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Intensity-dependent effects of repetitive anodal transcranial direct current stimulation on learning and memory in a rat model of Alzheimer's disease

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ABSTRACT

Single-session anodal transcranial direct current stimulation (tDCS) can improve the learning-memory function of patients with Alzheimer's disease (AD). After-effects of tDCS can be more significant if the stimulation is repeated regularly in a period. Here the behavioral and the histologic effects of the repetitive anodal tDCS on a rat model of AD were investigated. Sprague–Dawley rats were divided into 6 groups, the sham group, the β-amyloid (Aβ) group, the Aβ + 20 μA tDCS group, the Aβ + 60 μA tDCS group, the Aβ + 100 μA tDCS group and the Aβ + 200 μA tDCS group. Bilateral hippocampus of the rats in the Aβ group and the Aβ + tDCS groups were lesioned by $A\beta_{1-40}$ to produce AD models. One day after drug injection, repetitive anodal tDCS (10 sessions in two weeks, 20 min per session) was applied to the frontal cortex of the rats in the tDCS groups, while sham stimulation was applied to the Aβ group and the sham group. The spatial learning and memory capability of the rats were tested by Morris water maze. Bielschowsky's silver staining, Nissl's staining, choline acetyltransferase (ChAT) and glial-fibrillary-acidic protein (GFAP) immunohistochemistry of the hippocampus were conducted for histologic analysis. Results show in the Morris water maze task, rats in the Aβ + 100 μA and the Aβ + 200 μA tDCS groups had shorter escape latency and larger number of crossings on the platform. Significant histologic differences were observed in the Aβ + 100 μA and the Aβ + 200 μA tDCS groups compared to the Aβ group. The behavioral and the histological experiments indicate that the proposed repetitive anodal tDCS treatment can protect spatial learning and memory dysfunction of $A\beta_{1-40}$-lesioned AD rats.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder which is characterized by progressive loss of memory, perception, judgment and movement (Stuchbury & Munch, 2005). According to the statistics (Brookmeyer, Johnson, Ziegler-Graham, & Arrighi, 2007), there are more than 26 million AD patients around the world in 2006. But there are no effective clinical treatments can permanently cure AD (Freitas, Mondragón-Llorca, & Pascual-Leone, 2011; Shafqat, 2008). Nowadays, non-invasive brain stimulations, such as transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS) show great potentials in relieving the AD symptoms by neural modulation (Ahmed, Darwish, Khedr, El Serogy, & Ali, 2012; Bentwich et al., 2011; Freitas et al., 2011). These two techniques are similar in principle, but tDCS is more attractive because of the portability and the lower cost (Gandiga, Hummel, & Cohen, 2006). tDCS modulates the neural activity by injecting a weak direct current into cortex for further polarization of the neurons at the target (Hummel & Cohen, 2006). Studies of anodal tDCS in AD patients are reporting positive results. In a randomized cross-over sham-controlled study (Ferrucci et al., 2008), where anodal, cathodal and sham tDCS were applied to AD patients' temporoparietal cortex, recognition memory was improved by anodal tDCS whereas decreased by cathodal tDCS. Improvements in recognition memory of AD patients were also observed by applying single anodal tDCS over left dorsolateral prefrontal cortex (DLPFC) and left temporal

Abbreviations: AD, Alzheimer's disease; tDCS, transcranial direct current stimulation.

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cortex (Boggio et al., 2009). Furthermore, similar results were found and even maintained for at least 4 weeks after repetitive anodal tDCS (Boggio et al., 2012). Beyond human, one study based on AD rats suggested the application of anodal tDCS over the frontal cortex has beneficial effects on restoration of cognitive skill (Yu, Park, & Sim, 2014) which possibly resulted from the potential neuron-protective effect (Kim et al., 2010) and the wide-spread modulatory function in cortical–subcortical network of tDCS (Fregni et al., 2006).

The stimulation parameters, such as polarity, stimulation time and current intensity have diverse influences on the effects induced by tDCS (Nitsche et al., 2008). Based on previous findings, it is commonly agreed that tDCS-induced effects are modulated in a polarity-specific manner (Dockery, Liebetanz, Birbaumer, Malinowska, & Wesierska, 2011; Ferrucci et al., 2008; Fregni et al., 2006; Wachter et al., 2011). Animal studies have demonstrated that anodal stimulation increases and cathodal stimulation decreases the neural excitability, which is consistent with the findings in humans (Cambiaghi et al., 2010). Moreover, stimulation time also has influences on the effects. It was reported that with constant current density, the occurrence and duration of the effects depend on the stimulation time (Nitsche et al., 2008). When tDCS was applied for more than 10 min, a long-term effect in the neural excitabilities can last for more than 1 h (Nitsche & Paulus, 2000, 2001). This effect can last for even longer time if the stimulation was repeated (Benninger et al., 2010; Boggio et al., 2007). In rats, repetitive rather than single tDCS has been proven to improve motor function, and elicit inflammatory and regenerative processes in rat stroke models (Kim et al., 2010; Rueger et al., 2012). Similar repetitive stimulation was found to have antiepileptic effects in epilepticus rats (Kamida et al., 2011). However, the body of existing studies about the intensity-dependent effects of tDCS is rather small, especially in AD rats. Furthermore, the underlying mechanisms still remain unclear and need further investigation.

In this study, repetitive anodal tDCS was applied to the frontal cortex of Aβ-lesioned AD rats in order to evaluate intensity-dependent effects on spatial learning and memory in behavioral and histological levels. In addition, the possible mechanisms, safety and methodology considerations were also discussed.

2. Materials and methods

2.1. Animals

Female Sprague Dawley rats (8 weeks old and weigh 250–320 g) obtained from the Laboratory Animal Center of the Third Military Medical University (Chongqing, China) were employed for all experiments after one-week adaptive cultivation. They were housed in a humidity-controlled environment at 24 ± 1 °C with 12 h–12 h light–dark cycle, and permitted to take food and water freely. Rats were randomly divided into the sham group, the β-amyloid (Aβ) group, the Aβ + 20 μA tDCS group, the Aβ + 60 μA tDCS group, the Aβ + 100 μA tDCS group and the Aβ + 200 μA tDCS group, with six rats in each group.

All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animal of National Institutes of Health (Eighth Edition, NIH, USA).

2.2. Administration and hippocampus injection of $A\beta_{1-40}$

$A\beta_{1-40}$ (No. SCP0037, Sigma, St. Louis, USA) was dissolved into a sterile saline solution at a concentration of 2 μg/μl, and then incubated for one week at 37 °C. Aggregated $A\beta_{1-40}$ was stored in a refrigerator at 4 °C.

The rats in the Aβ group and the tDCS groups were anesthetized by intraperitoneal injection of 10% chloral hydrate (4 ml/mg), then fixed in a stereotaxic apparatus (No. 9084, Chengdu Instrument Plant, Chengdu, China). The hair and the scalp were cut and cleaned with alcohol. According to The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 1996), the stereotaxic coordinates of CA1 sub-region in hippocampus are: 3.3 mm posterior to bregma, 2 mm right and left of the sagittal, and 3.5 mm below skull (Figs. 1A and 2A). 5 μl $A\beta_{1-40}$ was injected bilaterally into hippocampus through two small holes (diameter: 0.8 mm) into the skull at a rate of 1.0 μl/min, then the needle was stayed for 5–10 min and withdraw at a rate of 1.0 mm/min. The fascia and skin were sutured and disinfected. The same operation was conducted to the rats in the sham group, but using 5 μl sterile saline instead of using $A\beta_{1-40}$.

2.3. Verification of the injection site

To verify the injection site, an injection test with black ink was conducted. The coronal rat brain section was used to show the injection site in the hippocampus (Fig. 1B).

2.4. Transcranial direct current stimulation

After the drug injection, the electrodes were installed. Based on the previous epicranial electrode protocol (Liebetanz et al., 2006a, 2006b), we modified the electrode with a plastic tube (inner diameter: 2 mm) filled with sponge and copper wire. The anodal

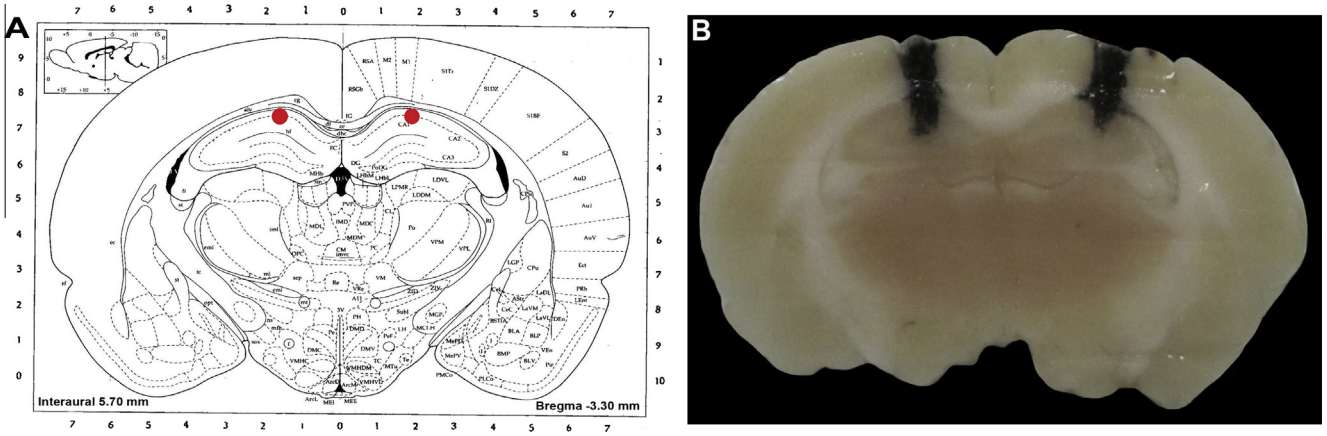

Fig. 1. (A) Schematic of a coronal section from 3.3 mm posterior to bregma, 2 mm right and left of the sagittal of the rat brain (Paxinos & Watson, 1996). It is showing the micro-injection sites (marked by red dots) of $A\beta_{1-40}$ and sterile saline. (B) Verification of CA1 sub-region stained by black ink in a coronal section of the rat's brain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

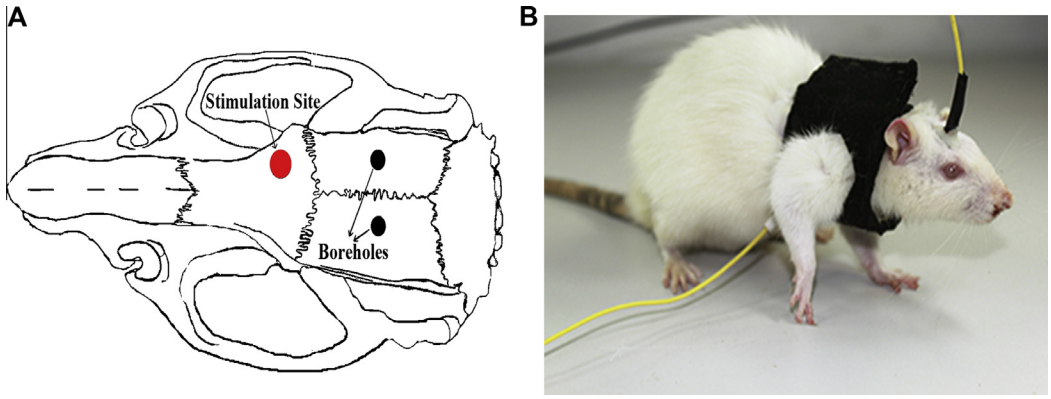

Fig. 2. (A) Schematic diagram displaying the boreholes above hippocampus and the location of stimulation electrode (anode) in the rat brain model. (B) Electrode configurations in an AD rat model. The anodal electrode was installed onto the skull above the right frontal cortex, while the cathodal electrode was placed onto the ventral thorax with a corset.

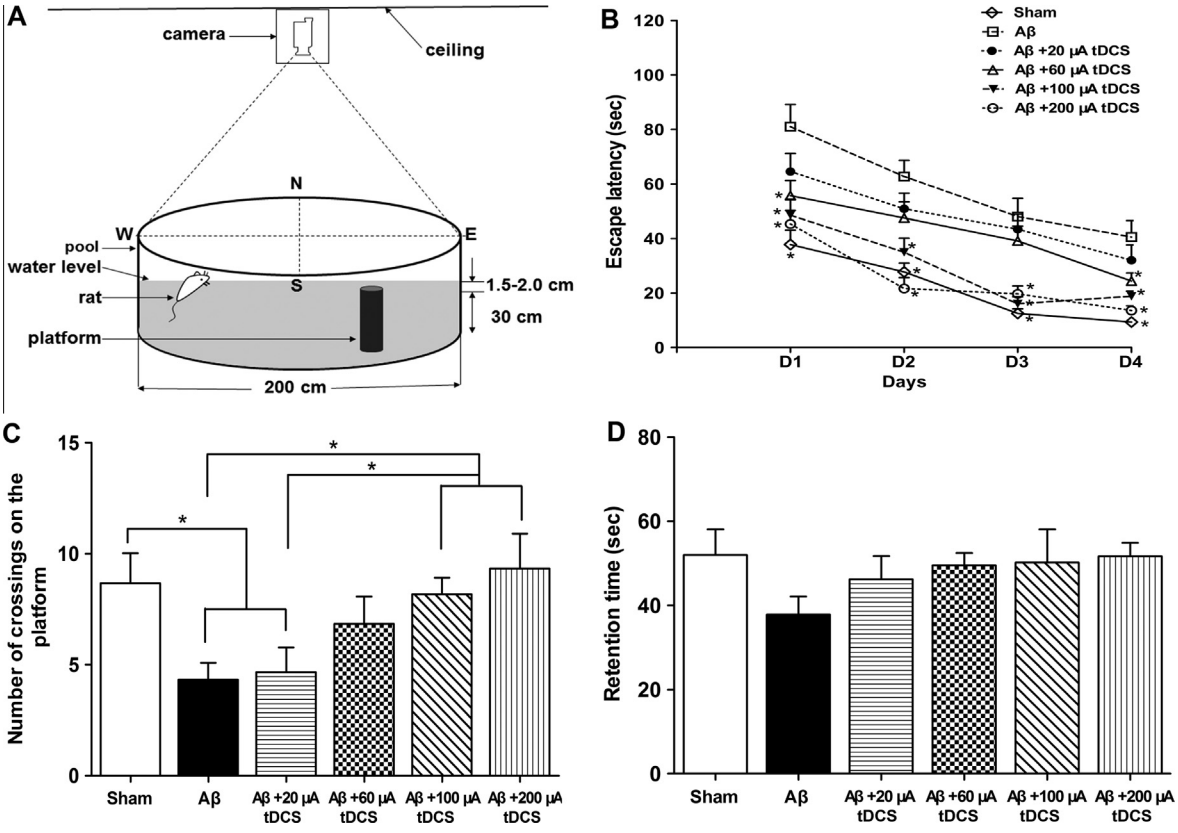

Fig. 3. The effects of repetitive anodal tDCS on spatial learning and memory of the rats in the MWM task. (A) Schematic diagram of the MWM task facilities. (B) Escape latency in the navigation trial. (C) The number of crossings on the platform in the probe trial. (D) Retention time in the probe trial. Data are presented as mean ± SEM. $n = 6$/group. $^{*}P < 0.05$.

electrode (contact area: 3.14 mm^2) was installed onto the skull above the right frontal cortex with glass ionomer cement (Fig. 2A). A larger sponge electrode (contact area: 10 cm^2) was used as the cathodal electrode which was placed onto the ventral thorax with a corset (Fig. 2B). Prior to tDCS, both anodal and cathodal electrodes were moistened with saline solution to reduce the contact impedance (Dundas, Thickbroom, & Mastaglia, 2007).

24 h after electrode installation, repetitive anodal tDCS was applied for 10 sessions in the following two weeks, 20 min per session, with current intensities of 20 μA, 60 μA, 100 μA and 200 μA respectively. Meanwhile, sham stimulation (anodal tDCS at 100 μA for 10 s) was performed in the sham and the Aβ groups. At the beginning and the end of tDCS, current was ramped up and down for 10 s to prevent damages to the brain tissue by suddenly changed current (Bindman, Lippold, & Redfearn, 1964).

2.5. Morris water maze

After all the 10 tDCS sessions, Morris water maze (MWM) task (Morris, 1984) was applied to measure the spatial learning and memory performance of the rats. As seen in Fig. 3A, the edges of the pool were divided into four quadrants by directions: E (east), S (south), W (west) and N (north). A circular platform, which was 1.5–2.0 cm below the water surface, was placed at the center of

the ES quadrant. During the process of the experiments, the surrounding environment was kept quiet and the reference substances were relatively unchanged.

2.5.1. Navigation trial

One day prior to the navigation trial, the rats were habituated to the pool (without platform) for 2 min. In every trial, the rats were placed into water at the start location with the heads facing toward the 1/2 radian of the quadrant. The time for the rats to climb on the platform within 120 s was recorded as the escape latency. If the rats failed to find the platform within 120 s, they were gently guided onto the platform with a stick, stayed there for 10 s, and then the escape latency was recorded for another 120 s. If the rats successfully found the platform within 120 s, they also stayed on the platform for 10 s to consolidate the memory. All rats were trained 4 times a day with a 20 min interval, and the trial was repeated for 4 days.

2.5.2. Probe trial

The probe trial was conducted 24 h after the navigation trial. Every rat was allowed to swim for 120 s in the pool (without platform), and at the same time, two variables were measured: (1) the number of crossings on the platform and (2) the retention time (the swimming time in the quadrant where the platform had been placed previously).

2.6. Histological procedure

One week later the last tDCS session, the rats were deeply anaesthetized, and perfused with about 200 ml room temperature saline solution, and then fixed with 300–500 ml 4% paraformaldehyde over 2 h at 4 °C. The brains were removed carefully from the skulls and placed into 30% sucrose in paraformaldehyde at 4 °C until they sink to the bottom. Coronal frozen sections of the brains were made by a freezing microtome (Leica CM1900, Germany) with a thickness of 25 μm.

2.7. Bielschowsky's silver staining

The frozen sections were hydrated in distilled water. Sections were incubated in a pre-warmed (37 °C) 2% silver nitrate solution away from light for 30 min. After rinsed 3 times, 5 min for each time (3 × 5 min) in distilled water, brain sections were reduced for 5 min with 10% formaldehyde, and then rinsed 3 × 5 min in distilled water again. Ammonium silver alcohol solution was added to the brain sections (200 μl/section) for 20–40 s in a wet box. Afterward, sections were directly reduced with 10% formaldehyde until they turned dark brown, and then rinsed 3 × 5 min in distilled water. Finally, the sections were fixed in a 5% sodium thiosulfate solution for 5 min and then rinsed 3 × 5 min in distilled water.

2.8. Nissl's staining

The frozen sections were hydrated by rinsing into 100%, 95%, 90%, 80%, 70% alcohol and distilled water in order (5 min for each). Then the sections were stained in a 1% toluidine blue solution for 5–10 min, and differentiated in 75% alcohol for seconds, then rinsed quickly in distilled water.

2.9. Choline acetyltransferase and glial-fibrillary-acidic protein immunohistochemistry

First of all, the frozen sections were rinsed 3 × 5 min in phosphate-buffered saline with tween 20 (PBST), and blocked with 3% H_2O_2 for 30 min to eliminate the activity of endogenous peroxidase. Then the sections were rinsed in PBST for 3 × 5 min again. Additionally, the sections were incubated with the goat serum at 37 °C for 1 h, then transferred directly to rabbit primary polyclonal antibody against choline acetyltransferase (ChAT) (1:100, No. BS6985, Bioworld, St. Louis, USA) and rabbit primary polyclonal antibody against glial-fibrillary-acidic protein (GFAP) (1:200, No. BA0056, Wuhan Boster Biological Technology, Wuhan, China) at 37 °C for 1 h, and then at 4 °C overnight. Thirdly, the sections were rinsed in PBST for 3 × 5 min and incubated with the biotinylated anti-rabbit secondary antibody at 37 °C for 1 h. In the end, after rinsed in PBST for 3 × 5 min, the sections were reacted with 0.05% diaminobenzidine for 6–8 min, and then rinsed in PBST for 3 × 5 min.

2.10. Hematoxylin and eosin staining

The coronal sections from the right frontal cortex (under the stimulation site) were used for hematoxylin and eosin (H&E) staining to see if there were tDCS-induced morphological changes in brain tissues.

2.11. Morphological analysis

The tissue slides were processed. All pictures were taken with an Olympus microscope (Olympus Co. Ltd., Japan). The average optical density of the positive immunoreactive cells of ChAT and GFAP immunohistochemistry in CA1, CA2-3 and dentate gyrus (DG) of the hippocampus of the rats in each group were measured using Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD) at 40× magnification.

2.12. Statistical analysis

Data were presented as the mean ± SEM and processed with SPSS19.0. For the navigation trial, we ran repeated measures analysis of variance (ANOVA) with the escape latency repeatedly measured on the four consecutive days (D1, D2, D3 and D4) as the repeatedly measured variable, and "group" (six levels: sham group, Aβ group, Aβ + 20 μA tDCS group, Aβ + 60 μA tDCS group, Aβ + 100 μA tDCS group and Aβ + 200 μA tDCS group) as the independent variable. For the probe trial, we ran a one-way ANOVA with the number of crossings on the platform and the retention time respectively as the dependent variables, and "group" (six levels: sham group, Aβ group, Aβ + 20 μA tDCS group, Aβ + 60 μA tDCS group, Aβ + 100 μA tDCS group and Aβ + 200 μA tDCS group) as the independent variable. For the ChAT and GFAP immunohistochemistry, we ran a two-way ANOVA with the average optical density of the ChAT and GFAP positive cells as the dependent variable, "group" (six levels: sham group, Aβ group, Aβ + 20 μA tDCS group, Aβ + 60 μA tDCS group, Aβ + 100 μA tDCS group and Aβ + 200 μA tDCS group) and "sub-region" (three levels: CA1, CA2-3, and DG) as the independent variables. When significant differences were observed, a post hoc test was made via LSD. Statistical significance referred to P value < 0.05.

3. Results

3.1. Changes of spatial learning and memory performance in Morris water maze

In the navigation trial, repeated measures ANOVA disclosed a significant main effect of group ($F_{(5,\ 120)}$ = 27.221, P < 0.001, Table 1) and day ($F_{(3,\ 120)}$ = 40.617, P < 0.001, Table 1) on the escape latency. However, group × day interaction was not significant ($F_{(15,\ 120)}$ = 0.553, P = 0.904, Table 1). As shown by post hoc test (Fig. 3B), the rats in the Aβ group spent a significantly longer time to find the

Table 1
F and *P* values of ANOVAs.

Experiments	Variables	Degrees of freedom	*F*	*P*
Navigation trial	*Escape latency*			
	Group	5	27.221	<0.001
	Day	3	40.617	<0.001
	Group × Day	15	0.553	0.904
Probe trial	*Crossing*			
	Group	5	3.257	0.018
	RT			
	Group	5	1.025	0.420
ChAT IHC	*AOD of ChAT*			
	Group	5	67.786	<0.001
	Sub-region	2	35.472	<0.001
	Group × Sub-region	10	1.332	0.231
GFAP IHC	*AOD of GFAP*			
	Group	5	24.029	<0.001
	Sub-region	2	9.097	<0.001
	Group × Sub-region	10	0.472	0.904

ChAT IHC, choline acetyltransferase (ChAT) immunohistochemistry; GFAP IHC, glial-fibrillary-acidic protein (GFAP) immunohistochemistry; Crossing, number of crossings on the platform; RT, retention time; AOD of ChAT, average optical density of the ChAT positive cells; AOD of GFAP, average optical density of the GFAP positive cells; Group × Day, Group × Day interaction; Group × Sub-region, Group × Sub-region interaction.

hidden platform than the rats in the sham group (all $P < 0.001$). Compared to the Aβ group, both 100 μA (D1: $P = 0.001$, D2: $P = 0.003$, D3: $P < 0.001$, D4: $P = 0.004$) and 200 μA (D1: $P < 0.001$, D2: $P < 0.001$, D3: $P < 0.001$, D4: $P = 0.001$) tDCS induced a significant decrease in escape latency across four days' MWM training. Interestingly, the significant differences in escape latency between the Aβ group and the Aβ + 60 μA tDCS group were observed only in the first ($P = 0.008$) and the fourth days ($P = 0.027$). 20 μA tDCS did not induce a significant change in escape latency compared to the Aβ group (all $P > 0.05$).

In the probe trial, the results of one-way ANOVA for the number of crossings on the platform indicated a significant difference among the groups ($F_{(5, 30)} = 3.257$, $P = 0.018$, Table 1). The rats in the Aβ group ($P = 0.013$) and the Aβ + 20 μA tDCS group ($P = 0.022$) had significantly decreased number of crossings on the platform compared to those in the sham group, while the rats with 100 μA ($P = 0.027$ vs. Aβ group; $P = 0.042$ vs. Aβ + 20 μA tDCS group) and 200 μA ($P = 0.005$ vs. Aβ group; $P = 0.008$ vs. Aβ + 20 μA tDCS group) tDCS showed significant increases in the number of crossings on the platform when compared to those in the Aβ group and the Aβ + 20 μA tDCS group (Fig. 3C). For the retention time, no significant differences were observed among the groups ($F_{(5, 30)} = 1.025$, $P = 0.420$, Table 1) (Fig. 3D).

3.2. Changes of nerve fibers in various sub-regions of hippocampus

Silver staining results (Fig. 4) show the nerve fibers of the hippocampus of the rats were stained as black filaments, and obvious dendrites and axons were seen in CA1, CA2-3 and DG. Several intracellular neurofibrillary tangles (NFT)-like changes in the hippocampus were found in the Aβ group and the tDCS groups, whereas not in the sham group. The intracellular NFT-like changes in the hippocampus between the Aβ group and the tDCS groups indicated no significant differences.

3.3. Changes of Nissl bodies in various sub-regions of hippocampus

As one of neural characteristic structures, the Nissl bodies can reflect the distribution of neurons. Therefore they are investigated as a marker of neurodegeneration in the hippocampus. Intraneural Nissl bodies of hippocampus in the Aβ group, the Aβ + 20 μA tDCS group, and Aβ + 60 μA tDCS group appeared in gritty shape, and exhibited sparsely arranged and lightly stained. However, deeper stained Nissl bodies with higher density in the hippocampus neurons were found in the Aβ + 100 μA tDCS group and the Aβ + 200 μA tDCS group (Fig. 5).

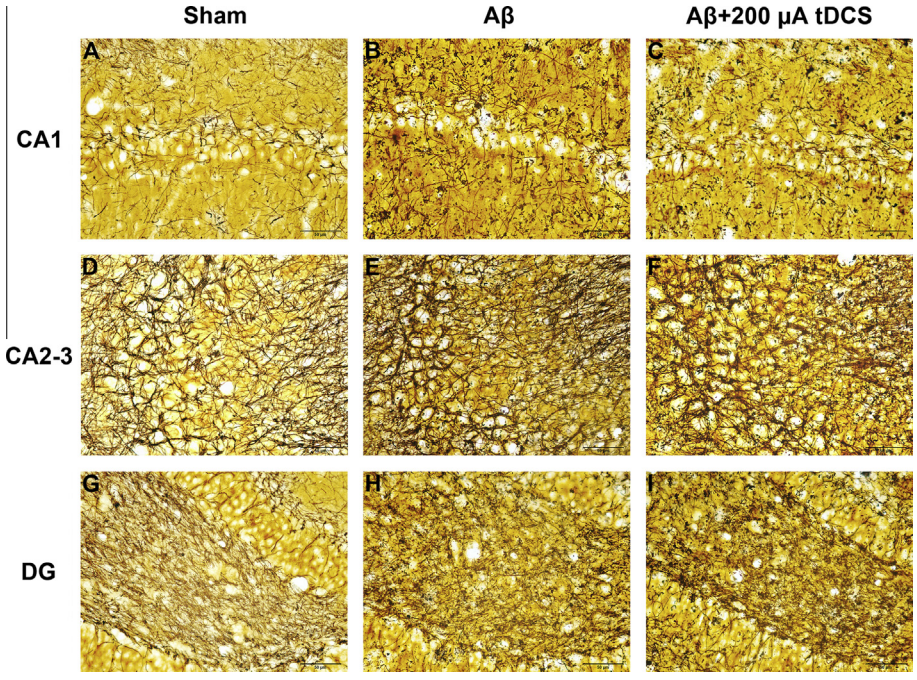

Fig. 4. Silver staining results of the nerve fibers in various sub-regions of hippocampus. (A) CA1 of the sham group, (B) CA1 of the Aβ group, (C) CA1 of the Aβ + 200 μA tDCS group, (D) CA2-3 of the sham group, (E) CA2-3 of the Aβ group, (F) CA2-3 of the Aβ + 200 μA tDCS group, (G) DG of the sham group, (H) DG of the Aβ group, (I) DG of the Aβ + 200 μA tDCS group. All stains were observed at 40×.

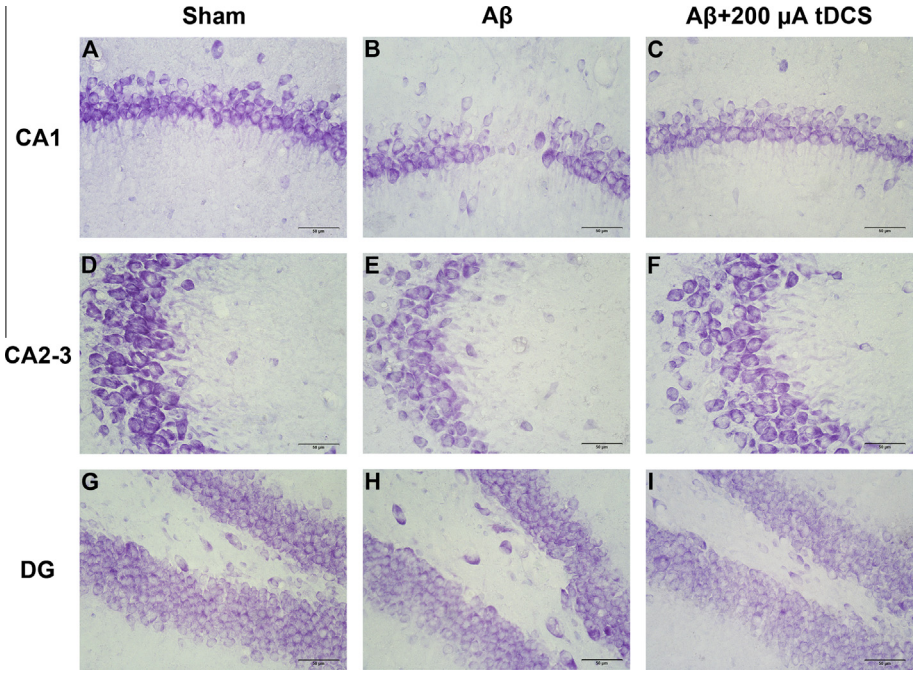

Fig. 5. Nissl's staining results of Nissl bodies in various sub-regions of hippocampus. (A) CA1 of the sham group, (B) CA1 of the Aβ group, (C) CA1 of the Aβ + 200 μA tDCS group, (D) CA2-3 of the sham group, (E) CA2-3 of the Aβ group, (F) CA2-3 of the Aβ + 200 μA tDCS group, (G) DG of the sham group, (H) DG of the Aβ group, (I) DG of the Aβ + 200 μA tDCS group. All stains were observed at 40×.

3.4. Changes of the expression of ChAT and GFAP in various sub-regions of hippocampus

Two-way ANOVA of the average optical density of the ChAT positive cells in the hippocampus showed a significant difference in term of group ($F_{(5,\ 90)} = 67.786$, $P < 0.001$, Table 1) and sub-region ($F_{(2,\ 90)} = 35.472$, $P < 0.001$, Table 1). No differences were found in term of group × sub-region interaction ($F_{(10,\ 90)} = 1.322$, $P = 0.231$, Table 1). The rats in the Aβ group and the Aβ + 20 μA tDCS group showed obviously decreased expression of ChAT than those in the sham group (all $P < 0.001$). Compared to the Aβ group and the Aβ + 20 μA tDCS group, tDCS with 60 μA, 100 μA and 200 μA led to significant increases in the expression of ChAT in all sub-regions (all $P < 0.001$, except $P = 0.013$ in CA2-3 (Aβ + 60 μA tDCS group vs. Aβ + 20 μA tDCS group); $P = 0.003$ in CA2-3 (Aβ + 100 μA tDCS group vs. Aβ + 20 μA tDCS group)). However, the increases were still significantly lower than those in the sham group (all $P < 0.001$ (Aβ + 60 μA tDCS group and Aβ + 100 μA tDCS group vs. sham group); $P = 0.008$ in CA1, $P = 0.038$ in CA2-3, $P = 0.045$ in DG (Aβ + 200 μA tDCS group vs. sham group)). Moreover, rats in the Aβ + 200 μA tDCS group had higher expression of ChAT in CA2-3 ($P = 0.036$) and DG ($P = 0.003$) than those in Aβ + 60 μA tDCS group (Fig. 6).

The expression of GFAP was indexed by the average optical density of the GFAP positive cells in the hippocampus. Significant effects of group ($F_{(5,\ 90)} = 24.029$, $P < 0.001$, Table 1) and sub-region ($F_{(2,\ 90)} = 9.097$, $P < 0.001$, Table 1) were observed. The expression of GFAP in the Aβ group and the Aβ + 20 μA tDCS group was obviously different from that in the sham group (all $P < 0.001$). Compared to the Aβ group and the Aβ + 20 μA tDCS group, the AD rats received 200 μA tDCS showed a significantly lower level of GFAP in all hippocampal sub-regions ($P = 0.003$ in CA1, $P = 0.009$ in CA2-3, $P < 0.001$ in DG (Aβ + 200 μA tDCS group vs. Aβ group); $P = 0.006$ in CA1, $P = 0.04$ in CA2-3, $P < 0.001$ in DG (Aβ + 200 μA tDCS group vs. Aβ + 20 μA tDCS group)). Rats in the Aβ + 100 μA tDCS group showed a significantly decreased expression of GFAP only in CA1 ($P = 0.014$ vs. Aβ group; $P = 0.022$ vs. Aβ + 20 μA tDCS group) and DG ($P = 0.012$ vs. Aβ group; $P = 0.026$ vs. Aβ + 20 μA tDCS group). Only subtle differences were found between the Aβ group, the Aβ + 20 μA tDCS group and the Aβ + 60 μA tDCS group (all $P > 0.05$) (Fig. 7).

3.5. Morphological changes of the cortex tissue under the stimulation site

As visualized by H&E staining of the brain slides from the sham group and the Aβ + 200 μA tDCS group (Fig. 8), cortical cells under the stimulation site were arranged in neat rows and had inerratic shape, and no karyopyknosis, karyolysis or rupture of cytomembranes were found.

4. Discussion

Consistent with previous observations (Bagheri, Joghataei, Mohseni, & Roghani, 2011; Nitta & Nabeshima, 1996), the only Aβ-injected rats in our study exhibited poor spatial learning and memory performance and histological deficiency in the density of Nissl bodies of the hippocampal neurons. Increased expression of GFAP was seen around the drug injection site, which means activated astrocytes. Reduced expression of ChAT was also observed in the AD rats when compared to the sham group. Rats in the Aβ + 100 μA tDCS group and Aβ + 200 μA tDCS group had better acquisition of spatial memory across the training days of the MWM task. Moreover, they maintained higher density of Nissl bodies and more expression of ChAT, as well as lower expression of GFAP in the hippocampal neurons. These observations indicated a protective effect of repetitive anodal tDCS on the neurons, keeping them from being damaged by the Aβ neurotoxicity. But no significant differences were found by using 20 μA and 60 μA repetitive anodal tDCS which revealed the intensity-dependent property of tDCS.

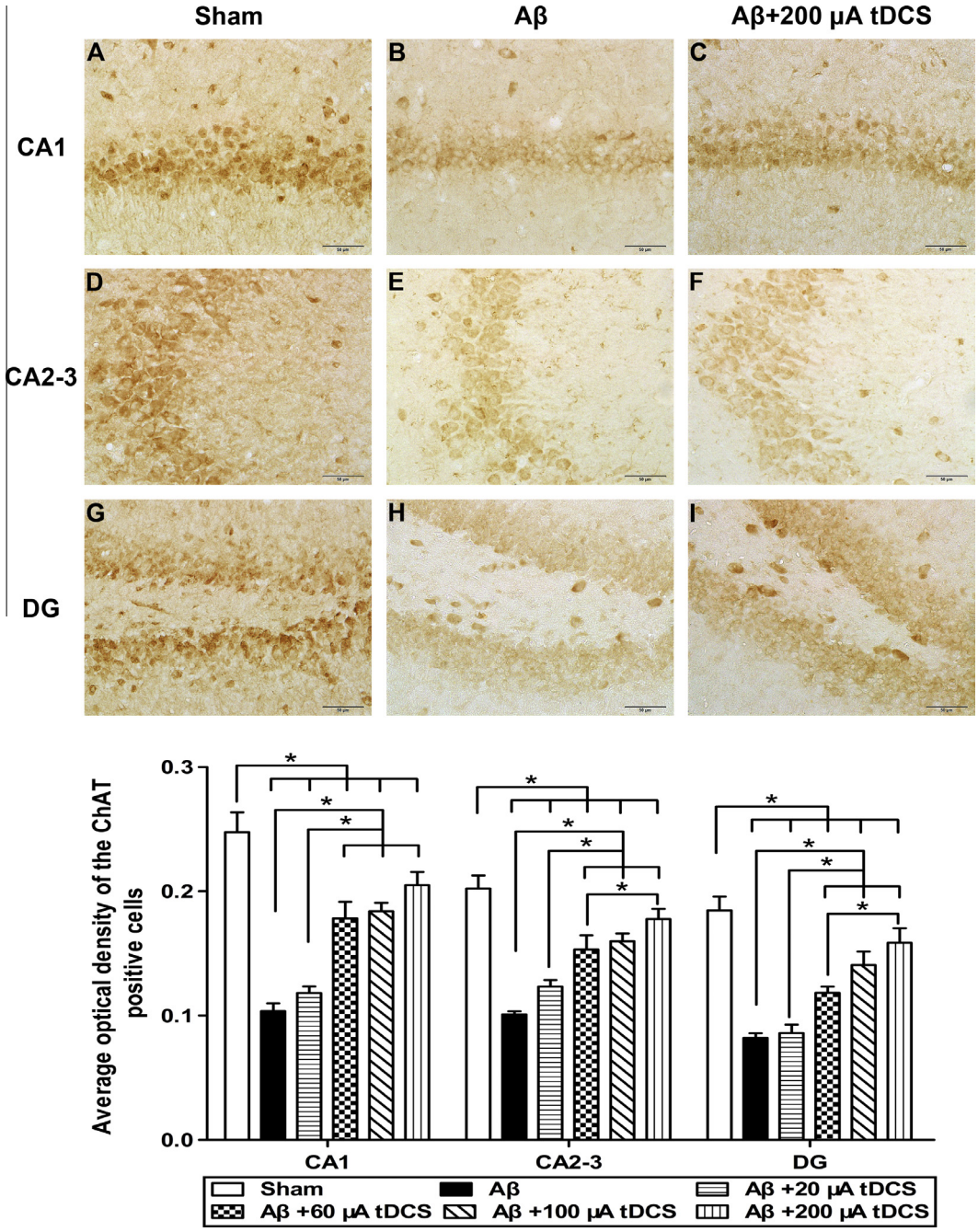

Fig. 6. The protective effects of repetitive anodal tDCS on the expression of ChAT in various sub-regions of hippocampus. The average optical density of the ChAT positive cells was measured at 40× using Image-Pro Plus 6.0. (A) CA1 of the sham group, (B) CA1 of the Aβ group, (C) CA1 of the Aβ + 200 μA tDCS group, (D) CA2-3 of the sham group, (E) CA2-3 of the Aβ group, (F) CA2-3 of the Aβ + 200 μA tDCS group, (G) DG of the sham group, (H) DG of the Aβ group, (I) DG of the Aβ + 200 μA tDCS group. All stains were observed at 40×. Data are presented as mean ± SEM. $n = 6$/group. $^{*}P < 0.05$.

4.1. Possible mechanisms of tDCS in improving learning and memory of the AD rats

Pathogenesis of AD has not been completely clarified. Generally, an abnormal deposition of Aβ in the hippocampus may activate inflammatory reaction, oxidative stress, destruction of intracellular calcium homeostasis and neuronal apoptosis (Verdile et al., 2004). These complications may result in neural circuitry dysfunction and further catalyze degeneration of the hippocampus area (Bagheri et al., 2011; Shankar & Walsh, 2009). In our study, aggregated $A\beta_{1-40}$ was injected into bilateral hippocampal CA1 sub-region to produce rat models of AD. Morphological results showed the hippocampal CA1 sub-region was damaged by $A\beta_{1-40}$ (Nitta & Nabeshima, 1996). It was shown in this study that repetitive anodal tDCS over the frontal cortex can relieve the spatial learning and memory deficits on behavioral and cellular levels in the rat models of AD. The primary hypothesis of anodal tDCS action is that the static electric fields, elicited by the weak direct current flowing from the cathode to the anode, can increase excitability of frontal cortex through the depolarization of neuronal resting membrane potentials (Liebetanz, Nitsche, Tergau, & Paulus, 2002). Hereby, the strength of the electric fields, proportional to stimulation intensity, determines the range and level of the neurons affected (Nitsche & Paulus, 2000). By using a stronger stimulation intensity (100 μA

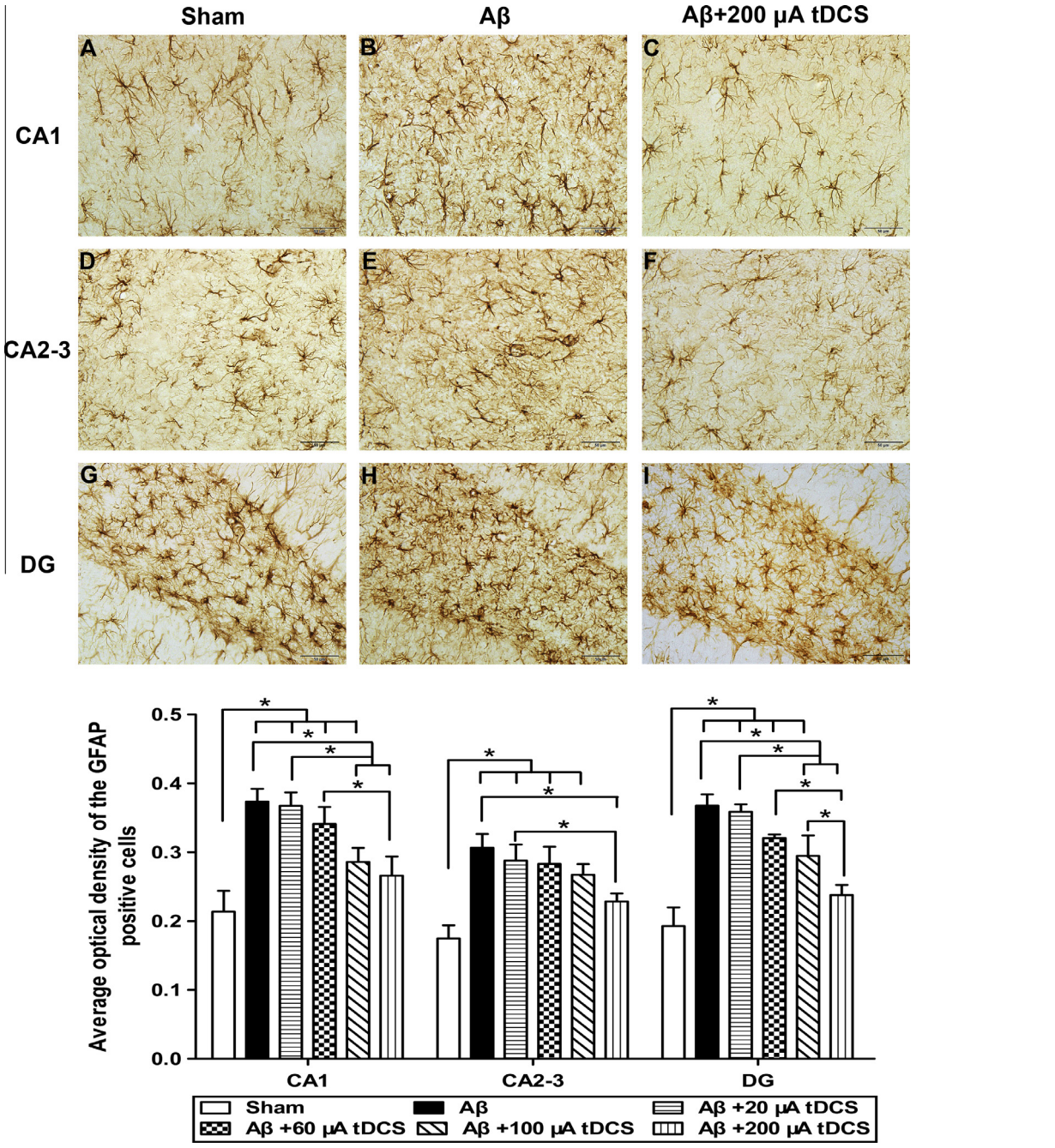

Fig. 7. The inhibitory effects of repetitive anodal tDCS on the expression of GFAP in various sub-regions of hippocampus. The average optical density of the GFAP positive cells was measured at 40× using Image-Pro Plus 6.0. (A) CA1 of the sham group, (B) CA1 of the Aβ group, (C) CA1 of the Aβ + 200 μA tDCS group, (D) CA2-3 of the sham group, (E) CA2-3 of the Aβ group, (F) CA2-3 of the Aβ + 200 μA tDCS group, (G) DG of the sham group, (H) DG of the Aβ group, (I) DG of the Aβ + 200 μA tDCS group. All stains were observed at 40×. Data are presented as mean ± SEM. $n = 6$/group. $^{*}P < 0.05$.

and 200 μA in our study), more current can penetrate the obstacles and affect more neurons. If the stimulation intensity is smaller (20 μA and 60 μA in our study), the current shunted by the scalp and skull becomes significant and thus the neurons cannot be thoroughly polarized. Moreover, increasing the stimulation intensity may affect more neurons in deeper cortical layers, e.g. the pyramidal cells are only affected by strong current. Significant increases of neural activities in both frontal cortex and nucleus accumbens after anodal tDCS in rats were observed by a functional magnetic resonance imaging (fMRI) (Takano et al., 2011). Similarly, it can be speculated that anodal tDCS over frontal cortex may cause a wide-spread influence over distant structures including basal forebrain and hippocampus throughout a distributed and interconnected cortical–subcortical network. Neurons directly stimulated by the anodal tDCS will in turn modulate the activity of nearby neurons and activate neuronal circuits related to learning and memory function. So the effects of the anodal tDCS are amplified. In addition, the release of neurotransmitters such as acetylcholine (ACh) and dopamine are possible candidates for these effects induced by anodal tDCS. The concentration of ACh, known to be associated with learning and memory capability, is controlled by the balance between ChAT and acetylcholinesterase (AChE) (Bagheri et al., 2011). Anodal tDCS can moderately protect the cholinergic neurons against damages caused by $A\beta_{1-40}$, thus

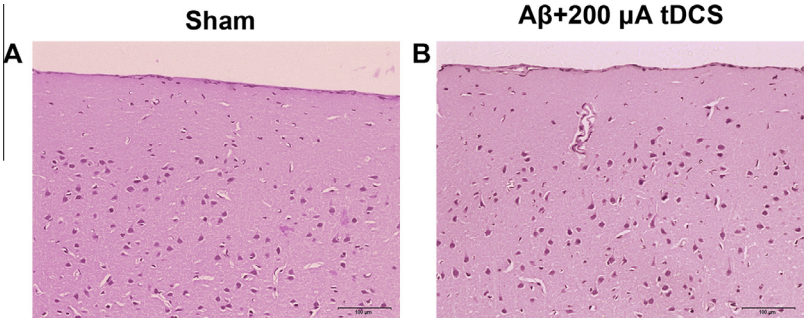

Fig. 8. H&E staining of the frontal cortex slides under the stimulation site. (A) The sham group, (B) the Aβ + 200 μA tDCS group. The stains were observed at 20×.

maintain the expression of ChAT and raise the ACh level in hippocampus. Also, the hypothesis that anodal tDCS induces increased dopamine concentration in the dorsal hippocampus as repetitive TMS does (Keck et al., 2002) is also considered as a contribution to the improvement of the spatial learning and memory deficits of AD rats (Nitsche et al., 2006).

The effects induced by anodal tDCS may not only depend on the depolarization of neuronal resting membrane potentials, but also be partly associated with long-term potentiation (LTP) of N-methyl-D-aspartate (NMDA) receptor-dependent synaptic plasticity in hippocampus (Liebetanz et al., 2002; Nitsche et al., 2003). Anodal tDCS can induce the release of glutamate (Glu) through depolarization of postsynaptic membrane potentials, and then activate NMDA receptors to combine with Glu via increasing intracellular Ca^{2+} level. The increased Ca^{2+} results in the production of LTP which plays an important role in the formation and strength of learning and memory (Stagg & Nitsche, 2011; Stagg et al., 2009). Anodal tDCS can modulate the synaptic plasticity by boosting the level of brain-derived neurotrophic factor (BDNF) (Fritsch et al., 2010) which can promote the formation of dendrite branches and the neuronal sprouts.

In a rat model of AD, abnormal aggregation of Aβ in the bilateral hippocampus led to activation of astrocytes, which were seen as an increase of GFAP level (Nitta, Fukuta, Hasegawa, & Nabeshima, 1997). Activated astrocytes act as a dual sword in the progression of AD (Verkhratsky, Olabarria, Noristani, Yeh, & Rodriguez, 2010). Once exposed to Aβ, activated astrocytes enhance the formation of neuronal synapse by releasing a variety of neurotrophic factors. Moreover, they can separate the normal neurons from Aβ. Therefore, they have neuroprotective effects on the neurons (Mohamed & Chaves, 2011). On the other hand, activated astrocytes can play a neurotoxic role through the release of inflammatory and neurotoxic factors and aggravate neuronal death (Verkhratsky et al., 2010). In the AD cases, the neurotoxic role of activated astrocytes dominates. Indicated by Ruohonen and Karhu, anodal tDCS can increase neurons' resistibility to Aβ toxicity, and modulate astrocytes (Ruohonen & Karhu, 2012). In theory, anodal tDCS is capable of continuously depolarizing transmembrane potentials of astrocytes to decrease their vulnerability to Aβ (Ruohonen & Karhu, 2012). In this study, the less activated astrocytes in the tDCS groups (100 μA and 200 μA) may result in decreased expression of inflammatory factors, such as nuclear factor-κB (NF-κB) and tumor necrosis factor-α (TNF-α), which may be responsible for the improvement of spatial learning and memory decline in AD rats.

Apart from the above assumptions, alterations in cerebral blood flow (CBF) by tDCS are also possible for relieving the spatial learning and memory degeneration (Wachter et al., 2011). Increased CBF induced by anodal tDCS has modulatory effects on neuronal metabolic activity and provides more blood flow to the lesioned brain regions, which alleviates neuron apoptosis.

So the anodal tDCS-induced improvement in spatial learning and memory of the AD rats is a consequence of a multi-factor neural modulation which includes different neural pathway interactions and blood-assisted neural protection.

4.2. Technical aspects

Rather than the symmetric electrode montage in human experiments (Fregni et al., 2006), we took the advantage of an asymmetric electrode montage similar to that employed previously (Liebetanz et al., 2006b): the stimulation electrode was installed onto the skull above the right frontal cortex, and the reference electrode was placed onto the ventral thorax. There are four advantages by using this montage: (1) It compensates for poor spatial resolution of tDCS by using smaller stimulation electrode and larger reference electrode (Nitsche et al., 2007). (2) Two distant electrodes decrease the bypassing of current on the scalp and skull, so higher current density is achieved under the electrodes (Miranda, Lomarev, & Hallett, 2006). (3) The improvement of spatial learning and memory dysfunction is considered to purely result from the increased excitability induced by anodal stimulation over the right hemisphere without any suppressing transcallosal inhibition induced by cathodal stimulation over the left hemisphere (Hummel et al., 2005). (4) The stimulation electrode installed onto the skull is solid and long-lasting that can avoid the process of tDCS from being interrupted by the rats.

Frontal cortex was selected as the stimulation site instead of primary motor cortex (M1) because it is well known that prefrontal cortex is essential for encoding and retrieval of spatial memory (Churchwell, Morris, Musso, & Kesner, 2010). Boggio et al. (2006) revealed an improvement in memory performance of the patients with Parkinson's disease after anodal tDCS over the left DLPFC. But no significant changes in memory performance occurred after anodal tDCS over the left M1. So it may be more effective to apply tDCS over DLPFC than over M1 for memory improvements.

The MWM task used in this study consists of the navigation trial and the probe trial. In the navigation trial, the platform, hidden beneath the water surface, remained the same location at the center of the ES quadrant. During the probe trial, the platform was removed from the pool. As a consequence, the platform designed in the present MWM should belong to the non-visible one. The present procedure required the rats to search for the non-visible platform using spatial cues in the environment, rather than using the self-position as a reference. So the memory measured in this study belongs to reference memory (Morris, Garrud, Rawlins, & Okeefe, 1982). Due to the encoding and retrieval of mnemonic information involved with limbic system (such as hippocampus) and relevant cerebral cortex regions (D'Hooge and De Deyn, 2001), accompanied with the Hebb synaptic modification (Lisman, 1989), the memory tested in this study is also the declarative memory (Morris et al., 1982).

In line with AD patients studies, this study confirms and extends the notion that repetitive anodal tDCS leads to significant improvements in spatial learning and memory deficits of AD rat models, moreover these effects are also intensity-dependent. In Dockery et al.'s study (Dockery et al., 2011), a single-session stimulation was applied over the frontal cortex in intact rats. Contrary to our results, long-term benefits of spatial working memory as compared to the controls were observed in the intact rats after cathodal rather than anodal tDCS. The controversial results may be caused by several differences between the two studies. First, the two studies tested different types of memory. The two major types of memory have been investigated in rats are reference memory and working memory (Frick, Baxter, Markowska, Olton, & Price, 1995; Givens & Olton, 1990; Morris, 1984; Morris et al., 1982). Reference memory is trial-independent. The acquired information, which is consistent from trial to trial, may be used in every trial rather than in just a single trial (Morris et al., 1982). But working memory is trial-dependent. Working memory is engaged to remember the current information because the information is changed from trial to trail (Frick et al., 1995). In present MWM task, the rats were tested to search for a fixed submerged or removed platform by spatial cues in the environment, which belongs to the reference memory testing procedure. Whereas, in the allothetic place avoidance alternation task (APAAT) used in Dockery et al.'s study, working memory was involved to support temporary storage of the location of to-be-avoided sector which was altered daily (Dockery et al., 2011). Since the effects of tDCS are task-dependent (Boggio et al., 2009, 2012), different tasks may result in different results between the two studies. Second, different stimulation time was used in the two studies. Repetitive tDCS sessions were applied in this study. Single session of tDCS used in Dockery et al.'s study may not be able to induce LTP of synaptic plasticity in hippocampus (Boggio et al., 2007). Third, the difference in brain physiology between intact rats and AD rats seems to impact the effects induced by tDCS. In human studies, it has been proven that with regard to working memory, anodal tDCS has positive effects on healthy subjects (Fregni et al., 2005), whereas causes no changes in AD patients (Boggio et al., 2009, 2012).

Moreover, Dockery's study showed paradoxical results in rats relative to the results reported in AD patients (Boggio et al., 2009, 2012). The mechanisms underlying these opposite effects are still unknown. Further systematic studies on polarity-dependent effects of tDCS on both reference and working memory are needed to answer this intriguing argument.

4.3. Safety of tDCS

The safety of tDCS is considered to be dependent on the current density (the ratio of current intensity and electrode size). When single cathodal tDCS over M1 at 500 μA for 10 min was applied, evident brain lesions were observed (Liebetanz et al., 2009). So the safe current density threshold in rat experiments was considered to be 142.9 A/m^2 (Liebetanz et al., 2009). Poreisz, Boros, Antal, and Paulus (2007) explored the safety limits of tDCS in human beings: when the current density was lower than 57 A/m^2, there were no severe side-effects observed, but only a slight itching under the electrode, a little fatigue and headache, etc. Moreover, in an MRI test (Nitsche et al., 2004), there were no remarkable changes found in brain structure, blood brain barrier and serum neuron-specific enolase level after tDCS, which means no neuronal damages created. Consequently, tDCS is considered to be safe if the applied current density is controlled within the suggested range. The largest current density of tDCS used in this study was 63.69 A/m^2 (200 μA/3.14 mm^2), which is lower than the safety threshold (142.9 A/m^2) (Liebetanz et al., 2009). On the other hand, H&E staining results support the assumption that the tDCS applied was safe to the rats.

5. Conclusions

In conclusion, our results show repetitive anodal tDCS can improve the spatial learning and memory dysfunction in AD rats in an intensity-dependent manner, indicating clinical application potentials in AD patients. More investigations on the duration of the effects induced by repetitive anodal tDCS, the effects of cathodal tDCS in AD patients should be concerned in subsequent studies.

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