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Investigation of The Learning and Memory Enhancing Effects of 0.25 mA and 0.5 mA Anodal and Cathodal Transcranial Direct Current Stimulations in Healthy Rats

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Abstract

Objective: Our aim in this study was to investigate the effects of tDCS, which is known to be effective on AMPA and NMDA, with different anodal and cathodal stimulation types and 0.25 mA and 0.5 mA current intensities on learning and memory by behavioral and molecular mechanisms.

Methods: 50 male Wistar rats weighing 290-310 g were divided into 5 groups as control, C1-tDCS, C2-tDCS, A1-tDCS and A2-tDCS. In the C1-tDCS group, 0.25 mA cathodal tDCS stimulation for 30 min per day for 5 days, in the C2-tDCS group for 30 min per day for 0.5 mA cathodal tDCS stimulation for 5 days, in the A1-tDCS group for 5 days with 0.25 mA anodal tDCS stimulation for 30 min per day and A2-tDCS group The tDCS group received 0.5 mA anodal tDCS stimulation for 30 minutes per day for 5 days. On the 6th and 7th days of the experiment, the locomotor activity, learning and memory behaviors of the rats were evaluated by open field test, y maze test and object localization test. In addition, glutamate levels were measured in hippocampus tissues by ELISA method.

Results: It was observed that there were non-significant decreases in the results of the C1-tDCS and C2-tDCS groups in which cathodal stimulation was applied compared to the control group in locomotor activity, learning and memory data. On the other hand, an increase was observed in the data of the A1-tDCS and A2-tDCS groups in which anodal stimulation was applied, and the increase in the data of the A2-tDCS group from these groups was found to be statistically significant compared to the control ($p<0.05$). Similar results were also seen in glutamate levels. A non-significant decrease in glutamate levels was observed in the C1-tDCS and C2-tDCS groups compared to the control, while an insignificant increase in glutamate levels in the A1-tDCS group was observed. On the other hand, there was a significant increase in glutamate level in the A1-tDCS group compared to the control group ($p<0.05$).

Conclusion: In conclusion, our data showed that 0.5 mA anodal tDCS stimulation for 30 min for 5 days can enhance learning and memory on the glutamatergic pathway.

Key words: Glutamate, Hippocampus, Learning, Memory, tDCS

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INTRODUCTION

The brain is the organ responsible for important physiological functions such as seeing, smelling, walking, running, thinking, feeling, attention, learning and memory (1). One of the most important functions of the brain is to obtain information about what is happening around people and to store the information they have acquired for later use. Learning is a process involving the acquisition of information about the environment and its changes on the behavior of such information (1). Memory, on the other hand, is the ability to store what is experienced and learned in the mind by establishing a relationship with the past, and it is shown as a trace of learning that remains in neural networks (1). Glutamate is the most important excitatory neurotransmitter in the central nervous system, which plays an active role in learning and memory formation in the hippocampus (1). Glutamate activity, the main neurotransmitter of learning, and N-methyl-D-aspartate (NMDA) receptor activity, which is the ionotropic glutamate receptor, are modulated by transcranial direct current stimulation (tDCS). Studies have reported that anodal tDCS improves behavioral performance and has an excitatory effect, while cathodal tDCS has an inhibitory effect on performance or neuronal activation (2-4).

tDCS, a non-invasive brain stimulation technique, is cheap and easy to use, and has no negative side effects (5). This technique is applied superficially to the cerebral cortex by applying constant and low-intensity current from the skull and the direct current given from the active electrode placed in the skull reaches the reference electrode by passing through the cerebrum tissue (5). tDCS is caused by the passage of a constant DC in the cerebrum after the two terminals of a battery-based stimulator are placed in the cerebrum (6). Weak current transmitted by tDCS, could not trigger a rapid depolarization of the electric field in the brain tissue, not directly create action potentials in cortical neurons (6). Therefore, tDCS can be considered to have a neuromodulatory effect. The current flow direction determines the effects of electrical excitation (6). tDCS has useful effects in an extensive variety of clinical pathologies like epilepsy (7), stroke (8, 9), and various pain conditions, and also psychiatric conditions such as depression and addiction (4, 10). In recent studies, it has been shown to affect cell excitability and epileptic discharges by changing membrane potential without creating an action potential in neurons (11, 12). Furthermore, tDCS has noninvasive clinical neuroprotective effects, which is preferred, especially for the treatment of learning and memory disorders (13, 14). In addition, tDCS is preferred in studies investigating the effects of cognitive functions such as learning, memory, and decision-making in healthy individuals (15). The duration, polarity and direction of the direct current applied determine the degree of change in the

brain (16). The current passing through the brain tissue creates increasing or decreasing excitable effects in the cortical area (17). The excitability is determined by the intensity of the current and the polarity with anodal stimulation or cathodal stimulation (17). There are two types of tDCS as anodal and cathodal tDCS. Anodal stimulation (A-tDCS) creates a depolarizing response with an excitatory effect, whereas cathodal stimulation (C-tDCS) induces a hyperpolarized response with an inhibitory effect (2-4). In studies related to the efficiency of tDCS stimulation, it has been shown the activation or inhibition effects last up to 90 minutes depending on the duration and location of the stimulation, and these effects continue even longer after regenerative stimulus (18, 19). It is known that A-tDCS application creates depolarization in neuron membranes and increases the excitability of cortical neurons by activation of Na+-Ca2+ dependent channels in neurons (17). Anodal tDCS shows its effects by modulation at both GABAergic (short interval intracortical inhibition) and glutaminergic (intracortical facilitation) synapses, while cathodal tDCS exerts its effects only through glutaminergic synapse modulation (16, 17) (Figure 1). It has been observed in the literature that the effects of tDCS are long-term rather than short-term (5, 20). It has been reported that tDCS activates NMDA receptors in the long term and that resulting effect can spread to neuronal networks in the area where it is applied (4, 17, 21). Nitsche and Paulus (22) reported that tDCS causes subthreshold stimulation in membrane polarization rather than presynaptic or postsynaptic cell stimulation. tDCS shows its effect through the activation of Na^{+}-$Ca2^{+}$ dependent ion channels and through long-term potentiation and depression-like changes through N-methyl-D-aspartate (NMDA) receptor activity (23).

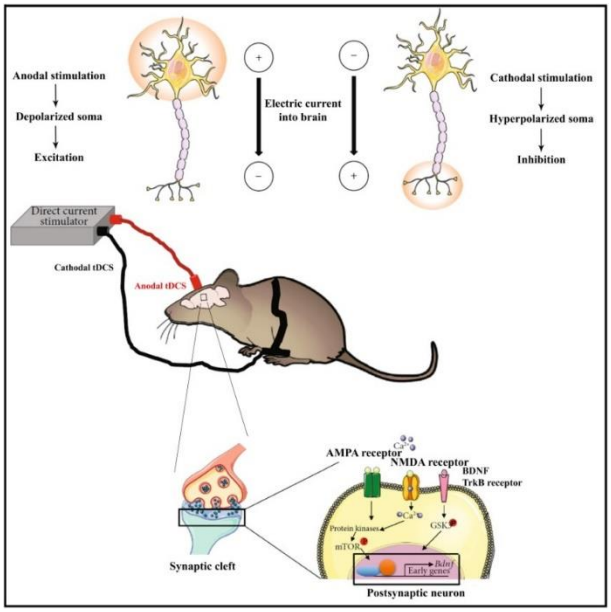

Figure 1. Schematic representation of transcranial direct current stimulation (tDCS). While A-tDCS increases excitability by acting on the neuronal membrane potential by depolarizing, C-tDCS decreases excitability by affecting hyperpolarization. A-tDCS depolarizes the presynaptic neuronal membrane and glutamate, and glutamate binds to AMPA and NMDA receptors. It regulates the neuronal signaling pathway that leads to transcriptional changes by activating protein kinases with the increase of intracellular Ca2+ in the postsynaptic neuron. Also, tDCS modulates the BDNF signaling pathway.

Abbreviations: AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; A-tDCS: anodal-tDCS, BDNF: brain-derived neurotrophic factor; CBP: CREB-binding protein; C-tDCS: cathodal-tDCS, CREB: cAMP response element binding protein; GSK3: glycogen synthase kinase 3; LTP: long-term potentiation; mTOR: mammalian target of rapamycin; NMDA: N-methyl-D-aspartate; TrkB: tropomyosin receptor kinase B. Adapted from Cavaleiro et. al. (30).

METHODS

Our study was carried out in Akdeniz University Experimental Animals Unit. Rats obtained from Akdeniz University Experimental Animals Application and Research Center with the approval of Akdeniz University Animal Experiments Local Ethics Committee (Decision No 40) were used in the study.

Experimental Groups and Protocol

Experiments were carried out by dividing 50 male Wistar albino rats, weighing 290-310 gr, into 5 groups:

Group 1: Control (n=10), sham tDCS stimulation was applied 30 min a day for 5 days (n=10),

Group 2: C1-tDCS, 0.25mA cathodal stimulation was applied for 30 minutes during the day (n=10),

Group 3: C2-tDCS, 0.5mA cathodal stimulation was applied 30 min a day for 5 days (n=10),

Group 4: A1-tDCS, 0.25mA anodal stimulation was applied 30 min a day for 5 days (n=10),

Group 5: A2-tDCS, 0.5mA anodal stimulation was applied 30 min a day for 5 days (n=10).

Throughout the experiment, animals were kept in a 12-hour dark/light cycle with 5 animals in each cage. During the experiment, animals were fed with commercial rat chow and tap water. Starting from the 3rd day of the experiment, handling was applied for 5 minutes, 3 times a day for 2 days. On the 6th day of the experiment, the rats were taken to open field (OF) and Y-maze tests, and the object localization test (OLT) was taken on the 7th day (Figure 2). On the 7th day of the experiment, the subjects were sacrificed and their brain tissues were taken, and glutamate measurement was made in the hippocampus tissue by ELISA method.

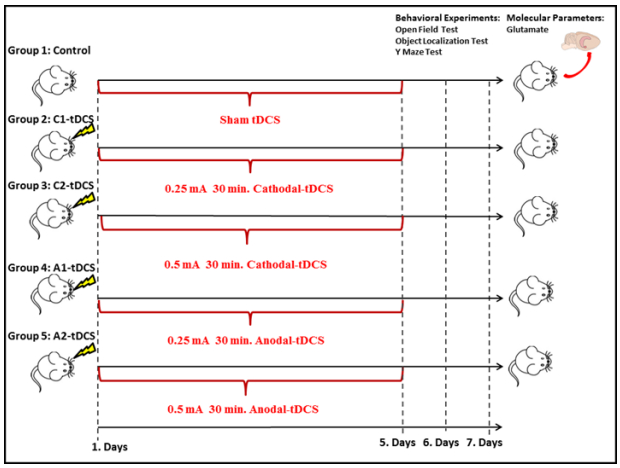

Figure 2. Experimental protocol

tDCS Stimulation

For tDCS application, Animal DCS Stimulator (model 2100) device with temporal resolution of 1 min was used. In our study, tDCS stimulation was applied to all groups, except the control group, for 30 minutes for 5 days. During the tDCS application, a superficial disc electrode was used, the maximum current intensity was ±1000 μA, and the current resolution was set as 0.01mA (Figure 3).

Behavioral Experiments:

Open Field Test

The open field test is used to evaluate locomotor activity. Open field experiments were performed in an 80x80x40 cm setup (Figure 4). Rats were placed in the central area of this area

and their movements were recorded for 5 minutes using a camera system. The odor cues were eliminated by cleaning the open field setup with 70% ethanol for each rat. Locomotor activity was evaluated with the parameters total distance (cm) and frequency (24).

Figure 3. tDCS applications (24)

Figure 4. Open field test (24)

Y-Maze Test

The Y-maze test is used to investigate short-term memory and spatial memory. The Y-maze test was performed on a black plastic assembly consisting of three arms (50 cm long, 20 cm wall high, 10 cm wide) at a 120° angle from each other in a room including a variety of distinct distal cues (Figure 5). In training phase, the rats were left at the end of the starting arm, and each rat was given 15 minutes to freely examine the other arms while the new arm was completely closed. IN testing phase, the rats were removed from maze and the Y-maze assembly was cleaned with 70% ethanol to prevent their movements according to the sense of smell during the experiment. One hour after the first session was opened arms and all three arms of rats were allowed to explore freely for 5 minutes. In this second session, subjects with spatial memory are expected to make the first turn into the "novel arm" and spend more time exploring this arm. The number of new arm entries and the time spent exploring the new arm were recorded. The behavior of the rats was monitored by a camera system.

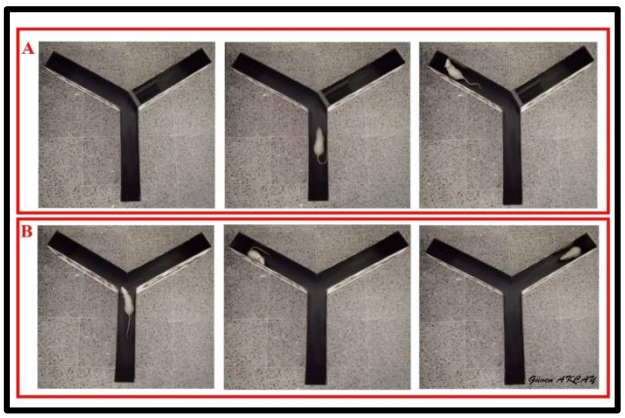

Figure 5. Y maze test procedure A) Training phase B) Testing phase

Object Localization Test

The object localization test is used especially in short-term and spatial memory studies. It consists of three stages: habituation, training and retention. On habituation phase, rats center of the arena (40 cm high, 80 x 80 cm in size) were placed and allowed to explore for 5

minutes without any object (Figure 6). In the training phase, the rats were allowed to set the media from the center and 5 minutes for the two objects are expected review. The time spent exploring each object was recorded. The maze was cleaned with 70% ethanol to prevent their movements according to the sense of smell during the experiment. In the testing phase, one of the objects was relocated and the rats were allowed to explore the objects for 5 minutes. It is expected to spend more time to examine the objects of the subjects relocated. The total exploration time and the total number of touches to the displaced object were recorded with the camera system (25).

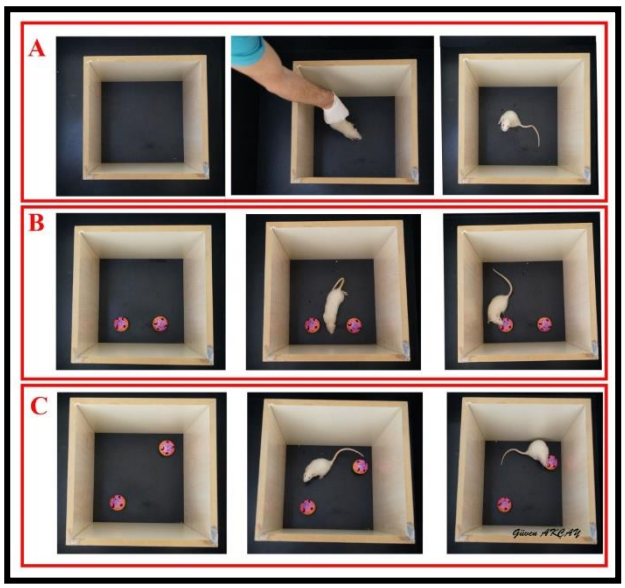

Figure 6. Object localization test procedure A) Habituation phase B) Training phase C) Testing phase

Biochemical Method:

Glutamate level

Measurements were performed according to the protocol specified in the commercially purchased solid phase sandwich enzyme immunoassay (Glutamate Assay Kit ab83389-ELISA) kit. Standard solutions and samples prepared in decreasing concentrations by serial dilutions were loaded into 96-well plates containing specific monoclonal antibodies against rat glutamate. In this method, which is based on antigen-antibody binding, glutamate molecules in the samples were bound to the antibody. It was incubated in an incubator set at 37 °C for 90 min. At the end of the incubation, concentrated biotinylated detection Ab was diluted 1/100 with biotinylated detection dilution solution and 100 µl was added to each well. After incubation at 37 °C for 1 hour, 750 ml of distilled water was added to the concentrated wash buffer and washed 3 times with a wash buffer solution, and unbound molecules were removed. Then, peroxidase (Horseradish Peroxidase-HRP) conjugate bound with 100 µl of streptavidin was added and incubated at 37 °C for 30 minutes. After incubation, this time it will be washed 5 times and substrate solution is added. Color change will be observed in direct proportion to the glutamate concentration in the samples and 50 µl of stop solution was added to each well to stop the reaction. The absorbance value of each well was determined by reading in a microplate reader at 450 nm.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software. Results are given as mean±SEM. Statistical significance was

accepted as p <0.05. Open field test data were evaluated with one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test was used as posthoc test.

RESULTS

The total distance and frequency in open field in A2-tDCS group (p<0.05) was significantly increased compared with that in Control group (Figure 7). When the locomotor activity parameters were compared between the control and A1-tDCS groups, no significant difference was found. Similarly, there was no difference between C1-tDCS and C2-tDCS groups.

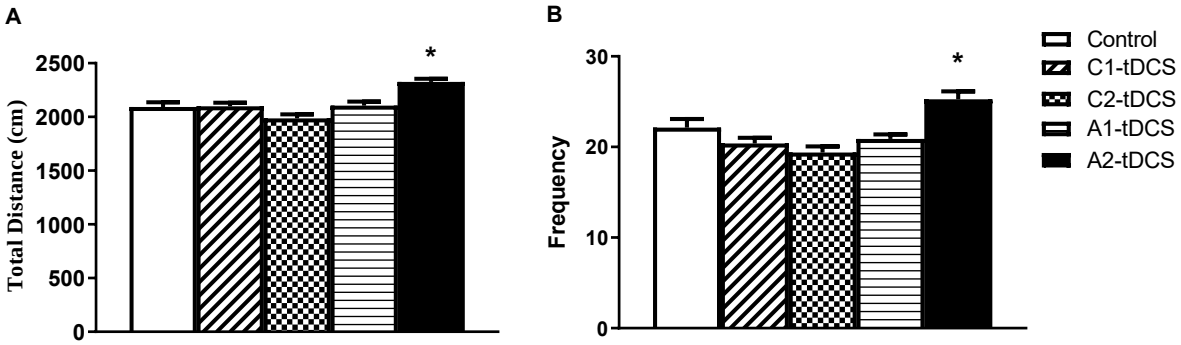

Figure 7. Open field test results of experimental groups. A) Total distance (cm), B) Frequency. (n=10, for each group; * p<0.05 shows the difference compared to the Control group, one-way ANOVA test, followed by Tukey post hoc test). All data are presented as means ± SEM.

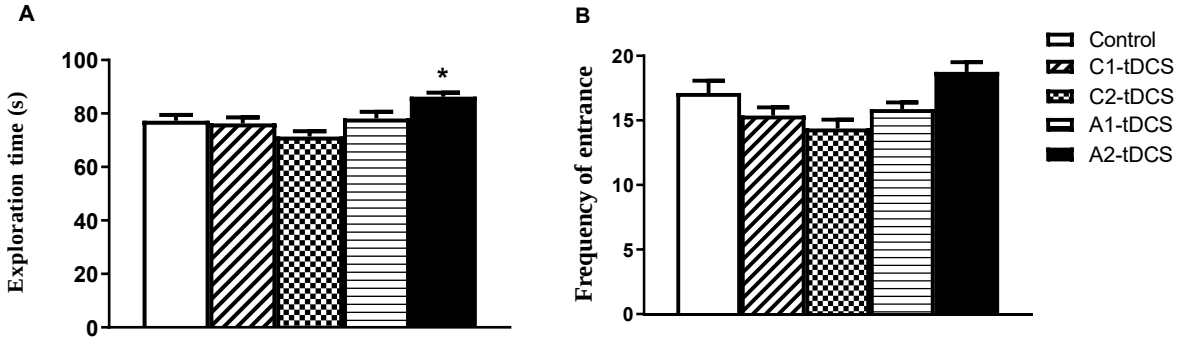

Figure 8. The effect of 5-day tDCS stimulation on the spatial memory. A) Exploration time to the novel arm (s) and B) Frequency of entrance to the novel arm (n = 10 for each group; *p < 0.05compared to Control, one-way ANOVA test, followed by Tukey post hoc test). All data are presented as means ± SEM.

The exploration time to the novel arm (s) and frequency of entrance to the novel arm were evaluated using Y maze (Figure 8). Our results showed that there was a decrease in the exploration time to the novel arm (s) and frequency of entrance to the novel arm in the C1-tDCS and C2-tDCS group compared to the Control group. Y-maze test values of the A2-tDCS group was significantly increased compared to the Control

group. Similarly, there was no difference between C1-tDCS and C2-tDCS groups.

The exploration time and number of touches to the relocated object were evaluated by object localization test (Figure 9). The results showed that there was not a significant decrease in both the exploration time and number of touches of the relocated in the C1-tDCS and C2-tDCS groups compared to the control group. The results showed that both exploration time and number of touches were significantly higher in A2+tDCS group as compared to control group (Figure 9).

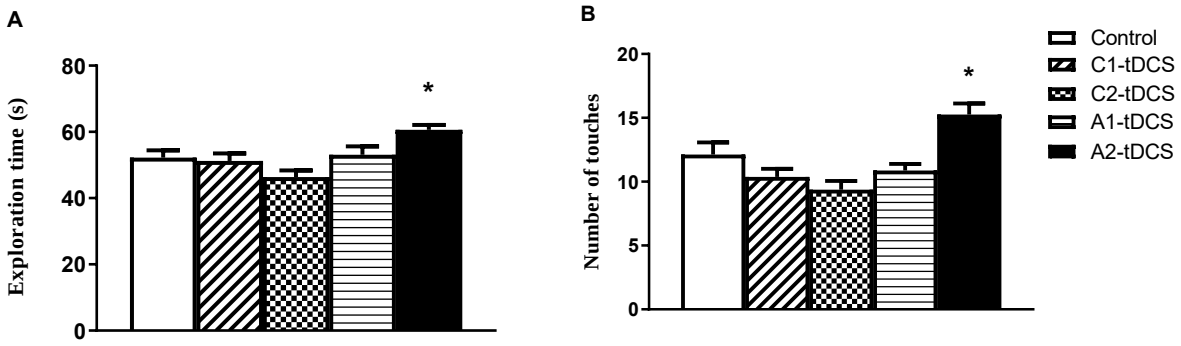

Figure 9. The effect of 6-day tDCS stimulation on the spatial memory. A) Exploration time of the relocated object (s), B) Number of touches (n = 10 for each group; * p < 0.05 compared to Control Sham, one-way ANOVA test, followed by Tukey post hoc test). All data are presented as means ± SEM.

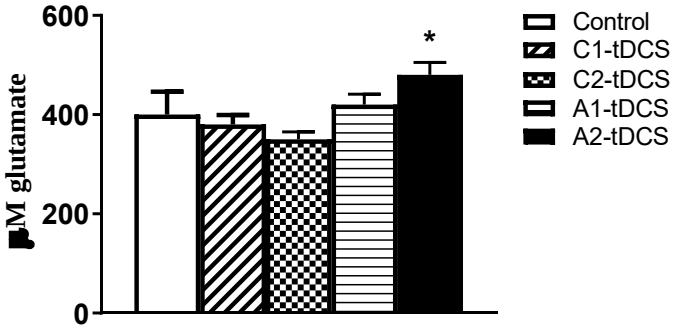

Figure 10. The effect of 7-day tDCS stimulation on the glutamate levels in the hippocampus tissue. * p<0.05 Control, (n =10). The data are means ± SEM. Statistical analyses are One-way ANOVA followed by Tukey's multiple comparison test against the indicated group.

Figure 10 shows glutamate levels in hippocampus tissue. When the glutamate levels in the hippocampus were evaluated, there was no difference between the Control and C-tDCS groups, while and glutamate (Figure 10) levels of the A2-tDCS group increased significantly compared to the Control group.

DISCUSSION

The brain is responsible for many important physiological functions such as learning, memory, speech, thinking and decision making. The learning and memory area of the brain is the hippocampal area, and the most important excitatory neurotransmitter in the central

nervous system is glutamate (1). There are different treatments and techniques to strengthen learning and memory. However, in recent years, neuromodulation and the regulation of the neuronal membrane potential of the brain have become widespread. One of these techniques transcranial direct current stimulation. tDCS is a non-invasive neuromodulation technique that delivers a constant low-intensity sub-threshold direct current to specific areas of the brain through electrodes placed on the scalp, thereby regulating cell transmembrane potential depolarization and hyperpolarization, and altering neuronal activity and excitability of the cerebral cortex (2, 26). Some clinical and basic studies have found that tDCS treatment can improve memory and cognitive dysfunction in patients and animals. Studies have shown that different tDCS current density and stimulus types have different effects on learning and memory (27-32). However, there are few studies investigating the effects of tDCS stimulation of different types and current intensity on learning and memory, and little is known about the mechanism of action. Preclinical studies investigating behavioral and molecular mechanisms are needed to fully understand the mechanisms of action of tDCS. Therefore, in our study, the effects of anodal and cathodal stimulation types of tDCS and different current intensities of 0.25 and 0.5 mA on learning and memory in the hippocampal glutamatergic pathway were investigated behaviorally and molecularly. Mehrsafar et al. conducted a study on 12 male archers on the dorsolateral prefrontal cortex for 20 minutes (33). They showed that the application of 2 mA anodal tDCS caused an increase in archers to feel more energetic and decreased anxiety feelings such as tension and fatigue. In their study on humans, Bogdanov and Schwabe reported that the application of 1.075 mA anodal tDCS could be a potential new method to prevent working memory disorders caused by acute stress (34). Luo et al. (28) reported that 0.15 mA Anodal tDCS stimulation for 2 weeks improves spatial learning and memory in the early stage of Alzheimer's disease in transgenic mice. Yu et al. (31) showed that 0.1 mA and 0.2 mA repetitive anodal tDCS can improve spatial learning and memory dysfunction in Alzheimer's mice, depending on current intensity. Au et al. (29) showed that 25 min with a current intensity of 2 mA tDCS on the left dorsolateral pFC strengthens long-term learning and memory consolidation in aging and improves performance in multiple memory areas. Zhang et al. (32) reported that after 0.5 mA 15 min and 5 days of cathodal tDCS treatment, it did not affect motor functions, learning and memory ability, and had no effect on neurotransmitter levels. Roostaei et al. (30) applied 0.2 mA 20 min 2-day anodal and cathodal tDCS stimulation in their study and reported that 0.2 mA anodal tDCS was effective

on memory via dopaminergic pathway, but cathodal tDCS had no effect on memory. Our findings are in line with the results of Roostaei et al. (30), and it was determined that 0.5 mA anodal tDCS application increased the locomotor activation in the open field test, and learning and memory parameters in the object localization tests with y maze. In our study, it was demonstrated that 5-day 0.25 mA and 0.5 mA cathodal stimulation had a reducing effect on both learning and memory behavior experiment results and glutamate level, and this activity may also be related to the current intensity of tDCS. As a matter of fact, 0.5 mA 30 min 5 days tDCS was used instead of 0.2 mA 20 min 2 days tDCS used by Roostaei et al. (30) in our study, and it was evaluated that the application time and current intensity of tDCS are important in the therapeutic efficacy associated with tDCS application.

Glutamate, a metabolite in the glutamatergic pathway and the main excitatory neurotransmitter of the central nervous system, is involved in learning and memory processes and can be affected by tDCS; thus, they present themselves as potential biomarkers for tDCS-induced behavioral gains due to neuroplasticity processes (27). Studies have reported that tDCS stimulation on learning and memory is beneficial by affecting AMPA and NMDA in the glutamatergic pathway (2, 24). Therefore, it has been stated that tDCS application to strengthen learning and memory may be related to the glutamatergic system, which is known to play a role in its pathophysiology. Although the reason for the shorter duration of action of NMDA receptor antagonists is not yet known, it is thought to be caused by changes in glutamate levels in the synaptic gap. In our study, it was concluded that anodal tDCS can consolidate learning and memory via the hippocampal glutamatergic pathway.

CONCLUSION

These results suggest that tDCS may have an enhancing effect on learning and memory, possibly via the glutamatergic pathway. It is thought that future studies researching especially glutamate transporters and receptor levels, as well as behavioral experiments and glutamate levels, will make great contributions in order to better understand the effects of tDCS.

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Ethics Committee Approval: Ethics committee approval was received for this study from local ethics committee at Akdeniz University Animal Experiments Local Ethics Committee (Decision No 40).

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Author Contributions: Concept: Design: Literature search: Data Collection and Processing: Analysis or Interpretation: Writing: G. A.

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