

# Mitochondrial function in Parkinson's disease cybrids containing an nt2 neuron-like nuclear background

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Received 3 December 2007; received in revised form 14 March 2008; accepted 31 March 2008

Available online 6 April 2008

## Abstract

Mitochondria likely play a role in Parkinson's disease (PD) neurodegeneration. We modelled PD by creating cytoplasmic hybrid (cybrid) cell lines in which endogenous mitochondrial DNA (mtDNA) from PD or control subject platelets was expressed within human teratocarcinoma (NT2) cells previously depleted of endogenous mtDNA. Complex I activity was reduced in both PD cybrid lines and in the platelet mitochondria used to generate them. Under basal conditions PD cybrids had less ATP, more LDH release, depolarized mitochondria, less mitochondrial cytochrome *c*, and higher caspase 3 activity. Equivalent MPP<sup>+</sup> exposures are more likely to trigger programmed cell death in PD cybrid cells than in control cybrid cells. Our data support a relatively upstream role for mitochondrial dysfunction in idiopathic PD.

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**Keywords:** Cybrids; MPP<sup>+</sup>; Mitochondria; Cytochrome *c*; Apoptosis

## 1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder. It is characterized by extensive loss of nigrostriatal dopaminergic neurons. Residual nigral neurons contain intraneuronal inclusions called Lewy bodies (LBs). Five to 10% of cases are postulated to have a genetic component, while the majority is categorized as idiopathic and sporadic. Sporadic PD prevalence is age-associated, with approximately 1% of the population affected at 65 years and 4–5% at 85 years of age (Reviewed in Cardoso et al., 2005).

While the etiology of the disease for most affected people remains unclear, mitochondrial dysfunction likely plays a key role in PD pathogenesis. Mitochondria are central not only to cell bioenergetics but also to apoptotic cell death (Shults, 2004). They are believed to play a fundamental role in ageing, and interact with specific proteins previously implicated in genetic forms of this neurodegenerative disease.

Several lines of evidence link sporadic PD to mitochondrial dysfunction. Complex I activity is reduced in both autopsy brain and platelets of sporadic PD subjects (Parker et al., 1989; Shapira et al., 1989, 1990; Mizuno et al., 1989). Recently, Keeney and co-workers observed complex I is oxidatively damaged in PD brain (Keeney et al., 2006). While the mechanisms underlying this are not entirely clear, in sporadic PD mutated or oxidatively damaged mitochondrial DNA (mtDNA) may play a contributory role (Ibeke et al., 1995; Cortopassi and Wang, 1995).

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Mitochondrial dysfunction in sporadic PD appears to arise at least in part from mtDNA (Swerdlow et al., 1996, 1998; Gu et al., 1998; Shults and Miller, 1998). Interestingly, relative to other brain areas the substantia nigra contains particularly high levels of large mtDNA deletions (Soong et al., 1992). These mtDNA deletions likely cause functional impairment in aged human substantia nigra neurons (Kraytsberg et al., 2006). PD brains also have discrete microheteroplasmic mutations in the ND5 gene that distinguish them from control brains (Smigrodzki et al., 2004; Parker and Parks 2005). Together, these studies suggest mtDNA may mediate mitochondrial dysfunction in PD and therefore contribute to PD neurodegeneration.

To address the relevance of mitochondrial function to PD, we used a cytoplasmic hybrid (cybrid) approach to generate a disease-specific, *ex-vivo* model of mitochondrial dysfunction. This approach has previously been used to produce cell culture models of PD mitochondrial dysfunction; successful studies utilizing SH-SY5Y and A549 human cell line nuclear backgrounds are reported (Swerdlow et al., 1996, 1998; Gu et al., 1998; Shults and Miller, 1998). This technique involves the transfer of platelet mitochondria from either PD or control subjects to mtDNA-depleted recipient cells (rho0 cells). The resulting cybrid lines express the nuclear genes of the recipient rho0 cell line and the mitochondrial genes of the platelet donor. If mitochondrial defects found in transferred platelet mitochondria persist in culture, it is hypothesized mtDNA should account for those persistent defects. Regardless of the role of mtDNA, though, cybrid models facilitate the study of downstream consequences of mitochondrial dysfunction in PD (Swerdlow et al., 1996; Ghosh et al., 1999).

We now report for the first time an analysis of PD cybrids prepared using a human teratocarcinoma (NT2) nuclear background. We further used these cell lines to study how PD and control (CT) cybrid cell lines respond to a mitochondrial toxin, 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>). MPP<sup>+</sup>, a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), induces Parkinsonism in humans, primates, and mice (Langston et al., 1983; Beal, 2001). It causes anatomically specific degeneration of substantia nigra pars compacta and locus coeruleus catecholaminergic neurons by inhibiting complex I of the mitochondrial respiratory chain (Przedborski et al., 2004; Mochizuki et al., 1996). In addition to finding the complex I defect of PD platelets perpetuates in NT2 PD cybrids, we describe for the first time that MPP<sup>+</sup> treated CT cybrids showed mitochondrial impairments similar to untreated PD cybrids. Furthermore, mitochondrial dysfunction observed in untreated PD cybrids renders them more susceptible to MPP<sup>+</sup> induced mitochondrial dependent apoptosis.

## 2. Materials and methods

Subject participation was approved by the Institutional Review Board of the University Hospital of Coimbra.

PD subjects were recruited from the Neurology Service at the University Hospital of Coimbra and met the Gelb and co-workers (1999) criteria for probable PD. They did not manifest signs or symptoms of an alternative neurodegenerative disease. The mean age of the CT group ( $n = 3$ ) was  $64.3 \pm 8.4$ , and for the PD group ( $n = 2$ ) was  $65.0 \pm 5$ .

### 2.1. Preparation of platelet mitochondria

Following informed consent, 60 ml of blood was collected through venipuncture in tubes containing acid–citrate–dextrose as an anticoagulant. Mitochondria were obtained from human platelets according to previously described methods (Krige et al., 1992). Platelet mitochondria protein concentrations were measured by the Bradford protein assay (Bradford, 1976), in which bovine serum albumin was used as the standard.

### 2.2. Cell culture

These experiments utilized NT2 human teratocarcinoma cells (Stratagene, La Jolla, CA) depleted of endogenous mtDNA (rho0 cells) via long-term ethidium bromide exposure (Swerdlow et al., 1997; Binder et al., 2005). Platelet mitochondria from either PD or CT subjects were used to repopulate NT2 rho0 cells with mtDNA as previously described (Swerdlow et al., 1997; Binder et al., 2005). Untransformed cells were removed by withdrawal of pyruvate and uridine from the culture medium and substitution of dialyzed, heat inactivated fetal calf serum for non-dialyzed, heat inactivated fetal calf serum (Swerdlow et al., 1996; Miller et al., 1996). Specifically, the selection medium consisted of Optimem (Gibco Life Technologies) supplemented with 10% dialyzed, heat inactivated fetal calf serum, penicillin (50 U/mL), and streptomycin (50  $\mu$ g/mL). Cells were grown in 75 cm<sup>2</sup> tissue culture flasks maintained in a humidified incubator at 37 °C and 5% CO<sub>2</sub>.

For the MTT assay, cells were plated in 24-well plates at a density of  $0.1 \times 10^6$  cells/well. For rhodamine 123 measurements, cells were plated in 24-well plates at a density of  $0.05 \times 10^6$  cells/well. For ATP and LDH determinations, cells were plated in 12-well plates at a density of  $0.2 \times 10^6$  cells/well. For measurements of caspase enzyme activities, Western blot analysis of  $\alpha$ -spectrin, and Western blot analysis of poly (ADP-ribose) polymerase (PARP) cells were plated in 6-well plates at a density of  $0.5 \times 10^6$  cells/well. For Western blot analysis of cytochrome *c* and determinations of electron transport chain (ETC) enzyme activities, cells were plated in petri dishes (10 cm) at a density of  $2.5 \times 10^6$  cells/dish.

### 2.3. MPP<sup>+</sup> treatment

MPP<sup>+</sup> was purchased from Sigma. Twenty-four hours after plating, cybrid cell lines were treated with 100, 500 or 1 mM MPP<sup>+</sup> (water diluted) and maintained at 37 °C for another 24 h. For each experimental parameter, a con-

control condition was run in which cells were not exposed to MPP<sup>+</sup>.

#### 2.4. Electron microscopy

Cell suspensions were fixed with 3% glutaraldehyde in 0.1 M phosphate-buffered pH 7.3 overnight at 4 °C. The fixed pellets were washed in 0.1 M phosphate-buffered. Pellets were then post-fixed with 1% osmium tetroxide during 2 h, dehydrated in grade ethanol and embedded in Spurr. The ultrathin sections were cut with an LKB ultra-microtome ULTRATOME III, and then contrasted with uranyl acetate and with lead citrate for transmission electron microscopy. Electron microphotographs were taken with JEOL JEM-100 SX electron microscope operated at 80 kV.

#### 2.5. Mitochondrial respiratory chain complexes

##### 2.5.1. NADH-ubiquinone oxidoreductase assay

Complex I activity was determined by a modified version of Ragan et al. (1987), which follows the decrease in NADH absorbance at 340 nm that occurs when ubiquinone (CoQ1) is reduced to form ubiquinol. The reaction was initiated by adding CoQ1 (50 μM) to the 30 °C reaction mixture. After 5 min, rotenone (10 μM) was added and the reaction was followed for another 5 min. Complex I activity was expressed both as nanomoles per minute per milligram of protein, as well as the ratio of complex I activity per citrate synthase activity.

##### 2.5.2. Succinate-cytochrome *c* oxidoreductase assay

Complex II/III activity was measured by following the formation of reduced cytochrome *c* at 550 nm (King, 1967). The sample was pre-incubated at 30 °C for 5 min with 20 mM succinate and 1 mM KCN prior to addition of the cytochrome *c*. The assay was performed at 30 °C. Activity of complex II/III was expressed both as nanomoles per minute per milligram of protein, as well as the ratio of complex II/III activity per citrate synthase activity.

##### 2.5.3. Cytochrome *c* oxidase assay

Complex IV activity was determined using the method of Wharton and Tzagoff (1967), which measures the oxidation of reduced cytochrome *c* by cytochrome *c* oxidase at 550 nm. To prepare reduced cytochrome *c*, cytochrome *c* was mixed with a few crystals of ascorbate and partitioned by a dialysis membrane overnight against 0.01 M phosphate buffer, pH 7.0 at 4 °C. The reduced cytochrome *c* concentration was then determined using 0.1 M potassium ferricyanide. The reaction mixture contained 0.01 M potassium phosphate, pH 7.0, and 50 μM reduced cytochrome *c*. The reaction was initiated by addition of the sample at 30 °C. The pseudo first order rate constant (*K*) was calculated, because the reaction is of first order with respect to cytochrome *c*. Results are expressed both as *K* per minute per milligram of protein, as well as the quotient of the cyto-

chrome oxidase activity divided by the citrate synthase activity.

#### 2.6. Citrate synthase assay

Citrate synthase activity was determined by the method of Coore et al. (1971), which spectrophotometrically follows the formation of 5-thio-2-nitrobenzoate at 412 nm. The assay was initiated by the addition of 100 μM oxaloacetate at 30 °C. Results were obtained in nanomoles per minute per milligram of protein.

#### 2.7. Analysis of adenine nucleotides

After the 24 h cell plating-incubation period, medium was removed. Cells were extracted, on ice, with 0.4 M perchloric acid. Cells were next centrifuged at 14,000 rpm for 5 min at 4 °C. The resulting pellets were solubilized with 1 M NaOH to further analyse protein content by the Bradford method. The resulting supernatants were neutralized with 5 M KOH and 2.5 M Tris, pH 7–8, and then centrifuged at 14,000 rpm for 5 min at 4 °C. These supernatants were assayed for adenine nucleotides (ATP, ADP and AMP) by reversed-phase HPLC as described by Stocchi et al. (1985). The chromatographic apparatus used was a Beckam Gold System, consisting of a 126 Binary Pump Model and a 166 Variable UV detector that was controlled by computer. The column was a Lichrospher 100 RP-18 (5 μm) from Merk (Germany). An isocratic elution with 100 mM KH<sub>2</sub>PO<sub>4</sub> buffer at pH 7.4 and 1% methanol was performed at a flow rate of 1.2 ml/min. The adenine nucleotides were detected at 254 nm for 6 min.

#### 2.8. Cell viability assays

##### 2.8.1. MTT reduction

Cell viability was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). In viable cells, the enzyme succinate dehydrogenase metabolizes MTT into a formazan that absorbs light at 570 nm. Following the cell treatment protocol the medium was aspirated and 0.5 ml MTT (0.5 mg/ml) was added to each well. The plate was then incubated at 37 °C for 3 h. At the end of the incubation period the formazan precipitates were solubilized with 0.5 ml of acidic isopropanol (0.04 M HCl/Isopropanol). The absorbance was measured at 570 nm. Cell reduction ability was expressed as a percentage of the CT cybrids.

##### 2.8.2. Lactate dehydrogenase release

In order to quantify the degree of plasma membrane injury, we determined the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the extracellular incubation medium. LDH activity was measured spectrophotometrically according to the method of Bergmeyer and Brent (1974), which follows the conversion of NADH to NAD<sup>+</sup> at 340 nm. LDH release was expressed as a per-

centage of total LDH activity, which was defined as the activity measured in the supernatant plus the activity measured after incubating the cells in a 15 mM Tris solution, pH 7.4.

## 2.9. Mitochondrial dependent apoptosis

### 2.9.1. Analysis of mitochondrial membrane potential ( $\Delta\psi_m$ )

**2.9.1.1. Rhodamine 123 probe.** Rhodamine 123, a fluorescent cationic dye, was used to monitor changes in mitochondrial membrane potential. Plated cells were washed with phosphate-buffered saline (PBS) and incubated with 1  $\mu$ M rhodamine 123 for 45 min at 37 °C. Basal fluorescence was read using excitation and emission wavelengths of 505 and 525 nm, respectively. Next, 1 mg/ml oligomycin and 6  $\mu$ M FCCP were added and the plate was read at 37 °C. Rhodamine 123 retention capacity was calculated as the difference between the final fluorescence (after oligomycin and FCCP exposure) and the initial fluorescence. Results are expressed as a percentage of the dye retained within the untreated CT hybrids.

**2.9.1.2. MitoTracker Red probe.** MitoTracker Red was used to assess mitochondrial membrane potential. Cells were grown on coverslips in 12-well plates. After treatment, cells were washed twice with PBS and incubated with 500 nm MitoTracker Red for 45 min at 37 °C. Cells were then washed twice with PBS and fixed for 30 min at room temperature in 4% paraformaldehyde. The fixed cells were washed again with PBS, and observed under a confocal microscope.

### 2.9.2. Western blot analysis of mitochondrial cytochrome c

Following MPP<sup>+</sup> treatment cells were washed with PBS, scraped in a buffer containing 250 mM sucrose, 20 mM Hepes, 1 mM EDTA, 1 mM EGTA, and protease inhibitors (0.1 M phenyl-methylsulfonyl fluoride (PMSF), 0.2 M dithiothreitol (DTT), and 1:1000 dilution of a protease inhibitor cocktail) and homogenized. Cells were frozen three times on liquid nitrogen and the lysate was centrifuged at 2200 rpm for 12 min at 4 °C. The resulting supernatant was centrifuged at 9600 rpm for 10 min at 4 °C. Pellets resulting from this step constitute a crude mitochondrial fraction. The amount of protein was measured by the Bradford method and 25  $\mu$ g of protein from each condition was loaded per well. The samples were resolved by electrophoresis in 12% SDS–polyacrylamide gels and transferred to PVDF membranes. Non-specific binding was blocked by gently agitating the membranes in 5% non-fat milk and 0.1% Tween in TBS for 1 h at room temperature. The blots were subsequently incubated with the respective primary antibodies [mouse purified anti-Cytochrome C (denatured) [(1:500) (BD Pharmingen)]] overnight at 4 °C with gentle agitation. Blots were washed with TBS containing 0.1% non-fat milk and 0.1% Tween three times (each time for 15 min), and then incubated with the secondary antibodies [anti-mouse (1:20000)] for 2 h at room tempera-

ture with gentle agitation. After three washes specific bands of interest were detected by developing with ECF detection reagent (Amersham). Fluorescence signals were detected using a Biorad Versa-Doc Imager.

### 2.9.3. Caspase activation assays

Caspase activation was measured using a colorimetric method described by Cregan et al. (1999), in which the substrate cleavage is monitored at 405 nm. After treatment with MPP<sup>+</sup>, cells were washed and placed in buffer containing 25 mM HEPES, 1 mM EDTA, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, and protease inhibitors (0.1 M PMSF, 2 mM DTT, and a 1:1000 dilution of a protease inhibitor cocktail). Cells were harvested by scraping and frozen three times on liquid nitrogen. The lysate was centrifuged for 10 min at 14,000, 4 °C. The resulting supernatant was stored at –80 °C. Protein concentrations were measured by the Bradford method. Lysates (50  $\mu$ g of protein) were incubated at 37 °C for 2 h in 25 mM HEPES, pH 7.5 containing 0.1% CHAPS, 10% sucrose, 2 mM DTT, and 40  $\mu$ M Ac-LEHD-pNA or 40  $\mu$ M Ac-DEVD-pNA (Calbiochem) to determine caspase 9 or caspase 3 activation, respectively.

### 2.9.4. PARP and $\alpha$ -spectrin cleavage

Following experimental treatments, cells were scraped in a buffer containing 25 mM HEPES, 1 mM EDTA, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, and protease inhibitors (0.1 M PMSF, 0.2 M DTT, and 1:1000 dilution of a protease inhibitor cocktail). Cells were then frozen three times on liquid nitrogen and stored at –80 °C. The amount of protein was measured by the Bradford protein assay. Equal amounts of protein samples (50  $\mu$ g per condition) were first resolved by electrophoresis in 10% (for PARP) or 7% (for  $\alpha$ -spectrin) SDS–polyacrylamide gels. The electrophoresed proteins were transferred to PVDF membranes. Non-specific binding was blocked by gently agitating the blots in 5% non-fat milk and 0.1% Tween in TBS for 1 h at room temperature. The blots were subsequently incubated with the respective primary antibodies [mouse anti  $\alpha$ -spectrin monoclonal antibody [(1:1000) (Chemicon)]] and mouse anti-PARP [(1:2000) (Cell Signaling)]] overnight at 4 °C with gentle agitation. All the blots were washed with TBS containing 0.1% non-fat milk and 0.1% Tween three times (each time for 15 min), and then incubated with the secondary antibodies [anti-mouse (1:20000)] for 2 h at room temperature with gentle agitation. After three washes specific bands of interest were detected following development with the ECF detection reagent. Fluorescence was detected using a Biorad Versa-Doc Imager.

## 2.10. Data analysis

Data provided are the mean  $\pm$  SE. At least three independent measurements were made for each experiment. Statistical analyses were conducted using one-way ANOVA. Post hoc Bonferroni testing was also performed.

Differences were considered statistically significant at  $p < 0.05$ .

### 3. Results

#### 3.1. Ultrastructure of mitochondria in cybrids

At the ultrastructural level, CT cybrids had a eukaryotic nucleus, and dark, oval mitochondria (Fig. 1A). However, PD cybrids contained pale and enlarged shaped mitochondria with disrupted cristae (Fig. 1A).

#### 3.2. Mitochondrial function in platelets and cybrids

We measured citrate synthase (CS) activities to ensure similar degrees of mitochondrial enrichment occurred between compared groups. PD and CT platelet CS activities were equivalent, as were PD and CT cybrid CS activities (Data not shown).

The results of our CS-corrected mitochondrial respiratory chain (MRC) complex activities are shown in Table 1. After correcting for CS activity, complex I activity was lower in PD platelet mitochondria than it was in CT platelet mitochondria. For NT2 cybrids prepared using mitochondria from the same platelet donors, a reduction in

the PD group complex I/CS activity was again observed. Additionally, complex IV/CS activity was lower in PD cybrids than it was in control cybrids.

We also assessed the effects of MPP<sup>+</sup> on PD and CT cybrid MRC enzyme activities. Both groups showed equivalent degrees of complex I and IV inhibition (Table 2).

We measured ATP levels in PD and CT cybrids at baseline and following 1 mM MPP<sup>+</sup> exposure. Basal ATP levels were lower in PD cybrids, but following MPP<sup>+</sup> exposure CT and PD ATP levels were equivalent. MPP<sup>+</sup> caused a significant reduction in CT but not PD cybrid ATP levels (Fig. 1B).

#### 3.3. MPP<sup>+</sup> toxicity in PD and CT cybrids

The ability of cells to reduce MTT is often used as a viability screen. Under basal conditions PD cybrids tended towards less MTT reduction than CT cybrids, but this parameter did not reach statistical significance (Fig. 2A). When exposed to varying concentrations of MPP<sup>+</sup> for 24 h, both PD and CT cybrids exhibited a dose-dependent, roughly equivalent decrease in MTT reduction capacity.

Cell viability was also assessed using an LDH release assay (Fig. 2B). Under basal conditions PD cybrids released more LDH than CT cybrids. Although MPP<sup>+</sup>

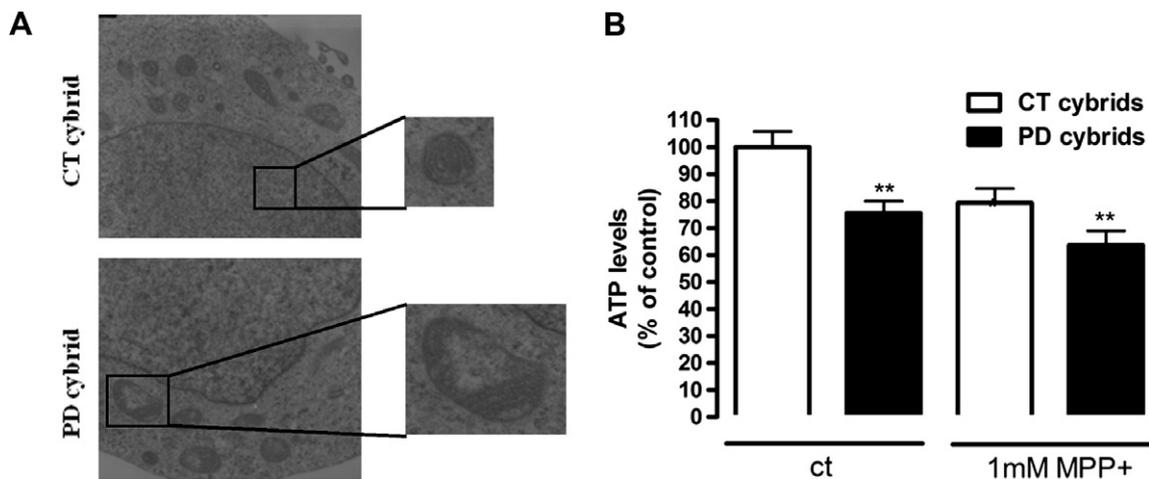


Fig. 1. (A) Electron microscopy of a PD and a CT cybrid cell. CT cybrid have a euchromatic nucleus (N) and rod-shaped mitochondria (M) with a dark matrix. PD cybrids have also a euchromatic nucleus (N) and oval mitochondria (M) with pale matrix. PD cybrids mitochondria are enlarged and with disrupted cristae (arrowhead) (8000 $\times$ ). (B) ATP levels in PD and CT cybrids. Cell ATP levels were determined by HPLC as described in Section 2. Mean  $\pm$  SEM ATP levels for each group were calculated from duplicate determinations from four different experiments. In the figure, data are reported as a percentage of each group mean ATP level as compared to the mean ATP level of the untreated CT cybrid group. \* $p < 0.05$ ; \*\* $p < 0.01$ , significantly different as compared to the untreated CT cybrid group.

Table 1

Activity of mitochondrial respiratory chain complexes in PD platelet mitochondria and PD cybrids

	CT platelet mitochondria	PD platelet mitochondria	CT cybrids	PD cybrids
Complex I/CS	2.35 $\pm$ 0.48	1.23 $\pm$ 0.34*	0.63 $\pm$ 0.02	0.31 $\pm$ 0.07**
Complex II/III/CS	2.27 $\pm$ 0.64	1.46 $\pm$ 1.18	0.06 $\pm$ 0.04	0.05 $\pm$ 0.02
Complex IV/CS	0.42 $\pm$ 0.15	0.37 $\pm$ 0.01	1,24 $\times 10^{-5}$ $\pm$ 0.08 $\times 10^{-5}$	0,55 $\times 10^{-5}$ $\pm$ 0.12 $\times 10^{-5}$ **

\*  $P < 0.05$ , significantly different as compared to CT platelet mitochondria.

\*\*  $P < 0.01$ , significantly different as compared to CT cybrids.

Table 2  
Mitochondrial respiratory chain complexes in MPP<sup>+</sup> treated cybrids

	CT cybrids	MPP <sup>+</sup> /CT cybrids	PD cybrids	MPP <sup>+</sup> /PD cybrids
Complex I (nmol/min/mg)	16.58 ± 2.86	9.53 ± 0.09**	11.65 ± 1.46**	9.38 ± 0.81**
Complex II/III (nmol/min/mg)	1.52 ± 0.58	LIS ± 0.31	2.09 ± 0.55	1.83 ± 0.57
Complex IV (nmol/min/mg)	0.39 ± 0.11	0.14 ± 0.04*	0.23 ± 0.14*	0.07 ± 0.02*

\*  $P < 0.05$ , significantly different as compared to CT platelet mitochondria.

\*\*  $P < 0.01$ , significantly different as compared to CT cybrids.

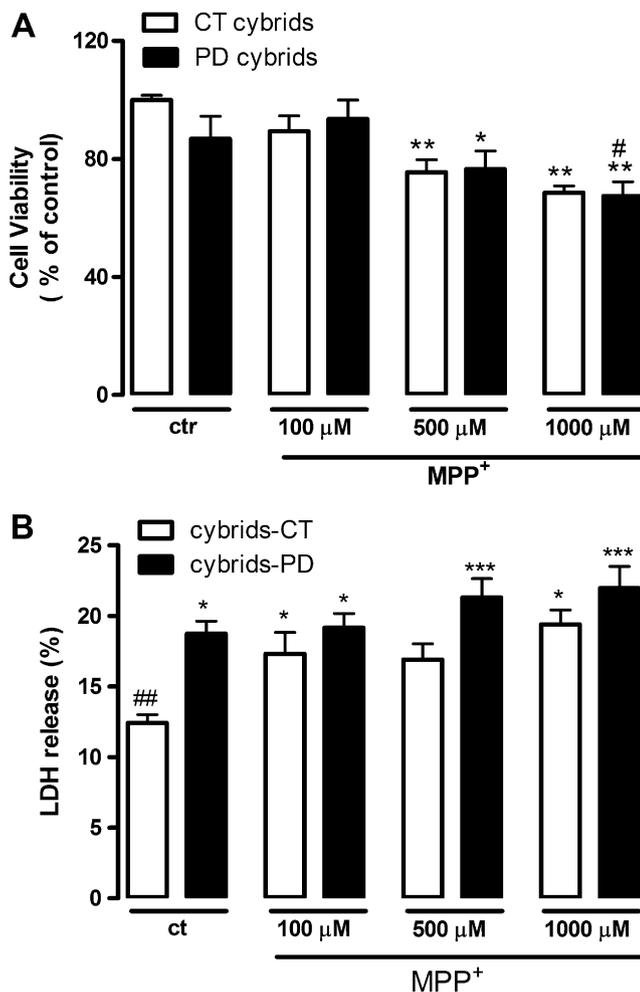


Fig. 2. Effects of MPP<sup>+</sup> on MTT reduction and LDH release. Cybrid cells were incubated in the presence of 100, 500, 1000 μM MPP<sup>+</sup>. (A) The ability of cells to reduce MTT was determined as described in Section 2. Data are expressed as a percentage of the untreated CT cybrid group, with the mean ± SEM from five independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ , significantly different from the untreated CT cybrid group; # $p < 0.05$ , significantly different from the untreated PD cybrid group. (B) The effect of MPP<sup>+</sup> on membrane integrity was evaluated by LDH quantification, as described in Section 2. Data are expressed as the mean ± SEM values from five independent experiments. \* $p < 0.05$ ; \*\*\* $p < 0.001$ , significantly different as compared to the untreated CT cybrid group; ## $p < 0.01$ , significantly different as compared to the untreated PD cybrid group.

increased LDH leakage in both groups, the relative magnitude of the MPP<sup>+</sup>-induced LDH release increase was greater in the CT cybrid group. Despite this, at the higher MPP<sup>+</sup> concentrations the amount of LDH released by CT

cybrids was still less than the amount released from PD cybrids.

### 3.4. Activation state of the intrinsic apoptosis pathway

The amount of rhodamine 123 taken up and retained by mitochondria is proportional to the magnitude of the mitochondrial membrane potential. Under basal conditions rhodamine 123 levels were lower in PD cybrids than in CT cybrids. This indicates the mitochondrial membrane potential was reduced in PD cybrids (Fig. 3A). Experiments performed with MitoTracker Red were consistent with those using rhodamine 123, in that under basal conditions the PD cybrid mitochondrial membrane potential was reduced relative to that of CT cybrids (Fig. 3B). Both approaches showed 1 mM MPP<sup>+</sup> exposure depolarised mitochondria in CT cybrids, but did not appreciably reduce PD cybrid mitochondrial membrane potentials. Following MPP<sup>+</sup> exposure, mitochondrial membrane potentials were similar between cybrid groups.

Mitochondrial fractions prepared from PD cybrids contained less cytochrome *c* than mitochondrial fractions prepared from CT cybrids (Fig. 3C). One millimolar of MPP<sup>+</sup> exposure reduced mitochondrial cytochrome *c* levels in both PD and CT cybrid cell lines. Levels of mitochondrial cytochrome *c* were decreased in MPP<sup>+</sup>-treated PD cybrids as compared to CT cybrids.

Under basal conditions, caspase 9 activities were equivalent between PD and CT cybrids (Fig. 4A). MPP<sup>+</sup> activated caspase 9 only at the highest dose studied (1 mM MPP<sup>+</sup>), and even then only for the PD cybrids. Caspase 3 activity was higher in PD cybrids under basal conditions, but not with 100 or 500 μM MPP<sup>+</sup> exposures (Fig. 4B). Neither of these MPP<sup>+</sup> concentrations was associated with increased caspase 3 activity in either PD or control cybrids. At the 1 mM MPP<sup>+</sup> concentration caspase 3 activity was increased in both PD and CT cybrids. At this MPP<sup>+</sup> concentration, caspase 3 activities were comparable between the PD and CT cybrid groups.

To further evaluate caspase 3 status, we immunohistochemically assessed PARP and alpha spectrin cleavage in PD and CT cybrids before and after MPP<sup>+</sup> treatment. Caspase 3-mediated cleavage of α-spectrin yields 120 and 150 kDa fragments. As demonstrated in Fig. 5A, under basal conditions levels of both of these fragments were increased in PD cybrids. One millimolar of MPP<sup>+</sup> induced α-spectrin cleavage in both PD and CT cybrid lines, and following MPP<sup>+</sup>

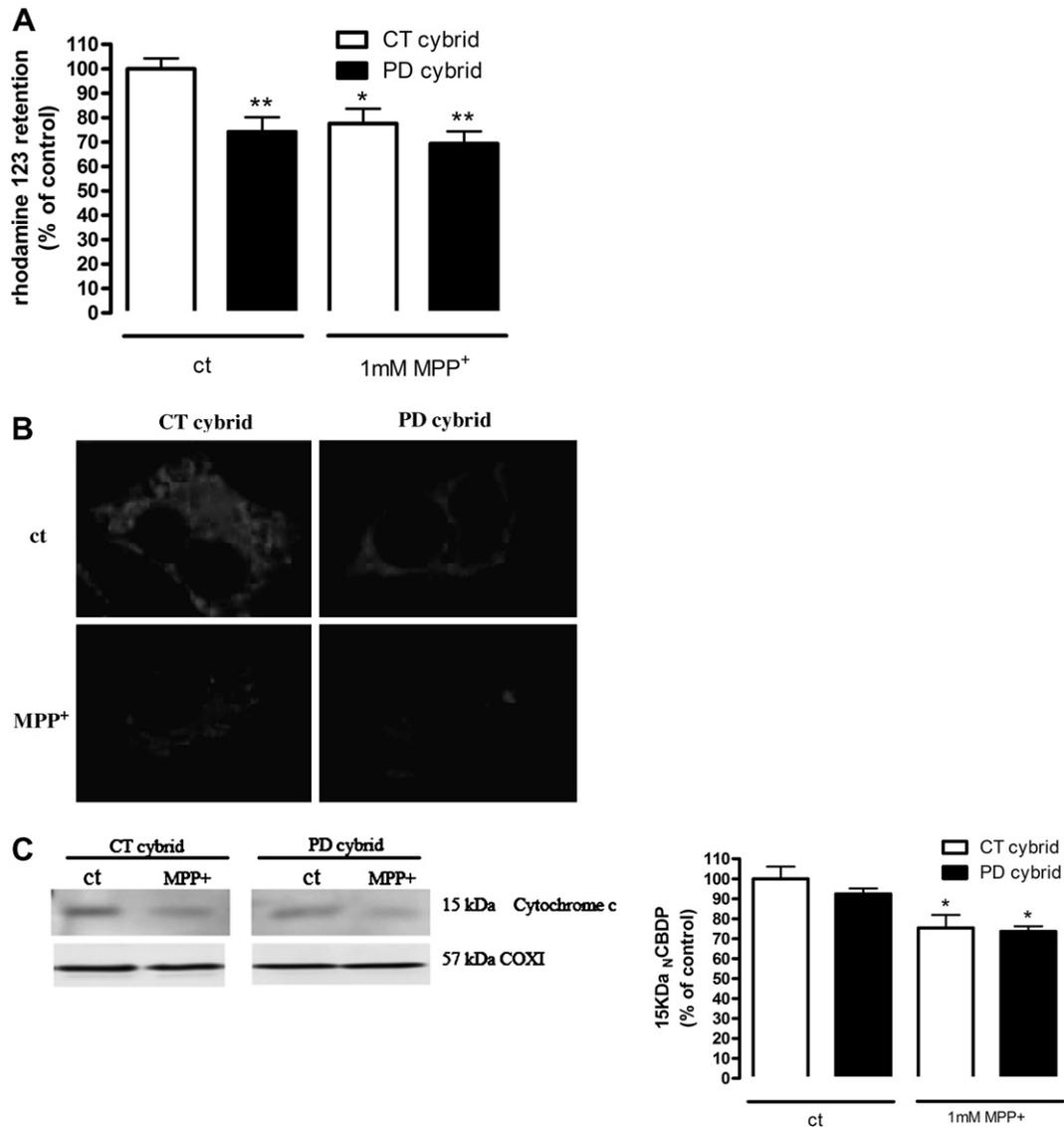


Fig. 3. Mitochondrial membrane potential and cytochrome *c* content. (A) Mitochondrial membrane potential was expressed as the percentage of rhodamine 123 retention in untreated CT cybrids, with the mean  $\pm$  SEM derived from five independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , significantly different as compared to CT untreated cybrids. (B) Mitochondrial membrane potential was also analysed using MitoTracker Red. Three independent experiments were performed for each cell line. The figure shows actual results from a representative experiment. (C) Basal mitochondrial cytochrome *c* levels were reduced in PD cybrids. Each cybrid cell line was assayed three times. The figure shows actual Western blot results from a representative experiment.

exposure both cybrid groups contained comparable levels of  $\alpha$ -spectrin cleavage products. PARP, a 116 kDa protein, is cleaved by caspase 3 to an N-terminally truncated 89 kDa derivative. Under basal conditions minimal PARP cleavage was observed in either PD or CT cybrids. MPP<sup>+</sup> increased PARP cleavage in both cybrid groups (Fig. 5B). As was the case with our  $\alpha$ -spectrin analysis, there was a trend towards greater activation (under both basal and MPP<sup>+</sup>-treatment conditions) of caspase 3 in PD cybrids.

#### 4. Discussion

Our data confirm prior studies showing complex I activity is reduced in PD subject platelets (Schapira and Mars-

den, 1994; Barroso et al., 1993; Parker et al., 1989; Yoshino et al., 1992; Benecke et al., 1993; Haas et al., 1995; Blandini et al., 1998). Our data further confirm and extend prior studies demonstrating mitochondrial transfer from PD subject platelets to rho0 cells produces cybrid cell lines in which complex I  $V_{max}$  activities are reduced relative to cybrid cell lines containing mitochondria from control subjects (Swerdlow et al., 1996; Cassarino et al., 1997). Complex I activity is reduced in PD brain (Shapira et al., 1989, 1990; Gu et al., 1998; Janetzky et al., 1994). It seems reasonable to postulate a common underlying factor causes complex I activity reductions in both these tissues. The fact that complex I dysfunction perpetuates in PD cybrids suggests mtDNA may account for systemic alteration of complex I in this disease.

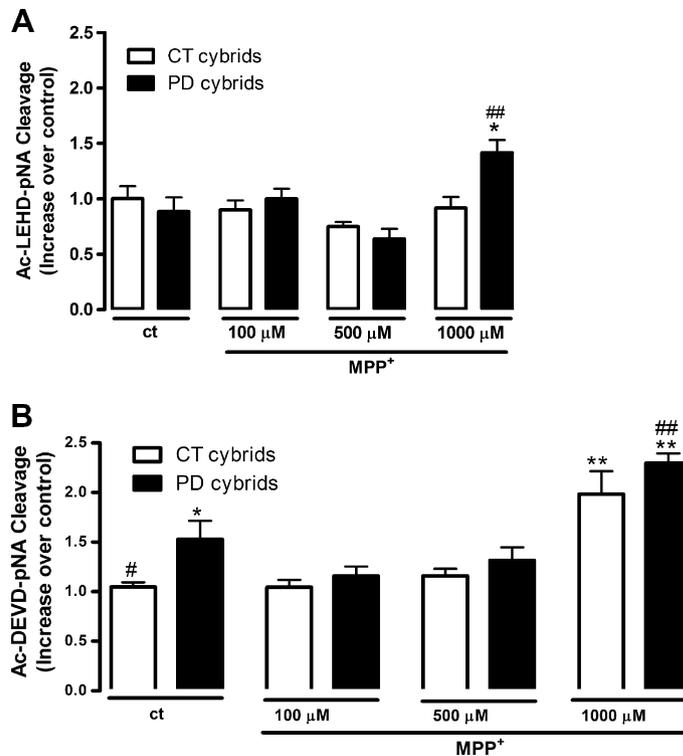


Fig. 4. Caspase 9-like and caspase 3-like activity in PD and CT cybrids. Cells were incubated in 0, 100, 500, or 1000  $\mu\text{M}$   $\text{MPP}^+$  over 24 h. Caspase activity was measured spectrophotometrically at 405 nm, as described in Section 2. (A) Caspase 9 activation was determined by Ac-LEHD-pNA cleavage. (B) Caspase 3 activation was determined by Ac-DEVD-pNA cleavage. Data represent mean  $\pm$  SEM values derived from six independent determinations. <sup>#</sup> $p < 0.05$ , <sup>##</sup> $p < 0.01$ , significantly different as compared to PD untreated cybrids; <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$ , significantly different as compared to CT untreated cybrids.

$\text{MPP}^+$  had a greater effect on CT cybrid than it did on PD cybrid MTT reduction capacity, LDH leakage, ATP levels, and mitochondrial membrane potential. Some of these endpoints were perturbed in PD cybrids even without  $\text{MPP}^+$  exposure, and these perturbations may have limited the amount of further change  $\text{MPP}^+$  could induce. It is interesting to note that in general,  $\text{MPP}^+$  caused CT cybrids and their mitochondria to resemble PD cybrids and their mitochondria. If mitochondrial function is truly relevant to PD pathogenesis, then these findings could help explain why MPTP causes Parkinsonism.

An intrinsic apoptotic cascade is associated with mitochondrial depolarisation, mitochondrial cytochrome *c* release, and caspase 9 activation. Our mitochondrial cytochrome *c* content, and caspase 9 activity data further suggest that when exposed to a mitochondrial toxin such as  $\text{MPP}^+$ , PD mitochondria are more likely than healthy mitochondria to activate the intrinsic apoptotic cascade. Our data extend the finding of Swerdlow and collaborators, showing that SH-SY5Y PD cybrids treated with  $\text{MPP}^+$  had a trend towards greater staining of apoptotic nuclei (Swerdlow et al., 1996). Substantial data from others argue environmental factors contribute to PD, conceivably by interfering with mitochondrial function. Persons with mitochondrial profiles similar to those observed in this study may be particularly susceptible to environmentally encountered mitochondrial toxins.

Data presented in this paper show evidences that allow us to propose a new unifying hypothesis for late onset, sporadic PD. Swerdlow and Khan previously proposed the “mitochondrial cascade hypothesis” for sporadic Alzheimer’s disease (AD), which postulates an upstream role for mitochondrial dysfunction in AD neurodegeneration (Swerdlow and Khan, 2004). This hypothesis is largely supported by data from cybrid models, as such models are produced through expression of mitochondrial genes from a non-degenerating tissue and therefore are unlikely to simply represent an artefact of tissue degradation. Based on data from this and prior studies of PD cybrids, we feel it is possible mitochondrial dysfunction in PD may represent a relatively upstream pathology and that a “mitochondrial cascade” paradigm may also apply in late-onset, sporadic PD as well.

Under our PD mitochondrial cascade hypothesis schema, inherited polymorphic mtDNA variation is postulated to influence age-related declines in mitochondrial function. This age-related mitochondrial dysfunction may itself arise through accumulation of somatic mtDNA mutations that decrease an individual’s mitochondrial ETC efficiency from an inherited set point. When a particular threshold of mitochondrial dysfunction is reached within the substantia nigra, neurodegeneration and PD-associated histopathologic changes result. In support of this view, Manfredi (2006) recently summarized data sug-

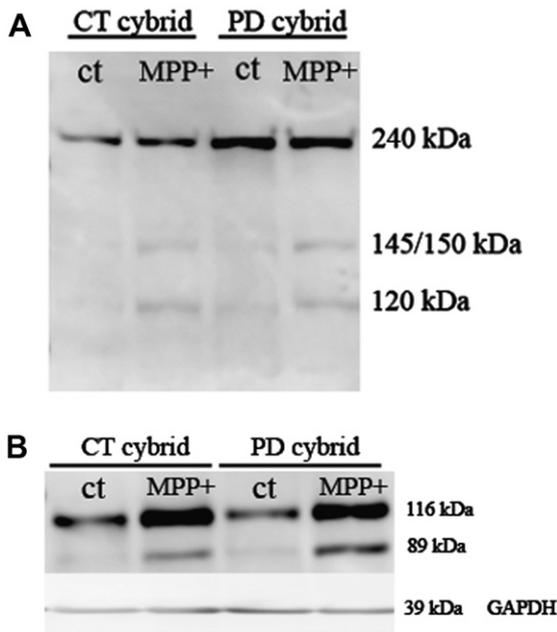


Fig. 5. Caspase 3 substrate cleavage. (A) Caspase 3-mediated  $\alpha$ -spectrin cleavage before and after 1000  $\mu$ M MPP<sup>+</sup> is shown. The Western blot in the figure represents results of an actual experiment. Each cybrid cell line was analysed three independent times. (B) Caspase 3-mediated PARP cleavage before and after 1000  $\mu$ M MPP<sup>+</sup> is shown. The Western blot in the figure represents results of an actual experiment. Each cybrid cell line was analysed three independent times.

gesting dopaminergic neurons of the substantia nigra accumulate high levels of mtDNA deletions. The extent of these deletions is arguably beyond the threshold required to cause respiratory chain mitochondrial dysfunction (Manfredi, 2006).

### Acknowledgement

We are grateful to Dr. Isabel Nunes Correia for cell culture support and to Dr. Luisa Cortes for Microscopy assistance.

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