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Research paper

# Decreased bladder contraction interval induced by periaqueductal grey stimulation is reversed by subthalamic stimulation in a Parkinson's disease model rat

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ABSTRACT

The medial prefrontal cortex (mPFC) regulates bladder contractions via the periaqueductal grey (PAG). Subthalamic nucleus deep brain stimulation (STN-DBS) modulates urinary afferent information from PAG in Parkinson's disease (PD). We do not know how STN-DBS modulates the activities of mPFC induced by PAG stimulation. We aim to clarify how STN-DBS modulates the neuronal activity of mPFC induced by PAG stimulation and its effects on bladder contraction Experiments were conducted under urethane anesthesia in normal (n = 9) and 6-hydroxydopamine hemi-lesioned PD rats (n = 7). Left-sided PAG stimulation and STN-DBS were applied with simultaneous bladder contraction monitoring. Local field potential (LFP) recording and collection of extracellular fluid in the mPFC were performed before stimulation, during PAG stimulation, during PAG+STN stimulation, and after stimulation. The bladder inter-contraction intervals significantly decreased with PAG stimulation with a concomitant decrease in mPFC LFP power in PD rats. Adding STN stimulation to PAG stimulation significantly increased the bladder inter-contraction intervals with a concomitant increase in mPFC LFP power in PD rats. Several mPFC catecholamine levels were modulated by PAG or PAG+STN stimulation in PD rats. The present study revealed that STN-DBS modulate the activities of mPFC induced by PAG, thereby leading to normalization of bladder contraction.

## 1. Introduction

Parkinson's disease (PD) is clinically characterized by bradykinesia, rigidity, and resting tremors (Kalia & Lang, 2015). It is also well known that PD patients usually suffer from urinary dysfunction characterized by urinary frequency and urinary urgency (Sakakibara et al., 2012). Urodynamic studies show that PD patients have reduced bladder volume and detrusor overactivity, which result in urinary frequency (Sakakibara et al., 2012). Several studies have suggested that subthalamic nucleus deep brain stimulation (STN-DBS) increases the maximum bladder volume, which in turn improves urinary frequency in PD patients (Witte et al., 2018; Winge et al., 2007; Mock et al., 2016; Seif et al., 2004). Positron emission tomography studies have reported that STN-DBS might normalize the afferent urinary information from the periaqueductal grey (PAG), thereby leading to improvement of the urinary frequency in PD patients (Herzog et al., 2006; Herzog et al., 2008), although the detailed mechanisms are not well understood. The medial prefrontal cortex (mPFC) plays an important role in regulating the micturition reflex based on the urinary afferent information conveyed by the PAG (Fowler et al., 2008; Griffiths, 2015). Urinary afferent projections from the bladder are received by the PAG and afferent information is sent to the thalamus, which projects to the mPFC (Sakakibara et al., 2012). The mPFC, which is known to have dense connections with the PAG, is thought to inhibit involuntary voiding via the PAG during the bladder relaxation phase (Fowler et al., 2008; Griffiths, 2015). The mPFC activates the pontine micturition center (PMC) via the PAG when voiding is both desired and socially appropriate. This leads to activation of the sacral parasympathetic neurons that result in bladder contraction. The mPFC receives dense dopaminergic, noradrenergic, and serotonergic projections from the ventral tegmental area, locus coeruleus, and

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raphe nucleus, respectively. This suggests that catecholamines play a significant role in micturition reflex regulation in the PFC (Ong et al., 2019). A previous positron emission tomography study revealed that significant neural activity correlations in the thalamus and the insular cortex with PAG neural activity were only observed during the STN-DBS switched ON phase, suggesting that STN-DBS might partially restore the basal ganglia circuit, which eventually leads to normalizing the PAG afferent projections illustrated by functional brain imaging studies (Fowler et al., 2008; Griffiths, 2015). Although, processing of afferent urinary information from PAG to thalamus is impaired in patients with PD, stimulation of STN leads to activation of GPi (internal segment of globus pallidus) which eventually restores the activity of thalamus receiving afferent urinary information from PAG, resulting in normalization of urinary afferent information. Afferent urinary bladder information is processed in the thalamus and the insular cortex, which is ultimately conveyed to the mPFC (Sakakibara et al., 2012; Fowler et al., 2008; Griffiths, 2015). It is important to understand the mPFC and PAG functional relationships with respect to bladder contraction because mPFC and PAG functional network might play a key role in regulating bladder contraction, and how STN-DBS modulates this network because significant urinary afferent activation is mandatory for the mPFC to decide whether urination is appropriate. Investigating how STN-DBS modulates mPFC and PAG network might ultimately contribute to the elucidation of the mechanisms of STN-DBS.

We have previously reported that STN-DBS increased the bladder inter-contraction intervals by increasing the alpha power in the mPFC in PD model rats, suggesting that STN-DBS might modulate efferent control of bladder contraction via mPFC (Yamamoto et al., 2020). STN-DBS might modulate mPFC activity, which is influenced by the urinary afferent information (PAG stimulation in this study) as STN-DBS was also reported to improve urinary frequency by normalizing the afferent projections from the PAG in PD patients (Herzog et al., 2008), and because the mPFC may decide whether voiding is appropriate depending on the urinary afferent information (Fowler et al., 2008).

We aim to clarify how STN-DBS modulates the neuronal activity (local field potential [LFP] and catecholamine levels) of the mPFC induced by PAG stimulation (increased urinary afferent projections), and its effects on the bladder inter-contraction intervals (which is equivalent to urinary frequency in humans) in normal and PD model rats.

## 2. Methods

### 2.1. Animals and ethics statement

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals on adult female Sprague–Dawley (SD) rats (14–16 weeks old, weighing 200–300 g). All efforts were made to minimize the animals' suffering and reduce the number of animals used. Animals were housed in a room under standard environmental conditions with an alternating 12-h light/dark cycle. The experimental protocol was approved by the Animal Ethics Committee, Chiba University Graduate School of Medicine (April 1, 2020, number 2-369). All experimental methods in this study are in accordance with ARRIVE guidelines.

### 2.2. 6-Hydroxydopamine (6-OHDA)-induced lesion (PD model)

Surgery was performed on SD rats under sodium pentobarbital anesthesia (40 mg/kg, intraperitoneally). The animals received a unilateral injection of 2 μg/mL 6-OHDA (Sigma–Aldrich, Japan) dissolved in 5 μL of 0.9 % sterile saline containing 0.1 % ascorbic acid into the left medial forebrain bundle at a rate of 1 μL/min. The stereotaxic coordinates of the injection site in relation to the bregma were as follows: anteroposterior, –3.6 mm; lateral, 2.0 mm; and dorsoventral, –8.8 mm.

### 2.3. Motor behavior

The extent of the DA neuron lesion was assessed 2 weeks following 6-OHDA injection through an apomorphine challenge (1 mg/kg, intraperitoneally; Sigma–Aldrich). The lesion was successful in animals that performed >80 net contraversive rotations in 20 min

### 2.4. Recordings of isovolumetric bladder contractions

The isovolumetric bladder contraction recordings were performed under urethane anesthesia (0.7 g/kg, intraperitoneally). Anesthesia depth was monitored by the absence of a response to toe pinch. A one-third of the initial dose of urethane was injected. Transurethral insertion of a single-lumen catheter into the bladder was used to measure bladder pressure. The catheter was attached to a syringe pump with an in-line pressure transducer. Saline was instilled (100 μL/min) to maintain the isovolumetric spontaneous bladder contraction and the bladder pressure was recorded (Urolab, Lifetech, USA). Bladder inter-contraction intervals were measured before stimulation, during PAG stimulation, during PAG+STN stimulation, and after cessation of stimulation.

### 2.5. PAG stimulation, STN-DBS, and extracellular mPFC recordings

PAG stimulation, STN-DBS and extracellular mPFC recordings were performed in normal (n = 9) and PD (n = 7) rats under urethane anesthesia (0.7 g/kg, intraperitoneally). The anesthesia depth was monitored by toe pinch response absence. About one-third of the initial dose of urethane was injected if a response to toe pinch was present. The experiments were performed 30–40 days after 6-OHDA injection in PD rats.

A concentric platinum/iridium bipolar stimulation electrode (outer diameter, 125 μm; Pt/Ir; FHC, USA) was stereotaxically inserted into the left PAG and left STN. We stimulated the ventrolateral portion of PAG. The stereotaxic coordinates in relation to the bregma were as follows: PAG: lateral 0.8 mm; anteroposterior, –7.8 mm; dorsoventral, –6.8 mm; STN: anteroposterior, –3.8 mm; lateral, 2.4 mm; and dorsoventral, –8.1 mm. The stimulation parameters were as follows: frequency, 130 Hz; intensity, 200 μA; pulse width, 80 μs; and stimulation time, 30 min. Electrical biphasic rectangular stimulation was applied using an STG-4004 stimulator (Multi Channel Systems, Germany).

Extracellular LFP recordings of the mPFC were performed before stimulation, during PAG stimulation, during PAG+STN stimulation, and after cessation of stimulation for 30 min using the same Pt/Ir electrode (outer diameter, 125 μm; tip impedance, 9–12 MOhm). The stereotaxic coordinates in relation to the bregma were as follows: anteroposterior, +2.2 mm; lateral, 0.8 mm; and dorsoventral, –4.0 mm. Extracellular recordings were performed between each pole of the concentric bipolar electrode, and extracellular signals were recorded (band-pass filtered, 0.3 Hz to 10 kHz) and amplified (×10,000) through a high-performance extracellular amplifier (Dagan 2400 A; Dagan, USA). An electrical lesion (cathodal stimulation at 400μA for 20 min) was created in the STN PAG, and mPFC at the end of each experiment. We used the Rat Brain in Stereotaxic Coordinates to determine the location of electrode insertion (Paxinos and Watson, 2008).

### 2.6. Power spectrum analysis

Lab Chart software (AD Instrument, Australia) was used to analyze the power spectrum of the mPFC. Fast Fourier transforms were performed to analyze the mPFC LFP in a frequency domain range of 0.3–50 Hz. The PSDs were estimated with 131072 Fast Fourier transform size, Hann window, and a 50 % overlap, and normalized by log10 (power spectral densities).

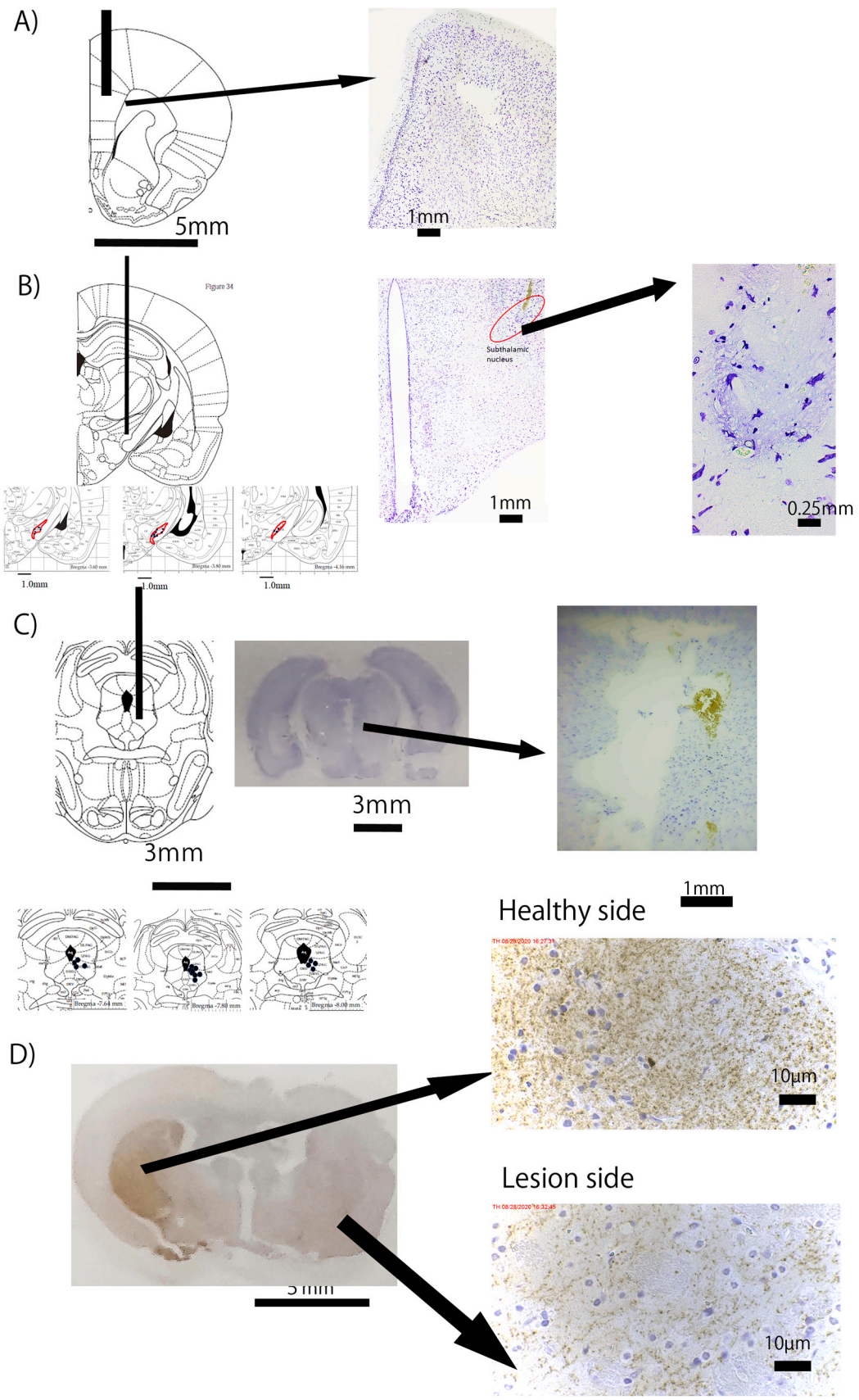



**Fig. 1.** Photographs of cresyl violet-stained coronal rat brain sections of the (A) medial prefrontal cortex (mPFC), (B) subthalamic nucleus (STN), (C) periaqueductal gray (PAG), and (D) a tyrosine hydroxylase-immunostained coronal rat brain section of the striatum. The location of the electrode in the (A) mPFC, (B) STN, and (C) PAG is presented. The number of dopaminergic fibers (brown stained) was significantly decreased in the lesion side of the (D) striatum.

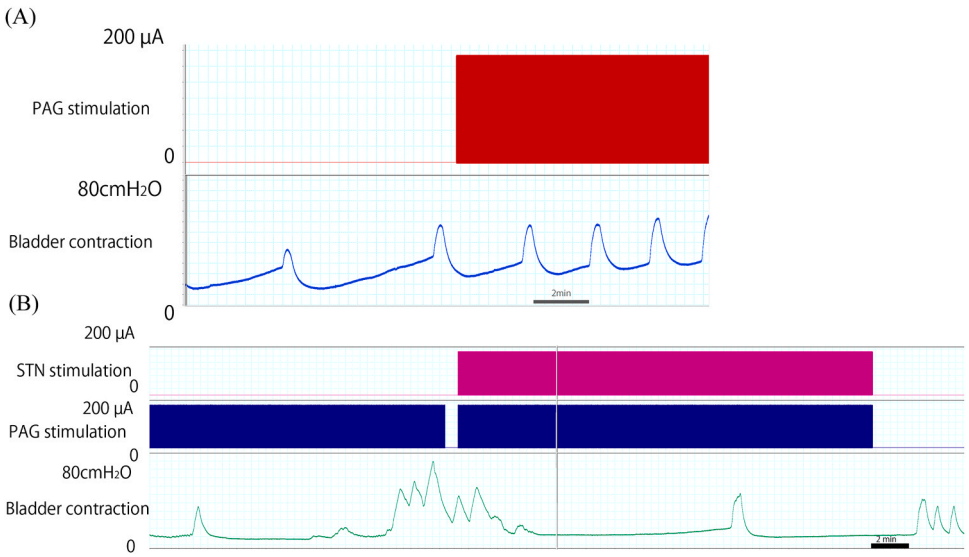



**Fig. 2.** Effect of PAG and PAG+STN stimulations on the bladder inter-contraction interval. (A) The upper and lower traces represent the PAG stimulation and bladder pressure, respectively. PAG stimulation decreased the bladder inter-contraction intervals in PD rats. (B) The upper, middle, and lower traces represent the STN stimulation, PAG stimulation, and bladder pressure, respectively. Adding STN stimulation to PAG stimulation increased the bladder inter-contraction intervals in PD rats.

### *2.7. In vivo microdialysis and high-performance liquid chromatography*

Extracellular fluid was collected from the mPFC before stimulation, during PAG stimulation, during PAG+STN stimulation, and after cessation of stimulation in normal (n = 9) and PD (n = 7) rats. A concentric I-type dialysis probe (diameter, 0.22 mm; exposed membrane, 2.0 mm; A-I-12–02; Eicom Inc., Kyoto, Japan) was inserted stereotaxically into the mPFC, ipsilateral to the STN stimulation site. The stereotaxic coordinates in relation to the bregma were as follows: anteroposterior, + 2.2 mm; lateral 0.8 mm; and dorsoventral –4.0 mm. The perfusion rate was maintained at 2 μL/min using modified Ringer's solution ($Na^+$, 147 mM; $K^+$, 4 mM; $Ca^{2+}$, 2.3 mM; and $Cl^-$, 155.6 mM). Dialysates were collected 1 h after implantation of the dialysis probe and at 10-min intervals for 2.0 h, and stored at –80 °C. The collection was performed before stimulation, during PAG stimulation, during PAG+STN stimulation, and after cessation of stimulation. The average levels of the catecholamines in the dialysates collected during the first 10, 20, and 30 min before stimulation were defined as the basal levels, and the levels at the following points were evaluated as the ratios to the basal levels conformed to our previous study (12). The high-performance liquid chromatography system used to determine the catecholamine levels was equipped with an electrochemical detector system (HTEC500; Eicom), and the mobile phase used was 0.1 M citric acid–0.1 M sodium acetate (pH 3.9) containing 140 mg/L sodium 1-octane sulfonate, 5 mg/L EDTA-2Na, and 15 % methanol at a flow rate of 0.23 mL/min. The samples were manually injected into an analytical column (EICOMPAK SC-5ODS; 2.1 μm × 150 mm; Eicom). The catecholamine levels was electrochemically detected using a graphite electrode (WE-3 G; Eicom) at 700 mV relative to a silver/silver chloride reference electrode.

The experimental timeline of this study is depicted in Fig. 6.

### *2.8. Histopathological examination*

All animals were sacrificed by intraperitoneal injection of 200 mg/kg sodium pentobarbital. The brain tissues were fixed in a 10 % neutral formalin solution, embedded in paraffin, and cut into 10-μm sections using conventional techniques. Cresyl violet staining was used to confirm the location of the electrode in the PAG and STN and of the probe in the mPFC.

### *2.9. Immunohistochemistry for tyrosine hydroxylase*

Using a primary anti-tyrosine hydroxylase antibody developed in rabbits (T8700; Sigma–Aldrich), immunohistochemical staining of the brain sections was performed according to the avidin–biotin complex method. Endogenous peroxidase activity was blocked by 0.5 % hydrogen peroxide ($H_2O_2$) for 15 min after deparaffinization with xylene and gradual dehydration. The tissue sections were then incubated with 10 % normal goat serum (G9023; Sigma–Aldrich) in phosphate-buffered saline with the diluted primary antibody (1:1000) overnight at 4 °C. They were then washed in phosphate-buffered saline containing 0.05 % Tween-20 (PBST), incubated overnight at 4 °C with a biotinylated anti-rabbit immunoglobulin G antibody raised in goats (BA-1000; Vector Labs; 1:1000) as the secondary antibody, and again washed in PBST. The sections were washed in PBST and then visualized by reaction with 3,3-diaminobenzidine tetrahydrochloride (Sigma–Aldrich) and 0.03 % $H_2O_2$ in Tris-buffered saline for 10 min after incubation with the Vectastain ABC Reagents (PK-6100; Vector Labs; 1:1000) for 2 h. Tyrosine hydroxylase positive nerve fibers were calculated by Image J 1.51 software (National Institutes of Health, USA).

### *2.10. Statistical analysis*

All data were expressed as mean ± standard error of mean. The SPSS version 22.0 software (IBM, Armonk, NY, USA) was used for the statistical analysis. Student's *t*-test was used to compare the number of TH-positive nerve fibers between the lesion and intact sides of the brains of PD rat.

To analyze the effect of PAG and PAG+STN stimulations on the catecholamine levels, we performed a two-way repeated measures ANOVA (animal: normal/PD rat, stimulation sites: PAG/PAG+STN). We aimed to examine the effect of stimulation on the levels of catecholamines. We used the Dunnett's test to compare the mPFC catecholamine levels during PAG stimulation, during PAG+STN stimulation, and after cessation of stimulation with those before stimulation (basal levels). *P*-values of $<0.05$ were considered statistically significant. To analyze the effect of PAG stimulation, PAG+STN stimulation on the mean logarithmic power in delta (0.5–3 Hz), theta (4–7 Hz), alpha (8–13 Hz), and beta (14–30 Hz) frequencies, two-way ANOVA was carried out (animal: normal/PD rat, stimulation sites: PAG/PAG+STN). Dunnett's test was

**Table 1**
Bladder inter-contraction interval. Results presented in seconds (s) and as mean ± standard error of mean.

| | Prestimulation (30 min) | PAG stimulation (30 min) | PAG+STN stimulation (30 min) | Post-stimulation (30 min) | *p*-value (pre vs. PAG stim) | *p*-value (PAG stim vs. PAG+STN stim) |
|---|---|---|---|---|---|---|
| PD rat (n = 7) | 336.20 ± 30.35 | 219.55 ± 23.62 | 334.45 ± 33.28 | 369.16 ± 41.59 | $p = 0.0002$ | $p = 0.03$ |
| Normal rat (n = 9) | 346.02 ± 23.87 | 320.59 ± 24.01 | 317.47 ± 23.27 | 281.28 ± 21.41 | $p = 0.46$ | $p = 0.92$ |
| p - value ( PD rat vs normal rat ) | 0.80 | 0.003 | 0.68 | 0.01 | | |

**Table 2**
The mean logarithmic power in mPFC with respect to the stimulation phase in normal and PD rats during bladder relaxation phase.

| | | Animal type: normal or PD rat | stimulation phase (* p < 0.05:vs pre-stimulation, †p < 0.05 vs PAG stimulation) | | | | F value | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | pre stim | PAG stim | PAG+STN stim | post stim | Animal type: normal or PD rat | stimulation phase | interaction (normal/PD vs stimulation phase) |
| bladder relaxation phase | delta frequency | normal | 10.06 | 9.66 * | 9.65 | 9.62 | 349.44(p < 0.01) | 14.12 (p < 0.01) | 6.17 (p < 0.01) |
| | | PD | 9.02 | 8.56 * | 8.93† | 9.00 | | | |
| | theta frequency | normal | 9.64 | 9.43 * | 9.27 | 9.38 | 1576.56 (p < 0.01) | 19.97 (p < 0.01) | 21.68 (p < 0.01) |
| | | PD | 8.58 | 8.28 * | 8.6† | 8.55 | | | |
| | alpha frequency | normal | 9.10 | 8.87 * | 8.7† | 8.82 | 2522.4 (p < 0.01) | 24.13 (p < 0.01) | 38.61 (p < 0.01) |
| | | PD | 7.91 | 7.68 * | 7.99† | 7.96 | | | |
| | beta frequency | normal | 8.41 | 8.03 * | 8.00 | 7.96 | 4039.53 (p < 0.01) | 32.33 (p < 0.01) | 52.32 (p < 0.01) |
| | | PD | 6.81 | 6.64 * | 6.91† | 6.99 | | | |

used to compare the mean logarithmic power in mPFC during PAG stimulation, PAG+STN stimulation, and after cessation of stimulation with those before stimulation (basal levels).

To analyze the effect of PAG stimulation, PAG+STN stimulation on the bladder inter-contraction intervals, a two-way ANOVA was carried out (animal: normal/PD rat, stimulation sites: PAG/PAG+STN). Dunnett's test was performed to compare the bladder inter-contraction intervals during PAG stimulation, PAG+STN stimulation, and after cessation of stimulation with those before stimulation (basal levels).

All methods in this study conform to our previous study (Yamamoto et al., 2020).

## 3. Results

### 3.1. Histological confirmation of the STN electrode and 6-OHDA lesion to the substantia nigra

Histological staining with cresyl violet was used to determine the locations of the mPFC microdialysis probe (Fig. 1A), STN electrode (Fig. 1B), and ventrolateral portion of PAG (Fig. 1C). Photographs of the tyrosine hydroxylase-immunostained coronal rat brain sections showed an apparent reduction in the number of dopaminergic fibers in the left striatum (Fig. 1D). The total number of TH-positive fibers in the lesioned side was 69.25 ± 17.8, whereas in the unaffected side was 387.5 ± 41.37 ($p < 0.01$).

### 3.2. Effects of PAG stimulation and STN-DBS on the bladder inter-contraction intervals

The typical bladder contraction responses induced by PAG and PAG+STN stimulation are represented in Fig. 2A and B, respectively.

#### 3.2.1. Normal rats

The bladder inter-contraction intervals changed after PAG stimulation from 346.02 ± 23.87 s (pre-stimulation) to 320.59 ± 24.01 s (during PAG stimulation) ($p = 0.46$; Table 1). Stimulating both with STN-DBS and PAG changed the bladder inter-contraction intervals from 320.59 ± 24.01–317.47 ± 23.27 s ($p = 0.92$; Table 1), and cessation of stimulation changed the bladder inter-contraction intervals from 317.47 ± 23.27–281.28 ± 21.41 s ($p = 0.25$). The bladder inter-contraction intervals showed no significant changes during PAG and PAG+STN stimulation in normal rats.

#### 3.2.2. PD rats

PAG stimulation significantly decreased the bladder inter-contraction intervals from 336.20 ± 30.35 s (pre-stimulation) to 219.55 ± 23.62 s (during PAG stimulation) ($p = 0.002$; Table 1). Stimulating both with STN-DBS and PAG significantly increased the bladder inter-contraction intervals from 219.55 ± 23.62–334.45 ± 33.28 s ($p = 0.02$; Table 1) and cessation of stimulation changed the bladder inter-contraction intervals from 334.45 ± 33.28–369.16 ± 41.59 s ($p = 0.50$).

#### 3.2.3. Comparisons of bladder inter-contraction intervals between normal and PD rats

The bladder inter-contraction intervals did not differ between the normal and PD model rats during pre- and PAG+STN stimulations. The bladder inter-contraction intervals were significantly reduced during PAG stimulation in the PD model rats compared to the normal rats (p = 0.003).

### 3.3. Effects of STN-DBS on the LFP power spectrum in the mPFC in relation to the bladder contraction/relaxation phase"

A two-way analysis of variance (ANOVA) showed a significant effect during the bladder relaxation phase (Table 2) and contraction phase (Table 3) depending on animal type (normal or PD rats), stimulation phase (pre stimulation, PAG stimulation, PAG+STN stimulation and post stimulation), and animal type and stimulation phase interaction on the mean logarithmic power of delta, theta, alpha, and beta frequency.

**Table 3**
The mean logarithmic power in mPFC with respect to the stimulation phase in normal and PD rats during bladder contraction phase.

| | | Animal type: normal or PD rat | stimulation phase (* $p < 0.05$:vs pre-stimulation, †$p < 0.05$ vs PAG stimulation) | | | | F value | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | pre stim | PAG stim | PAG+STN stim | post stim | Animal type: normal or PD rat | stimulation phase | interaction (normal/PD vs stimulation phase) |
| bladder contraction phase | delta frequency | normal | 10.06 | 10.3 * | 9.96† | 10.3 | 500.28 ($p < 0.01$) | 13.39 ($p < 0.01$) | 11.20 ($p < 0.01$) |
| | | PD | 9.4 | 9.09 * | 9.34† | 8.92 | | | |
| | theta frequency | normal | 10.21 | 9.9 * | 9.7† | 9.94 | 2075.40 ($p < 0.01$) | 26.92 ($p < 0.01$) | 34.06 ($p < 0.01$) |
| | | PD | 8.89 | 8.68 * | 8.98† | 8.56 | | | |
| | alpha frequency | normal | 9.69 | 9.32 * | 9.08 | 9.34 | 2739.83 ($p < 0.01$) | 47.36 ($p < 0.01$) | 44.39 ($p < 0.01$) |
| | | PD | 8.36 | 8.04 * | 8.37† | 8.01 | | | |
| | beta frequency | normal | 8.83 | 8.39 * | 8.24† | 8.46 | 4309.94 ($p < 0.01$) | 39.19 ($p < 0.01$) | 38.51 ($p < 0.01$) |
| | | PD | 7.12 | 7.00 | 7.18† | 7.03 | | | |

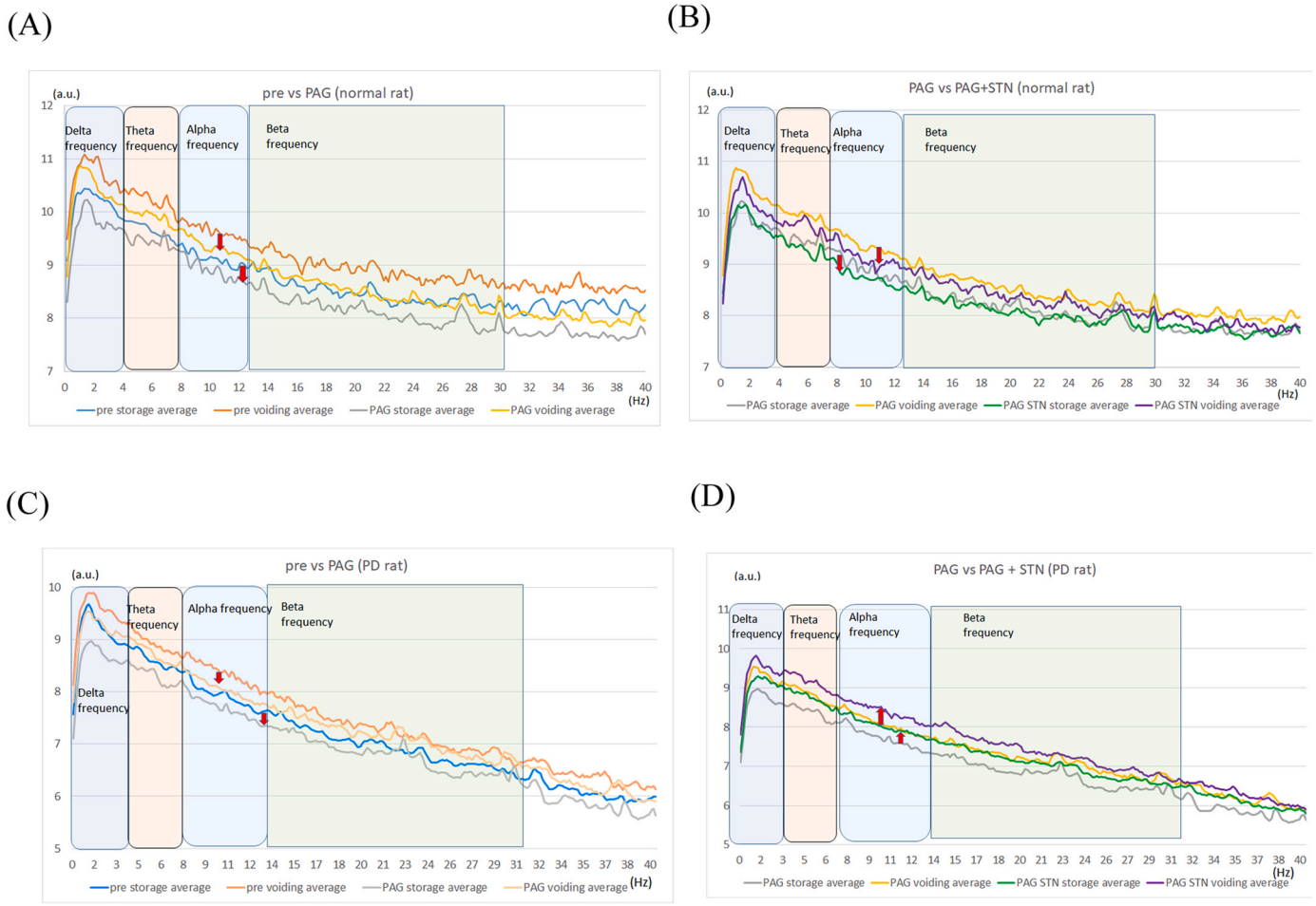



**Fig. 3.** Effects of PAG and PAG+STN stimulations on the LFP power spectrum of the mPFC in normal and PD rats. (A) In normal rats, the power spectrum analysis revealed that PAG stimulation significantly decreased the mean logarithmic power in the mPFC delta (0–4 Hz), theta (4–8 Hz), and beta frequencies during the bladder relaxation phase. PAG stimulation significantly decreased the mean logarithmic power in the mPFC delta, theta, alpha, and beta frequencies during the bladder contraction phase. (B) In normal rats, adding STN-DBS to PAG stimulation significantly decreased the mean logarithmic power in the mPFC delta, theta, alpha, and beta frequencies during the bladder relaxation phase. Adding STN-DBS to PAG stimulation significantly decreased the mean logarithmic power in the mPFC delta, theta, alpha, and beta frequencies during the bladder contraction phase. (C) In PD rats, the power spectrum analysis revealed that PAG stimulation significantly decreased the mean logarithmic power in the mPFC delta, theta, alpha, and beta frequencies during the bladder relaxation and contraction phases. (D) In PD rats, adding STN-DBS to PAG stimulation significantly increased the mean logarithmic power in the mPFC delta, theta, alpha, and beta frequencies during the bladder relaxation and contraction phases.

**Table 4**
Basal catecholamine levels. Results are presented as mean ± standard error of mean.

| (pg/μL) | PD rat (n = 7) | Normal rat (n = 9) | *P*-value (PD vs. SD) |
|---|---|---|---|
| LDOPA | 3.61 ± 1.18 | 5.23 ± 1.89 | $p = 0.48$ |
| MHPG | 52.38 ± 17.65 | 44.33 ± 16.4 | $p = 0.73$ |
| NE | 18.19 ± 4.05 | 23.13 ± 15.31 | $p = 0.76$ |
| DOPAC | 3.19 ± 1.34 | 4.00 ± 1.15 | $p = 0.65$ |
| DA | 3.47 ± 1.55 | 1.28 ± 0.46 | $p = 0.21$ |
| 5HIAA | 1.47 ± 0.79 | 1.57 ± 0.58 | $p = 0.92$ |
| HVA | 15.61 ± 4.65 | 12.52 ± 5.23 | $p = 0.66$ |
| 3MT | 10.09 ± 2.05 | 19.13 ± 15.03 | $p = 0.58$ |
| 5HT | 1.80 ± 0.89 | 0.80 ± 0.79 | $p = 0.32$ |

#### 3.3.1. Normal rats

PAG stimulation significantly decreased the mean logarithmic power in the mPFC delta frequency (0–4 Hz), theta frequency (4–8 Hz), and beta frequency (Fig. 3A, Table 2) during the bladder relaxation phase, as shown by power spectrum analysis. Adding STN-DBS to the PAG stimulation significantly decreased the mean logarithmic power of the mPFC delta, theta, alpha, and beta frequencies (Fig. 3B, Table 2).

PAG stimulation significantly decreased the mean logarithmic power during the bladder contraction phase in the mPFC delta, theta, alpha, and beta frequencies (Fig. 3A, Table 3). Adding STN-DBS to PAG stimulation significantly decreased the mean logarithmic power of the mPFC delta, theta, alpha, and beta frequencies (Fig. 3B, Table 3).

#### 3.3.2. PD rats

The power spectrum analysis revealed that PAG stimulation significantly decreased the mean logarithmic power of the mPFC delta, theta, alpha, and beta frequencies (Fig. 3C, Table 2) during the bladder relaxation phase. Adding STN-DBS to PAG stimulation significantly increased the mean logarithmic power in the mPFC delta, theta, alpha, and beta frequencies (Fig. 3D, Table 2).

PAG stimulation significantly decreased the mean logarithmic power during the bladder contraction phase in the mPFC delta, theta, alpha, and beta frequencies (Fig. 3C, Table 3). Adding STN-DBS to PAG stimulation significantly increased the mean logarithmic power in the mPFC delta, theta, alpha, and beta frequencies (Fig. 3D, Table 3).

### 3.4. Comparison of the mean logarithmic power of the delta, theta, alpha, and beta frequencies between the bladder contraction and relaxation phases

We compared the mean logarithmic power of the delta, theta, alpha, and beta frequencies between the bladder contraction and relaxation phase to determine whether the activity of mPFC is related to bladder contraction/relaxation cycle.

The mean logarithmic power of the delta, theta, alpha, and beta frequencies was significantly larger in the mPFC during the bladder contraction phase than that in the bladder relaxation phase in normal and PD rats.

### 3.5. Effects of PAG and PAG+STN stimulations on the levels of catecholamines in the mPFC

The basal levels (before subthalamic stimulation) of catecholamines in the mPFC are represented in Table 4. There was no difference in basal levels of catecholamines in the mPFC between the normal and PD rats. The ratios to the basal levels of catecholamines in mPFC with respect to the stimulation phase in normal and PD rats are shown in Table 4.

A Dunnet' s test revealed that PAG stimulation significantly decreased NE (norepinephrine) and HVA (homovanillic acid) levels compared to the baseline in normal rats. Stimulating both STN and PAG significantly decreased the levels of MHPG (3-methoxy-4-hydroxyphenylglycol), DA (dopamine), HVA, 3-MT (3-Mmethoxytyramine), and 5-HT compared with the baseline. The levels of L-DOPA (L-3,4-dihydroxyphenylalanine), MHPG, DA, HVA, 3-MT, and 5-HT (5-hydroxytryptamine) were significantly decreased after cessation of stimulation compared with the baseline (Fig. 4A, Table 5). For PD rats, PAG stimulation significantly decreased the levels of DA, 5-HIAA (5-hydroxyindole acetic acid) and 5-HT, significantly decreased those of L-DOPA, DA, 3-MT, and 5-HT during PAG+STN stimulation, and significantly decreased the levels of MHPG, HVA, DA, 3-MT, and 5-HT levels after cessation of stimulation compared with the baseline (Fig. 4B, Table 5).

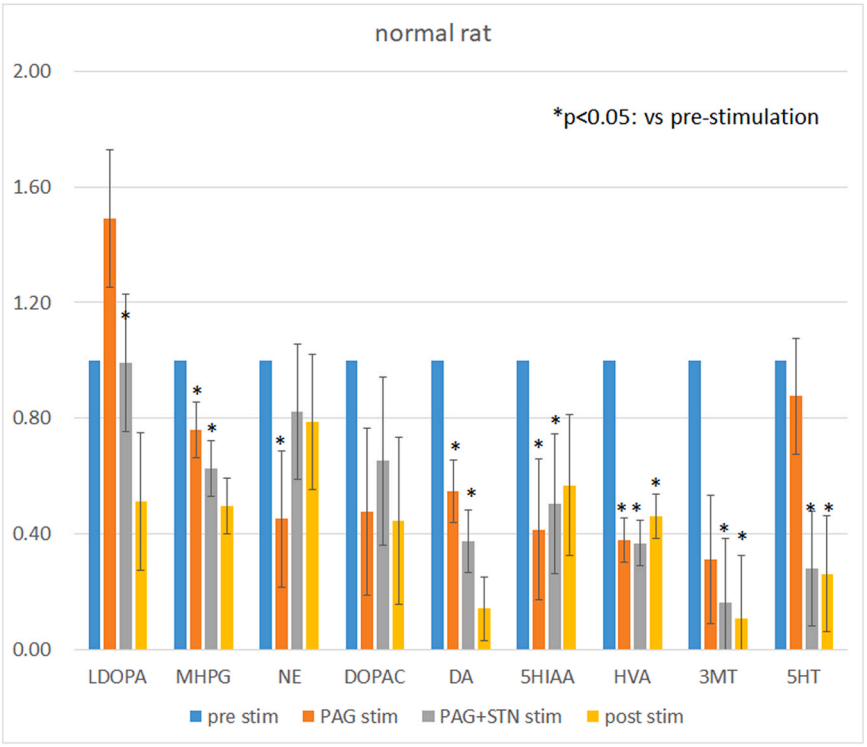
## 4. Discussion

In this study, we examined how STN-DBS modulates the neuronal activity (LFP and catecholamine levels) of the mPFC induced by PAG stimulation (increased urinary afferent projections) and its effects on the bladder inter-contraction intervals in normal and PD model rats.

The present study revealed that PAG stimulation significantly decreased the bladder inter-contraction intervals, and that adding STN stimulation to PAG stimulation significantly increased the bladder inter-contraction intervals, which was not significantly changed after cessation of PAG+STN stimulation in PD rats. On the contrary, PAG and PAG+STN stimulations did not seem to affect the bladder inter-contraction intervals in normal rats. The ventrolateral portion of PAG (vlPAG) was stimulated because the vlPAG receives afferent urinary information and relays them to the higher micturition center including the mPFC via the insula (Zare et al., 2019). This suggests that stimulation of the vlPAG might represent increased urinary afferent signals (Zare et al., 2019). Activation of the vlPAG also leads to detrusor contraction through activation of glutamatergic neurons in the vlPAG (Zare et al., 2019).

Regarding the power of LFP in the mPFC, PAG stimulation significantly decreased the mean logarithmic power in the mPFC delta, theta, alpha, and beta frequencies during the bladder relaxation and contraction phases. Adding STN stimulation significantly increased (reversed) the mean logarithmic power in the mPFC delta, theta, alpha, and beta frequencies during the relaxation and contraction phases in PD rats. The power of LFP in the mPFC did not change after cessation of PAG+STN stimulation during the relaxation phase. Cessation of PAG+STN stimulation during the contraction phase in PD rats significantly decreased the power of LFP in the mPFC. This suggests that the decreased LFP power in the mPFC induced by vlPAG stimulation was related to the decreased bladder inter-contraction interval, and adding STN stimulation to PAG stimulation increased the LFP power in the mPFC with a concomitant increase in the bladder inter-contraction intervals in PD rats. This result agrees with our previous findings showing that STN stimulation significantly increased the LFP power in the mPFC during the relaxation and contraction phases with a concomitant increase in the bladder inter-contraction intervals in PD rats (Yamamoto et al., 2020). This result suggested that increased afferent urinary information induced by vlPAG stimulation led to decreased bladder inter-contraction intervals with decreased LFP power in the mPFC was normalized by adding STN-DBS which resulted in increased inter-contraction interval with increased LFP power in mPFC in PD rat. The LFP power in the mPFC was significantly decreased by both PAG and PAG+STN stimulations, but significantly increased (reversed) by cessation of PAG+STN stimulation during the relaxation and contraction phases in normal rats. This result is also in agreement with our previous results showing that STN stimulation significantly decreased the LFP power in the mPFC during the relaxation and contraction phases in normal rats (Yamamoto et al., 2020). The mean logarithmic power of the LFP in the mPFC during the bladder contraction phase was significantly larger than that in the bladder relaxation phase in normal and PD rats when comparing the mean logarithmic power of LFP in the mPFC between the bladder contraction and relaxation phases. This suggests that mPFC is responsible for regulating the bladder contraction/relaxation cycle by changing the mPFC

(A)




(B)

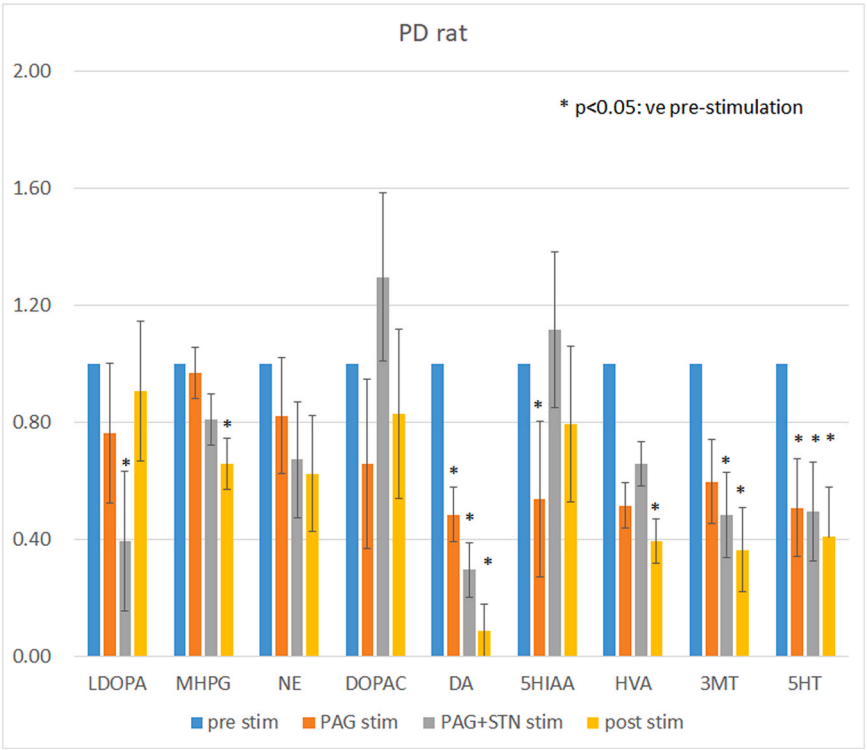



**Fig. 4.** Effects of PAG stimulation and PAG+STN stimulation on the levels of catecholamines in the mPFC. (A) In normal rats, PAG stimulation significantly decreased norepinephrine (NE) and homovanilic acid (HVA) levels compared to the baseline. Adding STN stimulation to PAG stimulation significantly decreased the levels of dopamine (DA), HVA, 3-MT, and 5-hydroxytryptamine (5-HT) compared to the baseline. The levels of L-3,4-dihydroxyphenylalanine (L-DOPA), MHPG, DA, 3-MT, and 5-HT were significantly decreased after cessation of stimulation compared to the baseline. (B) For PD rats, PAG stimulation significantly decreased the levels of DA and 5-HT in the PFC, the L-DOPA, DA, 3-MT, and 5-HT levels were significantly decreased during PAG+STN stimulation, and the HVA, DA, 3-MT, and 5-HT levels were significantly decreased after cessation of stimulation compared to the baseline.

**Table 5**
The ratios to the basal levels of catecholamines in mPFC with respect to the stimulation phase in normal and PD rats.

| | Animal type: normal or PD rat | stimulation phase (* $p < 0.05$:vs pre-stimulation) | | | | F value | | |
|---|---|---|---|---|---|---|---|---|
| | | pre stim | PAG stim | PAG+STN stim | post stim | Animal type: normal or PD rat | stimulation phase | interaction (normal/PD vs stimulation phase) |
| LDOPA | normal | 1.00 | 1.49 | 0.99 | 0.51 * | 1.28 ($p = 0.26$) | 1.51($p = 0.21$) | 2.30 ($p = 0.08$) |
| | PD | 1.00 | 0.76 | 0.39 * | 0.91 | | | |
| MHPG | normal | 1.00 | 0.76 | 0.63 * | 0.50 * | 3.00 ($p = 0.087$) | 5.06 ($p = 0.003$) | 0.22 ($p = 0.88$) |
| | PD | 1.00 | 0.97 | 0.81 | 0.66 * | | | |
| NE | normal | 1.00 | 0.45 * | 0.82 | 0.79 | 0.01 ($p = 0.94$) | 0.48 ($p = 0.70$) | 0.66 ($p = 0.58$) |
| | PD | 1.00 | 0.82 | 0.67 | 0.62 | | | |
| DOPAC | normal | 1.00 | 0.48 | 0.65 | 0.45 | 1.45 ($p = 0.23$) | 0.94 ($p = 0.42$) | 0.311 ($p = 0.81$) |
| | PD | 1.00 | 0.66 | 1.30 | 0.83 | | | |
| DA | normal | 1.00 | 0.55 | 0.38 * | 0.14 * | 0.36 ($p = 0.55$) | 15.84 ($p < 0.01$) | 0.031 ($p = 0.99$) |
| | PD | 1.00 | 0.48 * | 0.29 * | 0.09 * | | | |
| 5HIAA | normal | 1.00 | 0.42 * | 0.51 * | 0.57 | 1.32 ($p = 0.25$) | 1.04 ($p = 0.37$) | 0.44 ($p = 0.72$) |
| | PD | 1.00 | 0.54 * | 1.12 | 0.79 | | | |
| HVA | normal | 1.00 | 0.38 * | 0.37 * | 0.46 * | 1.79 ($p = 0.18$) | 10.2 ($p < 0.01$) | 1.94 ($p = 0.12$) |
| | PD | 1.00 | 0.52 | 0.66 | 0.39 * | | | |
| 3MT | normal | 1.00 | 0.31 | 0.16 * | 0.11 * | 2.06 ($p = 0.15$) | 3.86 ($p = 0.01$) | 0.168 ($p = 0.98$) |
| | PD | 1.00 | 0.60 | 0.48 * | 0.36 * | | | |
| 5HT | normal | 1.00 | 0.88 | 0.28 * | 0.26 * | 0.00 ($p = 0.98$) | 3.39 ($p = 0.02$) | 0.99 ($p = 0.39$) |
| | PD | 1.00 | 0.51 * | 0.49 * | 0.41 * | | | |

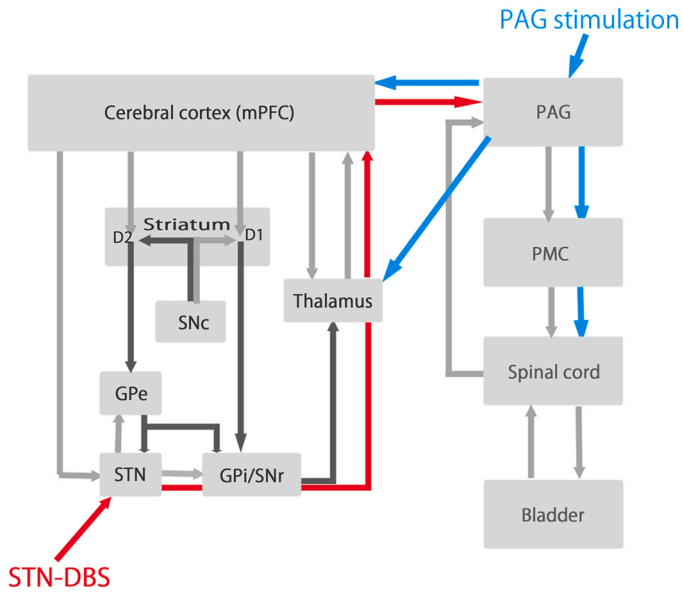



**Fig. 5.** The relationships between the circuit of the basal ganglia and the circuit of micturition in terms of the effect of STN-DBS on the activated micturition circuit induced by PAG stimulation. The figure represents the circuit of the basal ganglia, and the right side represents the circuit of micturition. PAG stimulation activates the micturition circuit via activation of PMC, which directly innervates the sacral parasympathetic nucleus, leading to bladder contraction. Because urinary sensory information is ultimately conveyed to the mPFC via PAG, PAG stimulation might represent increased urinary sensation, which might affect the thalamus and mPFC. STN-DBS influences the activity of the output signals of the basal ganglia (GPi/SNr) and sends signals (red line) to the mPFC via the thalamus. STN-DBS might normalize the abnormal neuronal activities (decreased alpha power) in the mPFC induced by PAG stimulation. MPFC: medial prefrontal cortex, SNc: substantia nigra pars compacta, SNr: substantia nigra pars reticulata, STN: subthalamic nucleus, GPe: globus pallidum externa, GPi: globus pallidum interna, PAG: periaqueductal gray, PMC: pontine micturition center.

LFP power, which conforms to our previous findings (Yamamoto et al., 2020).

It has been reported that noxious stimuli significantly reduced LFP power in rats' mPFC, although the relationship between PAG stimulation and the neuronal oscillatory activity in the mPFC are not well understood (Li et al., 2016). A recent review also reported that pain was associated with neuronal oscillations and synchrony at different frequencies in the brain, including the prefrontal cortex (Ploner et al., 2017). Both pain and urinary sensation are processed and modulated by the PAG, thalamus, insular cortex, and mPFC (Griffiths, 2015; Ong et al., 2019). It is plausible that PAG stimulation decreases the LFP power in the mPFC in the same way as noxious stimuli decrease LFP power (Li et al., 2016) because PAG stimulation might partially mimic increased urinary sensation. We must discuss the effect of PAG stimulation on bladder contraction in comparison to our previous study (Liu et al., 2004). vlPAG stimulation did not significantly change the bladder inter-contraction intervals in normal rats, whereas the vlPAG stimulation significantly decreased bladder inter-contraction intervals in PD rat. Our previous study revealed that high-frequency electrical stimulation of PAG inhibits bladder contraction in normal cats (Liu et al., 2004). The neurophysiological and neurochemical characteristics (Kitta et al., 2008) of PAG might be different between normal model and PD model rats, although we do not know this discrepant data. The reactivity of bladder contraction to electrical stimulation might also be different between normal rats and cats.

We also examined the effect of PAG and STN stimulation on the levels of catecholamines in the mPFC. In PD rats, PAG stimulation significantly decreased the HVA levels, PAG+STN stimulation significantly decreased the L-DOPA and DA levels, and cessation of stimulation significantly decreased the HVA, DA, and 5-HT levels compared with the baseline. PAG stimulation significantly decreased the NE and HVA levels in normal rats. Adding STN stimulation to PAG stimulation significantly decreased the DA, HVA, and 5-HT levels. The levels of L-DOPA, NE, DA, and 5-HT were significantly decreased after cessation of stimulation compared with the baseline. It may be difficult to discuss the relationship between the levels of catecholamines in the mPFC and bladder contraction with respect to PAG and PAG+STN stimulations. Both normal and PD rats had significantly decreased levels of DA and 5-HT after PAG+STN stimulation. This agrees with our previous study in which only STN stimulation significantly decreased DA and 5-HT levels in the mPFC in normal and PD rats. It has been reported that 5-HT in the mPFC may play an important role in regulating bladder contraction. Because there are many 5-HT receptor subtypes in the central nervous system, the effects of 5-HT on bladder contractions are very complex and

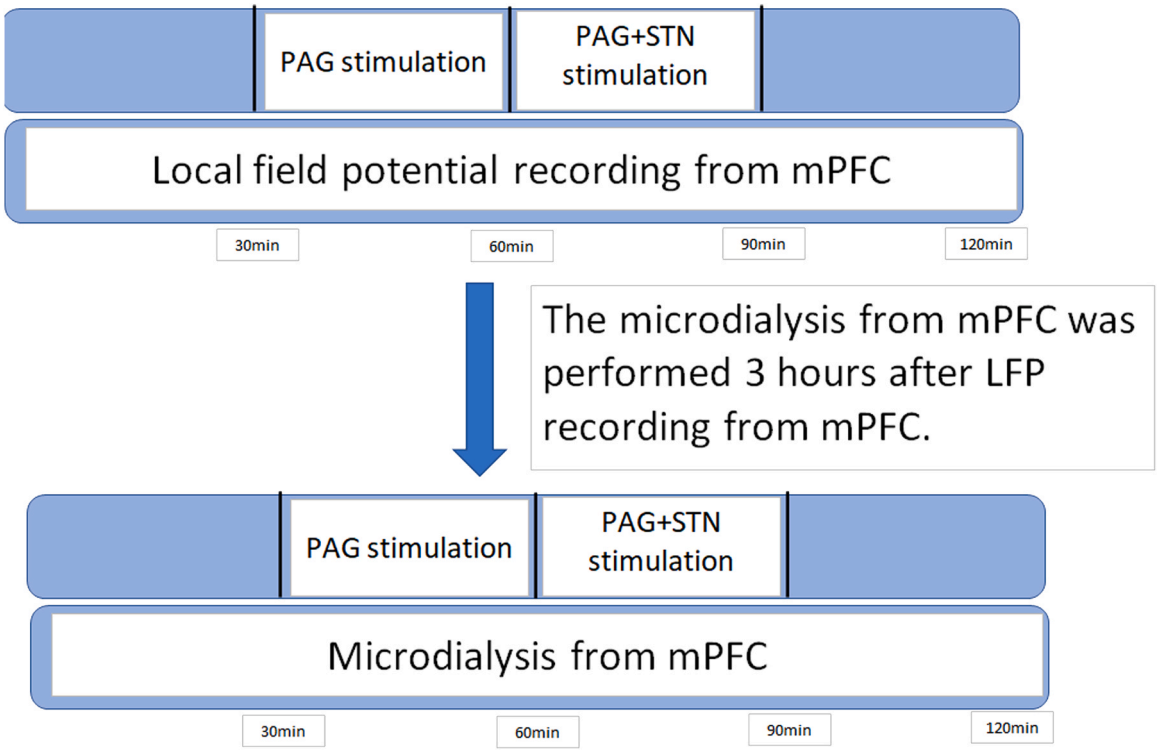



**Fig. 6.** The experimental timeline. This figure represents the experimental timeline of this manuscript. The microdialysis from mPFC was performed 3 h after LFP recording from mPFC.

depend on the 5-HT receptor subtype.

Chiba et al revealed that 5-HT2A receptors had an excitatory effect on micturition, whereas that of 5-HT7 receptors was inhibitory (Chiba et al., 2020). In this study, PAG+STN stimulation decreased 5-HT levels and increased bladder contraction interval in PD rats. Although it is difficult to discuss the mechanism by which 5-HT receptors are involved in the present results, it is possible that decreased 5-HT levels reduce activation of 5-HT2A receptors, leading to an inhibitory effect on bladder contraction.

The levels of DOPAC (3,4-dihydroxyphenylacetic acid) and 5-HIAA, which are metabolites of DA and 5-HT, respectively, did not change significantly following PAG and PAG+STN stimulations in normal and PD rats. The changes in the levels of catecholamines induced by PAG and PAG+STN stimulations were different between normal and PD rats. These differences might be attributable to the changes in the activity of the mPFC caused by dopamine depletion induced by 6-OHDA injection, which should be further examined in future studies.

There are several limitations in this study. The bladder inter-contraction intervals in PD rats did not decrease compared to those in normal rats during pre-stimulation, although PD patients usually show urinary frequency, which is equivalent to reduced bladder inter-contraction intervals. However, the bladder inter-contraction intervals during PAG stimulation were significantly reduced in the PD model rats compared with normal rats, reflecting a reduced threshold to electrical stimulation, which is evident in the 6-OHDA model (Buhidma et al., 2020; Domenici et al., 2019). Although we do not know why the present PD model did not show reduced bladder inter-contraction intervals during pre-stimulation, one plausible reason might be that the 6-OHDA rat model does not accurately represent the pathology of PD in humans. The present results might indicate the effect of the depletion of dopaminergic neurons on the bladder inter-contraction intervals and responsiveness of mPFC activity induced by PAG and STN stimulation because 6-OHDA is a selective neurotoxin for dopaminergic neurons. Even though the 6-OHDA model cannot accurately represent PD pathology, 6-OHDA is a widely used neurotoxin for constructing PD model rats. Appropriate PD model rats representing both PD pathology seen in humans and severe dopaminergic degenerations are currently not available.

Another limitation of this study is that PAG stimulation did not significantly change bladder inter-contraction intervals in normal rats, while PD model rats showed significantly decreased bladder inter-contraction intervals by PAG stimulation. The threshold to electrical stimulation to induce bladder contraction might be higher in normal rats than in PD model rats, although we do not know the exact reason why PAG stimulation did not induce a significant difference in the bladder inter-contraction intervals in normal rats. This is because 6-OHDA PD models usually show reduced threshold to electrical stimulation (Buhidma et al., 2020; Domenici et al., 2019). A previous study also revealed that unilateral 6-OHDA lesions in the substantia nigra changed GABAergic labeling in PAG, which might result in modulation of the nociceptive threshold (Domenici et al., 2019). We applied the same stimulation parameters (such as stimulation intensities, frequency, pulse width) to normal and PD model rats. Different stimulation parameters, such as stronger stimulation intensities or higher frequency stimulation, might change bladder contraction in normal rats, which should be examined in the future work.

Overall, our results indicate the relationships between bladder contraction and the neuronal activities in the mPFC (catecholamine levels and LFP) induced by PAG and STN stimulation. The present results suggested that STN-DBS might have partially contributed to the normalization of increased afferent urinary signals (induced by PAG stimulation) in the mPFC by increasing the power in the LFP and modulating catecholamine levels in the mPFC (Fig. 5). We have previously reported that STN-DBS increased the bladder inter-contraction intervals by increasing the alpha power in the mPFC during the relaxation and contraction phases in PD rats, which agrees with the results of the present study, suggesting that an increased LFP power in the mPFC might be key for the improvement of urinary frequency in PD patients (Yamamoto et al., 2020). The bladder inter-contraction intervals were unchanged by PAG and PAG+STN stimulations despite the significant

change in the LFP power and catecholamine levels in the mPFC induced by PAG and PAG+STN stimulations in normal rats. Therefore, it may be difficult to discuss the relationships between bladder contraction and the neuronal activities (LFPs and catecholamine levels) of the mPFC in normal rats. The responses of mPFC activity and the bladder inter-contraction intervals induced by PAG and PAG+STN stimulations were different between normal and PD rats, suggesting that dopamine depletion might alter the activity of the mPFC and PAG network.

## 5. Conclusion

PAG stimulation significantly decreased the bladder inter-contraction intervals with a concomitant decrease in the NE and DA levels, and LFP power in the mPFC. Adding STN stimulation to PAG stimulation significantly increased the bladder inter-contraction intervals by increasing the LFP power in the mPFC in PD model rats.

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## CRediT authorship contribution statement

Tatsuya Yamamoto: Conceptualization, Methodology, Writing – Original draft preparation. Ryuji Sakakibara: Conceptualization, Supervision. Tomoyuki Uchiyama: Conceptualization, Supervision. Satoshi Kuwabara: Supervision.

## Conflicts of interest

We have no conflict of interest.

## Data Availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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