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Behavioral tests predicting striatal dopamine level in a rat hemi-Parkinson's disease model

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ABSTRACT

Parkinson's disease (PD) is a frequent neurodegenerative disease causing bradykinesia, tremor, muscle rigidity and postural instability. Although its main pathology is progressive dopaminergic (DArgic) neuron loss in the substantia nigra, motor deficits are thought not to become apparent until most DArgic neurons are lost, probably due to compensatory mechanisms that overcome the decline of DA level in the striatum. Even in animal PD models, it is difficult to detect motor deficits when most DArgic neurons are functional. In this study, we performed various behavioral tests (apomorphine-induced rotation, cylinder, forepaw adjustment steps (FAS), beam walking, rota-rod, and open-field), using 6-hydroxydopamine (OHDA) and lipopolysaccharide (LPS)-induced hemi-PD model rats with various striatal DA levels, to find the best way to predict the DA level from earlier disease stages. Different from the 6-OHDA-induced model, reduction in the striatal DA levels in the LPS-model was less significant. Among the behavioral tests, data from cylinder and FAS tests, which evaluate forelimb movements, best correlated with decline of the DA level. They also correlated well with decreased body weight gain. The beam and apomorphine tests showed less significant correlation than the cylinder and FAS tests. Open-field and rota-rod tests were not useful. Expressional levels of mRNA encoding tyrosine hydroxylase (TH), a marker of DArgic neurons, correlated well with the DA level. Metabotropic glutamate receptor 4 mRNA expression correlated with the striatal DA level and may be related to compensatory mechanisms. These results suggest that motor impairments of PD should be evaluated by forelimb movements, or hands and forearms in clinical settings, rather than movement of the body or large joints. The combination of cylinder and FAS tests may be the best to evaluate the rat PD models, in which many DArgic neurons survive.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that frequently strikes aged people (Kalia and Lang, 2015; Twelves et al., 2003). Its main pathology is the progressive loss of dopaminergic (DArgic) neurons in the substantia nigra pars compacta (SNc). The DArgic neurons extend their axons to the striatum and secrete DA that binds to D1 and D2 receptors expressed by medium spiny neurons (MSNs) in the striatum (Kravitz et al., 2010). DA activates gamma-aminobutyric acid positive (GABArgic) MSNs expressing D1 receptors that constitute the direct pathway, while inhibiting basal ganglia outputs that are the substantia nigra pars reticulata (SNr) and globus pallidus pars interna (GPi). However, DA inhibits MSNs with D2 receptor expression that belong to the indirect pathway, leading to activation of the GP pars externa (GPe) followed by inhibition of subthalamic nuclei and the basal ganglia outputs. Thus, SNc-derived DA increases motor activity through the inhibition of basal ganglia outputs, which are inhibitory for the thalamus and the brainstem centers for muscle tone and locomotion (Takakusaki et al., 2003).

The main treatment for PD, administration of levodopa or DA agonists (Clarke, 2007; Kalia and Lang, 2015; Miyasaki et al., 2002), is just symptomatic therapy that increases DArgic actions in the striatum. These medicines strongly ameliorate the motor symptoms, which are bradykinesia, rigidity, resting tremor and postural instability. However, they do not slow or cease the progression of neurodegeneration in the SNc of PD patients. The critical goal of PD treatment is the development of therapeutic interventions to stop the neurodegenerative processes. Yet, when such an epoch-making intervention is successfully developed, it will inevitably be necessary to find ways to diagnose PD at the earliest opportunity, when substantial numbers of DArgic neurons are still

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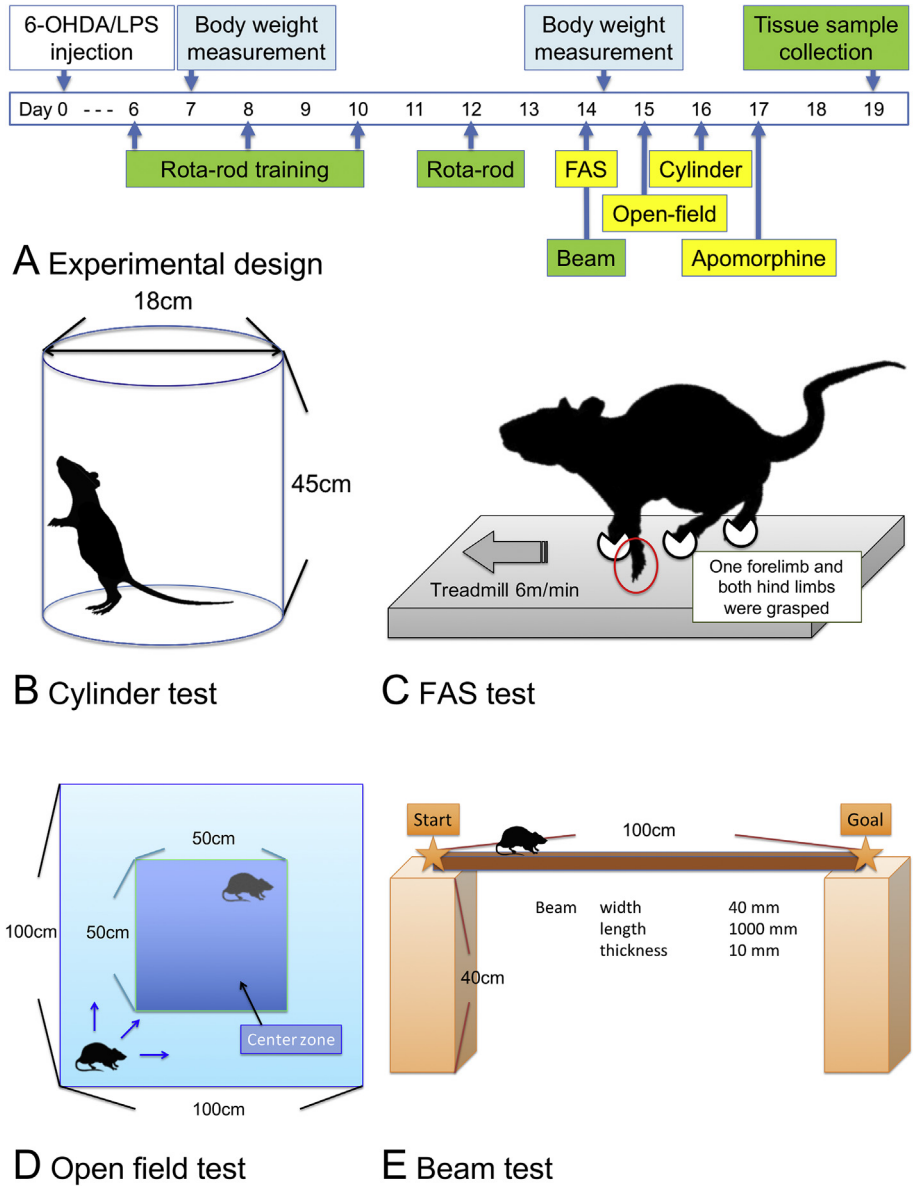

Fig. 1. Experimental design and diagrams for behavioral tests.
A) Experimental design of this study. Experimental items with green backgrounds were done only for 6-OHDA model. The beam test was done using a different 6-OHDA-treated rat group.
B–E) Diagrams for behavioral tests.

viable. However, it has long been thought that motor deficits cannot be observed until depletion of striatal dopamine and DArgic neuronal loss in the SNc reaches 70%–80% and 50%–60%, respectively, in not only human cases but also animal PD models (Choudhury et al., 2011b; Perez et al., 2008; Zigmond et al., 1990). This may be due to the presence of compensatory mechanisms that overcome the decrease of DA levels in the striatum (Aono et al., 2017; Bezard et al., 2003; Javor-Duray et al., 2017).

A DA receptor agonist, apomorphine, causes contralateral turning by stimulating both D1 and D2 receptors with increasing sensitivity to DA on the denervated side in PD model rats prepared by unilateral injection of 6-hydroxydopamine (6-OHDA) into the striatum or medial forebrain bundle (MFB) (Aono et al., 2017; Creese et al., 1977; Iancu et al., 2005). Although the apomorphine-induced rotation test has been employed as a standard method to evaluate the motor deficits of the 6-OHDA-induced hemi-PD model (Creese et al., 1977; Marin et al., 2006), the test can detect motor deficits only after the nearly complete disappearance of DArgic neurons, as described in this study and by others (Iancu et al., 2005; Metz et al., 2005; Schwarting and Huston, 1996; Warraich et al., 2009). Therefore, the apomorphine test may not be suitable for the evaluation of mild or early stage PD models. Many other behavioral tests have been employed to evaluate the severity of PD model animals, yet there is no conclusive literature that recommends the best behavioral test batteries to determine the severity. The aim of this study, therefore, was to find the most sensitive behavioral test battery to detect PD symptoms at the early stage using the 6-OHDA- and lipopolysaccharide (LPS)-induced hemi-PD model. Because we think that the severity of PD should be evaluated by striatal DA levels (Ando et al., 2018; Choudhury et al., 2011a, 2011b; Yuan et al., 2005), we tried to correlate the behavioral parameters with the decline of the striatal DA level, which was measured by high performance liquid chromatography (HPLC). For this purpose, we took advantage of inevitable variations in striatal DA decline in the 6-OHDA- and LPS-induced hemi-PD model rats.

Table 1
PCR primers.

Primers	Sense/anti-sense
GAPDH	GAGACAGCCGCATCTTCTTG TGACTGTGCCGTTGAACTTG
GFAP	CAGAAGCTCCAAGATGAAACCAA TCTCCTCCTCCAGCGACTCAA
Iba1	GTCCTTGAAGCGAATGCTGG CATTCTCAAGATGGCAGATC
NG2	TTACCTTGGCCTTGTTGGTC GATGATCTGTTTGGCCTGCT
mGluR4	AGGATAAGCCACACGGACAC GGGAGCGAAAGAAGACTGTG
TH	TGTGTCCGAGAGCTTCAATG GGGCTGTCCAGTACGTCAAT

2. Materials and methods

2.1. Animals

Male Wistar rats (n = 65; 7–8 weeks old; body weight 250–270 g) were maintained under standard laboratory conditions. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Ehime University Graduate School of Medicine that follows the National Institutes of Health Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf).

2.2. 6-OHDA-induced hemi-PD rat model

The 6-OHDA-induced hemi-PD rat model was prepared as described elsewhere (Conti et al., 2014). The 6-OHDA (Sigma-Aldrich, St. Louis, MO) was dissolved in saline containing ascorbic acid (Wako, Osaka, Japan) (10 mg/mL dissolved in 1% ascorbate-saline) and injected into the right MFB. Under deep anesthesia with isoflurane, the rat head was secured in a stereotaxic apparatus (Narishige, Tokyo, Japan) and 4 μl of 6-OHDA (0, 5, 10 and 20 μg; n = 34) was infused using a Hamilton syringe with a 26 gauge needle into the brain parenchyma in the vicinity of the right MFB. The location of the injection was at anteroposterior (AP) −4.5 mm, mediolateral (ML) +1.5 mm, and dorsoventral (DV) −8.0 mm. The injection speed was 1 μL/min. The needle was left after the injection for another 4 min and then slowly withdrawn. The skin of the head was sutured with surgical needle-equipped sutures (Alfresa Pharma Corporation, Osaka, Japan).

2.3. LPS-induced hemi-PD rat model

The LPS-induced hemi-PD rat model was prepared as described elsewhere (Kim et al., 2000). The LPS (strain O111:B4; Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate buffered saline (5 μg/μl in 2 μl) and infused into the right SNc using Hamilton as described above. Under deep anesthesia with isoflurane, the rat head was secured in a stereotaxic apparatus (Narishige, Tokyo, Japan) and 2 μl of LPS (10 μg; n = 23) was infused using a Hamilton syringe with a 26 gauge needle into the brain parenchyma in the vicinity of the right SNc. The location of the injection was at anteroposterior (AP) −4.8 mm, mediolateral (ML) +1.7 mm, and dorsoventral (DV) −8.2 mm. The injection speed was 1 μL/min. The needle was left after the injection for another 2 min and then slowly withdrawn. The skin of the head was sutured as described in the above.

2.4. Experimental design

Experimental design is summarized in Fig. 1. The 6-OHDA was injected on day 0 (Fig. 1). For the rota-rod test, the rats were trained three times on days 6, 7 and 8 and the rota-rod test was performed on day 12. Body weights were measured on days 7 and 14. Forepaw adjustment steps (FAS) and beam tests were done on day 14. Open-field, cylinder, and apomorphine tests was done on day 15, 16, and 17, respectively. On day 19, the ventral midbrain and striatal tissues were collected for biochemical analyses. Among the behavioral tests, the beam test was done using a different rat group from that used for the other tests. Normal male Wistar rats of the same age were similarly subjected to behavioral tests and body weight measurements, with the same schedule as for PD model rats. For the LPS-induced model, only body weight measurements, FAS, open-field, cylinder, and apomorphine tests were done.

2.5. Quantitative real-time RT-PCR (qPCR)

The ventral midbrain tissues containing the SN were dissected out on day 19 for qPCR experiments, as described elsewhere (Choudhury et al., 2011b). The tissues were homogenized in QIAzol Lysis Reagent (QIAGEN, Venlo, The Netherlands) using an ultrasonic cell disruptor and RNA was purified using an RNeasy Mini Kit (QIAGEN). Then, cDNA prepared by a method described previously (Islam et al., 2018) was diluted 1:3. Duplicate qPCR measurements were done using an MJ mini instrument (Bio-Rad, Hercules, CA, USA) with Fast Start Universal SYBR Green (Roche Diagnostic Japan, Tokyo, Japan). The mRNA expression was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level. The primer sequences for each gene are listed in Table 1.

2.6. Determination of DA concentration and DA metabolites in the striatum

The striatum was dissected on day 19 and the levels of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by HPLC (Choudhury et al., 2011b). Aliquots were independently prepared from tissues from both sides of the striatum, homogenized, and injected into a HPLC apparatus with a reversed-phase column (C18 phase, 150-mm length, 2.1-mm internal diameter; SC-5ODS, Eicom, Kyoto, Japan). The mobile phase was 15% (v/v) methanol containing 0.1 mol/L sodium acetate and 0.1 mol/L citric acid adjusted to pH 3.5, 180 mg/L sodium octydyl sulfate, and 10 mmol/L EDTA and pumped at a rate of 0.25 mL/min to determine the concentrations of DA and its metabolites. The results were evaluated by dividing the DA content (μg/mg tissue wet weight) in the right (ipsilateral) striatum by that in the left (contralateral) striatum. DA turnover rates were expressed as the ratio of the right (HVA + DOPAC)/DA value divided by the left turnover in the right striatum.

2.7. Behavioral tests

A diagram with a brief explanation of the cylinder, FAS, open-field and beam tests is shown in Fig. 1.

2.7.1. Rota-rod test

Motor coordination and balance were examined using a rota-rod (Ugo Basile, Rota-rod 7750, Italy) as described elsewhere (Choudhury et al., 2011b). The speed of the rota-rod was accelerated from 4 to 20 rpm in 1 min. The rats were trained for three days before the test (Fig. 1). Two days after the final training, the rota-rod test was performed by placing the rats on a rotating rod and measuring the duration until the animal fell off. The test was repeated three times and the data averaged.

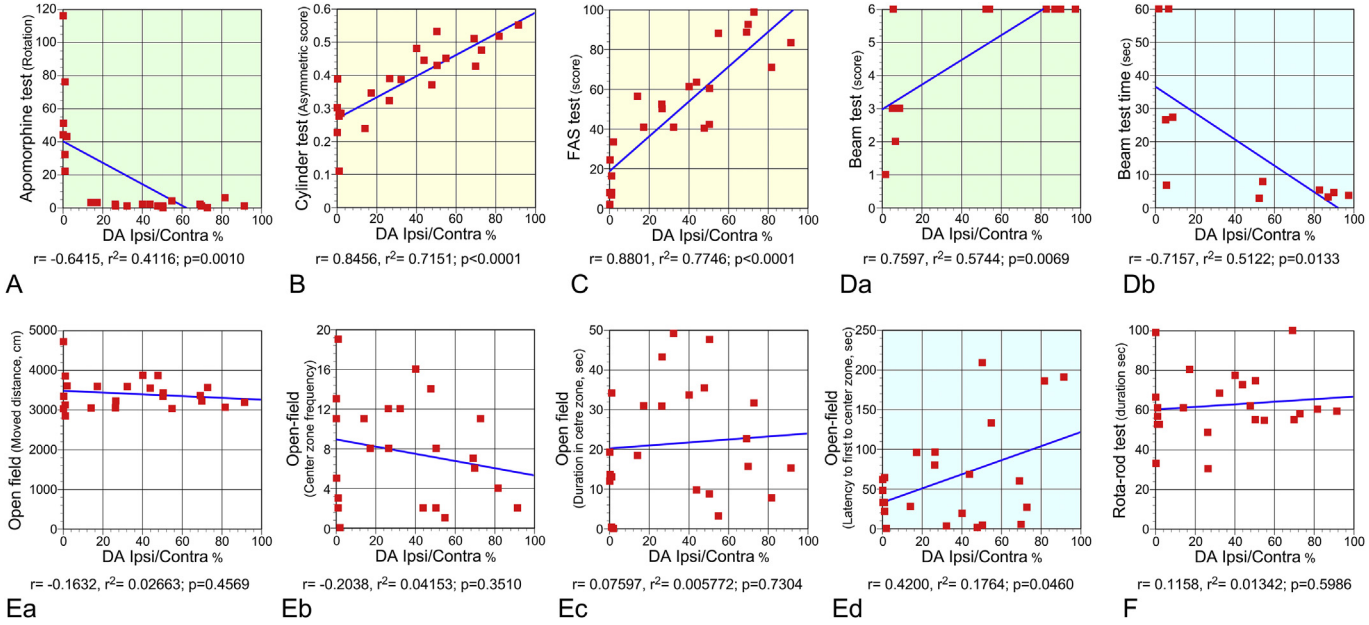

Fig. 2. Correlation between the decline of striatal DA and behavioral test data in 6-OHDA model.
The decline of striatal DA was expressed as the ratio of DA content in the right striatum to that in the left. DA content was measured by HPLC. Yellow, green and blue backgrounds respectively indicate highly ($p < 0.001$), moderately ($p < 0.01$) and weakly ($p < 0.05$) significant correlation between the behavioral test data and the DA ratio. Data were obtained from 23 hemi-PD model rats, except for the beam test. Beam test data were from 11 rats. The correlation between the DA ratios and the apomorphine test data (number of rotations; A), cylinder test (asymmetric score; B), FAS test (score; C), beam test (score; Da, time (sec); Db), open-field test (moved distance (cm); Ea, frequency of entering the center zone; Eb, duration in the center zone (sec); Ec, latency to first entrance into the center zone; Ed) and rota-rod test (duration (sec); F).

2.7.2. Open-field test

The open-field test was conducted at 20:00 and 22:00 h using a square open field (100 × 100 cm) with 50 cm high walls, equipped with a video-tracking system (Ethovision XT 7, Noldus Info. Tech., Wageningen, The Netherlands), as described elsewhere (Aono et al., 2017). The rats were placed in a corner of the open-field and allowed to move freely for 1 min. Then, the movement was video-recorded for 5 min and the experimenter sat at in a place not visible to the test animals. The total distance moved, the latency to the first entrance into and the duration in the center zone (50 × 50 cm) set in the middle of the field were measured. After each trial, fecal boli were removed, and the floor was wiped with a damp cloth and dried.

2.7.3. Forepaw adjustment steps test (FAS)

The FAS test was employed to evaluate the motor deficits in the left forelimb (Chang et al., 1999), because the hemi-PD model was prepared by causing degeneration of DArgic neurons in the right SN. An experimenter held the hindlimbs and one forelimb such that the other free forelimb was forced to support the body weight on a treadmill (Animal treadmill Exer 3/6, Columbus Instruments, Columbus, OH, USA) that moved at 9 cm/s. Rats were then moved laterally across the treadmill. The test was performed three times for 20 s each. The 'FAS score' was obtained by dividing the number of steps by the contralateral forepaw with that by the ipsilateral forepaw. Lower scores indicate a greater motor impairment. Triplicate measurements were performed.

2.7.4. Cylinder test

The cylinder test was employed to evaluate the motor deficits in the left forelimb (Lundblad et al., 2002; Schallert et al., 2000; Takarada-Iemata et al., 2018). The cylinder test assesses voluntary movement during exploratory activity in a transparent glass cylinder. Rats placed in an upright transparent cylinder stand up on their hindlimbs to explore the walls using their forelimbs. As the neurotoxin 6-OHDA was injected into the right MFB, hemi-PD model rats would preferentially use the ipsilateral (right in the present study) forelimb. An experimenter counted the number of wall contacts made by each forelimb respectively and by both forelimbs in 5 min. The cylinder test score was calculated by the formula [(contralateral side + 1/2 both)/(ipsilateral side + contralateral side + both)]. A ratio of 0.5 indicates both forelimbs were used with the same frequency, whereas scores less than 0.5 suggest motor impairment of the contralateral forelimb.

2.7.5. Beam walking test (Beam test)

The well-established beam walking test was utilized to assess motor coordination and balance (Baluchnejadmojarad et al., 2017; Glajch et al., 2012). The rats walked on a wooden beam suspended between a start platform and their home cage at a height of 50 cm (Fig. 1). A cushion was placed under the beam to protect animals that fell off. Rats were pretrained on day 14 to traverse the beam directly into their home cage. The following day (24 h later), the beam test was performed by putting rats on the start platform and measuring the time (up to 60 s) to reach the goal and the number of slips off the beam. The foot slips were evaluated by a 7-category rating system using a scale of 0–6 as follows: 0, the rat was not able to stay on the beam; 1, the rat did not move but was able to stay on the beam; 2, the rat tried to traverse the beam but fell; 3, the rat traversed the beam with multiple slips (4–6 times); 4, the rat traversed the beam with few foot slips (2–3); 5, the rat traversed the beam with only one slip of the hindlimb; 6, the rat traversed the beam without any slips of the hindlimb.

2.7.6. Apomorphine rotation test

Administration of apomorphine induces abnormal contralateral rotations in hemi-PD model rats (Creese et al., 1977; Iancu et al., 2005). Apomorphine (Kyowa Kirin, Tokyo, Japan) was subcutaneously administered to the 6-OHDA-treated rats at a dose of 0.2 mg/kg. Five minutes after the administration, the rats were individually placed into a 40 cm-diameter bowl and the counterclockwise (contralateral) rotations were monitored by video recording for 10 min. An experimenter counted the number of apomorphine-induced rotations on the recorded video.

2.8. Statistical analysis

Data expressed as mean ± standard error of the mean (SEM) or standard deviation (SD) were statistically analyzed using InStat3 software (GraphPad Software, La Jolla, CA). Correlations were performed using linear regression analysis. Data were subjected to two-tailed Student's t tests (unpaired) or a two-way ANOVA with Tukey's post hoc test. Significance was set at $p < 0.05$.

3. Results

3.1. Correlation between the decrease in DA content in the striatum and the behavioral deficits in 6-OHDA-induced PD model

Fig. 2 shows the correlation between behavioral test data and the ipsi/contralateral ratios of DA levels in the striatum of 6-OHDA-induced model. Apomorphine (Fig. 2A), cylinder (Fig. 2B), FAS (Fig. 2C) and beam (Fig. 2D) tests all correlated well with the decrease in DA level in the right striatum. Among these, the cylinder and FAS test data linearly correlated with high correlation coefficients, while apomorphine and beam test data correlated less significantly with the decrease of DA level. Although the apomorphine test has often been regarded as one of the essential behavioral tests to determine whether the 6-OHDA-induced rat PD model was correctly prepared, apparent apomorphine-induced rotation was observed only when DA was almost completely depleted in the striatum. The beam test also showed a similar tendency to the apomorphine test. The decrease in DA level did not affect the total moved distance (Fig. 2Ea) or the moving velocity for 5 min in the open-field test. The frequency of entering (Fig. 2Eb) and the duration of staying (Fig. 2Ec) in the center zone did not change. However, the latency to enter the center zone (Fig. 2Ed) was significantly shortened along with the decrease in DA level in the striatum. The rota-rod test has often been employed to evaluate the motor deficits of PD animal models (Higaki et al., 2016; Iancu et al., 2005; Jalewa et al., 2017), however it did not correlate with the decrease of DA level in the present 6-OHDA-induced model.

DA turnover in the striatum calculated as the (HVA + DOPAC)/DA ratio reflects the activity of the remaining DArgic neurons in the SNc (Perez et al., 2008). Apomorphine test was well positively correlated with the DA turnover rather than cylinder and FAS tests (Fig. 3).

3.2. Correlation between the decline in striatal DA and mRNA expression in the ventral midbrain

Not only DArgic neurons but also glial cells have been implicated in the pathogenesis of PD. Therefore, the right and left ventral midbrain tissues of 6-OHDA-induced model rats were divided, dissected and processed for qPCR, then the correlation between mRNA expression and the right/left ratios of striatal DA levels were investigated (Fig. 4). The mRNA for TH correlated well with the DA level decline (Fig. 4A). Involvement of astrocytes, microglia and NG2 glia (oligodendrocyte progenitor cells) in PD pathogenesis has been reported (Choudhury et al., 2011b; McGeer and McGeer, 2008), but there were no significant correlations between the mRNA expression of their marker genes (glial fibrillary acidic protein; GFAP, Iba1 and NG2) and changes in DA levels (Fig. 4B–D). Activated microglia in the SNr in the rat PD model have been reported to eliminate glutamatergic synapses by phagocytosis, while contributing to compensatory mechanisms that mask the motor symptoms (Aono et al., 2017). In accordance with this notion, expression of mRNA for metabotropic glutamate receptor 4 (mGluR4) was reduced along with the decrease in striatal DA level (Fig. 4E).

3.3. Indices that correlated with the inhibited increase in body weight

Changes in body weight may be a good marker reflecting the physical health of the PD model animals. Indeed, changes in the levels of striatal DA, TH mRNA and mGluR4 mRNA in the ventral midbrain correlated with body weight gain for 7 d (Fig. 5A–C). Apomorphine, cylinder, and FAS test data (Fig. 5D–F) also correlated well with the body weight gain. In the open-field test, the latency to enter the center zone but not the moving velocity (or total moved distance) weakly correlated with the body weight gain. Rota-rod test data were not correlated (Fig. 5H). There was no significant correlation between the body weight gain and the expression level of mRNA for the glial markers (data not shown).

3.4. Correlation between the striatal DA content and the behavioral test data in LPS-induced PD model

The decrease in DA levels in the stratum of LPS-induced hemi-PD model rats was not so marked as that of the 6-OHDA model (Fig. 6). The ipsi-/contralateral DA ratio did not decline to less than 20%, in contrast to 6-OHDA-induced model. As a result, apomorphine caused only one or two rotations even if it did (Fig. 6A). On the other hand, cylinder test data showed good correlation with the decline of DA. FAS test data (Fig. 6C) and the latency to enter the center zone (Fig. 6D) were also significantly correlated with the decrease in DA level in the striatum. Body weight change was not significantly correlated with the decline of DA (Fig. 6E).

3.5. Cylinder and FAS tests detected a moderate decline of the striatal DA level

The severities of the 6-OHDA-induced PD model rats were divided into mild, moderate and severe classes based on the severity of decline in the striatal DA. The ratio (x) of the mild class was $50 \leq x < 100\%$ (mean ± SD; 69.9 ± 14.2), that of the moderate class $10 \leq x < 50\%$ (36.1 ± 9.1) and that of the severe class $0 \leq x < 10\%$ (0.70 ± 0.67)

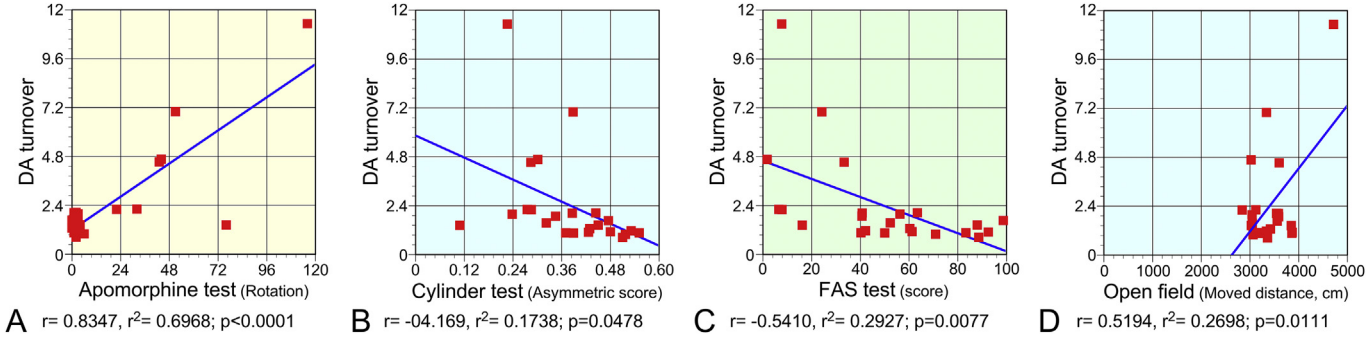

Fig. 3. Correlation between striatal DA turnover rate and behavioral test data in 6-OHDA model.
Striatal DA turnover rates calculated as (HVA + DOPAC)/DA were correlated with the behavioral test data. Yellow, green and blue backgrounds respectively indicate highly ($p < 0.001$), moderately ($p < 0.01$) and weakly ($p < 0.05$) significant correlation. Data were obtained from 23 rats.

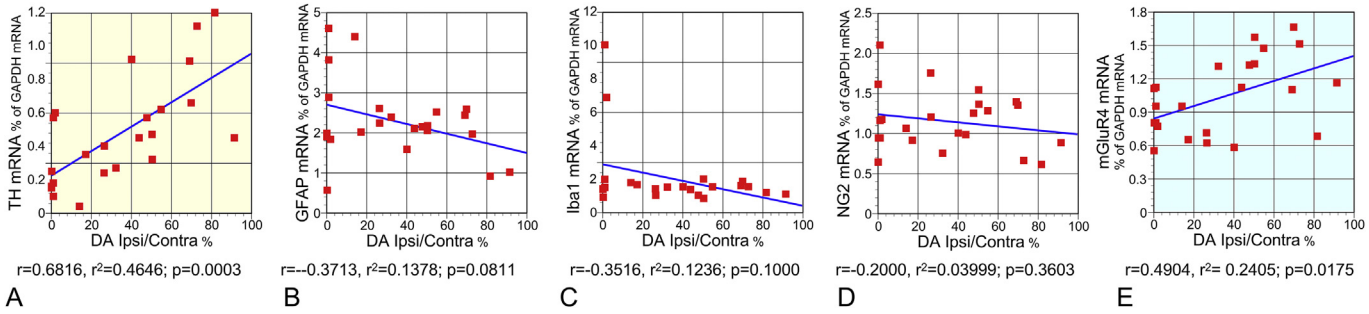

Fig. 4. Correlation between the decline of striatal DA and mRNA expression in the right ventral midbrain in 6-OHDA-induced PD model.
Expression levels of mRNA encoding tyrosine hydroxylase (TH; A), glial fibrillary acidic protein (GFAP; B), Iba1 (C), NG2 (D) and metabotropic glutamate receptor 4 (mGluR4; E) in the right (ipsilateral) ventral midbrain were investigated by qPCR and the data are expressed as percent of GAPDH mRNA levels. Yellow and blue backgrounds respectively indicate highly ($p < 0.001$) and weakly ($p < 0.05$) significant correlation between the data and the DA ratio. Data were obtained from 23 rats.

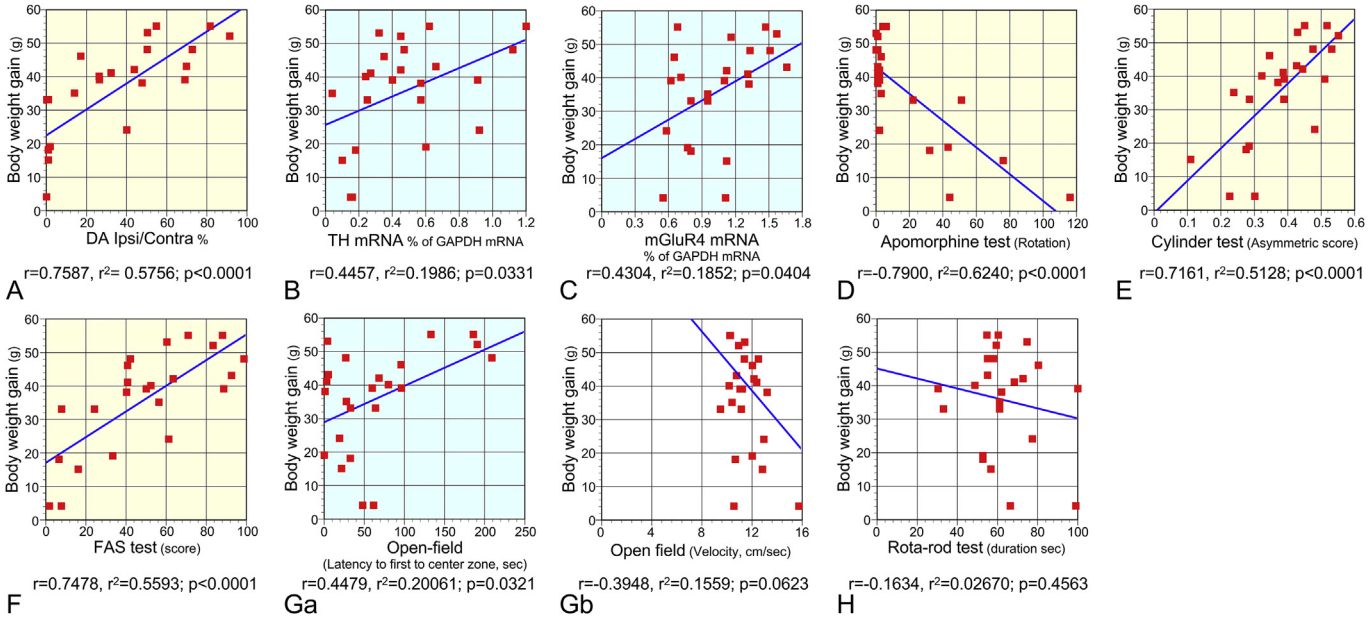

Fig. 5. Correlation between body weight gain and the DA ratio, qPCR and behavioral data.
Correlation between body weight gain for a week after 6-OHDA administration and the DA ratio (A), TH mRNA level (B), mGluR4 mRNA level (C), apomorphine test data (D), cylinder test (E), FAS test (E), open-field test (latency to first reach the center zone; Ga, velocity (sec/cm); Gb) and rota-rod test (duration (sec); H). Yellow and blue backgrounds respectively indicate highly ($p < 0.001$) and weakly ($p < 0.05$) significant correlation between the data and the DA ratio. Data were obtained from 23 rats.

(Fig. 7A). The apomorphine test (Fig. 7B) could detect only severe cases. Cylinder (Fig. 7B) and FAS (Fig. 7D) tests distinguished the three classes, although the tests could not discriminate between normal healthy rats and those with mild disease. Open field (Fig. 7E) and rota-rod (Fig. 7F) tests were useless in the discrimination. Suppressed body weight gain may predict the severe cases (Fig. 7G).

4. Discussion

Many reports have described behavioral test batteries for statistical

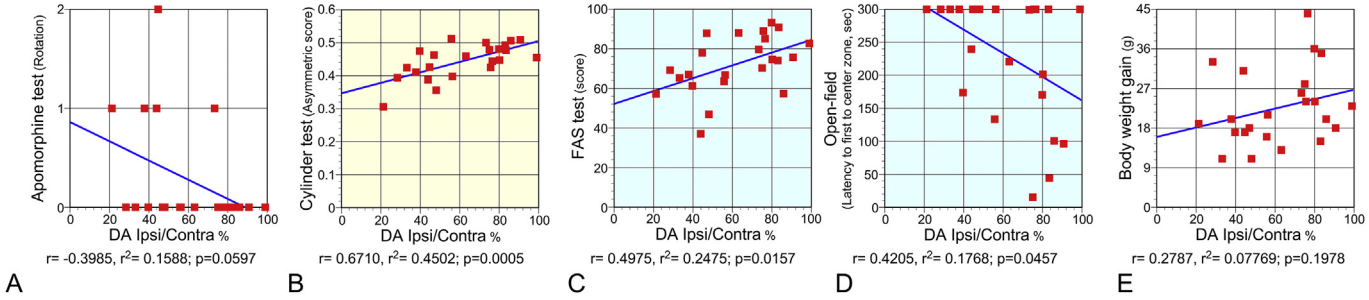

Fig. 6. Correlation of striatal DA with behavioral data and body weight gain in LPS model.
LPS caused only moderate or mild DA decline. Data were obtained from 23 rats. The correlation between the DA ratios and the apomorphine test data (number of rotations; A), cylinder test (asymmetric score; B), FAS test (score; C), open-field test (latency to first entrance into the center zone; D) and body weight gain (gram; E).

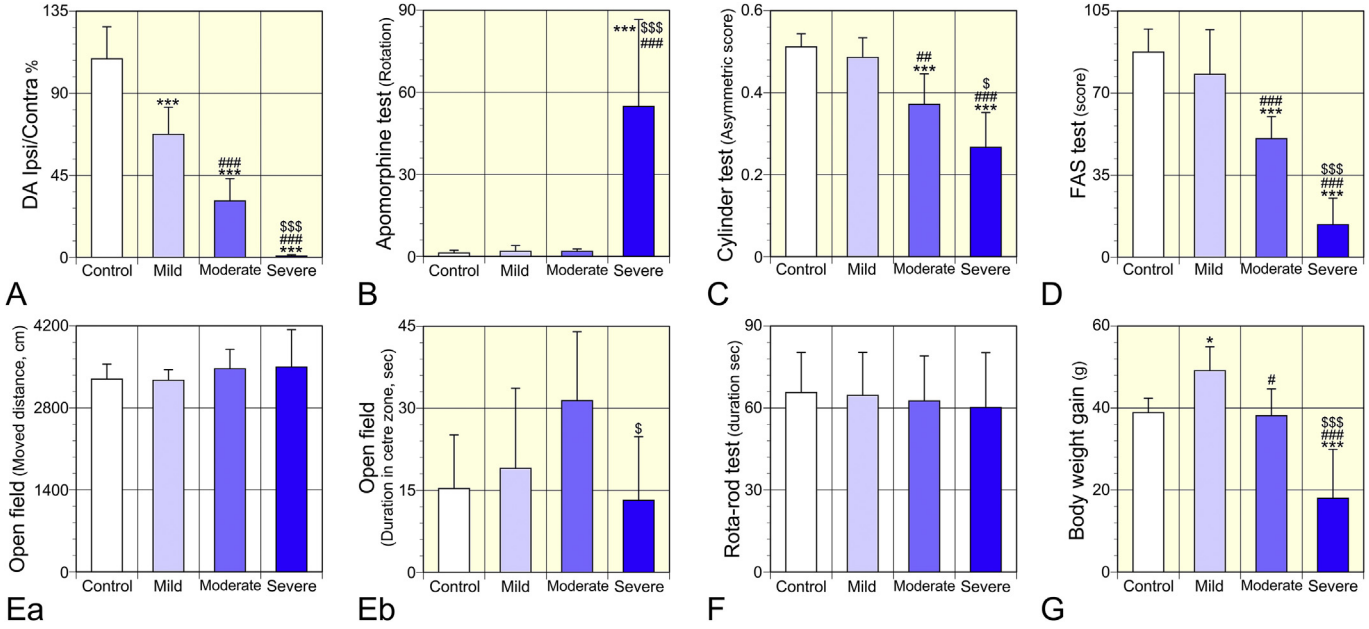

Fig. 7. Relationship between behavioral data and the severity of DA decline in 6-OHDA-induced model.
The 23 6-OHDA-induced PD model rats were divided into three classes (8 mild, 8 moderate and 7 severe rats) based on the severity of the decline of the striatal DA level. The data of the classified PD rats were compared with those of normal rats ($n = 8$) for the DA ratio (A), apomorphine test data (B), cylinder test (C), FAS test (D), open-field test (moved distance (cm); Ea, duration in the center zone (sec); Eb), rota-rod test (sec; F) and body weight gain (G). Data are shown as means ± SD. Statistical analyses were performed with ANOVA and Tukey's post-hoc test; *$p < 0.05$, ***$p < 0.001$ vs Control. ##$p < 0.01$, ###$p < 0.001$ vs mild, $ $p < 0.05$, $$$ $p < 0.001$, vs moderate.

evaluation of motor deficits in hemi-PD rodent models, because the evaluation of the severity of PD symptoms by behavioral tests is in particular critical for development of novel therapeutic interventions, as well as the study of PD pathophysiology (Iancu et al., 2005; Rumpel et al., 2015; Shi et al., 2004; Sleeman et al., 2012). Since the core pathology of PD is the progressive loss of DArgic neurons in the SNc, it would be useful if behavioral tests could predict the degree of neuron loss. Among the behavioral tests, the apomorphine test may be the most frequently employed to confirm that the PD model is correctly prepared: administration of this DA agonist stimulates hypersensitive DA receptors in the striatum of 6-OHDA-induced hemi-PD model animals, causing contralateral body turns (Creese et al., 1977; Marin et al., 2006). However, as shown in this study, apomorphine administration caused rotation of rats only when striatal DA was reduced to less than 10% of the contralateral DA level. In fact, apomorphine did not induce significant rotations in the LPS-induced PD model rats, of which striatal DA did not decline to less than the 20% level. LPS induces microglial activation that causes neuronal death at least partly through the release of nitric oxide (Higaki et al., 2016). Such neurotoxic activation of microglia has been implicated in DArgic neurons loss in animal models and human cases. (Higaki et al., 2016; Kim et al., 2000; McGeer and McGeer, 2008). However, compared to the direct neurotoxicity of 6-OHDA, LPS-induced microglia-mediated neurotoxic effects on DArgic neurons appeared to be less significant, taken the present results. Since the reduction in the striatal DA level correlated well with TH mRNA level in the ventral midbrain, the DA level may well reflect the number of viable neurons in the SNc. Thus, the apomorphine test may be able to diagnose the PD model only at the end stages, and therefore, it may not be suitable for the aim of developing novel interventions to delay or stop the progressive DArgic neuron loss. Because significant correlation between apomorphine-induced rotation numbers with DA turnover in the striatum, apomorphine test might be useful to evaluate the activities of the remaining DArgic neurons in the SNc.

In the present study, we aimed to find the best behavioral test batteries to diagnose PD at the early stage and to estimate the striatal DA level that reflects the number of functional DA neurons in the SNc. We administered three doses of 6-OHDA into the vicinity of MFB to prepare a 6-OHDA-induced hemi PD model with various striatal DA levels. Based on the level of striatal DA, the model rats were classified into three severities: mild, moderate and severe. Motor impairments of the model rats with various striatal DA levels were evaluated by various behavioral tests. As a result, the cylinder and FAS tests that evaluate forelimb movement in the hemi-PD model were found to be the most sensitive to detect the decrease in striatal DA level. The tests could distinguish moderate cases (10 ≤ DA levels < 50%, compared with the contralateral levels) from mild ones. So far, apparent motor deficits have been reported not to be recognized until the striatal DA level has declined to about 20% of normal, probably due to the existence of compensatory mechanisms (Bernheimer et al., 1973; Bezard et al., 2003; Perez et al., 2008; Zigmond et al., 1990). However, the present data suggest that both tests are able to detect motor deficits in the PD model with DA levels reduced to 36%, on average, of the contralateral level. The cylinder and FAS tests were found to be useful to evaluate the severity of the motor deficit also for the LPS-induced model, which only caused mild and moderate decrease in DA levels.

Contra/ipsilateral DA levels of the 6-OHDA-induced model rats can be estimated based on the data from cylinder and FAS tests. When cylinder test data are employed, the estimated DA level (y) can be calculated with the following equation: $y = 223 \times$ cylinder test data -50.4. For the FAS test, the equation is $y = 0.882 \times$ FAS test data -8.77. Furthermore, using multiple regression analysis, the decline of the striatal DA level can be more accurately estimated by the following equation: $y = 114 \times$ cylinder test data $+0.798 \times$ FAS Score -44.2. The multiple correlation efficient reaches 0.91. Collectively, the combined employment of cylinder and FAS tests may be useful for accurate estimation of the DA level in the hemi-PD rodent model.

The beam test showed less significance in detection of the weak decline in the striatal DA levels than cylinder and FAS tests in the 6-OHDA model rats. Total moved distance, moving velocity, and moving frequency were not significantly changed even in the severe models in the open-field test. However, latency to first reach the center zone of the open field was weakly correlated with the decrease in DA levels.

Preference for remaining in the center zone may suggest reduced anxiety. This may be correlated with changes in mental states observed in PD patients (Clarke, 2007; Postuma et al., 2012). Although the rota-rod test has often been employed to evaluate the motor deficits of PD model animals (Higaki et al., 2016; Iancu et al., 2005; Jalewa et al., 2017), there was no significant correlation with the decline of striatal DA levels in the present model. Taken together, motor impairment in 6-OHDA- or LPS-induced hemi-PD models should be evaluated by movement of the forelimbs but not by movement of the whole body. Decreased body weight gain may be another good index for decline of striatal DA levels in 6-OHDA-induced model. It correlated well with cylinder and FAS tests. It is likely that eating pellet foods requires movement of the forelimbs, therefore, the body weight changes may reflect the DA level decline similarly to cylinder and FAS tests. In addition, it should be noted that there are some differences in the usefulness of the behavioral tests dependently on the PD models. In our previous study, we prepared the PD model by injecting 6-OHDA into the striatum (Aono et al., 2017; Choudhury et al., 2011b; Higaki et al., 2016). The striatum model showed weak motor deficits, despite that they could be detected by open-field and rota-rod tests.

Glial cells become activated and/or proliferative in and around the SNc in PD models and patients (Choudhury et al., 2011b; Le et al., 2016; McGeer and McGeer, 2008). Astrocytes increase the expression of GFAP (Choudhury et al., 2011b). Although the number of NG2 glia may not change, activated microglia expressing NG2 increase in number (Choudhury et al., 2011b). Activation of microglia is observed not only in the SNc but also in the GP and SNr (Aono et al., 2017). These changes of glial cells have been observed by increased expression of mRNA encoding GFAP, NG2, and Iba1 in the ventral midbrain, which included both the SNc and SNr. In particular, activated microglia have been implicated in degeneration of DArgic neurons in the SNc (Higaki et al., 2016; McGeer and McGeer, 2008). However, there was no significant correlation between the decline in striatal DA level and mRNA expression for GFAP, NG2 or Iba1. This might suggest that the activation of glial cells is not simply a cause or a result of the DArgic neuronal degeneration. However, mGluR4 mRNA expression significantly correlated with the striatal DA decline. Synapses with mGluR4 expression may be eliminated by activated microglia in the SNr through phagocytosis as one of the compensatory mechanisms (Aono et al., 2017), and mGluR4 in the SNr may receive glutamatergic inputs from hyperactive subthalamic nuclei in the PD model. The decreased expression of mGluR4 mRNA along with the decline in striatal DA may reflect strengthened compensation.

The present study raises the possibility of diagnosing human PD patients at earlier opportunities by examining movement of hands and forearms rather than body or legs. Among the major motor symptoms of PD, bradykinesia is normally defined by a slow gait and slow body movement (Massano and Bhatia, 2012; Perlmutter, 2009). Rigidity is often assessed by examining movement of the neck, elbows, shoulders or knees. However, according to the present results, examination of finer movements using hands and forearms, such as tapping a smartphone, hitting letters on a keyboard or writing names on paper might be more effective for diagnosis of earlier stages of PD.

Conflicts of interest

Nothing to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2018.11.005.

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