



Research report

Time-course gait analysis of hemiparkinsonian rats following 6-hydroxydopamine lesion

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ARTICLE INFO

Article history:

Received 20 August 2010

Received in revised form 10 March 2011

Accepted 14 March 2011

Keywords:

Parkinson's disease

6-OHDA

Gait pattern

Dopaminergic neurons

ABSTRACT

Gait disturbances similar to those of human Parkinson's disease (PD) can be observed in animals after administration of neurotoxin 6-hydroxydopamine (6-OHDA) to induce unilateral nigrostriatal dopamine depletion. However, the relationship between gait disturbances and dopamine depletion following 6-OHDA infusion has not been determined. The present study investigated the longitudinal changes of spatiotemporal gait patterns using a walkway system to acquire footprints and lateral limb images over a 6-week period following unilateral 6-OHDA injection into the medial forebrain bundle of rats. Our results indicated that hemiparkinsonian rats exhibited changes in gait patterns, as compared to normal controls, and pre-lesion levels, including a significantly decreased walking speed and step/stride length as well as an increased base of support and foot angle. The relative percentage of the gait cycle was also altered, showing an increase in the stance to swing ratio, which was more evident in the affected hindlimb. Time-course observations showed that these gait disturbances occurred as early as 4 days post-lesion and gradually increased up to 42 days post-injury. The extents of gait disturbances were compared with conventional apomorphine-induced turning behavior and akinesia bar tests, which were also apparent at 4 days post-lesion but remained relatively unchanged after 28 days. Our time-course gait analysis of a unilateral 6-OHDA rodent model provides insight into the compensatory changes of motor functions during the 6-week development of a nigrostriatal lesion, which might be useful for future objective assessment of novel treatments for human PD subjects.

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1. Introduction

Gait disturbances are commonly observed in subjects with Parkinson's disease (PD) resulting from a degeneration of dopaminergic (DA) neurons in the substantia nigra (SN) [1]. Typically, the hallmark changes of gait following PD include temporal asymmetry, which manifests as an inability to maintain internal gait rhythm [2], reduced walking speed, increased cadence and increased double stance time [2]. In addition, PD subjects exhibit abnormal spatial indices of gait patterns, which typically include short steps [3], freezing gait [2,4] and decreased stride length [5]. These gait abnormalities become more pronounced in the advanced stages of PD, inducing further disability or limitation of mobility. To understand the development of PD and to further explore effective therapeutic strategies for improved management of gait disturbances, it is important to have relevant PD animal models, which can be obtained by systemic administration of 1-methyl-4-phenyl-

1,2,3,6-tetrahydropyridine (MPTP) or by infusion of the neurotoxin 6-hydroxydopamine (6-OHDA) in rats. Whereas MPTP injection causes acute and bilateral lesions in the nigrostriatal dopaminergic system, unilateral injection of 6-OHDA into the rat SN, medial forebrain bundle (MFB) or striatum (Str) has commonly been used to induce the changes of motor dysfunction observed in the hemiparkinsonian rat model [6,7].

Several animal behavior tests have been devised to assess the functional deficits and to quantify the behaviors that are similar to human PD symptoms, including a rotation test for severity of dopamine depletion [6,7], a bar test for akinesia [8–10] and a stepping test for rigidity [11]. Because gait impairment is the cardinal sign of PD in humans, gait analysis is used to quantify the multifaceted and complex motor functions in PD animal models [12–14]. Early studies investigating PD gait disorders in rats often used footprints to monitor abnormalities in the spatial parameters of gait. For example, the rodent hind paws were inked, and the rodent was then allowed to walk on paper strips. Based on footprint assessment, rats with a unilateral PD lesion were found to display a shuffling gait, motor asymmetries and short stride lengths that resemble the key features of the human PD gait [12,15]. How-

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ever, temporal data regarding the gait cycle in PD rats is insufficient due to the limitations of the inked footprint assessment system. The recent development of computer-assisted automatic gait analysis, such as CatWalk, provides objective quantification of static and dynamic gait parameters from footprint analysis and has been applied to bilateral 6-OHDA lesion rats [16]. Other simple video-based gait analysis systems have implemented a reflective mirror in a confined, transparent walking track for simultaneous recording of the plantar and sagittal views of the rat's hindlimbs; this allows assessment of spatiotemporal and kinematics data in varied, freely moving rats [17,18].

Although gait analysis in rats has been employed in various neuroscience studies [16], the literature is scant regarding the time-course changes of motor behaviors or locomotion functions during the development of the 6-OHDA hemiparkinsonian rat model. Understanding the relationships between development of motor disturbances and the degrees of DA cell loss might provide some insight into the quantitative assessment of novel therapeutic strategies for PD. Thus, the aims of the present study were to provide a detailed analysis of the time-course changes in gait spatiotemporal parameters and to observe the corresponding dopamine loss in the rat's brain for 6 weeks following unilateral 6-OHDA injection.

2. Materials and methods

2.1. Animals

Animal studies were conducted on 41 adult male Wistar rats with a body weight range of 350–450 g and age range of 8–12 weeks at experimental onset. The animals were separated into two groups for evaluating motor behaviors and DA cell loss. Sixteen rats (eight normal control and eight lesioned rats) were assigned for time-course assessment of motor behaviors for six weeks. The other 25 rats were separated into five subgroups, which were sacrificed at pre-lesion and at 1, 7, 21 and 42 days for evaluating the degree of DA neuron loss following the 6-OHDA lesion. All rats were obtained from the Laboratory Animal Center, National Cheng Kung University, Taiwan. The rats were housed at 25 °C with a 12/12 h light/dark cycle and continuous water and food. All experiments followed the Guide for the Care and Use of Laboratory Animals.

2.2. Chronic hemiparkinsonian rat model

For the 6-OHDA lesion, the rat was anaesthetized with intraperitoneal 400 mg/kg chloral hydrate and placed into a stereotaxic apparatus (Stoelting, IL, USA) to prevent head movement using a 45° non-puncture ear bar with the nose position at 3.3 mm below the interaural line. A 2-cm incision was made, and the area was carefully cleared to expose the line of bregma. To cause destruction of the nigrostriatal pathway, which results in near total depletion of dopamine in the ipsilateral Str and the SN [7], 2 µg/µl of 6-OHDA (dissolved in 0.02% ascorbic saline, Sigma Chemical Co., USA) was injected intra-cranially into the MFB (anterior–posterior: –4.3 mm from the bregma; lateral: 1.6 mm with respect to the midline and ventral 8.2 mm from skull surface;) according to the stereotaxic brain atlas of Paxinos and Watson [19]; this was done on left side of the brain using a 26-gauge 10-µl Hamilton microsyringe mounted vertically on the stereotaxic frame. The syringe was lowered through the burr hole, and the toxin was infused at a rate of 0.5 µl/min with a syringe pump, giving a total volume of 4 µl. The needle was left in the brain for at least 5 min to prevent back filling along the injection tract [6].

Rats with successful lesions were typically slower in their general activity and had a tendency to turn toward the ipsilateral lesion side, but had a tendency to turn toward the contralateral side after apomorphine injection [15]. The effectiveness of the MFB lesion was verified by an apomorphine-induced rotational test at 2 weeks following the lesion [15]. If apomorphine-induced contralateral rotation behavior did not occur, the rat was excluded from further statistical analysis.

2.3. Behavioral tests

Three motor behavior tests (gait, bar and drug-induced rotation) were performed in same sequence on same day. For each test, there were at least 2 h of resting time between each test.

2.3.1. Spatiotemporal analysis of gait patterns

A walking track equipped with a video-based system was modified from previous studies for acquiring more spatiotemporal parameters of gait in this study [17,18]. The walking track apparatus consisted of a plexiglass chamber 80 (l) × 6 (w) × 12 (h) cm with a mirror tilted at 45° underneath the walking track. The tilted

mirror reflected the image of the rat's paws for convenient observation with a digital camera (EX-F1, Casio, Japan). For image capture, the camera was set to record simultaneously a direct lateral view and a reflected underview of the walking track. For lateral kinematical data acquisition, the rats were shaved and marked with red on the skin of the lateral side of the bilateral hindlimbs before each test session. The marked landmarks included the lateral malleolus and the fifth metatarsal head as identified by palpation while moving the joints. Use of colored landmarks provided an easy way to determine the stance and swing phases of the gait cycle from heel contact to toe off.

Before the experiment, the rats were acclimated to the walkway by allowing them to walk freely on the track for 20 min before formal recording. The walking task was repeated in both directions, thus permitting the recording of the movement of each hindlimb. The walking task was repeated until five or six satisfactory walks of at least 4 steps without pause were obtained. Only the hindlimb stepping patterns were analyzed in our present video-based gait analysis system. The digital images obtained from each trial were processed with a threshold setting to detect the boundary of the soles, and critical points for derivation of paw indices were determined using Matlab software (MatWorks, version 7.6., R2008a). After identification of sequential footprints, four spatial parameters, including step length, stride length, base of support (BOS) and foot angle, and three temporal gait parameters, i.e., walking speed, stance/swing phase time and stance/swing ratio, were determined. Each gait parameter was averaged for at least 20 footsteps.

2.3.2. Bar test

Impairment of the initiation of movement or akinesia has been commonly characterized by bar tests for immobility, stepping or cylinder tests [8,20–22]. The bar test was adopted in this study to observe the akinesia phenomenon of PD rats. During the bar test, each rat was placed gently on a table. Each forepaw was placed alternately on a horizontal acrylic bar (0.7 cm diameter), which was suspended 9 cm above the table surface. The forepaw nearest the camera was recorded. The total time (in s) spent by each paw on the bar, i.e., the amount of time from the placing of the forepaw on the bar to the first complete removal of the paw from the bar, was recorded [9].

2.3.3. Analysis of apomorphine-induced spontaneous rotation

A conventional behavioral assessment using apomorphine-induced rotation was performed to quantify the unilateral nigrostriatal lesion-induced motor asymmetry after ipsilateral 6-OHDA injection [6,12,23,24]. The rotational tests were performed with apomorphine (0.5 mg/kg in 0.1% ascorbic acid, i.p.; Sigma) injection of PD rats. The rats were placed individually in a 30-cm-diameter round bowl and assessed over a 60-min period [24]. Round stickers of two different colors were pasted on the rat's back for easy identification of torso direction from the vector change derived from the centers of the color circles. For precise calculation of the number of rotations after apomorphine injection, the rotational behavior was recorded using a digital video camera, which was analyzed at 10-min intervals using an image analysis program written in Matlab. The net number of rotations was calculated as the difference between the number of contralateral rotations and the number of ipsilateral rotations with respect to the 6-OHDA injection side.

2.4. Immunohistochemistry

For evaluating DA neuron loss, tyrosine hydroxylase (TH) staining at five time points post-lesion was performed. The animals were deeply anaesthetized with an overdose of pentobarbital and perfused transcardially with 0.9% saline and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (PBS). Brains were removed and post-fixed for 3 days in the same fixative and dehydrated in 30% sucrose in 0.02 M PBS until the brain sank. The brains were cut into 30-µm sections containing the Str and the SN on a cryostat (Thermo Shandon Ltd., UK). Every fourth section was selected from the region spanning from –5.20 mm to –5.80 mm in the SN and from +1.70 mm to +2.30 mm in the Str with respect to the bregma [19]. The free-floating sections were quenched for 10 min in 0.3% H₂O₂/PBS and rinsed in a 1:200 dilution of concentrated IHC Wash Solution with distilled water for 5 min. Rinsing was repeated 3 times. All sections were soaked in nonspecific antibody binding solution that was blocked by Ready-To-Use IHC Blocking Solution for 15 min. The sections were subsequently incubated with a 1:1000 dilution of rabbit primary anti-TH (cat #AB125, Millipore) with Ready-To-Use IHC Antibody Diluent for 15–18 h at room temperature and then incubated in IgG anti-Rabbit IHC Antibody (Bethyl). Immunostaining was visualized by peroxidase reaction with stabilized, metal-enhanced diaminobenzidine for approximately 5–10 min with enhanced visualization by hematoxylin and, finally, bluing solution for 1–2 min. Sections were mounted on chromalum-coated slides, dehydrated in ascending alcohol concentrations, cleared in xylene and coverslipped in DPX. The TH-positive neurons in the SN from both hemispheres were counted manually in each section [6]. The loss rate of TH-positive cells in the lesion hemisphere was calculated and normalized as the percentage of TH-positive neurons with respect to the unlesioned side.

2.5. Experimental design and statistical analysis

For motor behavioral testing, eight PD-lesioned and another eight normal animals were pre-tested (gait, bar and rotation tests) at least two days before

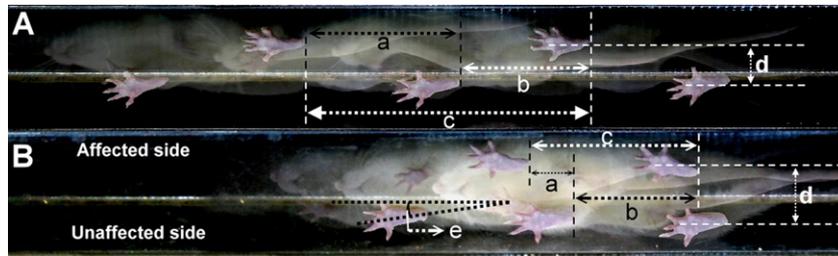


Fig. 1. Characteristics of stepping footprint during locomotion in (A) a pre-surgery and (B) a 42-day post-unilateral PD lesioned rat. Note the shorter step lengths of the affected side (a) and the unaffected side (b) in the lesioned rat relative to the pre-surgery rat. The stride lengths (c) are also shorter, in contrast to the wider BOS (d) and larger foot angle (e) relative to the pre-surgery level.

injection to establish baseline data. After induction of a unilateral 6-OHDA lesion, motor behavior test sessions were performed on the first and fourth day post-lesion, then at weekly intervals up to 6 weeks (i.e., 1, 7, 14, 21, 28, 35 and 42 days post-lesion) under the same environmental conditions. For immunohistochemistry analysis, five out of 25 lesioned rats were sacrificed at each of five specific time points (i.e., pre-lesion and 1, 7, 21 and 42 days post-lesion) for TH staining.

For statistical analysis of gait measurements, a two-way repeated measure analysis of variance (ANOVA) was used to test both group (PD versus normal) and time factors. Multiple within-subject comparisons were taken with the Bonferroni cor-

rection post hoc test when the main effect of time was significant. Also, a paired *t*-test was performed to investigate the differences between the ipsilateral (unaffected) and contralateral (affected) sides over the time-course. For bar and rotation tests, a one-factor analysis (time) repeated-measures ANOVA was used to compare pre- and post-values at each time point followed by a Bonferroni correction post hoc test in PD rats. For immunohistochemistry analysis, a one-way ANOVA was also performed to compare between groups followed by a Tukey's post hoc test. Data were analyzed using SPSS version 17.0 (SPSS Inc., USA) with the significance level set at *p* < 0.05 for each assessment. All data were presented as the average ± standard error of the mean (SEM).

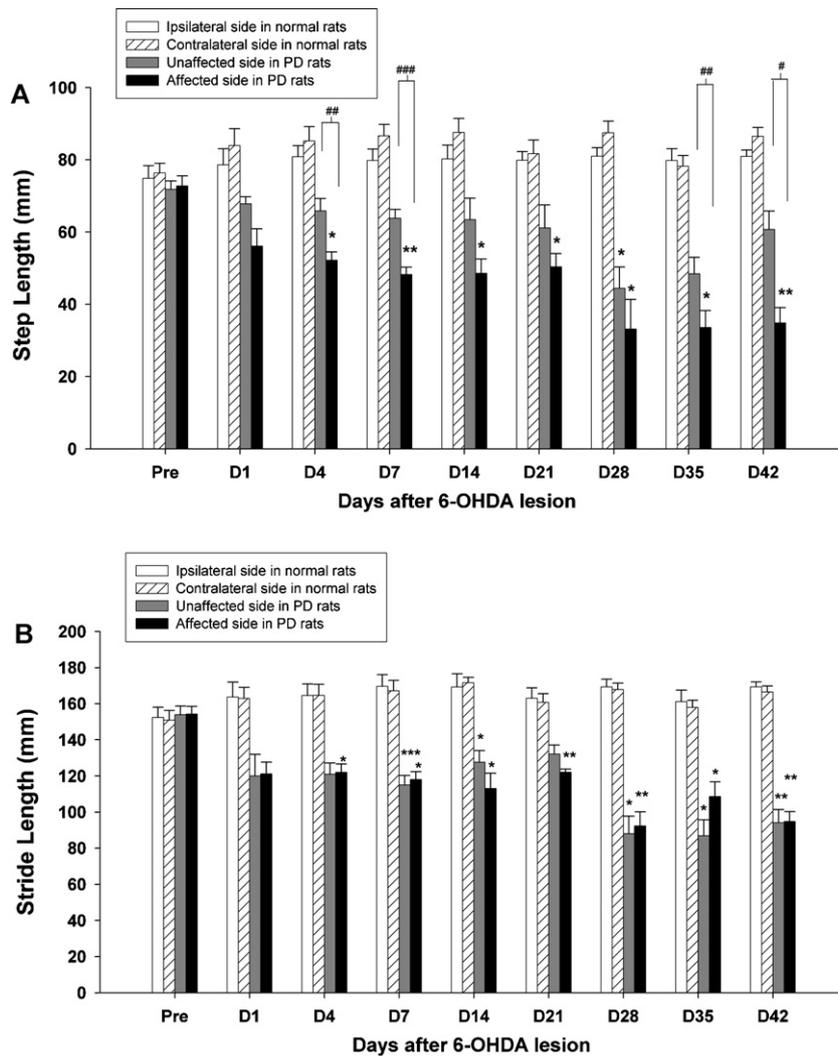


Fig. 2. Time-course changes in the step length (A) and stride length (B) of the ipsilateral (left) and contralateral (right) hindlimbs were observed over 42 days in hemiparkinsonian rats. Asterisks represent significant differences as compared to the baseline data before surgery by using a paired *t*-test with a Bonferroni correction (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). Significant changes between two hindlimbs are represented with a square bracket (paired *t*-tests, #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001). Note that the step length dropped significantly in the affected side (right) after 4 days post-lesion, and the stride length of the lesioned hemisphere (right) was particularly evident from the earliest (day 4) until the last (day 42) time points. Data represent the mean (±SEM) length (mm) of the hindlimb during gait.

3. Results

3.1. Spatiotemporal footprint analysis

Eight normal rats and eight PD rats completed gait analysis within 42 days post-lesion. Fig. 1 shows a representative series of footprint images captured from a pre-lesion rat (Fig. 1A) and a rat at 42 days post-lesion (Fig. 1B). The post-lesion footprints clearly showed shorter steps, especially on the affected side, whereas the pre-lesion animal exhibited a relatively consistent stride length. Furthermore, the ventral view of the footprints showed that post-lesion rats walked with a wider BOS than the pre-lesion rats. The bilateral stride length in the post-lesion animals was markedly shorter than that of the pre-lesion animals, as exemplified in Fig. 1B.

Compared to pre-lesion measurements, post-lesion data showed significant differences in the spatial gait parameters of the affected (contralateral) side of the lesioned rats. Fig. 2 illustrates the time-course changes of bilateral step length and stride length. A two-factor ANOVA on the step length over the 42 days showed a significant time \times group interaction in the ipsilateral limb ($F_{8,56} = 3.30$, $p = 0.004$) and the contralateral limb ($F_{8,56} = 8.24$, $p < 0.001$) as well as significant effects of time ($F_{8,56} = 2.44$, $p = 0.02$ in ipsilateral limb; $F_{8,56} = 3.93$, $p = 0.001$ in contralateral limb) and group ($F_{1,7} = 159.97$, $p < 0.001$ in ipsilateral limb; $F_{1,7} = 452.6$, $p < 0.001$ in contralateral limb). For stride length, a two-factor ANOVA showed significant effects of time in the ipsilateral limb ($F_{8,56} = 5.77$; $p < 0.001$)

and contralateral limb ($F_{8,56} = 4.89$; $p < 0.001$) groups ($F_{1,7} = 175.80$; $p < 0.001$ in ipsilateral limb and $F_{1,7} = 224.93$; $p < 0.001$ in contralateral limb) and a significant time \times group interaction ($F_{8,56} = 5.41$; $p < 0.001$ in ipsilateral limb and $F_{8,56} = 7.09$; $p < 0.001$ in contralateral limb).

In the normal control group, no significant differences were found in all gait parameters across the test time when compared to pre-surgery baseline data (all $p > 0.05$). In PD rats, a post hoc analysis with a Bonferroni correction in the time effect showed that the step and stride lengths in the affected side gradually decreased and reached a significant difference at 4 days post-lesion ($p < 0.05$) followed by a progressive increase of severity up to the sixth week of observation ($p < 0.01$). Furthermore, the difference in step length of the affected side revealed a decreasing time-course trend, with the unaffected side showing a slower trend than the affected side.

Regarding the BOS, there was a significant time \times group interaction ($F_{8,56} = 3.53$, $p = 0.00$). The main effect for time was significant ($F_{8,56} = 3.45$, $p = 0.003$). A significant group difference was also observed ($F_{1,7} = 59.48$, $p < 0.001$). Fig. 3A shows that post-lesion rats showed a significantly larger BOS at 7 days post-lesion compared to pre-lesion levels ($p < 0.001$), with a gradual but persistent increase up to the end of the measurement period ($p < 0.001$). Fig. 3B shows the time-course change of the foot angle. Much more external rotation was exhibited in both the ipsilateral and contralateral sides one day after the lesion in comparison with the pre-lesion state. Interestingly, the foot angle in the unaffected side reached a sig-

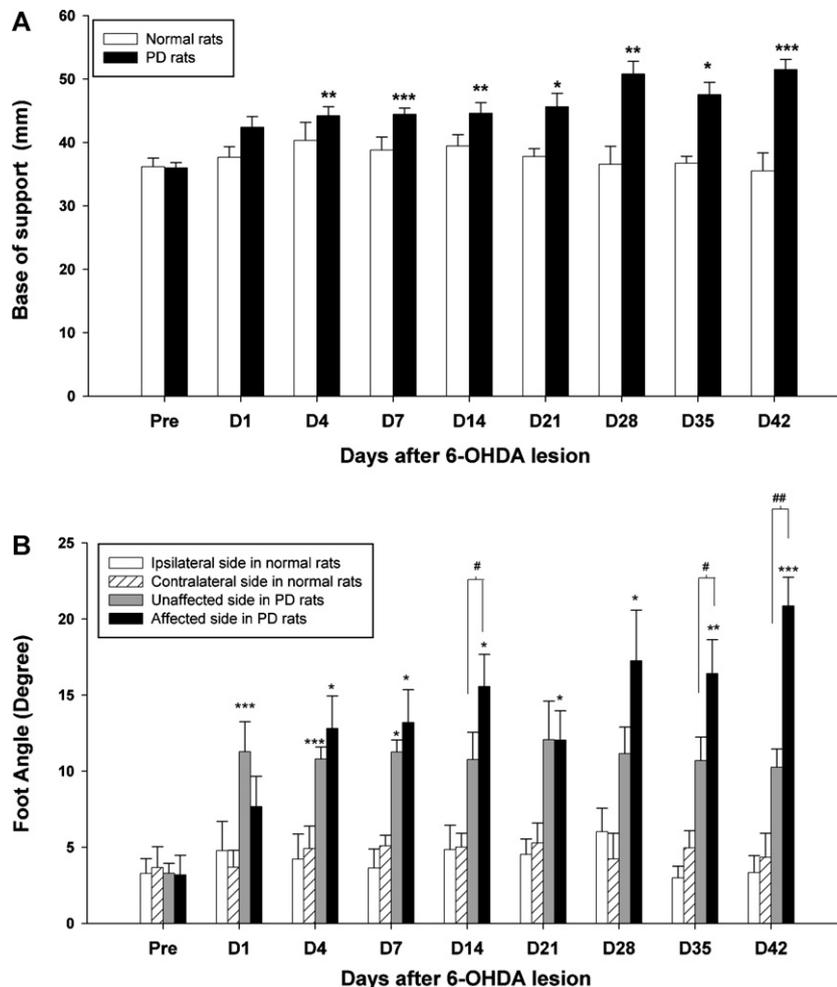


Fig. 3. Time-course changes of the BOS (A) and foot angle (B) of the hindlimbs after 6-OHDA injection through 42 days. Asterisks represent significant differences in the foot angle between measured values and pre-surgery data ($*p < 0.05$, $***p < 0.001$) by using Bonferroni correction post hoc tests. The bracket indicates significant differences between the hindlimbs of the two sides using a paired t -test ($\#p < 0.05$, $\#\#p < 0.01$).

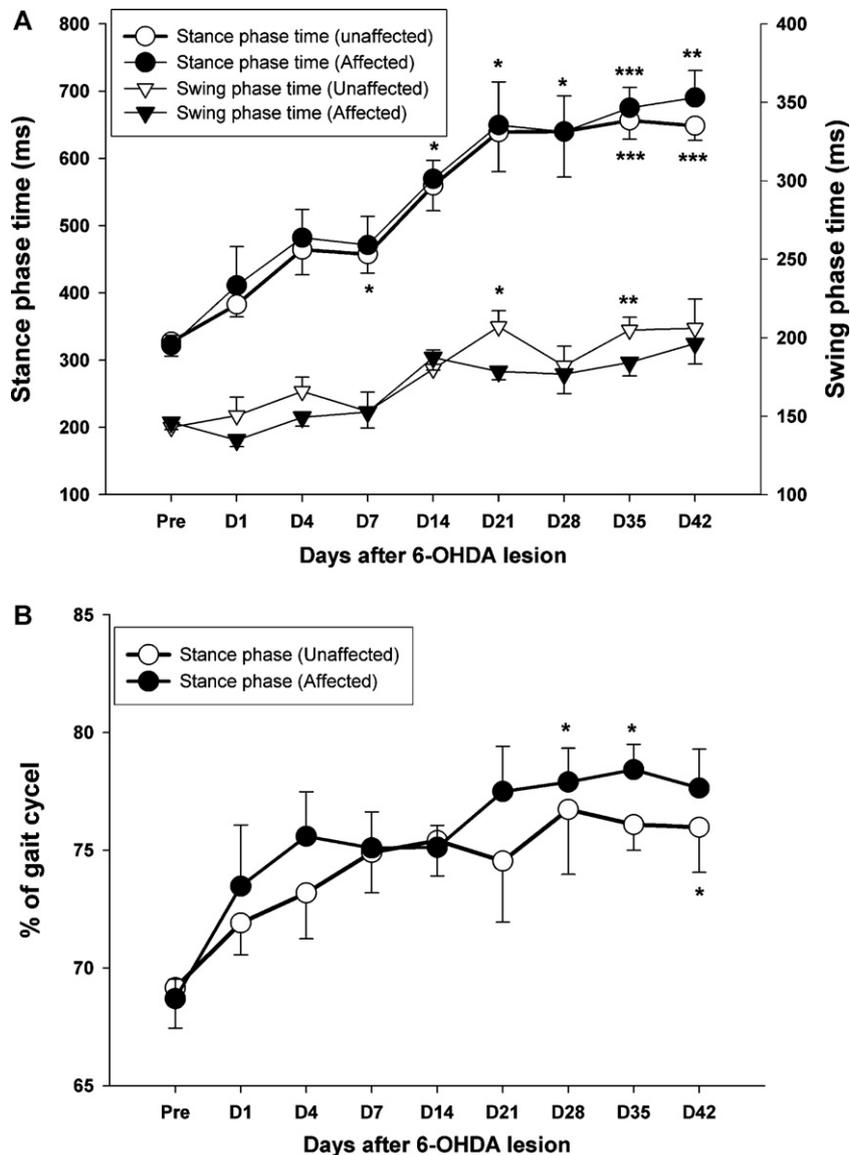


Fig. 4. (A) The time-course changes in the duration of stance and swing phase within 42 days of observation. Note the gradually increased trend in both phases, which was maintained at relatively high levels until 21 days after PD lesion. (B) The percentage of stance phase in the full gait cycle also shows a gradual increase either in the affected side (black circle) or the unaffected side (white circle) following 6-OHDA injection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to pre-operative values with a Bonferroni correction.

nificant difference at the second time point (4 days post-lesion) and remained substantially stable throughout the remaining time points. In contrast, the foot angle of the affected side exhibited a continuous increase over the full observation period.

With regard to temporal gait parameters after unilateral dopaminergic lesion, the data in Fig. 4A show that the precise duration of swing and stance phase can be clearly observed by high speed video recording. A significant increase in the stance phase time could be seen at 14 days post-lesion in the affected hindlimb, but no apparent change was seen in the swing phase time until 21 days post-lesion ($p < 0.05$). Furthermore, Fig. 4B shows that there was a significant difference in the percentage of stance phase as compared to the pre-lesion level in the affected side by 28 days post-lesion ($p < 0.05$). The time-course measurements also indicated progressive increase in the percentage of the stance phase in the affected hindlimb, from 69% at pre-surgery to 78% at 42 days post-lesion, as shown in Fig. 4B.

The walking speed of the post-lesion animals was also severely decreased. A significant difference was observed across time

($F_{8,56} = 6.51, p < 0.001$), as reflected in the main effect for test days. A significant group effect was also observed on the changes of walking speed ($F_{1,7} = 224.07, p < 0.001$). The time \times group interaction was found to be significantly different ($F_{8,56} = 320.69, p < 0.001$). For post hoc comparisons, Fig. 5 shows the time-course changes of walking speed. A significant difference clearly appears at day 4 post-lesion ($p = 0.011$). Interestingly, walking speed showed a continuously decreasing trend over the time-course of observation, although the most dramatic change in walking speed occurred by the first post-lesion test. From day 28 to 42, the decrease in walking speed seemed to reach a plateau. The average walking speed was 30.8 ± 2.3 cm/s at the pre-lesion state, which was quite different than the final speed (days 28–42) of approximately 8.0 ± 0.7 cm/s ($p = 0.004$).

3.2. Bar test

Fig. 6 shows the time-course changes of post-lesion paw immobility according to the bar test for both limbs. One-way

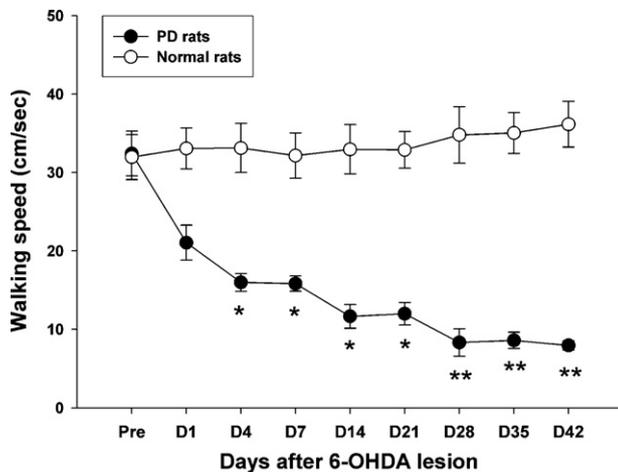


Fig. 5. Time-course changes of walking speed in normal and PD lesion rats while rats performed the walkway locomotion test during the 42 days. Note the gradual decrease in the walking speed, which started to reach significance at day 4 after 6-OHDA injection. Levels of significance: * $p < 0.05$; ** $p < 0.01$ as compared to pre-values with the Bonferroni correction. Data are expressed as the means \pm SEM.

repeated measures of ANOVA revealed a significant effect of time in the ipsilateral limb ($F_{8,56} = 18.27$, $p < 0.001$) and contralateral limb ($F_{8,56} = 10.65$, $p < 0.001$). Following 6-OHDA lesion, both limbs exhibited similar trends of increasing immobility. Compared with the pre-lesion level, the bar test scores showed a statistically significant increase ($p = 0.012$) at approximately 4 days after the PD lesion in the affected forelimb, but the unaffected side did not reach statistical significance until 7 days post-lesion ($p = 0.026$). The behavior asymmetry in the forelimbs became evident at day 4 post-lesion and remained statistically significant from day 4 until the end of observation (paired t -tests, $t = 3.07$, $p < 0.02$).

3.3. Apomorphine-induced rotation behavior

Fig. 7 depicts the time-course changes in rotation behavior for the test animals from pre-lesion until the end of the test period. Post-lesion animals revealed significant time-dependent contralateral rotation to the side of infusion following apomorphine challenge ($F_{8,56} = 21.65$, $p < 0.001$). At the first day after unilateral 6-OHDA administration, the rats displayed occasional rotation, but the behavior did not reach a level of significance ($p = 0.416$). However, at 4 days post-lesion, the injured animals revealed con-

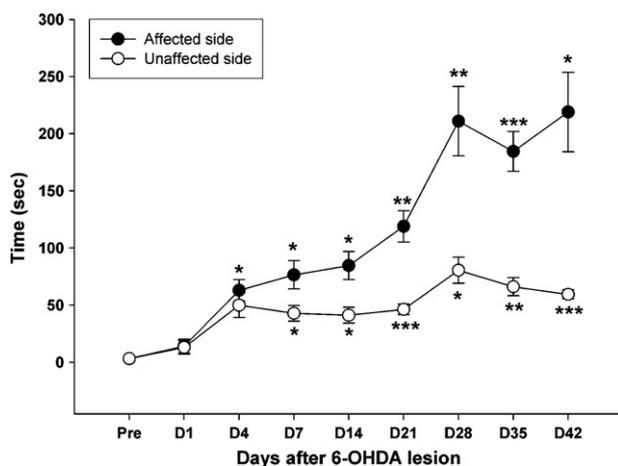


Fig. 6. Time-course changes of akinesia observed from the bar test scores of 6-OHDA lesioned rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different from the pre-lesion time point with the Bonferroni correction post hoc test.

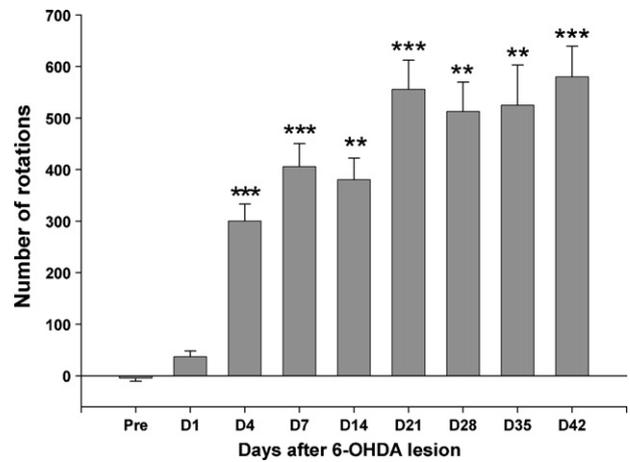


Fig. 7. Time-measurement changes in the rotational response to apomorphine. Bars represent the mean (\pm SEM) number of net contralateral rotations performed by the animals as the total number of full body rotations in 60 min. ** $p < 0.01$, *** $p < 0.001$ as the significant difference compared with the pre-lesion stage with the Bonferroni correction post hoc test.

sistent asymmetry in turning behavior difference ($p = 0.001$). The time-course measurement showed a gradual increase in the rotation number until 21 days post-lesion, after which a plateau was reached and the values remained basically unchanged.

3.4. Histological tests

The results of the TH-immunohistochemistry in the Str and the SN at pre-lesion and at 1, 7, 21 and 42 days post-lesion are shown in Fig. 8. At the Str and SN regions, infusion of 6-OHDA induced a progressive loss of TH-immunoreactive cells. A mild lesion was already apparent at 1 day post-injection in the ipsilateral Str and SN. A decrease in TH-immunoreactive density on the side of the infusion was markedly apparent at 1 week post-lesion, a trend which progressed throughout the course of observation. Six weeks after unilateral 6-OHDA infusion, tyrosine hydroxylase immunoreactivity in the Str and SN was less detectable on the side of the infusion. The quantification of DA neuron loss in the SN at each time point is presented in Fig. 9. In our longitudinal analysis, the average TH cell loss in the SN region was $11.30 \pm 1.10\%$, $67.13 \pm 5.28\%$, $88.66 \pm 4.68\%$ and $95.43 \pm 2.97\%$ at 1, 7, 21 and 42 days post-lesion, respectively. All lesioned groups had significant differences in the time factor (one-way ANOVA: $F_{4,20} = 58.29$, $p < 0.001$). The Tukey's post hoc analysis indicated that the survival rates of the TH-immunoreactive neurons reached a significant level after 7 days post-lesion ($p < 0.001$).

4. Discussion

The present study investigated time-course changes of motor behaviors, including gait spatiotemporal patterns, conventional bar and rotational behavioral and TH-immunohistochemistry patterns of DA neuron loss for 6 weeks following unilateral 6-OHDA lesion in rats. As compared to the pre-lesion state, animals with the development of unilateral dopamine depletion exhibited gradual reduction in step/stride length and walking speed but an increase in BOS and foot angle. The gait cycle of stance and swing phases were also affected. Additional behavioral tests, including apomorphine-induced rotation and the akinesia bar test further identified asymmetric motor behavior during the 6 weeks of gradual DA neuron loss following 6-OHDA injury.

The time-course observation of gait patterns and behavior tests helped confirm behavioral compensation and quantify the

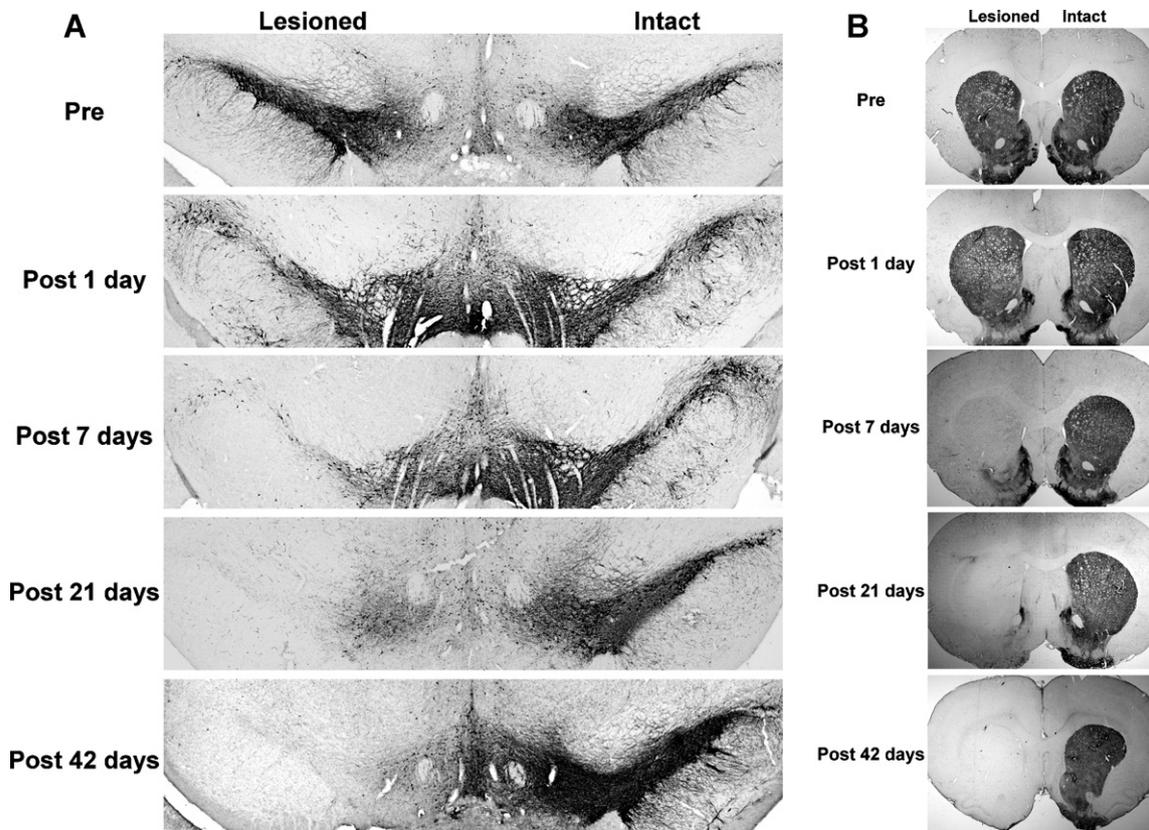


Fig. 8. Representative microphotographs demonstrating TH-immunoreactive fibers in the SN (A) and Str (B) from animals sacrificed at pre-surgery as well as at 1, 7, 21 and 42 days post-6-OHDA lesion. At pre-lesion, neurons in the bilateral hemisphere (darkly stained TH-positive cells), are present throughout the SN. Note that obvious reduction of TH-immunoreactive neurons in the lesioned hemisphere (left side) can be observed after 7 days of 6-OHDA lesion.

relative dopaminergic cell depletion at the measurement time points, which provided a clearer picture of the development of induced PD from pre-lesion to full symptom manifestation. After the unilateral injection of 6-OHDA, gradual impairment in gait performance reached a plateau at around 28 days post-lesion (Figs. 1–3). Similar observations could be found in the bar test and induced rotational behavior test. Across the 6 weeks of bar tests, the immobility duration of the unaffected side showed little

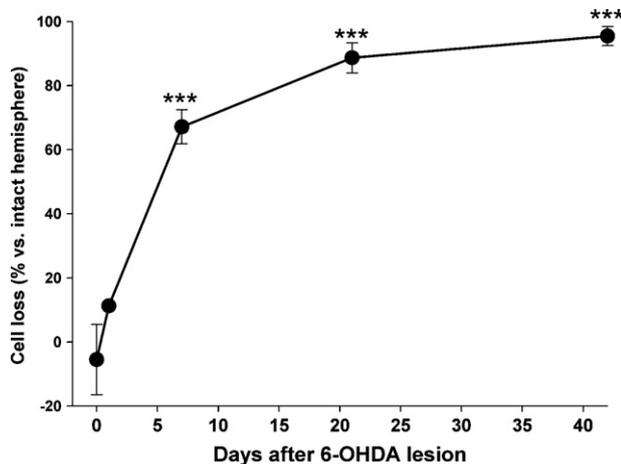


Fig. 9. Time-dependent histogram representing the percentage of neuron survival rates determined by quantification of TH-immunoreactive neurons of the SN in the lesioned hemisphere as compared to the SN of the intact hemisphere. Values are expressed as the mean \pm SEM. Note that TH-immunoreactive neuron loss becomes apparent after 7 days post-lesion as compared to the pre-lesion value ($***p < 0.001$, Bonferroni correction post hoc test).

variation after day 4, whereas the affected side showed a progressively increasing trend until day 28, followed by a plateau for the remainder of the test (Fig. 6). Similarly, the rotational response (Fig. 7) progressively increased up to day 21 and reached a relatively stable plateau. According to our time-course measurement of TH-immunoreactive neurons in the SN, the DA loss increased slowly from 89% at 21 days to 95% at 42 days post-lesion (Fig. 9), indicating that the depletion of DA neurons also reached a plateau at about the same time points of 21 or 28 days. Thus, rats with infusion of 6-OHDA in the MFB displayed a gradual development of difficulties in locomotion, which was well correlated to progressive loss of nigrostriatal DA neurons.

According to our detailed spatial gait analysis, our seven-day post-lesion data revealed significant modification of step/stride length and foot angle. At the same time point, the majority of DA depletion (about 67%) was observed from our TH staining. Interestingly, these results concurred with those of previous studies demonstrating that approximately 75% of DA cell loss in rats [6] and 68% of DA cell loss in PD patients [25] elicited pronounced locomotion deficits. Also, the asymmetric gait patterns became evident at 4 days post-lesion. The asymmetry in locomotion can be attributed to the significant reduction in step length, stride length and foot angle in the affected hindlimb of hemiparkinsonian rats. However, a smaller step length, stride length and foot angle than those of normal rats were also found in the unaffected hindlimb. Although the SN of the intact hemisphere did not experience dopamine depletion, the level of gait performance of the unaffected hindlimb also showed mild impairment, which has also been reported in a previous study using electromyographic (EMG) recordings [12]. Earlier work also indicated that the intact hindlimb plays an important compensatory role during locomotion, carrying more weight to support the unaffected side during propulsion [26]. Furthermore, a

significant increase in the BOS and foot angle of both hindlimbs was found in the PD rats. The progressive decrease in walking speed can explain a compensatory increase in the BOS and foot angle, which seems to be necessary to increase balance and stability during locomotion.

With regard to gait temporal information, our results showed that dopamine deficiency induced significant changes in stance and swing duration of the gait cycle after 7 days post-lesion. In addition to the elongation of swing and stance time as compared to pre-lesion measurements, the gait cycle showed a gradual increase in the percentage of stance phase (Fig. 4). The extension of both swing and stance duration indicated both the slowness of hindlimb movement and a strongly reduced walking speed [16]. The slow stepping pattern in the affected hindlimb might reflect akinesia or hypokinesia induced by dopamine depletion, which was supported by the forelimb akinesia observed during the bar test. The extended duration of hindlimb swing and stance during locomotion indicated that the PD rats had difficulty in initiating steps with the affected limb due to the 6-OHDA injury [16,27,28]. Similar observation of an elevated percentage of stance phase and a decreased percentage of swing phase has been demonstrated previously in both human [29] and animal studies [14], which can be explained by a delayed onset of the swing phase due to akinesia or muscle rigidity.

Compared to dynamic gait analysis, the bar test is useful for evaluating the motor asymmetry and akinesia of the forelimbs under static conditions [8,20]. The increase in the immobilized duration observed in our study confirms the well-known phenomenon that an established nigrostriatal lesion causes akinesia/bradykinesia in the bar test [8,20]. Compared with the values of pre-surgery and unaffected forelimb, we observed that the unilateral 6-OHDA lesion caused akinesia in bilateral limbs but was especially severe in the contralateral side. Regarding animal behavior, the rats tended to avoid the use of the affected forepaws after unilateral DA lesions. Clearly, the forelimbs play an important role in supporting the body weight during walking in four-legged animals [30]. Thus, avoiding the use of a forelimb would also contribute to the abnormal gait patterns, which could be confirmed from our time-course observation of bar test data showing gradual aggravation similar to our gait data.

Researchers often start to perform therapeutic examination or manipulation after confirmation of the PD rodent model from rotational behavior tested at 2–3 weeks post-lesion [6,11,12,31]. Our data showed that the rotational response presented at day 4 and progressively increased to day 21 post-lesion. These results agree with previous findings that the rotational response to apomorphine was present at about 3 days post-lesion [23], indicating that significant dopaminergic cell loss occurred at early time points. In addition, previous work reported that the minimal dopaminergic cell loss required to elicit a rotational response was about 40–50% for SN [6,7,32,33], which was very close to our TH-immunoreactive cell counts in the SN, interpolated from a DA neuron loss of 11% at day 1 and 67% at day 7 post-lesion (Fig. 9). Our results also showed that apomorphine-induced rotational changes correlated well with the progressive losses in dopamine neurons over time-course observations of six weeks.

Although the relationship and mechanisms between dopamine depletion and motor behavior are not fully understood, it is believed that the onset of gait pattern changes and reaching a plateau at 28 days after lesion is highly related to dopamine depletion [34]. Previous studies have also suggested that dopamine-depleted rats change their somatosensory and/or proprioceptive input [35,36] or undergo asymmetric reticulospinal tract activation [12,37], which may in turn result in an abnormal gait pattern. Furthermore, in addition to gait disturbances caused by the loss of dopamine in the SN, the involvement of the pedunculo-pontine nucleus (PPN) may play an important role in the control

of gait initiation, akinesia and locomotion. Deep brain stimulation of the PPN has been proposed as a new therapeutic means for gait restoration in animal models or PD patients [38–41]. However, the loss of neurons and the changes of neuronal activity in the PPN following 6-OHDA lesion in rodent studies still reveal certain discrepancies [39,42,43]. Understanding the mechanisms of impaired gait development from animal models may provide a basis for implementing new therapeutic approaches for functional recovery of PD subjects, such as repetitive transcranial magnetic stimulation (rTMS), a non-invasive brain stimulation technique, which may modulate brain activity and improve motor performance in PD [44–46].

5. Conclusions

The present study characterized the time-course development of rodent gait impairment and the extent of cell loss in the SN from the immediate post-lesion state to the stable plateau state following 6-OHDA injection. The unilateral 6-OHDA rat model study has generated interesting findings regarding the pre-lesion to full-symptom time-course development of gait impairment, rotational response, bar test behavior and TH-immunohistochemistry. The time-course changes in gait impairment started as soon as day four post-lesion and progressively increased to a peak level around four weeks post-lesion; they then persisted at a plateau state over the remaining 6 weeks of the test period. The rotational response to apomorphine and the bar test for akinesia also provided similar developmental curves. The video-based methodology and the experimental data provide new insight into the progressive changes affecting rodent gait pattern during the development of nigrostriatal lesions and suggest that the hemiparkinsonian rat model can be used as an animal model for human Parkinson's disease. Future researchers may use the presented methodology and data for enhanced understanding of the general mechanisms of PD and in the development of novel treatment protocols for functional recovery from PD.

Acknowledgements

The authors would like to thank the National Science Council and the National Health Research Institutes of Taiwan for financially supporting this work under contract numbers NSC 96-2628-E-269-001-MY3 and NHRI-EX98-9535EI. We also would like thank the anonymous reviewers for their constructive comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbr.2011.03.031](https://doi.org/10.1016/j.bbr.2011.03.031).

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