Neuronal and Mitochondrial Protection

In Chronic Parkinsonism

A Dissertation Presentation to

The Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of

The Requirement for the Degree

Doctor of Philosophy

By

Gaurav Deepak Patki

May 2010

NEURONAL AND MITOCHONDRIAL PROTECTION IN CHRONIC PARKINSONISM

A dissertation for the degree Doctor of Philosophy

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Gaurav Deepak Patki

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ABSTRACT

Among the several plausible neuropathological hypotheses underlying the progression of aging and neurodegenerative diseases such as Parkinson's disease (PD), oxidative stress and mitochondrial damage have emerged as one of the attractive biochemical mechanisms. However, most of the past studies linking mitochondrial dysfunction to neuronal degeneration have been carried out *in vitro* or in acute *in vivo* studies. It is not known whether mitochondrial dysfunction is an immediate response to cytotoxic-inducing agents or is sustained as a long-term consequence of neuronal death. The purpose of our research was to first validate the chronic mouse model of Parkinson's disorder (MPD), which has been developed and characterized by our laboratory as a suitable animal model for investigating neuronal and mitochondrial dysfunctions and then to examine the protective effects of endurance exercise and melatonin treatment on this model.

Male, C57/BL retired breeder mice at 6-10 months of age were used in the present study. The aged chronic MPD was treated with of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (15 mg/kg, s.c), twice a week for 5 weeks. MPTP was co-administered with probenecid (250 mg/kg, i.p), an adjuvant that is known to inhibit the peripheral and neuronal clearance of MPTP and potentiate the neurotoxicity of MPTP. The chronic MPD was previously shown to display neurochemical, histological,

behavioral and pathological features resembling those of PD-like neurodegeneration lasting for at least 6 months.

Six to twelve weeks after chronic MPTP treatment, aged mice showed sustained decrease in striatal mitochondrial respiration as well as loss of antioxidant enzymes, Cu-Zn superoxide dismutase (SOD), Mn SOD and cytochrome c (cyt c) expression. Striatal mitochondrial dysfunction correlated with dopamine neuron and behavioral deficits in the aged chronic MPD. When the chronic MPD was exercise-trained on a motorized treadmill 5 days/week for 18 weeks, the dopamine neuronal, mitochondrial and behavioral deficits as seen in the sedentary chronic MPD were prevented.

Melatonin (5 mg/kg, i.p), a known natural antioxidant and free radical scavenger was injected to the chronic MPD 5 days/week for 18 weeks. Melatonin alone did not alter the striatal neuronal, mitochondrial and motor functions in normal mice. However, melatonin was effective to reverse dopaminergic, mitochondrial and motor impairment as exercise did to the chronic MPD. We conclude that endurance exercise training and melatonin treatment are effective neuroprotective and mitochondrial protective measures in the chronic MPD. Exercise and melatonin treatment may have the potential to slow the progression of PD related neurodegeneration.

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LIST OF ABBREVIATIONS

ADP	Adenosine 5′-diphosphate
AFMK	N^1-acetyl-N^2-formyl-5-methoxykynuramine
AIFs	Apoptosis initiating factors
ANT	Adenine nucleotide translocator
ATP	Adenosine triphosphate
BG	Basal ganglia
BDNF	Brain derived neurotrophic factor
Ca^{++}	Calcium
CAT	Catalase
CoQ	Coenzyme Q
C_T	Cycle threshold
Cyt c	Cytochrome c
DA	Dopamine
DAT	Dopamine uptake transporter
DOPAC	3,4-dihydroxyphenylacetic acid
DNPH	2,4-dinitrophenylhydrazine
ETC	Electron transport chain

GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GDNF	Glial derived neurotrophic factor
GPe	Globus pallidus externa
GPi	Globus pallidus interna
GPx	Glutathione peroxidase
Grd	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
i.p.	Intra-peritoneal
3-OHMEL	3-hydroxymelatonin
LRRK2	Leucine-rich repeat kinase 2
MDA	Malondialdehyde
MEL	Melatonin
MOA-B	Monoamine oxidase B
MPD	Mouse model of Parkinson's disease
MPP^+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4phenyl-4-propionoxypiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSNs	Medium spiny neurons
MtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide
NGF	Nerve growth factor
NMDA	N-methyl-D-asparate
NO	Nitric oxide
$O_2^{\bullet-}$	Superoxide
$^{\bullet}OH$	Hydroxyl ion
$ONOO^-$	Peroxynitrile
PD	Parkinson's disease
PINK	Phosphatase and tensin homologue (PTEN)-induced putative kinase 1
PGC-1α	Peroxisome proliferator-activated receptor-coactivator
PTP	Permeability transition pore
qRT-PCT	Quantitative real-time RT–PCR
RCR	Respiratory control ratio
ROS	Reactive oxygen species
SAL	Saline
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata

s.c	Sub-cutaneous
SOD	Superoxide dismutase
STN	Subthalamic nucleus
TFAM	Mitochondrial transcription factor A
TH	Tyrosine hydroxylase
UCHL-1	Ubiquitin carboxy-terminal hydrolase L1
VMAT-2	Vesicular monoamine transporter-2

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1. INTRODUCTION AND STATEMENT OF PROBLEM

Parkinson's disease (PD) is a neurodegenerative disorder of the central nervous system characterized by progressive loss of dopamine (DA)-producing neurons in the substantia nigra pars compacta (SNpc) region of the brain, leading to impaired movement. With the exception of a few well-characterized familial cases in connection with specific gene mutations, PD is commonly a sporadic, adult-onset neurological disorder. Recent advances in PD research have reached a consensus suggesting that PD is not likely caused by a single factor but rather a combination of genetic and environmental factors leading to a complex clinical phenotype with underlying loss of nigrostriatal DA neurons (Bronstein et al., 2009). Hence, potential success for preventing and treating PD may not be easily achieved by a monotherapeutic approach.

The prevalence of PD tends to increase with age. In one study, it is estimated that 17.4 per 100, 000 people between 50 to 59 years of age, whereas 93.1 per 100, 000 people between 70 to 79 years of age have been afflicted with PD (Bower et al., 1999). In another study in Europe, PD prevalence ranges between 90-100 per 100, 000 of population over 65 years of age (de Rijk et al., 2000). There is no cure for the disease and only symptomatic treatments are available; thus, any preventive measure would be desirable. Although PD treatment has advanced in the last 40 years, the quality of life in

PD patients generally declines over time (Martinez-Martin, 1998), and eventually the PD patients suffer from immobility, dementia and death (Elbaz et al., 2003).

Many plausible genetic and environmental factors have been investigated and linked to the initiation and progression of PD neurodegeneration. Amongst them, neuronal oxidative stress and mitochondrial dysfunction have emerged as one of the mechanisms for instigating the demise of nigrostriatal DA neurons through a series of deleterious cellular and molecular events. It is generally believed that intrinsic genetic and extrinsic environmental factors may cause reactive oxygen species (ROS) accumulation and/or inadequate ROS disposition. This may result in oxidative damage to the neuronal mitochondria, depletion of antioxidant proteins, and activation of a series of caspase-dependent or caspase-independent molecular signaling events leading to apoptotic cell death (Dawson and Dawson, 2003a; Vila and Przedborski, 2003; Dawson and Dawson, 2003b). On the genetic basis, a number of published reports have associated point mutation of mitochondrial DNA (mtDNA) with PD neurodegeneration (Wallace, 1997; Gu et al., 2002; Bender et al., 2006; Kraytsberg et al., 2006). Furthermore, patients who are identified with mtDNA defects show progressive and disabling Parkinsonian features (Filosto et al., 2007; DiMauro and Schon, 2008).

Mitochondria are dynamic organelles that are present in all mammalian cells. They are functionally well known for generating adenosine triphosphate (ATP) as the

source of energy, for sequestering excess cytoplasmic Ca^{++}, for producing and disposing of ROS, for supporting cell survival and preventing cell death (Chan, 2006). Briefly, the generation of ATP by mitochondria involves oxidative phosphorylation of a number of protein complexes within the electron transport chain (ETC) system located in the inner mitochondrial membrane. During the process of mitochondrial respiration and ATP production, small amounts of molecular oxygen are naturally reduced to form ROS as byproducts, like superoxide free radicals. Under healthy conditions, the basal level of ROS produced within cells is quite low and not harmful. Furthermore, a number of mitochondrial anti-oxidant enzymes such as the superoxide dismutase (SOD) and cytochrome c (cyt c) can scavenge the abnormally elevated ROS from internal and external sources and prevent further damages to mitochondrial and cellular DNA, proteins, and lipids.

Neurons are extremely active cells requiring constant supply of energy in order to carry out highly specialized functions such as regulating the activities of neuronal transmission, receptors, ion channels, transporters and synapses. Mitochondria are vital for maintaining the homeostasis and integrity of neuronal functions; and mitochondrial damage and dysfunction may lead to neurological disorders (Schon and Manfredi, 2003; Chan, 2006).

The scientific evidence implicating exposure to environmental toxic chemicals as a possible contributing factor for causing cellular oxidative damage and mitochondrial dysfunction in association with PD has been widely reviewed (Sherer et al., 2002a). Self-administration of an illicit designer's drug, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that results in human Parkinsonism is an early indication of chemically induced toxicity to the nigrostriatal DA neurons (Langston et al., 1983). MPTP inhibits oxidative phosphorylation which is carried out by the mitochondrial nicotinamide adenine dinucleotide (NADH): quinone oxidoreductase (complex-1) of the ETC, increases mitochondrial ROS production, and depletes ATP (Ramsay and Singer, 1986; Ali et al., 1994). Infusion of other complex-I inhibitors, such as rotenone and trichloroethylene in rodents similarly results in loss of the nigral DA neurons and produces Parkinsonian phenotype suggesting mitochondrial dysfunction in PD pathogenesis (Betarbet et al., 2000; Gash et al., 2008). The concept of neuronal oxidative stress and mitochondrial complex-I inhibition has also been verified in the substantia nigra pars compacta (SNpc) from human PD (Parker et al., 1989; Schapira et al., 1990; Mann et al., 1992; Floor and Wetzel, 1998).

Genetically modified animal models are of particular interest as they provide evidence that reducing mtDNA expression and disrupting mitochondrial function can lead to respiratory chain breakdown and neuronal death reminiscent of PD phenotype

(Ekstrand et al., 2007). Over-expression of α-synuclein in cell culture and in transgenic mice impairs mitochondrial function and increases their vulnerability to MPTP neurotoxicity (Hsu et al., 2000; Song et al., 2004). Alternatively, α-synuclein knock-out mice are resistant to toxicity produced by respiratory chain inhibitors, such as MPTP (Dauer et al., 2002; Klivenyi et al., 2006). These findings strongly divulge a role of mitochondrial deficiency in α-synucleinopathy that is relevant to PD pathogenesis.

Most of the findings that associate mitochondrial dysfunction with PD-like DA neuron degeneration have been derived from in vitro experiments with isolated mitochondria and neuronal cells or from short-term investigations using an acute or subacute animal PD model. To further substantiate the functional role of mitochondria for the survival of nigrostriatal DA neurons, the objective of this research is to examine the long-term integrity of striatal mitochondria in a chronic MPD. We hypothesized that mitochondrial function is impaired in the striatum of the chronic MPD. The approach that we thought could prevent MPTP-induced mitochondrial dysfunction and neuronal deficits was using the treatment of long-term exercise. The beneficial effects of exercise have been well established in various disorders such as cardiovascular, metabolic, musculoskeletal and neurological conditions including PD (Al-Jarrah et al., 2007; Gobbi et al., 2009; Hirsch and Farley, 2009). Various laboratories, using the 6-hydroxydopamine (6-OHDA) induced MPD, have shown that forced exercise can reduce

the vulnerability of DA neurons to 6-OHDA (Smith and Zigmond, 2003). Various epidemiologic studies suggest an inverse relationship between the level of activity and the probability of developing PD (Chen et al., 2005). Regular exercise also delays the appearance of Parkinsonian features in patients already diagnosed with PD (Tsai et al., 2002). In this study, we examined the outcomes of long-term endurance exercise on mitochondrial and DA neuronal functions in the neostriatum of the aged chronic MPD that has been established in our laboratory (Lau et al., 1990; Petroske et al., 2001; Patki et al., 2009). We hypothesized that long-term endurance exercise protects DA neurons and mitochondrial function in the aged chronic MPD.

There have been recent studies demonstrating that exercise leads to an increase in the blood level of melatonin, a natural hormone produced by the pineal gland in brain (Reiter, 1991). In addition to its antioxidant property, melatonin is also known to scavenge both ROS and reactive nitrogen species (Korkmaz et al., 2009). We therefore hypothesized in this research that melatonin protects neuronal and mitochondrial function in the aged chronic MPD.

Following the completion of this dissertation research, our findings will have the following significances: (1) validating an integral chronic relationship between mitochondrial dysfunction and PD-like neurodegenerative disorder; (2) demonstrating the impact of long-term endurance exercise on mitochondrial and neuronal protection

in the chronic MPD; (3) revealing the protective effect of melatonin treatment on neuronal and mitochondrial function in the chronic MPD.

2. REVIEW OF LITERATURE

2.1. Parkinson's disease

Parkinson's disease (PD) is an age-related neurodegenerative disorder that primarily involves the loss of dopaminergic neurons and transmission in the nigrostriatal region of the brain. The disease is characterized by resting tremor, rigidity, bradykinesia, gait disturbance and postural instability (Olanow and Tatton, 1999). Current treatment strategies only ameliorate the symptoms; none halts or retards dopaminergic neuron degeneration. The barrier to developing neuroprotective therapies is due to the lack of understanding of molecular pathways that initiate neurodegeneration.

2.2. The basal ganglia

2.2.1. Anatomy of basal ganglia

The basal ganglia (BG) consists of a cluster of nuclei situated at the base of forebrain and is primarily linked with the cerebral cortex, thalamus and other brain areas. It is associated with a variety of functions, including motor control and learning (Obeso et al., 2008). The main components of the basal ganglia are the striatum (caudate and putamen), globus pallidus interna (GPi) and externa (GPe), SNpc, substantia nigra pars reticulate (SNpr) and subthalamic nucleus (STN) as illustrated in **(Figure 1).**

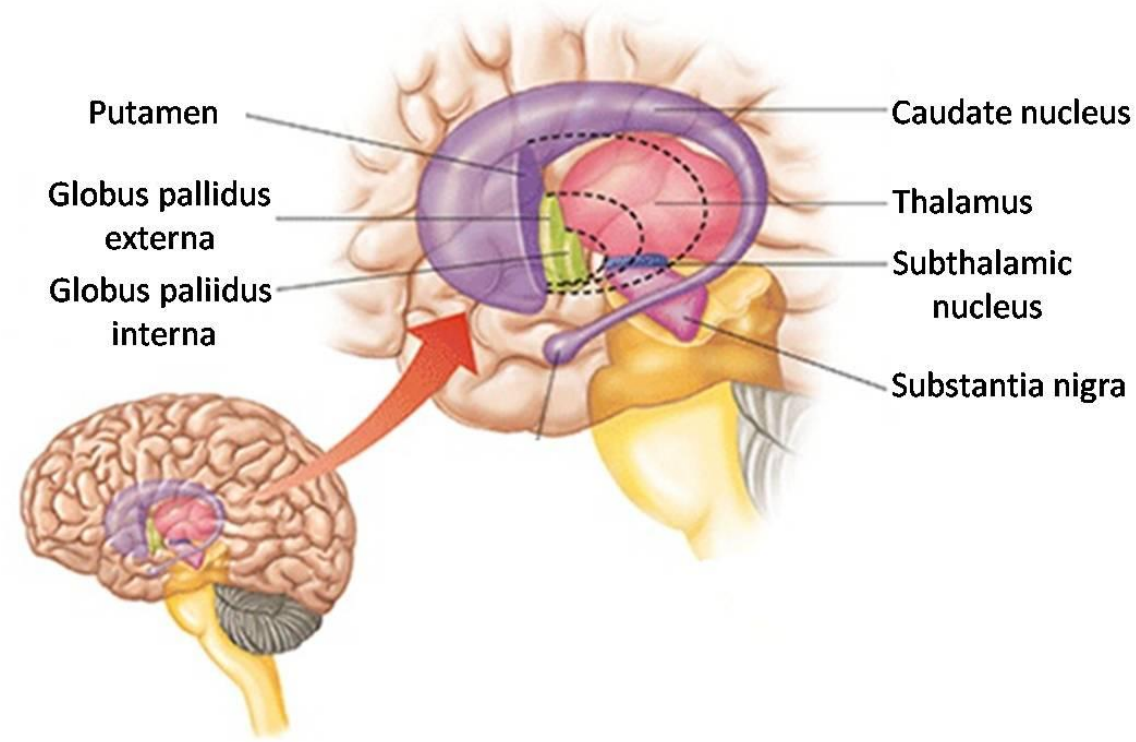

Figure 1: The human basal ganglia. The BG is connected with the cerebral cortex and the thalamus and also other brain areas (Rhawn, 2000).

2.2.2. BG circuitry in normal and PD state

The neuronal network in the BG comprises two major pathways - the direct and indirect pathways. The direct pathway is thought to facilitate movements while the indirect pathway is thought to suppress movements (Albin et al., 1989; DeLong, 1990).

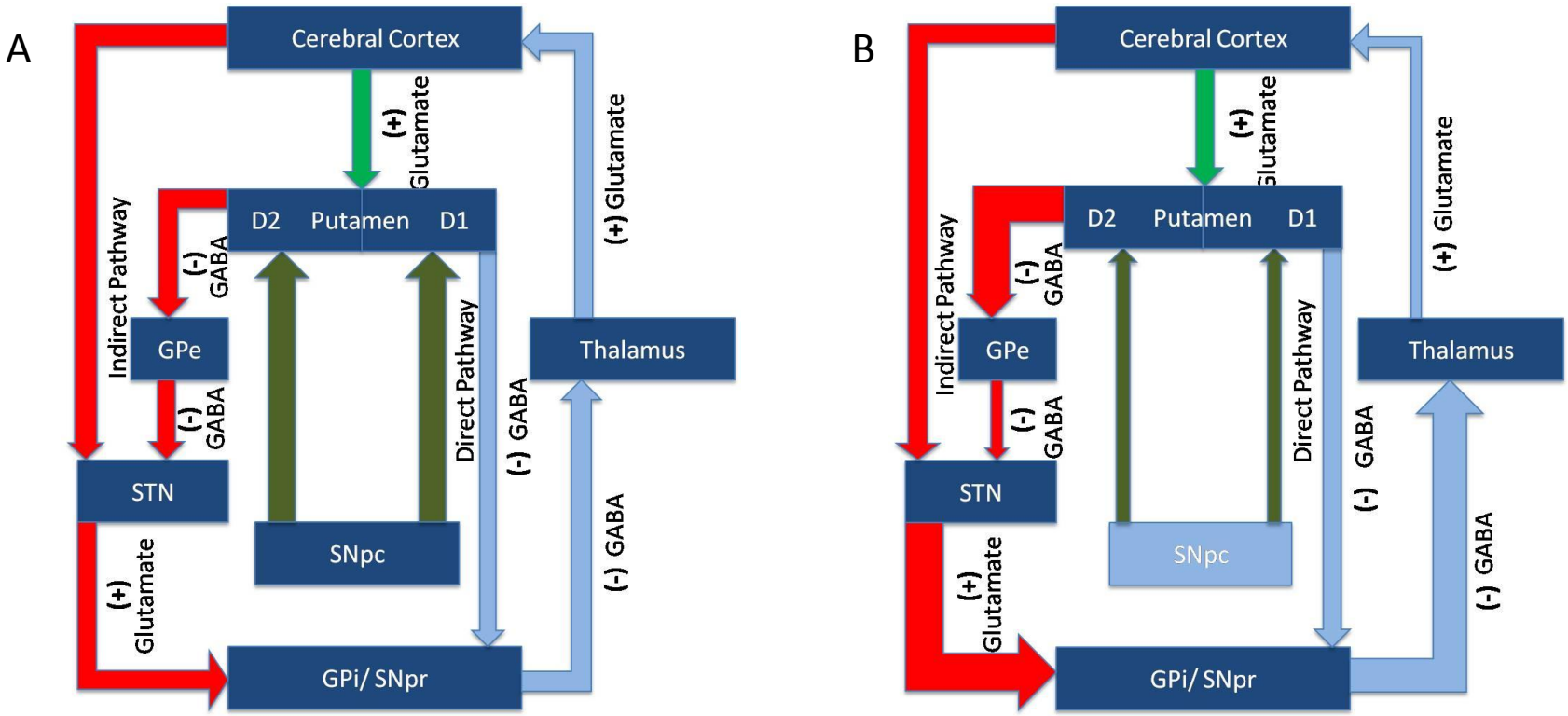

Figure 2: Proposed circuitry of the basal ganglia. (A) In normal state, corticostriatal stimulation excites the "direct" striato-GPi pathway (Blue arrows) and the "indirect" pathway (red arrows) striato-GPe–STN pathway in the medium spiny neurons (MSNs). The former inhibits, whereas the latter activates the GPi, which in turn, provides inhibitory innervations to thalamocortical projections. Thus the direct and indirect pathways serve to respectively facilitate or inhibit movement. (B) In Parkinsonian state, dopamine depletion in SNpc leads to increased activity in the "indirect" pathway and reduced activity in the "direct" pathway. This results in excessive GPi inhibitory output to the thalamus (modified from (Gale et al., 2008).

As illustrated in Figure 2A, most medium spiny neurons (MSNs) express either D1 or D2 receptors. It is generally accepted that the majority of D1-receptor expressing neurons comprise the “direct” pathway and project to the GPi/SNpr and use the inhibitory transmitter GABA, whereas D2-receptor bearing neurons project to the GPe and the STN to the GPi/SNpr and use two inhibitory GABAnergic links and one excitatory glutamatergic projection comprise the “indirect pathway” (Gerfen et al., 1990; Onn et al., 2000; West and Grace, 2002). Thus, DA exerts a dual effect on MSNs, inhibiting striatal D2-receptor bearing neurons by decreasing the synthesis of intracellular cAMP and exciting striatal neurons that express D1 receptors by increasing the synthesis of intracellular cAMP (Hernandez-Lopez et al., 1997). Thus, the “direct” and indirect pathways serve to respectively facilitate and inhibit movement.

The primary cause of PD is a selective loss of dopaminergic neurons in the SNpc. Loss of DA results in changes occurring in both pathways. Specifically, there is decreased excitation of D1-receptor bearing striatal neurons leading to reduced activity in the “direct” pathway. In contrast, there is reduced inhibition of D2-receptor bearing striatal neurons resulting in increased activity in GPe projections as illustrated in Figure 2B. This leads to increase in the STN neuronal activity which in turn causes excessive neuronal recruitment and augmentation of the inhibitory output of the GPi and SNpr. In conclusion, in parkinsonian state overactivity in the excitatory STN-GPi pathway predominates and overwhelms the inhibitory effects of the “direct” pathway. This leads

to excessive firing of the GPi in response to afferent signals, thereby impeding the normal movement (Boraud et al., 2000; Rubchinsky et al., 2003).

2.3. PD Pathogenesis

2.3.1. Historical background

Since the original description of the “Shaking Palsy” by James Parkinson in 1817, the knowledge about PD made a slow progress for almost a century. Beginning in the late 1950s, our understanding of PD progressed by leaps and bounds largely through advancement in biomedical technology and medical allied fields. The cause(s) of PD are still unknown, although various genetic and environmental factors have been proposed as causative factors for PD-like neurodegeneration.

2.3.2 Environmental factors and PD

Epidemiologic studies point to a number of factors that may contribute to the development of PD (Tanner and Langston, 1990). These factors include exposure to well water, pesticides, like paraquat and rotenone, wood pulp mills or simply living in a rural area. Also a number of exogenous toxins such as trace metals, cyanide, organic solvents have been associated with the possible development of Parkinsonism (Langston et al., 1983). Exposure to certain viruses and bacteria, although rare, has also been linked to PD (Duvoisin and Yahr, 1985).

2.3.3. Viral hypothesis

In early 1920's there was an epidemic of Von Economo's encephalitis. Several years later these individuals displayed PD-like symptoms. The viral disease progressed more slowly than PD. In these patients, there was a greater loss of nerve cells in substantia nigra, but without the development of Lewy bodies (Duvoisin and Yahr, 1985). In another report, Japanese researchers showed that certain strains of Influenza A virus were selective to destroy nigral neurons and could gain access to the brain via the nasal passages in mice (Takahashi and Yamada, 1999).

2.3.4. Chemical hypothesis

Carlsson et al. observed that systemic administration of reserpine causes depletion of brain catecholamines, leading to akinetic state in rabbits (Carlsson et al., 1957). Furthermore, L-DOPA administration alleviated reserpine-induced akinetic state indicating behavioral recovery was DA-dependent. The discovery that striatal DA deficiency resulted in PD-like symptoms prompted the development of reserpine animal model. The systemic reserpine administration causes depletion of DA at the nerve terminals and induces a hypokinetic state. These movement deficits are due to lack in the DA storage capacity in intracellular vesicles (Hornykiewicz, 1963).

The herbicide paraquat also induces a toxic model of PD. Paraquat has structural similarity to 1-methyl-4-phenylpyridinium (MPP^+) and is present in the environment (Liou et al., 1997). However, paraquat does not easily cross the blood brain barrier and its toxicity appears to be mediated by the excessive formation of superoxide radicals. Superoxide radicals are capable of reacting with lipids to induce lipid peroxidation, thereby altering membrane permeability and disrupting cellular functioning (Day et al., 1999). Systemic administration of paraquat to mice leads to SNpc dopaminergic neuron degeneration accompanied by α-synuclein containing inclusions (Manning-Bog et al., 2002; McCormack et al., 2002).

Rotenone is highly lipophilic and readily crosses the blood brain barrier (Talpade et al., 2000). Rotenone binds to and inhibits mitochondrial complex I of the ETC and its entry into the DA neurons does not depend on any transporter. Greenamyre and colleagues have reported that administration of low-dose intravenous rotenone to rats produces selective degeneration of nigrostriatal dopaminergic neurons accompanied by α-synuclein positive Lewy body-like inclusions (Betarbet et al., 2000). Rotenone-intoxicated animals developed abnormal postures and slowness in movement. The rotenone model was the first to link an environmental toxin to the pathologic hallmark of α-synuclein aggregation, an association previously reported only in cell culture studies (Uversky et al., 2001; Sherer et al., 2002b).

Around the early 1980's, a drug abuser intended to synthesize an illicit preparation of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), an analog of opioid drug, meperidine (Demerol). Unexpectedly due to careless synthesis of MPPP, an impure byproduct, MPTP was instead produced. Surprisingly, MPTP causes severe Parkinsonian syndrome in humans and monkeys, which is symptomatically indistinguishable from human PD (Langston et al., 1983).

MPTP is a lipophilic molecule. After systemic administration, it rapidly crosses the blood brain barrier. In the brain, MPTP is metabolized to $MPDP^+$ by the monoamine oxidase B (MAO-B) in non-DA cells. Then by further oxidation, $MPDP^+$ is converted to MPP^+, the active toxic metabolite of MPTP. MPP^+ is released into the extracellular space by an unknown mechanism. MPP^+ is a polar molecule. Although it cannot freely enter cells, it has a high affinity for the dopamine uptake transporter (DAT) (Mayer et al., 1986). Once taken up by the DAT, MPP^+ can (1) be further taken up by the vesicular monoamine transporter (VMAT) resulting in the translocation of MPP^+ into synaptosomal vesicles (Liu et al., 1992), (2) be concentrated by an active process within the mitochondria (Ramsay and Singer, 1986), or (3) remain in the cytoplasm and interact with different cytosolic enzymes (Klaidman et al., 1993) **(Figure 3A).**

Within the mitochondria, MPTP impairs mitochondrial respiration by initially inhibiting the NADH dehydrogenase (complex I) of the ETC. The inhibition of complex I

obstructs the flow of electrons along the ETC, leading to ATP deficiency **(Figure 3B)**. Another consequence of complex I inhibition by MPP^+ is an increased production of free radicals, especially the superoxides. Superoxide anion can be converted to hydrogen peroxide (H_2O_2) by mitochondrial matrix enzyme Mn SOD, and Cu-Zn SOD in the intermembrane space.

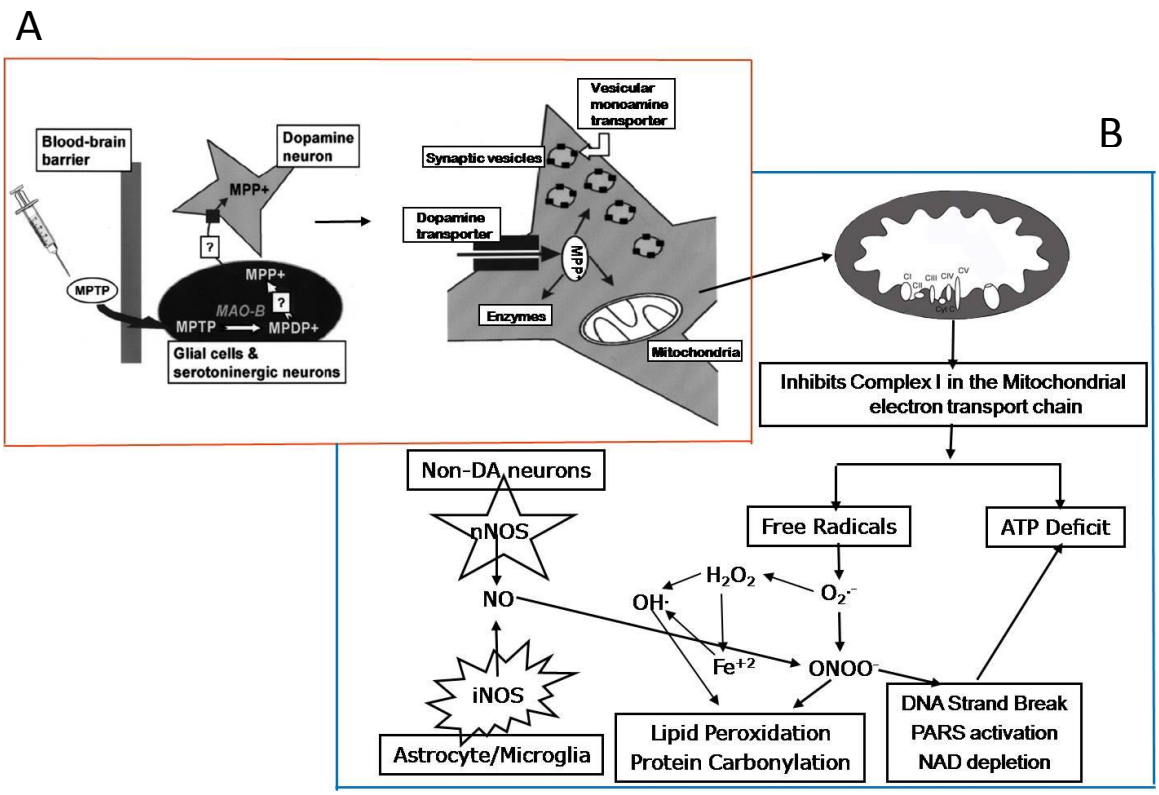

Figure 3: Proposed mechanisms of MPTP neurotoxicity. (A) After systemic administration, MPTP rapidly crosses the blood brain barrier. In the brain, MPTP is metabolized to $MPDP^+$ by the monoamine oxidase B (MAO-B) in non-DA cells. $MPDP^+$ is converted to MPP^+, the active toxic metabolite of MPTP. MPP^+ has a high affinity for DAT. Once taken up by the DA neurons, MPP^+ can (1) be taken up by the VMAT (2) be within the mitochondria or (3) remain in the cytoplasm. (B) In the mitochondria, MPTP impairs mitochondrial respiration by initially inhibiting the NADH dehydrogenase (complex I) of the ETC and leading to the formation of free radicals and eventually causing lipid peroxidation and DNA damage. (Modified from Przedborski and Jackson-Lewis, 1998).

However, H_2O_2 can be converted to highly toxic hydroxyl ion ($^{\bullet}OH$) by the Fenton reaction in the presence of transitional metals, such as iron **(Figure 3B).** Superoxides can also react with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$). NO, which is produced by the nNOS and/or iNOS outside DA-neurons can react with the elevated superoxide, to form $ONOO^-$ that can damage lipids, proteins and DNA **(Figure 3B)** (Przedborski and Jackson-Lewis, 1998).

2.3.5. Protein degradation pathways and genes involved in PD

Protein degradation and disposition is an important on-going process for maintaining the cellular protein homeostasis following various active stages of cell cycle, development and growth, transcription, signaling, and cellular toxicity and damage. There are two major protein clearance systems. (1) The ubiquitin proteosome system, which is responsible for degrading short-lived intracellular and plasma membrane proteins, as well as misfolded or damaged proteins in the cytosol, nucleus or endoplasmic reticulum under basal conditions. (2) The autophagy-lysosome pathway, which is responsible for the degradation of long-lived, stable proteins and by which organelles such as mitochondria can be recycled. Alteration in the function of these two systems for degrading and disposing of misfolded and aggregated proteins would be considered detrimental and could lead to neurodegeneration.

The UPS is responsible for degrading short-lived cellular proteins (Coux et al., 1996). In addition, misfolded, mutated, and oxidatively damaged proteins are also degraded by the UPS. Proteins that are destined for degradation are first marked by covalent attachment of a polyubiqutin chain to a lysine residue on the substrate. This polyubiquitinated protein is then degraded by the 26S proteasome. Monoubiquitinylation is important for transcription, translation, protein trafficking and DNA repair. Polyubiquitination of a target protein is achieved in systemic enzyme-mediated reactions that are necessary to ensure specificity and activate the ubiquitin moiety. The activated ubiquitin is produced by ubiquitin-activating enzyme (E1), which forms a thiol ester linkage between a cysteine residue and carboxy-terminal glycine in ubiquitin in an ATP-dependent manner (Hochstrasser, 1996). The activated ubiquitin is then transferred to one of many ubiquitin conjugating enzymes (E2) via the formation of another thiol linkage. Lastly, ubiquitin is ligated to the lysine residue of the protein substrate that is specifically bound to another member of the ubiquitin-protein ligase family of proteins (E3 ligase). Additional activated ubiquitin moieties can be attached to internal lysine residues within the ubiquitin to form polyubiquitin chains, which are recognized by the 26S proteasome complex for initiating protein degradation. A minimum of four ubiquitin moieties are required for efficiently targeting the ubiquinated protein at the proteasome. Selectivity for ubiquitination and recognition of substrates is mediated primarily by the E3s, thus the existence and distribution of UPS

enzymes are in the order of E3>E2>E1 in a normal biological system to ensure high selectivity and efficiency for protein degradation and disposition (Weissman, 2001).

Parkin, which is found defective in a familial form of PD, is an E3 ligase. Parkin is a 465 amino acid protein with two RING fingers separated by an in-between RING domain at the carboxyl end, which functions as an ubiquitin E3 ligase (Shimura et al., 2000). The E3 ubiquitin ligases are important constituents of the ubiquitin proteosome system (UPS), which is the main pathway for the removal of misfolded or unwanted protein from the apoptotic cells (Ciechanover and Schwartz, 1998). Mutations in the parkin gene (PARK2) on chromosome 6q25.2-27 have been reported to cause autosomal recessive early-onset PD (Kitada et al., 1998). Hyun et al. demonstrated that increasing the expression of parkin by gene transfection in NT-2 and SK-N- MC cells led to increased proteosomal activity, decreased levels of protein carbonyls, 3-nitrotyrosine-containing proteins, and a reduction in ubiquinated protein levels (Hyun et al., 2002). Mouse with parkin gene knockouts have shown extensive mitochondrial defects such as swollen mitochondria, fragmented cristae and reduced complex I and IV subunits of the ETC along with decreased mitochondrial respiratory capacity (Palacino et al., 2004).

Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) belongs to a family of deubiquitinating enzymes that are responsible for hydrolysis of polyubiquitin chains into monomeric ubiquitin. Missense mutation (Ile93M) in the gene coding for UCH-L1 on

chromosome 4p14-15 has been identified in two siblings of a German family with autosomal-dominant familial PD (Golbe, 1990). Mutations in the UCH-L1 gene results in 50% decrease of its catalytic activity, which may lead to reduced ubiquitination and in turn, decreased clearance of abnormal and misfolded proteins in diseases like PD (Leroy et al., 1998). UCH-L1 overexpression in rat primary cortical neurons is shown protective against H_2O_2-induced cell death and ROS generation (Kyratzi et al., 2008).

Autophagy is a catabolic process in which degradation of damaged cellular constituents such as membranes, proteins and organelles is brought about by lysosomes for maintaining the cellular integrity and homeostasis. Autophagy is an important survival mechanism and can be stimulated by nutrient deprivation. Since neurons are postmitotic in nature, they are innately prone to accumulation of damaged proteins. Thus, inability to dispose of them could lead to cell death. Three types of ALP have been described, namely (1) macroautophagy, (2) microautophagy and (3) chaperone-mediated autophagy (CMA) (Cuervo et al., 2004; Levine and Klionsky, 2004). Macroautophagy can be induced by short period of nutrient deprivation. On the other hand, CMA requires long period of nutrient deprivation for its induction and microautophagy involves a continuous turnover of cytosolic proteins (Klionsky and Emr, 2000; Levine and Klionsky, 2004).

Macroautophagy is the primary mechanism for the degradation of proteins before reaching the lysosomes. This is a multi-step process, which involves the formation of a double membrane structure called the autophagosome that fuses with the lysosomes to form the autophagolysosome. In the autophagolysosome the engulfed substrates are degraded by the hydrolytic enzymes by the disintegration of the inner membrane. The contents are recycled supplying amino acids and energy to the cells (Takeuchi et al., 2005). Microautophagy involves gradual but continuous turnover of cytosolic proteins which are pinched off from the lysosomes. This process takes place even in the resting conditions. Chaperone-mediated autophagy is responsible for degradation of ~30% of cytosolic proteins possessing a Lys-Phe-Glu-Arg-Gln (KFERQ)-like motif. There is a cytosolic protein-molecular chaperone (heat shock cognate, Hsc70) which recognizes such a motif and transfers it to the lysosome-assocaited membrane protein 2a (LAMP2a) (Crotzer and Blum, 2005). The resulting protein is taken up and digested in the lysosomal lumen. In PD, the autophagic process is believed to be dysregulated, which may prevent the normal uptake and removal of proteins containing KFERQ-like motif (e.g. myocyte enhancer factor 2D, MEF2D) and organelles (e.g. mitochondria). Toxic accumulation of damaged proteins may cause neurons more susceptible to stress stimuli. It is known that wild-type α-synuclein, but not the mutant α-synuclein, is selectively translocated into lysosomes for degradation by the CMA pathway (Cuervo et al., 2004). The failure of CMA to clear the mutant α-synucleins may

be explained by their high affinities for the LAMP2a than the wild α-synuclein. The binding of the mutant α-synuclein to the LAMP2a blocks the lysosomal uptake and inhibits the degradation of both wild and mutant α-synucleins (Cuervo et al., 2004).

2.3.6. Genetics and PD

Genetic factors have been identified linking to the etiology of PD (Golbe, 1990). Approximately 5% of PD patients have familial form of Parkinsonism with an autosomal-dominant pattern of inheritance (Wood, 1998). A number of candidate genes have been uncovered and screened.

α-Synuclein is a small protein, with 140 amino acids. It is normally expressed not only in the dopaminergic neurons but also in cortical and noradrenergic neurons (Li et al., 2002). The physiologic function of α-synuclein is for the maintenance, storage, and regulation of DA vesicles at synaptic terminals (Lotharius and Brundin, 2002a). As α-synuclein is a fibrillar aggregation prone protein, it has a high tendency to aggregate due to its hydrophobic non-amyloid beta component. This fibrillar form of α-synuclein becomes a major structural part of the Lewy bodies associated with PD pathogenesis. Recently it was shown that mitochondrial dysfunction and α-synuclein aggregation are interrelated and complementary to each other in PD pathogenesis (Martin, 2006). It has been reported that missense mutations along with its genomic triplications in the α-synuclein gene at chromosome 4q21-23 (PARK1 and 4 locus) is linked with autosomal

dominant PD (Polymeropoulos et al., 1997). Other mutations in the α-synuclein gene, such as G209A, A53T, G209A, G88C and A30P have been subsequently mapped contributing to autosomal dominant form of PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Markopoulou et al., 1999). Overexpression of human α-synuclein in mice increases their susceptibility to mitochondrial toxins, such as MPTP and paraquat (Yao and Wood, 2009). On the other hand, reduction of α-synuclein expression in animals shows resistance to these toxins (Klivenyi et al., 2006). This suggests that build up of mutant α-synuclein may facilitate mitochondrial damage and neuronal death (Dauer et al., 2002).

Phosphatase and tensin homologue (PTEN)-induced putative kinase (PINK1) gene contains 8 exons and encodes a 581 amino acid protein with an amino-terminal mitochondrial targeting sequence and a serine-threonine kinase catalytic domain (Silvestri et al., 2005). Wild-type PINK1 has been shown to protect against stress-induced mitochondrial dysfunction and neuronal apoptosis, whereas its mutant form negates this effect (Valente et al., 2004). Mutations in the PINK1 gene were first identified in three families on chromosome 1p35-p36 with an autosomal recessive PD (Valente et al., 2004). PINK1 mutations result in impaired phosphorylation of its substrates, which have been detected in the mitochondria of PD brains (Exner et al., 2007). Downregulation of PINK1 in human cell lines by RNA interference led to

morphological defects in the mitochondria such as, fragmented cristae, reduced mitochondrial membrane potential and increased sensitivity to oxidative stress (Gautier et al., 2008). Overexpression of wild-type, but not mutant, PINK1 in tumor cell lines was shown to prevent the activation of caspase-3 and release of cytochrome c from mitochondria, therefore apoptosis is arrested (Valente et al., 2004).

DJ-1 is a multifunctional protein and it functions as an oxidative stress sensor (Taira et al., 2004). DJ-1 (PARK 7) mutations on chromosome 1p36 are rare and have been linked to an autosomal recessive form of PD (van Duijn et al., 2001; Bonifati et al., 2003). DJ-1 knockout mice showed increased sensitivity to mitochondrial complex I inhibition, impaired mitochondrial ROS scavenging abilities and oxidative stress induced by MPTP (Kim et al., 2005). The Cys-106 residue on the DJ-1 gene seems to be important for the translocation of DJ-1 to the mitochondria, as mutations in this residue prevent the mitochondrial translocation of DJ-1 and impair the cell's response to mitochondrial oxidative stress (Canet-Aviles et al., 2004). Overexpression of human DJ-1 gene in mouse cerebral ganglion neurons considerably protects them against glutamate-induced cell death (Aleyasin et al., 2007).

Omi/HtrA2 is a mitochondrial serine protease that is released into the cytosol during apoptosis to antagonize the inhibitors of apoptosis (IAPs) and contribute to caspase-independent cell death (Suzuki et al., 2001; Martins et al., 2002). A G399S

mutation and A141S polymorphism in Omi/HtrA2 have been associated with increased risk of PD development (Strauss et al., 2005). These mutations affect the proteolytic activity of the enzyme. Omi/HtrA2 knockout mice have a Parkinsonian phenotype showing rigidity, tremor and striatal damage (Plun-Favreau et al., 2007). The phosphorylation of Omi/HtrA2 has been shown to be PINK1 dependent. The phosphorylation of Omi/HtrA2 at Ser142 demonstrates increased protease activity and enhanced protective effect under stressed conditions. In PD brains with PINK1 mutation, the phosphorylation of Omi at Ser142 is decreased (Plun-Favreau et al., 2007).

The leucine-rich repeat kinase 2 (LRRK2) gene provides instructions for making a protein called dardarin, but little is known about this gene or the dardarin protein. Part of the LRRK2 gene also provides instructions for making leucine. Proteins with leucine-rich regions appear to play a role in activities that require protein-protein interactions, such as transmitting signals or helping to assemble the cell's structural framework (cytoskeleton). Additional research findings indicate that dardarin has a kinase activity (Guo et al., 2006). Mutations in LRRK2 gene have been linked to an autosomal dominant PD (Paisan-Ruiz et al., 2004). LRRK2 mutations are found to account for 5-6% of familial cases of PD (Gilks et al., 2005). Some mutations increase its kinase activity while others decrease it. In primary cortical neuron cultures, overexpression of some LRRK2 mutations such as R1441C, Y1699C or G2019S can lead to neuronal degeneration (Smith et al., 2005; Greggio et al., 2006; Smith et al., 2006).

In conclusion, mitochondrial dysfunction has been implicated in both sporadic and familial forms of PD patients. Abnormal mitochondrial function has been replicated in several transgenic animal models with either overexpression or knockout of PD associated genes. Additionally, mitochondrial toxin-induced animal models have displayed some typical PD symptoms including loss of DA neurons and locomotor deficits. Deficits in mitochondrial complex I activity in combination with increased oxidative stress, and aging associated damage to the mtDNA are currently thought to be biochemical culprits for PD pathogenesis.

2.3.7. Oxidative stress and PD

Oxidative stress has received much attention in PD because DA can undergo oxidative metabolism to yield hydrogen peroxide (H_2O_2) and various other ROS (Halliwell and Gutteridge, 1984). Additionally, excess of DA may undergo hydroxylation and be converted to 6-OHDA, which is neurotoxic in nature (Stein and Wise, 1971). Oxidative stress and ensuing cell death could occur in the SNpc under circumstances in which there is (1) increased DA turnover resulting in increased production of H_2O_2 **(Figure 4a and c)**; (2) a deficiency in glutathione thus decreasing the brain's capacity to eliminate H_2O_2 **(Figure 4d)**; or (3) an increase in reactive iron, which would promote $^{\bullet}OH$ formation **(Figure 4e)**. Also, postmortem studies with PD brains reveal increased iron level, decreased GSH and increased oxidant damage to lipids, proteins and DNA

supporting the hypothesis that oxidative stress is involved in PD (Jenner and Olanow, 1996).

Various studies have been done showing that iron levels are elevated within the SNpc of PD patients (Dexter et al., 1989). Laser microprobe studies have shown that iron tends to accumulate within the neuromelanin granules of DA neurons (Good et al., 1992). Infusion of iron into the SNpc of rodents generates a model of PD characterized by a concentration-dependent and progressive loss of striatal DA, degeneration of SNpc neurons and locomotor deficits due to the formation of $^{\bullet}OH$ as seen in Figure 4e (Sengstock et al., 1994).

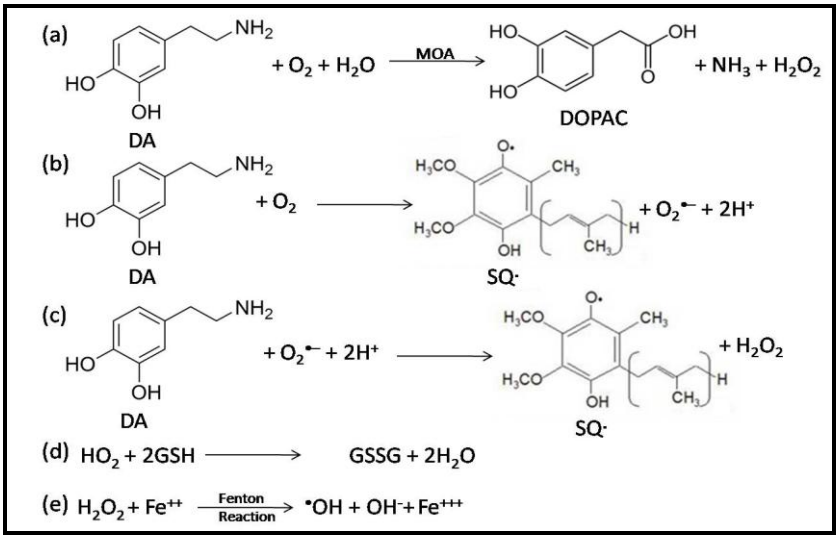

Figure 4: Enzymatic and the chemical metabolism of dopamine result in the formation of hydrogen peroxide (a and c). H_2O_2 is normally cleared by reduced glutathione (d). Increased levels of H_2O_2 can react with ferrous iron to generate the highly reactive $^{\bullet}OH$ radical according to the Fenton reaction (e). $SQ^{\cdot}$ – semiquinone, DOPAC - 3, 4-dihydroxyphenylacetic acid, GSSG – oxidized glutathione, GSH – reduced glutathione.

Reduced levels of GSH have been detected in the SNpc of PD patients (Sofic et al., 1992). A decrease in GSH level implicates that H_2O_2 clearance is impaired and $^{\bullet}OH$ radical formation is promoted. These events will be particularly enhanced in the presence of iron **(Figure 4d)**.

The overall oxidative damage in the brains of PD patients has been well demonstrated. Increased level of lipid peroxidation products such as malondialdehyde and lipid hydroperoxide have been detected in the SNpc but not in the cerebellum of PD patients (Dexter et al., 1994). Hydroxynonenal is a product of lipid peroxidation that has the capacity to modify proteins and to promote cellular toxicity. Increase in staining for hydroxynonenal has been detected in the DA neurons of PD brains (Yoritaka et al., 1996). A significant increase of protein carbonyls and 8-hydroxy-2-deoxyguanosine has been observed in various brain regions of patients with PD suggesting oxidative damage to neuronal proteins and DNA (Alam et al., 1997a; Alam et al., 1997b).

2.3.8. Mitochondrial dysfunction in PD

As shown in Figure 5, cellular energy generated through the transfer of electrons down the ETC is used to pump protons from the mitochondrial matrix into the intermembrane space, creating an electrochemical proton gradient across the inner mitochondrial membrane called mitochondrial membrane potential. This electrochemical proton gradient allows ATP synthase to use the flow of H^+ through the

enzyme back into the matrix to generate ATP from adenosine diphosphate (ADP) and inorganic phosphate. Complex I (NADH coenzyme Q reductase; labeled I) accepts electrons from the Krebs cycle electron carrier NADH, and passes them to coenzyme Q (ubiquinone; labeled CoQ), which also receives electrons from complex II (succinate dehydrogenase; labeled II). CoQ passes electrons to complex III (cytochrome bc_1 complex; labeled III), which passes them to cyt c. Cyt c passes electrons to Complex IV (cytochrome c oxidase; labeled IV), which uses the electrons and hydrogen ions to reduce molecular oxygen to water (Szeto, 2006).

Mitochondria are known to play a pivotal role in cellular apoptosis. Mitochondrial complexes I, III and IV of the respiratory chain are responsible for pumping protons out of the mitochondrial matrix across the inner mitochondrial membrane using the energy provided by the transfer of electrons along the electron transport chain creating voltage difference across the inner mitochondrial membrane, or MMP. A fall in MMP coupled with an increase in intramitochondrial calcium can jointly cause the opening of the permeability transition pore (PTP) and the release of apoptosis initiating factors, such as cyt c (Liu et al., 1996).

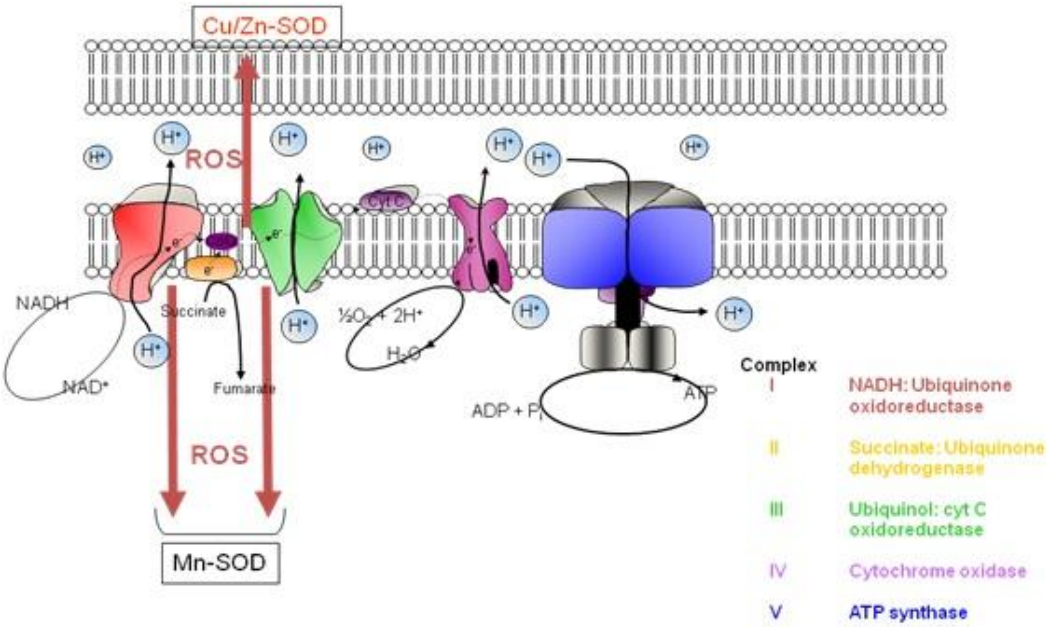

Figure 5: Schematic representation of the mitochondrial electron transport chain (modified from Szeto, 2006)

ETC, which is coupled to oxidative phosphorylation, provides cells with their important means for generating the required energy (Scheffler, 1999). Up to 95% of the ATP generated in aerobic cells is a result of mitochondrial oxidative phosphorylation. The ETC and oxidative phosphorylation must remain active to ensure mitochondrial and cellular survival.

In the SNpc of PD patients, around 30-40% decrease in complex I activity of the mitochondrial respiratory chain has been detected (Schapira et al., 1990). A mitochondrial complex I defect can lead to ATP depletion and cellular degeneration. The majority of O_2 inhaled is taken up by cells and processed in the mitochondrial ETC,

where oxygen is converted to water. However, during this reductive process, partially reduced species of O_2 such as superoxide anion ($O_2^{\bullet -}$), $^{\bullet}OH$, and H_2O_2 are also produced **(Figure 5)**. Collectively, these agents are referred to as ROS. Under normal conditions $O_2^{\bullet -}$ is less tissue damaging when formed, $O_2^{\bullet -}$ is quickly converted to H_2O_2 by cytosolic or mitochondrial superoxide dismutase (Cu-Zn SOD and Mn SOD). Since H_2O_2 is the immediate precursor of the highly damaging $^{\bullet}OH$, it is imperative that H_2O_2 be removed from the intramitochondrial environment as quickly as possible. The enzyme that achieves this is glutathione peroxidase (GPx), which metabolizes H_2O_2 to water; in this process GPx also converts GSH to GSSG. After continuous antioxidant effect, it is essential that GSSG be reduced back to GSH; this is accomplished by glutathione reductase (GRd) (Fernandez-Checa and Kaplowitz, 2005). The removal of H_2O_2 from the mitochondrial environment is never complete; thus via the Fenton reaction, some damaging $^{\bullet}OH$ are still formed. Cellular organelles have no enzymatic means to reduce $^{\bullet}OH$ unless it is removed by a free radical scavenger such as Cu-Zn SOD and Mn SOD, otherwise the free radical will accumulate and cause lipid peroxidation and DNA damage leading to neuronal death as summarized in **(Figure 6)**.

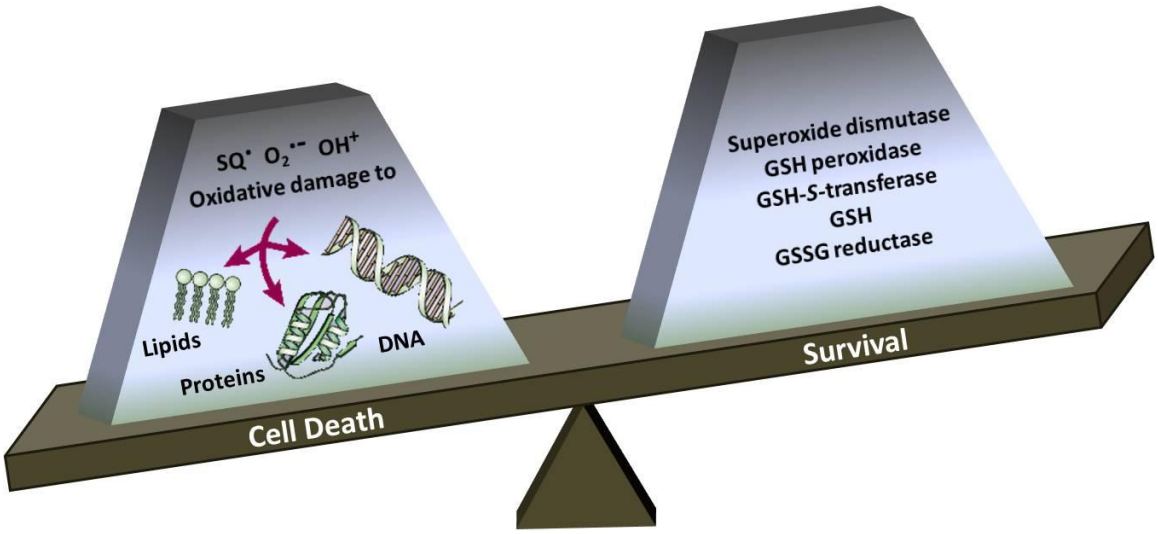

Figure 6: Reactive oxygen species and the proposed pathogenesis of PD. In PD, an abnormal elevation of ROS tilts the balance between the production and elimination, leading to enhanced oxidative stress (Lotharius and Brundin, 2002b).

In the immediate vicinity of where the $^{\bullet}OH$ is formed and accumulated due to its rapid reaction rate, the damage is likely site-specific. In addition to ROS, mitochondria also generate reactive nitrogen species; two of these are NO and the $ONOO^{-}$. $ONOO^{-}$ has direct toxic effects leading to lipid peroxidation, protein oxidation and DNA damage. It also can induce several transcription factors, including the nuclear factor-kappa B and activator protein-1, leading to cytokine-mediated inflammation (Korkmaz et al., 2009). Mitochondrial NO functions as a reversible antagonist of the complex IV in the ETC by competing with O_2 for its binding site. Usually tissue concentrations of NO and O_2 are in the ranges of 100–500 nM and 10–30 µM, respectively. In these concentration ranges, NO causes roughly half maximal inhibition of mitochondrial respiration. Thus, NO is a

physiological regulator of respiration and also of the rate of ATP synthesis (Brown, 1992). However, elevation of NO levels above the physiological concentrations as mentioned above would potentially inhibit not only complex IV but also complexes I and III. Consequentially, mitochondrial electron transfer reactions will be compromised causing more electron leakage and formation of $O_2^{\bullet -}$ and other ROS (Brown and Borutaite, 2004). Additionally, NO readily couples with the $O_2^{\bullet -}$ to produce the non-radical species, the $ONOO^-$, which irreversibly damages respiratory complexes (Lizasoain et al., 1996). The toxicity of the $ONOO^-$ is considered as important as the $^{\bullet}OH$ in terms of causing mitochondrial dysfunction and cell death (Brown, 1992).

There is a balance between the level of afferent glutamatergic stimulation and the ability of the neuron to cope with that stimulation. Neurons have threshold for excitotoxic glutamate stimulation, the threshold is not static and can be influenced by various neuronal parameters, such as mitochondrial function, calcium buffering and antioxidant levels. Under compromised conditions such as mitochondrial impairment, reduced calcium buffering or increased oxidative stress, less glutamatergic stimulation is required to produce toxicity. There have been reports demonstrating that N-methyl-D-asparate (NMDA) antagonists can prevent the neuropathological damage and behavioral consequences of MPTP (Turski et al., 1991; Zuddas et al., 1992). Blockade of this toxicity by NMDA antagonists suggests that excitotoxicity is involved in the rodent MPTP model and possibly in idiopathic PD (Heikkila et al., 1984).

2.4. Animal models for PD research

To represent the known human PD symptomatology, a desirable experimental model for PD research should display

1. Persistent depletion of at least 80% of striatal DA and its metabolites.
2. Pronounced reduction of striatal sites for DA uptake.
3. Significant and progressive loss of substantia nigra cells.
4. Marked impairment in the animal's motor performance.
5. The formation and accumulation of pathological inclusion bodies in nigral neurons (Lau, 2005).

Several animal models have been used in PD research such as the unilateral or bilateral, 6-OHDA lesion rat model (Ungerstedt, 1976; Schwarting and Huston, 1996). 6-OHDA is the first animal model of PD associated with SNpc dopaminergic neuronal death, was introduced more than 40 years ago (Ungerstedt, 1968). Although the neuropathology induced by the 6-OHDA model differs from PD, it is still widely used. 6-OHDA-induced toxicity affects mostly the monoaminergic neurons as the result of preferential uptake by DA and noradrenergic transporters (Luthman et al., 1989). There is a range of tissue sensitivity to 6-OHDA within the ventral midbrain dopaminergic

neuronal groups such that the SNpc neurons are greatly lost, whereas the tuberoinfundibular neurons are spared (Jonsson, 1980). Once in the neurons, 6-OHDA accumulates in the cytoplasm, generating ROS and causing neuronal damage (Cohen and Werner, 1994). As 6-OHDA cannot cross the blood brain barrier, it is commonly administered by local stereotaxic injection into the substantia nigra or striatum in order to target the dopaminergic pathway (Jonsson, 1983). After 6-OHDA injections in the substantia nigra, dopaminergic neurons start to degenerate within 24 hr and die without apoptotic morphology (Jeon et al., 1995). When injected in the striatum, 6-OHDA produces a more extended retrograde degeneration of the nigrostriatal neurons, which lasts for 1-3 weeks (Sauer and Oertel, 1994; Przedborski et al., 1995). However, it is not clear whether or not the mechanism by which 6-OHDA kills the DA neurons shares the same molecular features as in PD.

In mice MPTP is usually administered either by an acute or a subacute regimen. In most acute models, mice are injected with four i.p. doses of 20 mg/kg MPTP free base at 2 hr intervals (Sonsalla and Heikkila, 1986; Jackson-Lewis et al., 1995). In our hands the majority of the animals die after the third injection of MPTP therefore we routinely use only 2 doses of MPTP for our acute studies. Subacute MPTP treatment consists of five s.c. injections with 30 mg/kg MPTP on daily intervals for 5-10 days (Heikkila et al., 1984; Tatton and Kish, 1997; Turmel et al., 2001). Although the multiple-dose, acute

MPTP model can effectively cause rapid and drastic depletion of striatal DA, the DA depleting effect of MPTP tends to be short-lived and reversible (Lau and Meredith, 2003).

Many of the above mentioned models show either reversible or abrupt end-stage type of PD symptomatology, which are not likely suitable for long-term survival studies. MPTP has emerged as a leading neurotoxic agent for inducing parkinsonism in animal model (Lau and Meredith, 2003). The MPTP neurotoxicity in humans is irreversible, and the consequential clinical and biochemical traits closely resemble those seen in idiopathic PD (Ballard et al., 1985; Burns et al., 1985). After the discovery of MPTP, there was a boost in Parkinson's disease research. Since then MPTP has been tested in various species and the results are notably different.

Although similar symptoms can be replicated in nonhuman primates their sensitivity to the toxin is markedly different (Gerlach and Riederer, 1996). It has been reported that MPTP-induced Parkinsonism symptoms in primates tend to reverse spontaneously over time (Eidelberg et al., 1986). Additionally, the classical hallmark of PD, the Lewy bodies, are not detected in humans or non human primates (Forno et al., 1993; Langston et al., 1999). Although the use of nonhuman primate MPTP model would be invaluable for preclinical evaluations of new therapies for PD, it would be very

expensive and unjustifiable for investigating the neurological and pathological mechanisms underlying this disease.

The toxic effects of many substances after an acute exposure are different from those produced by repeated exposures over a prolonged time course. MPTP when given at regular intervals without providing sufficient time for cells to recover between successive doses can produce irreversible cellular damages even after the toxin has been eliminated from the brain (Langston et al., 1999). It is conceivable that a chronic small animal model of PD would be most practical for studying and testing neuroprotective and neurorestorative paradigms.

Our laboratory has established and utilized a modified, chronic mouse MPTP/probenecid model for Parkinson's disease, which is characterized by a slow, progressive loss of SNpc neurons coincident with persistent depression of striatal DA concentration and terminal DA uptake for at least six months after treatment (Lau et al., 1990; Petroske et al., 2001). Hence, in contrast to the acute and subacute MPTP models, this chronic MPD model affords the opportunity for elucidating the underlying cellular changes and mechanisms involved in dopaminergic neurodegenerative process resembling those of PD.

2.5. Exercise and PD

It is generally recognized that age-related physiological changes and keeping a sedentary lifestyle may increase the risk for developing cardiovascular, metabolic and neurological diseases (Hirsch and Farley, 2009). A recent report from the American Heart Association provides scientific evidence that regular exercise may decrease the vulnerability for the above mentioned conditions and promote healthy aging (Nelson et al., 2007). An epidemiological study has suggested an inverse relationship between the level of physical activity and the probability of developing PD (Chen et al., 2005). Regular exercise also tends to delay the progression of Parkinsonian symptoms in early diagnosed PD patients (Tsai et al., 2002). Regular aerobic exercise has been shown to promote neuronal plasticity-associated changes including synaptogenesis, enhanced glucose utilization and neurogenesis. In older individuals aerobic exercise has shown to reduce inflammation and stabilize calcium homeostasis (Cotman and Berchtold, 2002).

Exercise protocols used in laboratory studies have been extremely variable therefore it is essential to use standardized protocols (Call et al., 2008). Behavioral assessment of exercise effect has been reported in several animal models of Parkinsonism. In the rat model of Parkinsonism induced by 6-OHDA, either voluntary running or treadmill paced exercise attenuated DA loss in the striatum with or without significant recovery of behavioral deficits (Tillerson et al., 2003; Mabandla et al., 2004;

Poulton and Muir, 2005). The neuronal recovery in 6-OHDA-treated rats triggered by exercise is associated with an increase of the striatal GDNF (Cohen et al., 2003). In an acute mouse model of Parkinsonism induced by MPTP, treadmill exercise ameliorates behavioral deficits and reverses several striatal dopaminergic indices, including the loss of DA, tyrosine hydroxylase (TH)-immunoreactivity, and DA transporter levels when compared to the sedentary Parkinsonian animals (Tillerson et al., 2003). In another study, high-intensity treadmill exercise in acute MPTP-treated mice leads to behavioral recovery; however, the striatal expression of DA transporter is down-regulated and the expression of TH is not changed (Fisher et al., 2004). Differences in the exercise results obtained from animal models of Parkinsonism could be due to experimental variables such as the age and species of the animal, the method and severity of the induced nigrostriatal lesion, and the type and intensity of the exercise regimen.

In animal studies brain-derived neurotrophic factor (BDNF) is shown to enhance synaptic plasticity and memory through its actions on the TrKB receptor (Minichiello et al., 1999; Mattson et al., 2004). Physical exercise increases serum BDNF in humans and brain BDNF in rodents (Hirsch and Farley, 2009). This theory is supported by the fact that blocking BDNF signaling also prevents the cognitive function following physical training (Neeper et al., 1995; Vaynman et al., 2004). Long term exercise has also been shown to cause the release of neurotrophins such as BDNF, GDNF and nerve growth

factor (NGF) which are involved in enhancing synaptic plasticity, cognition, learning and memory (Colcombe and Kramer, 2003). Zigmond and colleagues demonstrated that when rodents and monkeys are exercised then followed with MPTP or 6-OHDA treatment, these animals show reduced behavioral impairments as assessed by PET imaging and biochemical or histochemical assessments (Zigmond et al., 2009). One of several possible explanations for the beneficial effects of exercise is the increase in the expression of neurotrophic factors, particularly the GDNF, suggesting that exercise-induced release of GDNF is neuroprotective in animal models of PD (Zigmond et al., 2009). These studies further implicates that the PD brain has an enormous capacity to reshape and remodel itself in response to physical activity. Thus, exercise is recommended in neurological diseases like PD to slow down the deterioration of symptoms and to regain lost function.

2.6. Melatonin and PD

In mammals, melatonin is a natural hormone primarily secreted from the pineal gland. Once released into the blood, melatonin maintains a circadian rhythm with highest concentration detected during the dark period in a nocturnal species (Quay, 1974; Reiter, 1991). Light-at-night quickly depresses pineal melatonin production via a photopigment melanopsin, which is present in the photoreceptive retinal ganglion cells in the lateral eyes (Panda et al., 2005). Age is also a factor that decreases melatonin

production. In all species, it has been shown that increasing age is associated with gradual waning of the nocturnal melatonin rhythm (Sack et al., 1986; Reiter, 1991). While melatonin activates its receptors for most of its biological functions, it can produce a receptor independent mitochondrial protective effect, through its antioxidant and free radical scavenging properties (Tan et al., 2002). Some preliminary studies show that mitochondrial melatonin concentration exceeds that found in blood or other portions of the cells under physiological conditions (Martin et al., 2000).

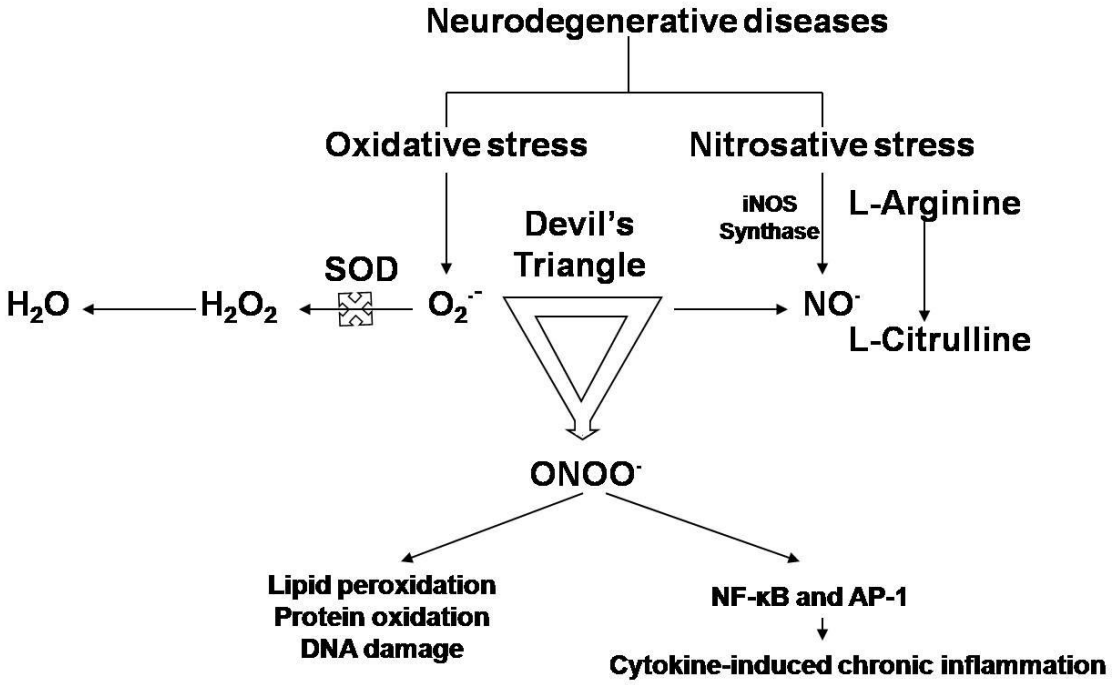

Figure 7: Organization of the devil's triangle within the targeted cell. Normally, $O_2^{\bullet-}$ is readily degraded to H_2O by intracellular SODs, but in the presence of increased $O_2^{\bullet-}$ and NO leads to the formation of $ONOO^-$ thereby reducing the degradation of $O_2^{\bullet-}$ by SOD (modified from Korkmaz et. al., 2009).

Melatonin is considered a free radical scavenger (Poeggeler et al., 1993) and is shown as a potent scavenger of $^{\bullet}OH$ (Valko et al., 2005; Fukutomi et al., 2006). It has been found that each molecule of melatonin scavenges two $^{\bullet}OH$ and in the process it is converted to 3-hydroxymelatonin (3-OHMEL). Like melatonin, its 3-OHMEL metabolite also possesses free radical scavenging properties and in doing so it gets converted into N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) (Tan et al., 2000). AFMK also has free radical scavenging properties. In this scheme it has been estimated that a single molecule of melatonin may remove up to ten toxic reactants. It is demonstrated that the antioxidant enzyme activities are increased in the presence of exogenously administered melatonin (Barlow-Walden et al., 1995; Pablos et al., 1995). Melatonin is also capable to inhibit nitric oxide synthase and to prevent the formation of NO (Korkmaz et al., 2009). As mentioned earlier when NO couples with $O_2^{\bullet-}$, the highly reactive and tissue damaging species, $ONOO^-$ is produced **(Figure 7)**. Hence $ONOO^-$ is considered a pro-oxidant and its rapid removal will cause less damage to the mitochondria (Pozo et al., 1997; Reiter et al., 2000; Acuna-Castroviejo et al., 2005; Leon et al., 2006). Melatonin is also capable to inhibit nitric oxide synthase and to prevent the formation of NO (Korkmaz et al., 2009). Studies using mutant mice lacking the nNOS and iNOS gene were resistant to MPTP-induced neurotoxicity when compared with wild-type mice. These results indicate that neuronally derived NO mediates, in part, MPTP-induced neurotoxicity (Liberatore et al., 1999; Klivenyi et al., 2000). These studies display the critical role of both NOS in

mediating dopaminergic neurodegeneration.

In 1999, Urata and colleagues also showed that under elevated oxidative stress, melatonin preserves intracellular GSH levels by stimulating the activity of γ-glutamylcysteine synthase. They also show that melatonin stimulates complexes I and IV of the mitochondrial ETC; therefore, the number of electrons that leak from the complexes is reduced and free radical generation is attenuated (Urata et al., 1999).

3. METHODS AND MATERIALS

3.1. Animals and housing conditions

Two different age groups of male, C57BL/6 mice, 6-10 weeks (young adult) and 6-10 months (aged retired-breeder), weighing 25-28 and 35-40 gm, respectively were used (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA). Mice 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. All animal treatments were carried out strictly in accordance to the National Institute 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. Our experimental procedures did not cause significant animal suffering.

3.2. Acute model for Parkinson's disease

In most acute models, mice are injected with four i.p. doses of 20 mg/kg MPTP free base at 2 hr intervals (Sonsalla and Heikkila, 1986; Jackson-Lewis et al., 1995). In our hands following this protocol, a majority of the animals die after the third injection of MPTP. Therefore in our acute model, we injected mice with 2 doses of MPTP

hydrochloride (25 and 15 mg/kg/injection in saline, s.c. for young and aged mice, respectively) 2 hours apart. Experiments were conducted 2 hours post-acute treatment.

3.3. Chronic mouse model of Parkinson's disease

To prepare the chronic MPD, mice were injected with a total of 10 doses of MPTP hydrochloride (25 and 15 mg/kg/injection in saline, s.c. for young and aged mice, respectively) in combination with an adjuvant, probenecid (250 mg/kg/injection dissolved in dimethyl sulfoxide, i.p.) as previously described (Lau et al., 1990; Petroske et al., 2001). The 10-dose regimen was administered on a five-week schedule with an interval of 3.5 days between injections. 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 procedures previously described (Lau et al., 2005). Control mice were treated with probenecid only. Probenecid was used to inhibit the rapid clearance and excretion of MPTP and its metabolites from the brain and kidney, and probenecid alone did not produce any significant neurotoxic effect; but in combination it potentiated the neurotoxicity of MPTP (Lau, 2005; Lau et al., 2005; Barber-Singh et al., 2009).

The chronic MPD used in this study has been well characterized. In contrast to the most commonly used acute and sub-acute MPTP MPD, in which neurological and behavioral deficits are short-lived and spontaneously reversed soon after the treatment,

the chronic MPD has long-term neurological deficits showing many features resembling those of the Parkinson's syndrome and lasting for at least 6 months (Lau, 2005). The observed features include marked depletion of DA content and terminal DA uptake in association with significant behavioral deficit and loss of DA cells in the substantia nigra (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). A comparison between the acute, sub-acute and chronic MPD is summarized in Table 1.

Table 1: Comparison between acute, sub-acute and chronic mouse models of PD

Factor	Acute and sub-acute	Chronic
Treatment	20mg/kg of MPTP 4 times/day at 1-2hr interval or 30mg/kg of MPTP daily for 5-10 days (Sonsalla and Heikkila, 1986; Petroske et al., 2001)	25mg/kg of MPTP and 250mg/kg of Probenecid 10 doses in a span of 5 weeks (Petroske et al., 2001)
Dopamine Content	Decrease striatal levels but reversible if survival times extended (Petroske et al., 2001)	>90% decrease of striatal levels for at least 6 months (Petroske et al., 2001)
Dopamine uptake	Decrease striatal uptake but reversible if survival times extended (Petroske et al., 2001)	>90% inhibited for at least 6 months (Petroske et al., 2001)
Neuronal loss	Neuronal loss in SN was not significant (Gorrell et al., 1996)	Neuronal loss was significantly detected for at least 6 months (Petroske et al., 2001)
Behavior	No long lasting motor deficit (Gerlach and Riederer, 1996)	Deficit in balance, ambulatory movement and gait performance (Pothakos et al., 2009)
Morphology	May induce apoptosis of dopaminergic neurons (Gorrell et al., 1996)	Induces apoptosis of dopaminergic neurons (Novikova et al., 2006)
Inclusions	Accumulation of α-synuclein and ubiquitin proteins not detected (Polymeropoulos et al., 1997)	Accumulation of α-synuclein and ubiquitin positive proteins detected (Meredith et al., 2002)

3.4. Isolation of striatal mitochondria

Due to tissue limitation, a crude striatal mitochondrial homogenate was prepared according to the protocol of Sullivan et al. (Sullivan et al., 2000). Briefly,

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 (BSA, 0.1%), EGTA (1 mM) and HEPES (20 mM) at a pH of 7.2. All subsequent procedures were carried out at 4^{o}C. The homogenate was centrifuged at 1,300 rpm 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 centrifuged again at 1,300 rpm for 3 min. The supernatants from both spins were combined and centrifuged at 13,000 rpm for 10 min. This last 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 (BSA, 0.1%), HEPES (20 mM), $MgCl_2$ (2 mM), and KH_2PO_4 (2.5 mM) at a pH of 7.2. The protein concentration in the mitochondria containing homogenate was determined with the Pierce micro BCA protein assay kit (Rockford, IL, USA) measured at an absorbance of 595 nm with a Beckman DU 640 spectrophotometer (Fullerton, CA, USA).

3.5. Mitochondrial respiration assay

Since mitochondria are vital organelles responsible for catalyzing the oxidation of various substrates through an electron transport chain system and for generating ATP to support cell function and survival, 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).

In the chamber, the mitochondrial homogenate preparation (0.5mg/ml) was first allowed to equilibrate with the respiration buffer at 30°C. After equilibration, the substrates (5 mM pyruvate and 2.5 mM malate) were added. The decrease in oxygen level was observed and recorded as the first state 4 respiration. After reaching a nadir, ADP (150 μM) was added to the incubation chamber and a rapid fall in oxygen level was detected as the state 3 respiration resulting in ATP synthesis **(Figure 8)**. After all the ADP was consumed, another fall in the oxygen level was seen that is designated as the second state 4 respiration **(Figure 8)**. Respiratory control ratio (RCR) is expressed as the ratio of state 3 over state 4 respiration. A normal mitochondrial preparation typically shows an RCR between 5-10, whereas an inhibited or defective mitochondrial preparation has an RCR less than 5.

Mitochondrial respiration

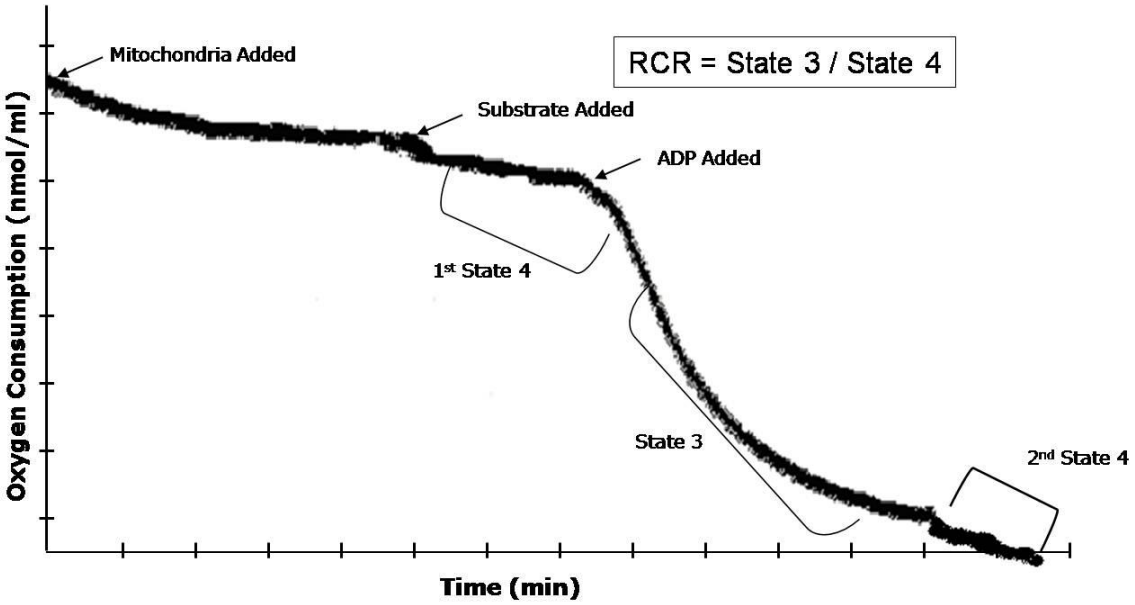

Figure 8: Representative polarographic tracing of mouse striatal mitochondrial homogenate using NAD-linked substrates (pyruvate and malate).

3.6. Measurement of mitochondrial ATP

Striatal mitochondrial homogenate was prepared under conditions identical to that of the respiration study. The reagents supplied in the ATP luminescence kit (PerkinElmer, Waltham, MA) were equilibrated at room temperature. The lyophilized substrate solution was reconstituted by adding 5 ml of substrate buffer solution. After the respiration experiment, 100 µl of striatal mitochondrial suspension from the chamber was recovered and mixed with an equal volume of the lysing buffer in each well of a microplate and shaken for 5 minutes at 700 rpm. Another 50 µl of the

substrate solution was added to the wells and continuously shaken for 5 min at 700 rpm. The sample was dark adapted for 10 min and the light emitted from luciferase-mediated reaction was captured with a luminometer (Wallac Victor II, PerkinElmer). The sample ATP content was calculated from a standard curve constructed with a series of known ATP concentrations (Tieu et al., 2003).

3.7. Western blot analysis

Besides serving as energy generating organelles, mammalian mitochondria also play a pivotal role in regulating the production and removal of ROS. Several mitochondrial antioxidant enzymes have been widely studied. Mn SOD is located in the mitochondrial matrix where it protects against the internally generated superoxides. Cu-Zn SOD and cytochrome c are capable to remove superoxides from external sources. These antioxidant enzymes are important for scavenging the elevated ROS and for preventing them from reaching a toxic level that causes mitochondrial damage and cell death (McCord, 1993; Pereverzev et al., 2003). Therefore, loss of antioxidant enzymes and release of cytochrome c in association with mitochondrial dysfunction in the DA neurons may lead to substantial ROS accumulation and cause PD-like neurodegeneration. Based on this neurotoxic mechanism, the level of mitochondrial antioxidant enzymes were also examined in the present study.

For Western blot analysis of antioxidant protein expression, crude striatal mitochondria were prepared according to the procedure as described above, except that the mitochondrial pellet was finally suspended in Tris-HCl buffer (50 mM, pH 7.4) containing a protease inhibitor cocktail (P-2174) purchased from Sigma Chemical Co. The mitochondrial homogenate concentration in each sample was determined with the Pierce micro BCA protein assay kit and diluted to an appropriate final concentration in a protein solubilization solution containing SDS (2%), Tris-HCl (62.5 mM, pH 6.8), glycerol (10%), 2-mercaptoethanol (5%), bromophenol blue (0.001%). The sample was boiled for 5 min and 25 μg of each sample was applied to SDS polyacrylamide (12%) gel electrophoresis. The resolved proteins were transferred to a PVDF membrane, which was probed with primary antibodies for various proteins. The immunoreactivity was visualized by using a species-matched horseradish peroxidase-conjugated secondary antibody with an enhanced NuGlo chemiluminescent substrate (Alpha Diagnostic International Inc., San Antonio, TX). The source and dilution for each antibody used are shown in Table 2.

Table 2: Antibodies, dilutions and sources: Summary of the primary and secondary antibodies that were used for the immunoblotting studies.

Proteins	Primary Antibody	Secondary Antibody
TH	Mouse anti-TH 1:1000 Millipore, Temecula, CA	Goat anti-mouse HRP 1:2000 Santa Cruz Biotechnology Inc., CA
DAT	Rat anti-DAT 1:1000 Chemicon, Temecula, CA	Goat anti-rat HRP 1:2000 Chemicon, Temecula, CA
VMAT-2	Polyclonal rabbit anti-VMAT-2 1:1000 Abcam, Cambridge, MA	Goat anti-rabbit HRP 1:2000 Chemicon, Temecula, CA
Mn SOD	Polyclonal rabbit anti-Mn-SOD 1:1000 Upstate, Lake Placid, NY	Goat anti-rabbit HRP 1:2000 Chemicon, Temecula, CA
Cu-Zn SOD	Polyclonal rabbit anti Cu/Zn-SOD 1:1000 Stressgen Bioreagents, B.C., Canada	Goat anti-rabbit HRP 1:2000 Chemicon, Temecula, CA
Cytochrome c	Monoclonal mouse anti-cytochrome c 1:750 Chemicon, Temecula, CA	Goat anti-mouse HRP 1:2000 Santa Cruz Biotechnology Inc., CA
GAPDH	Monoclonal mouse anti-GAPDH 1:3000 Chemicon, Temecula, CA	Goat anti-mouse HRP 1:2000 Santa Cruz Biotechnology Inc., CA
β-Tubulin	Monoclonal mouse anti-β-Tubulin 1:2000 Upstate, Lake Placid, NY	Goat anti-mouse HRP 1:2000 Santa Cruz Biotechnology Inc., CA
Cytochrome oxidase	Monoclonal mouse anti-cytc oxidase 1:1000 Molecular Probes, Carlsbad, CA	Goat anti-mouse HRP 1:2000 Santa Cruz Biotechnology Inc., CA

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 band was measured by densitometry (Fluorochem 8800, Alpha Innotech Corporation, San Leandro, CA) and expressed as a ratio to GAPDH, β-tubulin or cytochrome oxidase to ensure that changes of protein level are not simply due to differences in the amount of sample loading.

3.8. Assay for striatal dopamine and 3,4-dihydroxyphenylacetic acid content

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

3.9. Animal performance on a balancing beam

We monitored the ability of the aged chronic MPD in maintaining balance on a challenging beam 6 weeks post-chronic treatment as previously described (Pothakos et

al., 2009). The challenging beam as shown in **(Figure 9)** 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 (ITI) of 10-12 sec on traversing the beam. On the third day, each mouse was given 5 trials (ITI = 10-12 sec). The average number of videotaped limb slips per trial and the time latency for returning to the home cage were recorded and compared with control mice for statistical analysis. Slips were counted only while the mouse was in forward motion.

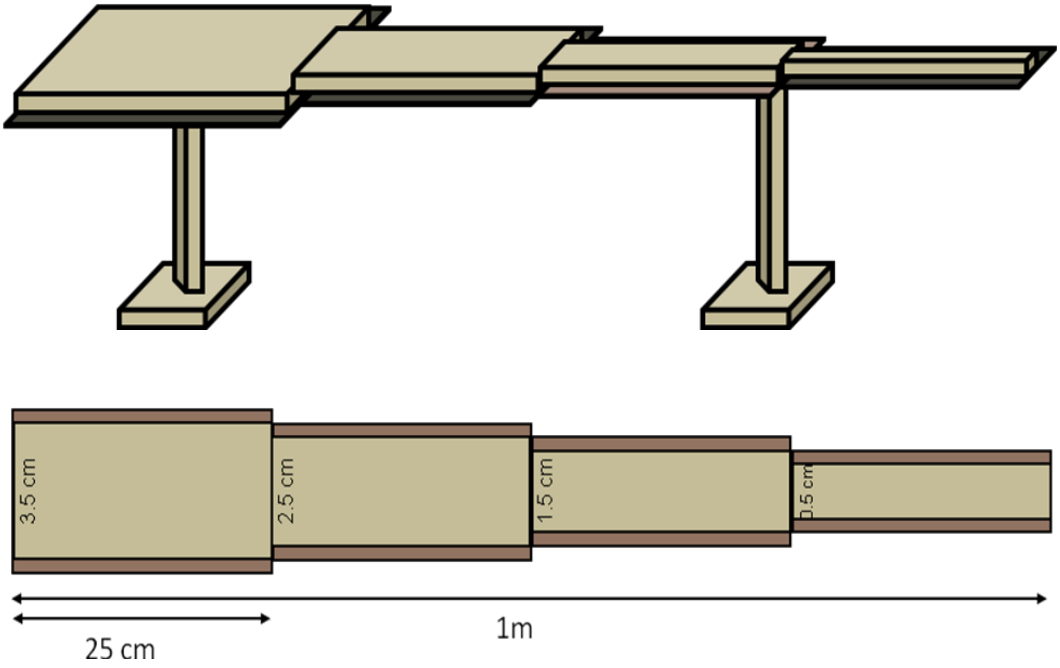

Figure 9: Schematic representation of the balancing beam.

3.10. Protein carbonylation

The level of carbonylated proteins was measured as a marker for oxidative stress in the striatum. The isolated striatum was homogenized in Tris-HCl buffer (50 mM, pH 7.4) containing the protease inhibitor cocktail purchased from Sigma Chemical Co. The tissue homogenate containing 20 µg of protein was reacted with 10 µl of 2,4-dinitrophenylhydrazine (DNPH) reagent at room temperature for 15 min, then by the reaction was terminated by adding 7.5 µl of a neutralization solution. Carbonylated proteins present in the sample reacted with DNPH to form DNP-hydrazone, which were

resolved by the SDS polyacrylamide (8-16%) gel electrophoresis (Pierce, Rockford, IL, USA). The resolved carbonylated proteins were detected by immunoblotting as per OxyBlot kit protocol (Millipore Billerica, MA). Monoclonal mouse anti-β-actin (1:2000, Millipore, Temecula, CA) was detected in membranes after stripping using an anti-mouse goat HRP (1:2000, Santa Cruz Biotechnology Inc., CA). Several conjugated proteins bands (43, 67, 94.2 kD) were included for quantification and were normalized against β-actin (Dalla Libera et al., 2009).

3.11. Striatal RNA extraction

Total RNA was isolated from striatum using an RNeasy kit (Qiagen Inc., Valencia, CA). Briefly the isolated striatum was flash frozen in liquid nitrogen and immediately transferred to -80°C for storage until used, Approximately 20 mg of tissue was thoroughly homogenized (usually 20-40 sec) in a conventional rotar-stator homogenizer containing 350 µl of lysis buffer (RLT) and 10 µl/ml of β-mercaptoethanol. The homogenate was centrifuged for 3 min at 10,000 rpm. The supernatant was carefully removed and transferred to a new microcentrifuge tube. One volume of 70% ethanol was added to the supernatant lysate, and immediately followed by pipettor mixing. Around 700 µl of the sample was transferred including any precipitate that might have formed to an RNeasy spin column placed inside a 2 ml collection tube. The lid was closed and the collection tube was centrifuged for 15 s at 10,000 rpm. The spin column

was first washed with 700 µl washing buffer (RW1) followed by 2 sequential washes with 500 µl of washing buffer containing 70% ethanol (RPE). All the flow-through solvent from each of the above steps was discarded. The RNeasy spin column was finally placed in a new 1.5 ml collection tube and RNA was eluted by adding 30 µl of RNase-free water and centrifuging for 1 min at 10,000 rpm. The integrity of the isolated RNAs was checked by running the samples on a 1% formaldehyde agarose gel. The final concentration of the extracted RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a U-2910 Hitachi spectrophotometer (Hitachi High Technologies America, Inc. USA) (Perier et al., 2007).

3.12. Quantitative real-time RT–PCR (qRT-PCR)

The total RNA extract (1 µg) was reverse-transcribed using random hexamers and multi-scribe reverse transcriptase supplied in an iScript cDNA synthesis kit in a two-step RT–PCR reaction (Bio-Rad Laboratories, Hercules, CA). The gene sequence was obtained from the genebank and primers for PCR were designed to span the intron/exon junctions using primer express software (Applied Biosystems, Foster City, CA) to minimize any amplification of residual genomic DNA. The primer sequences for mouse p53; mitochondrial transcription factor A (TFAM); peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC-1α) and GAPDH are listed in Table 3.

Table 3: Primers used for quantitative real time PCR

	Gene	Gene ID	Forward Primer	Reverse Primer
1	p53	NM_011640.3	5'-TTAAAAGAGTGCGCCGATAGG-3'	5'-GAATGCGTTAAGCAAGGGAAT-3'
2	TFAM	NM_009360.4	5'-GCACCCTGCAGAGTGTTCAA-3'	5'-CGCCCAGGCCTCTACCTT -3'
3	PGC-1α	NM_008904.2	5'-TGCGGGATGATGGAGACA -3'	5'-GCGAAAGCGTCACAGGTGTA -3'
4	GAPDH	NM_008084.2	5'-CTCATGACCACAGTCCATGC -3'	5'-CACATTGGGGGTAGGAACAC-3'

QRT-PCR was performed with the SYBR Green detection system (Bio-Rad Laboratories, Hercules, CA) with an ABI Prism 7300 sequence detector (Applied Biosystems). Thermal cycling conditions included pre-incubation at 50°C for 2 min; DNA polymerase catalyzed activation at 95°C for 1 min, which causes disruption of hydrogen bonds between complementary bases, yielding single strands of DNA (denaturation step); and 40 PCR cycles for 15 s at 95°C and for 1 min at 60°C that anneal of the primers to the single-stranded DNA template to from closely matched stable DNA-DNA hydrogen bonds. The transcript level for each gene was calculated at a cycle threshold value at which each fluorescent signal was first detected above the background and analyzed by the ABI Prism 7300 SDS software (Version 1.4) (Applied Biosystems). The mRNA level was normalized and expressed as a ratio to that of the housekeeping gene, GAPDH (Docter et al., 1982; Perier et al., 2007).

3.13. Endurance exercise training protocol

In literature the exercise protocols used in various disease models have been inconsistent. Hence, it was important to first standardize the exercise protocol best suited for our study. Initially normal and chronic MPD animals were tested in our laboratory on three types of exercise equipment such as voluntary exercise wheels, motorized exercise wheels and motorized treadmills. Although voluntary wheel exercise offers the great advantage of a more “environment enriched” type of exercise and the animals can exercise according to their will and individual capacity for running, it was found that there was a wide variation in the distance of running between individual mice, thus requiring a large number of animals to reach statistical significance. Motorized exercise wheels were also tested and we found that mice had a tendency to clasp onto the wheel while it rotated without getting real physical exercise. Motorized treadmill appeared to be better suited for our purpose of study. We had followed a running scheme according to the Bruce protocol for human physical fitness (Bruce et al., 1973). It is important to mention that the exercise protocol is comparable to an aerobic exercise-trained human individual and does induce stress or neuronal inhibition (Al-Jarrah et al., 2007).

A six-lane motorized rodent treadmill (Columbus Instruments, Columbus, OH, USA) was utilized. Mice were exercise trained one week before, 5 weeks during the

chronic MPTP/probenecid treatment, and exercise was continued for 12 weeks after the completion of the MPTP treatment **(Figure 10)**.

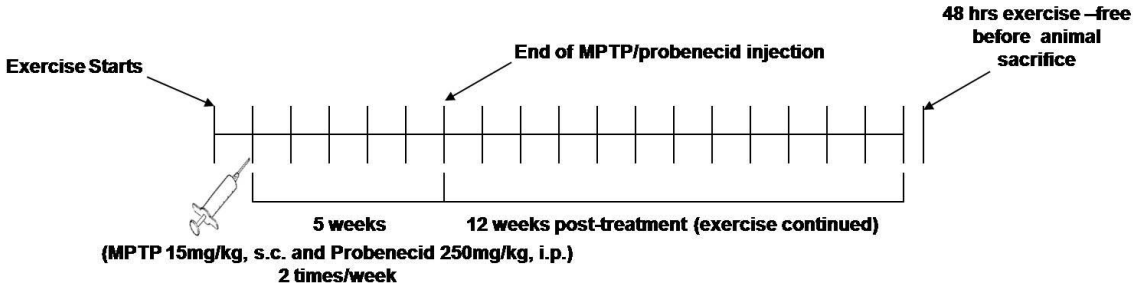

Figure 10: Schematic representation of the treadmill exercise in chronic MPD.

The exercised group of animals was trained on the treadmill running for 5 days/week, 40 min/day at a speed up to 15 m/min (5 min at 6 m/min, 5 min at 9 m/min, 20 min at 12 m/min, 5 min at 15 m/min, and 5 min at 12 m/min) with 0° of inclination. Using this treadmill exercise protocol in our laboratory, the chronic MPD mice were able to go through the training with minimal requirement for external stimuli or manual prodding, yet the animals developed physical endurance after 4 weeks showing cardiorespiratory and metabolic adaptations comparable to those seen in human subjects undergoing continuous exercise training (Al-Jarrah et al., 2007); and as summarized in Table 4. Sedentary mice did not exercise; however, they were transported daily to the training room so that they were exposed to the same environment as the exercised group of animals.

Table 4: Cardiovascular, metabolic and respiratory rehabilitations in chronic MPD after 4 weeks of treadmill exercise (Al-Jarrah et al., 2007).

Aerobic Exercise Indicators	Rehabilitations in Chronic MPD
Resting heart rate	Decreased
Left ventricular mass	Increased
Body heat generation	Decreased
O_2 consumption and CO_2 production	Decreased
Skeletal muscle citrate synthase	Increased

3.14. Melatonin treatment protocol

Male, 8-month old, retired breeder C57/BL mice were treated with 10 doses of MPTP (15 mg/kg, s.c.) and probenecid (250 mg/kg, i.p.) over five weeks as described in section 3.3. The chronic MPD group was injected with or without melatonin (5 mg/kg/day in DMSO and q.s. with saline, i.p.), for 5 days/week **(Figure 11).** Melatonin was made fresh every day and injected during the light phase when the melatonin levels in mice are low (around 10-11 am). Melatonin was injected 1 week before, 5 weeks during, and 12 weeks after the chronic MPD treatment. Control groups of mice were similarly treated with either saline or melatonin alone.

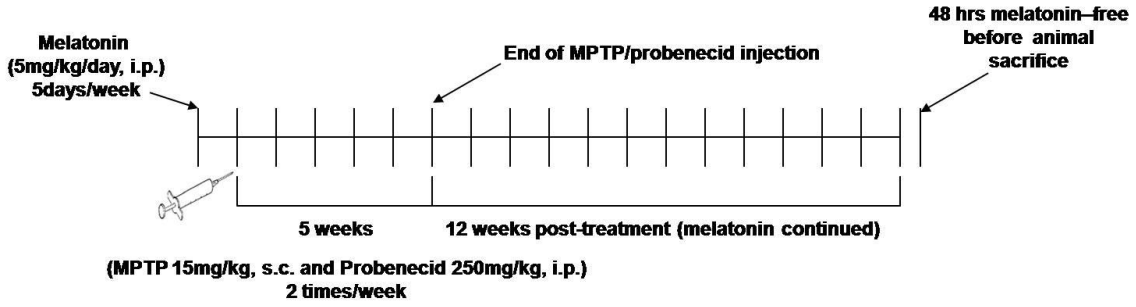

Figure 11: Schematic representation of chronic melatonin treatment in chronic MPD.

3.15. Statistical Analysis

Comparison of two group means was carried out with a Student's t-test. A comparison between more than two group means was carried out with one-way ANOVA followed by Tukey's post-hoc (Prism, Graphpad, La Jolla, CA). Minimal significance level was set at $P<0.05$. Results are expressed as means ± S.E.M.

4. RESULTS

4.1. Chronic MPTP treatment on neuronal and mitochondrial function in the striatum of young mice.

For studying the hypothesis on whether striatal mitochondrial dysfunction co-exists in the chronic MPD, we initially used the chronic model developed in our laboratory with young adult C57/BL mice at the age of 6-10 weeks. The mice were treated with 10-doses of MPTP (25 mg/kg) and probenecid (250 mg/kg) for 5 weeks (Lau et al., 1990; Petroske et al., 2001). In this model, six weeks after chronic MPTP/probenecid treatment, we detected a sustained 74.9% and 70.9% loss of striatal DA and DOPAC, respectively **(Table 5).** For comparison with an acute model, young mice were treated with two doses of MPTP (25 mg/kg), 2 hours apart. Two hours post-treatment, the acutely MPTP-treated mice showed a depletion of 54.1% DA and 48.3% DOPAC in the striatum **(Table 5).** These results indicate that MPTP has an acute effect in depleting the striatal DA; and when administered chronically with probenecid, MPTP produces a persistent loss of DA in the striatum.

Table 5: Striatal DA and DOPAC levels in acute and chronic young adult (6-10 weeks old) MPD

Animal group	N	DA (ng/mg tissue)	DOPAC (ng/mg tissue)
Young acute control	5	13.81 ± 1.73	1.43 ± 0.39
Young acute MPD[a]	5	6.34 ± 0.86*	0.74 ± 0.27*
Young chronic control	7	12.97 ± 1.33	1.65 ± 0.47
Young chronic MPD[b]	7	3.26 ± 0.41**	0.48 ± 0.36**

[a]The young acute MPD received 2 injections of MPTP hydrochloride (25 mg/kg/dose made in saline, s.c.) 2 hours apart. Experiments were conducted 2 hours post-acute treatment. The acute control group received saline only.

[b]The young chronic MPD received 10 doses of MPTP hydrochloride (25 mg/kg/dose in saline, s.c.) in combination with an adjuvant, probenecid (250 mg/kg/injection dissolved in dimethyl sulfoxide, i.p.). The 10-dose regimen was administered on a five-week schedule with an interval of 3.5 days between injections. Experiments were conducted 6 weeks post-chronic treatment. The chronic control group received probenecid only.*Significantly lower than the acute saline control group ($P<0.002$).**Significantly lower than the chronic probenecid control group ($P<0.0001$).

Mitochondrial respiration in the striatal homogenate was measured and compared similarly between the acute and chronic MPD. The basal rate of mitochondrial respiration (state 4 respiration) in young adult mice was not significantly different between acute control, acute MPD, chronic control, and chronic MPD groups **(Table 6).** The ADP-stimulated mitochondrial respiration (state 3 respiration) in young acute MPD was markedly reduced when compared with the acute control animals **(Table 6).** Surprisingly, the young chronic MPD showed no signs of respiratory depression in the striatal mitochondrial preparation when compared with that in the chronic control mice **(Table 6).** These data demonstrate that although MPTP has an

immediate toxic effect on mitochondrial respiration, this defect is not sustained 6 weeks after the chronic MPTP/probenecid treatment.

Table 6: Striatal mitochondrial respiration in acute and chronic young adult (6-10 weeks old) MPD[a]

Animal group[a]	N	State 3 respiration[b]	State 4 respiration[b]
Young acute control	5	9.62 ± 0.84	2.05 ± 0.39
Young acute MPD	5	4.20 ± 0.64*	1.40 ± 0.15
Young chronic control	6	10.55 ± 0.71	1.65 ± 0.12
Young chronic MPD	5	10.33 ± 0.69	1.80 ± 0.11

[a] See legends under **Table 5** for animal treatment protocols.

[b] Rate of mitochondrial oxygen consumption in presence (state 3) or absence (state 4) of ADP (150 μM). Data are expressed as mean ± S.E.M. in nmol/min/mg protein. *Significantly lower than the acute saline control group (P<0.001).

We further examined the expression of striatal mitochondrial antioxidant proteins by the Western blot analysis. In young acute MPD, the levels of Mn SOD, Cu-Zn SOD, and cytochrome c were not significantly altered in the striatal mitochondrial homogenate **(Figure 12, A-C)**. Similarly, the contents of these three proteins in young mice 6 weeks after chronic MPTP/probenecid treatment were not different from either the control or acute MPD group **(Figure 12, A-C).** Since the basal levels of these proteins in acute saline-treated and chronic probenecid-treated young mice were not statistically different, only the control data from chronic probenecid treated group were presented.

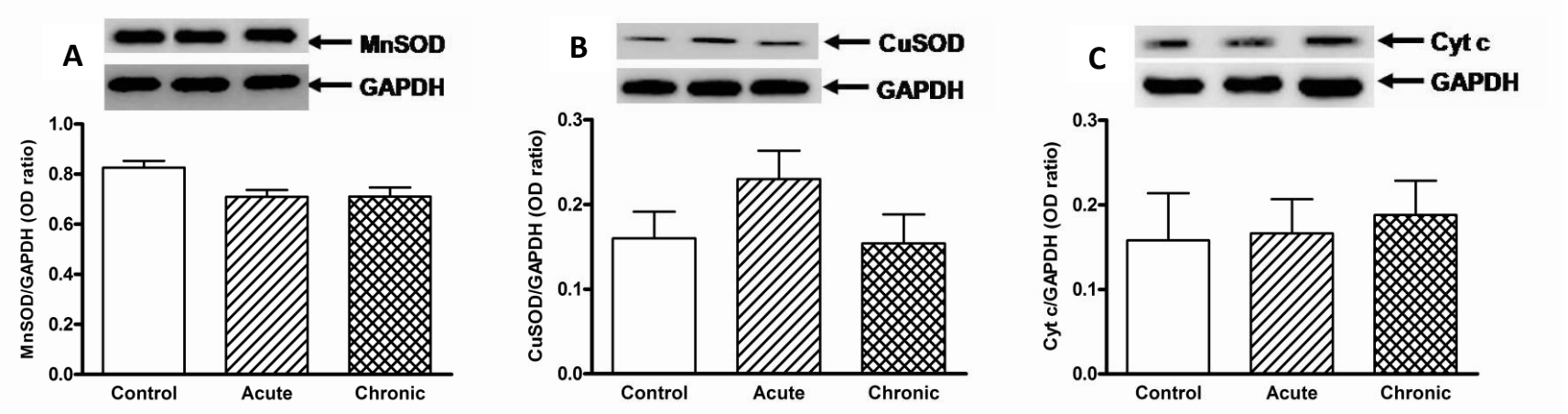

Figure 12: Protein expression of (A) Mn SOD, (B) Cu-Zn SOD and (C) cytochrome c in the striatum of young control, acute MPTP, and 6-week post-chronic MPTP/probenecid-treated parkinsonian (MPD) mice. Western blot technique and densitometry analysis were used to detect and quantify the respective anti-oxidant protein bands and GAPDH. The protein contents of Mn SOD, Cu-Zn SOD and cytochrome c were expressed as a ratio to that of GAPDH to ensure that the changes in protein levels were not due to sample loading variances. A representative image of Western blot protein bands was correspondingly shown above each set of bar graph. Each data point represents mean ± S.E.M. N = 6 per group of animals. The levels of Mn SOD, Cu-Zn SOD and cytochrome c in the striatum of young control, acute MPTP, and 6-week post-chronic MPD mice were not statistically different from each other.

4.2. Chronic MPTP treatment on neuronal function in the striatum of aged mice.

It is possible that the functionality of mitochondria may gradually recover in young adult mice after the toxic species of MPTP are dissipated from the brain. Before ruling out the possible association between Parkinson-like neurodegeneration and mitochondrial disorder, we were curious to investigate whether mitochondrial dysfunction may exist in aged chronic MPD, since aged mice are known to be more sensitive to MPTP neurotoxicity (Ricaurte et al., 1987). Based on our observations, aged mice do not survive the same chronic MPTP/probenecid regimen as in young adult mice; therefore we treated the aged chronic MPD with a reduced dose of MPTP (15 mg/kg, s.c) and probenecid (250 mg/kg, i.p), twice a week for 5 weeks in the present study. With this protocol, all aged mice (6-10 months old) survived displaying 64.6% and 53.3% loss of DA and DOPAC, respectively 6 weeks after the last injection **(Table 7).**

Table 7: Striatal DA and DOPAC levels in chronic aged (6-10 months old) MPD[a]

	Aged chronic control (N=5)	**Aged chronic MPD[a] (N=9)**	**P value**
DA (ng/mg tissue)	10.61 ± 0.71	3.76 ± 0.37*	<0.0001
DOPAC (ng/mg tissue)	1.67 ± 0.47	0.78 ± 0.16*	0.048

[a]The aged chronic MPD received 10 doses of MPTP hydrochloride (15 mg/kg/dose in saline, s.c.) in combination with an adjuvant, probenecid (250 mg/kg/injection dissolved in dimethyl sulfoxide, i.p.). The 10-dose regimen was administered on a five-week schedule with an interval of 3.5 days between injections. Experiments were conducted 6 weeks post-chronic treatment. The aged chronic control group received probenecid only.*Significantly lower than aged chronic control at P<0.05.

Analyses of TH and DAT expression in the striatal tissue further revealed that there were significant losses of TH and DAT in the aged chronic MPD 6 weeks after treatment when compared with that of the control animals **(Figure 13, A-B).** These results showed that chronic MPD treatment with a reduced dose of MPTP in aged mice generated a moderate and sustained loss of dopamine content in the striatum.

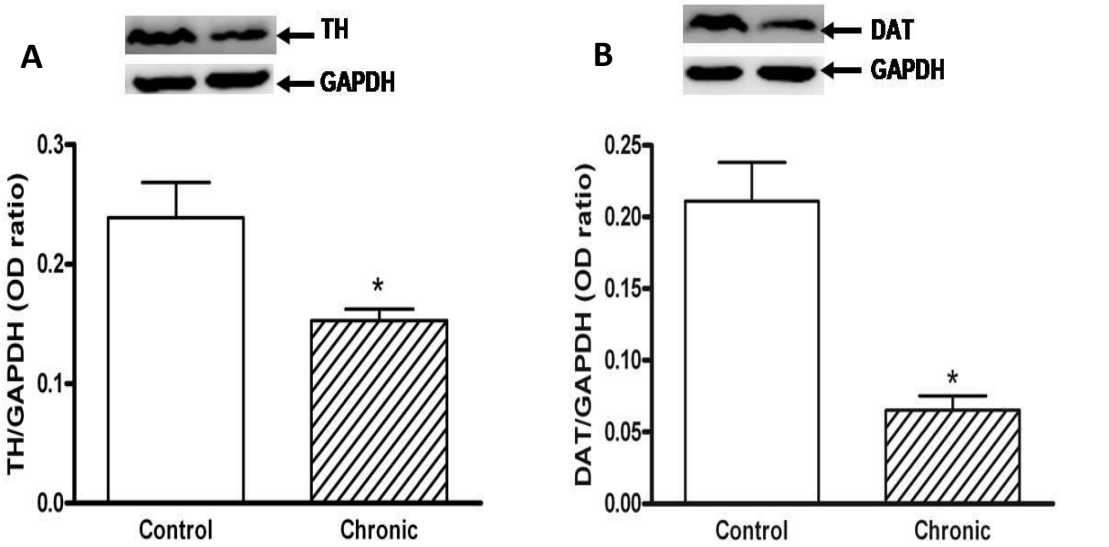

Figure 13: Western blot analysis of (A) TH and (B) DAT expression in the striatum of aged chronic probenecid control and aged chronic MPD mice. A representative image of Western blot protein bands was correspondingly shown above each set of bar graph. The protein contents of TH and DAT were expressed as a ratio to that of GAPDH and each data point represents mean ± S.E.M. (N = 5 for chronic control and N = 8 for chronic MPD). Western blots revealed that there were significant reductions of TH and DAT in the striatum of aged MPD 6 weeks after chronic treatment when compared with that of the control mice (*P<0.001).

4.3. Chronic MPTP treatment on balancing beam performance

We used the challenging beam performance test in this study to examine the animal's balance and motor coordination skills. Six weeks after MPTP/probenecid treatment, the aged chronic MPD group exhibited a significantly greater number of foot slips on the challenging beam than that of the control group **(Figure 14).** In addition, the aged chronic MPD mice were more hesitant and took longer time than the control animals on the balance beam before returning to the home cage **(Figure 14).**

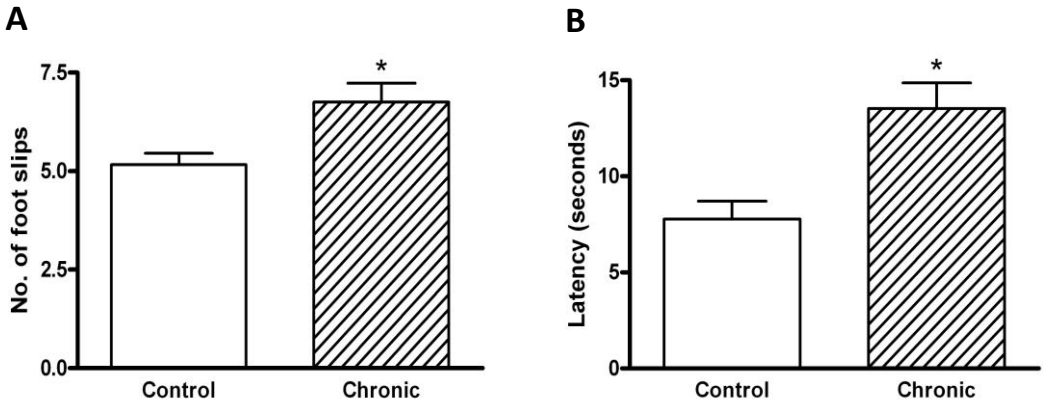

Figure 14: Challenging beam performance of chronic aged (6-10 months old) MPD[a]. Chronic MPD animals made greater number of slips (N = 31) (panel A) and were more hesitant to return to the home cage (panel B) as compared to the control group (N = 27).*Significantly higher than aged chronic control at P<0.05.

4.4. Chronic MPTP treatment on mitochondrial function in the striatum of aged mice.

Interestingly, both the basal (state 4) and ADP-stimulated (state 3) mitochondrial respiration in the striatal homogenate of aged chronic MPD mice were significantly impaired when compared to the chronic control animals **(Table 8).** There was a 50.8% and 41.0% reduction of state 3 and state 4 respiration, respectively 6 weeks after the chronic MPD treatment **(Table 8).** The mitochondrial ATP level was also decreased by 46.9% **(Table 8).** These data demonstrate that 6 weeks after chronic MPTP/probenecid treatment in the aged mice, there is a sustained attenuation of mitochondrial respiration and ATP production in the striatum.

Table 8: Mitochondrial respiration and ATP level in the striatum of chronic aged (6-10 months old) MPD[a]

	Aged chronic control (N=6)	**Aged chronic MPD[a] (N=5)**	**P value**
State 3 respiration (nmol/min/mg protein)	16.31 ± 0.39	8.03 ± 0.43*	<0.0001
State 4 respiration (nmol/min/mg protein)	2.61 ± 0.22	1.54 ± 0.11*	0.005
ATP (nM/mg protein)	12.85 ± 0.75	6.82 ± 0.64*	0.0002

[a]See legends under **Table 7** for animal treatment protocols.

*Significantly lower than aged chronic control at P<0.05.

Western blot analyses of antioxidant proteins additionally showed that the levels of Mn SOD, Cu-Zn SOD, and cytochrome c were significantly reduced in the striatal mitochondrial homogenate of the aged chronic MPD 6 weeks after treatment **(Figure 15, A-C).**

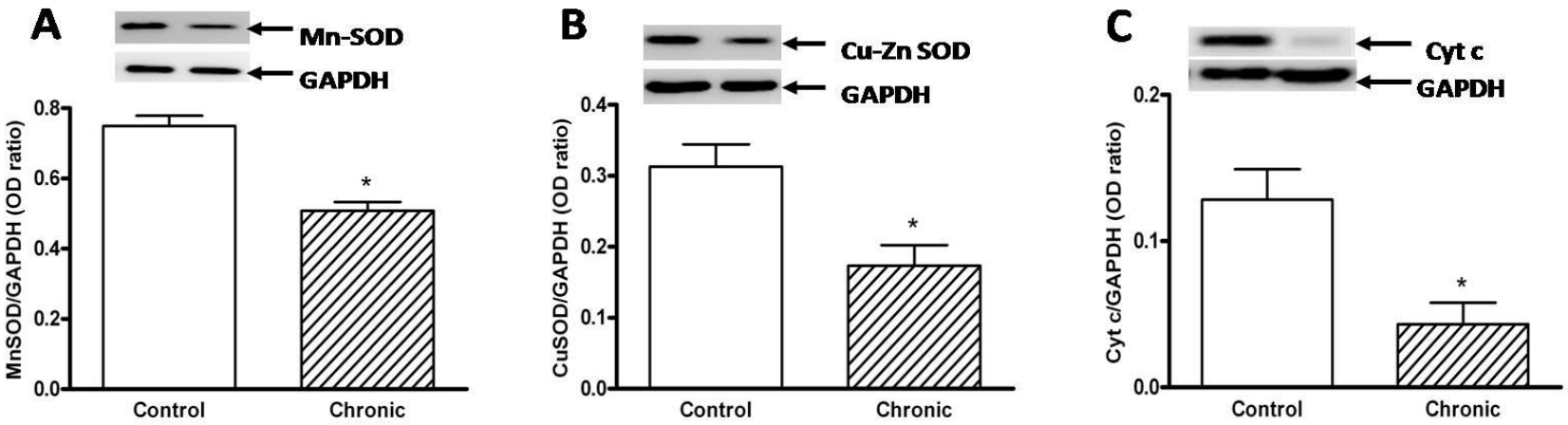

Figure 15: Protein expression of (A) Mn SOD, (B) Cu-Zn SOD and (C) cytochrome c in the striatum of aged chronic probenecid control, and aged chronic MPD mice. A representative image of Western blot protein bands was correspondingly shown above each set of bar graph. The protein contents of Mn SOD, Cu-Zn SOD and cytochrome c were expressed as a ratio to that of GAPDH and each data point represents mean ± S.E.M. N = 6 per group of animals. Statistical analyses revealed that the levels of Mn SOD, Cu-Zn SOD and cytochrome c in the striatum of aged chronic MPD 6 weeks after chronic treatment were significantly lower than that of chronic control animals (*P<0.05).

4.5. Effects of long-term exercise on dopamine neurons in the aged chronic MPD

We examined the effects of long-term endurance exercise on striatal DA neuron changes in the aged chronic MPD as previously described see section 3.3. The exercised group of animals was trained on a motorized treadmill one week before, 5 weeks during and twelve weeks after chronic MPTP/probenecid treatment (see section 3.13). After a total of 18 weeks of exercise, the aged chronic MPD showed a 35.1% loss of striatal DA, whereas the sedentary chronic MPD showed a 74% and 79% loss of DA in the striatum and SN, respectively when compared to the chronic probenecid-treated control group **(Table 9)**. These results confirm that chronic MPTP/probenecid treatment produces a persistent loss of DA in the striatum. Long-term (18 weeks) endurance exercise partially and significantly reduces MPTP neurotoxicity in the striatal DA neurons.

Table 9: Dopamine content in the striatum of chronic aged (6-10 months old) *MPD*[a]

	Aged chronic control (N=5)	**Aged Sedentary MPD[a] (N=9)**	**Aged Exercised MPD[a] (N=6)**
Striatal DA (ng/mg of tissue)	10.61 ± 0.7	3.76 ± 0.37*	6.9 ± 0.2**
SN DA (ng/mg of tissue)	3.91 ± 0.38	0.72 ± 0.17*	1.83 ± 0.33**

[a]The aged chronic MPD received 10 doses of MPTP hydrochloride (15 mg/kg/dose in saline, s.c.) in combination with an adjuvant, probenecid (250 mg/kg/injection dissolved in dimethyl sulfoxide, i.p.). The 10-dose regimen was administered on a five-week schedule with an interval of 3.5 days between injections. Experiments were conducted 12 weeks post-chronic treatment. The aged chronic control group received probenecid only.

[b]Rate of mitochondrial oxygen consumption in presence (state 3) or absence (state 4) of ADP (150 μM). Data are expressed as mean ± S.E.M. in nmol/min/mg protein.

[c]The exercised group of animals was trained on the treadmill running for 5 days/week, 40 min/day at a speed up to 15 m/min (5 min at 6 m/min, 5 min at 9 m/min, 20 min at 12 m/min, 5 min at 15 m/min, and 5 min at 12 m/min) with 0° of inclination. Mice were exercise trained one week before, 5 weeks during the chronic MPTP/probenecid treatment, and exercise was continued for 12 weeks after the completion of the MPTP treatment.

*Significantly lower when compared with control group ($P<0.05$).

**Significantly higher when compared with sedentary chronic MPD group ($P<0.01$).

We also compared the levels of TH, DAT and VMAT-2 in the striatum of the control, sedentary chronic MPD and exercised chronic MPD using Western blot technique. Significant losses of TH, DAT and VMAT-2 contents in the aged sedentary chronic MPD were observed 12 weeks after chronic MPTP/probenecid treatment when compared to the control group. The level of these proteins in the exercised chronic MPD was detected when compared with the sedentary chronic MPD group **(Figure 16, A-C)**.

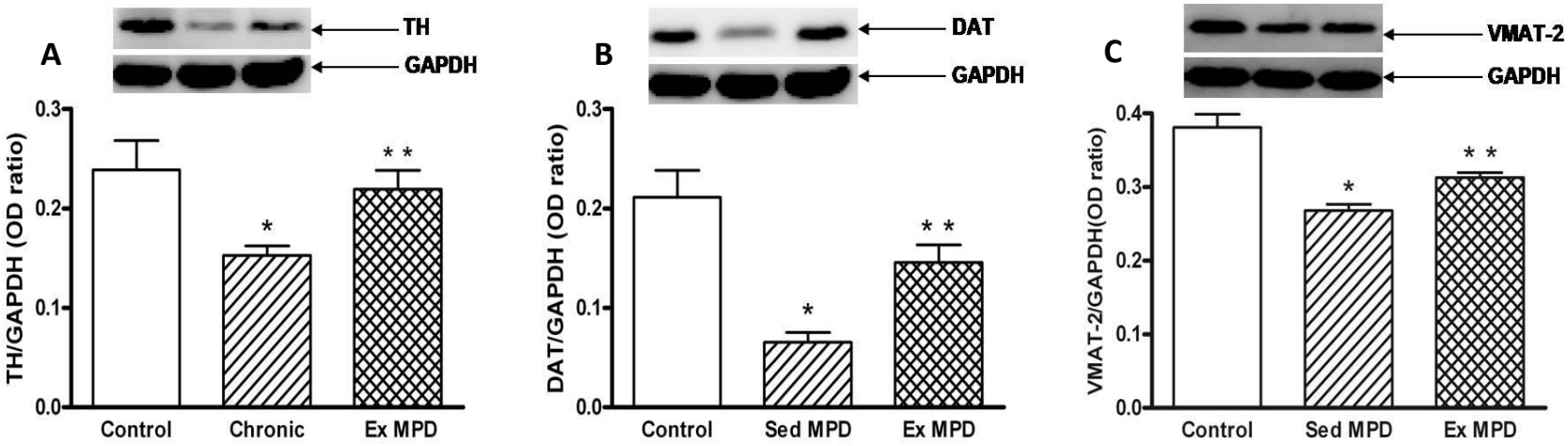

Figure 16: Western blot analysis of (A) TH, (B) DAT and (C) VMAT-2 expression in the striatum of aged chronic probenecid control and aged sedentary MPD and exercise MPD mice. A representative image of Western blot protein bands was correspondingly shown above each set of bar graph. The protein contents of TH, DAT and VMAT-2 were expressed as a ratio to that of GAPDH and each data point represents mean ± S.E.M. (N = 6 for chronic control and exercise MPD and N = 8 for chronic sedentary MPD). Western blots revealed that there were significant reductions of TH, DAT and VMAT-2 content in the striatum of aged sedentary MPD 12 weeks after chronic treatment when compared with that of the control mice. *Significantly different as compared to control ($P<0.05$). **Significantly different as compared to sedentary MPD. ($P<0.05$)

4.6. Effects of long-term exercise on balancing beam performance

We investigated and compared the animal's performance on the challenging beam for determining the balance and motor coordination skills between the chronic control, sedentary chronic MPD and exercised chronic MPD. Twelve weeks after MPTP/probenecid treatment, the chronic sedentary MPD mice exhibited a significantly greater number of foot slips on the challenging beam and time latency for returning to the home cage when compared with the control animals suggesting movement deficit in the sedentary chronic MPD [F (2, 81) = 20.39] **(Figure 17 A).** When the chronic MPD were exercised for 18 weeks, these animals displayed a marked decrease of foot slip errors and shortened time latency for returning to the home cage. Their challenging beam performance was similar to the control animals [F (2, 81) = 9.02] **(Figure 17 B).** These findings suggest an improvement in movement deficit in the exercised chronic MPD.

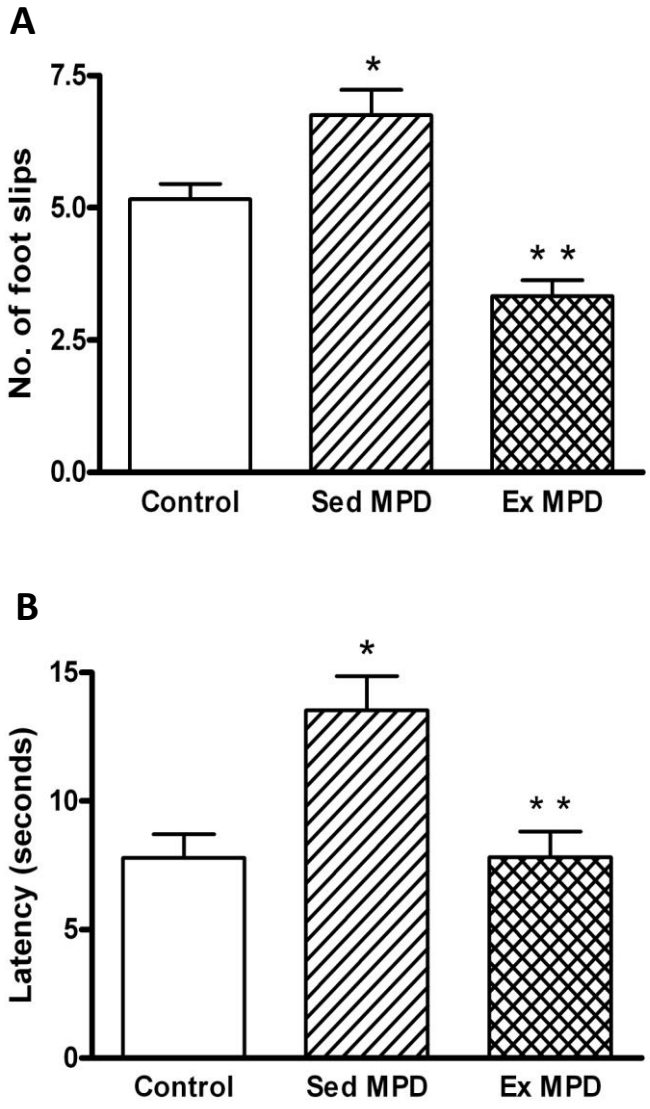

Figure 17: Challenging beam performance of chronic aged (6-10 months old) MPD. The sedentary chronic MPD showed greater number of foot slips (panel A) and were more hesitant to return to the home cage (panel B) as compared to the control mice.*Significantly higher than the chronic probenecid-treated controls. (N = 25 for chronic control, N =27 for exercise MPD and N = 31 for chronic sedentary MPD)**Significantly lower than the sedentary chronic MPD and not different from the chronic control ($P<0.05$).

4.7. Effects of long-term exercise on mitochondrial function in the aged chronic MPD

Mitochondrial respiration in the striatal homogenate preparation was measured and compared between sedentary chronic MPD and exercised chronic MPD. The basal rates of mitochondrial respiration (state 4 respiration) as well as the ADP-stimulated (state 3 respiration) rates were significantly impaired in the sedentary chronic MPD animals **(Table 10)**. The state 4 and state 3 respiration in the striatal mitochondria of the 18-week exercised chronic MPD animals were significantly higher than the sedentary chronic MPD and not different from the control animals **(Table 10).** The mitochondrial ATP level was also decreased by 47% in the sedentary chronic MPD group (**Table 10)** as compared to the aged control mice. Nevertheless, the mitochondrial ATP level in the striatum of 18-week exercised chronic MPD was only 22.2% lower than the control mice, which was significantly higher than that of the sedentary chronic MPD. These results clearly demonstrate that 18 weeks of endurance exercise significantly attenuated the dysfunction of mitochondrial respiration and the depletion of ATP as detected in the striatum of the sedentary chronic MPD.

Table 10: Mitochondrial respiration and ATP levels in the striatum of chronic aged (6-10 months old) MPD[a]

	Aged chronic control (N=5)	**Aged Sedentary MPD[a] (N=9)**	**Aged Exercised MPD[a] (N=6)**
State 3 respiration[b] (nmol/min/mg protein)	16.71 ± 0.8	9.03 ± 0.71*	16.27 ± 0.79**
State 4 respiration[b] (nmol/min/mg protein)	2.51 ± 0.13	1.85 ± 0.14*	2.57 ± 0.09**
ATP (nM/mg protein)	12.85 ± 0.75	6.82 ± 0.64*	9.99 ± 0.9**

See Table 9 for [a] [b] and [c] legends.

We further measured the level of various antioxidant enzymes in the striatal mitochondrial homogenate. In the sedentary chronic MPD, the protein expression of Mn SOD, Cu-Zn SOD, and cytochrome c were significantly decreased in the striatal mitochondrial homogenate when compared with that of the control animals (**Figure 18, A-C**). However, the protein levels of these antioxidant enzymes in the striatal mitochondrial preparation of 18-week, treadmill exercise-trained chronic MPD were not significantly different from the control mice but were significantly higher than that of the sedentary chronic MPD **(Figure 18, A-C).**

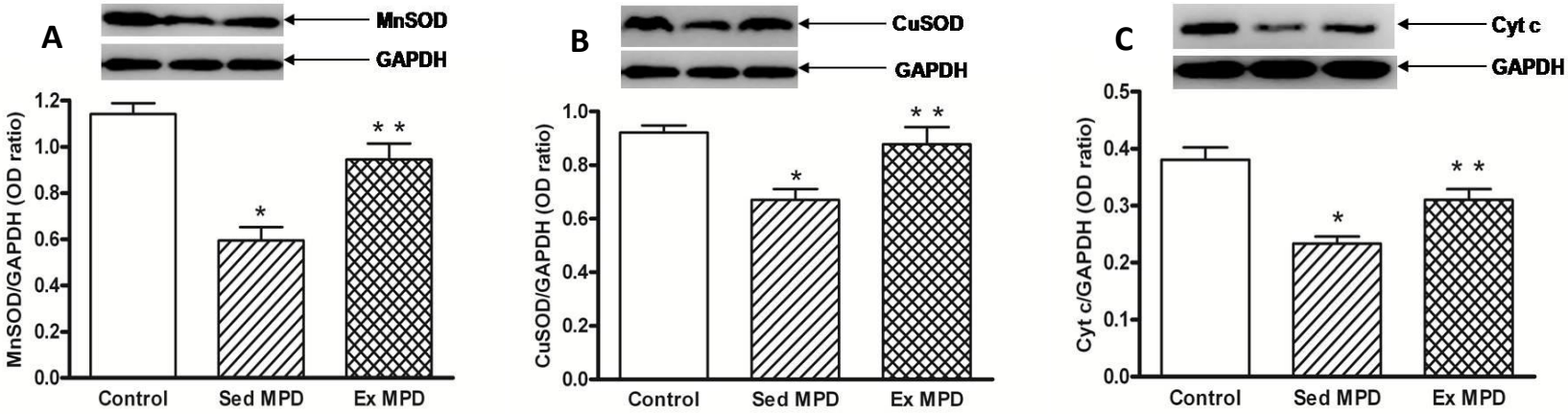

Figure 18: Protein expression of (A) Mn SOD, (B) Cu-Zn SOD and (C) cytochrome c in the striatal preparation of the aged chronic probenecid control, sedentary chronic MPD and exercise MPD mice. Western blot technique and densitometry analysis were used to detect and quantify the respective anti-oxidant protein bands and GAPDH. The protein contents of Mn SOD, Cu-Zn SOD and cytochrome c were expressed as a ratio to that of GAPDH to ensure that the changes in protein levels were not due to sample loading variances. A representative image of Western blot protein bands was correspondingly shown above each set of bar graph. Each data point represents mean ± S.E.M. N = 6 per group of animals. Statistical analyses revealed that the levels of Mn SOD, Cu-Zn SOD and cytochrome c in the striatum of aged sedentary chronic MPD 12 weeks after chronic treatment were significantly lower than that of control animals. *Significantly lower than the control ($P<0.05$). **Significantly higher than the sedentary MPD ($P<0.05$).

4.8. Effects of long-term exercise on other mitochondrial indicators in the aged chronic MPD.

4.8.1. On striatal carbonylated proteins

Protein carbonylation is a type of protein oxidation that can be promoted by reactive oxygen species. Carbonylated protein measurement is routinely used for detecting damaged proteins due to mitochondrial oxidative stress (Suzuki et al.; Wong et al.; Wong et al., 2008). The total level of carbonylated proteins present in the striatum of the sedentary chronic MPD was increased by 24.7% when compared with the control probenecid group **(Figure 19).** On the other hand, the amount of carbonylated proteins in the exercised chronic MPD was significantly lower than that in the sedentary chronic MPD group and not different from that in the control group **(Figure 19).**

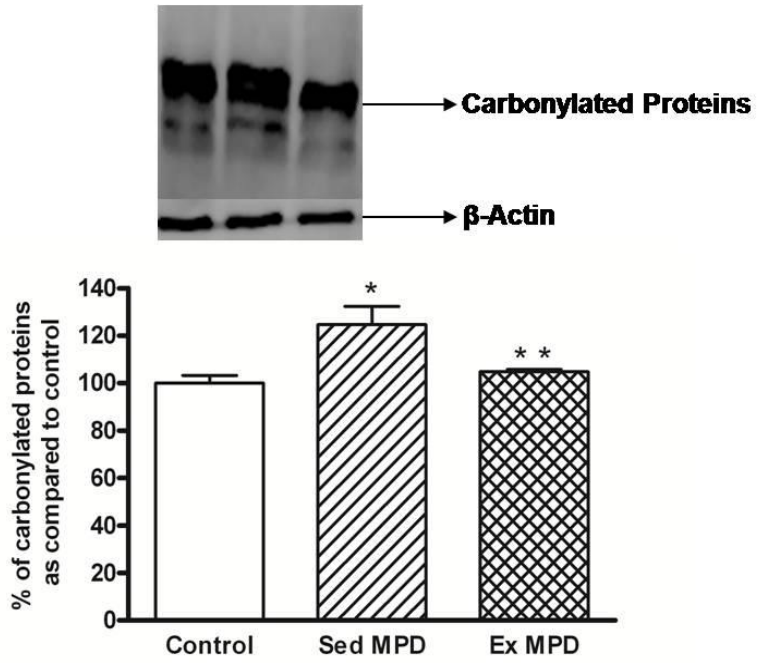

Figure 19: Protein carbonylation in the striatum of aged chronic probenecid control, aged sedentary chronic MPD and exercised chronic MPD mice was measured and compared. A representative image of Western blot protein bands was correspondingly shown above each set of bar graph. The carbonylated proteins were expressed as a ratio to that of β-actin and each data point represents mean ± S.E.M. (N = 6). The immunoblot revealed that there was a significant increase in the carbonylated proteins in the sedentary chronic MPD as compared to chronic control mice. The amount of carbonylated proteins in the exercised chronic MPD group was significantly lower than that in the sedentary chronic MPD, and not different from that in the chronic control mice. *Significantly higher when compared with the chronic control group ($P<0.05$). **Significantly lower when compared with the sedentary chronic MPD group ($P<0.05$).

4.8.2. On striatal p53 mRNA level

p53 is a tumor suppressor protein and it plays an important role in cell cycle control and apoptosis. In normal cells, the p53 protein level is low. DNA damage and other stress signals tend to increase p53 protein level in defense to growth arrest, DNA repair and apoptosis (Blagosklonny, 2000; Liu and Kulesz-Martin, 2001). Under neuronal

apoptosis and associated DNA damage, p53 is released to initiate DNA repair and remove the damaged cells (Perier et al., 2007). Therefore, p53 mRNA is expected to increase when DNA damage is evident. In this study, we observed a 48.6% increase of the p53 mRNA level in the sedentary chronic MPD when compared with the chronic control group. Exercise-training of the chronic MPD led to a significant reduction of p53 mRNA in the striatum when compared with sedentary chronic MPD suggesting lesser DNA damage **(Figure 20)**.

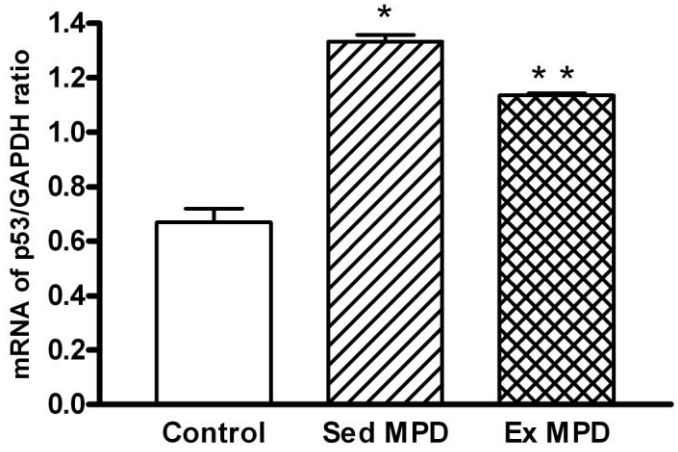

Figure 20: qRT-PCR of p53 in the striatum of aged chronic probenecid control, aged sedentary chronic MPD, and exercised chronic MPD mice. The mRNA was expressed as a ratio to that of GAPDH and each data point represents mean ± S.E.M. (N = 6). qRT-PCR revealed a significant increase in the p53 mRNA level in the sedentary chronic MPD when compared with the chronic control mice. The striatal p53 mRNA level in the exercised chronic MPD was still higher than the control group but was significantly lower than the chronic sedentary chronic MPD group. *Significantly higher than the aged chronic control at ($P<0.05$). **Significantly lower than the sedentary chronic MPD ($P<0.05$), but still higher than the chronic control group ($P<0.01$).

4.8.3. On PGC-1α and TFAM mRNA levels

One of the important aspects for maintaining the integrity of mitochondrial function is through biogenesis, the capacity for replenishing the damaged and degraded mitochondria (Wenz, 2009). The regulation of mitochondrial biogenesis is a complex process involving more than 1000 genes (Wenz, 2009). Despite the complexity of the various signaling pathways, they all seem to converge at the mitochondrial transcription factor A (TFAM) and peroxisome proliferator-activated receptor-γ coactivator (PGC-1α). These two transcription factors are considered essential for mitochondrial gene expression in mammals. The activation of PGC-1α and TFAM transcription is responsive to changes such as abnormal mitochondrial morphology, increased ROS and NO, ATP deficiency (Borniquel et al., 2006; Silveira et al., 2006; Irrcher et al., 2009). Therefore, PGC-1α and TFAM mRNAs are expected to increase when there is mitochondrial dysfunction. In this study we observed a 38.8% and four-fold increase of the PGC-1α and TFAM mRNA levels, respectively in the sedentary chronic MPD when compared with the chronic control group. Exercise-training of the chronic MPD led to significant reduction of the TFAM mRNA and PGC-1α mRNA when compared with the sedentary chronic MPD **(Figure 21, A-B).**

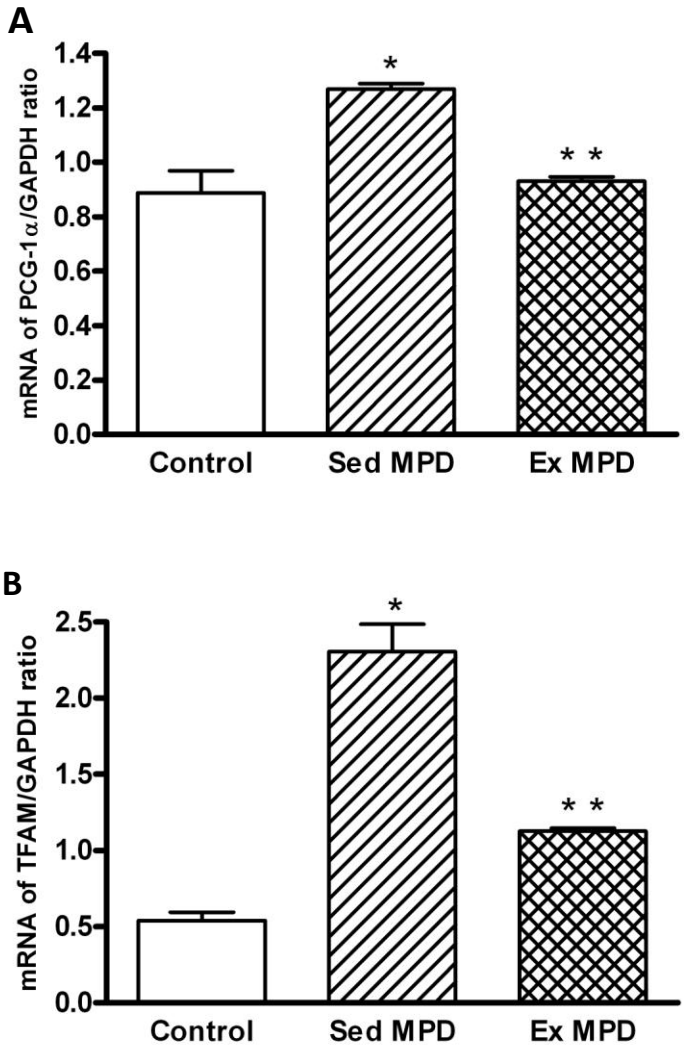

Figure 21: qRT-PCR of (A) PGC-1α (B) TFAM in the striatum of aged chronic probenecid control, sedentary chronic MPD and exercised chronic MPD mice. The mRNA was expressed as a ratio to that of GAPDH and each data point represents mean ± S.E.M. (N = 6). qRT-PCR revealed a significant increase in the mRNA levels of PGC-1α and TFAM in the sedentary chronic MPD when compared with the chronic control. The PGC-1α and TFAM mRNA level in exercised chronic MPD were significantly lower than the sedentary chronic MPD. *Significantly higher than aged chronic control at $P<0.05$. **Significantly lower when compared with sedentary chronic MPD ($P<0.01$).

4.9. Melatonin prevented the loss of dopamine neurons in the aged chronic MPD

We followed a similar treatment schedule for melatonin as for exercise-training in the chronic MPD. Briefly, melatonin (5 mg/kg, i.p) was injected 1 week prior to the first administration of MPTP (15 mg/kg, s.c) and probenecid (250 mg/kg, i.p) and was continued 5 weeks during and 12 weeks after the completion of chronic MPTP/probenecid treatment (see methods). Melatonin alone did not alter the normal neuronal, mitochondrial and motor functions in control animals. As expected, 12 weeks after chronic MPD treatment, we noticed a sustained 69.5% and 54% loss of DA in the striatum and substantian nigra, respectively when compared to the control **(Figure 22, A-B).** Interestingly, 18 weeks of melatonin treatment significantly prevented DA loss in the striatum and substantia nigra of chronic MPD mice **(Figure 22, A-B).**

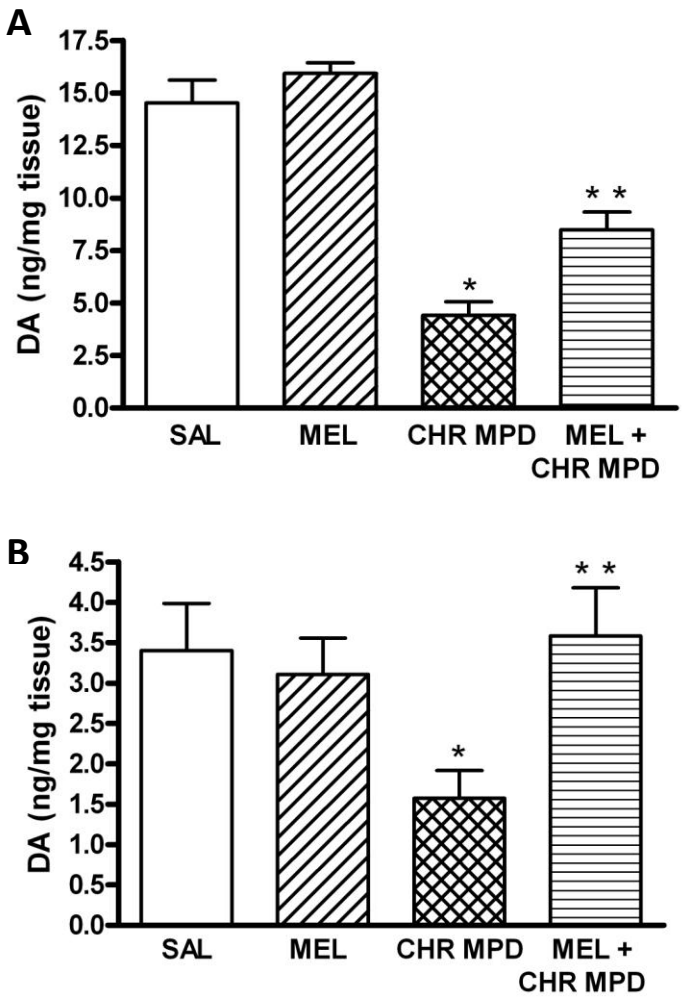

Figure 22: Dopamine content in the (A) striatum and (B) substantia nigra of chronic-saline control, chronic melatonin, chronic MPD, and melatonin-treated chronic MPD. The DA content was expressed in ng/mg of tissue weight and each data point represents mean ± S.E.M. (N = 8 for chronic saline, N = 4 for chronic melatonin and chronic MPD and N = 6 for melatonin-treated chronic MPD). There was a significant decrease in the DA content in the striatum and substantia nigra of chronic MPD respectively when compared with control. Eighteen weeks of chronic melatonin treatment significantly reduced DA loss in striatum and substantia nigra of chronic MPD mice. *Significantly lower when compared with saline or melatonin-treated mice ($P<0.05$). **Significantly higher when compared with chronic MPD ($P<0.05$). SAL=Saline, MEL=Melatonin, CHR=Chronic.

We also compared the protein level of TH, DAT and VMAT-2 in the striatum of the saline-control, melatonin, chronic MPD and melatonin-treated chronic MPD using Western blot analyses. Significant losses of TH, DAT and VMAT-2 proteins in the aged chronic MPD were observed 12 weeks after chronic MPTP/probenecid treatment when compared to the control group. In melatonin-treated chronic MPD, the loss of striatal TH, DAT, except VMAT-2 were significantly less than the non-melatonin-treated chronic MPD **(Figure 23, A-C).**

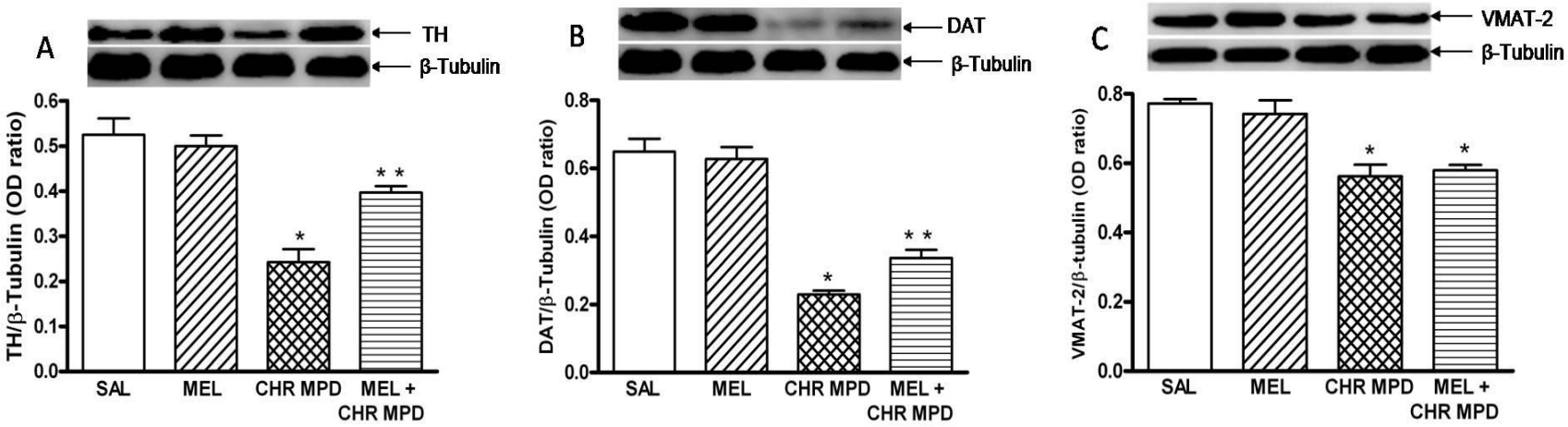

Figure 23: Western blot analysis of (A) TH, (B) DAT and (C) VMAT-2 expression in the striatum of aged chronic saline, chronic melatonin, chronic MPD and melatonin-treated chronic MPD. A representative image of Western blot protein bands was correspondingly shown above each set of bar graph. The protein contents of TH, DAT and VMAT-2 were expressed as a ratio to that of β-tubulin and each data point represents mean ± S.E.M. (N = 8 for chronic saline, N = 4 for chronic melatonin and chronic MPD, and N = 6 for melatonin-treated chronic MPD). Western blots revealed that there was a significant reduction of TH, DAT and VMAT-2 protein expression in the striatum of aged chronic MPD when compared with either saline or melatonin-treated mice. *Significantly lower when compared with saline or melatonin alone-treated mice ($P<0.05$). **Significantly higher when compared with chronic MPD ($P<0.05$).

4.10. Chronic melatonin treatment on balancing beam performance

We also used the challenging beam for evaluating animal's balance and motor coordination skills. Twelve weeks after MPTP/probenecid treatment, the chronic MPD group exhibited a significantly greater number of foot slips on the challenging beam than that of the control saline-treated or melatonin alone treated groups [$F(3, 29) = 27.91$] **(Figure 24, A)**. In addition, the aged chronic MPD mice were more hesitant and took greater time than the control groups on the balance beam before returning to the home cage [$F(3, 29) = 17.29$] **(Figure 24, B)**. The melatonin-treated chronic MPD mice displayed significant improvement in movement showing less number of foot slips and shorter latency time for returning to their home cage than the non-melatonin treated mice.

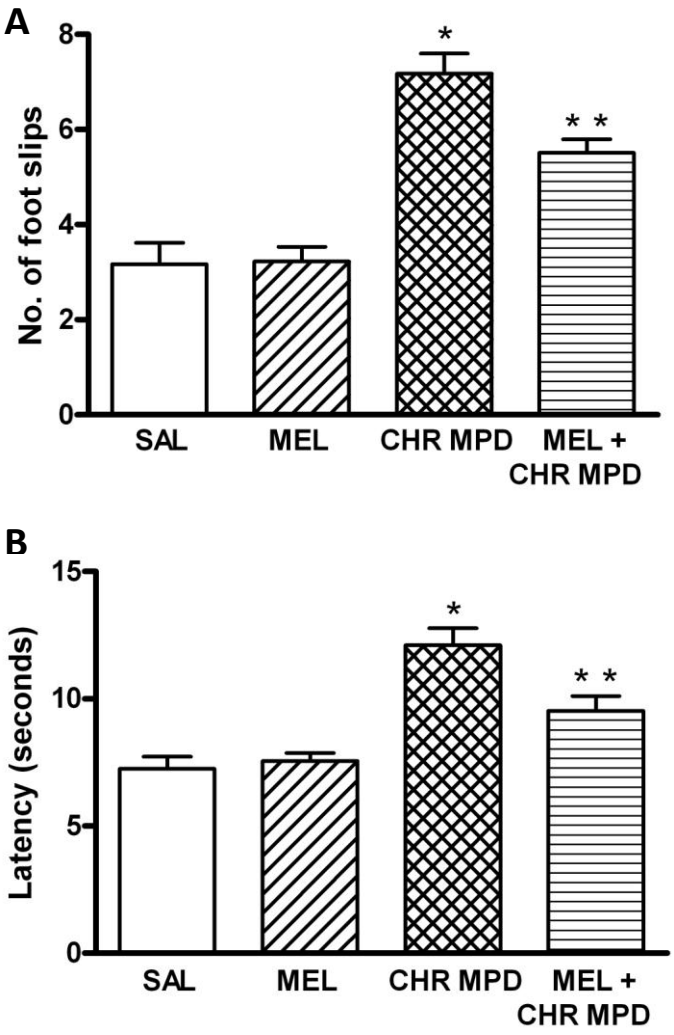

Figure 24: Challenging beam performance of chronic aged (6-10 months old) MPD. The chronic MPD animals showed greater number of foot slips (panel A) and were more hesitant to return to the home cage (panel B) when compared with saline or melatonin-treated mice. (N = 8 for chronic saline, N = 4 for chronic melatonin and chronic MPD, and N = 9 for melatonin-treated chronic MPD. *Significantly higher when compared with saline or melatonin-treated mice ($P<0.001$).**Significantly lower when compared with non-melatonin-treated chronic MPD ($P<0.05$).

4.11. Melatonin prevented the loss of mitochondrial function in the aged chronic MPD

Mitochondrial respiration in the striatal homogenate preparation was compared among four groups of animals. Chronic melatonin alone did not alter the normal mitochondrial respiration and ATP level. The basal mitochondrial respiration (state 4) as well as the ADP-stimulated (state 3) were significantly impaired in the chronic MPD **(Figure 25, A-B).** In contrast, the state 4 and state 3 respiration in melatonin-treated chronic MPD with melatonin were significantly higher than the chronic MPD and were not different from the control animals **(Figure 25, A-B).** The mitochondrial ATP level was decreased by 47.5% in the chronic MPD group as compared to the control mice **(Figure 25, C).** Nevertheless, the mitochondrial ATP level in the striatum of melatonin-treated chronic MPD was significantly higher than that of the chronic MPD, but not different from the control mice. These results clearly demonstrate that 18 weeks of chronic melatonin treatment significantly prevents the mitochondrial dysfunction induced by chronic MPTP.

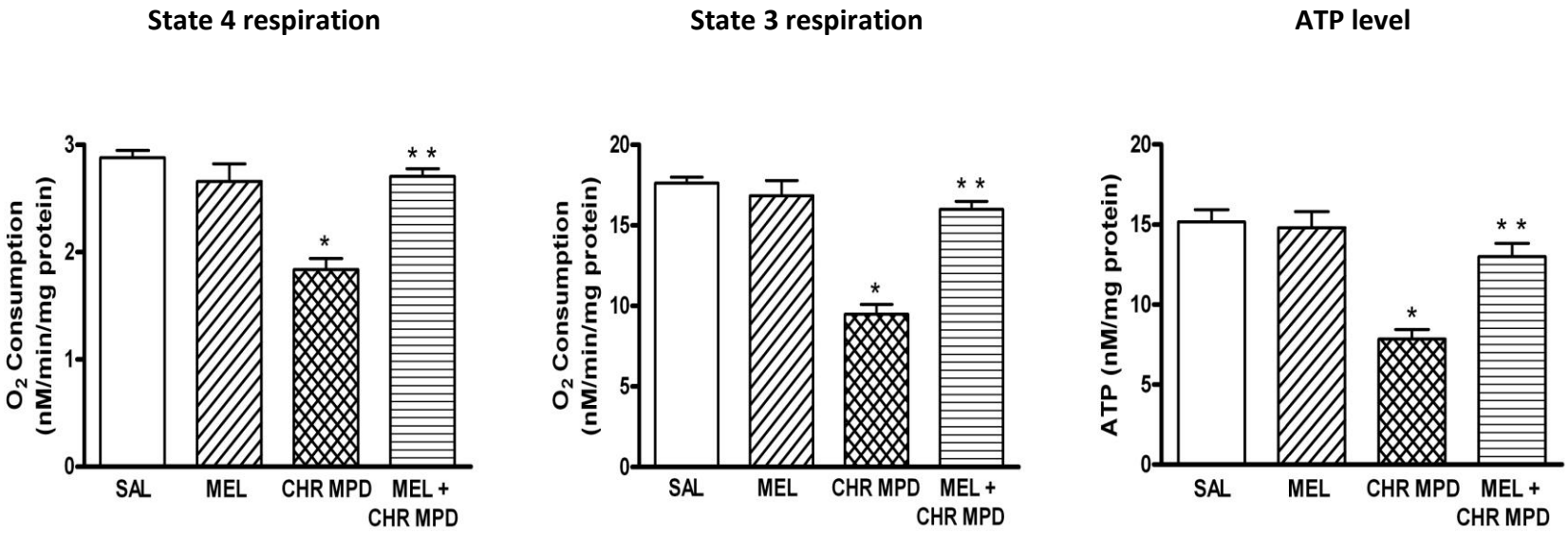

Figure 25: Effect of melatonin on mitochondrial functions in chronic MPD. (A) The basal level of respiration (state 4), (B) the ADP-stimulated respiration (state 3), and (C) ATP production in the striatal mitochondria preparation of the chronic MPD were all inhibited. These mitochondrial functions in the 18-week melatonin-treated chronic MPD were fully recovered. For mitochondrial respiration the data are expressed as mean ± S.E.M. in nmol/min/mg protein and for ATP level the data are expressed as mean ± S.E.M in nM/mg protein. (N = 8 for chronic saline, N = 4 for chronic melatonin and chronic MPD and N = 6 for melatonin-treated chronic MPD. *Significantly lower when compared with saline or melatonin-treated ($P<0.05$). **Significantly higher when compared to the chronic MPD ($P<0.05$); but not different from either saline or melatonin-treated mice ($P>0.05$).

Finally, we measured the level of various antioxidant enzymes in the striatal mitochondrial homogenate using Western blot technique. Chronic melatonin did not alter the levels of Mn SOD, Cu-Zn SOD, and cytochrome c in chronic MPD were significantly decreased when compared with that of the saline or melatonin alone-treated control animals (**Figure 26, A-C**). However, the protein levels of these antioxidant enzymes in 18-week melatonin-treated chronic MPD were not significantly different from the control mice but were significantly higher than that of the chronic MPD **(Figure 26, A-C).**

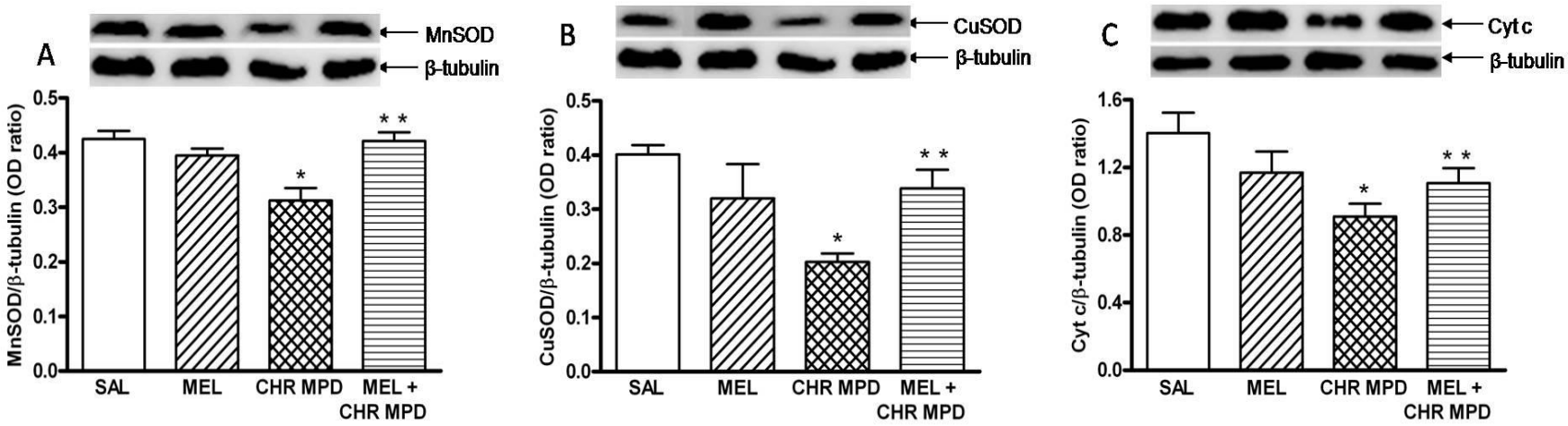

Figure 26: Protein expression of (A) Mn SOD, (B) Cu-Zn SOD and (C) cytochrome c in the striatal preparation of the chronic control, chronic melatonin, chronic MPD and melatonin-treated chronic MPD. A representative image of Western blot protein bands was correspondingly shown above each set of bar graph. The protein contents of Mn SOD, Cu-Zn SOD and cytochrome c were expressed as a ratio to that of β-tubulin and each data point represents mean ± S.E.M. (N = 8 for chronic saline, N = 4 for chronic melatonin and chronic MPD, and N = 6 for melatonin-treated chronic MPD). Statistical analyses revealed that the level of Mn SOD, Cu-Zn SOD and cytochrome c in the striatum of chronic MPD mice were significantly lower when compared to either saline or melatonin-treated mice. *Significantly lower when compared to either saline or melatonin alone treatment ($P<0.05$). **Significantly higher when compared to chronic MPD ($P<0.05$); but not different from either saline or melatonin-treated mice.

5. DISCUSSION

5.1. Striatal neurodegeneration and mitochondrial dysfunction in chronic mouse model of PD

There is a large body of *in vitro* and acute *in vivo* research findings suggesting a possible link between mitochondrial dysfunction and neurodegenerative disorders. However, very few investigations have been conducted and results are inconclusive involving long-term defects of mitochondria in chronic neurodegenerative models. In the present study, we investigated mitochondrial respiration and measured mitochondrial antioxidant enzyme levels 6 weeks after the induction of Parkinsonism in a chronic MPD. In contrast to our initial hypothesis stating that long-term mitochondrial deficiency is associated with chronic Parkinsonism, we failed to detect a significant inhibition of either the basal (state 4) or ADP-stimulated (state 3) mitochondrial respiration in the striatum of young adult chronic MPD mice at 6-10 weeks of age, although considerable loss of striatal DA neurotransmitter persisted in these animals. Furthermore, we did not ascertain any noticeable change in the levels of mitochondrial antioxidant enzymes including Mn SOD, Cu-Zn SOD, and cytochrome c. Our results may be interpreted as either mitochondrial impairment is not an obligatory pathophysiological factor for chronic neurodegenerative process or MPTP may instigate oxidative stress and inflammation to the mitochondria in other types of striatal cells in

addition to the DA neurons. Consequently, striatal mitochondria in the non-DA cells plus or minus DA neurons in young animals may have the capability to rejuvenate their functionality over time when the neurotoxin clears away from the brain. The latter possibility on cell specificity was not tested in the present study due to tissue limitation.

For comparison, we confirmed that acute injections of MPTP caused an immediate depletion of striatal DA and simultaneously suppressed the state 3 mitochondrial respiration. These observations are in line with the premise that neurodegeneration may be provoked by MPTP causing accumulation of excitotoxic mediators, production of oxidative free radicals, disruption of calcium (Ca^{++}) homeostasis, and ATP depletion in association with an inhibition of the neuronal mitochondrial complex I component of the electron transport chain (Przedborski and Jackson-Lewis, 1998; Blum et al., 2001). However, whether neurodegeneration follows a progressive course or not would depend on how rapid and how long the brain is exposed to neurotoxic chemicals. Indeed, the DA depleting effect of MPTP, when it is administered acutely or sub-acutely in mice, tends to be short-lived and reversible (Lau, 2005). In addition, as expected and shown in our results, we did not detect an immediate action of MPTP on the turn over of the antioxidant enzyme levels of SODs and cytochrome c in acutely treated young mice. We did not further carry out the long-term investigation of acute MPTP model. We do not anticipate that acute MPTP will

cause any sustained mitochondrial changes, because even chronically repeated administration of MPTP/probenecid in young mice did not generate any significant alterations in mitochondrial respiration and anti-oxidant enzyme levels 6 weeks after treatment.

To address the possibility that striatal mitochondria in young animals may have the potential for functional recovery over time when the neurotoxin is eliminated from the brain, we decided to examine the long-term neuronal and mitochondrial toxic effects of chronic MPTP/probenecid in an aged group of animals (6-10 months old). Resembling the observations from young adult animals, even with a reduced dose of MPTP (15 mg/kg/injection), the aged chronic MPD acquired moderate but significant losses of striatal DA, DOPAC, TH and DAT 6 weeks post-treatment. In contrast to the young animals, the aged chronic MPD consistently suffered an inhibition of the basal and ADP-stimulated respiration and a fall in ATP production in the striatal mitochondria. Chronic MPTP/probenecid treatment in aged mice also brought about a sustained diminution of striatal antioxidant enzyme levels including that of Mn SOD, Cu-Zn SOD, and cytochrome c. The striatal neurochemical and mitochondrial deficits in aged chronic MPD 6 weeks after treatment were further supported by behavioral observations showing that these animals made more foot slip errors and it took twice as long for them to complete the balance beam test.

It should be emphasized that a close correlation between neuronal, mitochondrial, and behavioral deficits 6 weeks after chronic Parkinsonism in the aged animals would suggest an important role of mitochondrial function in maintaining neuronal plasticity. However, such a positive correlation alone is not sufficient for establishing a cause-and-effect relationship between mitochondrial disorder and Parkinson-like neurodegeneration. Furthermore, our failure for demonstrating long-term mitochondrial deficiency in the young chronic MPD would not dispute the possibility that neurotoxins like MPTP can cause mitochondrial oxidative dysfunction and initiate the neurodegenerative process, but it is not required for sustaining neurodegeneration. This is well demonstrated in the present study that mitochondrial functions are fully intact whereas neuronal functions are severely impaired 6 weeks after chronic MPD treatment in young mice. Our observations support the belief that mitochondrial dysfunction is an early event of neurodegenerative process (Chan, 2006). The main significance of this investigation is the finding of coexisting phenotype between neurodegenerative and mitochondrial defects in the aged chronic MPD. This will provide an appropriate model for continuously establishing whether these two disorders are cause-and-effect related and for using these altered neurobiological indicators as biomarkers for potential neuroprotective targets in preclinical tests. We applied two approaches one with physical exercise (a non-pharmacological approach) and the other with melatonin (a pharmacological approach) to elucidate whether long-

term exercise or melatonin administration could simultaneously offer mitochondrial as well as neuronal protection in the neostriatum.

The topic on deterioration of mitochondrial functions associated with normal aging or correlated with age-related diseases has been widely reviewed (Calabrese et al., 2001; Bertoni-Freddari et al., 2004; Crompton, 2004; Martin, 2006; Swerdlow, 2009). In laboratory, neurons isolated from the brains of aged rats show deficiency in cytochrome c and inner mitochondrial membrane-associated cardiolipin levels that would preempt normal mitochondrial respiration and oxidative phosphorylation (Jones and Brewer, 2009). A single dose of MPTP (40 mg/kg) does not acutely generate ROS in young mice, but significantly releases ROS in old mice (Ali et al., 1994). Moreover, although attempts have been made to link mutations of mtDNA directly to normal aging or age-related disease states, it has not been confirmed that mtDNA mutations are either the primary cause or the result of aging or disease process (Arnheim and Cortopassi, 1992; Wallace, 1997; Kang and Hamasaki, 2005; Reeve et al., 2008). For translational consideration, one can envision that the capacity of mitochondria for carrying out oxidative respiration and generating ATP and for coping with acute oxidative insult in the neuron of a 20 year old would be much greater than that of a 70 year old individual. In other words, the mitochondria and neurons of an aged person would be more subject to irreparable injuries following chronic exposure to endogenous

or exogenous toxic chemical species. As implicated in the present study, our results are in agreement with the notion that the antioxidant capacity and the recovery potential for mitochondria and neurons post-injury are expected to decline with increase in age (Beal, 2005).

It is reasonable to presume that the normal level of physiologically released ROS from mitochondria is insignificantly low and is unlikely to cause neuronal death. Therefore, intentional reduction of endogenously produced ROS below its basal physiological level would not be considered health beneficial. However, if chronic exposure to neurotoxic chemicals like MPTP results in lasting depletion of antioxidant enzymes such as SODs and cytochrome c in the aged subject, as demonstrated in the current study, the loss of mitochondrial antioxidant protection can certainly exacerbate neurotoxicity and cell death initiated by the elevated ROS. Thus, developing new approaches for protecting the capacity of mitochondrial defense system, for eradicating the elevated ROS level, and for restoring the mitochondrial respiratory functions would be important strategies for preventing and treating progressive cell death under oxidative stress conditions.

5.2. Impact of endurance exercise on protection against neuronal and mitochondrial defects in aged chronic MPD

5.2.1. Long-term exercise on neuronal function

It has long been recognized that customary physical activities (e.g. walking and swimming) tend to reduce mental decline in elderly (Schuit et al., 2001) and help recovery and lessen the risk of further impairment due to stroke, PD and other degenerative diseases. It is not clear whether endurance exercise can prevent or delay the progression of PD-like neurodegeneration (Eldar and Marincek, 2000; Cotman and Berchtold, 2002; Hirsch and Farley, 2009). If it does, it would be imperative to determine the neuroprotective mechanisms of exercise.

Initial studies have been carried out in our laboratory examining the impact of endurance exercise in nigrostriatal DA neurons in the chronic MPD with severe neurodegeneration. In the severe chronic MPD where more than 80% of DA activities were lost, we failed to detect any significant recovery in the number of SNpc neurons or nigral and striatal TH expression, striatal DA content and DA uptake after 10 weeks of exercise training, although physical fitness and behavioral recovery could be established (Al-Jarrah et al., 2007). We reasoned that in the severely degenerated diseases or disease models, the feasibility for neuronal recovery would be unlikely without effective

neuroregenerative potentials and measures, especially dealing with aging neurons. Hence a moderately induced chronic MPD representing an early stage of Parkinsonism with moderate loss of DA neurons was used in this research. As shown under results, we validated that injecting the aged mice (6-10 months) with chronic MPTP (15 mg/kg) and probenecid (250 mg/kg), a moderate loss of DA neuron indicators and transmission was reproduced.

To examine neuronal damage as the result of chronic MPTP and to determine neuroprotection, we measured TH, the DA synthesizing enzyme, DAT, the DA terminal uptake transporter, VMAT-2, the vesicular DA transporter, along with the DA content in the SN and in the striatum. Our results demonstrated that there was 55-65% decrease in the level of DA along with TH, DAT, VMAT-2 protein expression in the striatum of sedentary chronic MPD group when compared with the control animals. Behaviorally, the sedentary chronic MPD group displayed greater locomotor deficits as compared to the control group. Following 18 weeks of treadmill exercise training, all of the above described deficits seen in the non-exercised chronic MPD were either partially or fully recovered, suggesting that the length and intensity of exercise regimen we used in this study is effective to achieve neuronal protection. Previous experiments in our laboratory showed only marginal, not statistical significant recovery could be detected after 10 weeks of exercise training. Taken all together, our research observations so far

conclude that exercise affords neuroprotection in moderate state of neurodegeneration and requires long-term exercise to attain an endurance level.

5.2.2. Long-term exercise on mitochondrial functions and regulation

To examine mitochondrial dysfunction as a result of chronic MPTP treatment and to determine mitochondrial protection, we measured mitochondrial respiration and ATP production along with various mitochondrial antioxidant enzyme levels, such as Mn SOD, Cu-Zn SOD and cytochrome c in the striatum. Our results demonstrated that there was a 40-50% decrease in the mitochondrial respiration and ATP production along with Mn SOD, Cu-Zn SOD and cytochrome c protein expression in the striatum of sedentary chronic MPD group when compared with the control animals. However, after 18 weeks of treadmill exercise-training, there was a significant improvement in both basal and ADP-stimulated respiration and ATP production in the striatal mitochondrial preparation. Additionally the mitochondrial antioxidant protein expression was partially or fully recovered when compared with the non-exercised chronic MPD.

Proteins are known to be the major targets for ROS. Toxic xenobiotics can cause oxidative modifications by introducing carbonyl groups to protein molecules through site-specific mechanisms. The presence of carbonyl groups is used as a biomarker for detecting abnormal oxidative proteins (Starke-Reed and Oliver, 1989). In this study, we detected a significant increase in the amount of carbonylated proteins in the sedentary

MPD group, an indication of sustained protein oxidation and damage due to chronic exposure to the neurotoxin, MPTP. This is the first time reported in a chronic model of PD that oxidative stress and protein carbonylation are the possible culprits. After 18 weeks of exercise, there was a significant reduction in the amount of carbonylated proteins in chronic MPD mice when compared with the non-exercised chronic MPD group.

p53 is a tumor suppressor protein and it plays an important role in cell cycle control and apoptosis. In normal cells p53 protein level is low, however, when there is DNA damage and/or stress signals the cellular p53 proteins are elevated (Liu et al., 2001). Neurotoxins, like MPTP cause DNA damage, which may activate protein kinases to phosphorylate p53 (Blagosklonny, 2000). Following neuronal apoptosis and associated DNA damage, p53 is released to initiate DNA repair and remove damaged cells (Perier et al., 2007). In our study, we observed a significant upregulation of the p53 mRNA in the sedentary chronic MPD group suggesting that chronic MPTP treatment results in heightened striatal DNA damage which may initiate neuronal apoptotic process and cell death. In chronic MPD, after 18 weeks of exercise, it is intriguing that p53 mRNA level in the striatum appears to be normal suggesting that exercised animals are protected from DNA damage.

The impact of endurance exercise on brain mitochondria in neurological diseases such as PD or PD-like disease models has not been reported. In this research, we investigated the outcome of chronic MPTP treatment on mitochondrial biogenesis in the aged MPD animals. PGC-1α and TFAM are the two transcription factors that are considered essential for mitochondrial gene expression and biogenesis in mammals (Borniquel et al., 2006; Wenz, 2009).

We demonstrated that PGC-1α and TFAM mRNA expression correlated with the neuronal and mitochondrial deficits seen in the sedentary chronic MPD mice as compared with the control group. These results suggest that sedentary chronic MPD animals develop sustained mitochondrial dysfunction and perhaps are attempting to compensate by repairing or regenerating the mitochondrial (Wenz, 2009). After 18 weeks of exercise-training, the chronic MPD mice displayed a significant reduction of TFAM mRNA, and PGC-1α mRNA, when compared with the sedentary chronic MPD, suggesting that less mitochondrial damage is seen in exercise-trained chronic MPD. Navarro et al. have also demonstrated that moderately exercised rats display increased mitochondrial biogenesis, electron transfer and bioenergetics (Navarro et al., 2004). Likewise, many investigators have reported that endurance exercise is a powerful inducer of mitochondrial biogenesis, for example, in skeletal muscles (Holloszy and Booth, 1976; Booth and Baldwin, 1997).

5.2.3. Long-term exercise on animal motor performance

The basal ganglia has been recognized for its control and refinement of motor performance and coordination (Grossman and Kelly, 1976). We examined the balancing skills in the chronic MPD animals by monitoring their walk on a balancing beam. The balancing beam task has been successfully used for demonstrating motor deficit in a transgenic Parkinsonian mouse model that overexpresses α-synuclein (Fleming et al., 2004). The balancing beam test confirmed that the sedentary chronic MPD mice exhibited movement deficits even 12 weeks after the MPTP/probenecid treatment when compared with the control group. Eighteen weeks of exercise training effectively prevented the motor deficit as seen in the chronic MPD.

Neuroprotection against MPTP-induced Parkinsonism has been demonstrated in mice when kept in an enriched environment and allowed voluntary exercise for 3 months; however, when these mice were only allowed to run for shorter periods of time, these animals lost a significant number of SNpc DA neurons (Faherty et al., 2005). Therefore, various components of the enriched environment including increased social interaction, introduction of environmental novelty and exercise would be beneficial for protecting SNpc DA neurons against oxidative insult and mitochondrial inhibition (Smeyne and Jackson-Lewis, 2005).

Published studies on impact of exercise in Parkinsonism have been inconsistent. While some studies have reported that exercise reverses both neurological and behavioral defects in acute MPD (Gerecke et al.), other investigators have shown exercise effects on behavioral improvement without evidence of preventing the loss of SNpc neurons (Steiner et al., 2006; O'Dell et al., 2007; Pothakos et al., 2009). The inconsistent findings from the published reports are highly attributed to the experimental designs such as, length of exercise training (Nishi et al., 1989; Fornai et al., 2000; Tillerson et al., 2003), differences in strain (Hamre et al., 1999; Sedelis et al., 2000), species (Terzioglu and Galter, 2008), the age of the chosen experimental animal (Tatton et al., 1992), toxins used to induce Parkinsonism (Smith and Zigmond, 2003), type of PD model (Tillerson et al., 2003; Pothakos et al., 2009), severity of neurotoxicity (Patki et al., 2009; Pothakos et al., 2009) and type and level of exercise applied (Mabandla et al., 2004; Petzinger et al., 2007).

5.2.4. Possible mechanisms of exercise on neuroprotection

It is conceivable that the neuroprotective effect of exercise is unlikely mediated by a single mechanism; instead it could be contributed by a constellation of processes that would be favorable for arresting cell death and for promoting cell survival. Exercise training in 6-OHDA model has resulted in elevation of prominent neurotrophic factors

such as BDNF and GDNF, prompted intracellular defense mechanisms against ROS, and restored the viability and transmission of DA neurons (Zigmond et al., 2009).

Other possible mechanisms by which exercise may lead to neuroprotection could involve increased circulation and blood flow to the brain, increased synaptogenesis, enhanced glucose utilization, increased vascularization (angiogenesis), proliferated progenitor cells (neurogenesis) and reduced inflammation in the brain. These are some of the areas that may be investigated in future studies on exercise neuroprotection in chronic MPD.

We have demonstrated here that mitochondrial dysfunction is associated with neurological and behavioral deficits in the chronic MPD. It is unquestionable that our study has proved the concept that long-term exercise has overarching benefits for prolonging cell survival and protecting against cell damage in presence of the neurotoxin, MPTP. However, it is uncertain whether these events are cause-and-effect related. With results showing that long-term exercise protects against neuronal, mitochondrial and behavioral deficits, it strengthens the intimate relationship that mitochondrial dysfunction may causally lead to neuronal loss in the chronic neurodegenerative MPD model.

In addition, exercise can apparently involve multifaceted cellular and molecular cascades for neuroprotection. With exercise, it is difficult to delineate whether

mitochondria protection is considered prerequisite to DA neuronal protection since exercise effect is not site-specific. Therefore, to further establish the link between mitochondrial dysfunction and neurological deficiency in the chronic MPD we elected to investigate a known antioxidant agent, melatonin to determine whether neuronal protection could be achieved by acting on mitigating the mitochondrial deficiency.

5.3. Effects of long-term melatonin treatment on neuronal and mitochondrial function in aged chronic MPD

Melatonin is selected for this research aim because it is known to cross the blood brain barrier after parental administration. It is a potent antioxidant and is capable of scavenging ROS and RNS by reducing electron leakage and free radical production. This would enhance the efficiency of the ETC and promote ATP synthesis (Leon et al., 2004; Reiter et al., 2005).

To examine the neuronal damage as the result of chronic MPTP and to determine whether melatonin offers any neuroprotection, we measured TH, DAT and VMAT-2 protein expressions along with DA in the SN and in the striatum. Our results demonstrated that there was around 50-60% decrease in the level of DA along with TH, DAT and VMAT-2 protein expression in the striatum of the chronic MPD group when compared to the control animals. Behaviorally, the chronic MPD group displayed greater motor deficits as compared to the control group. It is important to mention that

melatonin alone did not alter neuronal function in the SN and the striatum. Following 18 weeks of chronic melatonin treatment, all the above deficits, except on VMAT-2, seen in the chronic MPD were significantly prevented, suggesting that chronic melatonin treatment offers neuroprotection in chronic MPD mice. The reason on why melatonin failed to protect against VMAT-2 deficit is not clear.

Our observations that melatonin is neuroprotective in the chronic MPD are suggested by a recent study in the rotenone-induced PD model showing that melatonin attenuated the decreased TH and DAT protein expression levels in the striatum (Lin et al., 2008). The neuroprotective effect of melatonin in 6-OHDA and rotenone–induced models of PD have also been demonstrated by other laboratories (Sharma et al., 2006; Saravanan et al., 2007). In a study conducted by Ma et.al there were more SNpc cells shown in the melatonin-MPTP group when compared to the MPTP group demonstrating neuroprotection by melatonin (Ma et al., 2009).

To examine the impact of chronic melatonin treatment on mitochondrial dysfunction in the chronic MPD, we measured mitochondrial respiration and ATP production along with various antioxidant enzyme levels such as Mn SOD, Cu-Zn SOD and cytochrome c in the striatum. Our results demonstrated that melatonin alone did not alter the normal mitochondrial respiration. Furthermore, there was a 40-50% decrease in the mitochondrial respiration and ATP production along with Mn SOD, Cu-Zn

SOD and cytochrome c protein expression in the striatum of the chronic MPD group when compared with the control animals. The chronic MPD treated with 18-week melatonin displayed significant improvement in both basal and ADP-stimulated respiration and ATP production in the striatal mitochondrial preparation. Additionally, the endogenous antioxidant protein expression was significantly recovered. Elsewhere, related studies on isolated rat liver mitochondria and striatal synaptosomes have shown that melatonin prevents the inhibition of mitochondrial complex-I associated respiration when treated with MPP^+ (Absi et al., 2000). Melatonin is shown to protect the lipid bilayer, prevent DNA damage, and improve mitochondrial homeostasis (Costa et al., 1997). Melatonin stabilizes mitochondrial inner membranes (Garcia et al., 1999), and facilitates the ETC activity (Acuna-Castroviejo et al., 2001). Even the metabolites of melatonin, 3-OHMEL and AFMK are capable of scavenging free radicals (López-Burillo et al., 2003). Melatonin also increases the gene expression and activities of antioxidant enzymes, GPx, GRd, SOD and CAT (catalase) (Antolin et al., 1996; Pablos et al., 1998; Reiter et al., 2000; Kilanczyk and Bryszewska, 2003; Rodriguez et al., 2004). Elevation of GPx, GRd and CAT activities leads to increased conversion of H_2O_2 to water resulting in the detoxification of harmful $^{\bullet}OH$ from H_2O_2 by the Fenton reaction. Since melatonin stimulates GRd, it benefits the GSH recycling and also helps to maintain a high ratio of GSH: GSSG (glutathione disulfide) (Hara et al., 2001). Increase in SOD activity by melatonin will enhance the capacity of the mitochondria to get rid of the ROS and RNS

generated across the ETC.

Melatonin is shown to ameliorate the decrease of TH-positive fibers and lipid peroxidation in the striatum following after MPTP injection (Acuna-Castroviejo et al., 1997). Khaldy and colleagues have demonstrated that melatonin prevents the autooxidation of DA and prevents the inhibition of complex I activity induced by MPTP, however it does not restore the lost TH-activity (Khaldy et al., 2003). Melatonin inhibits mouse brain DNA fragmentation and apoptosis associated with acute and chronic MPTP treatment (Ortiz et al., 2001; Antolin et al., 2002). The redox potential of melatonin is high, suggesting that it could interact with the complexes of the ETC and also act as an donor and acceptor of electrons resulting in increased electron flow, an effect that is not exhibited by other antioxidants such as vitamin C and E (Tan et al., 2000; Martin et al., 2002).

In this research, we demonstrated that melatonin not only protected striatal mitochondria but also protected the striatal DA neurons in the chronic MPD. Our findings are significant and convincingly suggest that mitochondrial dysfunction is likely a proximal cause consequentially leading to neuronal and behavioral abnormalities in the chronic MPD. Melatonin and its related drugs may potentially serve as effective adjuvant therapies for slowing the progression of idiopathic PD. Melatonin may also be

valuable for modifying mitochondrial disorders where oxidative stress and complex I inhibition are the underlying etiology.

6. SUMMARY AND CONCLUSIONS

1. In the present study, we examined the role of striatal mitochondrial function in the chronic MPD. Although MPTP acutely suppressed mitochondrial respiration, it did not cause a long-lasting inhibitory effect or deplete antioxidant enzymes in the young chronic MPD. These data suggest that MPTP may initiate neurodegenerative process by blocking mitochondrial respiration, yet the mitochondria in the striatum of young animals may recover over time and may not be required for sustaining neuronal degeneration.

2. On the other hand, we demonstrated that aged mice were more vulnerable to chronic MPTP toxicity. The aged chronic MPD displayed long-term inhibition of mitochondrial respiration and depletion of antioxidant enzymes and ATP. These effects coincided with marked loss of striatal DA neuronal functions and impaired motor performance. These results validated that aged chronic MPD is a suitable investigative model for further elucidating the integral relationship between mitochondrial dysfunction and neurodegenerative disorder, and for assessing the therapeutic efficacy of mitochondrial protective agents as potential neuroprotective drugs.

3. At the neuronal and behavioral level, long-term exercise prevented the loss of striatal DA neuronal function in the aged chronic MPD. This was demonstrated by

the reversal of DA depletion, loss of TH, DAT and VMAT-2 protein expression as exhibited in the chronic MPD. Exercised mice performed better on the balancing beam as compared to the chronic MPD mice.

4. At the mitochondrial level, long-term exercise prevented the loss of mitochondrial function in the aged chronic MPD. This was confirmed by the reversal of suppressed mitochondrial respiration and ATP production as seen in the chronic MPD. The depleted level of antioxidant enzymes, such as Mn SOD, Cu-Zn SOD and cytochrome c were recovered in the exercise-trained chronic MPD.

5. We detected significant upregulation of p53 mRNA in the sedentary chronic MPD indicative of increased DNA damage that could lead to neuronal apoptosis. Long term exercise training partially reverted this phenomenon.

6. We observed a significant upregulation of PCG-1α and TFAM mRNA in the sedentary chronic MPD indicative of compensatory increase in mitochondrial biogenesis as an attempt to compensate for the dysfunctional mitochondria. Long-term exercise training significantly prevented this effect as well.

7. The findings from this study demonstrated that endurance exercise is mitochondria-protective as well as neuroprotective in the chronic MPD. Even though the exercise effect may involve in a wide range of possible mechanisms, it does point to the

possibility that dopamine neuron degeneration and mitochondrial disorder are integrally related in the chronic MPD.

8. Like in the case of long-term chronic endurance exercise, 18 weeks administration of melatonin treatment to the chronic MPD similarly prevented neuronal, mitochondrial and behavioral deficits. These experimental results implied that mitochondrial dysfunction may occur preceding the neuronal and behavioral impairments. Thus, melatonin may become a useful adjuvant agent for treating early onset of idiopathic PD.

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8. APPENDIX

Since GAPDH is a cytosolic enzyme in mitochondria, it would not be expected to be present in a pure mitochondrial preparation. However, GAPDH is detectable in a crude striatal mitochondrial homogenate preparation. In the melatonin study, we additionally used a mitochondria-specific enzyme, cytochrome oxidase as a protein loading control. However, before using cytochrome oxidase as control, we wanted to make certain that it was unaffected by animal treatments. The results are shown as in **(Figure A)**.

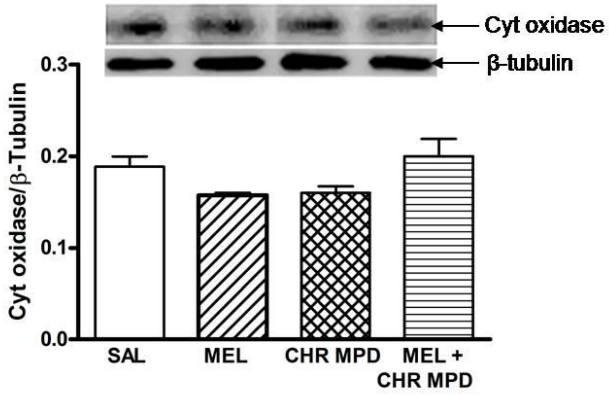

Figure A: Protein expression of cytochrome oxidase and β-tubulin in the striatal preparation of the chronic control, chronic melatonin, chronic MPD and melatonin-treated chronic MPD mice. A representative image of Western blot protein bands was correspondingly shown above each set of bar graph. Each data point represents mean ± S.E.M. (N = 8 for chronic saline, N = 4 for chronic melatonin and chronic MPD and N = 6 for melatonin-treated chronic MPD). Statistical analyses revealed that the ratio of cytochrome oxidase/β-tubulin was not different among the 4 experimental groups.