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Surgical Neurology 72 (2009) S2:55 – S2:65
SURGICAL NEUROLOGY
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Movement Disorder

Biochemical alteration in cerebrospinal fluid precedes behavioral deficits in Parkinsonian rats induced by 6-hydroxydopamine

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Received 1 April 2009; accepted 6 September 2009

Abstract

Background: Parkinson's disease, affecting at least 1% of population older than 65 years, is the most common neurodegenerative movement disorder. Up to now, no evidence has demonstrated that biochemical changes in CSF occur preceding the onset of Parkinson's symptoms. In this study, we tested the hypothesis that biochemical changes in CSF precede behavioral deficits in Parkinsonian animals.

Methods: We infused different doses of 6-OHDA into the MFB of rats bilaterally and examined the animals' movement behaviors, biochemical alterations in CSF, and dopaminergic neuronal number in the SNpc 1 week later.

Results: Our results indicated that animals with over 70% dopaminergic neuronal loss in the SNpc exhibited behavioral bradykinesia and rigidity, and a decrease of HVA in CSF. In contrast, animals with about 42% dopaminergic neuronal loss in the SNpc showed normal movement behaviors, but displayed a drastic decline of HVA in CSF. Furthermore, the number of dopaminergic neurons in the SNpc was positively correlated with the HVA level in CSF.

Conclusions: Our findings demonstrate that biochemical alteration in CSF foreruns behavioral deficits and the HVA level in CSF is positively correlated with the number of dopaminergic neurons in the SNpc of Parkinsonian rats induced by 6-OHDA. Our results strongly suggest that additional studies are

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; 6-OHDA, 6-hydroxydopamine; ANOVA, analysis of variance; CPu, caudate putamen; CSF, cerebrospinal fluid; DA, dopamine; DAB, 3,3′-diaminobenzidine; DOPA, 3, 4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; E, epinephrine; FRT, forelimb retraction time; GDNF, glial cell line-derived neurotrophic factor; HPLC, high-performance liquid chromatography; HRT, hindlimb retraction time; HVA, homovanillic acid; IP, intraperitoneal; MFB, medial forebrain bundle; MHPG, 3-methoxy-4-hydroxyphenylglycol; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; MRI, magnetic resonance imaging; NE, norepinephrine; NGS, normal goat serum; PB, phosphate buffer; PD, Parkinson's disease; PET, positron emission tomography; SN, substantia nigra; SNK, Student-Newman-Keuls; SNpc, substantia nigra pars compacta; SPECT, single photon emission computed tomography; TBS, Tris-buffered saline; TH-LI, tyrosine hydroxylase-like immunoreactivity; VMA, vanillylmandelic acid.

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doi:10.1016/j.wneu.2009.09.005

needed to evaluate usefulness of monitoring the HVA level in CSF for early detection of the loss of dopaminergic neurons in the SNpc that precedes the onset of Parkinsonian symptoms in humans.

Keywords: 6-Hydroxydopamine; CSF; Parkinson's disease; Parkinsonism; Substantia nigra; Tyrosine hydroxylase

1. Introduction

Parkinson's disease, affecting at least 1% of the population older than 65 years [20,21,38,53,58], is the leading neurodegenerative movement disorder. Parkinson's disease is characterized by loss of dopaminergic neurons in the SN, especially in the pars compacta (SNpc), and movement deficits including resting tremor, bradykinesia, gait disturbance, rigidity, and postural instability [2,19,53]. Various techniques have been used to study the pathophysiology of PD including MRI, SPECT, and PET imaging techniques [4,13,32,40,44]; however, these techniques are very costly and are still unable to estimate the degree of dopaminergic neuronal loss in the SN before the onset of Parkinson's symptoms. Thus, the development of a sensitive, reliable, and cost-effective method for early detection of dopaminergic neuronal loss in the SNpc before the emergence of movement disorders has become extremely important for slowing down the progression of PD.

Biochemical constituents of CSF differ between PD patients or animals and their respective controls [9,12,15,16,29,36,49]. Evidence shows that PD patients have a significantly lower level of HVA in CSF when compared with control subjects [15,16,31,33,49]. Other studies also indicate that PD patients have a higher epinephrine level in CSF than control subjects [16,43]. Intrastriatal application of 6-OHDA causes a significant decrease of HVA in CSF and a reduction of locomotor activity when compared with control rats 15 days after treatment [29]. Subcutaneous injections of MPTP also decrease motor activity and the concentrations of CSF monoamine metabolites including HVA and DOPAC in monkeys [9]. However, no evidence has demonstrated that biochemical changes in CSF occur preceding the onset of Parkinson's symptoms. Moreover, the relations among the levels of monoamines and their metabolites in CSF, the number of dopaminergic neurons in the SNpc and movement behaviors in presymptomatic and symptomatic Parkinsonian animals are still undetermined.

Several animal models have been used to study PD such as the 6-OHDA, MPTP and rotenone models [5,7,46]. In this study, we tested the hypothesis that biochemical alterations in CSF precede the behavioral symptoms in Parkinsonian animals by injecting different doses of 6-OHDA into the MFB of rats bilaterally and examining the number of dopaminergic neurons in the SNpc, movement behaviors and biochemical changes in CSF 1 week later. Our results demonstrate that biochemical alteration in CSF foreruns the onset of Parkinsonian symptoms in rats receiving 6-OHDA treatment and that a strong and positive correlation exists between the HVA level of CSF and the number of dopaminergic neurons in the SNpc.

2. Materials and methods

2.1. Animals

Forty-nine male Sprague-Dawley rats (9-10 weeks old) were purchased from National Laboratory Animal Center, Taipei, Taiwan. All animals were housed in groups of three in a room with 12:12 h light/dark cycle with lights on at 5:00 AM and the temperature and humidity of the animal room were maintained at 19°C to 21°C and 50% to 60%, respectively. All animals had free access to food and water at all times. After surgery, 5 mL of 0.9% sterile saline was administered subcutaneously to animals twice a day if they showed signs of body weight loss. For animal care and surgical procedures, we followed the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health and all surgical protocols were approved by the University Laboratory Animal Care and Use Committee of Taipei Medical University.

2.2. Surgical procedures

One week after arrival in our laboratory, animals were randomly assigned into 5 groups: Group A, B, C, D, and E (n = 10, 10, 10, 10, and 9, respectively). All animals were anesthetized by IP injections of Imalgene 1000 (ketamine, 50 mg/mL per kilogram, Merial, Lyon, France) and Rompun (xylazine, 10 mg/mL per kilogram, Bayer AG, Leverkusen, Germany). To increase the neurotoxic potency of 6-OHDA, pargyline (50 mg/kg) (Sigma-Aldrich Co., St. Louis, MO, USA) was injected intraperitoneally 30 to 60 minutes before application of 6-OHDA hydrobromide (Sigma-Aldrich Co.) [3]. The 6-OHDA was freshly prepared with 0.9% saline containing 0.02% ascorbic acid right before use. Animals in Group A, B, C, D, and E received 4 μL of 0, 0.25, 0.5, 1, and 2 μg/μL 6-OHDA into the MFB on both sides, respectively. The coordinates for the MFB (bregma: −4.0 mm, lateral: ±1.0 mm, and ventral: −7.8 mm) were determined empirically in our study with reference to "The Rat Brain in Stereotaxic Coordinates" by Paxinos and Watson [41]. Four μL of vehicle or 6-OHDA was stereotaxically injected into the MFB via a 27-gauge steel cannula connected to a 10 μL Hamilton microsyringe, which was driven by a motorized pump at a speed of 1 μL/min for 4 minutes. To avoid the backflow of vehicle or 6-OHDA along the needle track, the needle was left in place for additional 3 minutes after microinjection. Bone wax was applied to fill the drilled hole in the skull and the incision of the skin was closed with wound clips. Animals were kept warm under a lamp until they could move freely and then were transferred back to their home cages.

2.3. Behavioral tests

The movement behaviors of all animals were measured before and 7 days after vehicle or 6-OHDA treatment. Behavioral tests included the paw retraction test and adjusting steps test. The paw retraction test is a common measure used for testing bradykinesia in rats and has been reported in many studies [23,25]. This procedure was performed between 8 and 10 AM with a Plexiglass box, which was designed based on the information published by Ellenbroek et al. [25]. This Plexiglass testing box with dimensions 30 (length) × 30 (width) × 20 cm (height) had a triangle opening, 2 holes of 4 cm in diameter and 2 holes of 5 cm in diameter for the placement of the tail, forelimbs and hindlimbs, respectively. The experimenter held the rat by the body, put its hindlimbs into the 5-cm holes first, moved its body down, and then placed its forelimbs into the 4-cm holes. After the rat was placed in the testing box and released, the time for the rat to retract its first forelimb and hindlimb was defined as the FRT and HRT, respectively. The minimal time for the FRT and HRT was set as 1 second, and the maximal time was set as 30 seconds [25]. The FRT and HRT (in seconds) were recorded twice and the mean was calculated and used for further analysis.

The adjusting steps test is commonly used to measure the rigidity of movements in rats and has been described in previous studies [23,27,35]. In brief, healthy rats will move their forelimbs to adjust their posture when they are forced to move laterally. If the rats have the rigidity deficit, they will make fewer step adjustments. During the adjusting steps test, the researcher put one hand next to the left or right side of the rat and lightly moved the rat laterally (right and left) for 90 cm at a speed of about 20 cm/s on a smooth table surface. The experimenter, who was blind to the treatment of each animal, recorded the number of forelimb adjusting steps twice on both sides for each rat and calculated the mean of 2 tests for further analysis [23,35].

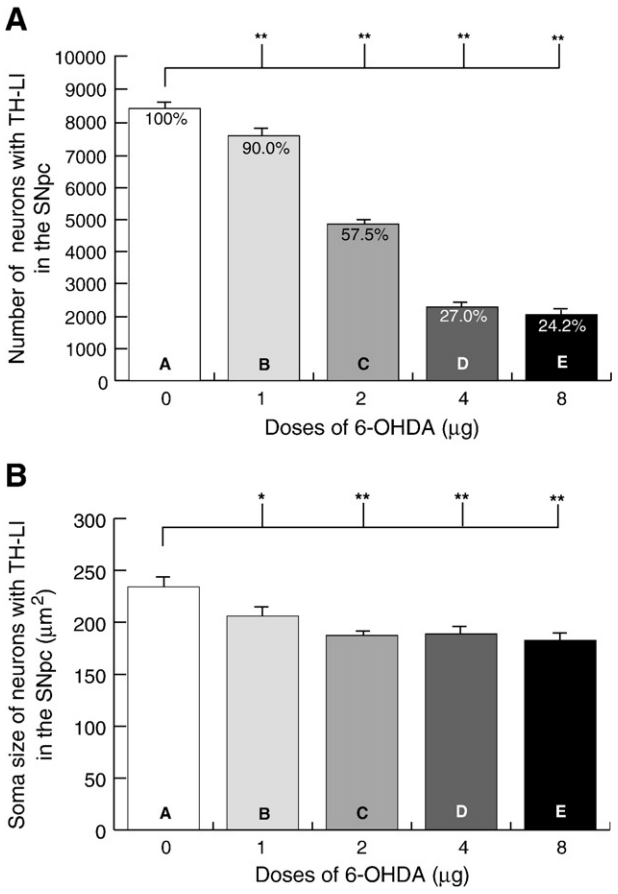
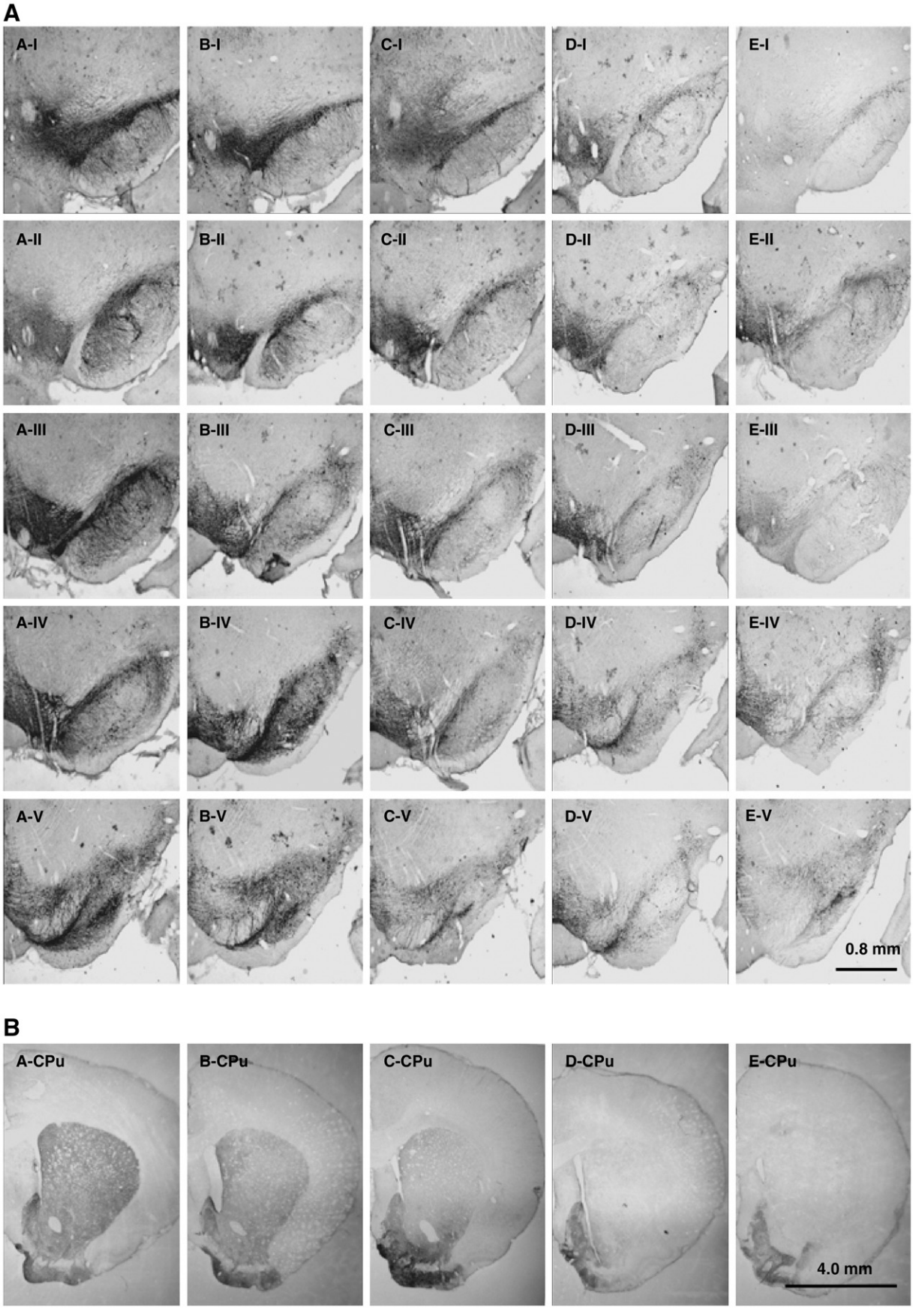


Fig. 1. The number and soma size of neurons expressing TH-LI in the SNpc of animals receiving different doses of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB). A: Application of 6-OHDA resulted in a dose-dependent loss of neurons expressing TH-LI in the SNpc. Animals receiving 1, 2, 4, or 8 μg 6-OHDA had significantly fewer neurons expressing TH-LI in the SNpc than control animals. B: Animals receiving 1, 2, 4, or 8 μg 6-OHDA showed a significant reduction in the soma size of neurons expressing TH-LI in the SNpc when compared with control animals. *$P <$ .05; **$P <$ .01 when compared with control animals in group A.

2.4. Tissue processing and immunohistochemistry

After behavioral tests 1 week after treatment, all rats were anesthetized with the same anesthetics described above. Cerebrospinal fluid was collected by a direct puncture through the cisterna magna with a 27-gauge scalp vein set connected to a 1-mL syringe. Cerebrospinal fluid was immediately aliquoted and stored in −80°C freezer. All animals were perfused transcardially with 0.9% heparinized saline followed by 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (0.1 mol/L PB). Brains were removed and postfixed in 4% paraformaldehyde solution at 4°C for overnight and then transferred into 20% sucrose solution (in 0.1 mol/L PB) at 4°C for cryoprotection until they sank.

Brains were sectioned transversely at a thickness of 50 μm on a freezing microtome and brain sections (from bregma +2.4 mm to −6.9 mm) including the CPu and the SNpc were collected. Every sixth brain section containing the SNpc and one section from the CPu (bregma +1.2 mm) in all 5 groups were processed for TH-LI simultaneously using the free-floating technique commonly used in our laboratory [22,56,57]. All immunohistochemical staining was performed at room temperature unless otherwise specified. All brain sections were rinsed 3 times with 0.05 mol/L TBS and then processed with a blocking solution, which consisted of 4% NGS, 1% H_2O_2, 0.3% Triton X-100, and 0.05 mol/L TBS, for 30 minutes. Subsequently, sections were incubated with rabbit anti-tyrosine hydroxylase antibody at a concentration of 1:4000 (Cat. No. AB152, Chemicon International, Inc., Temecula, CA, USA) in TX-100 solution (0.05 mol/L TBS, 0.3% Triton X-100, and 2% NGS) at 4°C for 48 h. Sections were then reacted with biotinylated goat anti-rabbit IgG (1:200, ABC Elite kit, PK-6101, Vector Laboratories, Burlingame, CA, USA) in TX-100 solution for 60 minutes. Next, sections were processed with ABC reagents (1:100, ABC Elite kit, PK-6101, Vector Laboratories) in 0.05 mol/L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TBS for 90 minutes. Brain sections were then incubated in 0.05 mol/L TBS solution containing 0.05% DAB (Sigma-Aldrich Co.) and 0.003% H_2O_2 until the staining was optimal. Brain sections were washed with 0.05 mol/L TBS three times (5 minutes each) between steps. Following three TBS rinses after DAB staining, brain sections were mounted on gelatin-coated slides, and subsequently covered with coverslips. Control brain sections including the CPu and the SNpc were also processed in the absence of either primary or secondary antibody along with the experimental brain sections and no positive TH-LI was observed in these control brain sections. One set of brain sections was mounted on gelatin-coated slides and counterstained with thionin for evaluation of the 6-OHDA injection site and only animals with the injection site located at the MFB were included for data analysis.

2.5. Microscopic analysis

The SNpc, spanning from bregma –4.80 mm to –6.30mm [41], included 5 sections when every sixth brain section was selected for immunohistochemical staining in this study. The experimenter, who was blind to the treatment conditions, bilaterally counted the number of neurons expressing TH-LI in the SNpc at five levels (I:-4.8 mm, II: -5.1 mm, III:-5.4 mm, IV:-5.7 mm, and V:-6.0 mm with respect to bregma) for all animals under the bright field illumination. The total number of dopaminergic neurons in the SNpc was calculated by the number of neurons expressing TH-LI in every sixth section multiplied by 6 and corrected for splitting of neurons by the commonly used Abercrombie method [1,6,22,34].

The soma size of 100 dopaminergic neurons in the SNpc for each rat was measured with Carl Zeiss microscope equipped with AxioCam HR and AxioVision software V.3.1. Ten neurons were randomly selected from the medial side to the lateral side and from the ventral side to the dorsal side of the SNpc on both sides for each of 5 sections mentioned above. The mean soma size of each group was calculated and used for further analysis.

2.6. Analysis of CSF by HPLC with photodiode detection

The CSF was prepared for HPLC analysis based on the protocol published by Wester et al. (1990) [55] with some modifications and all the procedures were performed on ice unless otherwise specified. In brief, 10 μL preparation solution, which contained 0.1 mol/L perchloric acid, 0.3 mmol/L Na_2EDTA, 0.5 mmol/L Na_2SO_3, and 1.25×10^{-6} mol/L isoproterenol, was added to 40 μL CSF after it was thawed on ice. The mixture of CSF and preparation solution was vortexed and centrifuged at 12,000 g at 4°C for 15 minutes, and the supernatant was transferred into the low volume insert (WAT072294, Waters Corporation, Milford, MA, USA) for HPLC analysis.

The standards of dopamine (DA), 3, 4-dihydroxyphenylalanine (DOPA), DOPAC, HVA, NE, VMA, MHPG, E, 5-HT, and 5-HIAA were purchased from Sigma-Aldrich Co. and the standard curve for each chemical was established by analysis of each chemical at various concentrations including 20000, 10000, 2000, 1000, 500, 200, 20, and 2 ng/mL. The 20 ng/mL standard solution was freshly prepared every day and was injected to calculate the retention time for each chemical before the HPLC analysis of CSF.

All the standard solutions and CSF specimens were autosampled for injection by Waters 717 plus autosampler (Waters Corporation). Three solvents (A: Milli-Q water, B: Acetonitrile, and C: 100 mmol/L formic acid, pH 3.0) constituting the gradient mobile phase were run and degassed by Waters 600E multisolvent delivery system (Waters Corporation) with an initial combination of 90% A and 10% C for the first 5 minutes and then switching gradually to the combination of 65% A, 25% B, and 10% C in the next 10 minutes. The machine was kept running in the latter combination of solvents for another 10 minutes. Ten μL of standard solution or specimen was injected into the HPLC system and the flow rate for the mobile phase was set at 1 mL/min. Between injections of samples, the system was washed with the initial combination of 90% A and 10% C for 10 minutes. The stationary phase consisted of a guard column measuring 4.6 × 20 mm (Part No.186001323, Waters Corporation) filled with micropore filters (5 μm in diameter) and a stainless steel analytical column measuring 4.6 × 150 mm (Part No.186001344, Waters Corporation) packed with Atlantis dC_{18} (5-μm particle size). The substances were detected by Waters 2996 photodiode array detector (Waters 2996 PDA, Waters Corporation) with absorbance at 280 nm [14]. The whole HPLC system operated at room temperature. The data were processed and analyzed by Empower Pro software Millennium version 4.0, an analysis software provided with Waters 2996 PDA. The peaks were identified by the software automatically with the same retention time derived from the standard solutions on the same day. The peak width and the threshold were determined by the standards and the integral area for each peak was recorded and calculated for further analysis.

2.7. Data analysis

The number and soma size of neurons expressing TH-LI in the SNpc and the levels of monoamines and their metabolites in CSF were analyzed by one-way ANOVA followed by SNK post hoc test. The paw retraction time and number of adjusting steps were analyzed by two-way

Fig. 2. Representative brain sections show the TH-LI in the SNpc and CPu of animals receiving various doses of 6-OHDA into the medial forebrain bundle. Application of different doses of 6-OHDA (0, 1, 2, 4, and 8 μg for groups A, B, C, D, and E, respectively) caused a dose-dependent decrease of TH-LI in (A) the SNpc at 5 levels (I: −4.8 mm, II: −5.1 mm, III: −5.4 mm, IV: −5.7 mm, V: −6.0 mm with respect to bregma) and (B) the CPu (one representative section at +1.2 mm with respect to bregma). Scale bar: 0.8 mm and 4.0 mm for Fig. 2A and Fig. 2B, respectively.

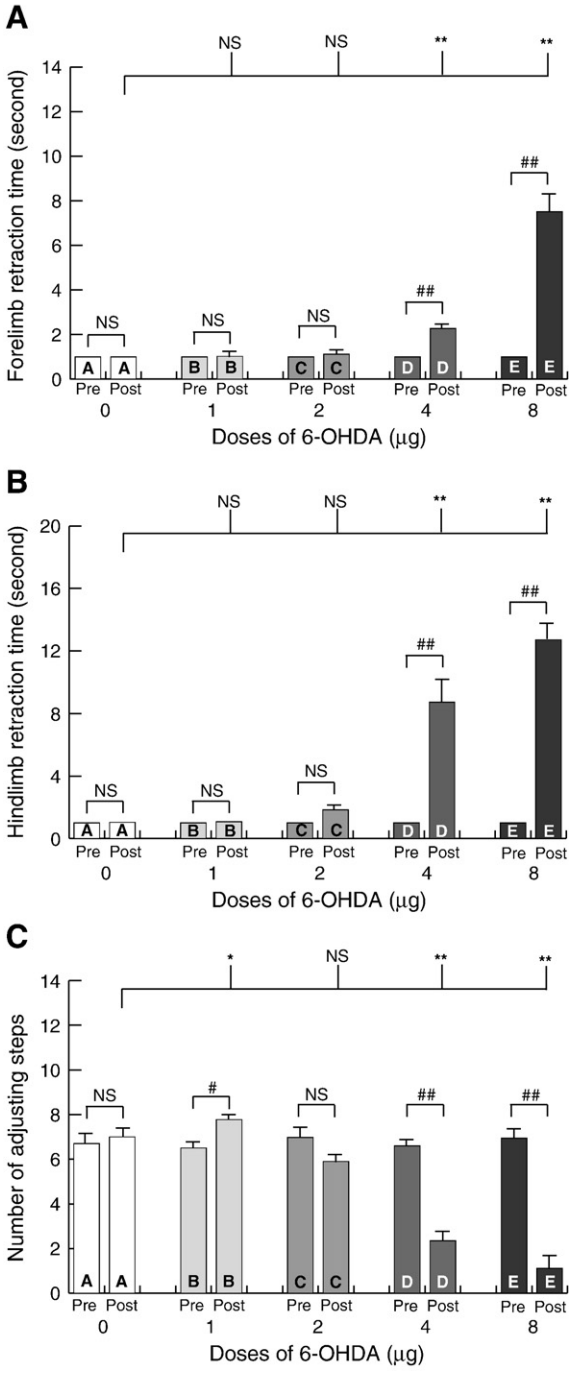

Fig. 3. The movement behaviors of animals before and after application of vehicle or 6-OHDA. A: Animals receiving 4 or 8 μg 6-OHDA showed a prolonged forelimb retraction time when compared with control animals. The post-lesion forelimb retraction time was significantly longer than the pre-lesion measure in animals receiving 4 or 8 μg 6-OHDA. B: The hindlimb retraction time in animals receiving 4 or 8 μg 6-OHDA increased significantly when compared with their respective pre-lesion score and the post-lesion score of control animals. C: The number of adjusting steps decreased dramatically in animals receiving 4 or 8 μg 6-OHDA when compared with their respective pre-lesion score and the post-lesion measure of control animals. In contrast, animals treated with 1 μg 6-OHDA showed a slight increase in their ability to adjust steps. Pre, pre-lesion; Post, post-lesion; NS, $P > .05$. *$P < .05$; **$P < .01$; $^{\#}P < .05$; $^{\#\#}P < .01$.

ANOVA with repeated measures followed by SNK post hoc test. There was no difference in the number of adjusting steps between the left side and the right side for each animal. Therefore, the mean number of adjusting steps on both sides was calculated and used for further analysis. The relation between the HVA level in CSF and the number of neurons expressing TH-LI in the SNpc was analyzed by the correlation analysis. The α level for all statistical analyses was .05. All data were expressed as mean ± SEM.

3. Results

Bilateral injections of 6-OHDA (0, 1, 2, 4 or 8 μg) into the MFB resulted in a loss of dopaminergic neurons in the SNpc in a dose-dependent manner. All groups of animals with 6-OHDA treatment showed a decrease in the soma size of dopaminergic neurons in the SNpc. In addition, animals receiving 4 or 8 μg 6-OHDA displayed movement deficits manifested by a prolonged paw retraction time and a decreased ability to adjust steps. Most importantly, animals that received 2 μg 6-OHDA did not show any observable movement deficits but had a drastic decrease of HVA level in CSF. Furthermore, the HVA level in CSF was positively correlated with the number of dopaminergic neurons in the SNpc.

3.1. Loss of dopaminergic neurons in the SNpc after 6-OHDA treatment

Bilateral injections of 6-OHDA into the MFB led to a dose-dependent loss of dopaminergic neurons in the SNpc. There was a significant difference in the number of dopaminergic neurons in the SNpc among animals receiving different doses of 6-OHDA injected into the MFB, [$F(4, 44) = 264.114$, $P < .0001$]. SNK post hoc tests indicated that animals receiving 1, 2, 4, or 8 μg 6-OHDA had significantly fewer neurons expressing TH-LI in the SNpc than the control animals ($P < .01$ for all 4 doses of 6-OHDA). The loss of dopaminergic neurons in the SNpc increased with the doses of 6-OHDA and the percentages of remaining dopaminergic neurons in the SNpc of animals receiving 1, 2, 4, or 8 μg 6-OHDA were 90.0%, 57.5%, 27.0%, and 24.2% of the control group, respectively (Fig. 1A).

3.2. Decrease of dopaminergic neuronal size in the SNpc of rats receiving 6-OHDA

The soma size of dopaminergic neurons in the SNpc decreased in all groups of animals receiving 6-OHDA. Statistical analysis showed a significant difference in the mean soma size of dopaminergic neurons in the SNpc among different treatments [$F(4, 44) = 6.862$, $P = .0002$]. The mean soma size of dopaminergic neurons in the SNpc of animals receiving 1, 2, 4, or 8 μg 6-OHDA was statistically smaller than that of the control group ($P < .05$ for Group B [the 1-μg group] and $P < .01$ for the remaining groups) (Fig. 1B).

Table 1
Concentrations of monoamines and their metabolites in CSF of rats receiving different doses of 6-OHDA

	Group A (0 μg 6-OHDA)	Group B (1 μg 6-OHDA)	Group C (2 μg 6-OHDA)	Group D (4 μg 6-OHDA)	Group E (8 μg 6-OHDA)
DOPAC	27.4 ± 4.6	15.0 ± 3.3	20.0 ± 4.8	22.1 ± 5.2	59.3 ± 10.5 **
NE	48.0 ± 8.3	22.3 ± 4.2	57.9 ± 5.0	96.5 ± 23.0	158.4 ± 20.8 **
MHPG	81.6 ± 14.7	95.0 ± 16.8	114.3 ± 30.4	108.1 ± 28.4	78.3 ± 12.4
VMA	928.2 ± 125.0	1038.4 ± 136.4	972.1 ± 123.2	1183.5 ± 116.9	1320.2 ± 85.7
5-HT	209.8 ± 17.4	180.3 ± 33.8	164.6 ± 8.0	323.0 ± 41.5 *	309.3 ± 50.8 *
5-HIAA	331.6 ± 21.7	208.7 ± 17.5	276.5 ± 31.8	311.3 ± 34.0	316.8 ± 81.0

Data are presented as mean ± SEM (ng/mL) and analyzed by one-way ANOVA followed by SNK post hoc tests. The number of animals for CSF analysis is 10, 9, 10, 10, and 9 for Group A, B, C, D and E, respectively.

* $P < .05$ when compared with the value in the control group (group A).

** $P < .01$ when compared with the value in the control group (group A).

3.3. Decline of TH-LI in the SNpc and CPu following 6-OHDA treatment

Application of 6-OHDA reduced the intensity of TH-LI in the SNpc and CPu. The intensity of TH-LI in both the SNpc and CPu decreased as the doses of 6-OHDA increased, with the maximal loss of TH-LI staining in the two groups receiving 4 or 8 μg 6-OHDA. Fig. 2A showed 5 representative brain sections of the SNpc (bregma: −4.8 mm, −5.1 mm, −5.4 mm, −5.7 mm, and −6.0 mm for section I, II, III, IV, and V, respectively) [41] in animals receiving 0, 1, 2, 4, or 8 μg 6-OHDA into the MFB (Group A, B, C, D, or E, respectively). The intensity of TH-LI in the SNpc reflecting the number of dopaminergic neurons decreased as the doses of 6-OHDA increased in all five sections (Fig. 2A). Fig. 2B showed one representative section of the CPu (bregma: +1.2 mm) for groups A, B, C, D, and E. The intensity of TH-LI reflecting the amount of dopaminergic fiber in the CPu decreased with the increasing doses of 6-OHDA (Fig. 2B).

3.4. Behavioral bradykinesia and rigidity induced by higher doses of 6-OHDA

Two higher doses of 6-OHDA lengthened the paw retraction time for both forelimbs and hindlimbs. Repeated measures ANOVA revealed a significant group effect [$F(4, 44) = 92.995$, $P < .0001$], lesion effect [$F(1, 44) = 138.275$, $P < .0001$], and group × lesion interaction effect [$F(4, 44) = 92.995$, $P < .0001$] on the paw retraction time of forelimbs among animals receiving 0, 1, 2, 4, or 8 μg 6-OHDA (Fig. 3A). SNK post hoc tests indicated that the post-lesion paw retraction time of forelimbs was significantly longer than the respective pre-lesion score in animals receiving 4 or 8 μg 6-OHDA ($P < .01$ for both doses), but there was no difference between the pre-lesion and post-lesion scores in animals receiving 2 μg or less 6-OHDA (Fig. 3A). In addition, the post-lesion paw retraction time of forelimbs was significantly longer in those animals receiving 4 or 8 μg 6-OHDA than in control animals ($P < .01$ for both doses) (Fig. 3A). Similarly, there were a significant group effect [$F(4, 44) = 35.146$, $P < .0001$], lesion effect [$F(1, 44) = 97.674$,

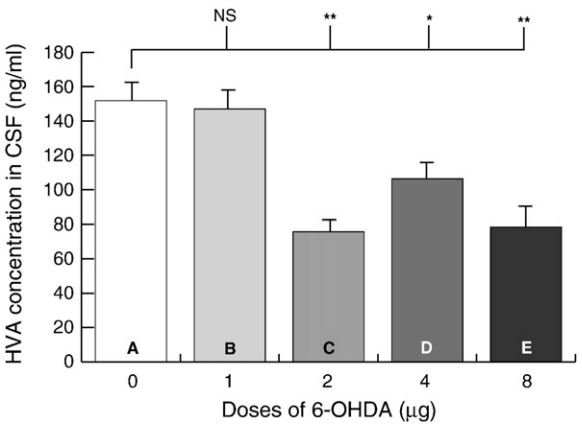

Fig. 4. The HVA level in CSF of animals receiving different doses of 6-OHDA. Animals receiving 2, 4, or 8 μg 6-OHDA showed a significant decrease of HVA in CSF when compared with control animals in group A. NS, $P > .05$; *$P < .05$; **$P < .01$.

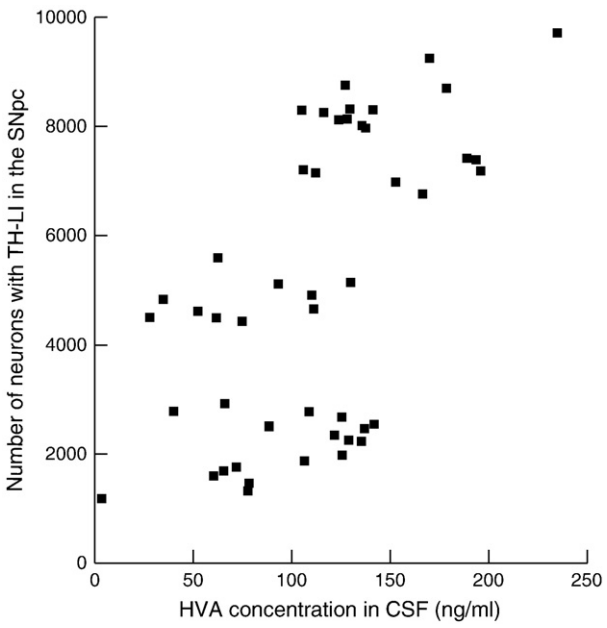

Fig. 5. The correlation between the number of neurons expressing TH-LI in the SNpc and the HVA level in CSF in animals receiving various doses of 6-OHDA. The number of neurons with TH-LI in the SNpc was positively correlated with the HVA level in CSF ($r = 0.582$, $P < .0001$).

$P < .0001$] and group × lesion interaction effect [$F(4, 44) = 35.146$, $P < .0001$] on the paw retraction time of hindlimbs among these 5 treatment groups (Fig. 3B). SNK post hoc analysis showed that the hindlimb retraction time increased significantly after lesion only in animals receiving 4 or 8 μg 6-OHDA but not in those receiving 0, 1, or 2 μg 6-OHDA (Fig. 3B). Moreover, only animals receiving 4 or 8 μg 6-OHDA had a significantly longer hindlimb retraction time when compared with control animals after lesion ($P < .01$ for both doses) (Fig. 3B).

Animals receiving 4 or 8 μg 6-OHDA exhibited a decreased ability to adjust their steps. Repeated-measures ANOVA showed a significant group effect [$F(4, 44) = 38.534$, $P < .0001$], lesion effect [$F(1, 44) = 114.400$, $P < .0001$], and group × lesion interaction effect [$F(4, 44) = 59.406$, $P < .0001$] on the ability to adjust steps among animals receiving different doses of 6-OHDA (Fig. 3C). SNK post hoc tests revealed that only animals treated with 4 or 8 μg 6-OHDA displayed a decreased ability to adjust steps after 6-OHDA treatment when compared with their respective pre-lesion score and the post-lesion score of control animals ($P < .01$ for both groups) (Fig. 3C). In contrast, animals receiving 1 μg 6-OHDA showed an increased ability to adjust their steps when compared with their pre-lesion score and the post-lesion score of control animals ($P < .05$) (Fig. 3C).

3.5. Biochemical changes in CSF after 6-OHDA treatment

Application of 6-OHDA affected concentrations of monoamines and their metabolites in CSF. The amounts of monoamines and their metabolites in CSF including DA, DOPA, DOPAC, HVA, NE, VMA, MHPG, E, 5-HT, and 5-HIAA were measured using the HPLC with known standards. All standards were detectable between 2 ng/mL and 20 μg/mL under our testing conditions. One CSF sample contaminated by venous blood was excluded from analysis. The levels of monoamines and their metabolites in CSF were reported in the following paragraphs and summarized in Table 1.

The HVA level in CSF decreased dramatically in animals receiving 2 μg or more 6-OHDA. One-way ANOVA showed a significant difference in the HVA level of CSF among animals receiving different treatments, [$F(4, 43) = 9.836$, $P < .0001$]. SNK post hoc tests further indicated that the HVA level in CSF was significantly lower in animals receiving 2, 4, or 8 μg 6-OHDA ($P < .01$, $P < .05$, or $P < .01$, respectively) than in control animals (151.9 ± 13.1 ng/mL) (Fig. 4).

The DOPAC level in CSF increased significantly in animals receiving the highest dose of 6-OHDA. One-way ANOVA revealed a significant difference in the DOPAC level of CSF among animals with various treatments [$F(4, 43) = 8.111$, $P < .0001$]. SNK post hoc tests showed that animals receiving 8 μg 6-OHDA had a significantly higher DOPAC level in CSF than control animals ($P < .01$) (Table 1). There was no difference in the DOPAC level of CSF between the control group and the groups receiving 1, 2, or 4 μg 6-OHDA (Table 1). Under the current testing conditions, the DA and DOPA levels in CSF were undetectable.

The highest dose of 6-OHDA increased the NE level in CSF. One-way ANOVA showed a major difference in the NE level of CSF among 5 treatment groups [$F(4, 43) = 12.431$, $P < .0001$]. SNK post hoc comparisons indicated that the NE level in CSF was significantly higher ($P < .01$) in animals receiving 8 μg 6-OHDA than in control animals (Table 1). There was no difference in the NE level of CSF between control animals and those receiving 1, 2, or 4 μg 6-OHDA (Table 1). No difference in the MHPG and VMA levels of CSF was found among 5 treatment groups (Table 1). The amount of E in CSF was undetectable under the current testing conditions.

Two higher doses of 6-OHDA led to an increase in the 5-HT level of CSF. One-way ANOVA revealed a statistical difference in the 5-HT level of CSF among 5 groups [$F(4, 43) = 4.938$, $P = .0023$]. SNK post hoc tests showed that animals receiving 4 or 8 μg 6-OHDA had a significantly higher 5-HT level in CSF than control animals ($P < .05$ for both doses) (Table 1). There was no difference in the 5-HIAA level of CSF among treatment groups (Table 1).

3.6. High correlation between dopaminergic neuronal number in the SNpc and the HVA level in CSF

The HVA level in CSF was positively correlated with the number of remaining dopaminergic neurons in the SNpc of animals receiving different doses of 6-OHDA. Correlation analysis showed a strong positive correlation between the HVA level in CSF and the number of dopaminergic neurons in the SNpc ($r = 0.582$, $P < .0001$) (Fig. 5).

4. Discussion

We demonstrate that a decreased HVA level in CSF precedes the emergence of behavioral deficits in the development of Parkinsonian symptoms in the 6-OHDA–treated rats. The paw retraction test [23,25] and adjusting steps test [23,35] have been commonly used for testing bradykinesia and rigidity in rats, respectively. Animals receiving 4 or 8 μg 6-OHDA into the MFB showed dramatic behavioral deficits manifested by a prolonged paw retraction time and a decreased ability to adjust steps, and also exhibited a significant decrease of HVA level in CSF when compared with control animals. In contrast, animals receiving 2 μg 6-OHDA into the MFB had no observable behavioral deficits; however, these animals had a significantly lower HVA level in CSF when compared with control animals. These findings demonstrate that biochemical alteration in CSF precedes the onset of behavioral deficits in Parkinsonian animals induced by 6-OHDA.

The HVA level in CSF decreased significantly in animals with approximately 42% or more loss of dopaminergic neurons in the SNpc, but the behavioral deficits became apparent only in animals with over 70% loss of dopaminergic

neurons in the SNpc. Animals with an average of 42.5% loss of dopaminergic neurons in the SNpc showed a significantly lower HVA level in CSF, but displayed normal movement behaviors evaluated by the paw retraction test and adjusting steps test. This result was consistent with the earlier finding that patients with an average of 52% loss of dopaminergic neurons in the lateral ventral part of SNpc have no signs of PD [30]. Animals with over an average of 70% loss of dopaminergic neurons in the SNpc exhibited both behavioral deficits and a significant decrease of HVA level in CSF. The decreased HVA level in CSF and behavioral deficits in animals with 70% loss of dopaminergic neurons in the SNpc were consistent with the previous findings that patients with Parkinson's disease have a significantly lower HVA level in CSF [11,15-17,31,33,39,48-50] and that clinical Parkinsonian symptoms become obvious when estimated 70% of dopaminergic neurons in the SN has died [47]. Our data were also in agreement with the finding that intrastriatal application of 6-OHDA induces a decrease of the HVA level in CSF of rats with decreased locomotor activity 15 days after treatment [29]. Moreover, our results revealed a strong positive correlation between the HVA level in CSF and the number of dopaminergic neurons in the SNpc. Furthermore, the HVA level in CSF decreases significantly in patients with severe rapid-onset dystonia-parkinsonism and in some gene carriers without symptoms [11]. The above findings together strongly suggest that the HVA level in CSF is a reliable measure for detecting dopaminergic neuronal loss in the SNpc before the onset of Parkinsonian symptoms.

The soma size of dopaminergic neurons in the SNpc decreased significantly following 6-OHDA treatment. All groups of animals receiving bilateral injections of 6-OHDA into the MFB showed a significant decrease in the soma size of dopaminergic neurons in the SNpc, even in those animals that received 1 μg 6-OHDA, had only 10% dopaminergic neuronal loss in the SNpc and showed normal movement behaviors and normal levels of monoamines and their metabolites in CSF. The cell shrinkage and neuronal death induced by 6-OHDA in this study were consistent with the previous findings that 6-OHDA decreases the soma size of PC12 cells [52] and induces apoptosis in different types of cells [10,51,52]. These findings together strongly imply that dopaminergic neurons affected by 6-OHDA suffer from its toxicity even though they are still alive.

The HVA level in CSF may better reflect the degree of dopaminergic neuronal loss in the SNpc if CSF is collected in humans with control intake of tyrosine in their diet. In this study, all animals had the same diet and the HVA level in CSF decreased significantly in rats with 42% or more loss of dopaminergic neurons in the SNpc. Evidence has shown that diet affects the HVA concentration in urine or serum of humans even under fasting conditions [24,37,54], implying that drastic variations in human diet may mask the biochemical changes in CSF and make it difficult to truthfully reflect the biochemical alterations in CSF of humans with increasing loss of dopaminergic neurons in the SNpc or with PD. To avoid the confounding effect of diet on the levels of monoamines and their metabolites in CSF, we strongly suggest that the CSF be collected and measured in patients on the same control diet with limited tyrosine (dopamine precursor) intake for the same period of time.

Early detection of dopaminergic neuronal loss in the SNpc and protection of dopaminergic neurons from various insults will make it possible to prevent or slow down the development of PD or Parkinsonism. Accumulating evidence indicates that intracerebral application of GDNF exerts neuroprotective effects against 6-OHDA or MPTP-induced neurotoxicity [8,18,26,28,42]. Continuous delivery of GDNF into the striatum by recombinant adeno-associated viral vectors protects nigrostriatal dopaminergic neurons and attenuates the behavioral deficits in common marmosets receiving intrastrital application of 6-OHDA [28]. Moreover, adenoviral delivery of the GDNF gene into the striatum safeguards nigrostriatal dopaminergic neurons against the 6-OHDA neurotoxicity in rats [8] and enhances the survival of transplanted fetal dopaminergic neurons in MPTP-treated monkeys [26]. Recent evidence also shows that long-term calcitriol treatment protects dopaminergic neurons against the neurotoxicity induced by intraventricular application of 6-OHDA [45]. In this study, we demonstrated that a decreased HVA level in CSF preceded the behavioral deficits in the development of Parkinsonian symptoms in the 6-OHDA-treated rats. These findings together provide compelling evidence that prevention or retardation of PD or Parkinsonism can be achieved by early detection of dopaminergic neuronal loss via routine biochemical analysis of CSF and protection of dopaminergic neurons from the toxic insults via delivery of neuroprotective agents such as GDNF and calcitriol.

Acknowledgments

This research was supported by Taiwan National Science Council NRPGM Grant NSC 95-3112-B-038-003 (LYY), Grant NSC 96-2320-B-038-022 (LYY), Grant NSC 92-2314-B-038-033 (JWL), TMU Grant 98TMU-SHH-01-4 (JWL), TMU Grant TMU97-AE1-B21 (JWL), Department of Health Grant DOH-TD-B-111-002 and Center of Excellence for Clinical Trial and Research in Neurology and Neurosurgery.

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Commentary

The absence of reliable markers in detection and staging of idiopathic PD in humans complicates medical management of PD patients. It particularly affects our ability to select appropriate surgical candidates forcing us to wait for the patients to develop functional deficits and disabilities before qualifying for established surgical interventions.

In the past, researchers tried to correlate PD progression with anatomical, radiographic and other markers. My group, for example, found some correlation between disease progression and the size of subthalamic nucleus [1]. The idea, however, has not been confirmed so far—and this is 1 of the reasons I found this paper from Lin et al, who detected a correlation between CSF concentration of HVA and degree of neuronal loss in dopaminergic area of the substantia nigra, interesting and potentially groundbreaking.

With this convincing set of preliminary data, I wonder if the authors' findings will be repeated and confirmed by others, and, most importantly, if the information from OHDA rat models of PD will correlate with multi-faceted, frequently unpredictable, human idiopathic PD. If this turns out to be true, the next steps in making this information clinically useful will be defining a correlation between HVA levels and clinical condition in human patients, and finding a way to make HVA measurements less invasive.

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