Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2023;48(3):919-928
DOI: 10.17826/cumj.1322361

RESEARCH

Transcranial direct current stimulation ameliorates motor and cognitive functions by regulating neuronal excitotoxicity in experimental Parkinson's disease model

Transkraniyal doğru akım stimülasyonu deneysel Parkinson hastalığı modelinde nöronal eksitotoksisiteyi düzenleyerek motor ve bilişsel işlevleri iyileştirir

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Abstract

Purpose: In the study, the therapeutic effects of early and long-term transcranial direct current stimulation (tDCS) in Parkinson's disease (PD) rats with 6-hydroxydopamine (6-OHDA) lesions of tDCS were investigated.

Materials and Methods: After early and prolonged tDCS administration in PD animals (starting 24 hours after PD lesion, 1000 mA anodal tDCS, 30 min/day, 13 days), the effects of tDCS on motor and cognitive function behaviors and glutamatergic neuron excitotoxicity were determined by Ca^{2+}, glutamate, and NMDAR1 levels.

Results: We found that the 13-day tDCS intervention significantly reduced 6-OHDA-induced motor deficits in locomotor activity, learning, and memory-like behavior. Biochemically, we showed that it also reduces Ca^{2+}, glutamate, and NMDAR1 levels, which cause hippocampal neuronal damage.

Conclusion: These results suggest that early and long-term tDCS may exert neuroprotective effects and reduce the exacerbation of motor and cognitive impairments in a rat model of 6-OHDA-induced PD. However, it also shows that tDCS has an effect on the glutamatergic pathway in PD and prevents neuronal excitotoxicity. Furthermore, this preclinical model may increase the potential use of therapeutic tDCS and serve as a translation platform to further define the therapeutic mechanism of tDCS for PD or other disorders.

Keywords:. Learning, locomotor activity, neuroprotective treatment, parkinson's disease, transcranial direct current stimulation

Öz

Amaç: Bu çalışmada, 6-hidroksidopamin (6-OHDA) Parkinson hastalığı (PH) sıçanlarında erken ve uzun süreli transkraniyal doğru akım stimülasyonunun (tDAS= terapötik etkileri araştırılmıştır.

Gereç ve Yöntem: PH hayvanlarında erken ve uzun süreli tDAS uygulamasından sonra (PH lezyonundan 24 saat sonra başlayarak, 1000 mA anodal tDCS, 30 dakika/gün, 13 gün), tDAS'nin motor ve bilişsel fonksiyon davranışları ve glutamaterjik nöron eksitotoksisitesi üzerindeki etkileri Ca^{2+}, glutamat ve NMDAR1 seviyeleri ile belirlendi.

Bulgular: 13 günlük tDAS tedavisi lokomotor aktivite, öğrenme ve hafıza benzeri davranışlarda 6-OHDA kaynaklı motor bozuklukları önemli ölçüde azalttığı görüldü. Biyokimyasal olarak, hipokampal nöronal hasara neden olan Ca^{2+}, glutamat ve NMDAR1 seviyelerini de azalttığı gözlendi.

Sonuç: Bu sonuçlar, erken ve uzun süreli tDAS'nin nöroprotektif etkiler gösterebileceğini ve 6-OHDA ile indüklenen PH sıçan modelinde motor ve bilişsel bozuklukların artmasını azaltabileceğini göstermektedir. Bununla birlikte, tDAS'ın PH'de glutamaterjik yolak üzerinde etkisi olduğunu ve nöronal eksitotoksisiteyi önlediğini de göstermektedir. Ayrıca, bu preklinik model terapötik tDAS'nin potansiyel kullanımını arttırabilir ve PH veya diğer bozukluklar için tDAS'nin terapötik mekanizmasını daha fazla açıklamak için bir tedavi yöntemi olabilir.

Anahtar kelimeler: Öğrenme, lokomotor aktivite, nöroprotektif tedavi, Parkinson hastalığı, transkraniyal doğru akım stimülasyonu

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Received: 05.07.2023 Accepted: 08.09.2023

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder involving the presence of Lewy bodies in the substantia nigra pars compacta and the degeneration of dopaminergic neurons[1]. s. More than 7 million people worldwide have PD. The frequency and symptoms of PD progress relatively according to age. Parkinson's disease is rarely encountered before the age of 50 and is 10 times more common in older people than in people aged 50. At the same time, the disease is 2 times more common in male individuals than in female individuals. Parkinson's patients exhibit motor symptoms such as bradykinesia, tremor, rigidity, and postural instability, as well as non-motor symptoms such as cognitive abnormalities, sleep and mood disturbances, pain, and sensory disturbances reflecting peripheral neurodegeneration[2,3]. Therefore, PD is a multisystem neurodegenerative disorder in which autonomic, psychiatric, and cognitive symptoms play a role and significantly affect the quality of life of patients. Dopaminergic drugs are still important in the treatment of PD, although their effects have diminished over time, and side effects have emerged[4].

Although the causes of neurodegeneration in PD are not fully known, many conditions such as abnormal protein accumulation in dopaminergic neurons, mitochondrial dysfunction, oxidative stress, microglial activation and inflammation, excitotoxicity, apoptosis, environmental and genetic factors are related[5-7]. Although the underlying mechanism of PD is known to be the loss of dopamine-producing neurons, abnormal glutamate release in the basal ganglia also plays a role. This is generally recognized as the result of decreased dopamine levels. Excitotoxicity, which plays an important role in the increase of nigrostriatal degeneration and plays a pathological role, is an important mechanism involved in the pathogenesis of PD[8]. The altered neurotransmission observed in the basal ganglia in PD affects the glutamatergic system, which plays a critical role in the pathogenesis of glutamate-mediated excitotoxicity. Direct and indirect neurotransmitter alterations in the nigro-striatal pathways occurring in PD include glutamatergic hyperactivity. Studies demonstrate that glutamate-mediated excitotoxicity may be the primary cause of dopaminergic neuronal loss and thus abnormal glutamate regulation may contribute to neurodegeneration in PD[9]. Glutamate is one of the most important excitatory neurotransmitters of the mammalian central nervous system and plays an important role in cognitive functions such as learning, memory, and synaptic plasticity.

The effect of glutamate released from the presynaptic terminal in the postsynaptic neuron is mediated by glutamate receptors embedded in the cell membrane[10]. Under pathological conditions, when excess glutamate is released from the presynaptic membrane or the reuptake function of glutamate is impaired, the extracellular glutamate concentration increases. Accumulation of extracellular glutamate and overstimulation of glutamatergic neurons increase ROS production. Activation of glutamate receptors involves Ca^{2+} homeostasis dysfunction, caspase activation, and an increase in cytotoxic transcription factors and free radical species[11]. In addition, intracellular calcium regulation, anti-inflammatory effects, and modulation of glutamatergic pathways play a role in neuroprotective activity[12]. Calcium signaling is involved in the selective degeneration of dopaminergic neurons[13]. Studies have shown that NMDA receptor antagonists treat motor symptoms, reduce dyskinesias, and slow progressive neurodegeneration[14-19].

The main medical treatment of PD is pharmacotherapy (especially levodopa), but long-term medication has side effects[4]. Treatment modalities such as deep brain stimulation especially improve motor and non-motor dysfunctions[20]. However, the high risk and cost associated with invasive neurosurgical procedures remain an important problem to be solved. On the other hand, transcranial direct current stimulation (tDCS) is a relatively easy and safe method to modulate cortical polarisation by applying low-intensity current (1.0-2.0 mA) to the scalp. anodal stimulation in tDCS increases cortical excitability, while cathodal stimulation decreases it[21]. tDCS exerts its effect through activation of Na_v^+, Ca_v^{2+}- ion channels and NMDA receptor activity[22]. Recent studies show that tDCS in combination with rehabilitation has long-term effects on the improvement of symptoms in various neurological disorders[23]. Especially in recent years, tDCS applications have been used to reduce pain in diseases such as traumatic spinal cord injury, fibromyalgia, central pain, and migraine; in psychiatric diseases, neurological diseases, and stroke rehabilitation research; and studies investigating the effects of cognitive functions such as learning, memory and decision-making in healthy people[24].

However, although there are antioxidant studies against PD-induced loss of motor and cognitive function, there is no study investigating the neuroinflammatory effects of tDCS treatment. The effect of tDCS, which has a neuroprotective effect, on PD motor and cognitive function and its relationship with excitotoxicity have not been fully elucidated. In this study, the therapeutic effects of transcranial direct current stimulation on learning and memory impairment as a result of glutamate and calcium excitotoxicity of tDCS were investigated in 6-OHDA-induced Parkinsonian model rats.

MATERIALS AND METHODS

Animals and experimental design

All animal use and experimental protocols were approved and implemented by the Animal Care and Ethics Committee of Akdeniz University (Akdeniz University, 20.06.2022/Decision number: 70). Thirty 3-month-old rats weighing 300-350 g were divided into 3 groups Sham, PD, and PD+tDCS. The animals were housed in separated cages and kept on a standard environmental condition (23–25 °C, 50 ± 5% relative humidity, 12 h light/dark cycle) with free access to food and water. Animals were acclimatized to the environment before the experiments and then were randomized and divided into five groups (i) Sham, (ii) PD, and (iii) PD+tDCS (n = 10 per group). 10 rats in each group were allocated to behavioral test groups and then were used for biochemical analysis. Researchers were blinded to the experimental groups. The size of experimental groups was determined by considering the accuracy and reproducibility of methods based on our previous data sets. Sham and PD+tDCS groups received 1 mA tDCS for 30 minutes at the same time every day for 13 days starting from the 2nd day of the experiment. Learning and memory were evaluated by a novel object recognition test and locomotor activity by open field test. After the behavioral experiments, the rats were sacrificed and hippocampus tissues were removed and Ca^{2+}, glutamate, and NMDAR1 activations were evaluated by ELISA.

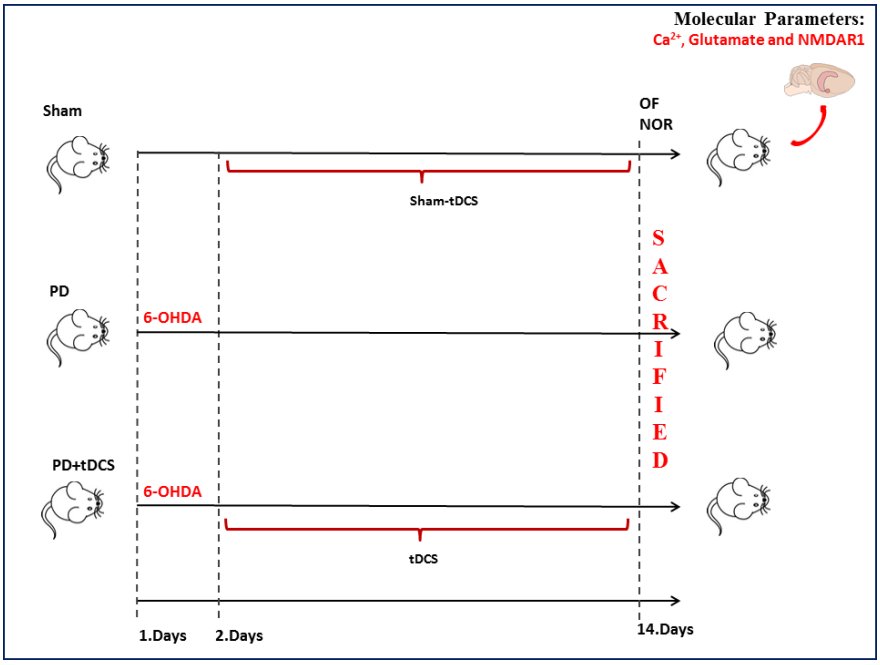

Figure 1. Experimental design

6-OHDA Parkinson Model

To prevent loss of noradrenergic neurons, 30 mg/kg i.p. desipramine and 10 mg/kg i.p. pargillin were given. The rats were then anesthetized with thiopental sodium at a dose of 25 mg/kg. The coordinates of the injection site were determined

unilaterally in the right hemisphere according to the rat brain atlas as anteroposterior (AP) -5.5 mm (from the bregma), lateral (L) 2 mm (from the midsagittal suture), and dorsaventral (DV) 8 mm (from the skull surface). A total of 8 μg/μl 6-OHDA (anteroposterior (AP) -5.5 mm, lateral (L) 2, and dorsaventral (DV) 8 mm) was injected unilaterally into the substansia nigra region of the animals in the PD and PD+tDCS groups with a Hamilton syringe in a volume of 4 μl dissolved in saline solution with 0.1% ascorbic acid using stereotaxic method[25].

tDCS stimulation

For tDCS stimulation, an Animal DCS Stimulator (model 2100) device with a temporal resolution of 1 min was used[26]. In our study, Sham and PD+tDCS groups received 1 mA tDCS for 30 minutes at the same time every day for 13 days starting from the 2nd day of the experiment. 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.

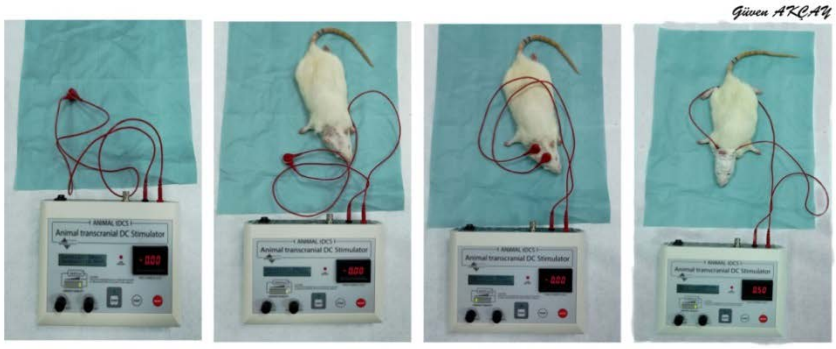

Figure 2. tDCS stimulation.

Behavioral Tests

Open-field test (OF)

Locomotor activity was carried out in a setup with a base of 80x80 cm and a wall height of 40 cm. The arena was divided into 16 equal squares (each 20 cm × 20 cm). The rats were individually placed in the central four squares of the arena and allowed to explore the environment for 5 min. The apparatus was thoroughly cleaned before each trial. For rats to explore the apparatus, they were placed in the center of the field and monitored and recorded by the video camera for 5 minutes. Total distance (cm), velocity (cm/s), and frequency were calculated to evaluate locomotor activity[27].

Novel Object Recognition Test (NOR)

The NOR test is particularly used in attention or short-term memory experiments and occurs in three stages: habituation, training, and retention. In the habituation stage, the rats were placed in a box with a wall height of 40 cm and a square base of 40 × 40 cm^2 with a black bottom and allowed to roam for five minutes without any objects in the environment. In the training stage, the rats were placed in the center of the box facing away from the objects and allowed to explore the objects for five minutes. After each trial, the maze and objects were cleaned with 70% ethanol to eliminate the smell of the rats. In the retention stage, one of the objects was replaced with a novel object, and the rats' behavior was recorded for five minutes. Rats are expected to spend more time examining the novel object. The time spent with familiar and novel objects was recorded[28]. In the NOR test, the discrimination index and the time spent in the novel object (sec) values will be analyzed. Discrimination Index = ((Time Spent at Novel Object-Time Spent at Old Object)/Total Time)*100.

Tissue collection

After the behavioral experiments, the brains of rats were perfused transcardially with heparinized saline and removed immediately. Then, hippocampi were dissected quickly and, frozen in liquid nitrogen and then stored at −80 °C until biochemical analysis. Tissues were homogenized in phosphate-buffered saline (PBS, pH 7.4) centrifuged at 12,000 rpm for 20 minutes at 4°C, and supernatants were used for biochemical analyses.

Biochemical analysis

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of Ca^{2+}, glutamate, and NMDAR1 were quantified using enzyme-linked immunosorbent assay (ELISA) kits. Commercially available ELISA kits (R&D Systems, Minneapolis, USA) were performed according to the manufacturer's instructions. Ca^{2+}, glutamate, and NMDAR1 concentrations in the samples were calculated from their corresponding absorbance values via the standard curve. Data were normalized to total tissue protein and expressed as pg·mg−1 tissue protein.

Protein measurements

Protein concentrations were measured in the hippocampus tissues at 595 nm by a modified Bradford assay using Coomassie Plus reagent with bovine serum albumin standard (Pierce Chemical Company, Rockford, IL, USA).

Statistical analysis

The SPSS software package 20.0 program was used for all analyses. The results were given as mean ± standard error of the mean (SEM). P values less than 0.05 were considered significant. The one-way ANOVA test was used in the analysis of data with normality conditions in the evaluation made with the Shapiro-Wilk test. The Tukey test was used for post-hoc analysis. If the data does not provide the normality condition, the non-parametric Kruskal-Wallis test was used for the comparison of the groups, and all pairwise multiple comparisons were performed by the Mann-Whitney U test. The results of the behavioral experiments and biochemical analyses were analyzed with the One-way ANOVA test after the Shapiro-Wilk test normality analysis.

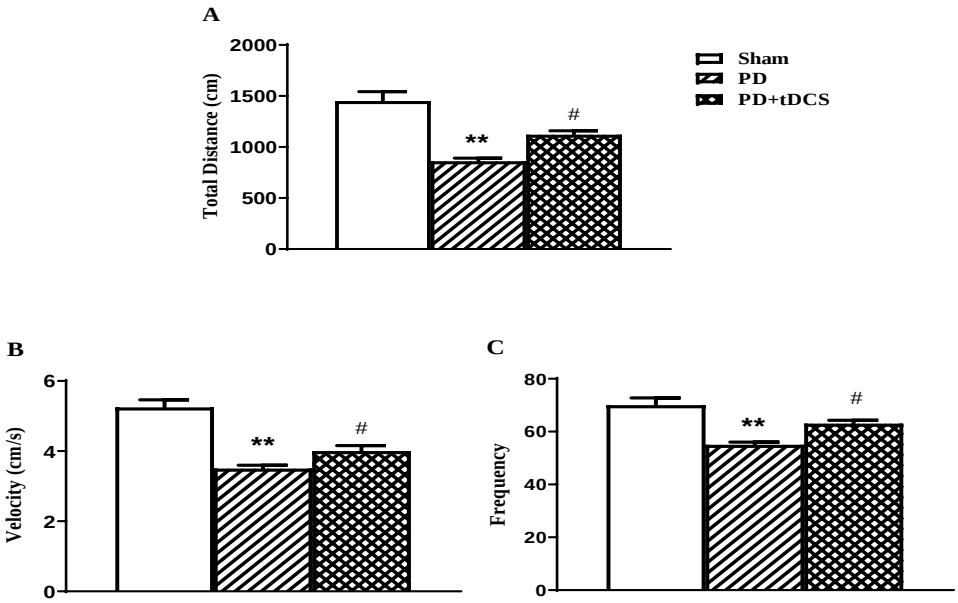

Figure 3. Locomotor activity results of experimental groups in OF. A) Total distance (cm), B) Velocity (cm/s), C) Frequency.

(n=10, for each group; ** p<.01 shows the difference compared to the Sham group, # p<.05 shows the difference compared to the PD group, one-way ANOVA test, followed by Tukey post hoc test). All data are presented as means ± SEM.

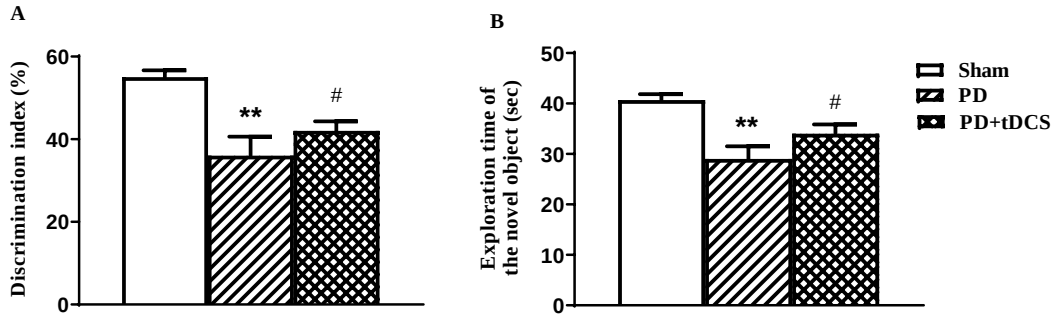

Figure 4. Learning results of experimental groups in NOR. A) Discrimination index (%) and B) Exploration time of the novel object (sec).

(n=10, for each group; ** p<.01 shows the difference compared to the Sham group, # p<.05 shows the difference compared to the PD group, one-way ANOVA test, followed by Tukey post hoc test). All data are presented as means ± SEM.

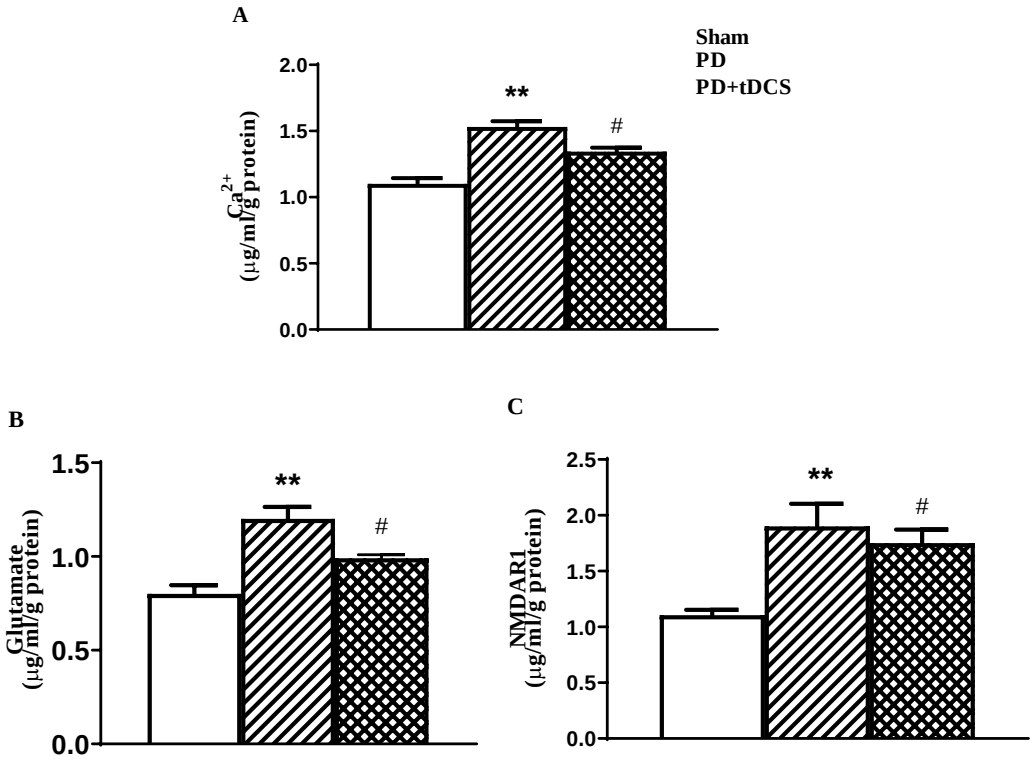

Figure 5. ELISA results. A) Ca^{2+} results in the hippocampus, B) Glutamate results in the hippocampus, C) NMDAR1 results in the hippocampus.

(n=10, for each group; ** *p*<.01 shows the difference compared to the Sham group, # *p*<.05 shows the difference compared to the PD group, one-way ANOVA test, followed by Tukey post hoc test). All data are presented as means ± SEM.

RESULTS

The behavioral experiments of the groups were measured in terms of locomotor activity in the OF (Figure 3) and learning in the NOR (Figure 4). Total distance, velocity, and frequency in the OF were significantly decreased in the PD group compared to the Sham group ($p<0.01$) (Figure 3). After tDCS treatment, a significant increase in locomotor activity was observed in the PD+tDCS group compared to the PD group ($p<0.05$) (Figure 3). Short-term memory experiments of the groups were evaluated in the NOR test (Figure 4). While there was a significant decrease in the learning of the PD group compared to the Sham group ($p<0.01$), there was a significant increase in the PD+tDCS group compared to the PD group ($p<0.05$).

Ca^{2+}, glutamate, and NMDAR1 ELISA results in hippocampus tissue are shown in Figure 5. There was a significant increase in the Ca^{2+}, glutamate, and NMDAR1 activation levels of the PD group compared to the Sham group, while there was a significant increase in the Ca^{2+}, glutamate, and NMDAR1 activation levels of the PD+tDCS group compared to the PD group after tDCS stimulation.

DISCUSSION

In this study, we used a tDCS rat model to examine the effect of long-term tDCS treatment for 13 days on PD rats with 6-OHDA lesions. We found that early and long-term tDCS intervention attenuated 6-OHDA-induced dysfunctions in motor and cognitive function behaviors. Furthermore, biochemical results showed greater preservation of glutamatergic hippocampal tissue in tDCS-treated groups compared to sham-tDCS-treated groups, suggesting a neuroprotective effect of tDCS intervention.

To date, the therapeutic efficacy and underlying mechanisms of tDCS treatment for PD are not fully understood. This is likely due to variability among therapeutic protocols, targeted brain regions, drug conditions, clinical heterogeneity, and disease severity. The few studies to determine the beneficial effects of tDCS on PD-related symptoms in animal experiments have focused on drug-induced rotational behavior[29-31]. In this study, the effects of 13-day tDCS treatment on locomotor activity and learning functions and the effects on Ca^{2+} and glutamate responsible for excitotoxicity were evaluated comprehensively and quantitatively.

We found that 13 days of tDCS in PD rats led to a reduction in locomotor activity and learning impairments. These results are similar to PD-related studies showing a reduction in locomotor and cognitive impairments after tDCS treatment and encouraging further investigation into its therapeutic potential. The mechanisms by which tDCS preserves various aspects of motor function in PD are not fully understood. Possible evidence supporting the efficacy of tDCS in PD is related to tDCS-induced dopamine release, but the glutamatergic pathway is known to be involved. The widespread activation of glutamatergic neuronal systems induced by anodal tDCS may release glutamine and may be the mechanism preventing or delaying the deterioration of motor dysfunction in PD rats. Furthermore, the reduction of 6-OHDA-induced impairments revealed in our comprehensive behavioral assessments obtained with long-term tDCS intervention further supports such neuroprotective glutamatergic effects.

Locomotor and cognitive impairments are common non-motor dysfunctions in PD. Compared to the sham treatment group, PD rats showed a decrease in the total distance, velocity, and frequency results of the open field test, while the decrease in motor function results was prevented in the tDCS treatment group. This result is consistent with the study showing that anodal tDCS alleviates impaired locomotor activity[29-31]. Our results also show that recognition memory also improved after tDCS treatment. We found a significant difference in recognition memory, discrimination index, and exploration time of the novel object, suggesting that recognition memory is affected by disease progression in our 6- OHDA PD rats.

Excitotoxicity is an important mechanism in the pathogenesis of PD[8]. Glutamate-mediated excitotoxicity may be the primary cause of dopaminergic neuronal loss, and therefore abnormal glutamate regulation leads to neurodegeneration in Parkinson's disease[9]. Increased glutamate causes overstimulation of glutamatergic neurons and ROS production. Calcium signaling is involved in the selective degeneration of dopaminergic neurons [13]. Studies have shown that NMDA receptor antagonists treat motor symptoms, reduce dyskinesias, and slow

progressive neurodegeneration[14-1119 14-19]. To examine the excitotoxicity of tDCS on the PD glutamatergic pathway, Ca^{2+}, glutamate, and NMDAR1 levels were analyzed by the biochemical ELISA method and it was found that tDCS reduced Ca^{2+} and glutamate excitotoxicity. These results suggest that 13 days of prolonged tDCS intervention may not only delay the progressive worsening of motor deficits but may also have neuroprotective effects against the reduction of significant hippocampal neurotoxin-induced damage. However, consistent with the neuroprotection effect of tDCS on glutamatergic neurons, most motor behavioral functions were significantly preserved after 13 days of tDCS treatment. Feng et al. reported that 3-4 weeks of anodal tDCS treatment showed neuroprotective effects against neuronal damage in dopaminergic neurons and reduced neuronal damage [29]. Winkler et al. demonstrated that 14-day anodal transcranial DCS significantly increased dopaminergic reinnervation of striatal tissue and enhanced motor function in PD[31]. Our study, together with behavioral and biochemical results, demonstrates that early (starting 24 hours after PD lesion) and prolonged (13 days) tDCS intervention can result in the preservation of various motor and cognitive functions through the protection of hippocampal glutamate excitotoxicity. Similar neuroprotective results were reported in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model using anodal tDCS[32,33]. The neuroprotective effects on dopaminergic cells may be related to the role of brain-derived neurotrophic factor (BDNF) in counteracting oxidative stress and inflammation, suppressing excessive mitophagy, and stabilizing mitochondrial dynamics induced by tDCS[32,34,35]. It has been shown that 0.1 mA anodal stimulation induced both improves motor functions and increases neuronal plasticity in the rat cerebral ischemia model[36]. Another study, it has been reported that anodal tDCS stimulation of 0.2 mA 20 min for 1 and 2 weeks in rat traumatic brain injury improves both motor functions and spatial memory, and decreases infarct areas with an increase in brain-derived neurotrophic factor (BDNF)[37]. Feng et al. (Feng et al., 2020) observed that 0.3 mA anodal tDCS stimulation improved motor function in Parkinson's rats[38]. In our study, similar to Feng et al.'s study, tDCS administration induced an enhancement of motor activity that was previously deteriorated by PD. The results of our study are consistent with the literature, we observed that the parameters of locomotor activity statistically decreased depending on PD in the open-field test. Although the exact mechanisms are still unclear, further research is needed to clarify the mechanisms underlying the neuroprotective effects of tDCS. tDCS shows its effect through membrane potential by activation of AMPA, NMDA and Na^{+}-Ca^{+2} dependent ion channels. The ionotropic glutamate receptors (NMDAR and KA/AMPA receptor) play a role for influx of extracellular ions such as Ca^{2+} and Na^{+} [39]. The NMDAR has a particular permeability to calcium, therefore overstimulation of the receptors causes an increase in the intracellular calcium, which activates the intracellular calcium-dependent signaling cascades that eventually lead to neuronal cell death several days after brain damage[39]. The hyperpolarisation effect of tDCS on NMDAR may have played a protective role against glutamate and calcium excitotoxicity. This study not only demonstrated the therapeutic effects of tDCS treatment on motor function and learning and memory impairment after PD, but also suggested that tDCS may be effective against neuronal damage caused by glutamate and calcium excitotoxicity such as stroke, epilepsy and traumatic brain injury.

This study has several limitations. Firstly, the use of only male rats and not female rats is among the limitations of the study. The effects of tDCS after PD could also be investigated, as this is important for the implementation of treatments in clinical settings. In addition, investigating the dopaminergic pathways that play an important role in PD would have made a great contribution.

This study provides information on the effect of tDCS treatment on the glutamatergic pathway in 6-OHDA-induced PD rats and documents the efficacy of tDCS on PD-related motor symptoms and learning impairment. Furthermore, tDCS shows a neuroprotective effect against Ca^{2+} and glutamate excitotoxicity leading to cell death. In addition, ionotropic glutamate was also found to be effective on the NMDAR1 receptor. Future preclinical studies are still needed to further define the underlying mechanisms, leading to improved protocols of tDCS in PD or other neurological disorders.

Author Contributions: Concept/Design : GA; Data acquisition: GA; Data analysis and interpretation: GA; Drafting manuscript: GA; Critical revision of manuscript: GA; Final approval and accountability: GA, ST; Technical or material support: ST; Supervision: GA; Securing funding (if available): n/a.
Ethical Approval: Ethical approval was obtained from the Local Ethics Committee of Akdeniz University Experimental Animal Application and Research Center Animal Experiments with the decision dated 20.06.2022 and numbered 70.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.

Financial Disclosure: The authors declare no competing financial interests.
Funding statement: This work was supported by the TÜBİTAK, Turkey (Project number: 1919B012103324).

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