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Neuroprotective effect of anodal transcranial direct current stimulation on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in mice through modulating mitochondrial dynamics

Sang-Bin Lee[a,b], Jinyoung Youn[d], Wooyoung Jang[e,*], Hyun Ok Yang[a,c,**]

[a] Natural Medicine Center, Korea Institute of Science and Technology, Gangneung, 25457, Republic of Korea
[b] School of Pharmacy, Sungkyunkwan University, Suwon, 16419, Republic of Korea
[c] Division of Bio-Medical Science &Technology, KIST School, Korea University of Science and Technology, Seoul, 02792, Republic of Korea
[d] Department of Neurology, Samsung Medical Center, School of Medicine, Sungkyunkwan University, Seoul, Republic of Korea
[e] Department of Neurology, Gangneung Asan Hospital, University of Ulsan College of Medicine, Gangneung, Republic of Korea

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the accumulation of protein inclusions and the loss of dopaminergic neurons. Abnormal mitochondrial homeostasis is thought to be important for the pathogenesis of PD. Transcranial direct current stimulation (tDCS), a noninvasive brain stimulation technique, constitutes a promising approach for promoting recovery of various neurological conditions. However, little is known about its mechanism of action. The present study elucidated the neuroprotective effects of tDCS on the mitochondrial quality control pathway in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model. We used the MPTP-induced neurotoxicity *in vivo* model. Mice were stimulated for 5 consecutive days with MPTP treatment. After observation of behavioral alteration using the rotarod test, mice were sacrificed for the measurement of the PD- and mitochondrial quality control-related protein levels in the substantia nigra. tDCS improved the behavioral alterations and changes in tyrosine hydroxylase levels in MPTP-treated mice. Furthermore, tDCS attenuated mitochondrial damage, as indicated by diminished mitochondrial swelling and mitochondrial glutamate dehydrogenase activity in the MPTP-induced PD mouse model. MPTP significantly increased mitophagy and decreased mitochondrial biogenesis-related proteins. These changes were attenuated by tDCS. Furthermore, MPTP significantly increased fission-related protein dynamin-related protein 1 with no effect on fusion-related protein mitofusin-2, and tDCS attenuated these changes. Our findings demonstrated the neuroprotective effect of anodal tDCS on the MPTP-induced neurotoxic mouse model through suppressing excessive mitophagy and balancing mitochondrial dynamics. The neuroprotective effect of anodal tDCS with modulation of mitochondrial dynamics provides a new therapeutic strategy for the treatment of PD.

1. Introduction

Parkinson's disease (PD) is the common neurodegenerative disorder with progressive dopaminergic neuron degeneration in the substantia nigra pars compacta (SNpc) in the midbrain. (Magrinelli et al., 2016). Although dopamine replacement treatment improves the symptoms of PD, there is no therapy for modifying the disease progression (Oertel and Schulz, 2016). Thus, there is an absolute unmet need for investigation of alternative treatment modalities aiming to halt or restore the disease course.

Although the mechanism of neurodegeneration in PD remains inconclusive, mitochondrial dysfunction has been regarded as one of the

Abbreviations: ATP, adenosine triphosphate; DAPI, 4′-6-Diamidino-2-phenylindole; Drp1, dynamin-related protein 1; GDH, glutamate dehydrogenase; LC3, microtubule-associated protein light chain 3; L-DOPA, L-3-4-dihydroxyphenylalanine; Mfn2, mitofusin; MPTP, 1-methyl-4-phenyl-1,2,3,4, tetrahydropyridine; NRF1, nuclear respirator factor 1; PD, Parkinson's disease; PGC-1α, peroxisome proliferator-activated receptor γ coactivator; PINK1, PTEN-induced putative kinase 1; PKC, protein kinase C; p62, sequestosome 1/p62; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; tDCS, Transcranial direct current stimulation; TFAM, mitochondrial transcription factor A; TH, tyrosine hydroxylase

* Corresponding author. Department of Neurology, Gangneung Asan Hospital, University of Ulsan College of Medicine, Bangdong-ri, Sacheon-myeon, Gangneung-si, Gangwon-do, 25440, Republic of Korea.
** Corresponding author. Natural Medicine Center, Korea Institute of Science and Technology, Gangneung, 25457, Republic of Korea.
E-mail addresses: neveu@gnah.co.kr (W. Jang), hoyang@kist.re.kr (H.O. Yang).

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central mechanisms in the pathophysiology of PD. In particular, the identification of pathologic variants in genes causing familial PD, such as Parkin (E3 ubiquitin ligase) and PINK1 (PTEN-induced kinase 1), supports this idea. Furthermore, many additional genes associated with familial PD have also been known to modulate the PINK1/Parkin pathway and mitochondrial quality control (Deas et al., 2011). Therefore, enhancing or restoring mitochondrial function could be a good and suitable candidate for therapeutic intervention in PD.

Transcranial direct current stimulation (tDCS) is a widely used noninvasive neuromodulatory technique in humans and in animal models. This therapeutic effect is based on the modulation of neuronal activity by small electrical currents that lead to polarization of the neuronal tissue (Giordano et al., 2017). Several studies in PD patients and rodent models demonstrated that tDCS alleviated the motor symptoms and cognitive impairments (Biundo et al., 2015; Giordano et al., 2017; Li et al., 2011; Manenti et al., 2018). Although the mechanism of tDCS in PD has not been fully investigated, Rae et al. reported that anodal tDCS exhibited an increased demand for adenosine triphosphate (ATP) and an increased pH (Rae et al., 2013). Considering that anodal tDCS increases neuronal excitability and that mitochondria are organelles that produce cellular energy through the electron transport chain, it could be assumed that anodal tDCS might affect mitochondrial dynamics. Recently, Lu et al. reported that anodal tDCS showed a neuroprotective effect on a 1-methyl-4-phenyl-1,2,3,4, tetrahydropyridine (MPTP)-induced toxic mouse model through inhibition of proteins associated with ROS (Lu et al., 2015). Our previous study also showed that anodal tDCS not only improved the enhancement of behavior and motor function but also modulated the autophagy molecule in 1-methyl-4-phenyl-1,2,3,4, tetrahydropyridine (MPTP)-induced parkinsonian mice (Lee et al., 2018). The aforementioned studies might have identified the crosslink between mitochondrial dysfunction and the targeted mechanism (Annepu and Ravindranath, 2000). However, there has been no study on the effect of anodal tDCS on mitochondrial function and quality control in pathological conditions. Therefore, we adopted an MPTP-induced parkinsonism mouse as a mitochondrial dysfunction model of PD and hypothesized that anodal tDCS could influence mitochondrial dynamics, mitophagy, and biogenesis and could show a neuroprotective effect through modulation of these mechanisms.

In the present study, we evaluated the neuroprotective effect of anodal tDCS using molecular markers of neurodegeneration and the efficacy of behavioral outcomes after applying anodal tDCS to untreated mice and MPTP-induced mouse models. We also investigated various markers related to mitochondrial biogenesis, dynamics, and mitophagy to elucidate the cellular or molecular effects of anodal tDCS on mitochondrial quality control.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice used in this study were obtained from Orient Bio Inc. (Seongnam, Korea) at the age of 7 weeks old. The mice were maintained on a 12-h light/dark cycle in a temperature- and humidity-controlled room (22 ± 3 °C and 50%, respectively) with water and food provided *ad libitum*. The protocols using mice in this study were performed in accordance with and granted (grant no. 2017018) by the Korea Institute of Science and Technology Animal Care Committee.

2.2. Surgery and transcranial brain stimulation

Under general anesthesia with ketamine and xylazine (55 and 7 mg/kg, i.p.), a custom-made plastic tube (American Plastics, CA, USA) was attached to the skull overlying the M1 area of the cortex using nontoxic dental cement. After sealing the tube with a custom-made screw cap to avoid the accumulation of debris, the mice were allowed to recover for five days. The mice were randomly distributed into four groups: (1) sham group (intraperitoneally vehicle-injected group), (2) tDCS group (tDCS plus intraperitoneally vehicle-injected group), (3) MPTP group (intraperitoneally MPTP-injected group), and (4) MPTP + tDCS group (tDCS plus intraperitoneally MPTP-injected group). To investigate the effects of tDCS on PD, mice were treated with anode tDCS for 30 min for five consecutive days with 30 mg/kg MPTP injection. According to our previous study, the cathode was positioned between the shoulders, while the anode was inserted in the saline-filled tube (Lee et al., 2018).

2.3. Motor coordination measurements

Seven days after the last MPTP injection, we performed the rotarod test to assess sensorimotor coordination. After 3 consecutive days of training for 3 min 3 times a day (rotation speed 2 rpm on the first day and increased speed to 16 rpm on the third day), we performed the test session in three trials with the same procedure on the third day of training. The rotating bar automatically recorded when each mouse fell off the rod.

2.4. Immunocytochemistry

After the rotarod test, the mice were sacrificed, and the SNpc was either kept at −80 °C or fixed with 4% paraformaldehyde for immunoassay. The entire midbrain was sectioned at 20 μm using a freezing cryostat (Thermo Fisher, MA, USA). The sections were placed in 3% hydrogen peroxide for 30 min to eliminate endogenous peroxidase and were subjected to free-floating immunohistochemistry with mouse Drp1 antibody and rabbit tyrosine hydroxylase (TH) antibody at 4 °C overnight. After washing the sections with PBS, the sections were incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibodies. 4′-6-Diamidino-2-phenylindole (DAPI) was used to stain cell nuclei in sections. Representative images were captured by a confocal microscopy (Leica, Solms, Germany) focused on the right area of the SNpc and analyzed using ImageJ software.

2.5. Immunohistochemistry

The SNpc and striatum (ST) sections were rinsed in PBS and then placed in 3% hydrogen peroxide for 15 min to remove endogenous peroxidase. Then, these were incubated at 4 °C overnight with rabbit TH antibody in 5% BSA and 0.3% Triton X-100 contained PBS. After incubation with a biotinylated anti-rabbit IgG (1:250), sections were incubated in ABC solution for 1 h at room temperature. TH immunoreactivity was visualized using 0.05% 3,3′-diaminobenzidine in PBS. The sections were dehydrated with ascending alcohol concentrations and cleared with xylenes before coverslipping. The images were captured on a microscope (Olympus, Tokyo, Japan).

2.6. Western blot immunoassay

The SNpc tissues were homogenized using a PRO-PREP (iNtRON Biotechnology Inc., Seongnam, Korea) and centrifuged at 13,000 rpm for 30 min at 4 °C. The fractionation lysates were separated by 6–15% SDS-polyacrylamide gel electrophoresis and were then transferred to PVDF membranes. The membranes were incubated with 5% skim milk or 3% BSA dissolved in TBST for 1 h. Then, the membranes were incubated with the primary antibodies overnight at 4 °C. The membranes were incubated with HRP-conjugated secondary antibodies for 1 h after washing with TBST. Immunoreactive bands were detected using a SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, Pierce Biotechnology, Rockford, Illinois, USA), and an LAS-4000 mini system (Fujifilm, Japan) was used for visualization. The data were normalized to the GAPDH intensity using Multi Gauge software (Fujifilm). The primary antibodies used were as follows: mitofusin 2

(Mfn2), nuclear respirator factor 1 (NRF1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), protein kinase C (PKC), phosphorylated PKC, PTEN-induced putative kinase 1 (PINK1), mitochondrial transcription factor A (TFAM), TH (Abcam, Cambridge, MA, USA), dynamin-related protein 1 (Drp1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-Synuclein, phosphorylated Ser616 Drp1, phosphorylated Ser637 Drp1, microtubule-associated protein 1 light chain 3 (LC3), Parkin, sequestosome 1/p62 (p62) and GAPDH (Cell Signaling Technology, Beverly, MA, USA).

2.7. Mitochondrial glutamate dehydrogenase (GDH) activity

GDH activity was detected by a commercial assay kit (Biovision) according to the manufacturer's recommendation. The homogenized tissues were mixed with prepared reaction reagents, and the absorbance was measured at 450 nm in a microplate reader. After 30 min of incubation, the absorbance was measured again at 450 nm to calculate the GDH activity.

2.8. ATP concentration

The ATP level was determined using a commercial ATP Assay Kit (BioVision). Briefly, homogenized SNpc tissues were deproteinized using a Deproteinization Sample Preparation Kit (BioVision) to rapidly block ATP consumption. Deproteinized samples were added to a 96-well plate with the reaction mixture. Then, the plate was incubated for 30 min at room temperature. The absorbance was measured at 570 nm by a microplate reader.

2.9. Statistical analysis

Data were analyzed with Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) using one-way ANOVA followed by the Newman-Keuls test. The results are expressed as the means ± S.E.M.

3. Results

3.1. tDCS ameliorates MPTP-induced behavioral deficits and neurotoxicity

First, we tested the effect of tDCS against MPTP-induced motor dysfunction. In the rotarod test, the latency to fall time was significantly reduced in the MPTP-treated group (38.7 ± 9.0) compared with that in the sham and tDCS groups (95.7 ± 13.9). However, tDCS increased the latency to fall time to –fold compared with MPTP alone (Fig. 1A). After the behavioral test, we confirmed the effect of tDCS on dopaminergic neuronal damage induced by MPTP. The protein expression of TH was significantly decreased to approximately 71% in the MPTP group compared with that in the sham group, while tDCS protected dopaminergic neurons (Fig. 1B). Consistent with the western blotting results, tDCS also showed many TH-positive fibers and TH-immunopositive neurons in ST and SNpc immunohistochemistry (Fig. 1C).

3.2. tDCS reduces MPTP-induced mitochondrial dysfunction

To investigate the effect of tDCS against MPTP-induced mitochondrial dysfunction, we measured mitochondrial GDH activity and ATP levels as markers of mitochondrial dysfunction. The mitochondrial GDH activity was 11.2 and 10.1 U/ml in the sham and tDCS groups. After MPTP treatment, the mitochondrial GDH activity significantly decreased to approximately 60% of that of the sham group. This decrease was attenuated by tDCS (Fig. 2A). The ATP level in the MPTP group significantly decreased to approximately 65% of that of the sham group, and tDCS attenuated this decrease (Fig. 2B).

3.3. tDCS regulates MPTP-induced abnormal mitophagy and mitochondrial biogenesis

Mitophagy and mitochondrial biogenesis are important events in controlling mitochondrial condition. Although many studies reported PD induced toxic agent such as MPTP and rotenone induced impaired autophagy, recently several studies have showed that excessive autophagic activation is induced by PD related toxic agent treatment (Bae et al., 2018; Xiong et al., 2013). The expression of mitophagy-related proteins, including PINK1, Parkin, and LC3-II, was significantly increased to 1.6, 1.7, and 1.4-fold, respectively, that of the sham group. Expression of another mitophagy-related protein p62, used as a marker of degradation phase of autophagy and decreased by autophagy activation was decreased to approximately 41% of that of the sham group. These changes were attenuated by tDCS (Fig. 3A–D). Expression of mitochondrial biogenesis-related proteins, such as PGC1α and NRF1, was significantly increased to 1.4 and 1.4-fold, respectively, that of the sham group, but expression of TFAM protein, which is also a mitochondrial-related protein, was significantly decreased to approximately 65% of that the sham group. tDCS also attenuated these changes (Fig. 4A–C). These results indicated that tDCS normalized mitophagy activation and impaired mitochondrial biogenesis induced by MPTP treatment.

3.4. tDCS regulates the MPTP-induced imbalance in mitochondrial fission and fusion

To determine the effects of tDCS on the MPTP-induced fission and fusion imbalance, we measured the protein expression of Drp1 and Mfn2, which are mitochondrial fission and fusion proteins. The protein expression of Drp1 was significantly increased to 1.6-fold in the MPTP group, but Mfn2 protein expression did not change. Increased Drp1 protein expression was ameliorated by tDCS (Fig. 5). The role of mitochondrial fission in the MPTP-induced behavior deficit and neurotoxicity was further confirmed by mdivi-1, which is a mitochondrial fission inhibitor. Mdivi-1 increased the latency to fall time and protein expression of TH and decreased the protein expression of Drp1 in MPTP-treated mice. Furthermore, cotreatment with tDCS and mdivi-1 attenuated the behavioral deficit and Drp1 protein expression (Fig. 6). Consistent with the western blotting results, tDCS and mdivi-1 also protected dopaminergic neurons in immunocytochemistry. After tDCS and mdivi-1 treatment, fluorescence intensity ratio of Drp1 and TH was decreased compared with that in the MPTP group (Fig. 7).

4. Discussion

Many causative genes linked to familial PD are associated with various aspects of mitochondrial quality control. For example, two genes causing autosomal recessive PD, Parkin and PINK1, are well known for their involvement in mitophagy (Deas et al., 2011). The serine protease HtrA2, which is a substrate for PINK1, causes autosomal dominant parkinsonism, and the α-synuclein gene, which causes autosomal dominant PD, has been reported to increase mitochondrial DNA damage and reduce mitochondrial biogenesis (Fu et al., 2018; Plun-Favreau et al., 2007). These findings strongly suggest that PD pathogenesis is closely linked with mitochondrial dysfunction.

The present study showed that anodal tDCS attenuated the behavioral abnormalities and dopaminergic neurotoxicity in an MPTP-induced PD mimic mouse model and that in parallel, anodal tDCS exerted modulatory effects on various mitochondrial quality control markers. Mitochondria play key roles in activating apoptosis and necrosis. Several PD human postmortem studies demonstrated that dopaminergic neurons die by inappropriate excessive apoptosis. However, non-apoptotic pathways have been discovered in several studies such as necrosis, necroptosis and autophagy (Niranjan et al., 2018; Wu et al., 2015; Yuan et al., 2019). Especially Hartmann et al. suggested that

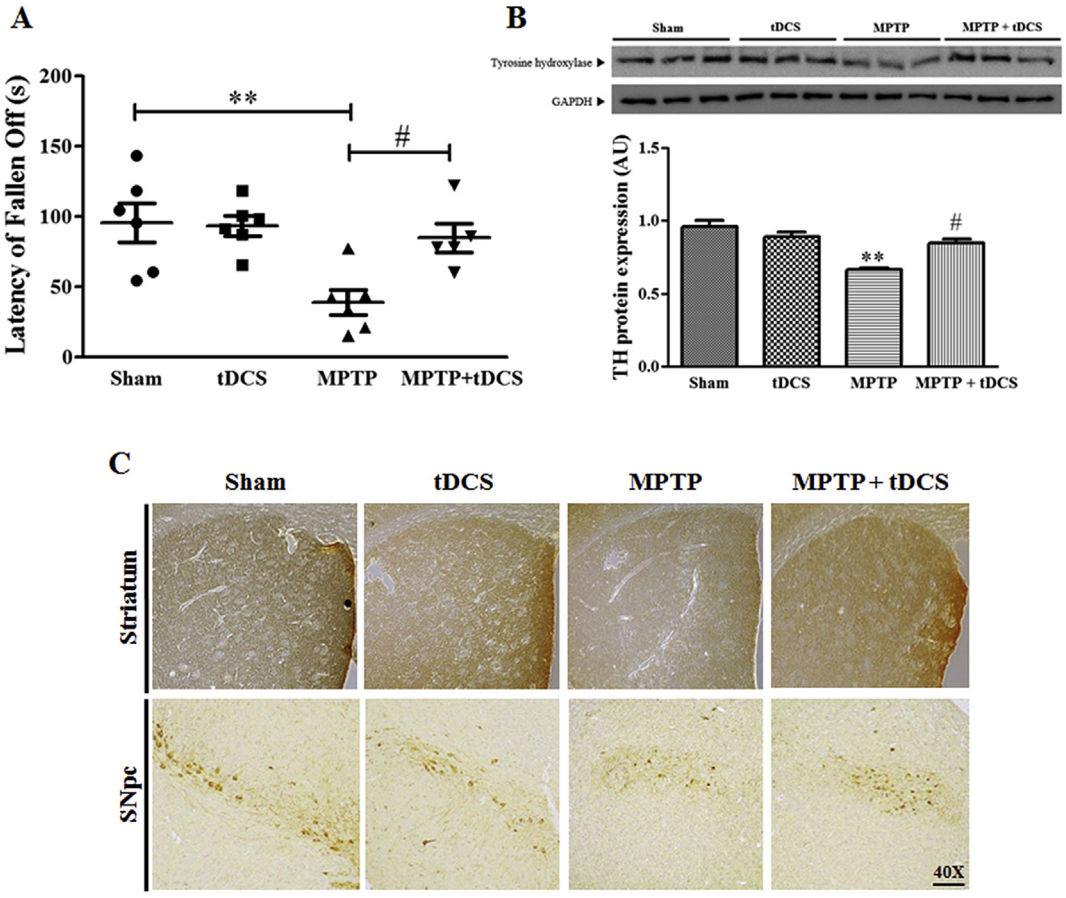

Fig. 1. Effects of tDCS on motor dysfunction and tyrosine hydroxylase (TH) expression in MPTP-induced parkinsonism. After 5 days MPTP treatment and tDCS, we examined the motor function of the mice by using the rotarod test (A) and then collect substantia nigra to measure the expression of TH protein by using western blotting (B) (n = 5–6). (C) Representative images showing TH staining neurons of the substantia nigra (SNpc) and striatum. The results are presented as the mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the sham group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the MPTP group.

MPP + induced mitochondrial dysfunction lead energy depletion, which shifted from apoptosis toward necrosis (Hartmann et al., 2001). Our data confirmed the mitochondrial dysfunction induced by MPTP treatment through estimating ATP levels and mitochondrial GDH activity, and anodal tDCS restored these changes. GDH, an enzyme located in the mitochondrial matrix, is a marker of mitochondrial membrane integrity and is closely related to mitochondrial function (Kravos and Malesic, 2008). Therefore, anodal tDCS clearly exerts a neuroprotective effect against MPTP toxicity, and the net effect of anodal tDCS reversed the mitochondrial dysfunction.

However, mitochondrial quality control is a very complicated process and includes multiple mechanisms, such as mitophagy, biogenesis, fission and fusion, for regulating mitochondrial homeostasis (Picca et al., 2018). The core machinery of mitophagy includes Parkin, PINK1, microtubule-associated protein light chain 3 (LC3) and p62 (Murakawa et al., 2015). Mitochondrial biogenesis is regulated by the coordinated expression of genes from the nuclear and mitochondrial genomes. The peroxisome proliferator-activated receptor γ coactivator (PGC-1α) acts as a master regulator modulating the expression of nuclear respiratory factors, followed by the induction of nuclear respiratory factor 1 (NRF1), intermediate transcription factor, and mitochondrial transcription factor A (TFAM), which is a final effector duplicating the mitochondrial DNA molecules (Cheng et al., 2012). Mitochondrial fission and fusion also affect mitochondrial morphology and function (Moore et al., 2016).

In the present study, MPTP treatment induced PINK1/Parkin upregulation and enhanced autophagy flux, and anodal tDCS attenuated these effects. In addition, mitochondrial biogenesis-related proteins,

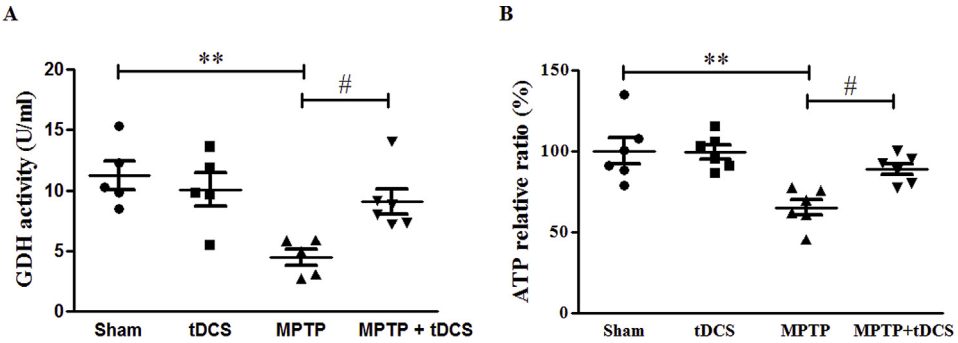

Fig. 2. Effects of tDCS on mitochondrial dysfunction in MPTP-induced parkinsonism. We measured the glutamate dehydrogenase (GDH) activity (A) and ATP content in the substantia nigra (B) to examination of mitochondrial function (n = 5–6). The results are presented as the mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the sham group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the MPTP group.

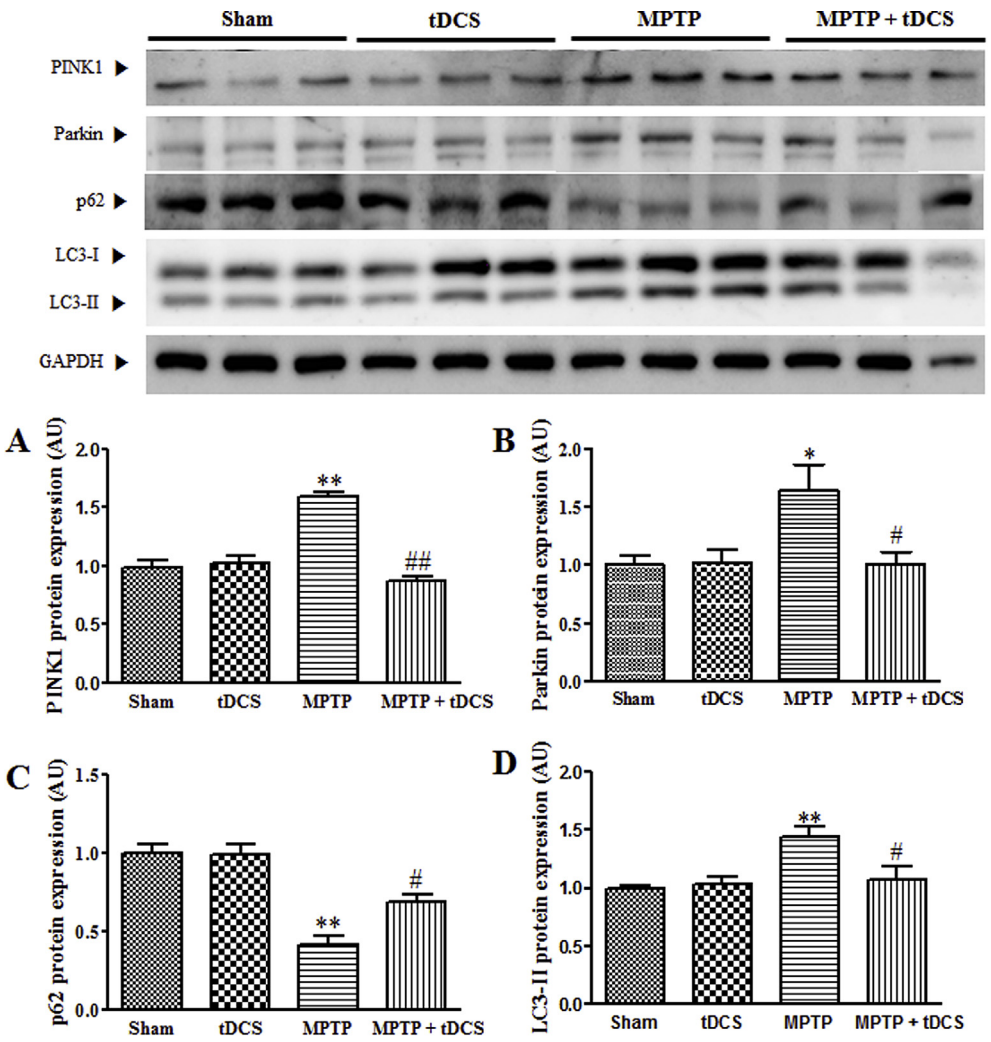

Fig. 3. Effects of tDCS on mitophagy in MPTP-induced parkinsonism. We measured the expression of mitophagy-related proteins, PTEN-induced putative kinase 1 (PINK1) (A), Parkin (B), sequestosome 1/p62 (p62) (C) and microtubule-associated protein light chain 3 (LC3) (D) in the substantia nigra using western blotting (n = 5–6). The results are presented as the mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the sham group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the MPTP group.

including PGC1α and NRF1, were also increased. Parkin can also play a role in mitochondrial biogenesis through ubiquitination of Parkin Interacting Substrate (PARIS), which is a transcriptional repressor of PGC-1α (Stevens et al., 2015). Torok et al. demonstrated that increased PGC-1α expression by MPTP treatment indicated a short-term compensatory protective mechanism against mitochondrial dysfunction (Torok et al., 2017). Therefore, the upregulation of Parkin in our study might lead to increased PGC-1α and NFR1 expression. However, TFAM protein expression was decreased in MPTP-treated mice. This finding indicates that increased mitophagy and organelle levels of mitochondrial quality control induced by MPTP could not compensate for the toxic effect of MPTP and led to cell death. Compared with the upregulation of PGC-1α and NRF1 levels, the reduced expression of TFAM reflects that the generation of de novo mitochondria could be impaired. Consistently, Wang et al. also reported that chronic MPP + treatment suppressed de novo mitochondrial DNA synthesis with decreased expression of TFAM (Wang et al., 2014). Therefore, the restorative effect of anodal tDCS could be mainly through increasing mitochondrial biogenesis at the level of transcription and replication of mitochondrial DNA, considering that NRF1 upregulates TFAM, which is directly involved in the replication and transcription of mitochondrial DNA.

Although several studies have demonstrated that activation of PINK/Parkin-mediated mitophagy protects against toxic and genetic PD models, our findings imply that balancing mitophagy and mitochondrial biogenesis might also be important for maintaining cell viability and homeostasis and that an imbalance between excessive mitophagy and inappropriate mitochondrial biogenesis could be linked to the development of neurodegeneration (Gao et al., 2017; Nardin et al., 2016). Shi et al. demonstrated that excessive mitophagy promotes cell death in a neonatal stroke mouse model (Shi et al., 2014). Additionally, pathologic mutation of α-synuclein could lead to mitophagy, mitochondrial DNA damage, and net mitochondrial loss, which were accompanied by excessive neuronal death (Wong and Krainc, 2017). Substantial genetic evidence suggests that pathologic variants of mitochondrial DNA could be related to the pathogenesis of PD. In particular, Luoma et al. reported that mutations in polymerase gamma (POLG) revealed multiple deletions of mitochondrial DNA and a parkinsonian phenotype (Luoma et al., 2007). Bender et al. also reported multiple mitochondrial DNA deletions in the postmortem human brain (Bender et al., 2008). Therefore, if anodal tDCS could have the ability to restore mitochondrial dysfunction in pathologic conditions through increasing the expression of TFAM, anodal tDCS could be a promising candidate for neuroprotective treatment in PD in addition to its well-known symptomatic benefit.

Our data, which represent mitochondrial fission and fusion in the MPTP model, also support this idea. Coordination between

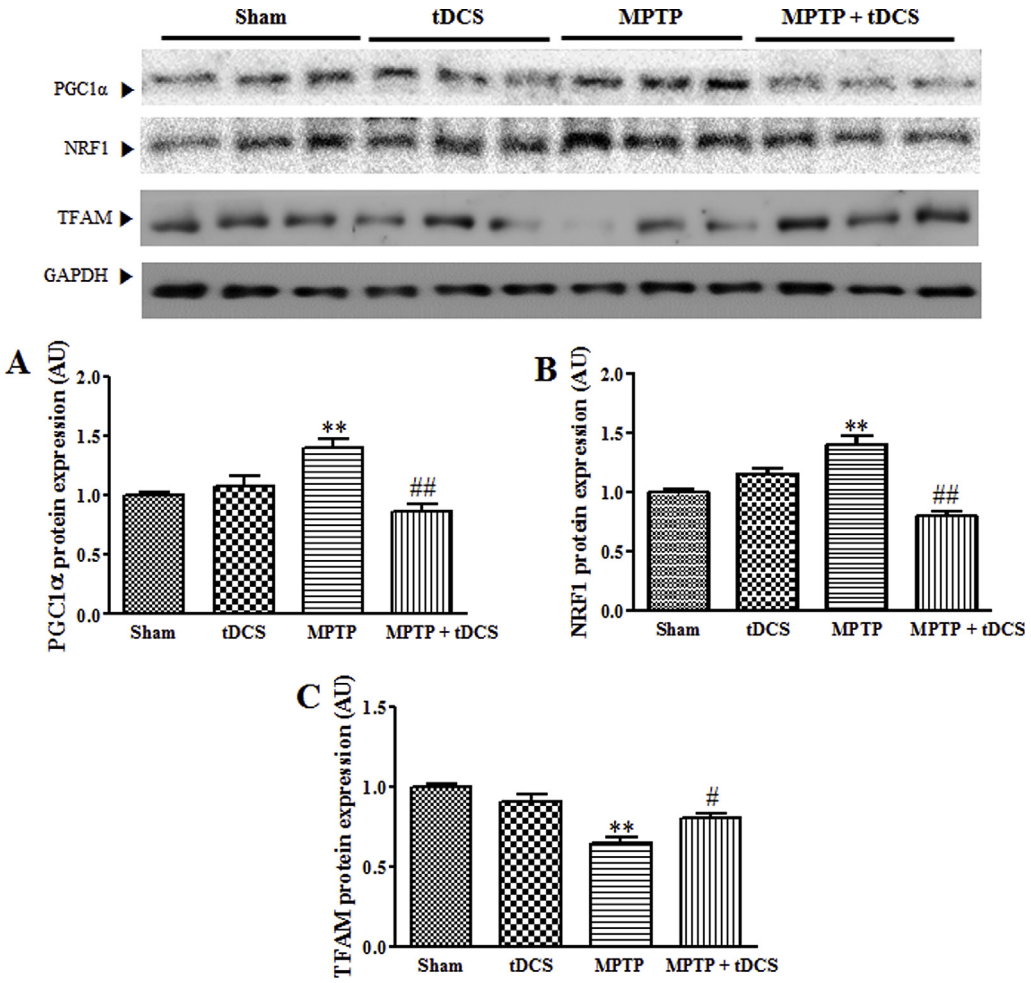

Fig. 4. Effects of tDCS on mitochondrial biogenesis in MPTP-induced parkinsonism. We measured the expression of mitochondrial biogenesis-related proteins, peroxisome proliferator-activated receptor γ coactivator (PGC1α) (A), nuclear respirator factor 1 (NRF1) (B) and mitochondrial transcription factor A (TFAM) (C) in the substantia nigra using western blotting (n = 5–6). The results are presented as the mean ± S.E.M. $^{**}p < 0.01$ compared to the sham group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the MPTP group.

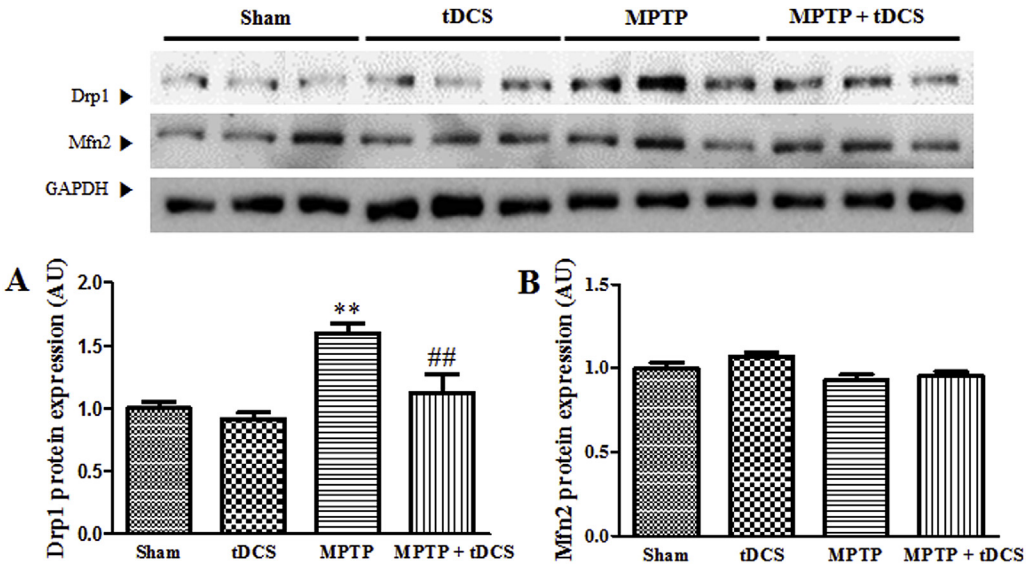

Fig. 5. Effects of tDCS on mitochondrial fission and fusion in MPTP-induced parkinsonism. We measured the expression of fission-related protein, dynamin-related protein 1 (Drp1) (A) and fusion-related protein, mitofusin 2 (MFN2) (B) in the substantia nigra using western blotting (n = 5–6). The results are presented as the mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the sham group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the MPTP group.

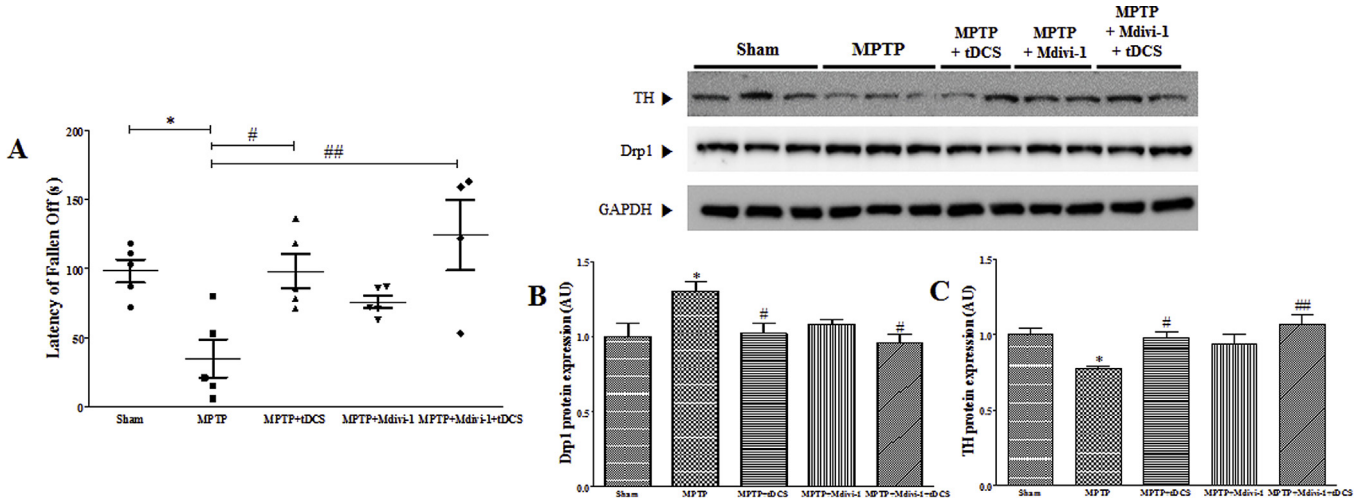

Fig. 6. Confirmation of the mitochondrial fission inhibitory effect of tDCS using the mitochondrial fission inhibitor Mdivi-1 in MPTP-induced parkinsonism. To confirm the protective and inhibitory effects of tDCS, we examined motor function (A) and protein expression of TH (B) and Drp1 (C) in the substantia nigra with Mdivi-1 treatment (n = 4–5). The results are presented as the mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the sham group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the MPTP group.

mitochondrial fission and fusion is also important for regulating mitochondrial number, morphology and distribution, the process of which is critical for maintaining mitochondrial bioenergetics activity and function. In particular, fission and fusion could regulate the activation of mitophagy (Abeliovich et al., 2013; Moore et al., 2016). Mitochondrial fission protein Drp1 and fusion protein Mfn2 indicate the balance of these opposing events. Overexpression of Drp1 under stressed condition triggers mitochondrial fragmentation and subsequently releases the pro-apoptotic protein including cytochrome *c* which is leading in turn to intrinsic apoptotic cell death (Filichia et al., 2016). Santos also reported that Inhibition of Drp1 attenuate neurotoxicity and restore dopamine release deficits in a PD animal model (Santos et al., 2015).

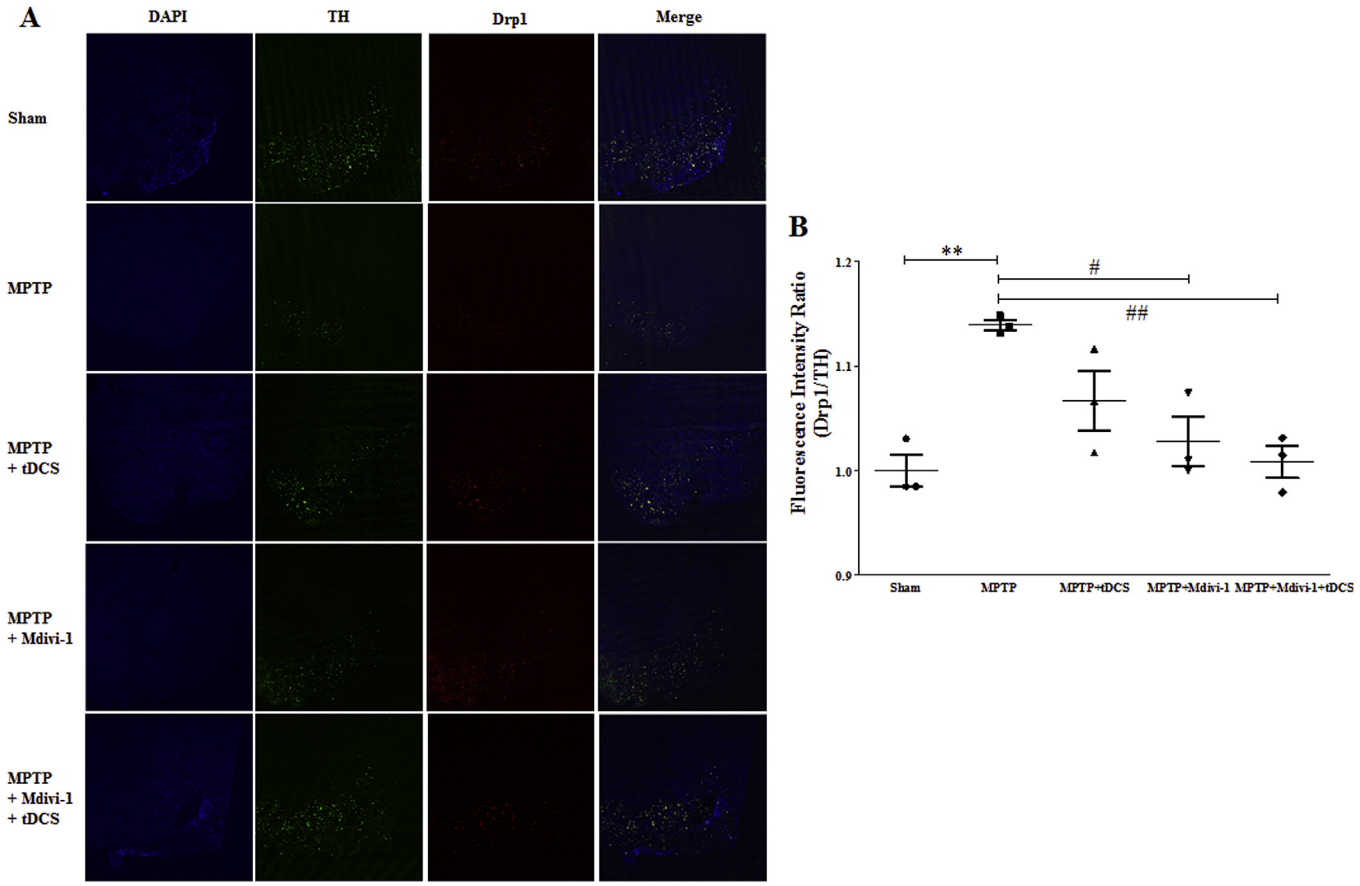

Fig. 7. Protective effects of tDCS on the fluorescence intensity of Drp1 using immunochemistry in MPTP-induced parkinsonism. Images of Drp1 (red), TH (green) and nuclei (blue) were collected in the substantia nigra tissues by fluorescence microscopy (A). Fluorescence intensity ratio of Drp1 and TH (B) (n = 3). The results are presented as the mean ± S.E.M. $^{**}p < 0.01$ compared to the sham group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the MPTP group.

Our data showed that MPTP treatment in mice revealed an increase in Drp1 expression and a decrease in the inactive form of Drp1 through PKC inhibition (supplementary data), which was recovered by anodal tDCS. However, mitofusin 2 expression showed no significant change among the MPTP mouse, control, and anodal tDCS mouse groups. Importantly, to confirm that the protective effect of anodal tDCS is directly mediated through Drp1, we administered Mdivi-1, a Drp1 inhibitor, to MPTP-treated mice. Drp1 inhibition through Mdivi-1 also ameliorated behavioral dysfunction, neurodegeneration, and α-synuclein accumulation. However, cotreatment of Mdivi-1 and anodal tDCS revealed synergistic improvement in the behavioral test, Drp1 expression, and α-synuclein clearance. This finding indicates that anodal tDCS could have additional mitochondrial quality control ability beyond the involvement of fission-fusion interactions, and the improvement of mitochondrial biogenesis induced by anodal tDCS may contribute to the synergistic effect with Mdivi-1.

There are several limitations to our experiment. First, we adopted the MPTP model as an *in vivo* mouse model of parkinsonism. Although the MPTP model is widely recognized and widely used as an animal model of PD, it does not completely reflect clinical PD and its pathogenesis. Many studies have reported that the impaired PINK1/Parkin pathway leads to a parkinsonian phenotype and mitochondrial dysfunction (Ahlskog, 2009; Koh et al., 2012). Therefore, to elucidate the mechanism of mitochondrial quality control underlying the neuroprotective effects of tDCS in PD, more elegant PD models, such as transgenic mouse models, are necessary. Second, although the anodal tDCS protocol in the present study is arbitrary, despite following methods used in previous studies, the polarity, simulation area, and the intensity at which tDCS is applied can greatly influence its efficacy. However, the M1 area, which is the montage we adopted, is both directly and indirectly connected to the striatum, and Li et al. reported that the anodal stimulation of M1 influenced the SN (Li et al., 2011). Thus, anodal M1 stimulation could be an appropriate montage for investigating cellular or molecular changes in the SN. Further studies will be necessary to discover an optimal protocol for tDCS.

To our knowledge, this study is the first to show that anodal tDCS attenuates neurotoxicity in an MPTP-induced mouse PD model through the regulation of damaged mitochondria, such as mitochondrial dynamics, mitophagy, and especially mitochondrial biogenesis. In conclusion, the neuroprotective effects of anodal tDCS provide an experimental basis for new PD treatment modalities, and further investigation is warranted.

Declaration of interests

The authors declare that they have no conflict of interest.

Author contributions

S.B.L and W.J designed this study. S.B.L conducted the experimental research and analyzed the data. W.J., J.Y. and H.Y.O contributed to the data discussion. All authors contributed to writing the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104491.

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