure of exogenous cannabinoids induces a LTD of synaptic strength and an impairment of spatial reference memory at the hippocampal CA3-CA1 synapses [25]. We have previously reported that EA administration induces release of endocannabinoids [26]. In accordance with our reports of repeated EA administration ameliorating AD-associated reference memory impairments, we speculate that EA administration could induce endocannabinoid release leading to LTD, attenuating reference memory impairment through modulation of hippocampal LTD.

Astrocytes, the most numerous cells in the brain, interact with neurons at synaptic junctions and modulate the survival of neurons via regulation of synapses, coupling synaptic activity to glucose utilization. Therefore, astrocytes affect long-term potentiation (LTP) and LTD, which allows them to influence information-processing abilities of neurons [27, 28]. Brain injury activates astrocytes, which results in a noticeable increase in GFAP expression in reactive astrocytes [29]. Upregulation of GFAP has been reported in animal models of AD [12], which suggests that astrogliosis is an important feature in the neuropathology of this disease. Furthermore, studies have demonstrated that A $\beta$  in the AD brain can trigger astrocyte reactivation and astrocytic alterations that are associated with Morris water maze test performance [30].

Hence, astrocytes are believed to play a central role in the pathogenesis of AD [31]. Upregulation of hippocampal GFAP in APP/PS1 double transgenic mice was also observed in our present study. These alterations of hippocampal astrocytes may attribute to AD-induced cognitive impairment. The observed upregulation in the APP/PS1 mouse model was attenuated by EA administration, which suggests that EA reduced the reactivity of astrocytes typically observed in AD.

The age- and gender-independent upregulation of NDRG2 mRNA and protein expression in AD brains have been associated with pathological lesions observed in AD brains [16]. Our APP/PS1 double transgenic mice revealed that NDRG2 upregulation in the hippocampus was accompanied by astrogliosis and reference memory impairment. Repeated EA administration partially reversed the NDRG2 upregulation and ameliorated reference memory deficits. Therefore, NDRG2 may not only be involved in AD, but may also participate in the development of cognitive impairments that are characteristics of this disease [16]. Therefore, the upregulation of astrocytic NDRG2 may increase astrocytic reactivity, which impairs AD-induced cognitive function. Our findings may serve as a new potential therapeutic target and offer the possibility of developing new pharmacological interventions to treat AD. Although reduction in NDRG2 was observed in wild-type and APP/PS1 mice undergoing EA treatment, the degree of attenuation observed in both groups was distinct. The NDRG2 attenuation was much more remarkable in APP/PS1 mice than in wild-type controls. This may be an interesting starting point for further studies aimed at identifying possible mechanisms of EA.

In summary, our current study demonstrates that repeated EA administration ameliorates cognitive impairment, which suggests a unique therapeutic role for repeated EA administration in the treatment of AD. In addition, our results show that repeated EA reverses the upregulation of astrocytic NDRG2, implying that a reduction of NDRG2 could alleviate AD risk via inhibition of reference memory decline. However, this is merely speculation and merits more research in order to confirm the role of NDRG2 in AD-induced cognitive impairment. Future investigations are also required to reveal the time course of GFAP/NDRG2 expressions and their relationship with AD-induced cognitive function. Furthermore, subsequent studies should investigate the effect of repeated EA on behavior performance and GFAP/NDRG2 expressions in the early and later phases of AD. Given its efficacy and safety, EA would offer a rational alternative therapeutic approach for the treatment of AD and potentially for other neurodegenerative disorders.

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