Gait analysis in three different 6-hydroxydopamine rat models of Parkinson's disease

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HIGHLIGHTS

• The gait readouts in the MFB and SNC 6-OHDA rats are more profound than the CPU.
• Many gait parameters show a close correlation with the protein levels of TH.
• CatWalk system can provide reliable and objective criteria to stratify gait changes arising from 6-OHDA rats.

ABSTRACT

Gait deficits are important clinical symptoms of Parkinson’s disease (PD) but are rarely studied. In this study we made three different rat PD models by administration of 6-hydroxydopamine into caudate putamen (CPU), medial forebrain bundle (MFB) and substantia nigra compact (SNC). We evaluated the gait changes in these models by using a computer-assisted CatWalk system. Correlations of gait parameters with tyrosine hydroxylase protein levels in the CPU and SNC were also investigated. The gait readouts were significantly impaired in both the MFB and SNC groups. However, the MFB group showed a more pronounced impairment than the SNC group. In contrast, only mild and incomplete gait impairment occurred in the CPU group. In addition, some gait parameters demonstrated close correlation with the protein levels of TH. This paper suggests that the 6-hydroxydopamine-induced MFB model is more propitious to study gait dysfunction than the other two models and the CatWalk system can provide reliable and objective criteria to stratify gait changes arising from 6-hydroxydopamine lesioned rats. These findings may hold promise in the study of PD disease progression and new therapeutic methods.

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1. Introduction

Parkinson's disease (PD), a pervasive motor disease, results from the depletion of dopamine (DA) in the nigrostriatal region of the central nervous system [1]. As PD progresses, gait disturbance and postural instability become increasingly prominent [2]. Thus, ameliorating gait disturbance is vital for PD patients to prevent unexpected injury, disability, and improve their quality of life [3].

The 6-hydroxydopamine (6-OHDA) induced rat model of PD has been extensively used as an animal model to mimic PD patients in basic science studies. The injection of 6-OHDA into different sites of the brain, i.e., the caudate putamen (CPU), the medial forebrain bundle (MFB) or the substantia nigra compact (SNC), causes different extent of the lesion in the corresponding brain area. Meanwhile, the lesion process in these three injection models can be quite different, leading to various kinds of performance of gait deficits [4–6]. To quantify these deficits, experimenters use behavioral tests including the open field test, the rotarod test, the treadmill test, the cylinder test, and the ladder walking test [7]. However, the accuracy of readouts can be affected by many factors. For example, timing of testing (day or night), training intention and frequencies, and testing environment are very important factors that affect the behavior performance. Animals behavior tested in an involuntary state are hardly repetitive. Test indicators that are limited to monitor either static or dynamic state cannot adequately display both readouts simultaneously [8], and so on. Therefore, although great efforts have been made to measure the gait deficits in these 6-OHDA PD models [5,9,10], the validation and characterization of gait variability is still lacking [11]. In this report we validated gait variability in three different rat PD models of

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unilateral 6-OHDA lesions using the CatWalk device. As the motor deficits are the direct consequence of DA loss in the nigrostriatal system [12], we evaluated the temporal expressions of tyrosine hydroxylase (TH) protein in the SNC and CPU of 6-OHDA treated rats and assessed the neuro-degeneration in the nigrostriatal regions by studying the correlation of TH expressions with changes of gait parameters.

2. Materials and methods

2.1. Animals

Adult male Sprague Dawley rats, weighing 290–310 g at the time of surgery, were housed 2–3 per cage with ad libitum access to food and water during a 12 h light/dark cycle. All procedures were approved and regulated by the Institutional Animal Ethics Committee of Southern Medical University of China.

2.2. Surgical procedure

The rats were randomly divided into three groups. Each group was comprised of 12 rats. All rats were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg). Rats were placed in a stereotaxic frame (Stoelting) and 6-OHDA was injected into the right brain using a 10 μl Hamilton syringe fitted with a glass capillary. Partial lesion of the nigro-striatal pathway was obtained by injection of 3 μl of 6-OHDA (3.5 μg/μl free base dissolved in a solution of 0.2 mg/ml l-ascorbic acid in 0.9% w/v NaCl) (Sigma) (0.5 μl/min) in the CPU at the following coordinates (flat skull position): antero-posterior: +1.2 mm, medio-lateral: −2.5 mm, dorso-ventral: −5.0 mm below dural surface, calculated relative to bregma according to the stereotaxic atlas of Paxinos and Watson (1986). Severe lesion of the nigro-striatal pathway was obtained by injection of the same dose of 6-OHDA in the MFB at the following coordinates: antero-posterior: −4.4 mm, medio-lateral: −1.1 mm, dorso-ventral: −7.8 mm or above the SNC at the following coordinates: antero-posterior: −5.3 mm, medio-lateral: −1.7 mm, dorso-ventral: −7.2 mm.

2.3. Gait analysis

The CatWalk XT (Noldus information Technology, Wageningen, Netherlands) was used to analyze gait of unforced moving rats. CatWalk XT consists of a hardware system of a long glass walkway plate, illuminated with green light that is reflected within the glass at points being touched, a high-speed video camera, and a software package for quantitative assessment of animal footprints [4,13]. Table 1 shows the definition of the gait parameters used in this study.

All rats were trained to cross the runway in a consistent manner at least six times a day for a week before any experimentation. A successful run is defined as an animal finishes running the tracks without any interruption or hesitation. Rats that failed the CatWalk training were excluded from the study. An average number of 5 replicate crossings made by each rat was recorded. Rats were subjected to computer-assisted CatWalk from day 26 to day 30 after administration of 6-OHDA.

2.4. Tissue processing and immunohistochemistry

All animals were sacrificed 30 min after the last behavior test. After being anesthetized, rats were perfused through the ascending aorta with 250 ml saline (0.9% w/v) at room temperature, followed by 250 ml ice-cold paraformaldehyde (4% w/v in 0.1 M phosphate buffered saline). The brain was removed, post-fixed for 12 h in 4% paraformaldehyde and cryoprotected overnight in sucrose (25% w/v in 0.1 M phosphate buffered saline) before being sectioned on a freezing microtome (Leica). Coronal sections were collected in 6 series at a thickness of 20 μm.

Immunohistochemical staining was performed on sections using antibodies raised against TH (mouse, 1:1000; sigma). Sections were rinsed three times in potassium-phosphate buffer (KPBS) between each incubation period. The sections were quenched for 10 min in 3% H2O2/10% methanol. One hour of pre-incubation with 5% normal goat serum was followed by incubation overnight with the primary antibody in 2% serum at 4 °C and incubation with 1:200 dilution of biotinylated goat anti-mouse antibody, followed with avidin–biotin–peroxidase complex, and visualized using 3,3-diaminobenzidine (DAB) as a chromogen.

2.5. Cell counting and optical densitometry analysis

Assessment of the total number of TH+ neurons in the SNC was made according to the optical fractionator principle, using the Image-Pro Plus (Media Cybernetics, Inc., USA). Every 6th section covering the entire extent of the SNC was included in the counting procedure. A coefficient of error of <0.10 was accepted. CPU TH+ fiber density was measured by densitometry at four coronal levels (+1.2, 0.8, 0.00 and −0.4 mm). The measured values were corrected for non-specific background staining by subtracting values obtained from the cortex. The data are expressed as a percentage of the corresponding area from the intact side.

2.6. Statistical analysis

All the data was expressed as mean ± standard error of mean (SEM). The comparison before and after surgery was performed with a paired-samples t-test. One-way ANOVA analysis followed by Bonferroni post hoc test was used to determine inter-group and intra-group differences. The correlations of TH expression levels in the SNC and CPU with the motor parameters in all tests were evaluated by Pearson’s product-moment correlation coefficient. A p value <0.05 was considered as to be statistically significant. All of the data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, USA).

3. Results

3.1. Cell loss and striatal fiber degeneration in the three models

One rat that failed to meet the CatWalk inclusion criteria and two rats that died were excluded from this study (n = 3). The final
number of animals included in the data analysis were: CPU n = 11; MFB n = 11; SNC n = 11.

Using a stereological approach, we estimated the number of TH+ neurons and compared it to that recorded from the contralateral intact side. Quantifications revealed that a severe loss of nigral neurons after injection of 6-OHDA in the MFB and SNC (7.7 ± 3.5% for the MFB group and 12.7 ± 8.1% for the SNC group; P < 0.05 compared to intact side; Fig. 3D). The lesion was less severe when 6-OHDA was delivered into the CPU (35.3 ± 15% for the CPU group; P < 0.05 compared to the intact side).

Degeneration of striatal DA fibers was assessed by optical densitometry of TH immunostained forebrain sections. Similar to the stereological cell counts, ANOVA analysis revealed a severe loss of terminals when 6-OHDA was injected in the MFB and SNC (7.7 ± 3.5% for the MFB group and 12.7 ± 8.1% for the SNC group; P < 0.05 compared to intact side; Fig. 3E), while CPU 6-OHDA injection induced a moderate loss of CPU fibers (35.4 ± 14.6% for the CPU group; P < 0.05 compared to intact side).

The lesion induced by injection of 6-OHDA in the MFB and SNC was significantly more remarkable than that in the CPU (P < 0.05), while no difference was observed between 6-OHDA injection in the MFB and SNC (P > 0.05; Fig. 3).

3.2. CatWalk test

The CatWalk system captures a substantial number of gait parameters, both dynamic and static (Fig. 1A). A brief overview of the injured print and gait patterns is presented in Fig. 1B–E. The most significant parameters analyzed using CatWalk are described in Fig. 2.

Gait analysis parameters collected by the CatWalk system were first analyzed to compare to the pre-test. The max contact area, mean intensity, stride length and swing speed decreased, whereas the stance, step cycle, duty cycle, terminal dual stance and bases of support increased in varying degrees in the three groups (Fig. 2).

On the other hand, all parameters in the MFB group took a more profound deterioration than the CPU group. Similar to the MFB group, there was also significant difference in all parameters

<table>
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<th>Parameters</th>
<th>Pearson correlationa</th>
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<th>Parameters</th>
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RF: right front, LF: left front, RH: right hind, LH: left hind.

* P < 0.05.

a CPU.
b SNC.
Several parameters of CatWalk were affected variously in three kinds of 6-OHDA lesioned rats. The max contact area (A), mean intensity (B), stride length (C) and swing speed (D) decreased after 6-OHDA injection and the columns indicating the parameters before surgery was covered with the columns indicating the parameters after surgery. In contrast, the stance (E), step cycle (F), duty cycle (G), terminal dual stance (H) and base of support (I) increased after 6-OHDA injection and the columns indicating the parameters after surgery was covered with the columns indicating the parameters before surgery.

* \( P < 0.05 \), ** \( P < 0.01 \) compared to the pre-test. \( \triangle P < 0.05 \), \( \triangle \triangle P < 0.01 \) between compared groups after surgery. 

\# \( P < 0.05 \), \( \# \# P < 0.01 \) compared between the right and left limbs after surgery.

between the SNC and CPU groups except the duty cycle. However, readouts of gait parameters of mean intensity, stride length, swing speed, step cycle, duty cycle and terminal dual stance of the MFB and SNC group were significantly different (all \( P < 0.05 \); Fig. 2).

Significant contralateral (‘lesioned’) vs. ipsilateral (‘nonlesioned’) differences were observed in the max contact area, mean intensity and terminal dual stance of the MFB and SNC groups (all \( P < 0.05 \); Fig. 2A, B, H). There was significant difference of max contact area between the contralateral and the ipsilateral fore paw (contralateral>ipsilateral, \( P < 0.05 \); Fig. 2A) and terminal dual stance (contralateral>ipsilateral, \( P < 0.01 \)) in the CPU group. Besides, other parameters were similar between the contralateral and the ipsilateral side (Fig. 2).

3.3. Correlation of TH with gait variability

Table 2 shows the analysis of the correlation between the levels of TH in the SNC and CPU of 6-OHDA treated rats and the
values derived from a variety of CatWalk tests after four weeks post 6-OHDA administration. Significantly positive correlations exist between TH levels and values of swing speed, stride length of all limbs, max contact area, and mean intensity in the left limbs. Substantially negative correlations between TH levels and values of stance, terminal dual support, step cycle, duty cycle in all limbs, and base of support were noted (Table 2).

4. Discussion

4.1. Quantitative assessment of gait

In a previous study [4], the parameters investigated displayed similar numerical output before and after surgery in the sham group, suggesting that all parameters in the study remain consistent and stable over time in neurologically intact rats.

There are a variety of tests that can be used to model bradykinesia. An increase in muscle rigidity and hypokinesia may cause remarkable decrease of stride length and swing speed in PD rats [14], which is similar to the clinical signs that is often seen in PD patients that they usually take smaller steps when trying to walk [15]. The measurement of stance, step cycle and duty cycle was increased in the severely impaired groups of rats of the study due mainly to the longer contact between the paws and the glass plate [16]. Clinically, PD patients demonstrate gait disturbances such as reduced overall velocity, decreased arm swing, reduced stride length, and increased duration of the stance phase [17]. The dynamic parameter stance and duty cycle illustrate the pitfalls as well, in which PD patients have increased stance duration of the part spend in double limb support [18].

In a previous study, Chuang et al. found a persistent reduction in paw pressure and maximal area of paw contact [5]. Similarly, we also found that a reduction in paw pressure and print area of paw contact in the affected limbs was due to unilateral dopamine deficiency. This was most likely because of the rigidity of muscle tone and altered use of paw surface in the lesioned side [19].

The terminal dual stance reflects the posture alterations. In our study, we found that, in the fully lesioned models, a significant increase of terminal dual stance in all limbs existed, suggesting an increased duration of the postural phase in the 6-OHDA-treated rats. We, therefore, postulated that the terminal dual stance is an important parameter mimicking delays of freezing of gait or gait.
hesitation in PD [20]. The data also agree with the reports of a longer double limb support time in PD, as a result of patients need to take longer time to prepare for the generation of propulsive forces [21,22].

The spatial parameters of base of support for hind limbs increased substantially in the MFB and SNC lesion group while remarkable difference between fore limbs in all rats still existed. This result, similar to the finding in a previous study [10], might be related to the fact that hind limbs playing a more sensitive role in the balance instability of 6-OHDA-treated rats tested. However, the same result was not seen in a bilateral 6-OHDA lesioned models [4]. This might be attributed to the difference of injected brain areas in animals of the two studies.

In our study, we cannot get the conclusion that gait deficits are not (parallelly) due to loss of LC-noradrenergic neurons, since we did not protect nor-adrenergic cell loss by the injection of desiminipramine before the injection of 6-OHDA, which is similar to a previous gait analysis study [4]. In addition, unilateral 6-OHDA infusions not only led to gait disturbance in the ipsilateral side paws but also in the contralateral side paws. We hypothesize that the rats adapted to the use of the contralateral side paws to compensate for the loss of the ipsilateral side paws in order to maintain a straight path down the narrow glass walkway.

4.2. The injection of 6-OHDA into different location of the brain produce different models

As described previously, injection of 6-OHDA into the MFB and SNC results in a rapid and profound loss of the nigrostriatal DA neurons accompanied by severe motor deficits [1]. In our study, the gait alteration in the CPU group is milder and incomplete. The SNC group has less stable loss of DA neurons compared with the MFB group on account of the narrow anatomical structure of SNC. In brief, the MFB group has a more evident and stable gait impairment than the other two models.

4.3. The correlation between the gait parameters and neurochemical expression

Various strategies have been employed to verify the correlation between the motor deficits and the impairment of the nigrostriatal system [10]. Here, we used correlation analysis of the motor parameters and TH protein levels in the SNC and CPU. Parameters of the max contact area, mean intensity, stride length, swing speed, stance duration, step cycle, duty cycle, terminal dual stance and base of support between limbs in the CatWalk test were strongly correlated with TH protein level in the SNC and CPU. This is one of the first studies to analyze correlation between gait measures and TH protein levels in the SNC and CPU to further confirm gait and posture deficits in the three classic 6-OHDA PD models.

5. Conclusion

This study compares the changes of gait dysfunction in three different 6-OHDA models and validates a novel and low-strategy method for analyzing the gait functions of rats. In future studies it seems advisable to test candidate therapeutic intervention in several models that complement each other with respect to individual and interacting mechanisms underlying the gait and pathogenesis of the human disease.

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References