MPTP toxicity causes vocal, auditory, orientation and movement defects in the echolocation bat

Wan-Jhen Wu, Chen-Wen Lu, Sheue-Er Wang, Ching-Lung Lin, Li-Yu Su and Chung-Hsin Wu

Introduction

Parkinson’s disease is a common neurodegenerative disorder characterised by movement difficulties such as rigidity, resting tremor, bradykinesia and postural instability [1]. These motor symptoms are reportedly correlated with the neural degeneration of the dopaminergic nigrostriatal pathway [2]. In addition to movement impairments, patients with Parkinson’s disease may be affected by several non-movement symptoms, such as sensorimotor deficits, which affect the voice and swallowing ability [3,4], and sensory impairments, which affect hearing function [5].

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is similar to common herbicides and pesticides, can damage dopaminergic neurons in the substantia nigra in many mammals. In animal models treated with MPTP, the biochemical and cellular changes are relatively similar to those observed in Parkinson’s disease [6]. Currently, MPTP is widely used to induce animal models in rodents and nonhuman primates because of its neurotoxicity to the dopaminergic system [7]. In particular, the MPTP-treated goldfish is used as a Parkinson’s disease animal model because of its simplicity and accessibility [8]. In MPTP-treated mouse models of Parkinson’s disease, the striatum and the substantia nigra in the brain are most sensitive to neurotoxicity, and therefore, susceptible to apoptosis and necrosis [9,10]. MPTP-treated mice may exhibit various symptoms resembling those in human patients with Parkinson’s disease, such as rigidity, akinesia, tremor and posture and gait disturbances [11]. However, research on whether MPTP treatment affects sensorimotor and sensory symptoms in MPTP-treated animal models of Parkinson’s disease is limited.

Although the cause of idiopathic Parkinson’s disease remains unclear, abundant evidence suggests that oxidative stress may contribute to dopaminergic neurodegeneration in MPTP-treated mice [12]. MPTP treatment also causes an inflammatory reaction because of the increasing expression of proinflammatory factors, such as tumour necrosis factor α (TNF-α) in dopaminergic neurons of MPTP-treated mice [13]. In addition, excitotoxicity and apoptosis rather than necrosis may contribute to MPTP-induced dopaminergic neurodegeneration in the substantia nigra of MPTP-treated mice [14,15]. Exposure to environmental pesticides has also been linked to human Parkinson’s disease [16]. Our recent study indicated that an imidacloprid pesticide may damage the neural connectivity of echolocation bats [17]. From both behavioural

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can damage dopaminergic neurons in the substantia nigra in many mammals with biochemical and cellular changes that are relatively similar to those observed in Parkinson’s disease. Our study examined whether MPTP-treated echolocation bats can cause changes in bat echolocation system. By considering ultrasound spectrums, auditory brainstem-evoked potentials and flight trajectories of normal bats, we observed that the vocal, auditory, orientation and movement functions of MPTP-treated bats were significantly impaired, and they exhibited various symptoms resembling those in patients with Parkinson’s disease. Our immunohistochemistry and western blot analyses further indicated that expression of vocal-related FOXP2 in the superior colliculus, auditory-related otoferlin in the inferior colliculus, dopamine synthesis-related aromatic L-amino acid decarboxylase in the substantia nigra and dopamine receptor in the striatum was significantly decreased. Furthermore, protein expression related to inflammation, oxidative stress and apoptosis in the substantia nigra was significantly increased in MPTP-treated bats. These results indicate that inflammation, oxidative stress and apoptosis may be instrumental in dopaminergic neurodegeneration in the substantia nigra. The vocal, auditory and orientation and movement dysfunctions of MPTP-treated bats are relatively consistent with symptoms of Parkinson’s disease. NeuroReport 32: 125–134 Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc.

Keywords: bats, dopamine, echolocation, FOXP2, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, otoferlin

Department of Life Science, School of Life Science, National Taiwan Normal University, Taipei, Taiwan

Correspondence to Chung-Hsin Wu, PhD, Department of Life Science, School of Life Science, National Taiwan Normal University, Taipei 11677, Taiwan

Tel: +886 2 77346363; fax: +886 2 29312904; e-mail: megawu@ntnu.edu.tw

Received 15 August 2020 Accepted 19 October 2020

This is an open access article distributed under the Creative Commons Attribution License 4.0 (CCBY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

0959-4965 Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc.

DOI: 10.1097/WNR.0000000000001574
and neurophysiological perspectives, we observed that imidacloprid toxicity may cause flight movement impairments and spatial memory defects in echolocation bats that resemble human Parkinson’s disease symptoms. By comparing the ultrasound spectrum and auditory brainstem-evoked potential, we revealed that imidacloprid toxicity may cause dysphonia and hearing loss, which are similar to symptoms in the sensorimotor and sensory systems of human Parkinson’s disease [18]. Thus, we are confident that the echolocation bat could be an ideal animal model for studying Parkinson’s disease pathological mechanisms.

This study investigated whether the MPTP-treated echolocation bat can be a new animal model for Parkinson’s disease. We compared the orientation, vocal and auditory functions between echolocation bats that underwent sham and MPTP treatment. We believe that the MPTP-treated bat can serve as a valuable supplementary animal model for Parkinson’s disease research.

### Materials and methods

#### Animal preparation

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of National Taiwan Normal University (no. 101017). Twelve insectivorous bats (Hippopposideros armiger terasensis, weighing 250–300 g) were obtained from mountain caves in Taipei’s suburbs. All bats were housed in cages with a fixed light environment (12 h light/dark cycle) at 25°C room temperature and 30–60% relative humidity. Bats had free access to food and vitamin-supplemented water at all times.

#### Experimental design

A total of 12 bats were allowed to adapt to the environment for six consecutive days. Subsequently, they were randomly divided into two groups, namely the sham and MPTP groups. The sham group received saline by intraperitoneal injection (10 mg/kg/day for 3 days), and the MPTP group was administered an intraperitoneal injection of 10 mg/kg MPTP for 3 days (once a day) to create the Parkinson’s disease bat model. MPTP (catalogue no. M0896) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). We treated the bats for 3 days under a constant temperature and dark conditions. Ultrasound spectrum, auditory brainstem-evoked potential and flight trajectory experiments were conducted every day. On the fourth day, all bats were decapitated, and brain tissue was isolated for use in immunohistochemistry and western blot assays.

#### Ultrasound spectrum analysis of bats

The ultrasonic waves of bats were separately recorded using an ultrasound detector (Pettersson D1000X; Pettersson Elektronik AB, Uppsala, Sweden). Ultrasound spectra of sham- and MPTP-treated bats were separately recorded at least three times for 180 s. Subsequently, sound amplitude and call bandwidth of the ultrasound spectra of each bat were analysed using BatSound pro 3.31b software (Pettersson Elektronik AB).

### Auditory brainstem responses analysis of bats

Auditory brainstem responses (ABRs) of each bat were separately recorded using the Tucker-Davis Technologies (Alachua, Florida, USA) system and the BioSig System III (Tucker-Davis Technologies). After anaesthetisation with pentobarbital sodium (15 mg/kg intraperitoneally; Sigma), a subdermal needle (Rochester Electro-Medical, Tampa, Florida, USA), was used as the recording electrode, was inserted at the vertex of each bat’s head. Auditory stimulation was delivered monaurally by using an EC-1 electrostatic speaker (Tucker-Davis Technologies), calibrated using SigCal software (Tucker-Davis Technologies) and an ER-10B+ low noise microphone system (Etymotic Research, Elk, Groove, Illinois, USA). The evoked ABR potentials of each bat were filtered (0.3–3.0 kHz), averaged (500 waveforms), and stored in a computer for offline analysis.

#### Assessment of bats’ flight trajectory

The bats’ flight trajectory was recorded in a completely dark acoustically isolated chamber. By using the continuous exposure of a digital camera (Canon EOS 600D, Canon Inc, Tokyo, Japan), we recorded light tracks generated by small bright light-emitting diodes on the bats. Subsequently, the light tracks were reconstructed offline as two-dimensional images of the flight trajectory. SDs from the midline of the flight trajectory and the velocity of each bat were calculated on a computer.

#### Immunohistochemistry staining

After anaesthetisation with pentobarbital sodium (15 mg/kg intraperitoneally; Sigma), the bats were sacrificed, their brains were removed and fixed with 4% formaldehyde, embedded in paraffin and then coronally sectioned into 5-μm-thick sections. These sliced sections were mounted on slides for immunohistochemistry staining with antibodies of vocal-related FOXP2 and auditory-related otoferlin proteins (Santa Cruz Biotechnology, Santa Cruz, California, USA), dopamine synthesis-related aromatic L-amino acid decarboxylase (AADC) and dopamine receptor proteins (Abcam, Cambridge, Massachusetts, USA), antioxidative stress-related SOD2, oxidative stress-related 3-NT, inflammation-related TNF-α, antiapoptosis-related Bcl-2, apoptosis-related BAX and caspase-3 proteins (Cell Signaling Technology, Danvers, Massachusetts, USA). Detection of immunohistochemistry staining was conducted using biotinylated secondary antibodies and avidin–biotin–horseradish peroxidase (HRP) complex (Novolink polymer detection system I; Leica Biosystems, Wetzlar, Germany). Visualisation of immunohistochemistry staining was performed using 3,3’-diaminobenzidine chromogen and counterstained with hematoxylin (Novolink polymer detection system I).
Western blotting analysis
Protein concentrations were determined using bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA). SDS-PAGE was performed using equal quantities of protein, which were transferred to a polyvinylidene fluoride membrane using standard protocols. Primary antibodies, at 1:1000 dilution, included antibodies of vocal-related FOXP2 and auditory-related otoferlin proteins (Santa Cruz Biotechnology), dopamine synthesis-related AADC and dopamine receptor proteins (Abcam), antioxidative stress-related SOD2, oxidative stress-related 3-NT, inflammation-related TNF-α, antiapoptosis-related Bcl-2, and apoptosis-related BAX and caspase-3 proteins (Cell Signaling Technology). Anti-rabbit IgG-HRP, anti-mouse IgG-HRP (1:5000 dilution; PerkinElmer, Waltham, Massachusetts, USA) and anti-goat IgG-HRP (1:5000 dilution; Enzo Life Sciences, Farmingdale, New York, USA) were used to detect the immune complexes and chemiluminescent substrate (GE Inc. Healthcare Life Sciences, Barrington, Illinois, USA) was used to detect the chemiluminescence. Densitometric assessments of bands were analysed using Image J software (version 1.48t, Wayne Rasnabd, Washington, District of Columbia, USA).

Statistical analysis
All data are presented as the mean ± SEM and were analysed using Student’s t-test or one-way analysis of variance followed by Student–Newman–Keuls multiple comparisons posttest. The information of the t value, degree of freedom and P value, as well as the F statistic were obtained and calculated by using SigmaPlot 12.5 version statistical software. Western blotting results were obtained from six independent experiments. P values <0.05 were considered significant.

Results
Neuroethological and electrophysiological evidence of vocal, auditory, orientation and movement dysfunctions in the MPTP-treated echolocation bat.
An echolocation bat uses its vocal system to emit sonar pulses, employs the auditory system to receive the reflected echoes, and uses the orientation and movement systems to locate targets (Fig. 1). MPTP treatment can cause vocal dysfunction in the Hipposideros armiger terasensis echolocation bat (Fig. 2A). We selected an echolocation bat that can emit constant frequency biosonar signals combined with a frequency-modulated sweep [Fig. 2A(a)]. By using ultrasonic sound spectrum analysis, we observed that the MPTP-treated echolocation bat exhibited an incomplete and smaller constant frequency–frequency-modulated ultrasound spectrum wave than did sham-treated bats [Fig. 2A(b–d)]. Figure 2A(e and f) exhibits the quantified and compared sound amplitude and call bandwidth of ultrasonic waves between sham- and MPTP-treated bats. Over time, MPTP-treated bats exhibited significantly decreased sound amplitude and shorter call bandwidth than did sham-treated bats (P<0.01).

MPTP treatment can cause auditory dysfunction in echolocation bats (Fig. 2B). These bats use information in the reflected ultrasound to analyse the auditory scene.

---

Fig. 1

(a) and (b) Echolocation behaviours and the related neuropathic nuclei of an insectivorous bat. (A) During echolocation, an insectivorous bat emits sonar pulses from the vocal system, receives the reflected echoes in the auditory system and finally locates targets in the orientation system. (B) A representative MRI and configuration diagram of echolocation behaviours related to neuropathic nuclei in the brain of an insectivorous bat, *Hipposideros armiger terasensis*. CA1, hippocampal CA1 area; CL, cerebellum; IC, inferior colliculus; SC, superior colliculus; SN, substantia nigra; Str., striatum.
MPTP treatment causes vocal, auditory and orientation dysfunction in echolocation bats. (A) Representative ultrasonic waves of sham- and MPTP-treated bats. The sham-treated bats exhibited complete constant frequency–frequency-modulated ultrasonic waves (a), whereas MPTP-treated bats exhibited incomplete and short constant frequency–frequency-modulated ultrasonic waves after 30, 60 and 90 min (b–d); MPTP-treated bats, after 30, 60 and 90 min, exhibited significantly decreased quantified sound amplitudes (e) and call bandwidths (f) in a dose–response manner compared with those in sham-treated bats. (B) Two representative ABRs of sham- and MPTP-treated bats; the arrows indicate the sound amplitude at which ABR reactions began (a). After 60 min, the ABR threshold of MPTP-treated bats was significantly increased compared with that of sham-treated bats (b). (B) Two representative flight paths of sham- and MPTP-treated bats. The sham-treated bats exhibited typical flight path patterns (a), whereas the MPTP-treated bats exhibited irregular patterns (b). MPTP-treated bats, after 30, 60 and 90 min, exhibited significantly increased quantified SDs from the midline of flight paths (c) and decreased flight velocity (d) in a dose–response manner compared with those of sham-treated bats. Results are expressed as mean ± SEM (*P < 0.05; **P < 0.01, one-way ANOVA followed by a Student–Newman–Keuls multiple comparisons posttest) and six experiments were conducted for each treatment. ABR, auditory brainstem response; ANOVA, analysis of variance; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

By using ABRs analysis, we recorded and compared the ABRs between sham- and MPTP-treated bats for 60 min. Sham-treated bats exhibited ABRs above 40 dB SPL in terms of sound stimulation intensity, whereas the MPTP-treated bats only exhibited ABRs above 80 dB SPL [Fig. 2(a)]. Figure 2(b) presents the quantified ABR threshold in sham- and MPTP-treated bats. MPTP-treated bats significantly increased their ABR threshold compared with sham-treated (P < 0.01).

Figure 2C reveals that MPTP treatment can induce orientation and movement dysfunction in the echolocation bat. By using a digital camera’s continuous exposure, we recorded and compared the light tracks between sham- and MPTP-treated bats for 60 min. Sham-treated bats exhibited fixed flight path patterns, whereas the MPTP-treated bats exhibited irregular patterns [Fig. 2(b)]. We compared the SDs from the midline of flight paths and the flight velocity between sham- and MPTP-treated bats. Over time, MPTP-treated bats exhibited significantly increased SDs from the midline of flight paths and decreased flight velocity than did sham-treated bats [Fig. 2(c and d)].

Histopathological evidence of vocal, auditory, orientation and movement-related protein defects in MPTP-treated bats

To clarify the possible pathological evidence of vocal, auditory, orientation and movement dysfunctions in MPTP-treated bats, we used immunohistochemistry to examine and compare vocal, auditory, orientation and movement-related proteins in the brain tissue between sham- and MPTP-treated bats. Figure 1B exhibits a representative MRI and the configuration diagram of the vocal, auditory, orientation and movement-related nuclei in the echolocation bat brain. Figure 3A and B illustrates histopathological evidence that MPTP-treated bats exhibit vocal- and auditory-related protein expression defects in the midbrain. We compared immunohistochemistry expression of vocal-related FOXP2 and auditory-related otoferlin proteins in the superior and inferior colliculus separately between sham- and MPTP-treated bats. Our data indicated that expression of the vocal-related FOXP2 protein in the superior colliculus [Fig. 3A(a)] and the auditory-related otoferlin protein in the inferior colliculus [Fig. 3B(a)] of MPTP-treated bats was obviously weaker than that in sham-treated bats.
MPTP toxicity causes echolocation defects in the bat. Wu et al.

Figure 3C presents histopathological evidence of expression defects of vocal-, auditory- and dopamine-related proteins in the brain of echolocation bats. (A) (a) Immunohistochemistry expression of vocal-related FOXP2 proteins in the superior colliculus of the midbrain in sham- and MPTP-treated bats. Scale bar = 30 μm. (b) Western blotting expression of FOXP2 proteins in the midbrain tissue of sham- and MPTP-treated bats. (c) Quantified FOXP2 expression in midbrain tissue of MPTP-treated bats was significantly decreased compared with that of sham-treated bats. (B) (a) Immunohistochemistry expression of auditory-related otoferlin proteins in the inferior colliculus of the midbrain in sham- and MPTP-treated bats. Scale bars = 30 μm. (b) Western blotting expression of otoferlin proteins in the midbrain tissue of sham- and MPTP-treated bats. (c) Quantified otoferlin expression in the midbrain tissue of MPTP-treated bats was significantly decreased compared with that of sham-treated bats. (C) (a) Immunohistochemistry expression of dopamine synthesis-related AADC proteins in the substantia nigra of the brain in sham- and MPTP-treated bats. Scale bar = 30 μm. (b) Western blotting expression of AADC proteins in the brain tissue of sham- and MPTP-treated bats. (c) Quantified AADC expression in the brain tissue of MPTP-treated bats was significantly decreased compared with that of sham-treated bats. (d) Immunohistochemistry expression of D1R proteins in the striatum of the brain in sham- and MPTP-treated bats. Scale bars = 30 μm. (e) Western blotting expression of D1R proteins in the brain tissue of sham- and MPTP-treated bats. (f) Quantified D1R expression in the brain tissue of MPTP-treated bats was significantly decreased compared with that of sham-treated bats. Data are exhibited as mean ± SEM (**P < 0.01, Student’s t-test), and three experiments were conducted for each treatment. AADC, aromatic L-amino acid decarboxylase; D1R, D1 receptor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.
Figure 4 illustrates histopathological evidence of oxidative-, inflammation- and apoptosis-related protein expression promotion in the substantia nigra of MPTP-treated bats. Our data indicated that immunohistochemistry expression of inflammation-related TNFα [Fig. 4A(a)] and oxidative stress-related 3-NT proteins [Fig. 4B(d)] in the substantia nigra of the MPTP-treated bats was obviously stronger than that in sham-treated bats, whereas immunohistochemistry expression of antioxidative stress-related SOD2 proteins in the substantia nigra [Fig. 4B(a)] of MPTP-treated bats was obviously weaker than that in sham-treated bats. Immunohistochemistry expression of apoptosis-related BAX [Fig. 4C(b)] and caspase-3 [Fig. 4C(g, h)] in the substantia nigra of MPTP-treated bats was obviously stronger than that in sham-treated bats, whereas immunohistochemistry expression of anti-apoptosis-related Bcl-2 proteins in the substantia nigra [Fig. 4C(c)] of MPTP-treated bats was obviously weaker than that in sham-treated bats.

Molecular evidence of vocal, auditory, orientation and movement-related protein defects in MPTP-treated bats

We further used western blotting to quantify the vocal, auditory, orientation and movement-related protein defects in MPTP-treated bats. Figure 3A and B illustrates molecular evidence of vocal- and auditory-related protein expression defects in the midbrain of MPTP-treated bats. We examined the protein expressions of vocal-related FOXP2 [Fig. 3A(b)] and auditory-related otoferlin [Fig. 3B(b)] in the midbrain tissue of sham- and MPTP-treated bats. Molecular expressions of FOXP2 [Fig. 3A(c)] and otoferlin [Fig. 3B(c)] in MPTP-treated bats were significantly decreased compared with that of sham-treated bats [P < 0.01; Fig. 3A, B(c)]. Figure 3C illustrates molecular evidence of dopamine-related protein expression defects in the brain tissue of MPTP-treated bats. Protein expressions of dopamine synthesis-related AADC [Fig. 3C(b and c)] and D1R [Fig. 3C(c and f)] in the brain tissue of the MPTP-treated bats was significantly decreased compared with that in sham-treated bats [P < 0.01; Fig. 3C(b, c, e and f)].

Figure 4 illustrates molecular evidence of oxidative-, inflammation- and apoptosis-related protein promotion in the brain tissue of MPTP-treated bats. Protein expressions of inflammation-related TNFα [Fig. 4A(b and c)] and oxidative stress-related 3-NT [Fig. 4B(e and f)] in the brain tissue of the MPTP-treated bats were significantly increased compared with that of sham-treated bats [P < 0.01; Fig. 4A(b, c) and B(e, f)], whereas protein expression of antioxidative stress-related SOD2 in the brain tissue of MPTP-treated bats was significantly decreased compared with that in sham bats [P < 0.01; Fig. 4B(b, c)]. Protein expressions of apoptosis-related BAX [Fig. 4C(b)] and caspase-3 [Fig. 4C(g, h)] in the brain tissue of MPTP-treated bats was significantly increased compared with that of sham-treated bats [Fig. 4C(b, g and h)], whereas expression of anti-apoptosis-related Bcl-2 proteins in the brain tissue of MPTP-treated bats was significantly decreased compared with that of sham-treated bats [P < 0.01; Fig. 4C(d)]. The quantified ratio of BAX/Bcl-2 was also significantly increased in MPTP-treated bats compared with that in sham-treated bats [P < 0.01; Fig. 4C(e)].

Discussion

The animal model of the MPTP-treated echolocation bat faithfully mimicked the biochemical and histological features observed in human Parkinson’s disease. As depicted in Fig. 1A, when flying, echolocation bats emit sonar pulses through their vocalisation system and then analyse the reflected echoes through their auditory system to locate targets or avoid obstacles [19,20]. To navigate efficiently, the echolocation bat must adjust the spectral and temporal parameters of the pulses they emit in response to message changes in returning echoes [21]. In this study, we observed vocal dysfunction in MPTP-treated bats resembling symptoms in patients with Parkinson’s disease (Fig. 2A). Studies have reported that the biologically relevant superior colliculus can trigger sonar call production and exhibit vocal-related FOXP2 expression in muroid rodents and echolocation bats [22–24]. In addition, FOXP2 causes severe language and speech disorders in humans [25] and is instrumental in the vocalisation system of echolocation bats [26]. Our immunohistochemistry and western blot analyses revealed significantly decreased FOXP2 expression in the superior colliculus of MPTP-treated bats compared with that in sham-treated bats (Fig. 3A). The results indicate that vocal dysfunction in MPTP-treated echolocation bats may be partly attributed to decreased vocal-related FOXP2 expression in the superior colliculus. A previous study has reported pulse acoustics of the echolocation bat can be regulated by the striatal dopamine [27]. It is possible that vocal dysfunction in MPTP-treated echolocation bats may be partly attributed to decreased striatal dopamine.

For echolocation bats, the fine auditory system rapidly and accurately processes messages carried by returning echoes to determine the position, size and features of targets [28,29]. We observed that MPTP-treated echolocation bats may exhibit auditory dysfunction resembling symptoms in patients with Parkinson’s disease (Fig. 2B). The inferior colliculus of echolocation bats contains many auditory-related neurons that respond well to returning echoes [30]. In addition, auditory-related otoferlin, an inner hair cell-specific protein, is highly expressed in the inferior colliculus of muroid rodents and echolocation bats [31–33]. Our immunohistochemistry and western blot analyses revealed significantly decreased otoferlin expression in the inferior colliculus of MPTP-treated echolocation bats compared with that of sham-treated bats (Fig. 3B). Because otoferlin is considered essential...
MPTP treatment causes enhanced expressions of oxidative stress-, inflammation- and apoptosis-related proteins in the brain of echolocation bats. (A) (a) Immunohistochemistry expression of inflammation-related TNF-α proteins in the substantia nigra of the brain in sham- and MPTP-treated bats. Scale bar = 30 μm. (b) Western blotting expression of TNF-α proteins in the brain tissue of sham- and MPTP-treated bats. (c) Quantified TNF-α expression in the brain tissue of MPTP-treated bats was significantly increased compared with that of sham-treated bats. (B) (a) Immunohistochemistry expression of antioxidative stress-related SOD2 proteins in the substantia nigra of the brain in sham- and MPTP-treated bats. Scale bar = 30 μm. (b) Western blotting expression of SOD2 proteins in the brain tissue of sham- and MPTP-treated bats. (c) Quantified SOD2 expression in the brain tissue of MPTP-treated bats was significantly decreased compared with that of sham-treated bats. (d) Immunohistochemistry expression of oxidative stress-related 3-NT proteins in the substantia nigra of the brain of sham- and MPTP-treated bats. Scale bar = 30 μm. (e) Western blotting expression of 3-NT proteins in the brain tissue of sham- and MPTP-treated bats. (f) Quantified 3-NT expression in the brain tissue of MPTP-treated bats was significantly increased compared with that of sham-treated bats. (C) (a) Immunohistochemistry expression of apoptosis-related BAX proteins in the substantia nigra of the brain in sham- and MPTP-treated bats. Scale bar = 30 μm. (b) Western blotting expression of BAX proteins in the brain tissue of sham- and MPTP-treated bats. (c) Immunohistochemistry expression of antiapoptosis-related Bcl-2 proteins in the substantia nigra of the brain in sham- and MPTP-treated bats. Scale bar = 30 μm. (d) Western blotting expression of Bcl-2 proteins in the brain tissue of sham- and MPTP-treated bats. (e) Quantified BAX/Bcl-2 ratio in the brain tissue of MPTP-treated bats was significantly increased compared with that of sham-treated bats. (f) Immunohistochemistry expression of apoptosis-related caspase-3 proteins in the substantia nigra of the brain in sham- and MPTP-treated bats. Scale bar = 30 μm. (g) Western blotting expression of caspase-3 proteins in the brain tissue of sham- and MPTP-treated bats. (h) Quantified caspase-3 expression in the brain tissue of MPTP-treated bats was significantly increased compared with that of sham-treated bats. Data are shown as mean ± SEM (**P<0.01, Student’s t-test), and three experiments were conducted for each treatment. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TNF-α, tumour necrosis factor α.
to the auditory function of the echolocation system, decreased auditory-related otoferlin expression in the inferior colliculus may contribute to hearing disorders in MPTP-treated echolocation bats.

When flying in a familiar environment, echolocation bats quickly develop fixed flight patterns with repetitive loops along a stable trajectory [34]. The typical patterns and velocity of bat flight can serve as a useful index for assessing orientation and movement ability in echolocation bats. In this study, we found that the flight paths of MPTP-treated echolocation bats deviated considerably from their originally learned paths [Fig. 2C(a)], and their flight velocity was significantly decreased compared with that of sham-treated echolocation bats [Fig. 2C(b)]. These results indicate that MPTP treatment may cause orientation and movement dysfunction in bats, which resemble symptoms in patients with Parkinson’s disease. For most mammals, the dopaminergic system plays a critical role in motor control. A part of the basal ganglia circuit-substantia nigra pars compacta (SNc) contains numerous dopaminergic neurons [35]. In dopaminergic neurons of the SNc, AADC is an essential enzyme for rapidly converted L-3,4-dihydroxyphenylalanine to dopamine [36]. In this study, our immunohistochemistry and western blot analyses revealed significantly decreased AADC expression in the SNc of MPTP-treated bats compared with that of sham-treated bats [Fig. 3C(a–c)]. However, MPTP-treated monkeys reportedly exhibit spine loss and dopaminergic denervation in the striatum [37]. The aforementioned study revealed the loss of D1R in the striatum [Fig. 3C(d–f)], which could be an early pathological event of Parkinson’s disease. Our study also observed obviously weaker expression of D1R in the striatum of MPTP-treated bats compared with that of sham-treated bats. Our results indicate that orientation and movement dysfunction in MPTP-treated bats may be partly attributed to reductions in the expression of AADC in the SNc and the D1R in the striatum.

After MPTP treatment, neurodegeneration of dopaminergic neurons in the SNc may occur [38]. Many investigators have reported that inflammation, apoptosis and oxidative stress play a role in the process of MPTP-induced death of dopaminergic neurons [14,15,39,40]. Our immunohistochemistry and western blot analyses further indicated that neurodegeneration of dopaminergic neurons in the SNc may be caused by inflammation, oxidative stress and apoptosis (Fig. 4). In this study, the MPTP-treated bats exhibited significantly increased expression of inflammation-related TNF-α protein and oxidative stress-related 3-NT protein, but significantly decreased antioxidative stress-related SOD2 protein.

Fig. 5

Comprehensive neuropathological defects in the brain of MPTP-treated bats. (a) MPTP treatment impairs vocal, auditory, and orientation and movement functions in the echolocation systems of bats. Vocal dysfunction may be partly attributed to decreased vocal-related FOXP2 expression in the SC. Hearing loss may be partly attributed to decreased auditory-related otoferlin expression in the IC. Orientation and movement disorder may be partly attributed to increasing inflammation-related TNF-α, oxidative stress-related 3-NT, apoptosis-related BAX/Bcl-2 and caspase-3 expression, but decreased antioxidative stress-related SOD2, and dopamine synthesis-related AADC expression in the SN. Notably, movement disabilities may be partly attributed to decreased DR expression in the Str. (b) MPTP-treated bats exhibited symptoms such as vocal, auditory, and orientation and movement dysfunctions similar to those observed in Parkinson’s disease. AADC, aromatic L-amino acid decarboxylase; DR, dopamine receptor; IC, inferior colliculus; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SC, superior colliculus; SN, substantia nigra; Str., striatum; TNF-α, tumour necrosis factor α.
decreased expression of antioxidative stress-related SOD2 protein (Fig. 4A and B). Furthermore, MPTP-treated bats exhibited significantly increased expression of apoptosis-related BAX and caspase-3 proteins, but significantly decreased expression of antiapoptosis-related Bcl-2 protein. Even the ratio of BAX/Bcl-2 of MPTP-treated bats was significantly increased compared with that of sham-treated bats (Fig. 4C).

Conclusion
As shown in Fig. 5, we observed that MPTP treatment may impair the vocal, auditory, orientation and movement functions in echolocation bats. Our immunohistochemistry and western blot analyses revealed that vocal dysfunction may be partly attributed to decreased vocal-related FOXP2 expression in the superior colliculus, and hearing loss may be partly attributed to decreased auditory-related otoferlin expression in the inferior colliculus. Moreover, orientation and movement disorder may be partly attributed to increased inflammation-related TNF-α, oxidative-stress-related 3-NT, apoptosis-related BAX/Bcl-2 and caspase-3 expression, and decreased antioxidative stress-related SOD2 and dopamine synthesis-related AADC expression in the substantia nigra. Furthermore, movement disabilities may be partly attributed to decreased dopamine receptor expression in the striatum. In sum, our results indicate that MPTP-treated bats exhibit diverse symptoms such as vocal, auditory and orientation, and movement dysfunctions that are relatively consistent with symptoms of human Parkinson’s disease. Thus, we recommend the use of the MPTP-treated bat as a novel animal model for Parkinson’s disease.

Acknowledgements
This research was supported by grants from the Ministry of Science and Technology, Taiwan (MOST 107-2320-B-003-003-MY3, 107-2321-B-003-001, 108-2321-B-003-001 and 109-2321-B-003-001) to C.H.W. 107-2320-B-003-003-MY3, 107-2321-B-003-001, 108-2321-B-003-001, 109-2321-B-003-001, 108-2321-B-003-001 and 109-2321-B-003-001) to C.H.W.

This article was edited by Wallace Academic Editing.

W.J.W., C.W.L., S.E.W., C.L.L. and L.Y.S. executed the experiment and analysed the data; C.H.W. designed the experiment and wrote the article.

Conflicts of interest
There are no conflicts of interest.

References
1 Hammond C, Bergman H, Brown P. Pathological synchronization in Parkinson’s disease: networks, models and treatments. Trends Neurosci 2007; 30:357–364.
2 Bergman H, Deuschl G. Pathophysiology of Parkinson’s disease: from clinical neurology to basic neuroscience and back. Mov Disord 2002; 17 (Suppl 3):S28–S40.
3 Ciucci MR, Ahrens AM, Ma ST, Kane JR, Windham EB, Woodlee MT, Schallert T. Reduction of dopamine synaptic activity: degradation of 50-4Hz ultrasonic vocalization in rats. Behav Neurosci 2009; 123:328–336.
4 John AR, Michelle RC, Nadine PC, Timothy S. Targeted exercise therapy for voice and swallow in persons with Parkinson’s disease. Brain Res 2010; 1341:3–11.
5 Vitele C, Marcelli V, Allocca R, Santangelo G, Riccardi P, Erro R, et al. Hearing impairment in Parkinson’s disease: expanding the nonmotor phenotype. Mov Disord 2012; 27:1530–1535.
6 Smeijne RJ, Jackson-Lewis V. The MPTP model of Parkinson’s disease. Brain Res Mol Brain Res 2005; 134:57–66.
7 Stephenson D, Ramirez A, Long J, Barrezeuta N, Hajes-Korsok E, Matheme C, et al. Quantification of MPTP-induced dopaminergic neurodegeneration in the mouse substantia nigra by laser capture microdissection. J Neurosci Methods 2007; 159:291–299.
8 Weireb O, Youdim MB. A model of MPTP-induced Parkinson’s disease in the goldfish. Nat Protoc 2007; 2:3016–3021.
9 Chan P, De Lanney LE, Irwin I, Langston JW, Di Monte D. Rapid ATP loss caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mouse brain. J Neurochem 1991; 57:348–351.
10 Meredith GE, Rademacher DJ. MPTP mouse models of Parkinson’s disease: an update. J Parkinsons Dis 2011; 1:19–33.
11 Sedelis M, Schwartz RK, Huston JP. Behavioral phenotype of the MPTP mouse model of Parkinson’s disease. Behav Brain Res 2001; 125:109–125.
12 Jenner P. Oxidative stress in Parkinson’s disease. Ann Neurol 2003; 53 (Suppl 3):S26–S36.
13 Kürkowska-Jastrzębska I, Błażkowiec-Isaka E, Cieśieliska A, Ironic I, Cudna A, Zaremba MM, et al. Decreased inflammation and augmented expression of trophic factors correlate with MOG-induced neuroprotection of the injured nigrostriatal system in the murine model of Parkinson’s disease. Int Immunopharmacol 2009; 9:781–791.
14 Turski L, Bresoler K, Rettig KJ, Löschmann PA, Wachtel H. Protection of substantia nigra from MPTP-induced neurotoxicity by N-methyl-D-aspartate antagonists. Nature 1991; 349:414–418.
15 Vyswanath V, Wu Y, Boonpleueang R, Chen S, Stevenson FF, Yantiri F, et al. Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson’s disease. J Neurosci 2001; 21:9519–9528.
16 Brouwer M, Huss A, van der Mark M, Nijssen PCG, Mulleners WM, Sas AMG, et al. Environmental exposure to pesticides and the risk of Parkinson’s disease in the Netherlands. Environ Int 2017; 107:100–110.
17 Hisao CJ, Lin CL, Lin TY, Wang SE, Wu CH. Imidacloprid toxicity impairs spatial memory of echolocation bats through neural apoptosis in hippocampal CA1 and medial entorhinal cortex areas. Neuroreport 2016; 27:462–466.
18 Wu CH, Lin CL, Wang SE, Lu CW. Effects of imidacloprid, a neonicotinoid insecticide, on the echolocation system of insectivorous bats. Pestic Biochem Physiol 2020; 163:94–101.
19 Neuweller G. Evolutionary aspects of bat echolocation. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 2003; 189:245–256.
20 Simmons JA, Fenton MB, D’Orell MJ. Echolocation and pursuit of prey by bats. Science 1979; 203:16–21.
21 Moss CF, Schnitzler HU. Behavioral studies of auditory information processing. Hearing Bats 1995; 5:87–145.
22 Campbell P, Reep RL, Stoll ML, Opip AG, Phelps SM. Conservation and diversity of Fo2 expression in murid rodents: functional implications. J Comp Neurol 2009; 512:84–100.
23 Valentine DE, Sinha SR, Moss CF. Orienting responses and vocalizations produced by microstimulation in the superior colliculus of the echolocating bat. J Comp Physiol 2002; 188:89–108.
24 Yin JX, Ruan YN, Liu JL, Zhang SY, Racey P. Foxp2 expression in an echolocating bat (Rhinolophus ferrumequinum): functional implications. Mammalian Biology 2017; 85:24–29.
25 Lai CS, Fisher SE, Hurst JA, Vargha-Khadem F, Monaco AP. A forkhead-domain gene is mutated in a severe speech and language disorder. Nature 2001; 413:519–523.
26 Li G, Wang J, Rossiter SJ, Jones G, Zhang S. Accelerated Foxp2 evolution produced by microstimulation in the superior colliculus of the echolocating bat. J Comp Physiol 2002; 188:89–108.
27 Tressler J, Schwartz C, Wellman P, Hughes S, Smotherman M. Regulation of bat echolocation pulse acoustics by striatal dopamine. J Exp Biol 2011; 214:3238–3247.
28 Suryljkee A, Moss CF. Echolocation behavior of big brown bat. J Accoust Soc Am 2000; 108:2419–2429.
29 Simmons JA. The resolution of target range by echolocating bats. J Comp Physiol A 2001; 197:157–173.
30 Poljak G, Marsh D, Bodenhamer R, Souther A. Echo-detecting characteristics of neurons in inferior colliculus of unanesthetized bats. Science 1977; 196:875–878.
31 Schug N, Braig C, Zimmermann U, Engel J, Winter H, Ruth P, et al. Differential expression of otoferlin in brain, vestibular system, immature and mature cochlea of the rat. Eur J Neurosci 2006; 24:3372–3380.
32 Shen YY, Liang L, Li GS, Murphy RW, Zhang YP. Parallel evolution of auditory genes for echolocation in bats and toothed whales. Plos Genet 2012; 8:e1002788.
33 Yasunaga S, Grati M, Cohen-Salmon M, El-Amraoui A, Mustapha M, Salem N, et al. A mutation in OTOF, encoding otoferlin, a FER-1-like protein, causes DFNB9, a nonsyndromic form of deafness. Nat Genet 1999; 21:363–369.

34 Barchi JR, Knowles JM, Simmons JA. Spatial memory and stereotypy of flight paths by big brown bats in cluttered surroundings. J Exp Biol 2013; 216:1053–1063.

35 Bergman H, Feingold A, Nini A, Raz A, Slovin H, Abeles M, Vaadia E. Physiological aspects of information processing in the basal ganglia of normal and parkinsonian primates. Trends Neurosci 1998; 21:32–38.

36 Hadjiconstantinou M, Neff NH. Enhancing aromatic L-amino acid decarboxylase activity: implications for L-DOPA treatment in Parkinson’s disease. CNS Neurosci Ther 2008; 14:340–351.

37 Villalba RM, Lee H, Smith Y. Dopaminergic denervation and spine loss in the striatum of MPTP-treated monkeys. Exp Neurol 2009; 215:220–227.

38 Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin IJ. A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Proc Natl Acad Sci USA 1983; 80:4546–4550.

39 Jenner P. The contribution of the MPTP-treated primate model to the development of new treatment strategies for Parkinson’s disease. Parkinsonism Relat Disord 2003; 9:131–137.

40 Kurkowska-Jastrzebska I, Wrońśka A, Kolutnicka M, Członkowski A, Członkowska A. The inflammatory reaction following 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine intoxication in mouse. Exp Neurol 1999; 156:50–61.