Effects of Subthalamic Nucleus Lesion and Electrical Stimulation in 6-OHDA-Induced Rat Parkinsonian Model

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Directed by Professor Jin Woo Chang

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Yong Sup Hwang

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This certifies that the Doctoral Dissertation of Yong Sup Hwang is approved

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Thanks to the wind .....

The wind is blowing strongly enough for you to strengthen. The wind would not blow, if you might fall over as a result of the root of the weak. Because the wind comes strongly your roots would not go down deeply and deeply not to come down. This one's for you is that all the wind. Actually, we have to thank the wind. 『Ho Seung Jeong, the fairy tale of love for age of twenty』

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Parkinson's disease (PD) is a neurodegenerative motor disease characterized by progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and a concomitant reduction of striatal dopamine. Abnormal activity of the STN, which sends hyperactivated glutamatergic neurotransmission to the substantia nigra pars reticular (SNpr) and globus pallidus internal segment (GPi), has been considered to be a pivotal function in the expression of PD symptoms. The present study was to investigate lesion and electrical stimulation of the STN ameliorated behavioral and electrophysiological functions with 6-OHDA lesioned rats. Behavioral changes were investigated after STN lesion by kainic acid in 6-hydroxydopamine (6-OHDA)-lesioned rats and measured levels of dopamine (DA) and its metabolites using tissue dissection. The results asserted that STN ablation induced behavioral improvement of parkinsonian motor deficit and DA was increased in the striatum and globus pallidus external segment (GPe).

The portable stimulators were made for animal PD model. STN-DBS

induced behavior improvement in PD models through the several behavioral tests. STN-DBS also induced change with distributions of ligands for dopamine D2 receptors ([11C]raclopride).

In the present study, The PD animals showed significant behavior improvements tested the several behavioral changes under the STN-DBS in PD models. A portable DBS stimulator and electrode fixation structure that could be useful tools to investigate the behavioral changes after STN-DBS in freely movable rat parkinsonian models. The results indicate that STN lesion in a 6-OHDA induced hemipakinsonian rat model may counteract some of the neurochemical changes within the striatum and GPe, and influence striatal dopaminergic metabolism.

Key words: Parkinson's disease, subthalamic nucleus, deep brain stimulation, High performance liquid chromatography, microPET

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I. INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative motor disease characterized by progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and a concomitant reduction of striatal dopamine.[1, 2] The subthalamic nucleus (STN) is an important structure in the basal ganglia circuitry and plays a critical role in regulating motor function. Abnormal activity of the STN, which sends hyperactivated glutamatergic neurotransmission to the substantia nigra pars reticular (SNpr) and globus pallidus internal segment (GPi), has been considered to be a pivotal function in the expression of PD symptoms such as akinesia, rigidity, and tremor.[3] During the past decades, ablation of the STN by lesions or high frequency stimulation has been shown to ameliorate motor dysfunction nonhuman primate and rodent models of PD.[4-6]

Various experimental studies indicated therapeutic lesion of the STN ameliorated behavioral and electrophysiological functions with 6-OHDA

lesioned rats.[6-8] But, Lesions of the STN, a considerably vascularized and small structure, may give rise to hemiballism and morbidity. During last years neurosurgeons have therefore developed deep brain stimulation (DBS) technique which become a fascinating intervention alternative in PD. Although the basic mechanisms underlying DBS are still unknown, the evidence that DBS of the STN has been shown to produce a dramatic alleviation of motor symptoms of PD, in both animals and humans experiments have been accumulated.[5, 9-16] During the recent years, deep brain stimulation (DBS) within basal ganglia has become a powerful therapeutic tool with advanced Parkinson disease (PD) in contrast to other stereotactic ablative surgery.[17, 18]

The advantages of DBS implicate its flexibility, the individual adjustment of stimulation parameter; safety, no permanent damage to brain tissue; and reversibility, the reduction of side effect and morbidity.[19, 20] In addition, it has been lately reported that STN-DBS seems to have neuroprotective effects as well as improving dopaminergic activity in experimental parkinsonian models.[21-23]

The aim of the present study was to investigate comparison between STN lesion and STN stimulation therapy in 6-OHDA lesioned rats. We provided comprehensive behavioral characterization of motor deficits by practicing variable behavioral tests, such as forepaw-adjusting steps, rotarod motor test, treadmill locomotor test, and amphetamine-induced rotational test.

In the present study, we report a portable DBS stimulator and electrode fixation structure that could be useful tools to investigate the behavioral changes after STN-DBS in freely movable rat parkinsonian models. As well, we tested the several behavioral changes under the STN-DBS in PD models. We also studied the tracer distributions of ligands for dopamine D2 receptors ([11C]raclopride) in the 6-OHDA lesioned rat during subthalamic electrical

stimulation to evaluate molecular changes for DA transmission in DBS. Although a few clinical studies were performed using radiolignad [11C]raclopride during DBS inpatients with Parkinson's disease,[24-26] their PET data didn't see any significant increased dopamine release under STN-DBS. However, none of in vivo PET studies on DBS in the 6-OHDA lesioned rats have elucidated how behavioral improvement relates to the alteration of DA transmissions for electrical stimulation in quantitative manner. To our knowledge, these are the first examples of [11C]raclopride PET imaging between DBS-ON and DBS-Off state in the rat brain in the progression of PD pathology.

II. MATERIALS AND METHODS

1. Subjects

Twenty five male Sprague-Dawley rats, weighing 230 to 250g each, were used at the beginning of the experiment. The rats were allowed to feed *ad libitum* during the experiment, and free access was given to water throughout the study. The cages were kept in a temperature- and humidity-controlled room with a 12 hour light-dark circle. Animal care and experiments were performed in a facility certified by the American Association for the Accreditation of Laboratory Animal Care. Rats were divided into 4 groups; (A) a control group (n=5); (B) a PD group (n=6) having a lesion of dopaminergic neurons caused by the 6-OHDA; (C) a STN group (n=7) receiving kainic acid in the STN with 6-OHDA lesioned rats; (D) a DBS group (n=7) subjected to electrical stimulation while the stimulation electrode was placed in the STN with 6-OHDA lesioned rats.

2. Surgical procedure for the medial forebrain bundle (MFB) and STN

lesion

The rats were anesthetized with a mixture of ketamine (75mg/kg), acepromazine (0.75 mg/kg), and rompun (4mg/kg). As previously described,[27] 8 μg(free base weight) of 6-OHDA(Sigma Chemical Co., St. Louis, MO) in 0.2% ascorbic acid with 0.9% normal saline was infused in 2 μl at a rate of 0.5 μl/min at the following coordinate of the medial forebrain bundle: -4.4mm AP, 1.2mm relative to the bregma ML, and 7.5mm from the dura mater DV. All Coordinates were taken from the atlas of Paxinos & Watson.[28] After injection of 6-OHDA, the cannula was left in place for 5 minutes before slowly retracting it. To prevent destruction of adrenergic neurons, 12.5 mg/kg desipramine was administered intraperitoneally 30 minutes before the infusion of 6-OHDA. The STN lesion was made with 2 μg kainic acid (Sigma) in 1 μl of saline solution injected at a rate of 0.5 μl/min (total 1 μl) into the STN (coordinates: AP -3.7 mm, ML 2.5 mm relative to bregma, and DV –8.0 mm from the dura). Upon recovery from the anaesthesia, many rats with STN lesions were diazepam (10 mg/kg, i.p.) to prevent seizures dissolved in sesame oil by warming and shaking. In the group of rats with paired lesions, the STN lesion was performed one week after the injection of 6-OHDA into the MFB.

3. Design of anchor

We design unique metal anchor to fix the electrode into skull of rat. We make side ditches to fix stimulation electrode to the anchor by dental cement and enable to bend the electrode and to connect with the connector cable. With this approach, we can close the scalp of the rat after the insertion of the electrode to minimize the inflammation.

4. Stimulation electrodes

We used tungsten electrodes (diameter 200μm) which are insulated by parylene coating of 5μm thickness for stimulation (Fig. 1). The tip of the all electrodes is tapered by electro-chemical etching to reduce the lesion effect of the target area. The tapered length of the electrode is 2mm and the area of the stimulation site is about 0.035mm^2, respectively. The impedance of the electrode is 15±1㏀ in saline and 1㎑ with potentiostat. (Zahner Elektrik IM6e, Germany)

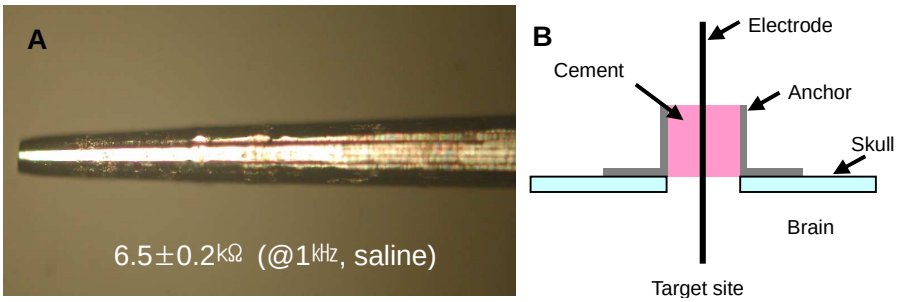

Figure 1. Photograph of electrode and diagram of implantation of electrode. We used tungsten electrodes (diameter 200 mm) that were insulated by parylene of a 5mm thickness (Fig. 1A). The tips of all electrodes were tapered by electrochemical etching to minimize the lesion effects of the targeted area. and We designed a unique metal anchor that was made of biocompatible stainless steel to fix the stimulation electrodes and to minimize infections and inflammations in the rats (Fig. 1B). A metal anchor was fixed on each skull with screws.

5. Portable deep brain stimulators

We used newly developed, small-sized portable stimulators to generate the current stimulus pulses (Table 1.). The main chip of the stimulator was an SX18AC microcontroller from Scenix. Stimulation parameters, such as duration and stimulation rate, could be changed with PC-based software. The parameters were stored in the internal ROM (Read Only Memory) of a chip through the RS-232C serial communication port. The amplitude of the stimulation voltage was controlled by the precision potentiometer. By adjusting the knob position of the potentiometer, the output current could be controlled from 14uA to 880uA. Finally, the stimulator was connected to the stimulation electrodes through the percutaneous connector that was the terminal of the subcutaneously implanted extension cable.

Table 1. Specification of SA(Stimulator for amplitude) version current stimulator

Amplitude	Max. 880uA 63 level 14uA step
Duration	60 us
Pulse rate	130 Hz
Channel number	1
Size	30mm X 30mm X 15mm
Supply voltage	5V
Current consumption	Max 180uA
Battery capacity	220mAh (6V)
Life time	1222 hours (50 days)

The total weight of each portable brain stimulator was 11±0.1g including battery and battery charge was sustained for 1222 hours.

6. Stereotaxic implantation of electrode into the STN and deep brain stimulation

Selected Animals were further unilaterally implanted with a metal electrode (tip diameter: 100μm) at the STN stereotaxic coordinates (AP-3.7mm, ML 2.5mm relative to bregma, and DV -8.2 mm from the dura) according to the stereotaxic atlas of Paxino & Watson.[28]

Stimulation for DBS group was applied one week after implantation of the electrode into STN. The stimulation parameters was determined on the basis of previous DBS studies by Maesawa et al.[23] Briefly, only current intensity was altered from 128 μA to 896 μA. The optimal intensity was chosen according to behavioral changes of each rat response below the threshold for apparent dyskinetic movement of contralateral forepaw, which was approximately 450 μA. Other parameters were fixed at a pulse rate 130 Hz and pulse width 60 μsec.

7. Behavior test

Forepaw-Adjusting Steps Contralateral forepaw adjusting steps were assessed immediately before the amphetamine-induced rotational test, as described in a previous study [29]. Briefly, the rats were held in a stationary position, while bearing weights on their forepaw, on the surface of the treadmill which moved at a rate of 90 cm/12 sec. During this interval, the number of forepaw adjusting steps involving the weight-bearing forepaw, which the rats made to compensate for the movement of their body, was counted. Each test consisted of 5 trials for each forepaw, alternating between forepaws. The contralateral forepaw adjusting steps were assessed on two occasions, 2 weeks after the 6-OHDA lesion and 1 week after the STN-DBS.

Elevated body swing test The elevated body swing test (EBST) was performed 1 day before the apomorphine-induced rotational test. Briefly, the rat was

placed in a Plexiglass box (40X40X35 cm), allowed to habituated for 2 min, and attain a neutral position. The rat was elevated approximately 2 cm above the surface by holding its tail. The direction of each swing was recorded when the rat moved, with its head directed more than 10° to either side of the vertical axis. Swings were counted for a period of 10 sec. One observer was responsible for timing the test session, determining, and recording the direction of swings, while another observer held the rat. All tests were conducted blind to the groups. The average performance in the five consecutive trials was used for within animal comparisons. The results of the swing test were displayed as percentage of right-biased swing for 10 seconds.

Rotarod motor test An accelerating rotarod (Ugo Basile, Comerio VA, Italy) was used to evaluate motor function of the rats. Each rat was placed on the rotating rod and received a training session on the rotarod at a continual speed of 8 rpm. The speed was slowly increased from 4 to 40 rpm over a period of 5 min. The time ended if the rat fell off the rung or gripped the device and spun around for two consecutive revolutions without attempting to walk on the rung. All tests were performed by observers blinded to each groups.

Treadmill locomotion test Locomotor ability was evaluated by treadmill locomotion test. Rats were trained to run on a straight treadmill enclosed 15cm wide, 40cm long conveyor belt chamber at a speed of 12cm/sec. The treadmill cycled between 20sec on and 20sec off periods. The trial was recorded average distance of the rats' nose position from the back wall of the chamber. A ruler was placed in view along with the treadmill belt to measure the nose position of the rat during the test. The average performance in the five consecutive trials was used for within animal comparisons.

Amphetamine-Induced Rotational Behavior Amphetamine-induced rotational behaviors were measured by using automated rotometer bowls with a tether attached to the torso of subjects. Amphetamine (5 mg/kg, i.p.) was

administered 2 weeks after medial forebrain bundle (MFB) lesioning. Rotational behavior was quantified every minute for 1 hour, after allowing the rats to become habituated for 20 min. Those rats that exhibited at least five full turns per minute, ipsilateral to the 6-OHDA lesioned side, were selected for the subsequent experiments. The total numbers of rotations during a period of one hour was measured and used for the analysis.

8. Tracer Synthesis

Functional images of the striatal dopamine system were obtained for each rat the D2 receptor radioligand [11C]raclopride(n=3). [11C]Raclopride was obtained by methylating the corresponding nor-precursor with [11C]methyltriflate. Approximately 18 MBq (0.5 mCi) of radioligand (specific activity range, 53– 760GBq/μmol; injection volume, 0.5mL) were injected into the tail vein using an infusion needle set.

9. Data Acquisition

All small-animal PET imagings were performed on a SIEMENS/Concorde Focus 120(Siemens Medical Solutions USA, Inc), which has an approximately $(1.3mm)^3$ resolution and plane-to-plane separation of 0.79mm. The coincidence window width was set at 6 ns. Before being imaged, the rats were anesthetized with isoflurane and N_2O/O_2. All rats were breathing spontaneously throughout the entire experiment. For [11C]raclopride, dynamic data were acquired during 120 min with frame duration increasing from 15sec to 300sec. All animals were scanned in the prone position with their brain centered in the field of view.

10. Data Reconstruction and image analysis

Small-animal PET studies were reconstructed using both filtered

backprojection and 3D-OSEM (3dimensional ordered subset expectation maximization). Compared with filtered backprojection, 3D-OSEM has been shown to result in improved spatial resolution and noise properties on small-animal PET images - an advantage for image registration. On the other hand, filtered backprojection may more accurately estimate radioactivity concentration and was used for quantification. After the reconstruction of data 2 mm round regions of interest (ROIs) were placed over the center of the bilateral striatum (2 slices) and cerebellum (2 slices), with image analysis software (ASIPro VM^{TM}, Siemens Medical Solutions USA, Inc). Decay-corrected time activity curves (TAC) in the ROIs were obtained from the dynamic PET images. The cerebellum was used as reference region, because it is nearly free of the D2 receptors.

11. HPLC-ECD analysis

High-pressure liquid chromatography (HPLC) with electrochemical detection was used for quantification of DA and DA metabolites (3, 4-dihydroxyphenylacetic acid, DOPAC, and homovanillic acid, HVA). When experiments were completed, rats were sacrificed by decapitation. The brains were rapidly removed and chilled in ice-cold 0.9% NaCl solution. After that, the brain was cut into coronal slice with the aid of a chilled brain mold (Rodent Brain Matrix, ASI Instruments, Warren, MI). The striatum and globus pallidus external segment (GPe) were immediately removed, placed onto dry ice, and tissue wet weight obtained. The tissue was homogenized in 1 ml of ice-cold 0.1 N $HClO_4$ for 30 sec, followed by centrifugation at 10,000 g and 4 ℃ for 10 min. Twenty μl of the supernatant was injected at 0.7 ml/min through a 80 x 4.6 mm HR-80 C-18 column (ODS, 3 μm particles; ESA, Chelmsford, MA, USA) with a mobile phase consisting of 75-mM Na_2HPO_4, 1.7-mM OSA, 0.01% [v/v] triethylamine, 10% [v/v] acetonitrile, and 0.1-mM

EDTA, adjusted to pH 3.0 with 2-mM phosphate buffer. Detection limit was 20 nA. Detection was performed with a coulometric detector (Coulochem Ⅱ, ESA, Chelmsford, MA, USA) coupled to a dual-electrode analytical cell (model 5014, ESA). Results were expressed as fmol/μl protein/mg wet weight of tissue.

12. Histology

Upon completion of an experiment, the rats were anesthetized and transcardially perfused with normal saline, followed by ice-cold 4% paraformaldehyde. Coronal 30μm tissue sections were cut at -20˚C using microtome cryostat the level of the striatum, the STN, and the substantia nigra pars compacta (SNpc) and mounted on gelatin-coated slides. Cresyl violet staining was performed to check the correct location of stimulation electrode in STN. Tyrosine hydroxylase (TH) immunohistochemistry was used to confirm the completion of the 6-OHDA lesions of the nigrostriatal pathway in rat PD models[30]. To assess loss of Dopamine (DA) cells and fibers in the SNpc and striatum on the side ipsilateral to 6-OHDA tissue sections were immunoreacted with primary polyclonal antibody against rat TH (PelFreeze, Rogers, AR) at a dilution of 1: 500 and biotinylated goat anti-rabbit IgG secondary antibody(Vector Laboratories, Burlingame, CA) secondary antibody. The signal was amplified by avidin and biotinylated horseradish peroxidase using the Elite ABC Vectastain kit (Vector Laboratories). We used 3, 3'-diaminobenzidine tetrahydrochloride as a chromogen, and cobalt chloride/nickel ammonium was used for intensification of the color changes.

13. Statistical analysis

Pared samples t-tests were used when comparisons involved only two groups. When three or more groups were involved, analyses of variance

(ANOVAs) were carried out and, if significant, follow-up LSD post hoc test was used to determine which pairs of groups were different from each other. The criterion for statistical significance was considered to be $P < 0.05$ in all statistical evaluations. All statistical analyses were performed Using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

III. RESULTS

1. STN lesion gives rise to behavioral improvement and dopamine elevation in rat parkinsonian model.

The extent and location of the lesions caused by the 6-OHDA were confirmed by assessing the loss of TH-immunoreactive cells and fibers in the substantia nigra pars compacta (SNpc) and striatum in the rat parkinsonian model with 6-OHDA (Fig. 2). The STN lesions were also evaluated following the completion of the experiments, and revealed evidence of local gliosis at the level of the STN (Fig. 3).

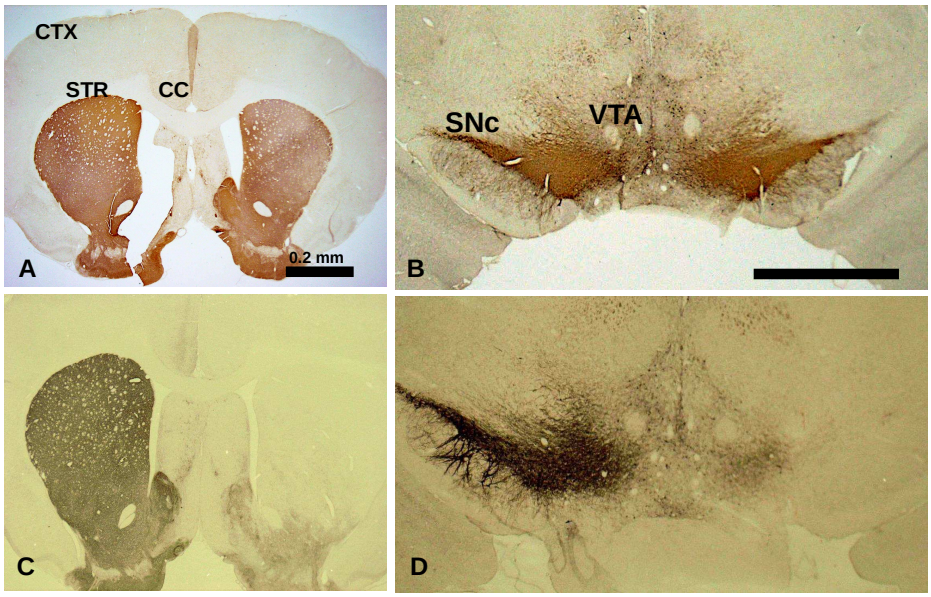

Figure 2. Bright field photomicrographs of histological sections obtained in rat PD models. Immunohistochemistry of tyrosine hydroxylase (TH) shows the total degeneration of dopaminergic fibers in the striatum (C) and dopaminergic cell bodies in the SNpc (D) ipsilateral to the 6-OHDA injection compared to normal rat(striatum;A, SNc(B)). Scale bar equals 0.2 mm. Abbreviation: Cx, cerebral cortex; Str, Striatum; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticular; VTA ventral tegmental area

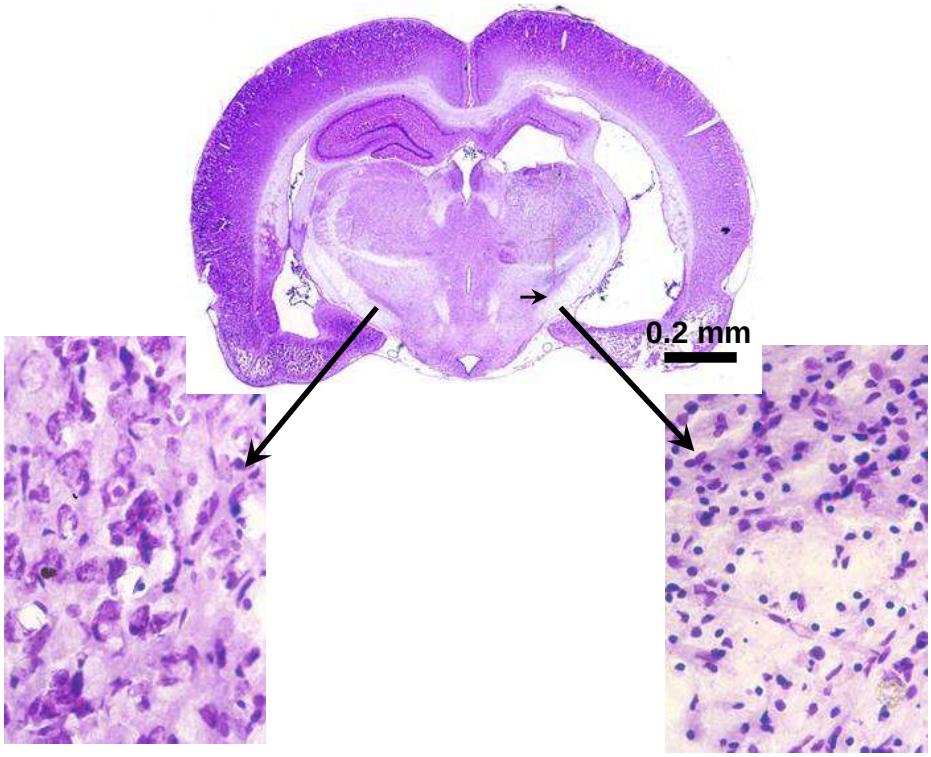

Figure 3. Cresyl violet-stained sections illustrating a unilateral kainic acid lesion in the subthalamic nucleus. The arrow indicates the location of the lesion (left: normal side, right: lesion side). Scale bar equals 0.2 mm

The levels of DA and its metabolites were measured in the striatum and GPe for each group of rats. Table 2 shows the change of neurotransmitter level in the striatum and GPe. There was a decrease of DA and DA metabolites (DOPAC and HVA) in these two regions in the 6-OHDA induced rat PD model, while the STN lesion group demonstrated a significant increase in these levels. After STN lesion induction the amount of DA in the striatum and GPe was significantly increased up to 20.14 ± 3.21 fmol/μl/mg and 45.02 ± 1.03 fmol/μl/mg, respectively (n=7, mean ± SEM, $P < 0.05$). The PD group followed by the STN lesion group demonstrated a significant increase in GPe DOPAC levels (62.279 ± 5.127 to 111.051 ± 15.163 fmol/μl/mg, $P < 0.05$), but did not show any statistical significance in the striatum. After STN lesioning in the rat PD model, HVA levels in the striatum and GPe increased significantly, 28.08 ± 3.29 fmol/μl/mg and 50.23 ± 6.88 fmol/μl/mg, respectively.

Table 2. Effects of STN lesioning on neurotransmitter levels and their metabolite/monoamine ratios in the striatum and GPe.

Site	Group	n	DA	DOPAC	HVA	DOPAC/DA	HVA/DA
Striatum	Normal	6	51.661±4.858	42.197±3.151	98.305±8.695	0.853±0.088	1.978±0.237
	PD	6	17.570±3.074*	33.736±3.241	66.043±2.359*	2.408±0.609*	4.796±1.281*
	PD+STN	7	37.711±6.286#	47.828±4.449	94.127±5.647#	1.744±0.591	3.414±1.049
	STN	7	65.077±4.722#	57.826±9.052#	112.572±7.291#	0.915±0.152#	1.795±0.174#
GPe	Normal	6	75.611±18.476	109.595±15.303	190.837±5.395	2.678±1.220	4.290±1.622
	PD	6	37.560±2.827	62.279±5.127*	67.482±3.405*	1.718±0.211	1.828±0.110
	PD+STN	7	82.582±3.852#	111.051±15.163#	177.707±10.280#	1.348±0.177	2.176±0.145
	STN	7	101.107±18.460#	124.986±8.561#	207.977±12.114#	1.739±0.543	3.202±1.249

. Data are expressed as fmol/ul protein/mg tissue or ratios of metabolite to monoamine (mean ± S.E.M.). Compounds are as follows: DA = dopamine, DOPAC = 3, 4-dihydroxyphenylacetic acid, and HVA = homovanillic acid. Statistical significance was determined by one-way ANOVA with LSD post-hoc comparisons (* $P < 0.05$ vs. Normal group. # $P < 0.05$ vs. PD group).

In 6-OHDA and STN lesioned rats, both striatal DOPAC/DA and HVA/DA ratios tended to increase but did not show any significant difference from the PD group.

The mean number of rotations in the rat PD model (n=6) for a period of one hour was 452.8 ± 45.82 ($P < 0.01$ compared with values control group, Fig. 4). After STN lesioning was performed in the rat PD models, the mean number of rotations for a period of one hour was significantly decreased, from 452.8 ± 45.82 to 97 ± 13.5 ($P < 0.05$). We also evaluated the effect of STN lesioning on forepaw adjusting steps. The mean number of forepaw-adjusting steps in each group is given in Fig. 5. After STN lesion induction, contralateral forepaw adjusting increased 5.0 ± 0.91, which was significantly higher than that of the 6-OHDA lesion group (2.6 ± 0.5, $P < 0.05$).

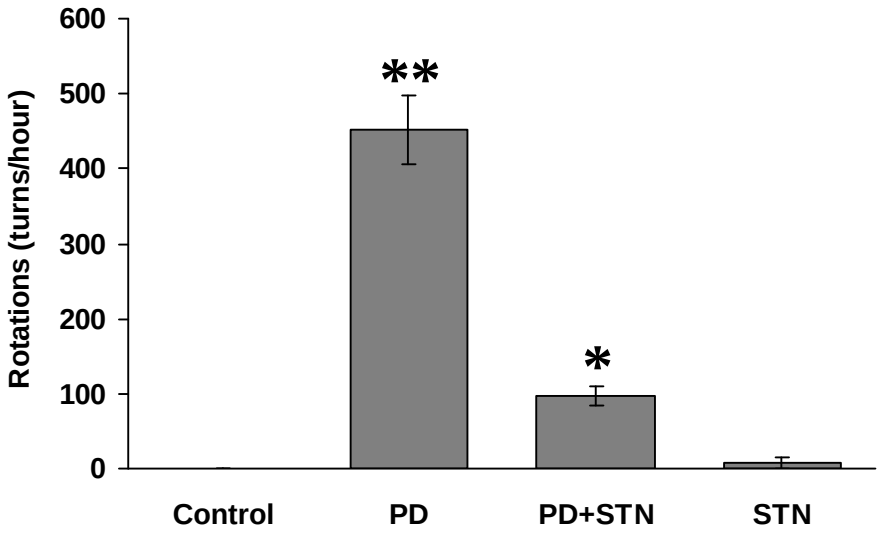

Figure 4. Apomorphine-induced rotational behavior in rats after 6-OHDA lesioning and ipsilateral kainic acid lesioning of the STN. Statistical significance was performed using one-way ANOVA followed by LSD post-hoc comparisons. (*P < 0.05 and **P < 0.01 compared to respective control group).

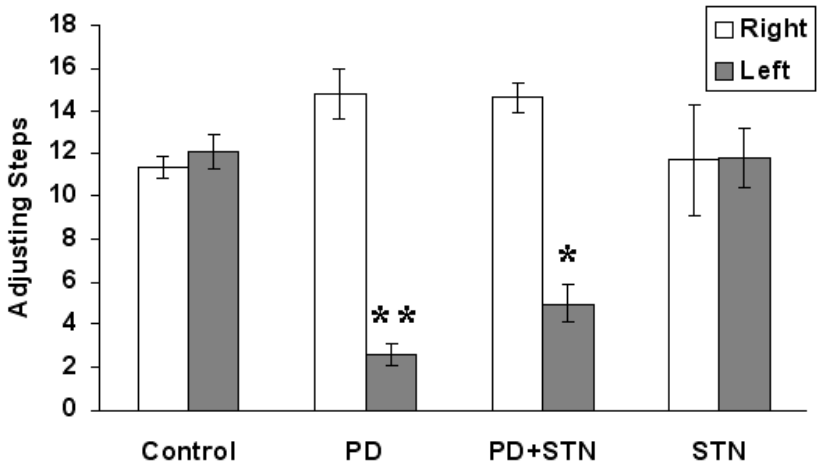

Figure 5. The average number of ipsilateral (open bars) and contralateral (filled bars) forepaw-adjusting steps in rats with unilateral 6-OHDA lesioning and kainic acid lesioning of the STN. Statistical significance was performed using one-way ANOVA followed by LSD post-hoc comparisons. (*P < 0.05 and **P < 0.01 compared to respective control group).

2. STN-DBS gives rise to spontaneous and drug-induced behavioral improvement in rat parkinsonian model.

The portable brain stimulator and the output biphasic pulse are shown in Fig. 6. The total weight of each stimulator was 11±0.1g including the battery, and the stimulators were positioned on the backs of the parkinsonian models with backpacks. In this study, a constant voltage stimulus, a bipolar biphasic square wave pulse with the duration of 60 us delivered to the STN through the electrodes. The intensity of the electrical stimulation was 450uA, and the high frequency of 130 Hz was applied.

The stimulation electrodes and their fixation method are shown in Fig. 1. The shape of the stimulation site was a flat circle with a diameter of 100±5 mm. The impedance of the stimulation electrodes was measured by electrochemical methods using a potentiostat (Zahner Elektrik IM6e, Germany), and the value was about 6.5±0.2KΩ with 1 KHz in saline. The scene of the electrode implantation in the STN with the holder is shown in Fig. 7. The metal anchor was fixed on the rat's skull with two screws, and its weight was about 0.9 g. After the electrode implantation, dental cement was added in the well structure of the anchor to secure the electrodes. We also verified there were no infections or inflammations six weeks after the anchor implantation. Also, we confirmed that there was no tissue damage caused by the metal anchor (Fig. 7).

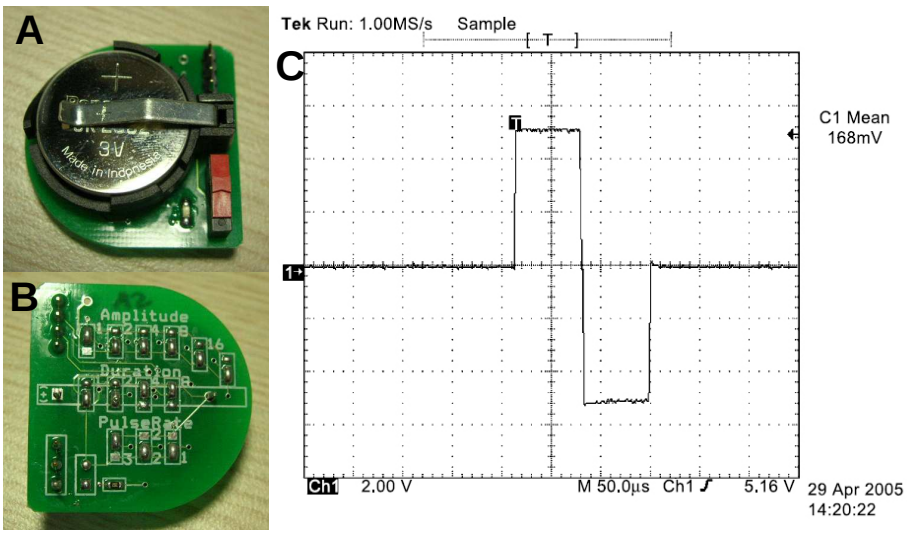

Figure 6. Portable brain stimulator (A;a top view, B; a bottom view) and biphasic output pulse of the stimulator(C)

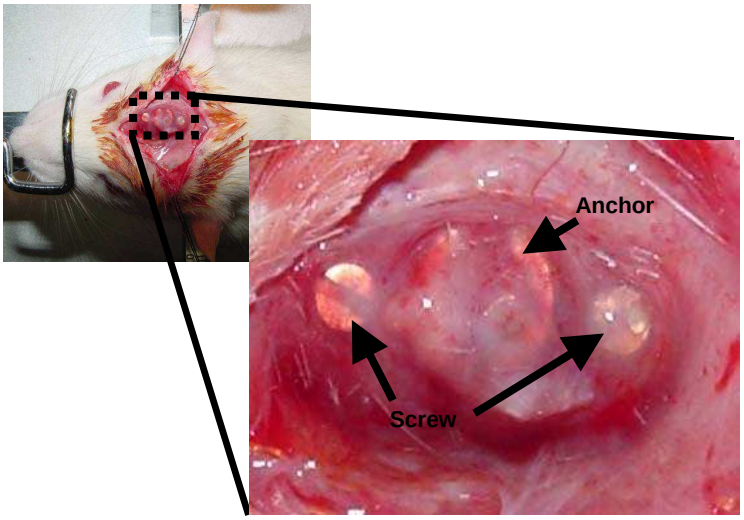

Figure 7. We verified there were no infections or inflammations six weeks after the anchor implantation. Also, we confirmed that there was no tissue damage caused by the metal anchor.

Forepaw –adjusting steps Consistent with previous reports [29, 30], the rat hemiparkinsonian 6-OHDA model produced a deficit in adjusting steps measured by the contralateral forelimb. The contralateral forepaw-adjusting steps appeared to have an all-or-none relationship to the conformity of the rats with the rat PD models (fig. 8). During STN-DBS, DBS group show increment of contralateral forepaw adjusting steps. Significant difference in the number of adjusting steps could be detected between PD and DBS group. After STN-DBS, contralateral forepaw adjusting increased 1.97 ± 0.23, which was significantly higher than that of the 6-OHDA lesion group (0.33 ± 0.11, $P < 0.05$).

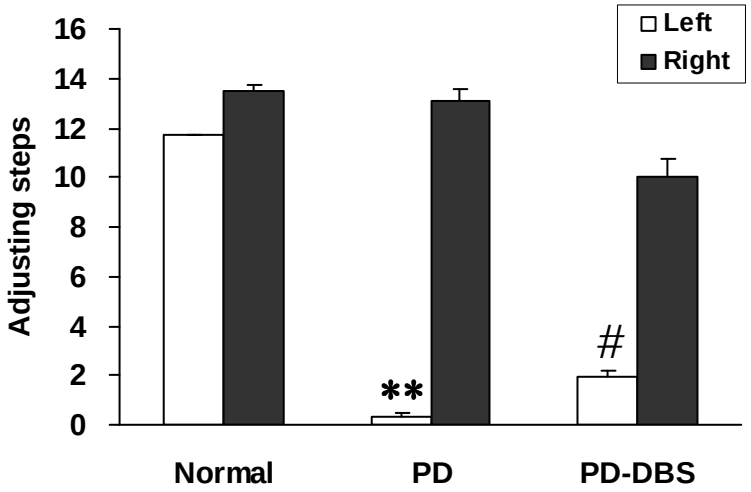

Figure 8. The average number of ipsilateral (open bars) and contralateral (filled bars) forepaw-adjusting steps in rats with unilateral 6-OHDA lesioning and DBS of the STN. Statistical significance was performed using one-way ANOVA followed by LSD post-hoc comparisons. (*$p < 0.05$ and ** $p < 0.01$ compared to respective control group, # $p < 0.05$ and ## $p < 0.01$ compared to respective PD group).

Elevated body swing test The effect of STN-DBS in 6-OHDA induced rat PD model was investigated on drug-free EBST after the 6-OHDA lesion and 2 weeks after the DBS electrode implant. PD group exhibited a 33.72 ± 2.97% significantly more right-biased swing behavior in the comparison with the control group on the test session (P<0.05), but during STN-DBS there was no significant improvement between the PD group and DBS group (results not shown).

Rotarod motor test The rotarod test was performed at 2 weeks after the 6-OHDA lesion and 2 weeks after the DBS electrode implant (Fig. 8). Prior to 6-OHDA lesion, The mean time spent on the accelerating rotarod in control group was 124.40 ± 8.36 sec (mean ± SEM). After lesion, the riding time was significantly decreased in the PD group (90.33 ± 10.73 sec, P<0.05). During STN-DBS, this impaired performance showed significant amelioration in the DBS group(118.25 ± 8.08 sec, P<0.05).

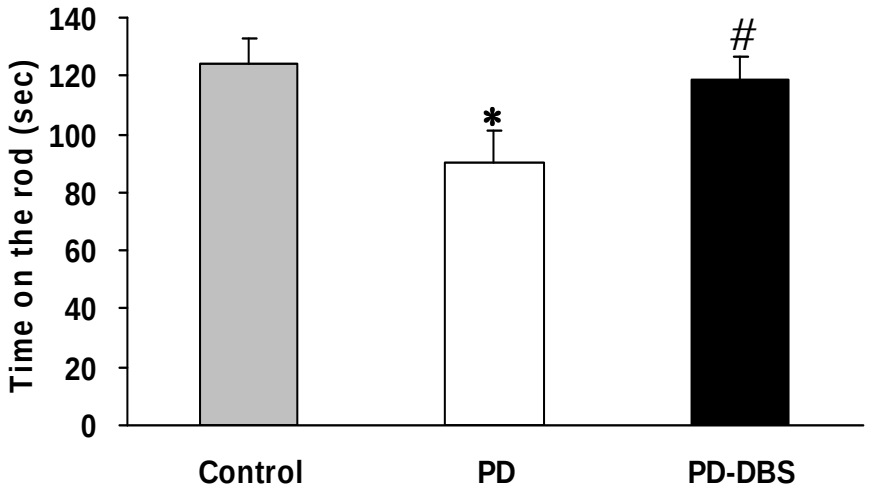

Figure 9. Effect of rotarod motor test during DBS in rat PD models. Each column represents the mean time spent on the accelerating rotarod (mean±SEM). Control: Normal Rats, PD: 6-OHDA lesion, and DBS: 6-OHDA lesion and DBS on STN. **P*<0.05, compared with values from normal group and #*P*<0.05, compared with values from PD groups.

Treadmill locomotion test The treadmill locomotion test was performed at 2 weeks after the 6-OHDA lesion and 2 weeks after the DBS electrode implant. FIGURE.3 shows the effect of STN-DBS on treadmill locomotion test measured by nose positioning the chamber. The PD group exhibited a 16.55 ± 0.61cm significantly less reduced distance compared with the control group (P<0.05) and showed inactive walking pattern. During the STN-DBS, there was significant increase in treadmill locomotion in the DBS group (20.96 ± 1.29cm, P<0.05). The pattern of walking movement displayed relatively more active during STN-DBS.

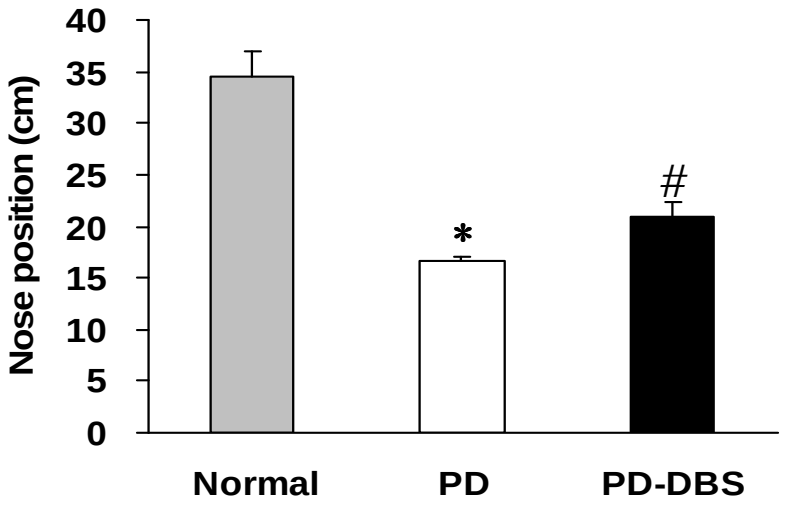

Figure 10. Effect of Treadmill locomotion test during DBS in rat PD models. Each bar represents the average distance between the rat's noses and posterior wall of chamber. Mean±S.E.M. **P*<0.05, compared with values from normal group and #*P*<0.05, compared with values from PD groups.

Rotational behavior Possible effects of STN-DBS on rotational behavior in 6-OHDA induced rat PD model were examined (Fig. 11). The rats passing the forepaw adjusting step test were selected for amphetamine induced rotational test. All rats that received the 6-OHDA injection met the criteria of the rat PD models in this study. The mean number of ipsilateral rotation in the rat parkinsonian model (n=6) for a period of one hour was 397.50 ± 139.92. After the STN-DBS is performed in rat PD models, the mean number of ipsilateral rotations for a period of one hour was significantly decreased, from 397.50 ± 139.92 to 165.00 ± 41.91 ($p<0.05$).

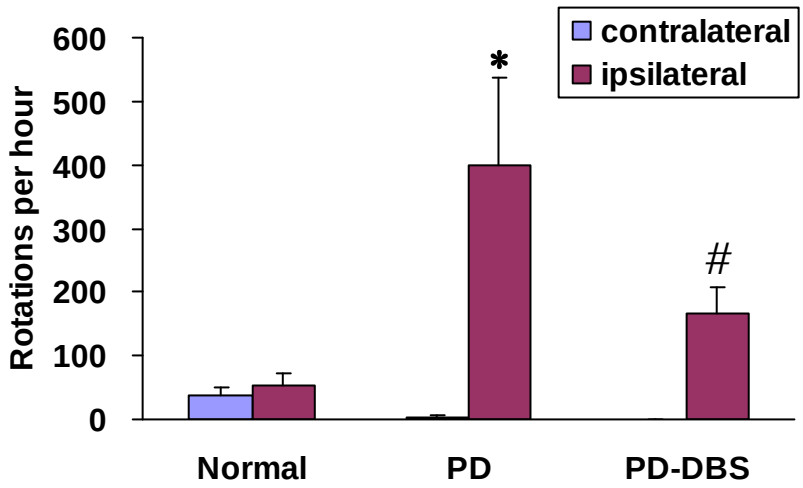

Figure 11. Effect of amphetamine-induced rotation test during DBS in rat PD models. A. Mean rotation showing amphetamine-induced ipsilateral rotational behavior. The sum of 360° rotations towards the ipsilateral sides recorded for 60 min are represented for each groups. Mean±SEM. **P*<0.05, compared with values from normal group and #*P*<0.05, compared with values from PD groups.

Histology After completion of the behavioral testing, all the animals were decapitated to confirm accomplishment of PD model and verification of accurate placement of stimulation electrode. The extent and location of the lesions caused by the 6-OHDA were confirmed by assessing the complete denervation of TH immunoreactive cells and fibers in the SNpc and striatum in the rat PD models with 6-OHDA (Fig 12. A and B). Only rats showing a correct localization of electrode were included in the data analysis. The localizations of the stimulation electrode in STN were confirmed by staining sections with cresyl violet (Fig 12. C). After histological verification, animals for stimulation electrode misplacement were discarded and only rats with accurate electrode placement in the STN were included in the data analysis. Figure 13 shows a schematic representation of coronal brain sections identifying the location of electrode in sham group, sham-DBS group and DBS group. No immune response and tissue damage within the STN was observed, whatever the electrode used.

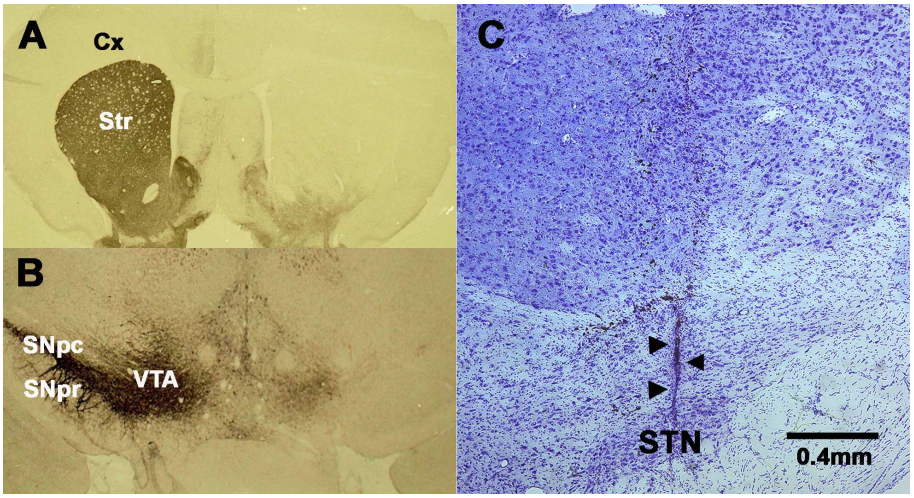

Figure 12. Bright field photomicrographs of histological sections obtained in rat PD models. Immunohistochemistry of tyrosine hydroxylase (TH) shows the total degeneration of dopaminergic fibers in the striatum (A) and dopaminergic cell bodies in the SNpc (B) ipsilateral to the 6-OHDA injection. (C) Photographs of cresyl violet-stained coronal section at subthalamic level. Arrow heads show the tract of the electrode in the subthalamic nucleus. Scale bar equals 0.4mm. Abbreviation: Cx, cerebral cortex; Str, Striatum; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticular; VTA ventral tegmental area; STN, subthalamic nucleus.

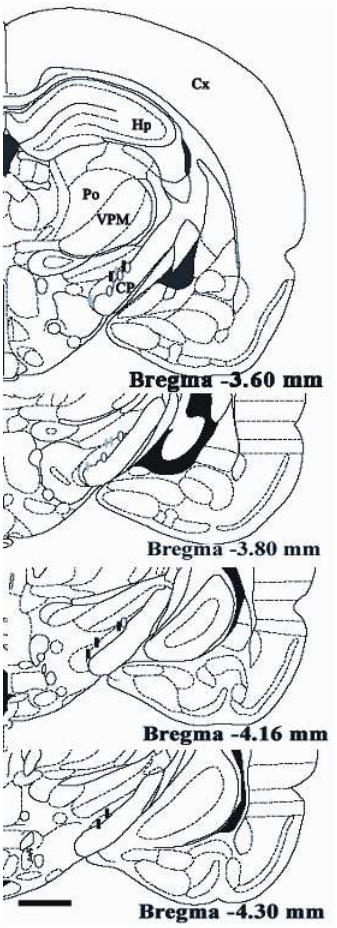

Figure 13. Target localization of the tips of stimulation electrodes within the STN (Graphic from Paxinos and Watson, 1998). One label is shown for each rat. Open circle indicate electrode located in the STN in sham-STN group, and black circle indicate electrode located in the STN in STN group. Relevant anatomical structures are: Cp, cerebral peduncle; Cx, cerebral Cortex; Hp, hippocampus; Po, posterior thalamic nuclear group; VPM, ventral posteromedial thalamic nucleus. Scale bar, 1mm.

3. STN-DBS does induce striatal dopamine release of rat parkinsonian model in micro PET study.

By time-radioactivity curves (TAC) specific [11C]raclopride binding values were computed using a graphic analysis method for assessing reversible radioligand–receptor interactions. The cerebellum served as the reference tissue, because it is nearly free of dopamine D2/D3 receptors.[31] Figure 14shows the metabolic, D2 receptor and microPET templates aligned in space with the rat brain atlas of Paxinos. The Logan analysis used the VOI (volume of interests) - specific and the cerebellar TAC of [11C]raclopride binding as a measure of the total radioligand distribution. Under DBS-off conditions (fig 15. A and C), the mean [11C]raclopride distributions in lesion side were higher compared with intact side in striatum. In a DBS-on condition (fig 15. B and D), they showed striatal change of similar condition between ipsi- and contralateral striatum. Table 3 show % control of [11C]raclopride radioligand between DBS-off and DBS-on condition. Disparity in DBS-off condition at parkinsonian state was considerably decreased during electrical stimulation in striatum compared to cerebellum.

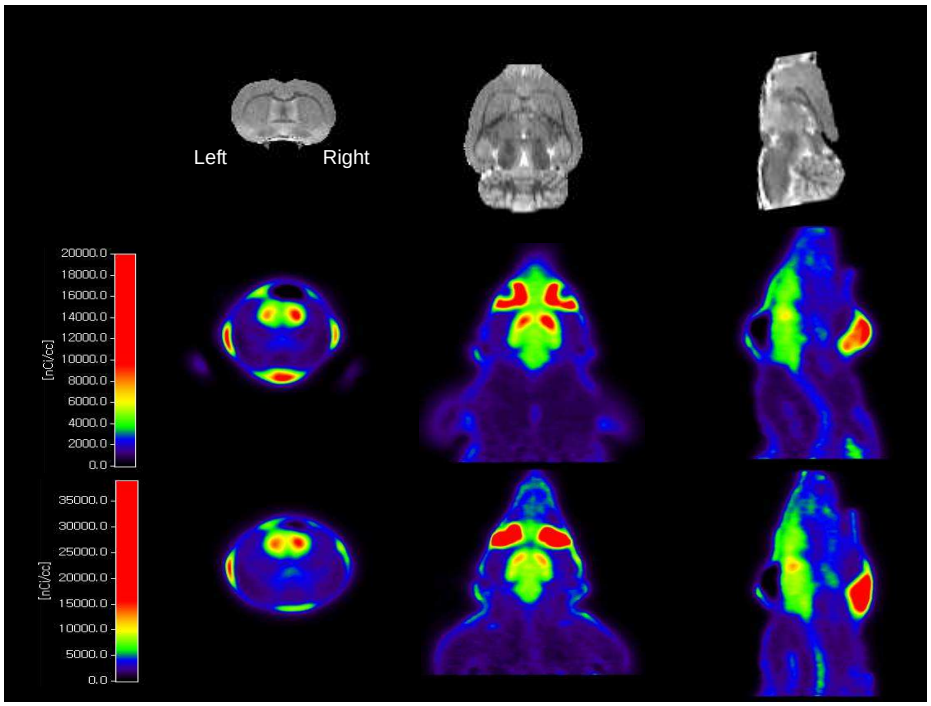

Figure 14. One representative coronal, horizontal and sagittal rat MR template through striatum area(top) and reconstruction image using 3D-OSEM algorithm(middle : DBS-off state in PD model and bottom: DBS-on state in PD model)

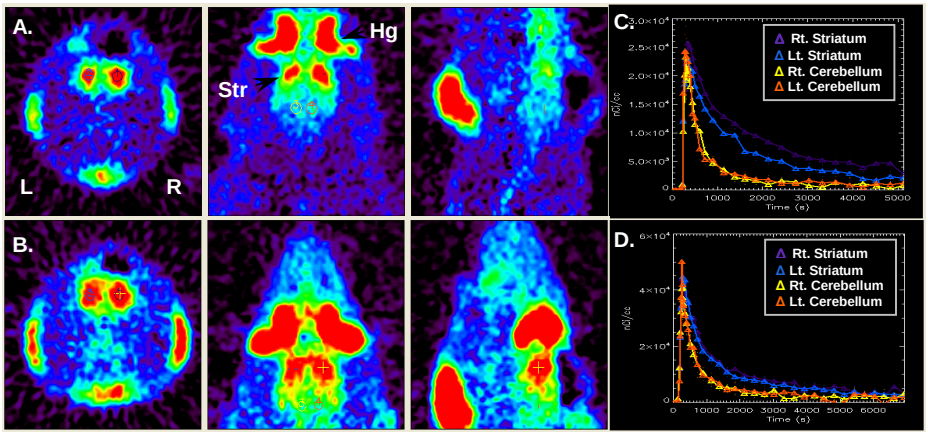

Figure 15. One representative summed microPET images of [11C]raclopride in coronal, horizontal and sagittal slice (radiologic orientation) through striatum area.(A : DBS-off state in PD model and B: DBS-on state in PD model), and representative example of time activity curves for [11C]RAC(C : DBS-off state in PD model and D: DBS-on state in PD model). Hg: harderian glands; L : left striatum; R: right striatum; Str: Striatum.

Table 3. Ratio of radioactivity (Ipsilateral/Contralateral X 100, %) of [11C]raclopride distribution in the DBS-on and DBS-off in rat PD model.

	DBS-off	DBS-on
Striatum	140.0	123.6
Cerebellum	97.2	101.3

IV. DISCUSSION

Output neurons from the STN are excitatory and use glutamate as a neurotransmitter. The degeneration of nigral dopamine neurons in PD leads to increased activity of glutamatergic neurons in the STN.[3] Therefore, inhibition of the STN plays a key role in the pathophysiological study of Parkinson's disease. We have previously demonstrated that ipsilateral STN lesioning by kainic acid in the rat hemi-parkinsonian 6-OHDA model induced behavioral amelioration of motor deficits and normalized overactivity of GABAergic SNpr neurons which amplified by increased excitatory input from the STN and the hypoactive firing rate of GP neurons, resulting in the depletion of dopaminergic neurons in the SNc.[6] Reductions in the circling induced by apomorphine and other DA agonists in the rat PD model following STN lesion induction have also been reported by others.[32] Recent studies have shown that STN lesions ameliorate parkinsonian motor symptoms in nonhuman primates.[4, 33]

In the present study, the obtained results provide the first evidence that STN lesions induced by kainic acid in the rat hemiparkinsonian 6-OHDA model produced increased dopamine neurotransmission in the striatum. A previous study indicated that stimulation of the STN produced an increased level of extracellular DA metabolites without an increase of extracellular DA in intrastriatal 6-OHDA lesioned rats.[22] Therefore, alteration in dopamine neurotransmission may reflect changes in nigrostriatal dopaminergic neuronal firing or dopamine release. Moreover, we observed increased levels of DA and DA metabolites after STN lesion induction in the GPe, suggesting that output neurons from the STN project to the GPe, GPi, and SNpr. Disinhibition of the STN neurons might increase thereby restoring the dopamine deficit.[3] Interestingly, STN lesions in the rat PD model produced decreased DOPAC/DA and HVA/DA ratios in the striatum. These findings prompt the

speculation that increased DA turnover and DA receptor sensitization (a compensatory mechanism in surviving neurons at parkinsonian condition) were normalized.[34]

Our finding that DA levels increased in the striatum after STN lesion production might explain the disinhibition of the thalamo-cortical projection. The striatum is known to be influenced along the nigro-thalamo-cortico-striatal connections. The decreased basal ganglia output would lead to disinhibition of the thalamo-cortical projection, which could result in an increased glutamatergic input from the cortical efferent projection to the striatum.[4, 35] The resulting increment of cortical afferent activation would account for some mediation of Parkinsonian symptoms. Alternatively, such a strong improvement in the DA level might be the consequence of a reduced availability of STN output.

Our findings might prompt speculation that a kainic acid lesion of the subthalamic nucleus alleviates disinhibition of nigral DA neurons. SNpc neurons are activated as a result of STN ablation in the rat hemiparkinsonian 6-OHDA model. This is supported by deep brain stimulation studies in which high frequency stimulation of the subthalamic nucleus increases the activity of nigral dopaminergic neurons.[23, 36]

A battery of behavioral tests susceptible to changing degrees of dopaminergic cell loss in nigrostriatal dopaminergic pathway was used in this study to evaluate motor function. The present study was carried out to see the effect of deep brain stimulation of 6-OHDA induced parkinsonian rat model. It was observed that 6-OHDA lesioning caused a decrease in motor function as evidenced by a decrease in forepaw-adjusting steps, rotarod motor test, and treadmill locomotor activity and increase in right-biased body swing, apomorphine-induced rotation. These parameters point toward neuronal deficit in the territory of SNc and striatum that regulate motor coordination.

This was also confirmed histologically as ipsilateral SNc and striatum were depleted after 6-OHDA injection as assessed by TH immunostaining.

We have previously demonstrated that ipsilateral STN lesioning by kainic acid in the rat hemi-parkinsonian 6-OHDA model induced behavioral amelioration of motor deficits.[6] Reductions in the circling induced by apomorphine and other DA agonist in rat PD model following STN lesions have also been reported by others.[32, 37, 38] We now show that STN-DBS has a similar effect in the rat PD models in apomorphine induced rotational test. The rat PD models with 6-OHDA showing an intense contralateral circling behavior were significantly reducing rotation by STN-DBS. This is similar to other observation.[39] When STN-DBS was applied, the beneficial improvements were assessed by decrease of contralateral rotational behavior induced by amphetamine in rat PD models. However, other reports showed controversial result. Chang et al.[40, 41] reported that there is no significant effect reducing rotation by STN-DBS. There is some contrary experimental procedure comparing with our experiment. We turned on the STN-DBS and continued for one hour during the rotation test, while in Chang's study,[40, 41] STN-DBS maintained for only 2 min after 20 minute apomorphine injection and measured the rotation immediately before and after stimulation. It could partially account for this discrepancy that extended period of time was different with the effect of DBS on rotation. Amphetamine-induced rotation is a useful parameter for evaluating imbalance of DA in asymmetrical hemiparkinsonian model. However, it is an artificial pharmacologically induced behavior may confound the interpretation while other important lesion-induced deficits remain basically unaffected.

Forepaw adjusting steps is an established test used for the simplified assessment of akinesia in unilaterally 6-OHDA lesioned rats. We found that forepaw adjusting steps showed the stepping deficits in the contralateral paw,

while having significant improvement on STN-DBS. We think the reason why restoration of motor deficit was showed in the STN-DBS is stimulation method attain to therapeutic threshold. We suggest that the adjusting steps measure a different behavioral deficit.[42]

We think that the EBST, first described by Borlongan and Sanberg[43] is not sensitive enough to discern asymmetrical motor behavior at STN-DBS of 6-OHDA induced hemiparkinsonian rat model. EBST has been reported applicable teat for the analysis of motor asymmetry in the 6-OHDA induced hemiparkinsonian rat model.[44] However, biased swing behavior by handling the animal by its tailing may lead to stressful stimuli and give rise to conflicting results. Some reports have been even described an ipsilateral instead of contralateral bias.[45, 46] In this respect, a similar condition may have occurred for no change or relative reduction in steps and swing in STN-DBS group. We conclude that this test is no useful in detecting amelioration of motor deficit on STN-DBS.

Rotarod test has showed to be useful for evaluating assessment of motor deficit. This test has usually been used as a drug-free for unilaterally 6-OHDA-lesioned rat to estimate for akinesia and postural instability.[46, 47] The present study shows that STN-DBS increase running time on the rod in 6-OHDA induced hemiparkinsonian rat model.

In the present study, using s treadmill locomotion test, we elucidated motor deficit after unilateral 6-OHDA lesion and then measured these restoration after STN-DBS. The treadmill locomotion test has previously been described as a appropriate tool of evaluating locomotion impairment following neurotoxin-induced motor deficit.[48] We didn’t use the electrical stimulation because the stress by electrical shock could affect movement during examination.[48, 49]

Interestingly, we observed abnormal behavior such as contralateral circling

and dystonia. Motor initiation deficit of contralateral circling by STN-DBS in a unilateral rat model of parkinsonism has been reported by Meissner et al.[50] And analogous tendency that subthalmic lesion induced the contralateral bias head position and body axis(‘curling’)has been reported by Henderson et al[51]. In differences with STN-DBS and STN lesioning, contralateral circling has also been described in cats after receiving local injection of gamma amminobutyric acid(GABA) receptor agonist muscimol into STN [52] and in rat PD model after administrating 5-hydroxytryptamine(5-HT; serotonin) into STN.[53] The contralateral circling may be explained by a decrease in the excitatory input from STN-SNpr indirect pathway. And we also observed dystonia symptom during STN-DBS. In previous study, we found that disabling of STN whether by lesion or stimulation in non-human primate showed side effect chorea, dyskinesia and hemiballism.[54, 55]

Using [11C]raclopride and microPET, We find that considerable effect of STN-DBS on striatal dopamine release. Previous PET studies in clinical humans have shown that did not provide evidence for an increased striatal dopamine using [11C]raclopride PET radiotracer.[24-26] But, other studies demonstrated that STN-DBS induce dopamine release with microdialysis data[21] and increase firing rate of SNc dopaminergic neuron.[36] These suggest that STN-DBS might have some effects on the striatal dopamine metabolism, such effects must be too small to be detected by PET in humans.

We suggest that DBS might have beneficial effects, mediating that STN-DBS function could lead to downstream changes in ipsilateral GPi, thalamus and cortical areas including supplementary motor area (SMA). The SMA projects bilaterally to STN and may mediate a bilateral effect from unilateral STN-DBS.[56, 57]

V. CONCLUSION

We conclude that STN lesions significantly influence the striatal DA system. The results indicate that STN ablation can mediate the pathophysiology of Parkinson's disease. However, the entire PD mechanism cannot be determined by the present experimental study. The present study investigated the effect of STN-DBS in a rat parkinsonian model produces an improvement in drug induced rotational behavior. But spontaneous behavior shows little enhancement. This hypothesis may assess the therapeutic strategy of STN stimulation on the PD patient. Further study is necessary to elucidate a crucial role for the stimulation’s mechanisms of action during STN-DBS.

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< ABSTRACT(IN KOREAN)>

6-hydroxydopamine에 의해 유발된 파킨슨 모형 백서의 시상하핵 손상과 전기 자극의 효과

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황 용 섭

파킨슨병은 중뇌흑질 치밀부의 도파민성의 점진적인 손실과 그에 따른 선조체의 도파민 감소를 야기하는 퇴행성 운동 질환이다. 시상하핵의 비정상적 활성은 중뇌흑질 그물부와 담창구내핵에 과활성된 글루타민성 신경전달을 일으키고 그 결과 파킨슨 증상의 주된 원인으로 여겨진다.

본 실험은 6-hydroxydopamine에 의해 손상된 백서에서 시상하핵을 손상시키거나 전기자극을 주었을 때의 행동학적 전기생리학적 기능의 회복을 탐구하였다. 6-hydroxydopamine에 의해 손상된 백서에서 kainic acid를 사용하여 시상하핵을 손상시킨 후에 행동검사와 조직절개를 통하여 도파민과 대사물질의 양을 측정하였다. 그 시상하핵을 파괴시킨 결과 파킨슨 병의 운동손상 징후의 행동학적 향상과 선조체와 담창구 외핵의 도파민 증가를 보였다.

파킨슨 동물모형을 위한 휴대용 자극기를 만들었으며, 파킨슨 동물 모형에서 시상하핵의 심부뇌자극 시 행동학적 향상과 도파민 D2 수용체의 방사성리간드인 [11C]raclopride의 분포의 변화를 유도하였다.

본 연구에서 파킨슨 모형에서 kainic acid 손상을 통해 운동결핍의 향상을 가져왔다. 휴대형 심부뇌자극 장치와 전극의 고정방법은 자유롭게 움직이는 파킨슨 백서 모형에서의 심부뇌자극 후 행동검사를 하는데 유용하게 쓰일 수 있다. 본 결과에서 6-hydroxydopamine으로 유도된 파킨슨모형의 시상하핵 손상은 선조체와 담창구외핵의 신경화학적 변화를 중화시키면 선조체의 도파민 대사과정에 영향을 준다.

핵심되는 말 : 파킨슨 병, 시상하핵, 심부뇌자극, 액체고속 크로마토그래피, 마이크로 양전자 방사 단선촬영

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