Pharmacology, Biochemistry and Behavior 99 (2011) 704–711

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Melatonin protects against neurobehavioral and mitochondrial deficits in a chronic mouse model of Parkinson's disease

Gaurav Patki [1], Yuen-Sum Lau *

Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX 77204, United States

ARTICLE INFO

Article history:
Received 13 May 2011
Received in revised form 21 June 2011
Accepted 24 June 2011
Available online 1 July 2011

Keywords:
Parkinson's disease
Chronic MPTP/probenecid model
Neurodegeneration
Neuroprotection
Mitochondrial dysfunction

ABSTRACT

Neuronal oxidative stress and mitochondrial dysfunction have been implicated in Parkinson's disease. Melatonin is a natural antioxidant and free radical scavenger that has been shown to effectively reduce cellular oxidative stress and protect mitochondrial functions in vitro. However, whether melatonin is capable of slowing down the neurodegenerative process in animal models of Parkinson's disease remains controversial. In this research, we examined long-term melatonin treatment on striatal mitochondrial and dopaminergic functions and on animal locomotor performance in a chronic mouse model of Parkinson's disease originally established in our laboratory by gradually treating C57BL/6 mice with 10 doses of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (15 mg/kg, s.c.) and probenecid (250 mg/kg, i.p.) over five weeks. We report here that when the chronic Parkinsonian mice were pre-treated and continuously treated with melatonin (5 mg/kg/day, i.p.) for 18 weeks, the defects of mitochondrial respiration, ATP and antioxidant enzyme levels detected in the striatum of chronic Parkinson's mice were fully preempted. Meanwhile, the striatal dopaminergic and locomotor deficits seen in the chronic Parkinson's mice were partially and significantly forestalled. These results imply that long-term melatonin is not only mitochondrial protective but also moderately neuronal protective in the chronic Parkinson's mice. Melatonin may potentially be effective for slowing down the progression of idiopathic Parkinson's disease and for reducing oxidative stress and respiratory chain inhibition in other mitochondrial disorders.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

Parkinson's disease (PD) is characterized by progressive degeneration of the dopamine (DA) neurons that project from the substantia nigra pars compacta (SNpc) to the corpus striatum. Clinically, nigrostriatal neurodegeneration leads to impaired movements, such as bradykinesia, rigidity, gait imbalance, and uncontrollable resting tremors. The exact etiology and process of PD neurodegeneration remain elusive. A combination of age, genetic and environmental factors has been implicated as likely causes for PD (Le Couteur et al., 2002). At the cellular and molecular level, one of the current hypotheses on PD neurodegeneration suggests that exposure to certain toxic xenobiotics could lead to non-physiological production and accumulation of endogenous oxidative free radicals, disruption of Ca^{2+} homeostasis, and ATP depletion in association with an inhibition of the mitochondrial complex I component in the neuronal electron transport system (Blum et al., 2001). Over time, mitochondrial dysfunction and release of proapoptotic stress factors will promote neural injuries and cell loss in conjunction with abnormal protein modification and accumulation, which are characteristics of PD neuropathology (Giasson et al., 2002).

In human PD, reduced mitochondrial complex I activity is found in the substantia nigra (SN) and platelets (Haas et al., 1995; Schapira et al., 1990), and mitochondrial DNA mutations have been detected (Bender et al., 2006). A link between mitochondrial dysfunction and PD-like neurodegeneration has also been demonstrated in vitro and in selected genetic animal models. An accumulation of α-synuclein in cultured human DA neurons induces reactive oxygen species (ROS)-mediated apoptosis (Xu et al., 2002). Parkinsonian symptoms are replicated in genetically engineered mice that are deficient of the mitochondrial respiratory chain (Ekstrand et al., 2007). Infusion of the mitochondrial complex I inhibitors rotenone or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces oxidative damage resulting in selective loss of SN DA neurons and buildup of cytoplasmic α-synuclein-immunoreactive inclusions (Betarbet et al., 2000; Fornai et al., 2005). Parkinsonism is shown in genetic models with point mutations of mitochondrial DNA (Thyagarajan et al., 2000). Administration of MPTP to α-synuclein overexpressed mice produces pathological features in mitochondria (Song et al., 2004). Parkin, a ubiquitin E3 ligase is associated with the outer mitochondrial membrane and is important for

* Corresponding author at: Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, 521 Science and Research Building 2, Houston, TX 77204, United States. Tel.: +1 713 743 1276; fax: +1 713 743 1229.
E-mail address: ylau2@uh.edu (Y.-S. Lau).
[1] Current address: Division of Biochemistry, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, United States.

0091-3057/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2011.06.026

the disposition of oxidatively damaged proteins. Mice that have parkin mutations or are parkin-deficient show elevated ROS levels and exhibit oxidative damage of the mitochondria and apoptosis of DA neurons (Palacino et al., 2004). In support of this mechanistic hypothesis, we have recently demonstrated discernible mitochondrial deficits in correlation with neurobehavioral deficits in an MPTP/probenecid-induced chronic mouse model of Parkinson's disease (MPD) (Lau et al., 2011; Patki et al., 2009).

Conceivably, one of the rational approaches for alleviating the drastic decline of mitochondrial and neuronal functions in PD should aim at reducing the risk of toxic xenobiotic exposure and also at preventing excessive cellular oxidative stress. The latter strategy may be attained by pharmacological agents that possess antioxidant and free radical scavenging properties. Several notable antioxidant agents, including creatine and coenzyme Q10, have been tested experimentally and clinically (Chaturvedi and Beal, 2008). However, results are somewhat varied. Melatonin (N-acetyl-5-methoxytryptamine) is a natural hormone primarily secreted by the pineal gland during darkness. It is known to produce a receptor-independent mitochondrial protective effect due to its antioxidant and free radical scavenging properties (Tan et al., 2002). Pharmacologically, melatonin readily crosses the blood–brain barrier, concentrates in the nuclear and mitochondrial compartments of the cell, and has relatively high efficacy and low toxicity (Acuna-Castroviejo et al., 2001). Melatonin has been tested as a potential neuroprotective agent in various experimental PD models. While some studies report that melatonin is neuroprotective in 6-hydroxydopamine (Sharma et al., 2006), MPTP (Capitelli et al., 2008; Ma et al., 2009), and rotenone-induced models of PD (Saravanan et al., 2007), other investigations using these in vivo experimental models either fail to detect neuroprotective effects of melatonin (Morgan and Nelson, 2001; van der Schyf et al., 2000) or even show potentiated neurodegeneration (Tapias et al., 2010). Most of these studies do not involve analysis of neurobehavioral and mitochondrial functions concomitantly. Furthermore, differences in the melatonin results obtained from animal models of PD could be due to experimental variability, such as the age and species of the animal model; the method, duration and severity of the induced nigrostriatal lesion; or the time, dosage, and duration of melatonin administration.

In the present investigation, we used a chronic MPD, which is gradually induced by a relatively low dose of MPTP over 5 weeks in aged C57BL/6 mice. It exhibits a long-lasting, moderate level of neurobehavioral and mitochondrial deficits (Lau et al., 2011; Patki et al., 2009). Melatonin was administered daily before, during and after MPD induction for a period of 18 weeks. For examining the impact of long-term melatonin treatment in the chronic MPD, paralleled measurements and analyses on neurobiological, behavioral and mitochondrial functions were conducted.

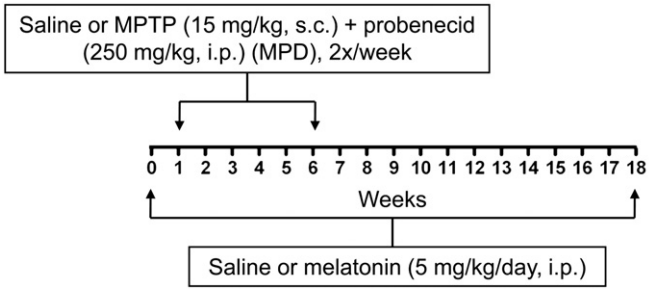

Fig. 1. Time scheme for animal treatments. Four groups of animals were treated. Chronic Sal/Sal: 18 weeks of daily saline, 5 weeks of 2× saline; chronic Mel/Sal: 18 weeks of daily melatonin, 5 weeks of 2× saline; chronic Sal/MPD: 18 weeks of daily saline, 5 weeks of 2× MPTP + probenecid; chronic Mel/MPD: 18 weeks of daily melatonin, 5 weeks of 2× MPTP + probenecid.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (6–10 months old, weighing 35–40 g) (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were housed in single cages with food pellets and water available ad libitum. The room was maintained at a constant temperature and humidity on a 12-h/12-h light/dark cycle (lights on at 7:00 h and off at 19:00 h). All animal treatments were carried out strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee from the University of Houston. A total of 48 mice were used in the present study.

2.2. Chronic mouse model of Parkinson's disease

To prepare the chronic MPD with moderate neurodegeneration, mice were injected with a total of 10 doses of MPTP hydrochloride (15 mg/kg/injection in saline, s.c.) in combination with an adjuvant drug, probenecid (250 mg/kg/injection dissolved in dimethyl sulfoxide, i.p.), which was originally established in our laboratory (Lau et al., 1990). The 10-dose regimen was administered on a 5-week schedule with an interval of 3.5 days between injections (Fig. 1). MPTP hydrochloride and probenecid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Safety precautions for the use of MPTP during chemical preparation and animal injections were taken according to the previously published procedures (Lau et al., 2005). Control mice were treated with saline only. Probenecid was used to inhibit the rapid clearance and excretion of MPTP and its metabolites from the brain and kidney. Alone, it did not produce any significant neurotoxic effect, but in combination it potentiated the neurotoxicity of MPTP (Barber-Singh et al., 2009; Lau, 2005; Lau et al., 2005).

The chronic MPD used in this study has been well characterized. In contrast to the most commonly used acute and subacute MPTP mouse models of PD, in which neurological and behavioral deficits are short-lived and spontaneously reversed soon after treatment, the chronic MPD has long-term neurological deficits showing many features resembling PD lasting for at least 6 months, long after MPTP has completely dissipated systemically from the animal (Lau, 2005). The observed phenotypic features in the chronic MPD include marked depletion of DA content and terminal DA uptake in association with significant behavioral deficits and loss of DA cells in the SNpc (Lau et al., 1990; Petroske et al., 2001; Pothakos et al., 2009). Early neuronal apoptosis and delayed appearance of α-synuclein-positive inclusion bodies along with ultrastructural neuronal damage in the SNpc have also been demonstrated (Meredith et al., 2002; Novikova et al., 2006).

2.3. Melatonin treatment

The chronic control and MPD animals were injected with melatonin (5 mg/kg, i.p.) around 12:00 noon daily. Melatonin (Sigma) was freshly prepared each day and protected from light exposure. The hormone was initially dissolved in a minimum amount of dimethyl sulfoxide and then was brought up to a desirable injection volume with saline. Melatonin was administered 1 week before, 5 weeks during, and 12 weeks after the chronic saline or MPD treatment (a total of 18 weeks) (Fig. 1). All neurochemical, mitochondrial, and behavioral measurements were performed at least 48 h after the last injection of melatonin to avoid any data misinterpretation due to acute melatonin residual effects.

2.4. Animal balance and motor coordination performance test

The ability of the chronic control or MPD mice to maintain balance and motor coordination on a challenging beam was carried out at 48 h

after 18 weeks of treatment with or without melatonin according to the procedures described by Drucker-Colin and Garcia-Hernandez (1991) and modified by Schallert et al. (2002). The challenging beam was a 1-m-long wooden beam suspended 23 cm above a bench top, which was covered with soft pads to protect the mouse in case of a fall. The beam was divided in four gradually narrowing sections (25 cm/section) leading to the mouse's home cage. The beam widths of the four sections were 3.5, 2.5, 1.5, and 0.5 cm in decreasing order. The beam was covered with surgical tape that provided sufficient surface traction for the animals to walk on. There were 1-cm-wide ledges hanging 1 cm below each side of the beam to encourage the mice to use their normal gait strategies even when their limbs slipped. All mice were pre-trained for two consecutive days (5 trials/day with an inter-trial interval of 10–12 s) on traversing the beam. On the third day, each mouse was given 5 trials (inter-trial interval of 10–12 s) and the average number of videotaped limb slips per trial and the time latency for returning to the home cage were recorded for statistical analysis. Slips were counted only while the mouse was in forward motion.

2.5. Assay for dopamine content

The DA contents in the SN and striatum were determined as previously described (Lau et al., 2005). Briefly, SN and striata from each animal were isolated, weighed and suspended in 0.2 ml and 0.5 ml of 0.2 N perchloric acid, respectively. Each sample was sonicated and centrifuged at 11,000 *g* for 15 min at 4 °C. The supernatant was filtered through a 4-mm nylon syringe filter with a pore size of 0.45 μm (National Scientific, Rockwood, TN, USA). An aliquot of the filtrate was injected into a high-performance liquid chromatography apparatus (Model 1525, Waters Corporation, Milford, MA, USA) equipped with a C_{18} reverse phase, 3 μm LUNA column (100 mm × 2.0 mm, Phenomenex, Torrance, CA, USA). The sample was eluted by a mobile phase made up of NaH_2PO_4 (25 mM), Na-citrate (50 mM), EDTA (0.03 mM), diethylamine HCl (10 mM), and sodium octyl sulfate (2.2 mM), at a pH of 3.2, plus methanol (30 ml/l) and dimethylacetamide (22 ml/l) at a flow rate of 0.4 ml/min. The DA peak was determined by the Coulometric electrochemical detector (Model Coulochem III, ESA, Inc., Chelmsford, MA, USA) and was calculated by extrapolating the peak area from a standard curve (0.05–1 ng of each chemical standard) constructed under the same conditions during each run.

2.6. Mitochondrial preparation

Due to tissue limitation, a crude striatal preparation was used for the mitochondrial respiration and protein assays in this study as previously described (Patki et al., 2009). Striata from each animal were isolated and homogenized with a dounce homogenizer in 1 ml of an ice-cold isolation buffer containing mannitol (215 mM), sucrose (75 mM), bovine serum albumin (0.1%), EGTA (1 mM), and HEPES (20 mM) at a pH of 7.2. All subsequent procedures were carried out at 4 °C. The homogenate was micro-centrifuged (Eppendorf Model 5402, Brinkmann Instruments, Westbury, NY, USA) at 138 *g* for 3 min. The supernatant was transferred to a new tube and the pellet was suspended in 0.5 ml of the isolation buffer and micro-centrifuged again at 138 *g* for 3 min. The supernatants from both spins were combined and micro-centrifuged at 13,800 *g* for 10 min. This latter supernatant was discarded and the pellet containing the mitochondrial fraction was suspended in 0.1 ml of a respiration buffer containing mannitol (215 mM), sucrose (75 mM), bovine serum albumin (0.1%), HEPES (20 mM), $MgCl_2$ (2 mM), and KH_2PO_4 (2.5 mM) at a pH of 7.2. The mitochondrial protein concentration was determined with the Pierce micro BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) measured at an absorbance of 595 nm with a Beckman DU 640 spectrophotometer.

2.7. Mitochondrial respiration assay

The respiratory activity of striatal mitochondria was measured polarographically with a Clark-type oxygen electrode in a sealed, thermo-controlled, and continuously stirred chamber (Oxytherm System, Hansatech Instruments, Norfolk, England) as previously described (Patki et al., 2009). The mitochondrial fraction at a protein concentration of 0.5 mg/ml of respiration buffer (see above) was initially equilibrated in the electrode chamber at 30 °C and then reacted with the addition of nicotinamide adenine dinucleotide-linked substrates, pyruvate (5 mM) and malate (2.5 mM). The state 3 respiration was initiated by adding adenosine 5′-diphosphate (150 μM) and the state 4 respiration was measured by adding oligomycin (1 μM) after adenosine 5′-diphosphate-dependent state 3 respiration had reached completion. The rate of oxygen consumption (nmol/min/mg mitochondrial protein) was calculated based on the slope of polarographic tracings obtained during state 3 and state 4 respiration. In this study, all mitochondrial preparations had demonstrated an average respiratory control ratio (state 3:state 4 respiration) of at least 5.

2.8. Measurement of mitochondrial ATP

Striatal mitochondrial samples were prepared under conditions identical to those of the respiration study. After the completion of respiration experiment, the mitochondrial suspension from the chamber was recovered and mixed with an equal volume of lysis buffer provided in the ATP bioluminescence assay kit (PerkinElmer, Waltham, MA, USA). The mitochondrial ATP content was measured according to the manufacturer's instructions. Light emitted from luciferase-mediated reaction was captured in a luminometer (Wallac Victor II, PerkinElmer) and the sample ATP content was extrapolated from a standard curve constructed with a series of known ATP concentrations (Patki et al., 2009).

2.9. Analysis of neuronal and mitochondrial protein expression

The protein expression of striatal DA synthesizing enzyme, tyrosine hydroxylase (TH), DA uptake transporter (DAT), mitochondrial antioxidant enzymes, Mn superoxide dismutase (SOD) and Cu–Zn SOD were determined following tissue solubilization, sodium dodecyl sulfate polyacrylamide (12%) gel electrophoresis and western blot transfer as described previously (Patki et al., 2009). The following primary antibodies and dilutions were used: monoclonal mouse anti-TH (1:1000, Millipore, Temecula, CA, USA), monoclonal rat anti-DAT (1:1000, Chemicon), polyclonal rabbit anti-Mn SOD (1:1000, Upstate, Lake Placid, NY, USA), and polyclonal rabbit anti-Cu–Zn SOD (1:1000, Stressgen Bioreagents, Victoria, BC, Canada). Immunoreactivity was visualized by a horseradish peroxidase-conjugated goat anti-rabbit (1:2000, Chemicon), goat anti-rat (1:2000, Chemicon) or goat anti-mouse (1:1500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) IgG with an enhanced NuGlo chemiluminescent substrate (Alpha Diagnostic International Inc., San Antonio, TX, USA). The exposure time of the blot on film was confined to the linear scale of detection without exceeding the saturation limit. The intensity of the protein bands was measured by densitometry (Fluorochem 8800, Alpha Innotech Corporation, San Leandro, CA, USA) and normalized as a ratio to the expression of β-tubulin with a monoclonal mouse anti-β-tubulin (1:2000, Upstate, Lake Placid, NY, USA) to ensure that changes of protein level are not simply due to differences in the amount of sample loading.

2.10. Statistical analysis

Statistical comparisons of values between two animal groups were carried out by unpaired Student's *t*-test and multiple group comparisons were conducted by one-way analysis of variance (ANOVA) with

Tukey's post-hoc test or two-way ANOVA with Bonferroni's post-hoc test using the PRISM software (GraphPad Software, Inc., La Jolla, CA, USA). Data are represented as mean ± S.E.M. In all cases, a P value of <0.05 was considered to be significantly different.

3. Results

3.1. Melatonin attenuates nigrostriatal dopaminergic deficit in the chronic mouse model of Parkinson's disease

We evaluated the dopaminergic biomarkers in the SN and neostriatum from four groups of chronically treated animals. When the 5-week chronic saline-injected mice were treated with 18 weeks of daily melatonin (chronic Mel/Sal), it did not alter the DA content in the SN and striatum when compared with the 5-week chronic saline-injected and 18-week daily saline-treated (chronic Sal/Sal) group ($P>0.40$ by unpaired Student's t-test) (Table 1). The abundance of striatal TH and DAT protein expression was not different between the two groups either ($P>0.65$ by unpaired Student's t-test) (Fig. 2). A moderate depletion of SN and striatal DA and loss of striatal TH and DAT levels were prominently detected in the daily saline and 5-week MPTP/probenecid-treated mice (chronic Sal/MPD) representing PD-like neurological deficit ($P<0.05$ by unpaired Student's t-test) (Table 1, Fig. 2). The nigral and striatal DA concentrations and striatal TH and DAT levels were significantly elevated in the 18-week melatonin-treated chronic MPD (chronic Mel/MPD) when compared with the non-melatonin-treated chronic MPD ($P<0.03$ by unpaired Student's t-test) (Table 1, Fig. 2). A significant difference due to treatment was also shown between chronic Mel/Sal, Sal/MPD and Mel/MPD groups when analyzed by a one-way ANOVA ($F_{2,11}=4.32$, $P=0.047$ for SN DA; $F_{2,11}=52.38$, $P<0.0001$ for striatal DA; $F_{2,11}=33.09$, $P<0.0001$ for striatal TH; $F_{2,11}=57.85$, $P<0.0001$ for striatal DAT) or for interaction among all 4 groups when analyzed by a two-way ANOVA ($F_{1,18}=4.78$, $P=0.043$ for SN DA; $F_{1,18}=4.44$, $P=0.049$ for striatal DA; $F_{1,18}=5.59$, $P=0.030$ for striatal TH; $F_{1,18}=4.54$, $P=0.047$ for striatal DAT) (Table 1, Fig. 2). These data suggest that long-term daily melatonin supplement using the current dosing scheme has a neuroprotective potential for preserving the nigrostriatal DA function in the chronic MPD.

3.2. Melatonin ameliorates locomotor deficit in the chronic mouse model of Parkinson's disease

We further assessed the animal's balance and motor coordination skills on a challenging beam. Eighteen weeks of daily melatonin treatment in the chronic Mel/Sal mice did not alter their performance on the challenging beam ($P>0.61$ by unpaired Student's t-test) (Fig. 3). As predicted, the chronic Sal/MPD mice performed poorly by committing more foot-slips while traversing on the balancing beam (Fig. 3A) and presented a delayed latency for returning to the home cage (Fig. 3B) when compared with the control Sal/Sal or Mel/Sal group of animals ($P<0.0001$ by unpaired Student's t-test). The deficit in balance and motor performance was significantly protected in 18-week melatonin-treated chronic Mel/MPD mice; the treated mice performed significantly better than the chronic Sal/MPD in both foot-slip and latency measures ($P<0.01$ by unpaired Student's t-test). A significant difference due to treatment was also shown between chronic Mel/Sal, Sal/MPD and Mel/MPD groups when analyzed by a one-way ANOVA ($F_{2,23}=31.81$, $P<0.0001$ for foot-slip and $F_{2,23}=16.90$, $P<0.0001$ for latency) or for interaction among all 4 groups when analyzed by a two-way ANOVA ($F_{1,29}=5.41$, $P=0.027$ for foot-slip and $F_{1,29}=6.96$, $P=0.013$ for latency) (Fig. 3A–B). These data suggest that long-term daily melatonin treatment prevents locomotor deficit in the chronic MPD.

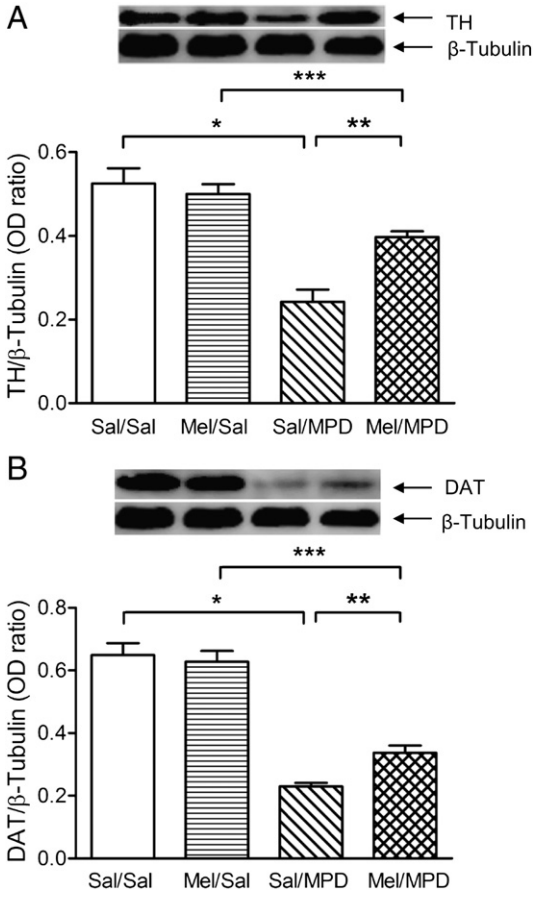

Fig. 2. Chronic melatonin treatment on striatal TH and DAT levels in the chronic MPD. Results are western immunoblot analyses of (A) TH and (B) DAT expression in the striatum of chronic Sal/Sal (N=8), Mel/Sal (N=4), Sal/MPD (N=4), and Mel/MPD (N=6) groups. A representative image of western blot protein bands was correspondingly shown above each set of bar graph. The abundance of TH and DAT was expressed as a ratio to that of β-tubulin. Each data point represents mean ± S.E.M. *Significantly lower than the chronic Sal/Sal-treated mice ($P<0.0005$, unpaired Student's t-test). **Significantly higher than the chronic Sal/MPD-treated mice ($P<0.009$, unpaired Student's t-test). ***Significantly lower than the chronic Mel/Sal-treated group ($P<0.004$, unpaired Student's t-test).

Table 1
Chronic melatonin treatment on DA content in the substantia nigra and striatum of the chronic mouse model of Parkinson's disease.

	Dopamine (ng/mg tissue)	
Animal group	Substantia nigra	Striatum
Chronic Sal/Sal (N=8)	3.40 ± 0.58	14.53 ± 1.07
Chronic Mel/Sal (N=4)	3.11 ± 0.45	15.94 ± 0.50
Chronic Sal/MPD (N=4)	1.57 ± 0.35[a]	4.43 ± 0.64[a]
Chronic Mel/MPD (N=6)	3.58 ± 0.60[b]	8.50 ± 0.84[c]

Data are presented as mean ± S.E.M.
[a] Significantly lower than either the chronic Sal/Sal or chronic Mel/Sal group by unpaired Student's t-test ($P<0.05$).
[b] Significantly higher than the chronic Sal/MPD group by unpaired Student's t-test ($P=0.03$), but not significantly different from the chronic Sal/Sal or Mel/Sal group ($P>0.50$).
[c] Significantly higher than the chronic Sal/MPD group ($P=0.008$) and significantly lower than the chronic Sal/Sal or Mel/Sal group by unpaired Student's t-test ($P<0.001$).

3.3. Melatonin blocks mitochondrial deficit in the chronic mouse model of Parkinson's disease

Next, we examined whether melatonin could avert mitochondrial dysfunction in the chronic MPD. Eighteen weeks of melatonin treatment in chronic Mel/Sal mice did not influence the rate of

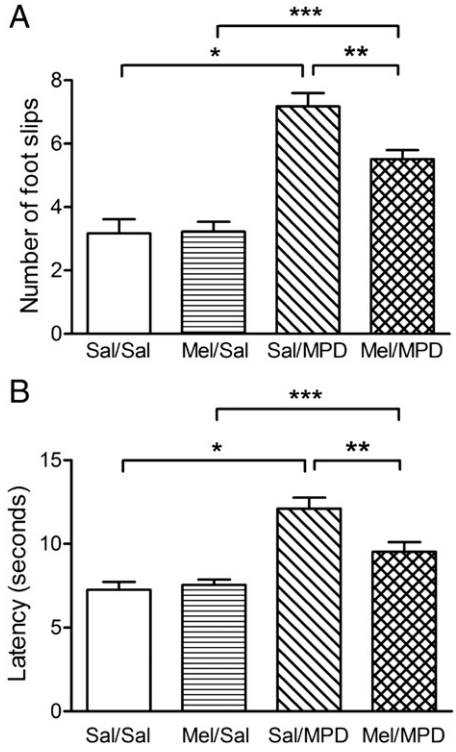

Fig. 3. Chronic melatonin treatment on behavioral deficit in the chronic MPD. The ability of the chronic MPD for maintaining balance and motor coordination on a challenging beam was carried out 48 h after the completion of last treatment. While traversing the challenging beam, the chronic Sal/MPD group of mice significantly made more limb slip errors (A) and took longer to complete the task (B) than the chronic Sal/Sal-treated control mice (*$P=0.0001$, unpaired Student's t-test). Following 18 weeks of melatonin injection, the chronic Mel/MPD mice made considerably less foot slips on the challenging beam (A) and spent less time for completing the traversing task (B) than the chronic Sal/MPD-treated mice (**$P<0.01$, unpaired Student's t-test); but these animals were still moderately impaired when compared with the chronic Mel/Sal-treated mice (***$P<0.02$, unpaired Student's t-test). N = 7–9 mice per group.

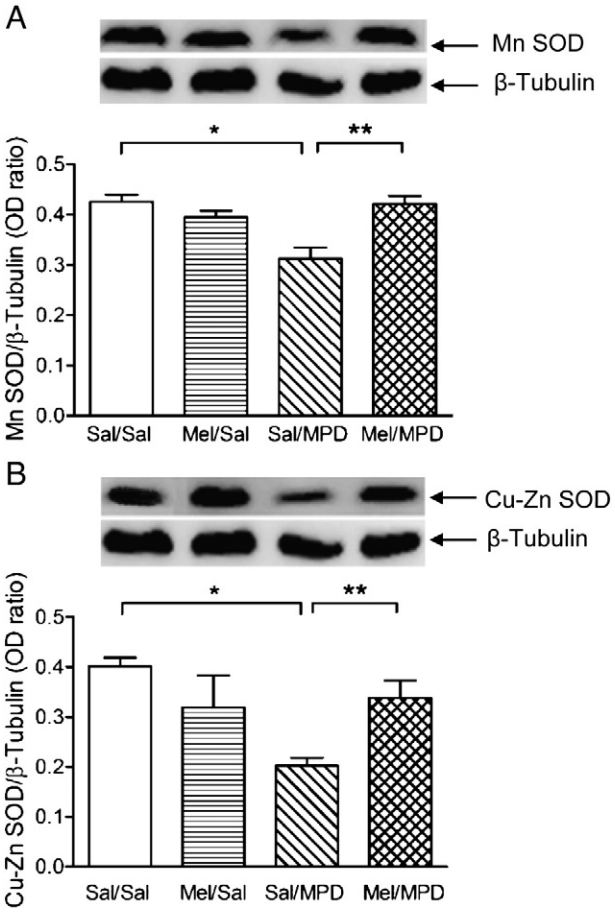

Fig. 4. Chronic melatonin treatment on mitochondrial antioxidant protein levels in the chronic MPD. Results are western immunoblot analyses of (A) Mn SOD and (B) Cu–Zn SOD expression in the striatum of chronic Sal/Sal (N = 8), Mel/Sal (N = 4), Sal/MPD (N = 4), and Mel/MPD (N = 6) groups. A representative image of western blot protein bands was correspondingly shown above each set of bar graph. The amount of Mn SOD and Cu–Zn SOD was expressed as a ratio to that of β-tubulin. Each data point represents mean ± S.E.M. *Significantly lower than the chronic Sal/Sal-treated mice ($P<0.002$, unpaired Student's t-test). **Significantly higher than the chronic Sal/MPD-treated mice ($P<0.02$) but not statistically different from the chronic Mel/Sal-treated group ($P>0.25$) by unpaired Student's t-test.

mitochondrial state 3 or state 4 respiration, ATP content ($P>0.20$ by unpaired Student's t-test) (Table 2) and the abundance of mitochondrial antioxidant enzymes (Mn SOD and Cu–Zn SOD) ($P>0.10$ by unpaired Student's t-test) when compared with the chronic Sal/Sal animals (Fig. 4). As anticipated, the mitochondrial function as represented by the rate of state 3 and state 4 respiration, ATP level (Table 2), and the mitochondrial Mn SOD and Cu–Zn SOD levels (Fig. 4) were all considerably reduced in the chronic Sal/MPD mice when compared with the chronic Sal/Sal or Mel/Sal animals ($P<0.05$ by unpaired Student's t-test). These observations collectively confirm that mitochondrial dysfunction persists in the chronic MPD in association with the neurodegenerative condition.

Table 2
Chronic melatonin treatment on mitochondrial respiration and ATP level in the striatum of chronic mouse model of Parkinson's disease.

Animal group	State 3	State 4	ATP
Chronic Sal/Sal (N = 5)	17.62 ± 0.38	2.88 ± 0.07	15.18 ± 0.74
Chronic Mel/Sal (N = 4)	16.84 ± 0.94	2.66 ± 0.16	14.80 ± 1.01
Chronic Sal/MPD (N = 5)	9.48 ± 0.62[a]	1.84 ± 0.10[a]	7.86 ± 0.59[a]
Chronic Mel/MPD (N = 6)	16.01 ± 0.49[b]	2.71 ± 0.07[b]	13.01 ± 0.81[b]

Mitochondrial state 3 and state 4 respiration values are measures of the rate of oxygen consumption in nmol/min/mg protein. ATP values are in nM/mg protein. Data are presented as mean ± S.E.M.

[a] Significantly lower than either the chronic Sal/Sal or chronic Mel/Sal group by unpaired Student's t-test ($P<0.05$).

[b] Significantly higher than the chronic Sal/MPD group ($P<0.002$), but not statistically different from the chronic Sal/Sal or Mel/Sal group ($P>0.05$) by unpaired Student's t-test.

Significantly, when the chronic MPD was treated daily with melatonin for 18 weeks, we were able to demonstrate that they maintained normal levels of mitochondrial respiration and ATP when compared with the chronic Sal/MPD ($P<0.002$ by unpaired Student's t-test). A significant difference due to treatment was also shown between chronic Mel/Sal, Sal/MPD and Mel/MPD groups when analyzed by a one-way ANOVA ($F_{2,12}=37.40$, $P<0.0001$ for state 3 respiration; $F_{2,12}=21.29$, $P=0.0001$ for state 4 respiration; $F_{2,12}=16.09$, $P=0.0005$ for ATP) or for interaction among all 4 groups when analyzed by a two-way ANOVA ($F_{1,16}=37.04$, $P<0.0001$ for state 3 respiration; $F_{1,16}=30.13$, $P<0.0001$ for state 4 respiration; $F_{1,16}=10.29$, $P=0.005$ for ATP) (Table 2). The contents of striatal antioxidant enzymes, Mn SOD and Cu–Zn SOD were also considerably higher in the chronic Mel/MPD than in the chronic Sal/MPD mice ($P<0.02$ by unpaired Student's t-test) (Fig. 4). While the difference due to treatment between chronic Mel/Sal, Sal/MPD and Mel/MPD was significant for Mn SOD level ($F_{2,11}=10.47$, $P=0.003$), the difference for Cu–Zn SOD was not significant due to high experimental variations among the chronic Mel/Sal group ($F_{2,11}=3.01$, $P=0.091$) when analyzed by a one-way ANOVA (Fig. 4). However, when all 4 groups of animals were analyzed by a two-way ANOVA, significant

interactions for both Mn SOD ($F_{1,18}=15.92$, $P=0.0009$) and Cu–Zn SOD ($F_{1,18}=9.79$, $P=0.006$) were demonstrated (Fig. 4). These data illustrate that 18 weeks of daily melatonin treatment prevented the loss of striatal mitochondrial integrity and function in the chronic MPD, suggesting that long-term melatonin is mitochondrial protective.

4. Discussion

The purpose of this study was to determine whether melatonin, a known antioxidant and free radical scavenger, could intervene with the neurobehavioral and mitochondrial deficits in a chronic experimental model of PD. We found that when melatonin was administered daily for a long period spanning one week before, 5 weeks during, and 12 weeks after the chronic MPD induction, the amount of nigrostriatal DA and the abundance of striatal TH and DAT were significantly less impaired than in the non-melatonin-treated chronic MPD. The neuroprotective effect of melatonin was corroborated by behavioral improvement as well. Thus, the melatonin-treated chronic MPD performed better than the non-melatonin-treated chronic MPD on the challenging beam. We also found that long-term melatonin treatment protected against mitochondrial dysfunction and loss of antioxidant proteins that were detected in the chronic MPD. As demonstrated in the chronic MPTP/probenecid-induced MPD, we conclude that mitochondrial dysfunction is at least in part intimately related or cause-and-effect related to neurological and behavioral deficits, and long-term melatonin treatment is both neurobehavioral and mitochondrial protective.

Our present findings on prevention of neurotoxic xenobiotic-induced, oxidative stress-associated DA neuron degeneration and behavioral impairment in the chronic MPD are consistent with some recent investigations using 6-hydroxydopamine, MPTP and rotenone models of PD (Capitelli et al., 2008; Ma et al., 2009; Saravanan et al., 2007; Sharma et al., 2006). On the contrary, other studies have reported that melatonin either does not protect or even exacerbates neuronal loss in the experimental PD models (Morgan and Nelson, 2001; Tapias et al., 2010; van der Schyf et al., 2000). In this research, we compared with the published in vivo melatonin studies that use MPTP as the neurotoxic inducing agent and designed our experiments by incorporating the following approaches.

First, most published studies use a series of rapid and multiple-dose MPTP injections within a few hours that tend to cause a robust DA neurotoxicity acutely (Ma et al., 2009; Morgan and Nelson, 2001). In our present study, we employed a chronically induced PD model with 10 low doses of MPTP for a duration of 5 weeks. This model has displayed persistent neurobiological, pathological, and behavioral phenotypes resembling that of PD for many months after the parkinsonism induction (Lau, 2005; Novikova et al., 2006; Pothakos et al., 2009). In terms of melatonin administration, high doses (20–50 mg/kg/day) have been given in most studies (Capitelli et al., 2008; Ma et al., 2009; Tapias et al., 2010), whereas a lower daily parenteral dose (5 mg/kg/day) was administered in our 18-week chronic study. In an initial pilot study, higher doses of melatonin were also tested; at 10 mg/kg/day we began to detect adverse reactions of melatonin in the chronic MPD.

Second, by reviewing the published reports, the endpoint of treatment analyses in many studies were typically conducted within a week after neurotoxic induction or melatonin treatment (Capitelli et al., 2008; Ma et al., 2009; Morgan and Nelson, 2001), during which MPTP may still have residual neurotoxic effects, and melatonin may still possess a single dose effect from the last administration. Melatonin is reported to rhythmically suppress TH synthesis and DA release in the striatum, which may indirectly reduce the production of toxic hydroxyl radicals (Khaldy et al., 2002; Lemmer and Berger, 1978). Melatonin is also shown to down-regulate the DAT, which may cause inhibition of MPTP uptake and lead to less toxicity (Lin et al., 2008). The diurnal circadian secretion of melatonin is also synchronized with the animal's locomotor activity (Nakahara et al., 2003). Therefore, to alleviate any residual melatonin effect that may cause misanalysis and misinterpretation of the data in a chronic study, we examined the pharmacological outcome of melatonin over 18 weeks of treatment and 12 weeks after parkinsonian induction. In addition, all data analyses were carried out at least 48 h after the last pharmacological manipulation. Because of this experimental design, we demonstrated in this study that after 18 weeks of daily low dose of melatonin administration, the baseline levels of striatal DA, TH, DAT, animal's balance and coordination performance, mitochondrial respiration, ATP, and antioxidant proteins were all unaltered, when compared with the non-melatonin-treated animals.

Third, age is also an imperative factor that links melatonin secretion, mitochondrial bioenergetics and neurodegenerative disorders. In all species, it has been shown that increase of age is associated with a gradual decline of the nocturnal melatonin rhythm (Sack et al., 1986). The aging process is also evidently connected to a reduced capacity of mitochondrial and neuronal responses to metabolic stress resulting in their structural and functional changes (Conley et al., 2007; Toescu et al., 2000). It is well recognized that neurodegenerative diseases, like idiopathic PD and Alzheimer's disease generally display late-age symptomatic onset and progression. Considering the entwined relationship between the above mentioned factors, melatonin has been suggested as a potential therapeutic agent for slowing down normal brain aging and for treating neurodegenerative conditions (Sharman et al., 2008). Based on laboratory findings, old age C57BL/6 mice are shown to be more susceptible to exogenous neurotoxic xenobiotics, such as MPTP, for inducing an experimental form of parkinsonism (Ricaurte et al., 1987). In our laboratory, we also found that co-existence of parkinsonism and mitochondrial dysfunction induced by the chronic MPTP/probenecid protocol is better demonstrated in the aged rather than in the young adult C57BL/6 mice to provide a suitable model for concurrently studying neurological and mitochondrial deficits relevant to PD (Patki et al., 2009). With this model, our findings that long-term melatonin treatment attenuates neurobehavioral and mitochondrial deficits in the chronic MPD further provide strong evidence illustrating an effective therapeutic role of melatonin in preserving mitochondrial and neurological functions.

Although melatonin produced a significant protection against neurobehavioral and mitochondrial deficits in the chronic MPD, melatonin appeared to safeguard mitochondrial functions to a greater extent than to the dopaminergic and behavioral manifestations. We believe that in addition to the prevalent mechanism on mitochondrial oxidative stress and bioenergetic defects underlying MPTP toxicity, MPTP-induced chronic parkinsonism may trigger other neurodegenerative mechanisms, like the release of proinflammatory cytotoxic factors involving microglial and astroglial reactions (Kurkowska-Jastrzebska et al., 1999) and inhibition of the ubiquitin–proteasome system (Fornai et al., 2005) that may not be protected by melatonin treatment alone. In other words and based on our results, mitochondrial deficiency may play a partial role in neurotoxin-induced chronic parkinsonism.

Customary exercise in human subjects has also been reported to influence the endogenous secretion of melatonin that may vary according to time of day or exercise intensity (Buxton et al., 1997). In a separate study, we observed that following 18 weeks of treadmill exercise training in the chronic MPD, the MPTP/probenecid-induced protein oxidation, perturbed mitochondrial function, loss of dopaminergic neurons and transmission, and locomotor impairment are similarly intervened as shown here with melatonin (Lau et al., 2011). A link between exercise, melatonin, and antioxidant protection in the chronic MPD is being further investigated in our laboratory.

5. Conclusions

In this research we employed pharmacological approaches to examine the long-term effect of melatonin as an antioxidant and free

radical scavenger on neurobehavioral and mitochondrial deficits in a chronic MPTP mouse model of PD. Our results indicate that when melatonin is administered daily for 18 weeks, striatal mitochondrial respiration, ATP and antioxidant enzymes in the chronic MPD are maintained at normal levels. At the same time, the dopaminergic and locomotor defects seen in the chronic MPD are partially and significantly reduced. These results implicate that long-term melatonin is not only mitochondrial protective but also moderately neuronal protective in the chronic MPD. Melatonin and its related drugs may potentially serve as an effective adjuvant therapy for slowing down the progression of idiopathic PD. Melatonin may also be valuable for modifying other mitochondrial disorders in which oxidative stress and respiratory chain inhibition are the underlying causes.

Abbreviations

ANOVA	analysis of variance
DA	dopamine
DAT	dopamine uptake transporter
Mel	melatonin-treated
MPD	mouse model of Parkinson's disease
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
ROS	reactive oxygen species
Sal	saline-treated
SN	substantia nigra
SNpc	substantia nigra pars compacta
SOD	superoxide dismutase
TH	tyrosine hydroxylase

Acknowledgments

This work was supported by a grant from the US National Institute of Neurological Disorders and Stroke (NS 47920 to Y.S.L.). Preliminary results of this study were presented in abstract form at the 40th Society for Neuroscience Annual Meeting, November 13–17, 2010 in San Diego, CA, U.S.A. Data presented in this manuscript are in partial fulfillment of the requirement for the Doctor of Philosophy Degree submitted by G.P. to the University of Houston.

References

Acuna-Castroviejo D, Martin M, Macias M, Escames G, Leon J, Khaldy H, Reiter RJ. Melatonin, mitochondria, and cellular bioenergetics. J Pineal Res 2001;30:65–74.

Barber-Singh J, Seo BB, Nakamaru-Ogiso E, Lau YS, Matsuno-Yagi A, Yagi T. Neuroprotective effect of long-term NDI1 gene expression in a chronic mouse model of Parkinson disorder. Rejuvenation Res 2009;12:259–67.

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, Jaros E, Hersheson JS, Betts J, Klopstock T, Taylor RW, Turnbull DM. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet 2006;38:515–7.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000;3:1301–6.

Blum D, Torch S, Lambeng N, Nissou M, Benabid AL, Sadoul R, Verna JM. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. Prog Neurobiol 2001;65:135–72.

Buxton OM, L'Hermite-Baleriaux M, Hirschfeld U, Cauter E. Acute and delayed effects of exercise on human melatonin secretion. J Biol Rhythms 1997;12:568–74.

Capitelli C, Sereniki A, Lima MM, Reksidler AB, Tufik S, Vital MA. Melatonin attenuates tyrosine hydroxylase loss and hypolocomotion in MPTP-lesioned rats. Eur J Pharmacol 2008;594:101–8.

Chaturvedi RK, Beal MF. Mitochondrial approaches for neuroprotection. Ann N Y Acad Sci 2008;1147:395–412.

Conley KE, Marcinek DJ, Villarin J. Mitochondrial dysfunction and age. Curr Opin Clin Nutr Metab Care 2007;10:688–92.

Drucker-Colin R, Garcia-Hernandez F. A new motor test sensitive to aging and dopaminergic function. J Neurosci Methods 1991;39:153–61.

Ekstrand MI, Terzioglu M, Galter D, Zhu S, Hofstetter C, Lindqvist E, Thams S, Bergstrand A, Hansson FS, Trifunovic A, Hoffer B, Cullheim S, Mohammed AH, Olson L, Larsson NG. Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. Proc Natl Acad Sci U S A 2007;104:1325–30.

Fornai F, Schluter OM, Lenzi P, Gesi M, Ruffoli R, Ferrucci M, Lazzeri G, Busceti CL, Pontarelli F, Battaglia G, Pellegrini A, Nicoletti F, Ruggieri S, Paparelli A, Sudhof TC. Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin–proteasome system and alpha-synuclein. Proc Natl Acad Sci U S A 2005;102:3413–8.

Giasson BI, Ischiropoulos H, Lee VM, Trojanowski JQ. The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. Free Radic Biol Med 2002;32:1264–75.

Haas RH, Nasirian F, Nakano K, Ward D, Pay M, Hill R, Shults CW. Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. Ann Neurol 1995;37:714–22.

Khaldy H, Leon J, Escames G, Bikjdaouene L, Garcia JJ, Acuna-Castroviejo D. Circadian rhythms of dopamine and dihydroxyphenyl acetic acid in the mouse striatum: effects of pinealectomy and of melatonin treatment. Neuroendocrinology 2002;75:201–8.

Kurkowska-Jastrzebska I, Wronska A, Kohutnicka M, Czlonkowski A, Czlonkowska A. The inflammatory reaction following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine intoxication in mouse. Exp Neurol 1999;156:50–61.

Lau YS. Progressive neurodegeneration in the MPTP/probenecid model of Parkinson's disease. In: Ebadi M, Pfeiffer R, editors. Parkinson's disease. Boca Raton, FL: CRC Press; 2005. p. 109–15.

Lau YS, Trobough KL, Crampton JM, Wilson JA. Effects of probenecid on striatal dopamine depletion in acute and long-term 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. Gen Pharmacol 1990;21:181–7.

Lau YS, Novikova L, Roels C. MPTP treatment in mice does not transmit and cause Parkinsonian neurotoxicity in non-treated cagemates through close contact. Neurosci Res 2005;52:371–8.

Lau YS, Patki G, Das-Panja K, Le WD, Ahmad SO. Neuroprotective effects and mechanisms of exercise in a chronic mouse model of Parkinson's disease with moderate neurodegeneration. Eur J Neurosci 2011;33:1264–74.

Le Couteur DG, Muller M, Yang MC, Mellick GD, McLean AJ. Age–environment and gene–environment interactions in the pathogenesis of Parkinson's disease. Rev Environ Health 2002;17:51–64.

Lemmer B, Berger T. Diurnal rhythm in the central dopamine turnover in the rat. Naunyn Schmiedebergs Arch Pharmacol 1978;303:257–61.

Lin CH, Huang JY, Ching CH, Chuang JI. Melatonin reduces the neuronal loss, downregulation of dopamine transporter, and upregulation of D2 receptor in rotenone-induced parkinsonian rats. J Pineal Res 2008;44:205–13.

Ma J, Shaw VE, Mitrofanis J. Does melatonin help save dopaminergic cells in MPTP-treated mice? Parkinsonism Relat Disord 2009;15:307–14.

Meredith GE, Totterdell S, Petroske E, Santa Cruz K, Callison Jr RC, Lau YS. Lysosomal malfunction accompanies alpha-synuclein aggregation in a progressive mouse model of Parkinson's disease. Brain Res 2002;956:156–65.

Morgan WW, Nelson JF. Chronic administration of pharmacological levels of melatonin does not ameliorate the MPTP-induced degeneration of the nigrostriatal pathway. Brain Res 2001;921:115–21.

Nakahara D, Nakamura M, Iigo M, Okamura H. Bimodal circadian secretion of melatonin from the pineal gland in a living CBA mouse. Proc Natl Acad Sci U S A 2003;100:9584–9.

Novikova L, Garris BL, Garris DR, Lau YS. Early signs of neuronal apoptosis in the substantia nigra pars compacta of the progressive neurodegenerative mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/probenecid model of Parkinson's disease. Neuroscience 2006;140:67–76.

Palacino JJ, Sagi D, Goldberg MS, Krauss S, Motz C, Wacker M, Klose J, Shen J. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. J Biol Chem 2004;279:18614–22.

Patki G, Che Y, Lau Y. Mitochondrial dysfunction in the striatum of aged chronic mouse model of Parkinson's disease. Front Aging neurosci 2009;1:1–9.

Petroske E, Meredith GE, Callen S, Totterdell S, Lau YS. Mouse model of Parkinsonism: a comparison between subacute MPTP and chronic MPTP/probenecid treatment. Neuroscience 2001;106:589–601.

Pothakos K, Kurz MJ, Lau YS. Restorative effect of endurance exercise on behavioral deficits in the chronic mouse model of Parkinson's disease with severe neurodegeneration. BMC Neurosci 2009;10:6.

Ricaurte GA, Irwin I, Forno LS, DeLanney LE, Langston E, Langston JW. Aging and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced degeneration of dopaminergic neurons in the substantia nigra. Brain Res 1987;403:43–51.

Sack RL, Lewy AJ, Erb DL, Vollmer WM, Singer CM. Human melatonin production decreases with age. J Pineal Res 1986;3:379–88.

Saravanan KS, Sindhu KM, Mohanakumar KP. Melatonin protects against rotenone-induced oxidative stress in a hemiparkinsonian rat model. J Pineal Res 2007;42:247–53.

Schallert T, Woodlee MT, Fleming SM. Disentangling multiple types of recovery from brain injury. In: Krieglstein J, Klumpp S, editors. Pharmacology of cerebral ischemia. Stuttgart, Germany: Medpharm Scientific Publishers; 2002. p. 201–16.

Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 1990;54:823–7.

Sharma R, McMillan CR, Tenn CC, Niles LP. Physiological neuroprotection by melatonin in a 6-hydroxydopamine model of Parkinson's disease. Brain Res 2006;1068:230–6.

Sharman EH, Sharman KG, Bondy SC. Melatonin causes gene expression in aged animals to respond to inflammatory stimuli in a manner differing from that of young animals. Curr Aging Sci 2008;1:152–8.

Song DD, Shults CW, Sisk A, Rockenstein E, Masliah E. Enhanced substantia nigra mitochondrial pathology in human alpha-synuclein transgenic mice after treatment with MPTP. Exp Neurol 2004;186:158–72.

Tan DX, Reiter RJ, Manchester LC, Yan MT, El-Sawi M, Sainz RM, Mayo JC, Kohen R, Allegra M, Hardeland R. Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. Curr Top Med Chem 2002;2:181–97.

Tapias V, Cannon JR, Greenamyre JT. Melatonin treatment potentiates neurodegeneration in a rat rotenone Parkinson's disease model. J Neurosci Res 2010;88:420–7.
Thyagarajan D, Bressman S, Bruno C, Przedborski S, Shanske S, Lynch T, Fahn S, DiMauro S. A novel mitochondrial 12SrRNA point mutation in parkinsonism, deafness, and neuropathy. Ann Neurol 2000;48:730–6.
Toescu EC, Myronova N, Verkhratsky A. Age-related structural and functional changes of brain mitochondria. Cell Calcium 2000;28:329–38.
van der Schyf CJ, Castagnoli K, Palmer S, Hazelwood L, Castagnoli Jr N. Melatonin fails to protect against long-term MPTP-induced dopamine depletion in mouse striatum. Neurotox Res 2000;1:261–9.
Xu J, Kao SY, Lee FJ, Song W, Jin LW, Yankner BA. Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. Nat Med 2002;8:600–6.