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MITOCHONDRIAL FUNCTION IN PARKINSON'S DISEASE AND OTHER NEURODEGENERATIVE DISEASES

Thesis submitted for the degree of Doctor of Philosophy
in the Faculty of Science, University of London

MEI GU

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ABSTRACT

The cause of neuronal loss in the substantia nigra from Parkinson's disease (PD) brain is unknown. A deficiency of mitochondrial respiratory chain (MRC) complex I has been found in PD substantia nigra. Normal complex I function in multiple system atrophy suggested neither neuronal degeneration nor L-dopa therapy was the cause of this defect in PD. Normal MRC function in other specific brain areas from Alzheimer's disease (AD), dementia with Lewy bodies (DLB) and PD suggested that neither cholinergic cell loss nor the presence of Lewy bodies *per se* was associated with complex I deficiency.

The increased ApoE $\varepsilon 4$ allele frequency in AD and DLB was not observed in PD patients with dementia. These results suggested that the ApoE $\varepsilon 4$ allele influenced neither the development of Lewy bodies nor the dementia associated with PD, and that the risk factors for dementia in PD differed from that of AD and DLB at least with respect to ApoE.

In Huntington's disease (HD) caudate nucleus, severe defects of complexes II, III and complex IV activities of the MRC were demonstrated, supporting the role of abnormal energy metabolism in HD.

The feasibility of the platelet-A549ρ° cell fusion technique to study the involvement of mitochondrial DNA (mtDNA) in PD was demonstrated using platelets with the A3243G mtDNA mutation (MELAS). Platelets from seven PD patients with low complex I activity were fused with A549ρ° cells. Mixed cybrid analysis demonstrated a selective 25% deficiency of complex I activity. Furthermore, the analysis of 16 A549ρ°-PD fusion cybrid clones from one of the patients expressed complexes I (25%) and IV (20%) deficiencies. These results point to abnormal mtDNA as the underlying cause of the MRC dysfunction in at least a proportion of PD patients.

The MRC inhibitors piericidin A and antimycin A induced much greater levels of apoptosis in A549 than in A549ρ° cells implying apoptosis was induced via a mechanism that involved inhibition of the MRC, this contrasted with the toxic effects of rotenone which affected both cell types equally and therefore must be mediated via a pathway independent of the MRC.

Declaration of author's contribution

All of the experimental work was performed by the author. The content of the majority of this thesis has been published (see Publications).

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Abbreviations

AD	Alzheimer's disease
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CNS	central nervous system
CPEO	chronic progressive external ophthalmoplegia
CYP2D6	cytochrome P450 2D6 gene
DCPIP	6,6-dichlorophenolindophenol
ddH_2O	double distilled water
DLB	dementia with Lewy body disease
ΔmtDNA	deleted mitochondrial DNA
DMSO	dimethyl-sulfoxide
DNA	deoxyribonucleic acid
DPI^+	diphenyleneiodonium
DTNB	5-5'-dithiobis-nitrobenzoic acid
ε	molar extinction coefficient
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
$FADH_2$	flavin adenine dinucleotide (reduced)
Fe-S	iron-sulphur centre
GABA	gamma aminobutyric acid
FP	flavoprotein fraction of complex I
GSH	reduced glutathione
GSHpx	GSH peroxidase
GSSG	oxidised glutathione
GSSGrd	GSSG reductase
HBSS	Ca^{2+}-Mg^{2+}-free Hank's buffered saline solution
HD	Huntington's disease
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HP	hydrophobic fraction of complex I
hr	hour
6-OHDA	6-hydroxydopamine
IP	iron protein fraction of complex I

KSS	Kearns-sayre syndrome
LHON	Leber's hereditary optic neuropathy
MAO-B	monoamine oxidase B
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy and ragged-red fibres
MiMyCa	mitochondrial myopathy and cardiomyopathy
min	minute
$MPDP^+$	1-methyl-4-phenyl-dihydropyridine
MPP^+	1-methyl-4-phenyl-pyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine
MRC	Mitochondrial respiratory chain
MRS	magnetic resonance spectroscopy
MSA	multiple system atrophy
mtDNA	mitochondrial DNA
mtTERM	transcription termination signal
NADH	nicotine adenine dinucleotide (reduced)
NARP	neuropathy, ataxia and retinitis pigmentosa
nDNA	nuclear DNA
NMDA	N-methyl-D-aspartate acid
3-NP	3-nitropropionic acid
OPCA	olivopontocerebellar atrophy
PAGE	SDS-Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PEG	polyethyleneglycol
PET	positron emission topography
PI	propidium iodide
PM-delay	post-mortem delay
PMF	proton motive force
PMSF	phenylmethylsulfonyl fluoride
PRP	platelet-rich plasma
PSP	progressive supranuclear palsy

PUFA	polyunsatuated fatty acid
Q_1	ubiquinone-1
SDS	sodium dodecyl sulphate
sec	second
TCA	tricarboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TIQ	tetrahydroisoquinoline
$\Delta\psi_m$	transmembrane potential
Tris	tris(hydroxymethyl)aminomethane
TTFA	2-thenoyltrifluoroacetone
Tween 20	polyxyethylenesorbitan monolaurate
UV	ultraviolet

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CHAPTER 1 INTRODUCTION

1.1 Background of Parkinson's disease (PD)

1.1.1 Definition of Parkinson's disease

Nearly two hundred years have passed since Parkinson's disease (PD) was described by James Parkinson in his "An Essay on the Shaking Palsy" in 1817. PD is a progressive neurodegenerative disorder clinically characterised by resting tremor, loss of movement (akinesia) or extremely slowness of movement (bradykinesia), muscular rigidity and often postural instability. In the UK, it affects approximately 1 in every 1000 of the population, increasing to 5 in every 1000 in those aged over 50 years. It is one of the most common neurological disorder affecting the elderly.

1.1.2 Pathology of PD

The pathological hallmarks of PD include: (1) the loss of dopaminergic neurons in the substantia nigra (pars compacta), affecting those neurons projecting to the striatum with the consequent loss of dopamine in this motor and sensory integration and control centre; (2) the presence of Lewy bodies (intracytoplasmic inclusions) in surviving pigmented neurons of the substantia nigra; and (3) the loss of neurons in other brain areas *e.g.* substantia innominata (cholinergic) and locus coeruleus (adrenergic).

In 1960, reporters first showed that the striatal dopamine concentration was decreased in PD, which is the distinctive biochemical marker of PD (Ehringer and Hornykiewicz, 1960). This reflects degeneration of dopaminergic neurons in the substantia nigra. The loss of nigral neurons exceeds 60% in PD patients, and dopaminergic neurons may be virtually absent in patients with a long history and advanced disease. Indeed, clinical symptoms only develop when more than 70% of nigral neurons are lost and the levels of striatal dopamine are reduced by 80% (Marsden, 1990). The loss of the nigral neurons is accompanied by evidence of increased dopamine turnover in surviving neurons, reflected by an increased ratio of homovanillic acid to dopamine in the striatum, or an increased striatal dopamine receptor binding and supersensitivity.

Lewy bodies stain with haematoxylin and eosin showing a spherical lammellar eosinophilic structure with a dark centre, a surrounding halo and fibrillary processes radiating from the centre. Lewy bodies are composed of proteins including neuronal proteins (neurofilaments, tubulin), fatty acid and sphingomyelin (Roberts *et al.* 1993b). Although Lewy bodies are characteristic of PD and essential for a pathological diagnosis, they are also seen in about 5% of neurologically normal individual over 65 years, *e.g.* incidental Lewy body disease. Furthermore, Lewy bodies are not restricted to the monoaminergic neurons nor are they unique to PD, since they have been observed in brain stem and spinal motor neurons in amyotrophic lateral sclerosis and are also present in conditions such as cortical-basal ganglionic degeneration (Hallevorden-Spatz disease) (Roberts *et al.* 1993b). Lewy bodies are occasionally detected in other akinetic rigid syndromes such as multiple system atrophy (MSA) and Alzheimer's disease (AD), but are more commonly observed in dementia with Lewy body disease (DLB) (Roberts *et al.* 1993b).

1.1.3 Genetics of PD

The majority of cases of PD are sporadic. It was long thought to be caused by environmental factors, but there is increasing evidence that genetic factors may play an important role or interact with as yet unknown environmental factors. A large kindred with autosomal dominant PD has been reported (Golbe *et al.* 1990), suggesting that a single gene without additional environmental insults can cause the clinical and pathological changes typically observed in PD. Duvoisin and Jonhson (1992) reported that 41 members of this family were affected with PD and 2 cases had typical Lewy body PD. The screening of this large Italian family including more than 500 members over 11 generations has recently been completed (Polymeropoulos *et al.* 1996). They confirmed the significant familial aggregation of PD and the genetic marker on chromosomal 4q21-q23 was the only one to show linkage to the PD phenotype. This fundamental finding of a highly penetrant genetic locus linked to PD suggested that abnormalities of a single gene may be sufficient to cause the PD phenotype, and also lead to identification of the gene and research on the pathogenesis of PD. Recently, in this same Italian kindred and in three

unrelated families of Greek origin with autosomal dominant inheritance of PD, a mutation was identified by the same group of researchers in the α-synuclein gene which codes for a presynaptic protein thought to be involved in neuronal plasticity, (Polymeropoulos *et al.* 1997). This single base pair change at position 209 (G209A) results in a substitution at position 53 (Ala53Thr) (Polymeropoulos *et al.* 1997). However, mutations of α-synuclein gene have not been found in 70 sporadic PD (Warner and Schapira, 1998).

An additional mutation mapped on the chromosome 2p13 has been described in a group of families of European origin which appears to be involved in the development of parkinsonism closely resembling sporadic PD including a similar mean age of onset (Gasser *et al.* 1998). Another mutation mapped to the long arm of chromosome 6 (6q25.2q27) has been recently reported in several unrelated Japanese families with autosomal recessive juvenile Parkinson disease (AR-JP) (Kitada *et al.* 1998). By positional cloning within this microdeletion, a complementary DNA clone of 2960 base pairs has been obtained, which encoding a protein of 465 amino acids with moderate similarity to ubiquitin at the amino terminus and a RING-finger motif at the carboxyl terminus (Kitada *et al.* 1998). The mutations in this newly identified gene appear to be responsible for the pathogenesis of AR-JP, therefore this gene has been named the "parkin gene" by the reporters (Kitada *et al.* 1998).

The use of twins is one of the best models to identify whether genetic factors play a role in the cause of PD. However, the reports from different twin studies are contradictory. In one twin-study (Ward *et al.* 1983), it was reported that among 43 monozygotic and 19 dizygotic twin pairs, only one of the 43 pairs of monozygotic twins was definitely concordant for PD. The frequency of PD in monozygotic co-twins of cases with PD was similar to that expected in an unrelated control group matched for age and gender. It was concluded that genetics do not play a major role in the aetiology of PD. This finding was supported by Marsden *et al.*, who reported that the prevalence of PD in the co-twin was no greater amongst 11 identical twin-pairs than amongst 11 non-identical twin-pairs in the UK (Marsden, 1987). Similar results were reported (Marttila *et al.* 1988) in 41 twins (18 monozygotic and 14 dizygotic pairs) in

Finland. Therefore, they suggested that PD may be an acquired disease not caused by hereditary processes.

Others have implied that maternal inheritance may play a part in PD at least in some families. Wooten *et al.* (1997) found that the age of onset of PD in 5 families whose mothers were affected, was younger than the age at onset in the parental generation. Similar results were reported previously (Lazzarini *et al.* 1994; Payami *et al.* 1995). These data support the suggestion that both genetic factors and genetic anticipation play a role in at least some patients with PD. However, Maraganore *et al.* (1991) reported that their data did not support the possibilities of either maternal (mitochondrial) or polygenic inheritance in familial PD, although the latter cannot be excluded. Therefore, the role of genetic factors in PD remains unclear.

A number of mutations/polymorphisms have been reported which may suggest the involvement of susceptibility genes in PD. These include polymorphism in the cytochrome p450 debrisoquine hydroxylase (CYP2D6) gene (Barbeau *et al.* 1985); monoamine oxidase B (MAO-B) gene (Sturman *et al.* 1990).

The function of the CYP2D6 gene encoded protein helps protect against toxic environmental compounds by hydrogenation. Individual variations in the CYP2D6 gene might influence susceptibility to environmental linked diseases. CYP2D6 gene function in PD was first reported by Barbeau *et al.* (1985). They studied 40 PD patients and 40 controls and found that the incidence of partially or totally defective CYP2D6 enzyme activity was significantly greater in PD than controls subjects (Barbeau *et al.* 1985). In 1992, there were two groups of researchers classified individuals as poor metabolises of debrisoquine if they were homozygous for either a G→A transition at the intron 3/exon 4 junction, a base pair deletion within exon 5 or a whole gene deletion of the CYP2D6 gene. Individuals with a CYP2D6 poor metabolises genotype had a 2.54-fold increased risk of PD (Smith *et al.* 1992). Others reported that the CYP2D6 poor metabolised genotype was twice as common in patients with PD as in controls (Armstrong *et al.* 1992). However, it has been reported that there was no association between CYP2D6 polymorphism and IPD, although

the frequency of CYP2D6 polymorphism was significantly higher in Caucasians compared with Japanese (Nicholl *et al.* 1995). More recently, others (Maeda *et al.* 1997; Lima *et al.* 1997) reported that there were not a statistically significant difference in the various CYP2D6 alleles between 63 PD and 51 controls and suggested that a mutation at CYP2D6 locus is not the major genetic determinant of PD.

Monoamine oxidase (MAO-B) exists in at least two forms designated as type A and B. Both MAO-A and B are enzymes primarily responsible for the metabolism of amine neurotransmitters, such as dopamine, norepinephrine, and serotonin. Serotonin is predominantly deaminated by MAO-A, whereas MAO-B has a higher affinity for dopamine. The MAO-A and B genes are located next to each other in the p11.3-11.4 region of the human X chromosome. A single-stranded conformational polymorphism in the MAO-B gene was shown to be A or G, 36 bases upstream from the intron13-exon14 boundary and an allelic association study revealed that there was no significant associations between this single-base polymorphism and PD (Ho *et al.* 1995). One particular haplotype at MAO-A was three times more frequent in PD patients as compared with control, but no associations were observed between individual MAO-B alleles and the disease state (Hotamisligil *et al.* 1994). These findings supported the suggestion that polymorphism of MAO genes may be among the hereditary factors that influence susceptibility of individuals to PD.

The search for susceptibility genes in PD is complicated because the aetiology of PD may involve genetic or environmental factors, or a combination of both. The interaction of a number of genes and environmental factors may contribute equally to the aetiology of PD and equally various factors may be differentially important in the cause of the disease in different individuals.

1.1.4 Environmental toxicity in PD

In the absence of a clear genetic cause of PD, other factors must be involved. Several studies support the involvement of environmental agents in the cause of PD.

Several demographic factors including lifetime histories of places of residence, source of drinking water, and occupations such as farming were taken into account. Rural living and drinking well water were significant factors in the PD patients, but there were no significant differences between PD patients and controls for farming or exposure to herbicides or pesticide (Koller *et al.* 1990). These data provide further evidence that an environmental toxin could be involved in the aetiology of PD.

1.1.4.1 MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)

Neurotoxins have intrigued scientists investigating the aetiology of PD for some time. For many years, the best model of PD has been that of MPTP-induced parkinsonism. MPTP was inadvertently discovered, as it was mistakenly injected by heroin addicts as a contaminant of a narcotic drug in northern California in 1982 (Langston *et al.* 1983). A number of young people who took the drug rapidly developed a severe, permanent parkinsonian syndrome, including the rest tremor, rigidity and akinesia, which responded to L-dopa therapy. Pathological studies in one of these patients revealed neuronal loss limited to the substantia nigra (Langston *et al.* 1983).

The MPTP animal model has helped our understanding of the neuropathological features of PD, although its effects are limited to nigral degeneration and corresponding lose of dopamine in the caudate and putamen. MPTP also induced degeneration in the locus coeruleus in five out of six monkeys (Mitchell *et al.* 1985; Forno *et al.* 1986). Lewy body formation is an important neuropathological feature of PD. However, such pathology was absent in the MPTP-induced parkinsonism animal model, although ageing monkeys treated with MPTP developed cytoinclusions resembling immature Lewy bodies (Forno *et al.* 1986).

The mechanism of MPTP induced dopaminergic cell death

The precise molecular mechanisms that bring about cell death after MPTP administration are not well understood, and two major hypotheses have arisen. It has been postulated that MPTP-induced cell death may be caused by either

decreased ATP synthesis or increased free radical production (Adams *et al.* 1996) (see later section 1.3.3.1 and section 1.5.2).

MPTP readily crosses the blood brain barrier where it is converted to MPP^+ via an intermediate 1-methyl-4-phenyl-dihydropyridine ($MPDP^+$) and expresses its neurotoxicity in the brain. The first step of this reaction, MPTP to $MPDP^+$, is mediated by MAO-B (Chiba *et al.* 1984) which is particularly active in astrocytes in catecholaminergic regions of the brain where it is located on the outer mitochondrial membrane. MAO-B inhibitors block this transition in rat brain mitochondria *in vitro* (Chiba *et al.* 1984) and *in vivo* (Langston *et al.* 1984), and prevent parkinsonism and nigral cell death in primates (Langston *et al.* 1984) as well as dopamine depletion in rodents induced by MPTP (Markey *et al.* 1984). However, there was a report that overexpressing neuronal levels of MAO-B in transgenic mice did not cause increased sensitivity to MPTP, and therefore neither conversion of MPTP to its active form MPP^+ by MAO-B nor MPP^+ uptake by the dopaminergic transporter are likely to be the rate-limiting step in the toxicity of MAO-B (Andersen *et al.* 1994) (Fig. 1.1).

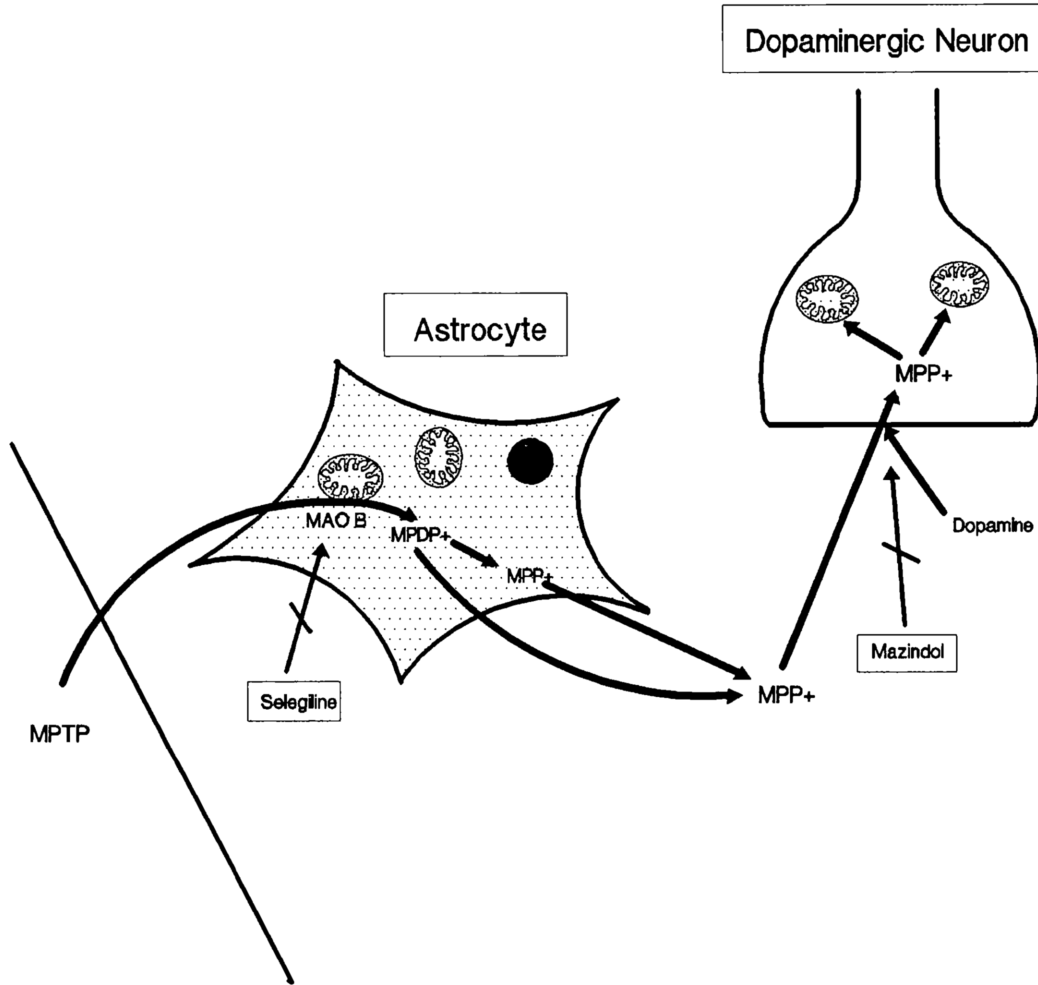


Fig. 1.1 The conversion of MPTP to MPP^+ and its uptake by dopaminergic neurons. Reproduced by kind permission of Dr. J. M. Cooper.

1.1.4.2 Other environmental toxins

Iron

Injection of iron into the substantia nigra of rodents resulted in the loss of neurons in this region and decreased striatal dopamine levels which suggested that iron overloading may be involved in the neuronal cells loss in brains (Ben-Shachar *et al.* 1991). Increased iron concentrations may stop PC12 cells dividing and cause a decrease in the activities of complex I and IV (Hartley *et al.* 1994).

Manganese

Manganese has been suggested to contribute to the aetiology of PD. The clinical symptoms of manganese toxicity are similar to those of PD, although unlike idiopathic PD patients, affected individuals do not develop the on-off fluctuations and dyskinesia (Huang *et al.* 1993). The observation that manganese-induced encephalopathy can result in parkinsonism which with marked damage to the globus pallidus and relatively little destruction of the substantia nigra and was not L-dopa responsive, suggested that manganese induced parkinsonism is distinct from both idiopathic PD and MPTP induced parkinsonism.

Isoquinolines

Isoquinolines (IQs) are structurally related to MPTP and can be formed within the brain by the condensation of biogenic amines, including dopamine, with an aldehyde. Unlike MPTP, Isoquinoline has been shown to be present in human brain (Niwa *et al.* 1987) whereas MPP^+ has not (Irwin and Langston, 1993) and the selective toxicity of isoquinoline to dopaminergic neurons indicates that it is a potential candidate in PD. For example, long term administration of tetrahydroisoquinolines to monkeys produced a parkinsonian syndrome causing decreased dopamine levels and decreased tyrosine hydroxylase activity in the nigral region (Yoshida *et al.* 1990). The administration of IQs to primates (Nagatsu and Yoshida, 1988) and rats (Naoi *et al.* 1996) induced parkinsonism with the depletion of striatal dopamine. Thus, these potential parkinsonian-inducing neurotoxins may be more appropriate agents to employ

in the search for the aetiology of PD. Indeed, IQs specifically inhibit complex I in isolated rat brain mitochondria, showing a similar mechanism of action to MPP^+ (McNaught *et al.* 1995) (see section 1.3.2.1).

In vitro studies showed that a variety of isoquinoline derivative compounds are toxic to PC12 cells (McNaught *et al.* 1996) with their toxicity reflecting their affinity for the dopamine transporter suggesting their ability to concentrate into the neurons is more important than their ability to inhibit mitochondrial function (McNaught *et al.* 1996)

β-carbolines

β-carbolines are potential neurotoxins which have been detected in human brains (Matsubara *et al.* 1993). These compounds can be produced by the condensation of tryptophan and aldehydes. β-carboline is a substrate for the dopamine reuptake system of dopaminergic neurons (Drucker *et al.* 1990). There is some doubt as to whether β-carbolines directly inhibit MRC activities. β-carboline has been shown to be a stronger inhibitor of NADH-linked mitochondrial respiration than MPP^+ (Collins et *al.* 1992). β-carbolines have also been reported to inhibit the activities of complexes I and II (Fields *et al.* 1992). However other reports suggested that β-carbolines preferentially inhibited NADH-linked substrate oxidation (Krueger *et al.* 1993). Therefore, β-carbolines were more likely acting indirectly upon the MRC, *i.e.* affecting substrate transport.

1.1.5 Treatment of PD

1.1.5.1 L-Dopa

The discovery of the degeneration of substantia nigra dopaminergic neurons in PD brain led to a new area of treatment of the disease with the metabolic precursor of dopamine, L-dopa. L-dopa is the most effective therapy for PD patients, particularly ameliorating akinesia and bradykinesia, as well as improving rigidity and tremor. In the early stages of PD, L-dopa is often dramatically effective in reversing the symptoms but within 3-5 years of starting the treatment most patients develop side-effects such as dyskinesia, dystonic reactions, and psychotic symptoms. Moreover, L-dopa does not halt

the progression of the disease and dopaminergic neurons continue to die. However the cause of the complications is poorly understood.

Orally administered L-dopa is metabolised to dopamine by the enzyme dopa decarboxylase. The combination of L-dopa with peripheral decarboxylase inhibitors such as carbidopa can minimise the side-effects arising from L-dopa metabolism in peripheral tissues and increases L-dopa availability to cross the blood-brain barrier.

The potentially toxic metabolites of dopamine , such as 6-hydroxydopamine (6-OHDA), can impair the nigral protective mechanisms (decreased nigral glutathione, glutathione peroxidase and catalase) and increase generation of free radicals in the nigra (increased nigral iron with decreased ferritin and increased nigral lipid peroxidation) (Roberts *et al.* 1993c). However, there is no clear evidence that long-term use causes or accelerates dopaminergic neurone death in patients.

1.1.5.2 Selegiline (L-Deprenyl)

Selegiline is a selective irreversible MAO-B inhibitor, and acts by decreasing the metabolism of dopamine, thereby indirectly increasing the concentration of dopamine in the brain. Evidence from a number of studies in experimental animals has shown that inhibition of MAO-B by selegiline confers protection against the damaging effects of several neurotoxins, including MPTP and 6-OHDA (Rinne *et al.* 1991).

Clinical trials have suggested that preservation of nigral neurons was greater in PD patients treated with selegiline in combination with L-dopa than in those with L-dopa alone (Rinne *et al.* 1991) and a recent study has suggested that selegiline delayed the need for L-dopa therapy and slowed the progression of PD signs and symptoms in newly diagnosed, untreated PD patients (Parkinson study group, 1993).

1.2 Other neurodegenerative diseases

1.2.1 Multiple system atrophy (MSA)

1.2.1.1 Definition of MSA

MSA is a pathological entity distinct from idiopathic PD (IPD) which is responsible for 5-10% of patients with parkinsonism and represents the most common cause of parkinsonism after PD (Quinn, 1995). MSA includes the Shy-Drager syndrome, striatonigral degeneration, olivopontocerebellar atrophy (OPCA) and progressive supranuclear palsy (PSP) (Roberts *et al.* 1993c). Most MSA cases are sporadic, but familial forms of OPCA can occur and show an autosomal dominant mode of inheritance.

1.2.1.2 Pathology of MSA

Pathological changes are common to all forms of MSA, including loss of nigral, striatal, pallidal, brain stem and cerebellar neurons with gliosis, and white matter degeneration (Papp *et al.* 1989). Some MSA patients may respond well to treatment with L-dopa and patients with these features are often initially diagnosed as having IPD. Correct diagnosis may be made only some years later when cerebellar and brain stem atrophy become apparent .

1.2.1.3 Aetiology of MSA

The cause of neuronal cell death in MSA remains unknown but MSA and PD share some features, especially progressive nigral dopaminergic cell loss, suggesting the possibility of similar mechanisms of cell death.

Deficiency of glutamate dehydrogenase activity has been reported in some individuals with the OPCA form of MSA (Plaitakis *et al.* 1984). It has been suggested that a deficiency of glutamate dehydrogenase would allow toxic levels of glutamate to accumulate in nervous tissue thus causing cell death (Plaitakis *et al.* 1982).

1.2.2 Alzheimer's disease (AD)

1.2.2.1 Definition of AD

AD is a progressive neurodegenerative disorder characterised by an insidious onset with dementia in the absence of other systemic causes or CNS disease,

which would account for the progressive cognitive deficit and personality change (Burns, 1991). The neuropathological characteristics of AD include extensive neuronal cell loss and formation of neurofibrillary tangles and amyloid plaques in the cortex. The neuronal damage and loss normally approaches 25% of the neurons in the cortex (Roberts *et al.* 1993a). More specific cell loss in discrete subcortical nuclei is also associated with dementia.

1.2.2.2 Pathology of AD

Senile plaques and neurofibrillary tangles are the two major pathological features observed in the AD brain. There are three characteristic sites of abnormal fibrous protein deposits (amyloid) within the AD brain: (1) extracellular senile plaques in the neuropil; (2) intraneuronal neurofibrillary tangles; and (3) deposits of amyloid in the walls of cerebral blood vessels (Hardy and Allsop, 1991). Plaques are roughly spherical structures and are usually located in the cerebral cortex. These plaques are the first and also the most abundant types of pathology seen in the AD brain. The tangles appear as thickened, twisted fibrils which may occupy a large part of the cytoplasm of neurons. They may disrupt protein synthesis and cellular processes, and neurons do not survive indefinitely with these large intracellular inclusions. The precise protein composition of tangles is unknown but one of most important components is an abnormally phosphorylated form of tau proteins.

The relationship between plaques and tangles is not clear, but tangles may represent a response to the local toxic effect of plaques.

1.2.2.3 Genetics of AD

Many cases of autosomal dominant inherited forms of early onset AD are caused by mutations in the genes encoding amyloid precursor protein (APP, chromosome 21q), presenilin 1 (PS1, chromosome 14q) and presenilin 2 (PS2, chromosome 1q). A polymorphism, apoplipoprotein E gene (apoE, chromosome 19q) has also been linked to the pathogenesis of AD.

APP

A proportion of early onset familial AD have been shown to have point mutations in the APP gene (Goate *et al.* 1991). Although calculations indicated that these mutations account for less than 0.01% of all AD cases, the finding that the APP gene mutations were tightly linked to these AD families was a hallmark in the understanding of the cause of this disorder. The fact that an APP gene mutation can give rise to all of the neuropathological features strongly suggests that amyloid deposition is a primary, or at least a seminal, event in the pathogenesis of all cases of AD (Roberts *et al.* 1993a). This proposal was strongly supported by the observation that transgenic mice, produced by overexpression of APP constructs containing the human APP gene carrying the AD mutation, showed deposits of β-amyloid protein similar to those seen in the human brain (Games *et al.* 1995).

Presenilins (PSs)

The PS1 gene was identified by a purely genetic approach. Linkage of early-onset familial AD to chromosome 14q locus was found in the majority of published AD pedigrees (Levy-Lahad and Bird, 1996). The PS1 gene is predicted to encode for a protein containing 467 amino acids. Nearly all PS1 gene mutations are missense mutations, which result in a single amino acid substitution rather than premature termination or a truncation of the protein. This suggests that PS1 mutations most likely cause AD by change or gain in the protein's function rather than by loss of function (Levy-Lahad and Bird, 1996).

The PS2 gene was identified by studying a group of 8 pedigrees in Volga Germans in which familial AD was a result of a founder effect (Levy-Lahad *et al.* 1995). The PS2 gene was predicted to encode a 448 amino acid protein that was 67% identical to PS1. A single mutation segregated in all the Volga Germans pedigrees that had strong linkage to chromosome 1. The PS2 mutations were also missense mutations, similar to PS1.

PSs are expressed in neurons throughout the nervous system, with differences in abundance among cell populations. PS1 and PS2 each have

6-8 transmembrane domains and are localized mainly in the endoplasmic reticulumn of cell bodies and dendrites (Mattson and Guo, 1997).

The normal function of PSs is unknown. There is evidence supporting their role in membrane trafficking, APP processing and regulation of the endoplasmic reticulumn calcium homeostasis (Mattson *et al.* 1998).

Apoplipoprotein E (apoE) gene

The apoE gene was identified as a risk factor, or susceptibility locus for AD by genetic analysis of late-onset familial pedigrees (see section 1.2.6 for more detail).

1.2.3 Dementia with Lewy body disease (DLB)

1.2.3.1 Definition of DLB

DLB is characterised by the presence of motor and psychiatric symptoms, the presence of Lewy bodies in cortical neurons and the sharing of some pathological features of both AD and PD.

Extensive examination of the neuropathology of AD has shown that as many as 20% of cases have Lewy bodies. These observation are judged to be of a separate category termed, DLB which has also been called cortical Lewy body disease or diffuse Lewy body disease. Whether DLB is a discrete disease entity is still under consideration since it has been regarded as a variant of AD (Hansen *et al.* 1990).

Ultrastructural studies show that the filaments of cortical Lewy bodies are randomly arranged. They are almost always found within neurons and it is rare to find them lying free in the neuropil. This is in contrast to tangles, which are thought to disrupt protein synthesis and cellular processes and neurons containing these large intracellular inclusions do not survive. Plaques containing β-amyloid seen in AD are also found in all DLB cases and approximately 30-40% of DLB cases will also show significant numbers of tangles in the cortex.

1.2.3.2 Comparison of DLB with AD

The aetiology of DLB is not known. At present, the only factor to be clearly linked to the occurrence of DLB is the β-amyloid expression. AD and DLB cases have been reported to contain very similar amounts of β-amyloid in the medial temporal lobes and both contain increased APP compared to controls (Gentleman *et al.* 1992). This may suggest that DLB is a morphological variant of AD.

1.2.3.3 Comparison of DLB with IPD

Cell loss in the substantia nigra is present in all cases of DLB with classical Lewy bodies in the remaining neurons. These changes are indistinguishable from those cases with IPD. Approximately 40% of DLB cases exhibit the symptoms and signs which are typical of IPD (see Table 1.2.1 for summary of PD, AD and DLB pathological features).

Table 1.2.1 Distributions of Lewy bodies, plaques and tangles in substantia nigra and cortex of PD, AD and DLB

	SN	Cortex
PD		
Lewy bodies	(+++)	(-)
plaque	(±)	(-)
tangle	(±)	(-)
cell loss	(+++)	(-)
AD		
Lewy bodies	(-)	(±)
plaque	(-)	(+++)
tangle	(-)	(+++)
cell loss	(-)	(±)
DLB		
Lewy bodies	(-)	(+++)
plaque	(-)	(++)
tangle	(-)	(++)
cell loss	(++)	

(+++): strong;(++): moderate; (±): occasional; (-): none

1.2.4 Huntington's disease (HD)

1.2.4.1 Definition of HD

HD is a neurodegenerative disorder with autosomal-dominant inheritance. Clinically, the disease presents with chorea and cognitive impairment in young

adults. Pathologically HD is characterised by severe neuronal loss, gliosis, and atrophy of the neostriatum and pars reticulata of the substantia nigra (Roberts *et al.* 1993d).

Chorea is a form of involuntary movements typified by brief muscle jerks and affects virtually all muscle in HD patients. Cognitive impairment may be present when the patients present with chorea. As the disease progresses, the severity of chorea may diminish, whilst dystonic and parkinsonian features and abnormal eye movements become obvious. The average age of death of HD patients is 51-55 years (Roberts *et al.* 1993d).

1.2.4.2 Pathology of HD

Histopathological changes in the neostriatum include loss of neurons and gliosis. Neuronal degeneration begins in the tail of the caudate nucleus, and progresses in the medial to lateral areas. There is relative selective neuronal loss within HD brain with marked depletion of median spiny neurons and relative preservation of cholinergic interneurons and medium-size aspiny neurons in which neuro-peptide Y, somatostatin, and NADPH-diaphorase (NOS) are co-localised. The depletion of the GABA-synthetic enzyme, glutamate decarboxylase, is the most striking neurochemical abnormality in HD (Roberts *et al.* 1993d)

1.2.4.3 Genetics of HD

Genetic linkage analysis has indicated that a single locus is involved in HD pathogenesis. Linkage was first established by Gusella and co-workers using a linked polymorphic marker (Gusella *et al.* 1983). The mutant gene (IT15) has been identified on the short arm of chromosome 4, encoding a 348kD protein product (huntingtin) with unknown function.

The IT15 gene contains a CAG repeat which is highly polymorphic in the general population, with more than 99% people having less than 30 repeats. However, almost all patients with HD (>99%) have more than 36 repeats with a median of 44 CAG repeats (Kremer *et al.* 1994). Thus, CAG expansion within the HD gene is a specific marker for the inheritance of HD. The IT15

gene is widely expressed throughout the body. Within the brain, the gene is expressed in tissue of both neuronal and glial origin and it is not enriched in the basal ganglia (Strong *et al.* 1993).

A number of studies have shown that there is an inverse correlation between the CAG repeat size and the age of onset of HD, *i.e.* longer repeats are associated with an earlier age of onset (Duyao *et al.* 1993; Andrew *et al.* 1993). CAG repeat lengths associated with disease chromosomes show a predisposition to increase in length from one generation to the next, particularly when the disease is transmitted from the father which explains why patients with early onset HD more often inherit the disease from their father (Telenius *et al.* 1993).

Studies have shown that the HD gene is fully penetrant. Homozygous for the HD gene mutation are phenotypically identical to the heterozygots (Wexler *et al.* 1987). However, little is known as to how the expanded CAG results in HD. It has been shown that the expanded CAG trinucleotide is translated into an expanded polyglutamine tract presumably resulting in a gain-of-function for the HD protein, leading to neuronal cell death which is a feature of HD (Ross, 1995).

1.2.4.4 Biochemical features of HD

The biochemical basis of HD remains unclear, although several studies suggest that abnormal MRC function may be involved (see section 1.3.4 for details). A positron emission topography (PET) study has shown that patients with symptomatic HD had reduced glucose metabolism in the caudate nucleus implying that deficient energy metabolism may be involved in neuronal death (Beal, 1992). A recent study demonstrated elevated occipital cortex lactate levels in HD brain and the magnetic resonance spectroscopy (MRS) study of resting muscle in HD patients showed elevated inorganic phosphate levels suggesting defective oxidative phosphorylation (Koroshetz *et al.* 1997). However, these results were not confirmed by Huoang *et al.* who reported that there were no increase in cerebral lactate or decrease in phosphocreatine and

ATP, therefore energy metabolism was normal in the 4 cerebral locations measured by MRS (Hoang *et al.* 1998).

Excitotoxicity has been proposed as an important factor in neuronal death in HD, although it is unclear what role mutant huntingtin plays in this process. One study demonstrated increased glutamate levels in HD caudate, supporting the hypothesis that glutamate toxicity may be important (Taylor-Robinson *et al.* 1994). Agents that block glutamate receptors may prevent the progression of the disease. The CAG repeat encodes a polyglutamine stretch which has been shown to increase huntingtin binding to proteins including huntingtin-associated proteins and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Li *et al.* 1995; Burke *et al.* 1996), a feature which could determine the selectivity of neuronal cell death in HD. Mutant huntingtin could interact with specific proteins making the neurones more susceptible to excytotoxicity thus leading to a variety of effects including inhibition of MRC functions and cell death (Beal, 1992).

1.2.5 Dystonia

Dystonia is a movement disorder caused by the degeneration of the basal ganglia. Idiopathic torsion dystonia (ITD) is the commonest form of primary dystonia. Genetic linkage analysis has suggested that the gene mapped to 9q34 (DYT1) (Ozelius *et al.* 1989) is responsible for the diseases in the majority of patients with childhood and generalized ITD (Warner *et al.* 1993). Focal ITD is usually of adult onset and sporadic, and to date there is no genetic linkage in focal ITD.

1.2.6 Apolipoprotein E (ApoE) and neurodegeneration

ApoE is a single glycosylated 37kDa protein containing 299 amino acids and is synthesized in a variety of different tissues including liver (Hixson and Vernier, 1990). The major function of ApoE is as a carrier for both the low density lipoprotein receptor and the hepatic E receptor. Therefore, ApoE plays a important role in determining the metabolic fate of circulating lipoproteins, especially the hepatic fate of cholesterol-enriched species (Hixson and Vernier, 1990). The human apoE gene is on the long arm of chromosome 19

and contains 4 exons (Das *et al.* 1985). Three apoE alleles, apoE ε2, ε3 and ε4 differ from each other by a single amino acid at codons 112 and 158 of the apoE gene, giving rise to the 3 homozygous phenotypes (ε2/2, ε3/3 and ε4/4) and 3 heterozygous phenotypes (ε2/3, ε2/4 and ε3/4) (Utermann *et al.* 1980). The 3 alleles differing by a single unit of net charge (Table 1.2.2) can be detected by isoelectrofocusing.

Table 1.2.2 ApoE polymorphisms and biochemical properties

genotype			**ε4**	**ε3**	**ε2**
phenotype		relative charge	isoelectronic profile (-)		(+)
homozygotes	ε4/4	+2	•		
	ε3/3	+1		•	
	ε2/2	0			•
heterozygotes	ε4/3		•	•	
	ε4/2		•		•
	ε3/2			•	•
protein coded by each allele					
site 112			ARG	CYS	CYS
site 158			ARG	ARG	CYS

1.2.6.1 ApoE and AD

The apoE protein has been shown to be a component of both plaques and tangles (Wisniewski and Frangione, 1992), which are hallmarks of AD. The ApoE ε4 allele is a well-established risk factor for AD and is associated with both late-onset familial AD and sporadic AD (Strittmatter *et al.* 1993; Corder *et al.* 1993). It has been suggested that apoE protein binds avidly to plaques and tangles in AD brain, which may be mediated by β-amyloid and tau protein, respectively the main protein components of plaques and neurofibrillary tangles (Richey *et al.* 1995). The ApoE ε4 binds more avidly to β-amyloid than ε3 that it may encourage more plaque formation (Richey *et al.* 1995).

1.2.6.2 ApoE and DLB

There is a strong clinical and pathological overlap between AD and DLB (see section 1.2.3). Indeed in both AD and DLB, the ε4 frequency was significantly

higher than that observed in non-demented controls, which provided further evidence that AD and DLB share apoE ε4 as a genetic risk factor (Galasko *et al.* 1994).

1.2.6.3 ApoE and PD

PD is one of the most common cause of dementia in the elderly (Kokmen *et al.* 1988). PD shares some features with DLB, such as Lewy bodies, and progressive dementia is a common feature in PD, AD and DLB. However, there is conflicting evidence concerning the overrepresentation of apoE ε4 allele in these three diseases. There was a report suggested that ε4 allele frequency was increased in 35 AD and 22 DLB patients, but not in 23 PD compared to 55 controls. Since DLB and PD appear to differ genetically in relation to ε4 allele frequency, this suggests the cause of Lewy body formation in these two disorders is not related to apoE genotype (Benjamin *et al.* 1994). Others reported that there was a significant increase in the frequency of apoE ε4 in 14 PD patients with dementia compare with 49 PD patients without dementia, and therefore the apoE ε4 allele may be a risk factor for dementia in PD and therefore may have a genetic background closely related to that seen in AD (Arai *et al.* 1994b). However, Marder *et al.* reported that there was no significant difference in the frequency of apoE ε4 allele between 22 demented PD and 57 nondemented PD patients, suggesting that there was no association between apoE ε4 and dementia in PD patients. Therefore they concluded that the genetic basis for dementia in PD may differ from that of AD (Marder *et al.* 1994).

1.3 Mitochondrial respiratory chain function

1.3.1 The mitochondrion

Mitochondria are intracellular organelles present in the cytoplasm of aerobic cells. They play a major role in cellular energy supply, as their main function is the conversion of energy derived from the oxidation of substrates to cellular. ATP synthesis. Structurally, mitochondria consist of four regions, the outer and inner membranes and two spatial regions delineated by these membranes, namely the inter membrane space and matrix. These structural regions are

associated with specific and different functions. The inner membrane and matrix are associated with most of the functional activities of mitochondria.

1.3.2 Mitochondrial respiratory chain (MRC)

One of the main functions of mitochondria is the oxidative phosphorylation of ADP to ATP. The MRC catalyses the oxidation of NADH and $FADH_2$, which are the reduced products from dicarboxylic, tricarboxylic and fatty acid oxidation by the enzymes of the tricarboxylic acid (TCA) cycle and β-oxidation, respectively.

Structurally, the MRC consists of 4 multi-subunit enzyme complexes which are situated in the inner mitochondrial membrane: NADH-ubiquinone reductase (complex I); succinate-ubiquinone reductase (complex II); ubiquinol-cytochrome c reductase (complex III); and cytochrome c oxidase (complex IV): and two mobile electron carriers: ubiquinone and cytochrome c. The first four complexes form the electron transport chain (ETC). Together with complex V (ATPase) they constitute the components of the oxidative phosphorylation system (Fig .1.2).

NADH is oxidised by complex I, but $FADH_2$ is oxidised by complex II or the electron-transferring flavoprotein. The reducing equivalents are transported via a series of electron carriers to the terminal electron acceptor, oxygen. As electrons are transferred there is a decrease in electropotential which is conserved in the translocation of protons (H^+) across the inner membrane from the matrix to the outside space by complex I, complex III and complex IV, thus generating a proton motive force (PMF). The PMF is used for a variety of functions including the transport of charged molecules (proteins, carboxylic acids and iron) and drives mitochondrial ATPase to phosphorylate ADP to ATP. Together the MRC and ATPase constitute the components of the ATP-synthesising machinery. The important of this system to cellular ATP generation is emphasised by the fact that mitochondrial oxidative phosphorylation is much more efficient (36 mol ATP/mol glucose) than anaerobic glycolysis (2 mol ATP/mol glucose).

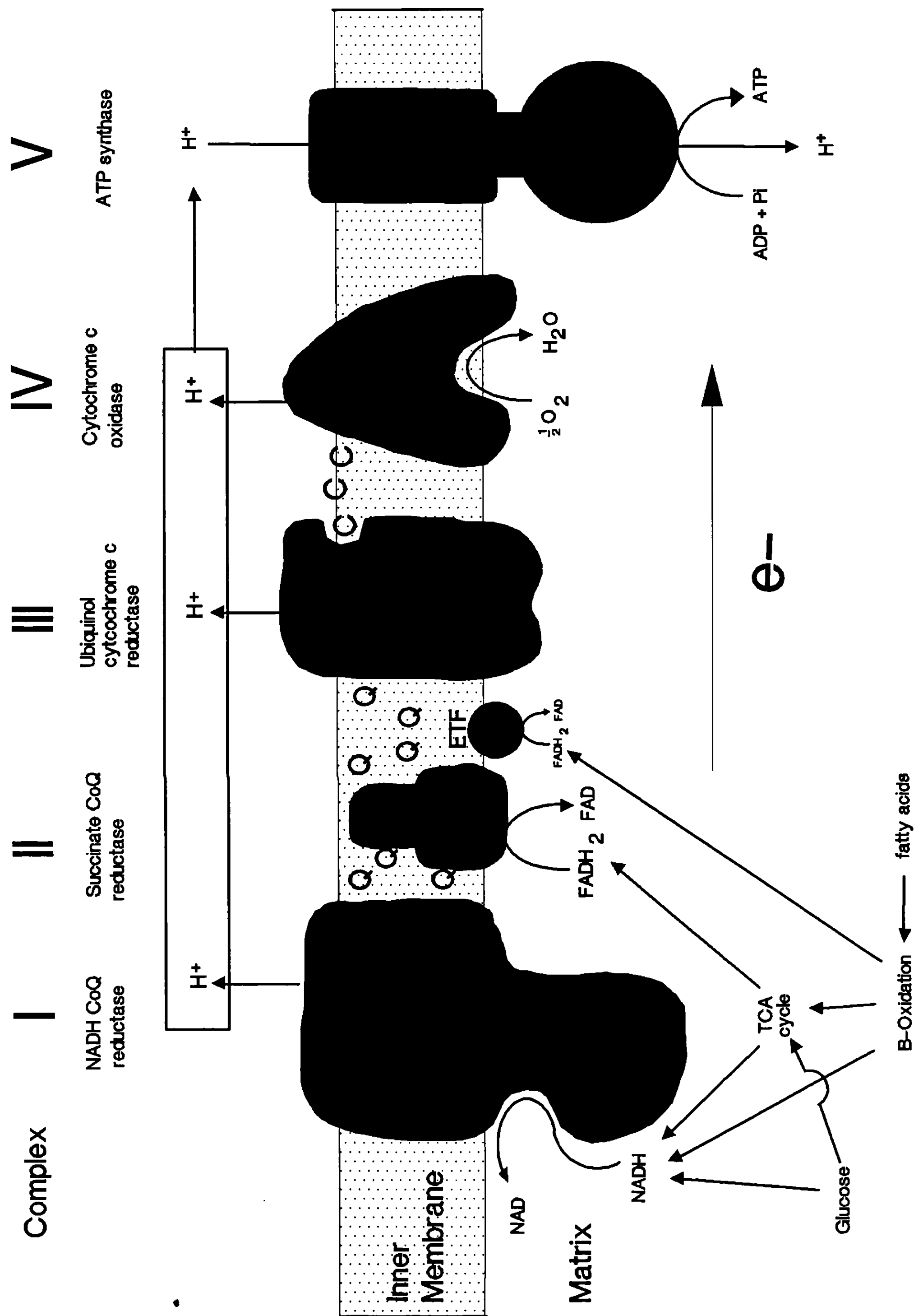


Fig. 1.2 The mitochondrial respiratory chain. Q: ubiquinone; C cytochrome c; ETF: electron-transferring flavoprotein. Reproduced by kind permission of Dr. J. M. Cooper.

1.3.2.1 Complex I

Structure and function

Complex I, also known as the rotenone-sensitive NADH-dehydrogenase, is the first enzyme in the MRC and is the largest of the respiratory complexes. It contains at least 41 different subunits and an unknown number of redox groups, including flavin mononucleotide (FMN) and 22-24 atoms of iron in the form of various iron-sulphur (Fe-S) clusters. The Fe-S groups are either binuclear (2Fe-2S) or tetranuclear (4Fe-4S). The enzyme catalyses the oxidation of NADH, to reduce ubiquinone, protons are translocated from inner membrane to intermembrane space and the resulting electrons are transferred via these redox centres. Within the 41 subunits, complex I has 7 polypeptides which are coded by mtDNA, namely ND1, 2, 3, 4, 4L, 5, and 6 (Chomyn *et al.* 1986) and the remaining 34 by nuclear DNA (nDNA). The whole molecular weight of complex I has been estimated to be ~ 900 KDa (Walker, 1992).

The nomenclature of the complex I subunits is confused, but subfracturation of complex I from bovine heart has been used to clarify matters. There are two major preparation methods for the subfractionation of complex I from bovine heart. With the 'Hatefi' method (Hatefi *et al.* 1962), complex I is subfractionated into three fractions using chaotropic agents: two soluble fractions, the flavoprotein (FP) and iron-protein (IP) fraction, and one insoluble hydrophobic (HP) fraction. The FP fraction comprises three subunits: 51, 24 and 10KDa. The 51 KDa subunit which binds FMN and NADH, probably contains a tetranuclear (4Fe-4S) cluster (Ragan *et al.* 1987). The IP fraction consists of six subunits: 75, 49, 30, 18, 15 and 13 KDa. The 75KDa may also be involved in the NAD^+-binding site (Ragan *et al.* 1987). The HP fraction is a less well defined fraction containing, presumably, all seven mtDNA-encoded subunits (ND 1, 2, 3, 4, 4L, 5 and 6).

However, how the three subfractions of complex I (FP, IP and HP) relate to each other and whether they have any structural significance is uncertain. Studies on mitochondria and inverted submitochondrial particles using cross-linking, hydrophilic and hydrophobic probes and antisera to specific subunits

have shed light on the membrane orientation and interrelationship with other constituent complex I polypeptides.

More recently, another fractionation of complex I has been described (Finel *et al.* 1992). Treatment with the detergent, N,N-dimethyldodecylamine N-oxide, splits complex I into two sub-complexes, 1α and 1β. The 1α subfraction catalyzes electron transfer from NADH to ubiquinone-1, contains 22 hydrophilic subunits including all the FP and IP subunits, 9 other nDNA-encoded subunits and 1 mtDNA-encoded subunit ND2. The sub-fraction 1β has about 15 subunits including 2 mtDNA products, ND4 and ND5, some of which appear to be hydrophobic. No biochemical function has yet been attributed to subcomplex 1β.

Inhibitors of complex I

A variety of toxins have been identified that inhibit complex I activity, including rotenone, piericidin A, MPP^+, and diphenyleneiodonium (DPI^+). Rotenone, a plant toxin, has been shown to inhibit between the highest potential iron-sulphur cluster and ubiquinone (Earley *et al.* 1987). A 33KDa subunit identified as ND1 has been confirmed as the rotenone binding site (Earley *et al.* 1987). MPP^+, which is a less potent complex I inhibitor, is readily reversible (Ramsay *et al.* 1987). It has been reported that rotenone, piericidin A and MPP^+ inhibit at the same or similar sites in complex I (Ramsay *et al.* 1991). However, the inhibition by DPI^+, another complex I inhibitor, binds to a 23-25 KDa subunit, which is enhanced by the presence of rotenone, suggesting that its inhibition site is different to that of other inhibitors (Ragan and Bloxham, 1977).

1.3.2.2 Complex II

Complex II is the simplest of all complexes, composed of four nDNA-encoded protein subunits, with molecular masses of 70, 27, 15 and 13kDa, and one covalently bound FAD, three Fe-S centres and a cytochrome b. This enzyme is the only complex in the MRC which does not contain mtDNA products. It is also the only complex that does not involve proton translocation.

Functionally, complex II catalyses the oxidation of succinate to fumarate in the TCA cycle with transfer of reducing equivalents to ubiquinone. The compounds 2-thenoyltrifluoroacetone (TTFA), 3-nitropropionic acid (3-NP) and malonate are complex II inhibitors.

1.3.2.3 Complex III

Complex III is composed of 11 subunits including the low-potential cytochrome b566 and high-potential cytochrome b562, cytochrome c1 and a high-potential 2Fe-2S centre (the Rieske iron-sulphur centre). Of all of these, only cytochrome b is a mtDNA-encoded protein. Functionally, complex III catalyses the oxidation of ubiquinol and reduction of cytochrome c, and this reaction is associated with the translocation of protons across the inner membrane. Complex III requires an interaction with the mobile electron carrier, ubiquinone, which functions by transferring one of its electrons to the Fe-S centre, which passes via cytochrome c1 to cytochrome c. Another electron is transferred to cytochrome b566 and b562, which reduces the semiquinone radical to quinone (the Q-cycle).

There are a number of complex III inhibitors, including antimycin A, myxathiozol and stigmatellin. The antimycin A binding site is in the vicinity of the high-potential haem b group which was affected by a mutation of cytochrome b (Roberts *et al.* 1980). Myxathiozol binds to or near the low-potential haem b, as suggested the fact that cytochrome b mutants are resistant to myxathiozol (Thierbach and Michaelis, 1982).

1.3.2.4 Complex IV

Complex IV is the final MRC complex and reduces the terminal electron acceptor O_2 to H_2O. It contains 13 subunits in mammals, three of which are mtDNA-encoded proteins (COX I, II and III), and the catalytic core of the enzyme. The remaining polypeptides are nuclear DNA-encoded subunits (IV-VIII). COX I contains the a and a3 haem, the Cu_B site and the oxygen biding site, while COX II contains the Cu_A site and the cytochrome c binding site (Tsukihara *et al.* 1995; Taanman *et al.* 1996).

The function of the nuclear DNA-encoded subunits (IV-VIII) is less well defined. Null mutants in subunits IV, Va, Vb, VIc or VIIa fail to assemble the complex, implying that these subunits are either needed structurally or involved in the importation/assembly process (review see Taanman, J-W., 1999, paper in press).

Functionally, complex IV catalyses the oxidation of cytochrome c, the reduction of oxygen and the translocation of protons. Electrons enter the complexes through cytochrome a/Cu_A, which are in rapid electron-transfer equilibrium, and then pass to the binuclear centre in what is considered to be the rate-limiting step. Oxygen binds to the binuclear centre only when both haem a3 and Cu_B are reduced. At this stage, the oxidation of reduced substrate by oxygen is complete.

The inhibitors of complex IV are cyanide, azide and carbon monoxide (vanGelder and Muijers, 1966).

1.3.2.5 Complex V

Complex V is composed of two fractions, the F1 catalytic centre (coupling factor) and the F0 hydrophobic domain (membrane segment) concerned with proton translocation. The F1 fraction contains α, β, γ, δ and ε subunits and the F0 fraction contains ATPase 8, oligomycin sensitivity conferring protein, coupling factor 6, inhibitor protein, a (ATPase 6), b, c, d and e. The F0 fraction is embedded in the inner membrane and is concerned with proton translocation. The eukaryotic complex V consists of 14 subunits, 2 of which are mtDNA-encoded, they are ATPase 6 and ATPase 8 (A6L).

Functionally, the F1 fraction which forms the 'head' of the lollipop structure seen under electron microscopy contains the catalytic centre for ATP synthesis (hydrolysis). The F1 is bound by the stalk to the membrane sector, the hydrophobic F0, the latter forming a specific proton-conducting pathway which is intimately involved in the production of ATP from ADP and Pi (Hatefi, 1985).

A brief summary of the composition of the MRC complexes and oxidative phosphorylation system is listed in Table 1.3.1.

Table 1.3.1 Composition of MRC and phosphorylation system

MRC complex	Total no. of subunits	No. of mtDNA-encoded
complex I (NADH-CoQ reductase)	41	7: ND1, 2, 3, 4, 4L, 5, 6
complex II (succinate-CoQ reductase)	4	0
complex III ($CoQH_2$-cytochrome c reductase)	11	1: cytochrome b
complex IV (cytochrome oxidase)	13	3: COX, subunits I, II, III
complex V (ATP synthase)	14	2: ATPase subunits 6, 8

1.3.3 MRC function in PD

The discovery that MPP^+ may induce parkinsonism in men and primates, possibly via inhibition of mitochondrial complex I activity, focused attention on the function of the MRC in idiopathic PD. There is increasing evidence that complex I deficiency may be involved in the pathogenesis of IPD. The MRC function has been studied in various PD brain regions, skeletal muscle and platelets.

1.3.3.1 The effect of MPTP on MRC in PD

MPTP is converted to its neurotoxic metabolite MPP^+ which is selectively accumulated into dopaminergic neurons of the substantia nigra is explained by its high affinity uptake by the dopamine-reuptake system (Javitch *et al.* 1985). MPP^+ is accumulated into mitochondria via an uptake mechanism driven by the mitochondrial membrane electrochemical potential (Ramsay *et al.* 1986). In the mitochondria, MPP^+ has been reported to inhibit the complex I activity of the MRC by preventing electron flow from NADH to ubiquinone at the same or similar site to the classical complex I inhibitors; rotenone and piericidin A (Ramsay *et al.* 1991). The rotenone binding site includes the mitochondrial DNA (mtDNA) encoded subunit ND1 protein (Earley *et al.* 1987). The stereotactic injection of MPP^+ or rotenone into rat brain caused specific

damage to the dopaminergic area (Heikkila *et al.* 1985), implying that specific inhibition of MRC function can cause neuronal degeneration.

It is not clear how MPP^+ actually kills neurons. It has been suggested that MPP^+ associates with neuromelanin maintaining higher concentrations in the nigral region (Irwin and Langston, 1985) and inhibition of complex I activity leading to decreased ATP levels, leading to cell death. Oxidative stress induced by MPP^+ may also contribute to MPP^+-induced cell death (Nicklas *et al.* 1985; Di Monte *et al.* 1986) (see section 1.5.2)

1.3.3.2 MRC function in PD brain

Mitochondrial complex I activity was significantly decreased (37%) in substantia nigra from 22 PD patients compared with 17 age-matched controls (Schapira *et al.* 1989; Mann *et al.* 1992a). A second group has demonstrated a 33% decrease in complex I activity (Janetzky *et al.* 1994) in the substantia nigra of PD patients. However, normal complex I activity but significantly decreased complex III activity has been detected in the striatum of 5 PD patients (Mizuno *et al.* 1990).

The complex I activity was not significantly different in the caudate nucleus, cerebral cortex, cerebellum, medial globus pallidus and lateral globus pallidus suggesting it was anatomically specific to the substantia nigra (Schapira *et al.* 1990a). Moreover, this decrease in complex I activity was not present in the substantia nigra from MSA patients (section 1.3.4), suggesting it was not secondary to neuronal degeneration in the substantia nigra.

Several groups have used western blotting with complex I antisera to look at the composition of complex I in PD brain. By using substantia nigra homogenates probed with antisera to the complex I holoenzyme, Schapira *et al.* (1990a) were unable to detect any differences in molecular weight or staining intensity of cross-reactive complex I subunits. However, another group observed less intense staining in low molecular weight complex I subunits in PD striatum but these subunits were not identified (Mizuno *et al.* 1989). Hattori *et al.* reported that a proportion of the nigral neurons showed reduced

immunohistochemical staining with antisera against complex I (Hattori *et al.* 1991).

Table 1.3.2 Summary of MRC function in substantia nigra of controls and PD.

Table 1.3.2 MRC function in substantia nigra of controls and PD

Study	Numbers	Notes	Complex I results
Mann (1992a)	C=17, PD=22	enzyme assay	37%↓ CxI, S
Janetzky (1994)	C=7, PD=7	enzyme assay	33%↓ CxI, S

C: control, Cx I: complex I

S: significant difference.

↓: defect

1.3.3.3 MRC function in PD skeletal muscle

Muscle weakness, fatigue and the presence of ragged-red fibres on muscle biopsy are characteristic features of patients with classical MRC defects. Although these features are not typical features in PD, skeletal muscle provides a convenient tissue for analysis of MRC function in PD patients while they are still alive.

There have been several conflicting reports of mitochondrial MRC function in PD muscle. Bindoff *et al.* (Bindoff *et al.* 1991) detected significant defects of complex I, II and IV (41, 51 and 60%, respectively) compared to matched controls. Shoffner *et al.* (1991) reported that, relative to the control mean, the activities of complex I in the 4 PD patients showed a defect ranging from 86-92%. Mann *et al.* (1992a) compared MRC function in 9 PD and 6 controls by both spectrophotometry and polarography, but no differences with matched controls were detected. Cardellach *et al.* (1993) detected significant decreases of 26% and 68% in the mean activities of complex I and IV respectively in muscle mitochondria in 8 PD patients compared to 10 controls. Di Donato *et al.* (1993) reported that there were no differences in citrate synthase (CS) corrected MRC enzyme activities in muscle crude homogenates between 16

PD and 8 controls or purified mitochondria between 6 PD and 6 controls (Table 1.3.3).

Table 1.3.3 MRC function in controls and PD muscles

Study	Numbers		Notes	Results(CxI-IV)	Significance
	C	PD			
Bindoff (1991)	4	5	enzyme assay	Cx I 41%↓ CxII 51%↓ CxIV 60%↓	S S S
Shoffner (1991)	11	6	enzyme assay*	CxI 86-92%↓	S
Mann (1992a)	6	9	enzyme assay		NS
			polarography		NS
Cardellach (1993)	10	8	mitochondria	Cx I 26%↓	S
				CxIV 68%↓	S
DiDonato (1993)	6	6	mitochondria		NS
	8	16	homogenates		NS

C: control, Cx I, II, III and IV: complex I, II, III and IV.
S: significant difference. NS: no significant difference
↓: defect. *: not age-matched

The choice of age-matched controls may be crucial for comparison. Although the controls and patients were age matched in the studies of Bindoff *et al.* (Bindoff *et al.* 1991), the results still showed differences in terms of both the decline of enzyme function with age and the mitochondrial function in PD muscle.

There may be a problem with the diagnosis of PD in the absence of brain pathology as MSA patients generally account for ~15% of PD cases. Therefore some patients classified as PD patients may be MSA patents with a different aetiology.

1.3.3.4 MRC function in PD platelets

Platelet structure and function

Megakaryocyties, which are found in the bone marrow, give rise to 1000-1500 platelets which are enucleated cytoplasmic fragments. Normal blood contains between 150,000-750,000 platelets/ml. Not all platelets are present in the circulating blood since up to a third are sequestered elsewhere, principally in the spleen. The life of a platelet is about 4-5 days and aged or damaged platelets are removed from the circulation by the reticular endothelial system.

Structurally, platelets have a trilaminar covering membrane with a series of surface-connecting channels which greatly expand the surface of the platelet and are used for extrusion of secretary products. There are at least three different types of granules; (1) electron-dense granules containing ATP, ADP, calcium and serotonin; (2) α-granules containing platelet factor IV, fibrinogen and β-thromboglobulin; (3) glycogen granules.

The most distinguishing feature of platelets is the absence of a nucleus. It has been estimated that there are about 4 mitochondria per platelet and that there is only one copy of mtDNA per mitochondrion (Shuster *et al.* 1988). The fact that platelets have no nucleus makes them an ideal model for fusion with ρ° cells (platelet fusion is described in details in chapters 5 and 6).

Functionally, the platelets have a critical role in haemostasis and thrombus formation, sealing endothelial defects and forming plugs which initially arrest bleeding. This plug is formed via platelet adhesion and aggregation. Adhesion is followed by endothelial damage, platelets adhering to subendothelial connective tissue structures, such as collagen. Aggregation occurs following adhesion of platelets.

The use of platelets in PD studies

Despite having an important role in haemostasis, platelets have been considered as a useful model system for central serotonergic neurons (DaPrada *et al.* 1988). The platelets have an active uptake system for

serotonin (5-hydroxytryptamine, 5-HT) which is concentrated into dense granules. Not only does this transport system concentrate MPTP/MPP$^+$ and its analogues, but since human platelets contain MAO-B (Chen *et al.* 1993), they are also capable of activating toxins such as MPTP.

MRC function in PD platelets

The first report of MRC enzyme analysis in PD platelets revealed a specific 55% deficiency of complex I activity in 10 PD patients compared with 8 controls (Parker *et al.* 1989). This observation raised the possibility that platelets could be used to investigate the complex I defect in PD, and could possibly be used as a presymptomatic test for PD. However, 7 further studies have been reported (Table 1.3.4). Of the eight studies, five of them showed significant complex I defects in PD platelets (Parker *et al.* 1989; Krige *et al.* 1992; Yoshino *et al.* 1992; Benecke *et al.* 1993; Haas *et al.* 1995), which were accompanied by complex II defect in one (Yoshino *et al.* 1992), complex II+III defect in one (Haas *et al.* 1995) and complex IV defect in two (Yoshino *et al.* 1992; Benecke *et al.* 1993).

Table 1.3.4 MRC function in controls and PD platelets

Study	Numbers C	PD	Results (Cx I-IV)	Significance
Parker (1989)	8	10	CxI 55%↓ ,	S
			CxIV	NS
Mann (1992a) *	15	14	Cx I, II+III and IV	NS
Yoshino (1992) **	15	20	CxI 27%↓	S
			CxII 20%↓	S
Krige (1992)	15	25	CxI 16%↓	S
			CxII+III and IV	NS
Benecke (1993)	27	27	CxI 52%↓	S
			CxIV 30%↓	S
Haas (1995)	18	18	CxI 23%↓	S
			CxII+III 20%↓	S
			CxIV	NS
Blake (1997)	9	13	CxI, III and IV	NS

C: control, Cx I, II, III and IV: complex I, II, III and IV. S: significant difference
NS: no significant difference. ↓: defect; *: homogenate;**: not age matched

In general, the results showed a spread of platelet complex I activities in PD patients which overlapped those of the controls, thus making it impossible to use the complex I deficiency in platelets as a diagnostic test for PD (Krige *et al.* 1992; Blake *et al.* 1997).

1.3.4 MRC function in other neurodegenerative diseases

MSA

MSA is characterised by cell death in many areas of the brain, including the substantia nigra where cell death is similar or even greater than that seen in PD. However, in contrast to PD, normal MRC function in substantia nigra and platelets from MSA patients, has been reported (Schapira *et al.* 1990a; Mann *et al.* 1992a; Gu *et al.* 1997).

AD

A severe defect in complex IV in mitochondria isolated from AD platelets was first reported by Parker *et al.* (1990a). Since then, more reports have supported the complex IV deficiency either in AD platelets (Parker *et al.* 1994a) or brain cortex (Mutisya *et al.* 1994; Parker *et al.* 1994b; Chagnon *et al.* 1995). Although complex IV abnormality in AD is the most common finding, normal complex IV function has been reported (Cooper *et al.* 1993). Furthermore, a 40% decrease in complex II+III activity in AD temporal cortex but a normal complex I activity has been reported (Reichman *et al.* 1993).

DLB

DLB is pathologically thought to be as a variant of AD. To date, there are no reports of MRC dysfunction in this disorder.

HD

In HD caudate nucleus, the biochemical basis of neuronal death in HD remains unclear, although several studies suggest that abnormal MRC function may be involved. There were decreased complex II+III and complex IV activities in HD caudate (Brennan *et al.* 1985) and a preliminary study also showed a deficiency in complex II+III in HD caudate nucleus (Mann *et al.* 1990). Furthermore Parker *et al.* (1990b) reported that there was a defect of

complex I activity in the platelets from HD patients which may suggest a wide tissue distribution of MRC abnormality in HD patients, although the site of the defect of MRC in platelet contradicts that seen in the brain.

Dystonia

A 62% reduction in complex I activity in the platelets of segmental/generalized dystonic patients was reported (Benecke *et al.* 1992). Recently, Schapira *et al.* (1997) confirmed the results of a complex I defect in platelet mitochondria from patients with sporadic focal dystonia but normal complex I activity in patients with familial generalized dystonia (linked or not linked to 9q34).

Ageing

Ageing is associated with a gradual decline in normal tissue function and is also an important risk factor for many neurodegenerative disorders. It has been suggested that the MRC function declines with age and has been suggested to be a factor in the ageing process. Indeed, organs which are highly dependent on oxidative phosphorylation for energy requirements exhibit a decrease in MRC function with age. The decline in MRC function with age may contribute to the onset of PD.

A decline of MRC function with age has been found in primate brain (Bowling *et al.* 1993), human liver (Yen *et al.* 1989) and skeletal muscle (Trounce *et al.* 1989; Cardellach *et al.* 1989; Cooper *et al.* 1992). Trounce *et al.* (1989) reported that there was negative correlation between the MRC function and ageing in skeletal muscle from all 29 subjects whose ages ranged from 16-92, the 90 years old subject had a significant decrease in complexes I, II and IV activities. Subsequently, Cooper *et al.* (1992) demonstrated similar results in skeletal muscle, with a decrease of 59% for complex I and 47% for complex IV activities in patients aged around 70 years.

1.4 Mitochondrial DNA

1.4.1 Mitochondrial DNA (mtDNA)

Human mtDNA contains 37 genes: 13 polypeptide-coding genes (mRNA), 22 tRNA genes and 2 rRNA genes (12S and 16S rRNA). All 13 polypeptides are

components of the MRC and oxidative phosphorylation system: 7 constitute subunits of complex I (ND1-6 and 4L); cytochrome b is in complex III; 3 subunits are in complex IV (COX I-III); and 2 subunits in complex V (ATPase 6 and ATPase 8). All 5 complexes contain proteins encoded by nuclear DNA (nDNA) which are synthesised as precursors on cytosolic ribosome, imported from the cytoplasm and for complexes I, III, IV and V, they assemble together with the mtDNA-encoded subunits.

The tRNA and rRNA encoded by mtDNA are required for its translation, however all the other machinery for replication, translation and also proteins which regulate mtDNA replication are encoded by nuclear genes and imported into the mitochondrion.

Replication

MtDNA can be separated by alkaline caesium chloride density-gradient centrifugation into 2 bands termed heavy (H) and light (L) strands. There are 2 origins of replication: one for the H strand (O_H) and another for the L (O_L) strand. O_H is located in the D-loop (Fig 1.3). MtDNA replication begins at a site close to the 5'-end of the 12S rRNA gene (Crews *et al.* 1979) and displacement synthesis of a daughter H-strand extends away from the rRNA genes. When the H-strand is approximately two thirds complete, the O_L is exposed, synthesis of the complementary L-strand begins and continues asymmetrically until the two daughter molecules are complete (Crews *et al.* 1979).

Replication of mtDNA appears to be cell-cycle regulated, with about one round of mtDNA synthesis cell cycle which usually takes ~ 90mins (Clayton, 1982). The number of mitochondria in each cell varies, generally from several hundred to thousands per cell, and the mtDNA is polyploid (*i.e.* several copies of mtDNA in each mitochondrion).

Transcription

The D-loop contains both H-strand and L-strand promoters (HSP and LSP, respectively), which initiate transcription of their respective strands to produce

giant nearly-full-circle polycistronic transcripts. The tRNA genes are located at strategic points on the mtDNA circle, which separate all but one of the individual rRNA and mRNA genes. These precursor RNAs are then cleaved by endogenous nuclease at the 5' and 3' ends of the tRNA, releasing the transcript that is then processed to yield mRNA for 12 of the 13 structural genes, 2 rRNAs and 14 tRNAs. The L-strand transcript gives rise to the remaining 1 mRNA and 8 tRNAs (Fig. 1.3).

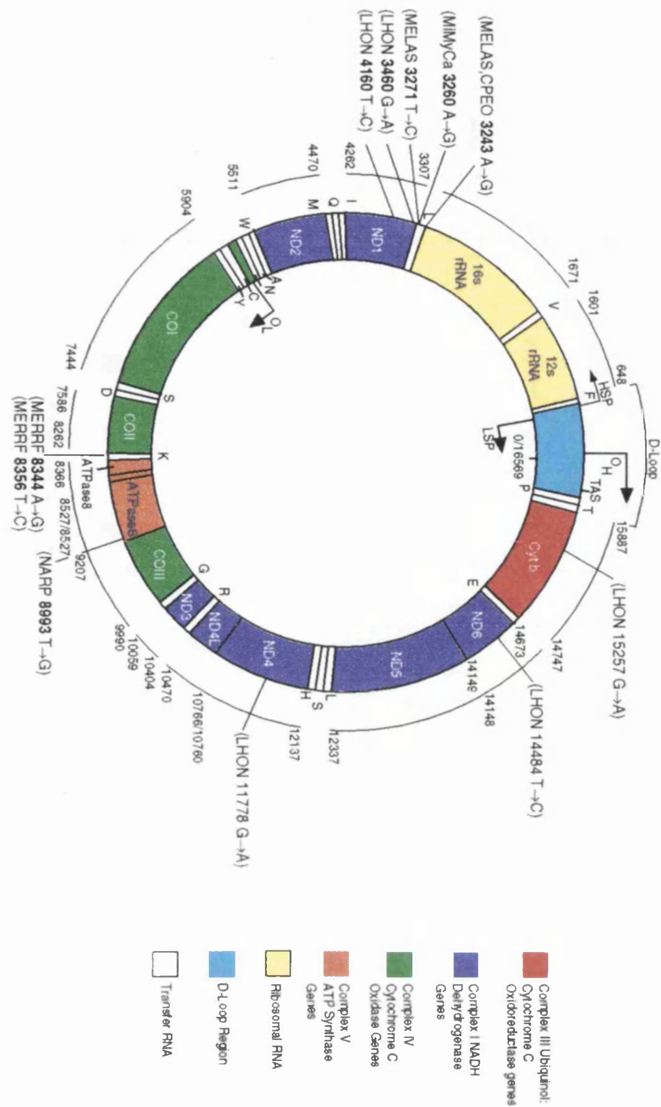


Fig. 1.3 The human mitochondrial genome. The structural genes for the mitochondrial-encoded 12S and 16S rRNAs, the subunits of NADH-cozyme Q oxidoreductase (ND), cytochrome c oxidase (CO), cytochrome b (cyt b) and ATP synthase (A), and 22 tRNAs (one-letter amino acid nomenclature) are shown. The origins of light-strand (O_L) and heavy-strand (O_H) replication, and of the promoters for initiation of transcription from the light strand (LSP) and heavy strand (HSP), are shown by arrows. D-loop is a non-coding regulating region. Common mutations of mtDNA associated with mitochondrial diseases are shown. Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS); myoclonic epilepsy and ragged-red fibres (MERRF); chronic progressive external ophthalmoplegia (CPEO or PEO); Leber's hereditary optic neuropathy (LHON); neuropathy, ataxia and retinitis pigmentosa (NARP); mitochondrial myopathy and cardiomyopathy (MiMyCa).

Mitochondria need to synthesis enough rRNA to satisfy the translation requirements of 13 mRNAs. To overcome this problem, transcription of HSP, which encodes the rRNAs, is made in short and full-length transcripts. The short transcript extends from HSP to the end of the 16S rRNA gene, and the synthesis rate for the short transcript is made at approximately 25 times faster than that for the full-length transcript (Attardi *et al.* 1989). Therefore, sufficient amounts of 12S and 16S rRNA are produced for all 13 mRNAs. The short rRNA transcript is terminated at a site within the $tRNA^{Leu(UUR)}$ gene, immediately downstream of the end of the 16S rRNA. A specific 'terminator' protein (mtTERM) binds to a DNA region within the $tRNA^{Leu(UUR)}$ gene, terminating transcription (kruse *et al.* 1989).

Translation

Translation of mitochondrial mRNAs occurs on the mitochondrial ribosome, which contains 12S and 16S rRNAs. It has been shown that the 13 mtDNA-encoded subunits are synthesized at different rates, which do not necessarily reflect the abundance of their mRNAs, suggesting different translational efficiencies (King and Attardi, 1993).

The mammalian mitochondrion does not always follow the universal genetic codon usage, relating each triplet to a particular amino acid. Mitochondrial mRNAs lack an AUG start codon; UGA is not a stop codon but codes for tryptophan; AUA codes for methionine rather than isoleucine, AGA and AGG are stop codons rather than coding for arginine.

1.4.2 The genetics of mtDNA

Mitochondria and mtDNA are inherited maternally (Giles *et al.* 1980), although there is evidence that paternal mitochondria can be inherited at an extremely low level (Gyllensten *et al.* 1991). Therefore, genetic diseases caused by a primary mtDNA mutation are only inherited maternally.

Several features including heteroplasmy, complementation, threshold effects and mitotic segregation are specific to mitochondrial genetics (Wallace, 1987).

The occurrence of both wild type and mutant mtDNA within the same tissue (heteroplasmy) of affected patients complicates the interpretation of how certain mutations may be expressed. This is particularly important in those mutations in tRNA genes which are required to translate the whole genome. If the tRNAs from the wild type genomes can complement the loss of functional tRNAs from the mutant mtDNA, a functional defect may not be observed, This would differ markedly if complementation did not occur.

There is evidence for complementation between wild type and mutant mtDNA in cells with a mtDNA deletion (Hayashi *et al.* 1991; Hammans *et al.* 1992). Translation was inhibited if the percentage of deleted mtDNA was over a threshold (approximately 60%) (Hayashi *et al.* 1991). However, the threshold of wild type mtDNA required to rescue the biochemical function of cells with either the $tRNA^{Leu}$ or $tRNA^{Lys}$ point mutations was found to be very low (approximately 10-15%) (Chomyn *et al.* 1994), suggesting that the mutant mtDNA could be complemented by a small number of wild type mtDNA genomes.

Based on the fact that both mtDNA replication and mitochondrial division are stochastic processes unrelated to the cell cycle or the timing of nuclear DNA replication, a term, mitotic segregation, has been used to describe mtDNA distribution into daughter cells. Thus, two daughter cells may potentially have mitochondrial components and mtDNA genomes which vary quite considerably from each other and the parent cells. If a dividing cell contains both wild-type and mutant mtDNAs, the phenotypic expression in daughter cells is based on how the mutant and wild type mtDNA segregate.

1.4.3 Disease-associated mtDNA mutation

There is increasing evidence that a decline in mitochondrial function may contribute to the pathogenesis of a variety of neurological and neurodegenerative disorders and the ageing process. Abnormalities of the MRC often clinically present with muscle fatigue either alone (mitochondrial myopathy, MM), such as chronic progressive external ophthalmoplegia

(CPEO) or with central nervous system (CNS) dysfunction (mitochondrial encephalopathy), such as Kearns-sayre syndrome (KSS), myoclonic epilepsy and ragged-red fibres (MERRF), and mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS). Other clinical features include diabetes, optic atrophy and cardiomyopathy.

The patients with respiratory chain defects and mtDNA mutation usually exhibit muscle mitochondrial proliferation which stains red with the modified Gomori trichrome method and results in so-called ragged-red fibres (RRF).

MtDNA mutations have been identified in many of these patients. These mutations can be divided into large scale rearrangements; point mutations; multiple deletions and depletion.

1.4.3.1 Large-scale rearrangements

Deletion

Patients with CPEO and KSS are both characterised as MM with ragged red COX negative fibres in muscle. Abnormal mtDNA associated with disease was first reported by Holt *et al.* (1988). They found deletions of mtDNA (mtDNAs) in 9 of 25 patients with MM. Further studies confirmed that spontaneously occurring large-scale deletions (~9kb) were a hallmark of KSS (Zeviani *et al.* 1989) and some cases of CPEO (Lestienne and Ponsot, 1988; Holt *et al.* 1989; Gerbitz *et al.* 1990). All patients with deletions were heteroplasmic. However, one particular deletion termed the 'common' deletion has been found in approximately one-third of all patients with mtDNA deletion (Holt *et al.* 1989). This common deletion is 4977bp long and occurs at a 'hot spot' between directly repeated sequences of 13bp in length (Schon *et al.* 1989).

Deleted mtDNAs have been shown to be transcribed but not translated, even the genes which are not within the deleted region (Nakase *et al.* 1990). However, it has been observed that there is a threshold number of deleted mtDNAs required before translation is inhibited (Hayashi *et al.* 1991). Hayashi *et al.* (1991) re-populated ρ° cells (lacking mtDNA) with mitochondria from a patient with wide type and partially deleted mtDNAs. Analysis of several clones

indicated that if the levels of deleted mtDNA exceeded 65%, then, translation was not observed, but clones with up to 50-60% mutant genomes displayed the full complement of translation products. The lack of translation in those clones with deleted mtDNA above the threshold level may be explained by the fact that the deletion removes essential tRNAs and there is competition for the remaining tRNAs for protein synthesis. Another explanation suggested that the deletion may be dominant. By competing for the decreased tRNAs for translation, the deleted mtDNAs may prevent overall translation even if the mtDNA population within the mitochondrion is heteroplasmic (Shoubridge *et al.* 1990).

Partial duplication

Partial duplications of mtDNA have only been properly documented in a few cases KSS (Poulton *et al.* 1989) and diabetes mellitus (Dunbar *et al.* 1993). The low frequency may reflect misclassification of a proportion of such mtDNA rearrangements as partial deletions.

Depletion

Two related mtDNA depletion diseases (the early-onset and late-onset forms) have been described as inherited as Mendelian traits. The early-onset type presents at birth with weakness, hypotonia, lactic acidosis and RRF on muscle biopsy. Approximately 2-17% of the mtDNA remains in muscle and is associated with a severe decline in complex IV activity. The infants rarely survive more than 12 months after birth. The late-onset form of mtDNA depletion presents after 1 year, with similar histological and biochemical abnormalities on muscle biopsy as the early-onset ones. MtDNA levels in the later onset group have been reported as 14-34% of normal. Immunohistochemical staining has revealed that these patients lack mtDNA-encoded subunits (COX I, II and III) but have normal amounts of nuclear DNA encoded proteins (Schapira, 1994). In general, the patients were characterised by MRC defects and severe depletion of mtDNA. However, there was no observable biochemical or genetic abnormality in unaffected tissues.

1.4.3.2 Point mutations

Five maternally inherited mitochondrial diseases have been identified that are associated with point mutations in mtDNA, and neurodegenerative disorders.

MELAS

MELAS is a typical mitochondrial myopathic disease. There are two different point mutations associated with the MELAS phenotype. One is an A→G transition at 3243 bp within the $tRNA^{Leu(UUR)}$ gene, which was found in the majority of MELAS cases (about 80%) (Goto *et al.* 1990). Another is also an A→G transition at 3271 bp within the $tRNA^{Leu(UUR)}$ gene, which has been found only in 10% of MELAS cases (Goto *et al.* 1991). These mutations are heteroplasmic, with a high proportion of mutated mtDNAs in muscle (>80%) but a relative low level (<50%) in blood of the same patients.

A severe decrease in overall protein synthesis in MELAS patients has been observed, which is associated with reduced oxygen consumption by cells harbouring the mutated mtDNAs (Chomyn *et al.* 1992). In a heteroplasmic situation *in vitro*, fewer than 10% wild-type genomes are sufficient to maintain respiratory competency (Chomyn *et al.* 1992). However, the underlying cause of the decreased mitochondrial translation has not be resolved. There is no relationship between the $tRNA^{Leu(UUR)}$ gene content of individual proteins and their rate of synthesis. Therefore, the defect is unlikely merely to be due to dysfunction or instability of the $tRNA^{Leu(UUR)}$ gene. It has been suggested that this mutation may decrease the binding affinity of purified termination factor (mtTERM) *in vitro* (Chomyn *et al.* 1992), thereby affecting rRNA levels and therefore translation.

The MRC function of skeletal muscle from MELAS patients usually has a complex I defect (Koga *et al.* 1988), often in combination with complex IV dysfunction (Byrne *et al.* 1988). Western blot analysis of mitochondria with antisera against complex I has revealed a general decrease in all cross-reactive subunits in a number of patients (Ichiki *et al.* 1988).

It has been found that some patients with CPEO harbour the MELAS-3243A→G mutation (Ciacci *et al.* 1992). The level of this mutation is usually only at ~ 50-70% in muscle from these patients, compared to MELAS patients, who usually harbour more than 80% mutated mtDNA, implying that the threshold effects in these two kinds of disease are different.

MERRF

The A→G transition at 8344bp in the TψC loop of the $tRNA^{Lys}$ gene is the mutation most commonly associated with MERRF (Shoffner *et al.* 1990). A second mutation of this gene at position 8356bp (T→C transition) has also been found in one MERRF patient. The 8344bp mutation causes decreased protein synthesis of mtDNA-encoded subunits with the subunits of complex I and IV being the most affected. The 8344bp mutation has also been identified in patients with Leigh's syndrome (Hammans *et al.* 1991).

LHON

LHON is associated with optic atrophy leading to acute blindness. Several point mutations of protein-coding regions of mtDNA including the G→A transitions at positions 3460bp (ND1) and 11778bp (ND4) and T→C transition at positions 14484bp (ND6), have been reported in LHON. The point mutation (11778bp) in ND4 accounts for over 50% of cases of LHON. The mutation causes the replacement of an arginine with a histidine residue. The link to mtDNA was the exclusive maternal transmission of this disorder. However, it is not always 100% penetrate and the predominance of male cases has still not been explained (Sweeney *et al.* 1992). The mutations at the positions of 11778bp, 3460bp and 4160bp of mtDNA have been linked to a functional defect of complex I in platelet or lymphocyte mitochondria (Howell *et al.* 1991).

NARP/Leigh disease

NARP (neuropathy, ataxia and retinitis pigmentosa) is a maternally inherited disease, associated with a heteroplasmic point mutation (T→G) at 8993bp of the ATPase 6 gene (Holt *et al.* 1990). When the proportion of NARP mutation is higher than 90%, it produces another clinically phenotype, termed Leigh syndrome (Tatuch *et al.* 1992).

The most common point mutations of mtDNA and their predominant clinical presentations are listed in Table 1.3.5.

Table 1.3.5 Selected examples of point mutations of mtDNA associated with mitochondrial diseases.

Clinical phenotype	Mutations	Gene	References
MELAS	A→G at 3243bp	$tRNA^{Leu(UUR)}$	Goto 1990
MELAS	T→C at 3271bp	$tRNA^{Leu(UUR)}$	Goto 1991
MERRF	A→G at 8344bp	$tRNA^{Lys}$	Hammans1991
	T→C at 8356bp	$tRNA^{Lys}$	
LHON	G→A at 3460bp	ND1	Huoponen1991
	T→C at 4160bp	ND1	Howell1991
	G→A at 11778bp	ND4	Wallace1988
	T→C at 14484bp	ND6	
	G→A at 15257bp	Cyt b	John 1991
NARP	T→G at 8993bp	ATPase6	Holt 1990

1.4.4 MtDNA and PD

1.4.4.1 Deletion in PD

Using the polymerase chain reaction (PCR), early reports suggested there was an increased level of the 5kb 'common deletion' in PD striatum (Ikebe *et al.* 1990). However, using age matched PD patients and controls, Mann *et al.* (Mann *et al.* 1992b) detected no increase in mtDNA bearing the 'common deletion' in substantia nigra from PD patients in comparison to age matched controls. The levels of deleted mtDNA was extremely low (about 0.01%) and therefore it is unlikely that such a small proportion of mutation could cause a significant biochemical defect in PD. The findings (Mann *et al.* 1992b) suggested that the common deletion was not specific to PD brain, but was simply an ageing phenomenon. This has subsequently been confirmed in the substantia nigra (Di Donato *et al.* 1993) and in platelets (Sandy *et al.* 1993).

1.4.4.2 Point mutation in PD

A search for specific mtDNA point mutations in PD has generally proved unrewarding. In many studies, mitochondrial DNA polymorphisms have been identified in individual PD patients. Schapira *et al.* (1990b) observed two different novel polymorphisms, a RsaI restriction site and a CfoI site polymorphism, in two PD patients that were not present in control brain (n=6) or blood (n=34) samples. However, the significance of these two polymorphisms was unclear, as they each occurred in different regions of the mtDNA in single cases. Ozawa *et al.* reported that two transitions at nt150 (T-C) in the D-loop region and at nt8071 (A-G) in the COX II gene were found in two PD patients (Ozawa *et al.* 1991). However, both polymorphisms were silent mutations, as they neither changed conserved nucleotide among animal species nor converted amino acids. In 1993, an A→G transition at position 4336bp of the mtDNA $tRNA^{Gln}$ gene was detected in a cohort of PD patients (Shoffner *et al.* 1993). This gene variant altered a moderately conserved nucleotide, which connected the amino acid acceptor stem with the TψC stem of $tRNA^{Gln}$ and is presumed to affect its function. The mutation was present in 5.3% (2/38) of PD patients surveyed but in only 0.7% (12/1691) of controls. However, the increased frequency of this mutation was not specific to PD and was also detected in 3.2% (2/62) of AD samples (Shoffner *et al.* 1993). The $tRNA^{Gln}$ 4336bp polymorphism appears to be mild and seems likely that individuals who inherit this mutation will occasionally manifest disease symptoms which will appear in a sporadic manner (Shoffner *et al.* 1993). These results have been recently confirmed by Egensperger *et al.*, they reported that mtDNA A4336G mutation was found in 2 of 23 PD and 1 of 28 AD, but not found in 100 age-matched controls (Egensperger *et al.* 1997). Ikebe *et al.* (1995) analysed the total nucleotide sequences of mtDNA and reported that there were no predominant point mutations/polymorphisms in the brains of 5 patients with IPD. However, each patient had several polymorphisms that would result in a change in the gene products. They suggested that some of these polymorphisms could be regarded as a risk factor accelerating degeneration of the nigrostriatal pathway in PD (Ikebe *et al.* 1995). More recently, studies demonstrated the presence of multiple deletions and 48 discrete polymorphic sites including 23 missense, 2 tRNA and 1

nonsense polymophism in substantia nigra mtDNA from 4 elderly and 2 PD patients (Kapsa *et al.* 1996).

1.4.5 MtDNA and ageing

Due to the absence of a histone coat on mtDNA for protection and the lack of excision repair and recombinational repair, mtDNA is susceptible to a variety of mutagenic effects, such as free radical attack. The high rate of spontaneous mutations may effect the mtDNA-encoded subunits. Age-dependent accumulation of a hydroxyl-radical adduct of deoxyguanosine, 8-hydroxydeoxyguanosine which is a marker of free radical-mediated DNA damage, has been observed in mtDNA in the diaphragm (Hayakawa *et al.* 1991) and in the heart concomitant with the increase in deleted mtDNA (Hayakawa *et al.* 1992). There was an increased level (1/8000 bases) in mtDNA compared with 1/130,000 bases in nuclear DNA in the ageing human brain (Mecocci *et al.* 1993). Evidence supports the suggestion that age-associated accumulation of mitochondrial deficits due to oxidative damage is likely to be a major contributor to cellular and tissue dysfunction with increasing age (Ames *et al.* 1995).

There is evidence that the 4977bp mtDNA common deletion accumulates with age in human skeletal muscle (Cooper *et al.* 1992) and liver (Yen *et al.* 1991). Tissues that turn over slowly, such as skeletal muscle and heart, contained more deleted mtDNA than more rapidly dividing tissues, such as liver (Simonetti *et al.* 1992). Cooper *et al.* (1992) demonstrated that increased common deletion in skeletal muscle is purely an ageing process and less than 1 in 5000 mtDNA molecules were affected, although Simonetti *et al.* (1993) reported that the maximum amount of common deletion observed in aged muscle was higher (approximately 1 in 1000). Such a low mutated frequency alone is unlikely to account for the decline in MRC function with age, but other mtDNA deletions and point mutations accumulating with age could account for a significant proportion of the mtDNA and contribute to decreased MRC function.

1.5 Oxidative stress in PD and other neurodegenerative diseases

1.5.1 Free radicals and oxidative stress

Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance (Sies, 1985). It can arise from an absolute lack of antioxidants or increased levels of free radical generation resulting in an imbalance between them. A free radical is any species with one or more unpaired electrons which are capable of an independent existence. It is generally unstable, highly reactive, taking part in self-perpetuating or autocatalytic reactions and has a very short half-life which is variable from nanoseconds for the hydroxyl radical to seconds for peroxyl radical or nitric oxide.

The superoxide radical ($O_2^{-\bullet}$) can be formed by many biological systems, including the MRC or endoplasmic reticulum. It is believed that superoxide radical generating systems are damaging mainly because of the formation of a more reactive species, the hydroxyl radical ($OH^\bullet$). H_2O_2 can decompose to generate $OH^\bullet$ (Haber-Weiss reaction), a reaction catalysed by a transition metal *i.e.* iron (Fig. 1.4).

Free radicals have important roles in normal human metabolic pathways but have also been implicated in human pathology. They can attack DNA to cause DNA damage, *e.g.* ^{8}OH-deoxyguanline. When $OH^\bullet$ reacts with a polyunsaturated fatty acid (PUFA), a hydrogen atom is abstracted from a methyl group ($-CH_2-$) leaving behind an unpaired electron on the carbon atom ($-HC^\bullet-$). PUFA carbon-centred radical tend to become stabilized by undergoing electronic rearrangement to form a conjugated diene radical which can react with O_2 to form a peroxyl radical ($-COO^\bullet-$). A peroxyl radical is then capable of abstracting hydrogen from another lipid molecule creating a cycle of lipid peroxidation with the formation of a lipid hydroperoxide product (-CHOOH-) with final products including malodialdehyde (MDA) (Fig. 1.4). Free radicals also attack proteins in both the cytoplasm and cell membranes leading the generation of protein carbonyls. However, cells possess protective mechanisms (micronutrient antioxidants and scavenging enzymes) to prevent the occurrence of free radical induced injury. The major defence systems include the non-enzymatic metabolites including vitamins C and E, β-carotene,

reduced glutathione and metal sequestering agents (ferritin, transferrin), and enzymatic systems including superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHpx) and glutathione reductase (GSSGrd).

The thiol-containing tripeptide glutathione (GSH), present in all cells especially in organs with high metabolic turnover, together with GSHpx and GSSGrd represents one of the most important antioxidant defence systems. GSH is capable of oxidising H_2O_2 and lipid hydroperoxides, forming the oxidised form of glutathione (GSSG). GSSG is then reduced to GSH by the action of GSSGrd. This regeneration system prevents depletion of intracellular thiols and requires NADPH (Fig. 1.4).

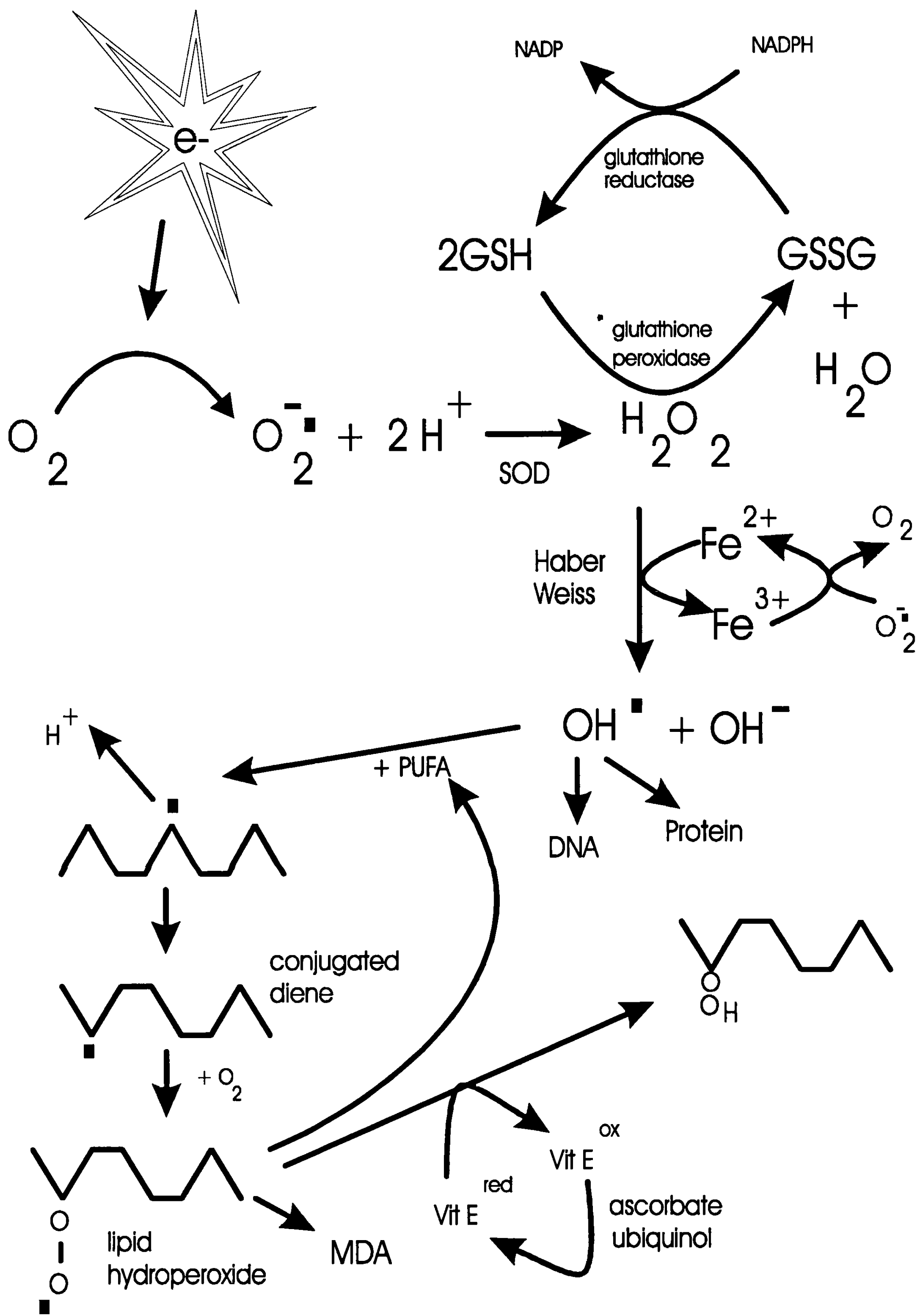


Fig. 1.4 Oxidative stress and cellular defence mechanisms. Reproduced by kind permission of Dr. J. M. Cooper.

There is increasing evidence that oxidative stress in PD substantia nigra suggesting free radical mediated damage plays a role in nigral cell death (Dexter *et al.* 1989, 1990, 1991 and1992). The origin of free radical generation could be either exogenous or endogenous.

1.5.2 Evidence of oxidative stress in PD

The basal ganglia are believed to be particularly vulnerable to free radical generation (Olanow, 1992). It has a high lipid content and high levels of metals, such as iron and copper which in the free form may catalyse the decomposition of H_2O_2 to the highly reactive $OH^{\bullet}$. One of the antioxidant enzyme systems, catalase, is virtually absent in the brain (Chance *et al.* 1979) but Cu/Zn SOD, which catalyses the conversion of the superoxide radical to H_2O_2, has been reported to be increased in PD substantia nigra (Saggu *et al.* 1989). Furthermore, the nigral dopaminergic cell seems to be extremely susceptible to radical processes. As indicated above, it has a high dopamine turnover and dopamine autooxidation processes leading to the production of free radicals and neuromelanin which have been shown to bind iron with a high affinity (Ben-Shachar *et al.* 1991).

A role for oxidative stress in the pathogenesis of PD has been postulated (Jenner *et al.* 1992; Jenner and Olanow, 1996). Indeed, post-mortem studies of PD substantia nigra support this hypothesis. Evidence includes decreased GSH levels (Riederer *et al.* 1989; Sian *et al.* 1994a), increased total iron levels (Riederer *et al.* 1989; Dexter *et al.* 1992), decreased ferritin content (Dexter *et al.* 1990 and 1991), decreased copper levels, and an increase in zinc content (Dexter *et al.* 1991), increased levels of malondialdehyde (a lipid peroxidation product) (Dexter *et al.* 1986 and 1989), decreased levels of polyunsaturated fatty acid (Dexter *et al.* 1989), and alterations in the antioxidant protective systems, such as SOD (Saggu *et al.* 1989), in the substantia nigra in PD. The increased free iron could facilitate decomposition of lipid peroxides and H_2O_2, leading to the formation of reactive oxygen species such as hydroxyl radicals and peroxyl radicals which could contribute to cell damage. To combat this,

excess iron is bound to ferritin, which is a protein for storage. Consequently any variations in iron levels should be reflected by variations in ferritin levels.

Ferritin levels have been reported to be increased (Riederer *et al.* 1989), decreased (Dexter *et al.* 1991) and unchanged (Mann *et al.* 1994) in PD substantia nigra. Consequently it is not clear what effect the increased iron levels would have upon the level of oxidative stress in PD substantia nigra. In addition, increased iron levels are not specific to PD substantia nigra, and have also been observed in the substantia nigra of MSA and PSP patients (Dexter *et al.* 1991).

Levels of the antioxidant vitamins, such as α-tocopherol (Dexter *et al.* 1992) and ascorbate (Riederer *et al.* 1989) are both normal in the PD substantia nigra. One of the most important antioxidant defence systems in the brain is the glutathione recycling system. This system reduces H_2O_2 and other peroxide products, thereby preventing the iron catalysed decomposition of H_2O_2 to the highly reactive $OH^{\bullet}$. Perry and Young (1986) found decreased total GSH+GSSG in the substantia nigra of PD which has since been supported by other research groups (Sian *et al.* 1994a; Riederer *et al.* 1989b). The loss of GSH was selective to the substantia nigra, specific to PD, and not linked to drug therapy. The cause of GSH depletion in PD substantia nigra is unknown but could be related to alteration in its synthesis, utilisation or degradation. Increased γ-glutamyltranspeptidase activity (Sian *et al.* 1994b) suggested that increased efflux of GSH mainly from glia, promoted by γ-glutamyltranspeptidase, may result in the decreased GSH levels. These changes were not a reflection of nigral cell death because they were not evident in the substantia nigra in MSA patients with similar cell loss in this area, nor were they due to the L-dopa therapy, since MSA patients were also treated with this drug.

1.5.3 Possible origins of free radicals in PD

1.5.3.1 Evidence for exogenous free radicals generation in PD

MPP^+

As described previously, MPP^+ is a neurotoxic metabolite of MPTP converted via the action of MAO-B. The mechanism by which MPP^+ kills dopaminergic neurons was clarified by the discovery that MPP^+ causes disruption of ATP production by inhibiting the oxidation of mitochondrial NAD-linked substrates (Ramsay *et al.* 1986). The decreased ATP synthesis may not be the only effect of MPP^+ inhibition on complex I activity. There is also a body of evidence that suggests the involvement of free radicals in the pathogenesis of MPTP-induced cell death. Free radicals may be produced directly during the redox cycling between $MPDP^+$ and MPP^+ and indirectly via the inhibition of complex I function (see section 1.3.3.1).

Mouse brain mitochondria incubated with MPTP induced superoxide formation (Rossetti *et al.* 1988) and transgenic mice overexpressing the Cu/Zn SOD gene were resistant to MPTP (Przedborski *et al.* 1992) suggesting that oxidative stress contributes to MPTP toxicity. Although MPP^+ is a reversible inhibitor of complex I, prolonged incubation of mitochondria with MPP^+ under reducing conditions caused a progressive irreversible inhibition of complex I activity which could be protected by the addition of antioxidants such as ascorbate, glutathione or catalase (Cleeter *et al.* 1992).

There are conflicting reports as to whether oxidative stress plays a primary role in MPTP/MPP^+-induced neuronal loss. There was a report suggested that impairment of cellular function by MPP^+ was not due primarily to increased production of free radicals by electron transport chain, but is more likely to be due to impaired capacity for ATP production caused by specific binding of MPP^+ to the rotenone-sensitive site of mitochondrial complex I (see section 1.5.2), which in turn decreased rather than increased oxidative stress (Bates *et al.* 1994).

There is increased evidence that nitric oxide synthase (NOS) may play an important role in MPTP/MPP^+ induced neuronal death. A study showed that a

selective inhibitor of neuronal NOS, 7-nitroindazole, protected against MPTP-induced dopamine depletion in mice (Schulz *et al.* 1995), which was further supported by Hantraye *et al.* who reported that 7-nitroindazole also protected against dopamine depletion and loss of tyrosine hydroxylase positive neurons in the substantia nigra in baboons (Hantraye *et al.* 1996). More recently, there is evidence that 7-nitroindazole may act by inhibiting MAO-B, therefore preventing MPTP toxicity by inhibiting MPP^+ synthesis (Di Monte *et al.* 1997). However, the role of NOS and NO in the toxicity of MPTP/MPP^+ was confirmed and extended by showing that neuronal NOS knockout mice were resistant to MPTP neurotoxicity (Przedborski *et al.* 1996).

L-Dopa

L-dopa is the most effective and commonly used drug treatment for PD, but it has been suggested that it may contribute to the side-effects of PD. Oxidation of dopamine by MAO-B results in the production of H_2O_2, an active oxygen species which can decompose to give the highly reactive hydroxyl radicals which may cause oxidative damage and accelerate nigral cell death in PD brain (Cohen, 1990). L-dopa was toxic to neuroblastoma cell cultures, a process which was partially protected by free radical scavengers, such as ascorbic acid (Pardo *et al.* 1993), indicating the involvement of free radicals. It has also been shown that L-dopa can produce a mild complex I defect in rat nigra, which was also protected by free radical scavengers (Przedborski *et al.* 1993), implying that L-dopa treatment for PD may involve free radical generation and exacerbate an underlying complex I abnormality.

1.5.3.2 Evidence for endogenous free radicals generation in PD

One of the most important biological source of free radicals in vivo is the electron transport chain in the mitochondrial inner membrane. Mitochondria could produce superoxide anions at two sites of the electron transport chain: The first site of superoxide anion formation has been suggested to be via NADH dehydrogenase (complex I) (Turers and Boveris, 1980) while the second site is via complex III or ubiquinone (Turrens *et al.* 1985).

The MRC has been known for some time to be a significant source of cellular superoxide as single electrons leak from the respiratory chain to molecular oxygen (Boveris and Chance, 1973). When the MRC is inhibited by rotenone (Takeshige and Minakami, 1979) or MPP^+ (Hasegawa *et al.* 1990) there is a marked increase in superoxide generation. However, a report suggested that while toxin inhibition of complex I may result in lipid peroxidation, superoxide originated from the interaction of the inhibitor with a separate site either within the complex or elsewhere within the MRC (Ramsay and Singer, 1992).

1.5.4 Oxidative stress in other neurodegenerative diseases

AD

The mechanisms responsible for neuronal loss in AD remain unknown. There is some evidence that oxidative stress may contribute to the pathogenesis of AD (Markesbery, 1997). This hypothesis was based on the toxicity of β-amyloid and the finding that aggregation of β-amyloid can be induced by metal-catalysed oxidation and that free radicals may involved in the APP metabolism (see section 1.2.2.2).

Several studies suggested that increased iron levels and lipid peroxidative products in corticies (LeVine, 1997), inferior temporal cortex (Palmer and Burns, 1994) and in the ventricular fluid (Lovell *et al.* 1995) and elevated levels of hydroxyl radical, decreased SOD in blood (Ihara *et al.* 1997) and catalase activity in parietotemporal cortex, basal ganglia and amygdala (Gsell *et al.* 1995) from AD patients. As for oxidative protein damage, there was an increased level of protein carbonyls, a general assay of oxidative protein damage, in the parietal lobe of AD (Lyras *et al.* 1997). As for oxidative DNA damage, there were increased levels of some (including 8-hydroxyadenine and 8-hydroxyguanine) but not all oxidized DNA bases in parietal, temporal, occipital, and frontal lobe, superior temporal gyrus, and hippocampus in AD brain (Lyras *et al.* 1997). The findings of increased oxidative damage to lipids, proteins and DNA strengthen the possibility that oxidative damage may play a role in the pathogenesis of AD in at least some key brain regions. However, because tissue injury itself can induce free radicals generation, it is not known whether this is a primary or secondary event.

DLB

There is not much evidence to support the involvement of oxidative stress in the pathogenesis of DLB. The levels of iron, copper, manganese, or zinc, and ferritin in the substantia nigra and other brain areas were unaltered (Dexter *et al.* 1994).

MSA

Increased iron levels in the striatum but not in the substantia nigra (Dexter *et al.* 1992) and normal GSH levels (Sian *et al.* 1994a) were observed in the cerebral cortex, caudate nucleus, putamen, globus pallidus and substantia nigra in the brain from MSA patients. However, up to now, there is little evidence to support the involvement of oxidative stress in the pathogenesis of MSA.

HD

A few studies have looked at evidence for oxidative stress in HD brain. Reduced GSH levels were found in HD cortex, caudate and substantia nigra, and oxidized glutathione levels were increased significantly in caudate (Sian *et al.* 1994a). In a recent study, the level of 8-hydroxydeoxyguanosine was increased in HD caudate and cytosolic Cu/Zu SOD activity was mildly decreased in HD parietal cortex and cerebellum, but not in the striatum or frontal cortex. Mitochondrial Mn-SOD activities were unchanged in all regions studied (Browne *et al.* 1997).

1.6 Genomic transplantation (fusion)

1.6.1 The rho zero (ρ^o) cells

Early studies on human cells with ethidium bromide (EB), an inhibitor of mtDNA replication, demonstrated a 10-fold reduction in mtDNA content after 3 cell doublings at a concentration of 20ng/ml of EB (Wiseman and Attardi, 1978). Subsequently, there was a report showed a complete depletion of mtDNA in chicken embryo fibroblasts following long-term exposure to 400ng/ml of EB (Desjardins *et al.* 1985). However, the cells had to be supplemented with media containing pyruvate and uridine. By modifying the

protocol of Desjardin *et al.* (1985), King and Attardi generated the first human cell line depleted of mtDNA from a human osteosarcoma cell, named 143B.206ρ° cells (206ρ°) (King and Attardi, 1988). Such ρ° cells did not have cellular MRC function, with less than 5% of the O_2 consumption of the parent cells, and relied on pyruvate and uridine supplement media for survival. Because of their deficiency in thymidine kinase activity and inability of converting bromodeoxyuridine (BrdU) to toxic products, these ρ° cells were resistant to BrdU. Other ρ° cells have been generated in similar ways including A549.B2 ρ° (A549ρ°) (Holt, personal communication) and SHSY5Yρ° cells (MitoKor Corporation, San Diego, CA). These A549ρ° cells also rely on pyruvate and uridine supplement media for survival, and because they have a geneticin resistant gene, they can grow in a medium containing geneticin.

1.6.2 ρ° cell fusion studies

Using ρ° cells, it is possible to transfer mitochondria into a new cellular environment, either by directly microinjecting isolated mitochondria into ρ° cells (King and Attardi, 1988) or fusing the ρ° cells with cytoplasts (*i.e.* enucleated cells) from the mtDNA donor cell line to form cybrids (*i.e.* cytoplasmic hybrids) (Fig 1.5).

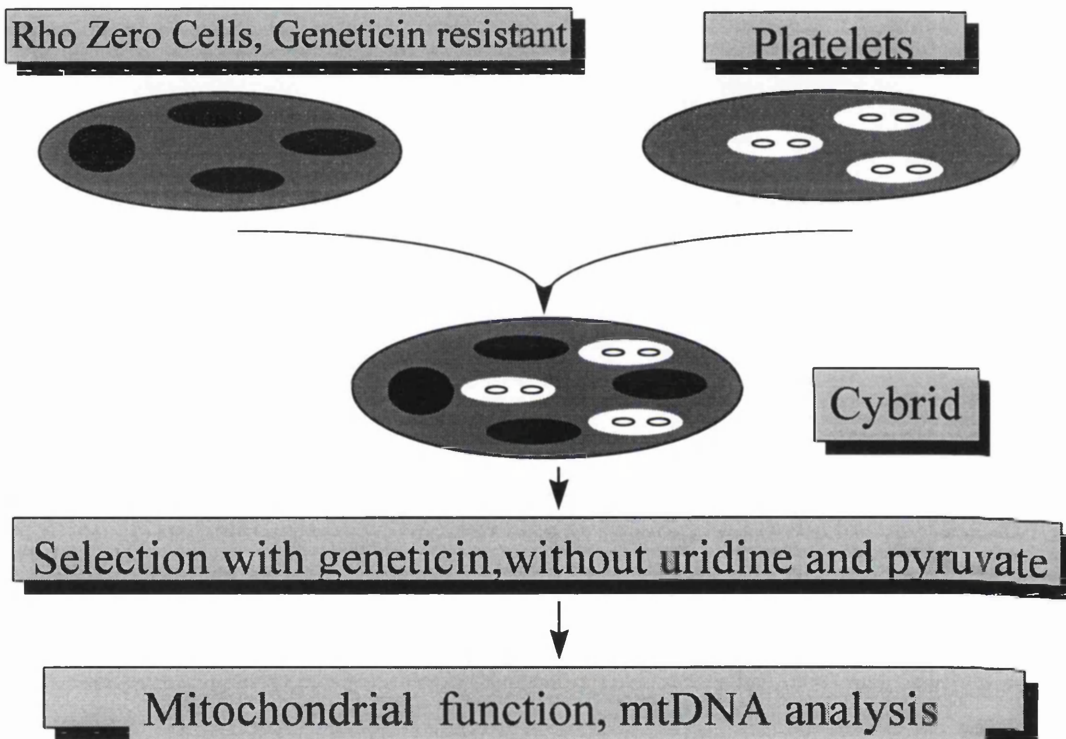


Fig 1.5 Scheme for performing a fusion study

This *in vitro* selective system has been a very useful model to study mtDNA diseases. Mitochondria from patients who have a specific mtDNA mutation can be used to repopulate ρ° cells generating cybrids which have been used for two main proposes: firstly, to help localise any MRC defect to a mutation of mtDNA or suggest a nuclear DNA mutation or combination of both; secondly, to generate cell lines containing various proportions of mutant and wild-type mtDNA or either purely mutant or purely wild-type genome, therefore to investigate the molecular mechanism of the diseases.

The ρ° cell fusion system has been used to investigate several mitochondrial disorders, including point mutations A3243G (King *et al.* 1992; Chomyn *et al.* 1992; Yoneda *et al.* 1992; Dunbar *et al.* 1996), A8344G (Chomyn *et al.* 1994), 11778 (Hofhaus *et al.* 1996), deletion (Hayashi *et al.* 1991) and a mitochondrial DNA depletion disorder (Bodnar *et al.* 1993; Taanman *et al.* 1997).

Transfer of the mitochondria in myoblast cell from a MERRF patient to a recipient 206ρ° cell line allowed the establishment of the mutant mtDNA in permanent culture. Chomyn *et al.* (1992) reported that altered mitochondrial protein synthesis and respiratory deficiency in the transforming clones provided strong evidence that the mtDNA mutation was the cause of the MRC defect, which was shown to be an A to G at 3243bp.

In order to investigate the mitochondrial segregation of mutant and wild-type mtDNA in patients with the 3243bp mutation, Yoneda *et al.* (1992) used enucleated myoblasts from a patient with MELAS harbouring the A3243G mutation to fuse with 206ρ° cells and isolated the cybrids. They showed that 5 of 13 cybrids containing mixtures of wild-type and mutant mtDNAs (heteroplasmic) underwent a rapid shift of their genotype towards the pure mutant type, but that the remaining cybrids maintained a stable genotype. These results suggested that the mutant mtDNA had a replication advantage which was responsible for the genotype shift observed in the unstable cell cybrids.

By using the platelet mediated mitochondria transfer procedure in patients harbouring that MERRF A8344G mutation, Chomyn *et al.* observed a certain variability in respiratory capacity among the MERRF platelet-derived 206ρ° cell transformants. This MRC function variation was unrelated to their mtDNA content, but was found not only to be related to the presence and amount of mitochondrial $tRNA^{Lys}$, but also reflected, at least in part, differences in nuclear gene content and/or activity among the original recipient cells (Chomyn *et al.* 1994). This experiment was performed in the platelets which is an ideal natural enucleated cytoplast for fusion studies. The procedure is simple, quick, economic and efficient, but only useful if the mtDNA mutation is present or likely to be present in blood (platelets).

Recently, a report suggested that MRC complex I and IV deficiency was associated with the MELAS A3243G mtDNA mutation expressed in 206ρ°-A3243G cell cybrids. Cybrids with various proportions of the mutant and wild-type mtDNA were selected, and the biochemical studies revealed a decrease in oxygen consumption with pyruvate in cybrids harbouring 60-90% mutant mtDNA. Greater deficiencies in complex I and IV activity were observed in a cybrid containing 95% mutant mtDNA. *In vitro* translation demonstrated that these cybrids had decreased synthesis of a number of mitochondrial complex I polypeptides such as ND6 (Dunbar *et al.* 1996).

Hofhaus *et al.* (1996) established a cytoplast fusion model between skin fibroblasts from a patient with the LHON-A11778G mutation and 206ρ° cells. They observed mitochondrial DNA-linked biochemical defects underlying the LHON-A11778G phenotype in a foreign nuclear environment and demonstrated that several cybrids harbouring the LHON-A11778G mutation, which is in the gene encoding ND4 of complex I, did not affect the synthesis, size, or stability of ND4, nor its incorporation into the enzyme complexes. Complex I activity was approximately 40% decreased in digitonin-permeabilized cells determined by polarography in these cybrids, but there was no decrease in rotenone-sensitive NADH dehydrogenase activity. The authors suggested that this was a reflection of the NADH possibly delivered directly to the binding site closely associated with complex I. The 11778bp

ND4 mutation could conceivably interfere with the interaction of complex I with NAD-linked dehydrogenases, and therefore with the channelling of NADH to complex I and consequently cause a decrease in polarography due to decreased NADH level, but this effect would not be observed in the enzyme assay as excess NADH is added to the mitochondrial fraction (Hofhaus *et al.* 1996).

To exclude the involvement of the nuclear genome in the expression of the mitochondrial dysfunction, characteristic of CPEO, Hayashi *et al.* (1991) introduced mtDNA, with a large-scale deletion from a CPEO patient, into a mtDNA-less HeLa cell (HeLaρ°). The results showed that the cybrids containing higher levels of deleted mtDNA correlated well with a slower cellular growth rate. In these cybrids, if deleted mtDNA was less than 60% of the total mtDNA, a translational complementation of missing tRNA occurred. However, if the deleted mtDNA was more than 60%, an inhibition of overall mitochondrial translation resulting in a decrease in complex IV activity was observed due to competition for the remaining tRNAs. This suggested that deletion of mtDNA alone was sufficient to cause the mitochondrial dysfunction in CPEO.

Bodnar *et al.* (1993) described two siblings with mtDNA depletion and a severe decrease of respiratory chain activity in cultural fibroblasts. The cells were enucleated and fused with A549ρ° cells, thereby placing mitochondria with depleted levels of mtDNA in a controlled nuclear environment. Interestingly, a restoration of mtDNA levels as well as recovery of MRC function were observed in these cybrids. To confirm this observation, a reverse experiment was performed, where normal mitochondria were inserted into the patient's cells. Cells failed to grow which helped to confirm the involvement of the nuclear genome in the expression of the defect in this patient. Although the localisation of the abnormality of a nuclear-encoded factor has yet to be identified, it has been suggested that either a factor involved in the regulation of mtDNA copy number or mtDNA replication may be involved (Bodnar *et al.* 1993). More recently, this fusion strategy has been used to show that the nuclear influences on the biochemical expression of complex I defect in

cybrids with the A3460G mtDNA mutation of LHON's disease (Cock *et al.* 1998).

1.7 Apoptosis

1.7.1 Apoptosis

Apoptosis has been applied to a mechanism of cell death which is controlled by cell suicide machinery. Most cells have the ability to undergo apoptosis. The dying cell is phagocytosed by neighbouring cells thus the unwanted cell is removed cleanly without releasing its potentially harmful contents.

Apoptosis differs from random cell necrosis in several biochemical and morphological features, not least of which is the initiation of the apoptotic pathway which is regulated by genetic events (Ellis *et al.* 1991). Morphologically, apoptosis is characterised by nuclear and cytoplasmic condensation (shrinkage) followed by the loss of the nuclear membrane, the fragmentation of the nuclear chromatin, and the subsequent formation of multiple fragments of condensed nuclear material and cytoplasm known as apoptotic bodies (Cotter *et al.* 1992). Biochemically, the pathogenesis of apoptosis involves nuclear chromatin fragmentation which involves activation of endonuclease(s) resulting in cleavage of DNA at linkage regions between nucleotides to form fragments of double-stranded DNA at multiples of 180-200bp. DNA degradation in this fashion gives a 'ladder' pattern on agarose gel electrophoresis (Wylie, 1980). However, apoptosis can occur without degradation so that lack of a ladder does not necessary demonstrate that apoptosis has not occurred. Necrosis, an alternative mechanism of cell, involves uncontrolled cellular swelling followed by rupture of the plasma membrane. The nuclear changes occur at a later stage and DNA degradation tends to be non-specific. The release of cellular contents to the surrounding environment results in an inflammatory response which often causes further damage (Buja *et al.* 1993).

There are two kinds of process that can lead to apoptosis, one involving cell cycle perturbation and another the activation of cytokine receptors (Thompson, 1995).

1.7.2 Cell cycle and apoptosis

1.7.2.1 Cell cycle

A typical eucaryotic cell cycle consists of four successive phases: mitosis (M), gap 1 (G1), DNA synthesis (S) and gap 2 (G2). Interphase starts with the G1 phase. The S phase begins when DNA synthesis starts, and ends when the DNA content of nucleus has doubled and the chromosomes have replicated. The cell then enters the G2 phase, continuing until mitosis starts, which initiates the M phase. During the M phase, the replicated chromosomes condense and the nuclear envelope breaks down, the sister chromatids separate, two new nuclei form, and the cytoplasm divides (cytokinesis) to generate two daughter cells. The daughter cells then enter the interphase of a new cycle.

The crucial features of the cell cycle are the existence of two transition control points, at the G2/M boundary and during the G1 phase. The M phase is characterised by activation of a kinase which is regulated by phosphorylation and dephosphorylation. All cell cycle transitions are initiated by a clock apparatus, which receives a wide variety of growth-controlling signals. By integration and processing these signals, the 'clock' responds and cells undergo a growth cycle or enter a quiescent growth state (G0). Damage to genomic DNA, such as ionising radiation, prolonged hypothermia, toxic agents exposure, viral infections *etc*, can generate a negative feedback, resulting in the shutdown of the clock's forward motion, and consequently inducing apoptosis (Willingham and Bhalla, 1994).

1.7.2.2 Cytoskeleton and apoptosis

The cytoskeleton is a complex network of protein filaments that extends throughout the cytoplasm. The activities of the cytoskeleton depend on three principal types of protein filaments: actin, microtubules and intermediate filaments. Actin is the most abundant protein in many eucaryotic cells and its main function is the determination of cell surface movements. Microtubules can regulate cell shape, control cell movement and regulate the cell division by forming the mitotic spindle. The assembly of microtubules is affected by drugs

that bind to tubulin. Colchicine binds tightly to tubulin preventing tubulin polymerization causing the disappearance of the mitotic spindle thus blocking the cell in mitosis. There is evidence that colchicine induces the cytoskeleton alterations, directly initiating the genetic and structural modifications that result in apoptotic cell death of rat cerebellar granule cells (Bonfoco *et al.* 1995) and pheochromocytoma (PC12) cells (Lindenboim *et al.* 1995). The drug taxol has the opposite effect. It binds to microtubules and stabilizes them, causing most of the free tubulin to assemble into microtubules resulting in cell cycle blockage at mitosis. Consequently, interfering in cytoskeletal function itself results in apoptosis (Willingham and Bhalla, 1994; Wahl *et al.* 1996).

1.7.3 Cytokine and apoptosis

The process of apoptosis is regulated by signals generated when cytokines bind to their receptors. There are two types of cytokine-induced signals. The first is an inductive signal that initiates apoptosis. The second is an inhibitory signal that suppresses apoptosis (Nagata, 1997).

Apoptosis proceeds through a group of proteases called caspases, termed Cytosolic aspartate-specific cysteine proteases, which cleave vital intracellular proteins (Alnemri *et al.* 1996; Nagata, 1997). Caspases are inactive until a signal initiates activation of one, starting a cascade in which a series of other caspases are proteolytically activated (Nagata, 1997).

1.7.4 The genes and gene products in apoptosis

Bcl-2

The bcl-2 proto-oncogene is a unique cellular gene because of its ability to block apoptotic death induced by a variety of stimuli (Korsmeyer, 1992). The bcl-2 gene was first discovered in B-cell malignancies, where chromosomal translocations activated the gene in the majority of follicular B-cell lymphomas (Tsujimoto *et al.* 1984). In these translocations t(14;18)(q32;q21), the bcl-2 gene was moved from its normal chromosomal location at 18q21 into juxtaposition with a powerful enhanced element in the immunoglobulin heavy-chain (IgH) locus at 14q32. Vaux and colleagues first observed that bcl-2 can prolong immature pre-B-cell survival in the absence of interleukin-3, which is

an essential factor for maintenance of cell survival *in vitro* (Vaux *et al.* 1988). By microinjection of bcl-2 expressing plasmids into neurotrophic factor-dependent CNS-derived sensory neurons, the rate of apoptotic cell death was markedly delayed without this growth factor (Allsopp *et al.* 1993). Jacobson *et al.* (1993) initially demonstrated that a transformed human fibroblast cell line did not require protein synthesis for apoptosis induced by staurosporine, but the overexpression of bcl-2 was capable of protecting the cells in both the presence and absence of mtDNA. To date, the induction of apoptosis by diverse stimuli such as radiation, hypothermia, growth factor withdrawal, glucocorticoids and multiple classes of chemotherapeutic agents, can be inhibited by Bcl-2 *in vitro* models (Hockenbery *et al.* 1993; Hockenbery, 1995).

The Bcl-2 protein is associated with intracellular membranes including the mitochondrial membrane (Hockenbery *et al.* 1990, 1993 and Hockenbery, 1995). In addition, Bcl-2 has also been localised to the nuclear membrane (Monaghan *et al.* 1992; Hockenbery, 1995).

The mechanism by which Bcl-2 prevents apoptosis is not clear. Two possible mechanisms of Bcl-2 action have been proposed by Hockenbery and co-workers: (1) Bcl-2 acts as a signalling mechanism that might activate downstream genes or enzymes leading to damage; (2) Bcl-2 is a crucial target being directly damaged by oxidative stress. It has been proposed that Bcl-2 regulates an antioxidant pathway at sites of free radical generation (Hockenbery *et al.* 1993). As an antioxidant Bcl-2 may act after the generation of $O_2^{-\bullet}$ and its conversion to peroxides to prevent cellular damage including lipid peroxidation (Hockenbery *et al.* 1993). One possibility for the function of Bcl-2 would be as a free radical scavenger serving as a non-reactive free radical trap, *i.e.* sacrificed target. Another intriguing observation is that Bcl-2 may function as a pro-oxidant regulating levels of reactive oxygen intermediates and controlling early entry into apoptosis (Steinman, 1995). However, it remains controversial whether the antioxidant-like effect of bcl-2 expression is a requisite property for its anti-apoptotic effect, since bcl-2 expression has also been shown to inhibit hypoxia-induced apoptotic death (Shimizu *et al.* 1995; Jacobson and Raff, 1995). Hockenbery *et al.* (1993)

proposed a model in which Bcl-2, through protein-protein interactions would focus as well as regulate an antioxidant pathway at the selective sites of reactive oxygen species generation. However, it is unknown whether the effect of bcl-2 expression requires interaction with other cellular proteins.

p53

A wide variety of genes and their products have been implicated in the control of the apoptotic process. The tumour suppresser gene, p53, is one of the most commonly mutated genes in human cancer, being inactivated in almost half of all tumours (Lane, 1992). The p53 gene product is a transcriptional activator regulating many targets including the cellular response to DNA damage as well as cell cycle progression. The role of p53 has been recognised in part by the inability for cells lacking functional p53 to arrest the cells in G1 phase after DNA damage. This arrest is believed to be necessary for DNA repair, to maintain chromosomal fidelity, and to increase survival in normal cells (Hartwell, 1992). In the absence of functional p53, there is an increase in uncontrolled cell cycling and tumourigenesis. In cells with a functional p53, if repair fails, p53 is involved in the induction of apoptosis to prevent the risk of uncontrolled cell cycling.

c-fos is a proto-oncogene that has been associated with a variety of biological responses such as cellular proliferation, differentiation and cancer. Prolonged *c-fos* expression is capable of inducing apoptosis in a variety of neuronal cell types which may suggest a potential role in neurodegenerative diseases such as AD and amyelotrophic lateral sclerosis (Smeyne *et al.* 1993). *c-myc* is the cellular homologue of a viral oncogene which has been shown to be involved in cellular proliferation and, in a mutated form, cancerous growth. Like *c-fos*, when expressed to abnormal levels, *c-myc* is capable of causing cell death (Evan *et al.* 1992).

1.7.5 Mitochondria and apoptosis

Since 1994, it has become clear that even enucleated cells can undergo apoptosis and that non-nuclear cytoplasmic structures participate in the control of apoptosis (Jacobson *et al.* 1994). This observation raised the question as to

whether or not mitochondria play a important role in the control of apoptosis. Indeed, cells undergo changes in mitochondrial structure and function well before nuclear or chromatin condensation (Vayssiere *et al.* 1994; Petit *et al.* 1995). Before cells exhibit common signs of nuclear apoptosis, they undergo a reduction of the mitochondrial transmembrane potential ($\Delta\psi_m$) that may be due to the opening of mitochondrial permeability transition (PT) pores (Zamzami *et al.* 1996). These pores are multiprotein complexes that are present at sites where the inner mitochondrial membrane contacts the outer mitochondrial membrane. The relationship between the transition pores and members of the Bcl-2 family remain to be determined. However, several pieces of evidence suggest that bcl-2 product inhibits apoptosis by preventing PT and over-expression of Bcl-2 directly abolishes the induction of mitochondrial PT (Zamzami *et al.* 1996). Under normal conditions, the inner mitochondrial membrane is quasi-impermeable for small molecules, thus facilitation the electrochemical gradient which is indispensable for mitochondrial function. However, opening of PT pores or "megachannels" allows for the free distribution of solutes of <1500 Dalton and of some proteins, thereby disrupting the $\Delta\psi_m$ (Bernardi *et al.* 1994). The opening of the mitochondrial PT has been suggested to constitute a critical early event of the apoptotic process (Petit *et al.* 1995; Zamzami *et al.* 1996).

Other observations have shown that cells without mtDNA undergo apoptosis induced by staurosporine, a protein kinase C inhibitor, or withdrawal of serum (Jacobson *et al.* 1993). Marchetti *et al.* (1996) also demonstrated that mtDNA was not important for the regulation of apoptosis although they suggested that mitochondria may be involved in apoptosis. Based on these observations, it has been assumed that mtDNA and by extension mitochondria might not be involved in the regulation of apoptosis in all cases (Marchetti *et al.* 1996).

There is evidence which suggests that inhibition of normal mitochondrial channel function causes the mitochondrial matrix to swell. Swelling of the matrix extends the inner membrane, which then appears to cause rupture of the outer membrane, releasing cytochrome c into the cytosol, and inhibition of Bcl-2 activity leads to this release (Reed, 1997). In the cytosol, cytochrome c is

bound by Apaf-1, a protease activating factor, which binds and triggers the activation of caspases, which then accelerates apoptosis by activating other capases (Li *et al.* 1997).

1.7.6 MRC inhibition and apoptosis

Several toxins have been identified to inhibit MRC function. As mentioned in section 1.3.2.1-1.3.2.4, these toxins include the complex I inhibitors, rotenone, piericidin A and MPP^+, the complex III inhibitor, antimycin A, and the complex IV inhibitors, cyanide and azide. There are several reports suggesting that apoptosis plays a role in cell death induced by MRC inhibition with MPP^+ (Hartley *et al.* 1994), rotenone or antimycin A (Wolvetang *et al.* 1994). Rotenone and antimycin A have been reported to induce apoptotic death in cultured human lymphoblastoid cells but not in cells depleted of mitochondrial DNA (ρ° cells) (Wolvetang *et al.* 1994) indicating apoptosis results specifically from their effects upon MRC function.

2.1 Materials

2.1.1 Equipment

Unless otherwise stated, the following equipment was used.

Spectrophotometers (split-beam)

Hitachi U-3210 (Hitachi Scientific Instruments, Wokingham, Berks, UK) and Kontron Uvikon 940 (Kontron Instruments, Watford, Herts, UK) split-beam spectrophotometers.

Centrifuges

Beckman GPR bench-top centrifuge with GH-3.7 horizontal rotor (Beckman Ltd, High Wycombe, Bucks, UK.), Kontron T-124 high-speed centrifuge with A 8.24 8x50ml fixed angle rotor (Kontron Instruments, Watford, Herts, UK) and Biofuge 13 with 18x1.5ml fixed angle rotor (Heraeus, Germany)

Cell and tissue homogenisers

Uni-form 5ml and 10ml glass/teflon homogeniser (Jecons Ltd., Leighton Buzzard, Bedfordshire, UK.), 5ml glass homogeniser and Glass-Col stirrer (CamLab Ltd., Cambridge, Cambridgeshire, UK.) and a cell disruption bomb (45ml volume) (Parr Intrument Co., Moline,Illinois, USA)

Cell and tissue cultural equipment

ICN-Flow Automatic CO_2 Incubator Model 320 (ICN-Flow, Ltd., High Wycombe, Bucks, UK.) and Envair Class II Microbiological Safety Unidirectional Laminar Cabinet (Envair Ltd., Rossendale, Lancashire, UK)

Molecular biological equipments

Hybaid OmniGene PCR machine (Hybaid Ltd., Middlesex, UK), Hybaid mini hybridisation oven (Hybaid Ltd., Middlesex, UK) and Infors-HT orbital shaker (Infors Ltd., Crewe, Cheshire, UK)

Electrophoresis

BioRad 200/2.0 constant voltage power packs (BioRad Lab. Ltd., Hemel Hempstead, Herts, UK), BRL horizontal system for agarose gel electrophoresis (Bethesda Res. Lab., Life Technological Inc., Gaithsburg, MD 20887, USA) and BioRad vertical system were used for polyacrylamide gel electrophoresis (BioRad Lab. Ltd., Hemel Hempstead, Herts, UK) and UV transilluminator (GRI Ltd., Dunmow, Essex, UK)

Microscopy

Zeiss axiophot fluorescence microscope with FITC and rhodamine filters (Carl Zeiss Microscope Division, Oberkochen, Germany), Zeiss 902A transmission electron mycroscope (Carl Zeiss Microscope Division, Oberkochen, Germany), MRC 600 confocal microscope (Bio-Rad Microscience Divission, Hertfordshire, UK) and Zeiss 902A electron microscope (Carl Zeiss Electron Microscope Division, Oberkochen, Germany)

2.1.2 Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma, Poole, Dorset, UK or Merck Ltd, Dagenham, Essex, UK.

2.2 Data analysis

The text was typed with Microsoft Word, figures were drawn using Microsoft Excel or Sigmaplot-40 software and statistical analysis was performed with *Instat2* software.

All the data obtained in this study did not necessarily exhibit normal distribution, therefore non-parametric ranking tests were used for the analysis of all data. The non-specific alternative hypotheses were applied to all analyses (2-tailed test). Differences between the groups of patient and controls were analysed by the Mann-Whitney U test for unpaired samples. Differences between values were considered to be statistically significant when $p<0.05$.

The chi-square test was performed when the data consisted of numbers of subjects classified by two discrete schemes.

The Spearman's linear regression module was used to analyse the relationship between two subjects and therefore to predict one variable as a function of another.

2.3 Patients

All samples were obtained with informed consent, except those used for post-mortem studies where tissues were donated to the Brain Bank, and the studies were performed with the approval of the Joint Ethics Committee of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology, London, UK.

2.4 Enzyme analyses

Enzymatic analyses of brain samples were performed at 27°C while platelet mitochondrial analyses were performed at 30°C, unless otherwise stated.

2.4.1 NADH-CoQ_1 oxidoreductase (complex I activity)

Determination of ubiquinone-1 (Coenzyme Q_1, CoQ_1) concentration: The CoQ_1 was a gift from the Eisai Chemical Co, Japan. The concentration of CoQ_1 was calculated as described (Redfearn, 1967). Briefly, a dilution of CoQ_1 stock was made in ethanol and the absorbance at 275nm of CoQ_1 was recorded. An excess of sodium borohydride was added to the reference cuvette to completely reduce the quinone to the quinol and the absorbance change used to calculated the CoQ_1 concentration using a molar extinction coefficient for CoQ_1 of 2.25×10^3.

Principle: Based on the method of Ragan, the assay measures the rotenone sensitive CoQ_1 dependent oxidation of NADH at 340nm (Ragan, 1987).

Assay: Two identical cuvettes were set up containing: 20mM potassium phosphate buffer, pH 8, 150μM NADH, 1mM KCN and sample (5-15μg protein for homogenised brain, or 0.5-1.5μg protein for platelet mitochondria) in a final

volume of 1ml. The reaction was initiated by the addition of CoQ_1 to give a final concentration of 50μM to the sample cuvette. The rate of NADH oxidation was monitored at 340nm. Rotenone was added to a final concentration of 10μM and the rotenone insensitive rate was measured. Complex I activity was defined as the rate which was sensitive to rotenone. A molar extinction coefficient (ε) for NADH of 6.81×10^3 was used to allow for the contribution of reduced CoQ_1 to the absorbance at 340nm. Enzyme activity was expressed as nmol NADH oxidised per minute per mg of protein (nmol/min/mg protein) or a ratio with citrate synthase (CS).

To optimise the complex I activity in platelet mitochondrial fractions, modified reagents were used: 20mM potassium phosphate buffer, pH 7.2 including 8mM $MgCl_2$ and 2.5mg/ml BSA (essentially fatty-acid free). The assay was performed as above.

2.4.2 Succinate cytochrome c oxidoreductase (complex II+III)

Principle: Based on the method of King (King, 1967), the assay determined the activity of complex II+III of the mitochondrial electron transport chain by detecting the succinate dependent reduction of cytochrome c at 550nm.

Assay: Two identical cuvettes were set up containing: 0.1M potassium phosphate buffer pH 7.4, 0.3mM EDTA potassium salt (K_2-EDTA), 0.1mM cytochrome c, 1mM KCN, 2.5mg/ml BSA in a final volume of 1ml. A mixture of sample, 40μl of 500mM sodium succinate and 10μl of 100mM KCN was pre-incubated at 30°C for 5mins to fully activate the enzyme. The complex II+III activity was calculated as the rate which was sensitive to the addition of antimycin A (final concentration 20μM). The molar extinction coefficient of cytochrome c, $\varepsilon = 19.2 \times 10^3$, was used. Enzyme activity was expressed as nmoles of cytochrome c reduced per minute per mg of protein (nmol/min/mg protein) or a ratio with CS.

2.4.3 Succinate-ubiquinol oxidoreductase (complex II)

Principle: Based on the method of Hatefi *et al.* (Hatefi *et al.* 1962). This enzyme activity was followed by measuring the reduction of the dye 6,6-

dichlorophenolindophenol (DCPIP) at 600nm in the presence of succinate and ubiquinone-2 (CoQ_2). Enzyme activity was taken as the rate which was inhibited by 2-thenoyltrifluoroacetone (TTFA).

Assay: Two identical cuvettes were set up containing 50mM potassium phosphate buffer pH 7.4, 0.1mM K_2-EDTA, 20mM sodium succinate, 74μM DCPIP, 1mM KCN, 10μM rotenone and sample in a final volume of 1ml. Ubiqunol-2 (50μM, preparation as section 2.4.4) was added to initiate the reaction which was then inhibited by the addition of 1mM TTFA. A molar extinction coefficient of DCPIP, $\varepsilon = 2.1 \times 10^4$, was used to calculate enzyme activity which was expressed as nmol of DCPIP reduced per minute per mg of protein (nmol/min/mg protein) or a ratio with CS.

2.4.4 Ubiquinol-cytochrome c reductase (complex III)

Principle: Based on the method of Birch-Machin *et al.*, this enzyme catalyses the oxidation of ubiquinol and reduction of cytochrome c which was monitored at 550nm (Birch-Machin *et al.* 1989). The rate of reaction is dependent upon the concentration of both ubiquinone-2 and cytochrome c, and therefore the concentration of these must be consistent and determined prior to assay.

Preparation of ubiquinol-2: 10mM of ubiquinone-2 (ε=12.25mM) was prepared in ethanol and acidified to pH 2 with 1-2μl 1M HCl. A few granules of sodium borohydride and 1ml of H_2O were added to reduce quinone to the quinol. The quinol was extracted into 3ml of diethylether:cyclohexane (2:1, v/v) and the upper phase was collected. 1ml of 2M NaCl was added, and the upper phase (diethylether) collected again which was evaporated to dryness under a stream of nitrogen gas. Finally the residue was dissolved in 1ml of ethanol, acidified to pH 2 with HCl and 100μl aliquots were stored at -20°C under nitrogen gas.

Determination of ubiquinol-2 concentration: This was performed by measuring the amount of ubiquinol-2 oxidised by the addition of potassium hydroxide (KOH). 10μl of 5M KOH were added to 10μl of ubiquinol-2 and 980μl of ethanol and the increase in absorbance, due to oxidation of ubiquinol-2 to ubiquinone-2, was used to calculate ubiquinol-2 concentration using ε=12.25.

Determination of cytochrome c concentration: This was based on the amount of cytochrome c that can be reduced by ascorbate. Approximately 15μM of cytochrome c were added into both cuvettes and a few granules of ascorbate were placed in the reference cuvette. The absorbance change was noted and the concentration of cytochrome c was calculated (ε=19.2mM).

Assay: The mixture consisted of identical cuvettes containing (final concentrations): 35mM potassium phosphate buffer pH 7.2, 1mM K_2 - EDTA, 5mM $MgCl_2$, 2mM KCN, 5μM rotenone, 15μM cytochrome c. Ubiquinol-2 (15μM) was added to the test cuvette to initiate the reaction which was recorded at 550nm for 5mins.

Non-enzymatic reduction of cytochrome c: There was a non-enzymatic reduction of cytochrome c, which was dependent upon the concentrations of both ubiquinone-2 and cytochrome c. This rate was determined prior to the analysis of the samples and was subtracted from the observed enzyme rate. Identical cuvettes were set up containing all assay solutions except ubiquinol and sample. The non-enzymic rate was determined by the addition of ubiquinol-2 into the test cuvette and the rate of absorbance change was noted.

Calculation of the pseudo first order rate constant (k): The calculations were performed by extrapolation of the absorbance back to time=0 and determination of the change in absorbance at various time points up to 2mins. The non-enzymatic rate of absorbance change was subtracted from this value and k/min/ml calculated as:

k=[ln0.288-ln(0.288-change in absorbance at time t) x 1000/sample volume(μl) x dilution factor], where 0.288 is the absorbance of cytochrome c fully reduced.

The k/ml values for 5 time points (0, 0.5, 1.0, 1.5 and 2.0mins) were plotted against t and the gradient of this line calculated as k/min/ml using linear regression analysis. Results were expressed as k/min/mg protein.

2.4.5 Cytochrome c oxidase (complex IV)

Principle: The assay was based on the method of Wharton, which monitored the oxidation of reduced cytochrome c at 550nm (Wharton and Tzagoloff, 1967).

Preparation of reduced cytochrome c: A 1% (w/v) solution of cytochrome c (horse heart, Boehringer Mannheim Ltd, Lewes, Sussex, UK) was fully reduced by the addition of an excess of ascorbate (about 12mg in 100ml of 10mM potassium phosphate buffer). The reduced cytochrome c solution was transferred to size 1 dialysis tubing (Medicell International Ltd. London, UK) and dialysed against 5L of 10mM potassium phosphate buffer pH 7.0 at 4°C for 1-3hrs to remove the ascorbate. To check that no excess ascorbate remained, oxidised cytochrome c was added to the dialysed reduced sample. The assay should show that the excess oxidised cytochrome c could not be further reduced, *i.e.* no change in absorbance. To check that cytochrome c was fully reduced, the assay should show that the addition of more ascorbate acid failed to reduced the cytochrome c further.

Assay: The cuvettes contained 50mM potassium phosphate buffer pH 7.0 and 50μM reduced cytochrome c. Potassium ferricyanide (1mM) was added to the reference cuvette to fully oxidise the cytochrome c. The initial absorbance was noted (0.96 being equivalent to 50μM cytochrome c. The reaction was initiated by addition of the sample to the test cuvette and monitored at 550nm. The assay was first order with respect to cytochrome c, so the pseudo first order rate constant k was calculated (similar to that described in section 2.4.4) Enzyme activity was expressed as k/min/mg protein.

2.4.6 Citrate synthase

Principle: This assay was based on the method of Coore *et al.* The enzyme catalyses the condensation of acetyl-CoA and oxaloacetate to form citrate, producing CoA whose free thiol group combines with 5-5'-dithiobis-nitrobenzoic acid (DTNB), resulting in an increase in absorbance at 412nm (Coore *et al.* 1971).

Assay: Identical cuvettes contained 100mM Tris-HCl buffer pH 8.0, 200μM DTNB, 0.1% (v/v) Triton X-100 and sample (10-30μl) in 1ml. The reaction was initiated by the addition of 100μM oxaloacetate to the sample cuvette and the increase in absorbence followed at 412nm. Enzyme activity was calculated using the molar extinction coefficient of 13.6×10^3 for the DTNB-CoA-SH complex and was expressed as nmol DTNB reduced per minute per mg protein.

2.4.7 Protein assay

The method described by Lowry *et al.* (Lowry *et al.* 1951) was used. The protein sample (0-80μg) was diluted to 1ml with H_2O to which 5ml of solution I [(2% Na_2CO_3 + 0.4% NaOH): 1% $CuSO_4$: 2% NaK-Tartrate in a ratio of 100:1:1] were added. The mixed samples were incubated for 20mins and then 0.5ml of 50% (v/v) Folin/Ciocalteau's reagent were added to each sample at 15secs intervals. The sample was mixed and incubated for a further 45mins, after which time sample absorbencies were recorded at 750nm. Protein concentrations were calculated against standard samples of 0, 20, 40, 60, 80 and 100μg of BSA.

2.4.8 Preparation of brain homogenates

Aliquots of frozen (-70°C) brain samples were quickly weighed (~50mg) and homogenized in 9 volumes of ice-cold homogenization buffer (Appendix 1.1) using a 5ml glass homogenizer. After three cycles of freeze-thawing, mitochondrial enzymes were immediately analysed blind and in triplicate at 27°C.

2.4.9 Preparation of platelet mitochondrially-enriched fraction

Platelet mitochondrially-enriched fractions were prepared from 60ml of venous blood taken without a tourniquet via a 19-gauge butterfly into a plain syringe. The blood was immediately transferred into two 50ml universals and mixed at the ratio of 9:1 with 3.8% (w/v) tri-sodium citrate to avoid platelet aggregation. The platelet-rich plasma (PRP) was separated at room temperature by two centrifugation steps at 200g for 10mins. The PRP was carefully removed and prostacyclin I_2 (0.07nM final concentration) was added before the PRP was

centrifuged at 1000g for 20mins. The PRP pellet was gently resuspended with an equal volume (to the PRP) of modified Tyrodes buffer (Appendix 1.2) and was centrifuged at 1000g for 10mins. The pellet was washed with Tyrodes buffer and centrifuged before being resuspended with 6ml of ice-cold homogenization buffer (Appendix 1.1). The cell suspension was transferred to the cell disruption bomb which was charged to 1200psi with oxygen free nitrogen gas (BOC Ltd, Guildford, Surrey, UK) and left on ice for 20mins. After this time the pressure was released and the cell homogenate was collected in a plastic universal. Any nuclei or unbroken cells were pelleted by centrifugation at 1000g for 15min at 4°C. The post nuclear supernatant was transferred to a 30ml centrifuge tube on ice and the nuclear pellet was resuspended with 6ml ice-cold homogenisation medium and re-homogenised in the cell disruption bomb. The second post nuclear supernatant was pooled with the first, and the mitochondrially-enriched fraction was pelleted by centrifugation at 8500g for 10mins at 4°C. The supernatant was removed and the mitochondrially-enriched fraction resuspended with 600μl homogenisation medium.

2.5 DNA extraction

2.5.1 Brain and skeletal muscle genomic DNA extraction

Extraction buffer (2ml, Appendix 2.1) was added to 10-50mg of brain or muscle tissue and crudely homogenised with a glass/teflon hand-held homogenizer. Proteinase K (2mg/ml) and 0.5% (w/v) SDS were added to the homogenates and incubated at 56°C for 3hrs with shaking at 150rpm on an Infors-HT Shaker, before a second addition of proteinase K was made, to make a final concentration of 4mg/ml. The sample was subsequently incubated overnight at 37°C with shaking at 150rpm.

An equal volume of Tris-saturated phenol-buffer was added to the sample and mixed on a whirly-wheel for 20mins, followed by centrifugation at 3000g for 20mins. The aqueous phase was carefully removed to a fresh tube, and the phenol extraction repeated twice more with equal volumes of phenol buffer. An equal volume of chloroform:isoamyl alcohol (24:1, v:v) was added to the aqueous phase to remove phenol and mixed on a whirly-wheel for 5mins, followed by centrifugation at 3000g for 20mins. The aqueous phase was

removed to a fresh tube and ice-cold 0.3M sodium acetate was added. Ice-cold 100% ethanol was added to two volumes of sample plus sodium acetate and precipitated at -70°C for at least 30mins. The DNA was pelleted at 17,000g for 30mins at 4°C, then was washed with 2ml of cold 70% ethanol, air dried for at least 1hr, and finally dissolved in 200μl of TE buffer (Appendix 2.2).

2.5.2 Blood genomic DNA extraction

The procedure was the same as that for the extraction of DNA from brain, with the following modification. Blood (5ml) was mixed with 15ml of double distilled H_2O (dd H_2O) to lyse erythrocytes and centrifuged at 3000g for 20mins at 4°C. The pellets were resuspended with 25ml of 0.1% (v:v) Nonidet P40 and centrifuged at 3000g for 20mins at 4°C. The above procedures were repeated 3 times. The pellets were resuspended in 2ml of extraction buffer (Appendix 2.1) containing 2mg/ml proteinase K and 0.5% SDS. The sample was incubated overnight at 37°C with shaking at 150rpm. Then DNA was extracted with phenol as described above.

2.5.3 Calculation of DNA concentration and purity

Each DNA sample (5μl) was added to 995μl of ddH_2O in a silica cuvette. The reference cuvette only contained ddH_2O. The absorbances at 260 and 280nm were recorded. The concentration of DNA was determined, based on the observation that a 1mg/ml DNA solution has an absorbance of 20 at 260nm. A value of A_{260}/A_{280} between 1.7-2.3 was considered to be of sufficient purity.

2.5.4 Cell lysis for PCR analysis

Cells ($1x10^6$) were harvested and were pelleted by centrifugation at 200g for 10mins. The cell pellet was resuspended in TE buffer (600μl, Appendix 2.2) and centrifuged at 12,000g for 5mins. This procedure was repeated. After resuspension in 200μl of lysis buffer (Appendix 2.3) containing 0.4mg/ml proteinase K, the sample was incubated at 55°C for 20mins. Finally, 100μl of ddH_2O was added to the sample and was further incubated at 90°C for 10mins.

2.6 PCR analysis

DNA primers were synthesised by R&D System Europe Ltd. (Abingdon, UK); dNTPs (dATP, dGTP, dCTP and dTTP) were supplied by Pharmacia Ltd. (Milton Keyned, Bucks, UK); Taq DNA polymerase and polymerase buffer were supplied by Promega (Southampton, Herts, UK).

The PCR reaction mixture (100μl) contained 500ng of total DNA, 0.5μM forward and reversed primers, 200μM dNTPs, 2.5 units Taq DNA polymerase and polymerase buffer (10mM Tris, 50mM KCl, 1.5mM $MgCl_2$, pH 8.8) in 0.5ml eppendorf tubes. This solution was overlaid with 80μl paraffin oil, and tubes were placed into the thermal cycler (OmniGene-PCR Hybaid).

The reaction condition consisted of an initial denaturation temperature of 94°C for 4mins, followed by 30 cycles of: 1min for primer annealing at various temperatures according to the different primers, 1min for primer extension at 72°C and 1min for DNA denaturation at 94°C. The reaction was followed by a final extension of 10mins at 72°C.

2.6.1 PCR detection of A3243G point mutation

Based on the method of Mann *et al.*, which generated a 630bp fragment of mtDNA encompassing the 3243bp position, the A to G mutation at the position of 3243bp creates an ApaI restriction site, cutting the 630bp product into two 315bp fragments (Mann *et al.* 1992b).

In details, PCR primers (forward primer at nt 2928 of mtDNA: 5'-CCT AGG GAT AAC AGC GCA AT-3' and reverse primer at nt 3558 of mtDNA: 5'-TAG AAG AGC GAT GGT GAG AG-3') were designed to amplify a 630bp fragment of mtDNA. PCR conditions were as described earlier except that the annealing temperature was 60°C. For restriction, 8μl of PCR product were added to 10units ApaI and the appropriate restriction buffer (Promega), and incubated at 37°C for at least 1hr.

Determination of ApaI digestion efficiency: To determine the efficiency of ApaI digestion, a pair of primers (forward primer at nt 8756 of mtDNA: 5'-CCA CAA

CTA ACC TCG GA-3' and reverse primer at nt 9756 of mtDNA: 5'-TGA AGG GAG ACT CGA AGT-3') were used to amplify a 1000 bp mtDNA fragment which included one ApaI restriction site at the position of mtDNA 9256bp. The PCR conditions were the same as described above. With complete ApaI digestion, only one fragment of 500bp was visualised. However, if after digestion two fragments (500 and 1000bp) were visualized the percentage of undigested 1000bp product could be calculated by quantification using image analysis (see later section 2.7.8). This product was used to spike the '3243' reaction prior to ApaI digestion.

Agarose gel electrophoresis: Agarose gels (1.2%, Life Tech. Ltd., Paisley, UK) were prepared in 1xTBE buffer (Appendix 2.4) containing 1μg/ml ethidium bromide. 9μl of PCR products as well as 500ng of DNA standards (φx174/HaeIII) were mixed with 1μl 6x loading buffer (Promega) and loaded into the wells. Electrophoresis was performed in 1xTBE buffer containing 1μg/ml ethidium bromide at 40-60 volts for 1-2hrs, and the sample was visualised using a UV transilluminator and photographed with a Polaroid camera.

2.6.2 A549 cell polymorphism

A549 cells were from a patient who had a rare 4 **CA**-repeat sequence at the position of 514nt in mtDNA rather than a 5 **CA**-repeat sequence. To confirm the origin of the mtDNA in the cybrids, a pair of primers (forward primer at nt 371 of mtDNA: 5'-CTA ACA CCA GCC TAA CAA GA-3' and reverse primer at nt 535 of mtDNA: 5'-GGT TAG CAG CGG TGT GTG AG-3') were designed to amplify a 164bp fragment of mtDNA. The reverse primer had one mismatched nucleotide (underlined) to create an AluI restriction site in the presence of 4 **CA**-repeat. When A549 cell mtDNA was amplified and, after AluI digestion, two fragments 142bp and 20bp were present; otherwise a uncut single fragment of 164bp was present after digestion. PCR conditions were as described earlier except that the annealing temperature was 56°C. For restriction, 8μl of PCR product were added to 8 units AluI and the appropriate restriction buffer (Promega), and incubated at 37°C for at least 1hr. The gel

electrophoresis was performed as described in section 2.6.1, except using ϕx174/HinfI DNA standards.

2.6.3 PCR detection of apolipoprotein E (apoE) isoforms

A PCR based method (Wenham *et al.* 1991) was used to detect apoE allelic variations, based on the nucleotide substitutions that account for ApoE allelic resulting in polymorphic restriction sites for CfoI. PCR primers (forward primer: 5'-TAA GCT TGG CAC GGC TGT CCA AGG A-3' and reverse primer: 5'-ACA GAA TTC GCC CCG GCC TGG TAC AC-3') were designed to amplify a 244bp fragment of ApoE gene. PCR conditions were as described earlier except that annealing temperature was 63°C. For restriction, 8μl of each PCR product were added to 8 units CfoI and the appropriate restriction buffer (Promega), and incubated at 37°C for at least 1hr.

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis was used to analyse the apoE isoforms. 1mm thick of 8% polyacrylamide gels were prepared using 30% acrylagel and 2% bis-acrylagel solution at the ratio of 2.6:1 (National Diagnostics, Atlanta, USA) (Appendix 2.5) and were run using a BioRad vertical system in 1xTBE buffer at 30mA for 1.5-2hrs. The gel was stained with 1μg/ml ethidium bromide in 100ml 1xTBE buffer, visualised using a UV transilluminator and photographed with a Polaroid camera.

2.7 Genomic transplantation (cell fusion)

2.7.1 Cell culture for fusion studies

All cell culture reagents were supplied by Life Technologies Gibco BRL, unless otherwise stated.

A human lung carcinoma cell line (A549) and its derivative cell line (A549ρ^{o}), which lacked mitochondrial DNA by exposure to ethidium bromide were supplied by Dr I Holt (Ninewells Hospital, Dundee). Cells were cultured in standard medium containing Dulbecco's Modified Eagle Medium (DMEM) containing 25mM glucose, supplemented with 10% foetal calf serum (FCS), 50 units/ml penicillin, 50 μg/ml streptomycin, 0.2mM uridine, 2mM glutamine and 1mM sodium pyruvate.

2.7.2 Platelet fusion

A549ρ° cells ($1x10^6$) were seeded on a 100mm-plate and incubated at 37°C in a humidified gas mixture containing 7.8% CO_2. After 48-72hrs culture, the cells were harvested using 2ml 0.1% (v/v) trypsin at 37°C for 2mins. The number of cells were counted using a haemocytometer, and $1x10^5$ - $1x10^6$ of cells used for the fusion experiments.

Platelets ($1x10^7$ - $4x10^7$) were prepared as previously described (section 2.4.9) and resuspended in 2ml of Ca^+-free DMEM. The suspension was pipetted onto A549ρ° cell pellets. The mixture of cells was centrifuged at 160g for 5mins. The supernatant was aspirated and the pellet resuspended in 100μl freshly made PEG-DMEM solution [5g of PEG (polyethyleneglycol 1500, NBS Biol., Hatfield, Herts, UK) in 4ml Ca^+ free DMEM and 1ml DMSO]. The mixture of platelets and A549ρ° cells was incubated at room temperature for exactly 1min, followed by the addition of 10ml of normal growth medium onto the mixed cells which were transferred into a 100mm-plate and incubated at 37°C. The medium was changed the following day.

2.7.3 Selection of clonal cybrids

After 3 days in normal medium, the fused cells were split onto ten 100mm-plates and cultured in selection medium (standard medium but with 5% dialysed FCS, 300μg/ml geneticin and lacking uridine and pyruvate). In this selection medium, only fused cybrids were expected to survive and divide, since A549ρ° cells would not grow in medium without uridine and pyruvate (King and Attardi, 1989). After about 20 days in selection medium, the cloned cybrids (5-10 clones on each plate) were ready for ring-cloning using a plastic ring.

2.7.4 Ring-cloning

The bottom of the sterile plastic rings (cut from the tops of 0.5ml eppendoff tubes) was covered with sterile grease (high vacuum silicone grease, UV sterilised) and then placed onto the individual clones. 100μl trypsin (1mg/ml)

were added into each ring to harvest individual clones which were then transferred to 35mm plates and cultured in normal medium.

2.7.5 Mixed cybrids

For mixed cybrids the procedure was identical except the ring-cloning steps were omitted. In addition the cybrids were cultured in selection medium until 10 plates of confluent cells were obtained A549ρ° cells were grown in parallel for comparison.

2.7.6 Cell growth

The cells from each 35mm-plate were transferred to a 100mm-plate. Fifteen 100mm-plates of cells were seeded measuring cell growth rates. Cells grew for 5 days with every 3 plates of cells was harvested each day to count the number of live cells using haemocytometer.

2.7.7 Preparation of mitochondrial pellets

To prepare mitochondrial pellets from cybrids, 5-7 plates of confluent cybrid cells were harvested, centrifuged at 750g for 10mins and washed with PBS three times. The cell pellet was frozen at -70°C for 1hr, and the pellet was resuspended in 2ml of homogenization buffer (Appendix 1.1). All remaining procedures were performed at 4°C. Cell pellets were homogenised at 1000rpm for 20 strokes using a Glas-Col stirrer and centrifuged at 1800g for 10mins. The post nuclear supernatant was collected into a fresh tube. The nuclear pellet was resuspended in 2ml of homogenization buffer and the above procedure was repeated twice. All supernatants were combined in one tube and centrifuged at 6000g for 12mins. The mitochondrial enriched fraction was resuspended in 200-500μl of homogenization buffer and snap frozen in liquid nitrogen and then stored at -70°C for 2 days before analysis.

2.7.8 Quantification of mutant mtDNA

Cybrid cells ($1x10^6$) were harvest and lysed for PCR analysis of the $tRNA^{Leu}$ A3243G mutation (section 2.5 and 2.6). PCR products were electrophoresed on 1.2% agarose gels. The sample was visualised under a UV transilluminator and photographed with a Polaroid camera. Agarose gels were analysed on a

Kontron Vidas AT image analysis system using a monochrome Sony 77CE 768x512 pixel CCD camera. The Vidas 2.5 Gel analysis software (Imaging Associates) package was used. Video images were digitised and the gel lanes defined. A background subtracted image was analysed and specific bands were identified and measured. Values for mean, sum and percentage peak optical density were recorded. Image analysis was kindly performed by Mr. J. Muddle in the department using densitometry.

2.7.9 Recloning the high mutant-load cybrid (89% mutant-load)

After the percentage of mutant mtDNA in cybrids was determined, the clone with the highest levels of mutant mtDNA (85% mutant-load) was seeded at a density of 2000-3000 cells per 100mm-plate. After three weeks, the cell clones were ring-cloned and mutant-loading was determined as previously described (section 2.7.3 and 2.7.4).

2.7.10 Western blot - horseradish peroxidase conjugated (HRP) detection

This technique was used to detect subunits I, II and IV of cytochrome oxidase in A549 cybrid cell lines.

Preparation of total proteins from cells

Cybrid cells (1×10^6) were harvested by trypsinization and washed twice with PBS. The number of cells were counted using a haemocytometer. Cell pellets were resuspended in extraction buffer (cold PBS supplemented with lauryl maltoside, PMSF, leupeptin and pepstatin A, Appendix 3.1). Cell numbers were adjusted to $1 \times 10^4/\mu l$ in extraction buffer and cells were mixed at 4°C for 30mins, prior to being centrifuged at 13,000g for 20mins at 4°C. The supernatant was denatured in 5 times concentrated denaturation buffer (Appendix 3.2) at 37°C for 20mins.

SDS-Polyacrylamide gel electrophoresis (PAGE)

A 12.5% SDS polyacrylamide separating gel (Appendix 3.3) was cast using a minigel system (BioRad, UK). After the separating gel had set, the 5% stacking gel (Appendix 3.4) was formed on the top of the separating gel. 10μl of each denatured protein sample (equivalent extracted from 5,000-50,000

cells) was loaded into each well. Molecular weight markers were prepared in loading buffer (18kd - 48kd, BioRad, UK) and also included in the same gel. Gels were immersed in a running buffer (Appendix 3.5) and electrophoresed at 100 volts for 30mins until the bromophenol blue entered the separating gel and then at 200 volts for about 1.5hrs until bromophenol blue reached the bottom of the gel.

Western blotting

Following separation of the proteins, the gel, PVDF-p transfer membrane (Millipore, Bedford, MA) and 2 sheets of Whatman No.1 blotting paper (Whatman Ltd., Maidstone, Kent, UK) were pre-soaked in transfer buffer (Appendix 3.6). The gel and membrane were placed between 2 sheets of blotting paper and placed in a BioRad mini blotting tank containing transfer buffer. While stirring, the blotting was performed at 100 volts, with an ice box in the transfer cell to cool the running system for 1hr. Coomassie brilliant blue staining of the blotted gel confirmed the efficient transfer of the protein to the membrane.

Antibody and detection

The wet PVDF membrane was blocked in 10% (w/v) fat-free milk in PBS-Tween (0.3% Tween-20 in PBS) for 1-2hrs at room temperature. After washing twice with PBS-Tween, the membrane was incubated overnight at room temperature with the first antibody diluted in PBS-Tween (Appendix 3.7) (see Taanman *et al.* 1996, for the details of using these antibodies). The membrane was washed for 10mins at room temperature three times with PBS-Tween, followed by incubation with the secondary antibody for 2hrs (1:3000 dilution of blotting grade affinity purified goat anti-mouse IgG conjugated to horseradish peroxidase, BioRad). The membrane was washed three times for 10mins with PBS-Tween and a final wash with PBS only. The membrane was then developed for 1min with an equal volume of chemiluminescence oxidizing reagent and enhanced luminol reagent (Renaissance Kit "Dupont NEN", Boston, MA). The membrane was exposed to X-ray film (ECL, Amersham) for variable times (30secs, 1min, 2mins and 4mins) developed and then fixed.

2.7.11 Cytochemical and immunocytochemical staining

Cytochemical staining of COX

Cells ($0.2x10^6$) were cultured on cover slips for 24-48hrs, washed with PBS and then incubated with 2ml COX activity dye solution (Appendix 4.1) at 37°C for 1.5hrs. Cells were washed with PBS and then incubated in nuclear counter staining solution (heamatoxylin) for 2.5mins. After washing with tap water the cover slips were dehydrated in 100% ethanol and cleared in 100% xylene at room temperature and finally mounted in DPX (contain xylene, Agar Scientific Ltd. Cambridge, UK).

Immunocytochemical staining of COX I

Cells ($0.2x10^6$) were cultured on cover slips for 24-48hrs. The cells were incubated in medium containing 2μM MitoTracker CMXRos-H_2 (Molecular Probe, Eugene, Oregon, USA) for 45mins in a tissue culture incubator followed by culturing in plain medium for 30mins. Cells on the cover slips were washed subsequently in PBS, fixed in 4% paraformaldehyde in PBS for 15mins, permeabilized in pure methanol at -20°C for 15mins and then blocked with 10% goat serum in PBS (GS-PBS) at 37°C for 30mins. The cells were incubated in 10% GS-PBS solution containing the monoclonal antibody against COX I subunit (α-I) (1:25 dilution) and 10μg/ml of 33258 Hoechst dye at 37°C for 45mins. Finally, the cells were incubated with goat anti-mouse IgG-FITC in PBS at 37°C for 45mins before being washed and mounted in Citiflour-glycerol PBS.

2.8 Analyses of apoptosis

2.8.1 Cell culture

A549, A549ρ° cells and fibroblasts: A549 and A549ρ° cells line were the same as those used for platelet fusion. Normal human fibroblasts were obtained from a skin biopsy of a six year old healthy female and were cultured in standard medium (see previous section 2.7.1).

Dorsal root ganglion (DRG) neuronal cultures: Sprague-Dawley rats aged 1-4 days old were killed by cervical dislocation and their DRG dissected out under aseptic conditions and placed in cold Ca^{2+}-Mg^{2+}-free Hank's buffered saline

solution (HBSS). The ganglia were coarsely freed of connective tissue and washed in HBSS. They were then incubated in 0.2% collagenase type IV in HBSS for 20mins, followed by a brief wash and centrifuged, and a further incubation with 0.25% trypsin in HBSS for 18mins at 37°C. After rinsing in buffer, the trypsin reaction was arrested by immediate addition of culture medium. The ganglia were carefully triturated by 10 passages and immediately plated on 12 cover slips (pre-coated with 0.5mg/ml poly-D-lysine and 20mg/ml laminin) in two 6-well plates. The monolayer culture was incubated in a humid environment at 37°C in 5% CO_2. The cells were maintained for up to 9 days in DMEM/RPMI medium, supplemented with 10% FCS, 0.625% glucose, 2mM glutamine, 100U/ml penicillin, $3x10^{-8}$ M sodium selenite and 30ng/ml nerve growth factor. In order to inhibit proliferation of non-neuronal cells, 10µM cytosine arabinoside was added 2hrs after plating and removed after 48hrs.

2.8.2 Toxin treatment

A549, A549ρ° cells and fibroblasts: Cells (except DRG neurones) were seeded onto cover slips ($0.2x10^6$ cells) which were placed in 6-well plates and cultured as described in section 2.7.1. After 24hrs in culture, rotenone (0-10µM, in ethanol), piericidin A (1µM in DMSO), antimycin A (20µM, in ethanol), colchicine (0.1µM in PBS) or staurosporine (1µM in DMSO) were added to the culture medium. As a control, cells were treated with an equal volume of ethanol or DMSO.

DRG neurones: DRG neurones of one rat were plated onto 12 cover slips, following the removal of cytosine arabinoside, the neurones were treated with 1µM rotenone, 1µM piericidin A, 1µM antimycin A or 0.1µM colchicine for 24hrs.

2.8.3 Cell staining

Staining with antibodies (except DRG neurones)

For immunofluorescent analysis of α-actin and ß-tubulin, cells on cover slips were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10mins, permeablised in a 1% Triton X-100 solution for 10 min, incubated in 10% normal goat serum (NGS) for 30mins, followed by 1hr incubation with a

monoclonal antibody to either α-actin (1:50 dilution in 0.1% BSA-PBS, Boehringer Mannheim, Germany) or ß-tubulin (1:50 dilution in 0.1% BSA-PBS, Amersham, UK).

For immunofluorescent analysis of Bcl-2, cells on cover slips were fixed in acetone for 10 min, incubated in 10% normal goat serum (NGS) for 30mins, followed by a 2hrs incubation with a monoclonal antibody to Bcl-2 (1:20 dilution in 1% NGS, DAKO, Santa Barbara, CA).

Following a PBS wash, all cells on cover slips were incubated with FITC conjugated goat anti-mouse IgG antibody (1:20 dilution in 0.1% BSA-PBS, Southern Biotechnology Associated Inc., Birmingham, USA) for 1hr. This process was performed for all antibody cross-reactions.

Staining with propidium iodide

If the cell had already been stained with antibodies, the steps for fixation and permeabilisation were omitted. Otherwise, cells floating in the medium were collected by centrifugation at 500g for 10mins and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 10mins and cell pellets smeared onto cover slips and dried at 37°C for 10mins. Cells adhering to the cover slips were also fixed with 4% paraformaldehyde for 10mins. Both floating and adhering cells on cover slips were permeabilised in a solution of HCl:ethanol:H_2O (1:70:29) at -20°C, and then incubated with a propidium iodide (PI) solution (2µg/ml) containing 0.1mg/ml of DNase-free RNase at 37°C for 20mins. The cover slips were washed with PBS between each step and finally mounted in Citifluor (glycerol/PBS solution, Cambridge Lab, UK).

In one study, the cells on coverslips were stained only with Bcl-2 antibody where PI staining disturbed the interpretation of the immunostaining.

Staining with Apotaq Kit (Tunel, apoptosis detection kit, Appligene Oncor, Durham, UK)

Cells adhering to the coverslips were fixed with 4% paraformaldehyde for 10mins and permeablized with ethanol: acetic acid (2:1) at -20°C for 5mins. Then the cells were incubated with labelling reaction mixture containing

terminal deoxynucleotidyl transferase (TdT) at 37°C for 1hr. After stopping the reaction with blocking solution (37°C for 30mins), the cells were incubated with the anti-digoxigenin-dUTP-fluorescein antibody at room temperature for 30mins. The coverslips were washed with PBS between each step and mounted with citifluor.

DRG neurones immunostaining and PI staining

DRG neurones on cover slips were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10mins, permeablised in a 1% Triton X-100 solution for 10mins, blocked with 5% BSA for 30 min followed by a 1hr incubation with a monoclonal antibody specific to neuronal ß-tubulin diluted 1:250 (TuJ1, supplied by Dr. T. Cowen, Department of Anatomy, Royal Free Hospital School of Medicine, London) in 1% NGS-PBS for 1hr. Following this, the cells were incubated in FITC conjugated goat anti-mouse IgG antibody (1:20 dilution, Southern Biotechnology Associated Inc. Birmingham, AL 35226 USA) for 1hr. Finally. the coverslips were stained with a PI solution as described above and mounted in citifluor. Fluorescently labelled cells were observed under a confocal microscope (BioRad, MRC 600).

All procedures were at room temperature and cover slips were washed with PBS between each step.

2.8.4 Cell counting

The number of mitotic, apoptotic and endoreduplicated cells were determined in at least three independent experiments by manually counting 500 cells stained with PI from at least five randomly selected fields. Mitotic cells were taken as those cells with highly fluorescent nuclei, with condensed chromatin or chromosomes and usually with a regular shape (see chapter 7, results). Apoptotic cells were taken as those highly fluorescent cells which were usually much smaller in size, and with characteristic polarised chromatin aggregates or fragmented nuclei. For Tunel staining, the cells with bright yellow/green colour were counted as apoptotic cells. The endoreduplicated cells were classified as those cells having more than two nuclei usually distinguished by their highly irregular shape.

Cells were also harvested and counted using a haemocytometer, live cells being taken as those cells that exclude trypan blue. In immunochemical studies, when primary antibody was omitted, no fluorescence was observed.

CHAPTER 3 MITOCHONDRIAL FUNCTION IN NEURODEGENERATIVE DISEASES

3.1 Aim

To study: the role of L-dopa in the complex I dysfunction in PD substantia nigra; the relationship between either Lewy body formation or cholinergic cell loss and complex I dysfunction; and any relationship between apoE genotype and dementia associated with PD, dementia with Lewy bodies type (DLB) and Alzheimer's disease (AD).

3.2 Introduction

The aetiology of PD remains undefined. Complex I deficiency (Schapira *et al.* 1989) and oxidative stress (Sian *et al.* 1994a) have both been identified in PD substantia nigra but their place in the sequence of events resulting in dopaminergic cell death is uncertain.

The neurotoxin 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) has been shown to destroy substantia nigral neurons in primate models (Langston *et al.* 1984) probably through its ability to inhibit mitochondrial complex I activity and/or to generate free radicals. The discovery of complex I deficiency in the substantia nigra in PD (Schapira *et al.* 1989; Janetzky *et al.* 1994) suggested a direct biochemical link between the idiopathic disease and the MPTP model. The relevance of this biochemical defect to the cause of dopaminergic cell death in PD was enhanced by the finding that the complex I deficiency was confined to the substantia nigra within the brain and was not present in the caudate nucleus, putamen, globus pallidus, cerebral cortex, or cerebellum (Schapira *et al.* 1990a). Activities of other respiratory chain complexes (II+III and IV) were not significantly different from age-matched controls in any of the brain areas examined, including the substantia nigra. Mitochondrial respiratory chain dysfunction has also been considered as a potential cause for the neuronal loss in MSA (Gu *et al.* 1997).

MSA is a distinct usually sporadic adult-onset neurodegenerative disorder characterised clinically by autonomic failure, parkinsonism, cerebellar ataxia

and pyramidal signs in any combination, and pathologically by nigral cell loss. Glial cytoplasmic inclusions were described as a characteristic cytological marker for MSA (Papp *et al.* 1989). MSA accounts for approximately 10% of patients with parkinsonism in specialized brain banks, and represents the most common cause of parkinsonism after PD (Quinn, 1995).

In order to investigate the potential contribution of mitochondrial dysfunction to the pathogenesis of MSA, and to determine its tissue specificity, the brain mitochondrial respiratory chain function in MSA patients and matched controls was investigated. In addition, most MSA patients are given L-dopa and so this disease provides information on whether this drug can induce the complex I defect observed in PD.

Although the substantia nigra is the site of severe neuronal loss in PD and its degeneration is thought to be the cause of the major clinical features of this disorder, other areas of the central nervous system are also affected in the pathology of PD and any hypothesis of aetiology and pathogenesis must encompass this observation. For instance, the substantia innominata is the site of cholinergic cell loss and also exhibits Lewy body formation in PD. If the cause of the cell loss and Lewy body formation in the innominata and the nigra were the same, and if mitochondrial dysfunction and oxidative stress are involved, then it would be expected that the same biochemical abnormalities should exist in both tissues. Lewy body formation also occurs in the cingulate cortex of patients with DLB, although without the profound cell loss seen in the innominata or nigra of PD. Thus, the cingulate cortex provides a useful internal 'control' to judge whether Lewy body formation *per se* is associated with complex I deficiency and oxidative stress. In contrast, the cingulate cortex of AD patients shows neuronal loss but few or no Lewy bodies, thereby providing the means to dissect further the relationship between the pathology of neuronal degeneration, Lewy body formation and the biochemistry of PD. We have therefore analysed respiratory chain activity, iron and GSH concentrations (in collaboration with King's College) in PD substantia innominata and in the cingulate cortex of patients with PD, AD and DLB to investigate their association with neuronal death and Lewy body formation (Gu *et al.* 1998b).

Recent studies have identified the apoE ε4 allele as a high risk factor for the development of late-onset familial and sporadic AD (Strittmatter *et al.* 1993; Corder *et al.* 1993). It has been suggested that apoE ε4 itself may play a role in plaque tangle formation (Poirier, 1994; Richey *et al.* 1995). We hypothesised that if dementia in PD were due to concomitant AD, the allele frequency of ε4 would be higher in PD with dementia than in PD patients without dementia. The study of patients with AD, DLB and PD might provide insight into whether the formation of Lewy bodies are influenced by apoE genotypes. On this basis, the apoE genotype were examined in patients with AD, DLB, PD and controls from the same ethnic population.

3.3 Methods

3.3.1 Enzyme assays

Frozen brain samples were homogenized and MRC enzyme assays were analysed as described in chapter 2, section 2.4.

3.2 Selection of patients and controls

All controls were age-matched and showed no evidence of primary neurodegenerative diseases in this study.

Substantia nigra: small group (8 controls, 8 PD and 8 MSA) and combined group (22 controls, 16 PD and 15 MSA) (Table 3.1-3.2)

Caudate nucleus: 7 controls and 8 PD (Table 3.3)

Substantia innominata: 11 controls and 12 PD (Table 3.4)

Cingulate cortex: 10 controls, 10 PD, 10 AD and 10 DLB (Table 3.5)

ApoE study: 57 PD (14 with dementia and 43 without dementia), 20 AD and 10 DLB patients with 57 controls (Table 3.6)

Data analysis see chapter 2, section 2.2.

3.4 Results

3.4.1 Results for MRC function study

Substantia nigra (SN)

The complex I function was determined in the SN from PD, MSA and controls. Samples were matched for age and post mortem delay (PM-delay) (Table 3.1).

The complex I activity in the SN from PD and MSA were not significant different from the control values when enzyme results were expressed per unit total protein (Table 3.1, group B). However, after citrate synthase correction, the complex I activity showed a significant decrease in PD compared to the control (p<0.001).

Table 3.1 Mitochondrial respiratory chain activity (complex I) in substansia nigra from age and PM-delay matched control, PD and MSA patients

	Control		PD		MSA	
	A	B	A	B	A	B
n	14	8	8	8	7	8
Age (years)	66.2±10.4	69.4±11.0	72.3±8.5	75.0±6.0	67.7±8.9	62.6±10.8
PM-delay (hrs)	17.6±6.7	22.1±14.3	15.4±10.3	11.8±10.1	21.0±6.4	21.3±12.1
Cx I	3.73±1.12	3.38±1.29	2.87±0.43**	2.54±0.48	2.81±0.98	2.88±0.80
CS	114±32	102±31	103±30	111±31	91±13	98.4±23.2
Cx I/CS (x100)	3.43±1.39	3.67±0.76	2.89±0.63*	2.37±0.44***	3.42±1.26	2.98±0.79

Enzyme activities expressed as nmol/min/mg protein or as CS ratio. Values are mean±SD. Cx I: complex I

*:p<0.05, **:p<0.01, ***:p<0.001, compared with relative controls, as judged by Mann-Whitney U test.

A: previously reported (Schapira *et al.* 1990a); B: this study.

The complex I activity in SN from PD (n=8, group A) previously showed a significant defect but not in SN from MSA (n=7, group A) compared with controls (n=14) (Schapira *et al.* 1990b).

There were no significant differences between the data from group B and the data from group A (Table 3.1). The cumulative data were presented for complex I activity in age and PM-delay matched SN samples from controls (n=22), PD (n=16) and MSA (n=15). There was a significant decrease in

complex I activity in the PD group, both when the results were expressed per unit protein or as a citrate synthase ratio ($p<0.05$, Table 3.2).

Table 3.2 The cumulative data on complex I activity in substantia nigra from age and PM-delay matched control, PD and MSA patients

	Control	PD	MSA
n	22	16	15
Age (years)	68.6±11.4	71.3±5.8	65.0±10.3
PM-delay (hrs)	18.7±7.6	14.5±10.3	21.2±9.4
Complex I	3.53±1.10	2.56±0.56*	2.85±0.86
CS	105±31	112±32	92±19
Complex I/CS (x100)	3.40±1.21	2.50±0.99*	3.18±1.02

Enzyme activities expressed as nmol/min/mg protein or as CS ratio.

Values are mean±SD.

*: $p<0.05$, as judged by Mann-Whitney U test.

The cumulative data were analysed to investigate the correlation between complex I activity and L-dopa intake in MSA (n=14) (Fig. 3.1) and PD (n=16) (Fig. 3.2). There was no correlation with this feature and the daily L-dopa intake over the last 3 months of life by PD and MSA patients as judged by Spearman's correlation study.

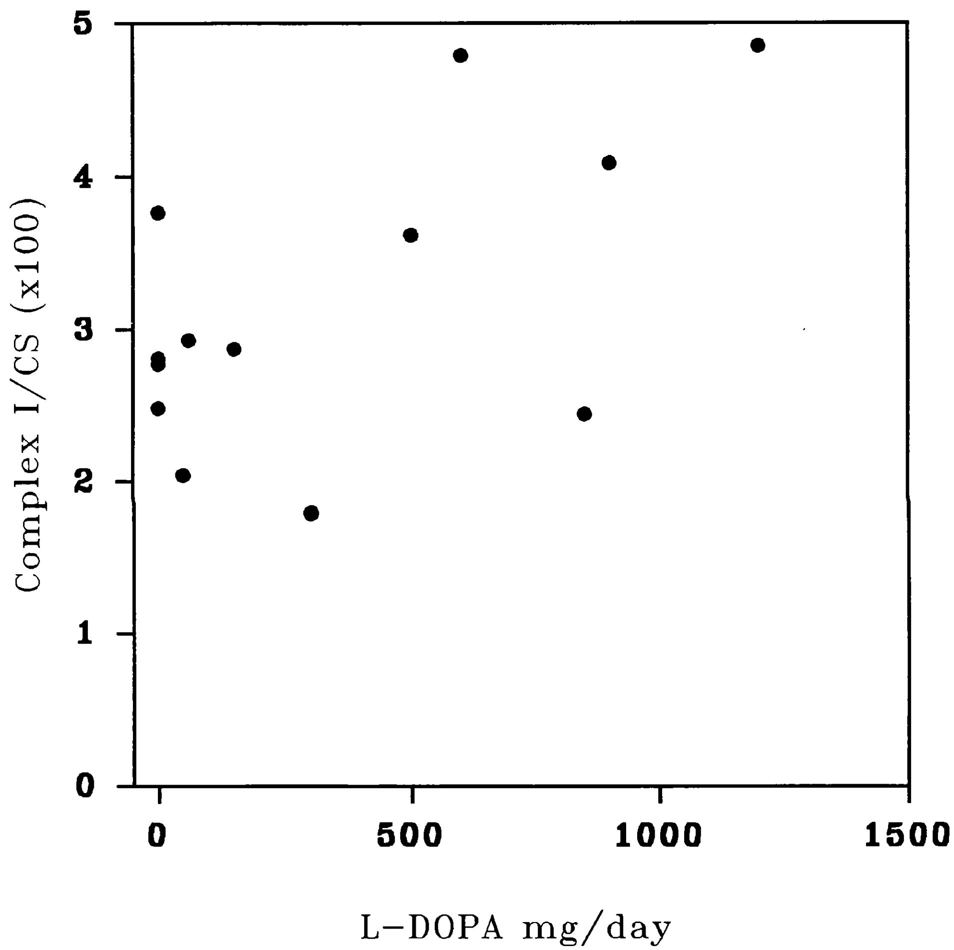


Fig. 3.1 Effect of mean daily L-dopa dose on complex I/CS ratio in substantia nigra samples of 14 MSA patients during the last 3 months of life (Spearman r=0.408, p>0.05).

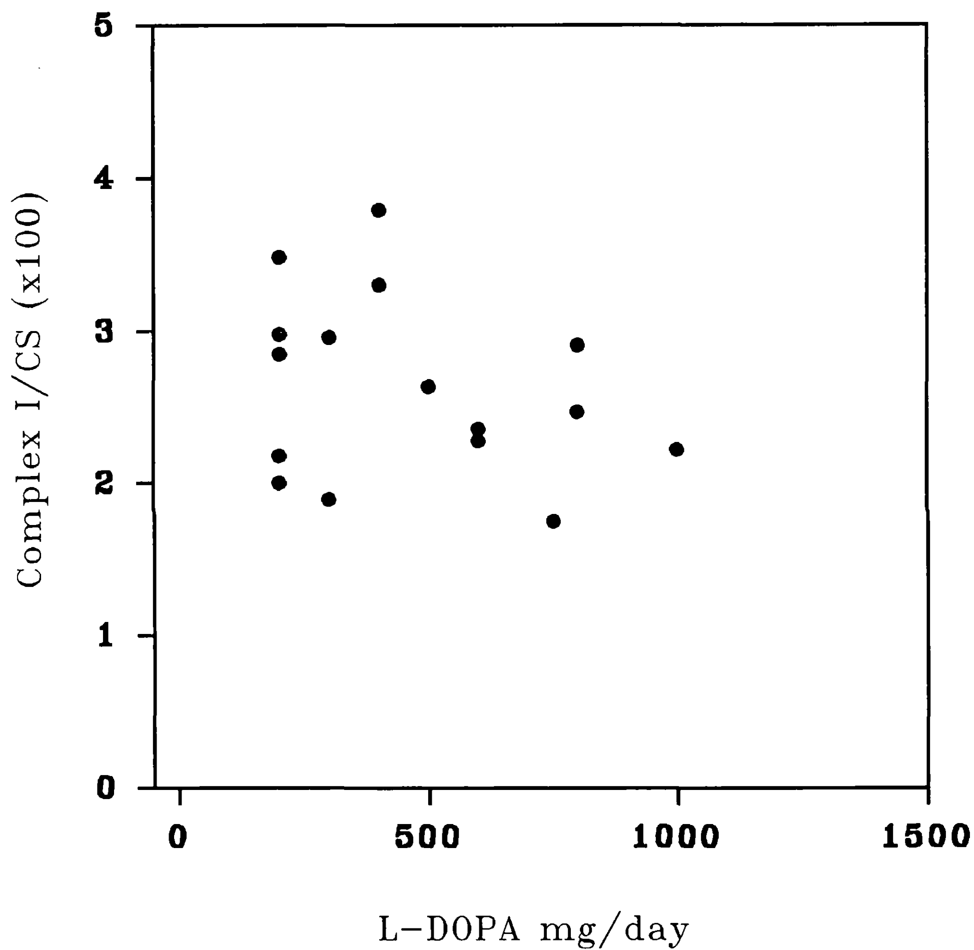


Fig. 3.2 Effect of mean daily L-dopa dose on complex I/CS ratio in substantia nigra samples of 16 PD patients during the last 3 months of life (Spearman r=-0.201, p>0.05).

Caudate nucleus (CN)

The MRC enzyme activities were determined in the CN from PD and controls. Samples were matched for age and PM-delay. There were no significant difference from controls in the activities of all MRC enzymes (Table 3.3).

Table 3.3 Mitochondrial respiratory chain activities in caudate nucleus from age and PM-delay matched control and PD patients

	Control	PD
n	7	8
Age (years)	77.0±9.6	73.0±3.9
PM delay (hrs)	16.9±5.7	17.7±5.4
Complex I	4.14±0.86	3.56±0.67
Complex II+III	24.5±6.72	22.2±8.56
Complex IV (k)	1.37±0.42	1.00±0.31
CS	187±28	163±39
Complex I/CS (x100)	2.39±0.65	2.34±0.82
Complex II+III/CS (x10)	1.39±0.28	1.32±0.25
Complex IV/CS (x100)	0.70±0.25	0.52±0.19

Enzyme activities expressed as nmol/min/mg protein or CS ratios, except for cytochrome oxidase (complex IV) which is k/min/mg where k is the first order rate constant. Values are mean ± SD. No significant difference from controls was found for any values, as judged by Mann-Whitney U test.

Substantia innominata(SI)

Iron and GSH levels (performed by Drs. Dexter and Owen, King's college) and MRC enzyme activities were determined in SI samples from age and PM-delay matched PD (n=12) and controls (n=11). MRC enzymes, iron and GSH in SI from the PD showed no significant difference from the controls (Table 3.4).

Table 3.4 Mitochondrial respiratory chain activities, iron and GSH levels in substantia innominata from age and PM-matched controls and PD patients

	Control	PD
n	11	12
Age (years)	71.6±10.5	78.6±7.40
PM delay (hrs)	17.4±12.4	20.2±11.7
Complex I	3.41±0.67	3.38±1.22
Complex II+III	15.1±7.08	14.9±5.40
Complex IV (k)	0.93±0.38	0.86±0.19
CS	150±40.7	126±30.4
Complex I/CS (x100)	2.46±0.91	2.72±0.80
Complex II+III/CS (x10)	1.00±0.28	1.19±0.31
Complex IV/CS (x100)	0.62±0.13	0.70±0.16
Iron	6.99±1.63 (n=10)	8.96±2.95 (n=11)
GSH	0.51±0.19 (n=8)	0.66±0.24 (n=8)

Enzyme activities expressed as nmol/min/mg protein or CS ratios, except for complex IV which is k/min/mg where k is the first order rate constant. Values are mean ± SD. Figures in parenthesis are sample numbers where they differ from the rest of the column. Iron and GSH results are expressed as μmol/g dry weight and wet weight respectively.

No significant difference from controls was found for any values, as judged by Mann-Whitney U test.

Cingulate cortex (CC)

Iron and GSH levels (performed by Drs. Dexter and Owen, King's college) and MRC enzyme activities were determined in CC samples from age and PM-delay matched PD (n=10), AD (n=10), DLB (n=10) and controls (n=10).

Mitochondrial enzyme activities in cingulate cortex of PD, AD and DLB were not significantly different from that in controls. There were significantly increased iron levels in both AD (41%) and DLB (35%). However, the decreased GSH levels (49%) were only observed in AD cingulate cortex (Table 3.5).

Table 3.5 Mitochondrial respiratory chain activities, iron and GSH levels in cingulate cortex from age and PM-matched controls and PD, AD, and DLB patients

	Control	PD	AD	DLB
n	10	10	10	10
Age (years)	82.2±4.9	76.2±7.4	80.9±6.0	78.9±6.6
PM delay (hrs)	46.1±17.0	39.2±19.7	34.1±17.6	35.1±22.6
Complex I	4.34±1.89	3.41±1.15	3.31±1.17	3.66±1.54
Complex II+III	11.3±4.98	13.7±6.38	11.0±5.43	12.0±6.43
Complex IV	0.93±0.50	1.29±0.78	0.80±0.37	0.84±0.42
CS	186±70.6	158±61.1	139±48.6	150±49.5
Complex I/CS (x100)	2.47±0.86	2.42±0.89	2.51±0.70	2.44±0.55
Complex II+III/CS (x10)	0.72±0.32	0.80±0.39	0.81±0.27	0.81±0.31
Complex IV/CS (x100)	0.48±0.18	0.67±0.24	0.55±0.15	0.53±0.21
Iron	3.35±0.62 (n=11)	2.93±0.84 (n=13)	4.72±1.35* (n=12)	4.51±1.38* (n=11)
GSH	0.97±0.35 (n=11)	0.93±0.50 (n=11)	0.49±0.25*	0.75±0.29 (n=12)

Enzyme activities expressed as nmol/min/mg protein or CS ratios, except for complex IV which is k/min/mg where k is the first order rate constant. Values are mean ± SD. Figures in parenthesis are sample numbers where they differ from the rest of the column. Iron and GSH results measured as μmol/g dry weight and wet weight respectively.
*$p<0.05$, as judged by ANOVA one-way analysis of variance test.

3.4.2 ApoE genotypes

ApoE genotypes were determined in AD (n=20), DLB (n=10), PD (n=57) and controls (n=57). All the patient groups and control group were age-matched (Table 3.6). The apoE ε4 allele was over-represented in patients with AD and DLB pathology: ε4 allele frequencies were 47.5% and 40.0%, respectively, which were significantly higher than patients with PD (total frequency 15.8%) and neuropathological controls (total frequency 20.2%). There was no significant difference in the frequencies of ε4 allele in AD compared with DLB, PD with dementia compared with PD without dementia, or PD (dementia and nondementia) compared with controls. Three of 20 AD patients and one of 10 DLB patients were ε4 homozygous for the allele. None of PD patients without dementia was homozygous for the ε4 allele, while one PD patient with dementia and one control were homozygous for the ε4 allele.

Table 3.6 ApoE genotypes and frequencies in AD, DLB, PD (demented and nondemented) patients and controls

AD	DLB	PD		PD	Controls
		demented	nondemented	total	
No of patients					
20	10	14	43	57	57
Age					
81.1±7.5	78.9±6.6	72.5±6.8	72.7±10.3	72.6±8.5	71.5±16.6
ApoE* genotypes(%)					
ε4/4					
15.0(3)	10.0(1)	7.1(1)	0.0(0)	1.8(1)	1.8(1)
ε4/3					
55.0(11)	60.0(6)	21.4(3)	16.3(7)	17.5(10)	24.6(14)
ε4/2					
10.0(2)	0.0(0)	12.5(1)	16.7(5)	10.5(6)	12.3(7)
ε3/3					
5.0(1)	10.0(1)	64.3(9)	51.2(22)	42.9(31)	59.6(34)
ε3/2					
15.0(3)	20.0(2)	0.0(0)	23.3(10)	17.5(10)	2.5(1)
ε2/2					
0.0(0)	0.0(0)	0.0(0)	0.0(0)	0.0(0)	0.0(0)
No of alleles					
40	20	28	86	114	114
ApoE alleles frequencies (%)**					
ε4					
47.5(19)#^	40.0(8)#^	21.4(6)	14.0(12)	15.8(18)	20.2(23)
ε3					
40.0(16)	50.0(10)	75.0(21)	68.6(59)	70.2(80)	72.8(83)
ε2					
12.5(5)	10.0(2)	6.2(1)	17.4(15)	14.0(16)	7.0(8)

Comparison for statistical significance between all these four groups (AD, DLB, PD, control). Using chi-square test (X^2) on ε4 allele frequencies revealed significant differences only between the following: #; AD versus controls, ^;AD versus PD, #; DLB versus control and ^; DLB versus PD, all at $p = 0.0001$ (X^2=17.8, df=2).

*:Figures in parenthesis are the number of patients with the genotype.

**: Figures in parenthesis are the number of alleles.

3.5 Discussion

3.5.1 MRC function in MSA and PD

This study has found normal complex I activity in MSA substantia nigra. A previous smaller study by this group also failed to show any citrate synthase corrected mitochondrial defect in MSA substantia nigra (Schapira *et al.* 1990a). Although mitochondrial respiratory chain function in substantia nigra from MSA patients has not been reported. Blin *et al.* (1994) found a 30% decrease in complex I activity in the muscles of MSA, but complex I activity was normal in the platelets of MSA (Benecke *et al.* 1993; Gu *et al.* 1997).

It is possible that to some extent such a discrepancy might be explained by the difficulty in diagnosing MSA ante-mortem and the likelihood of alternate pathologies confounding the data. In this respect, post-mortem studies do have the advantage of pathological confirmation of diagnosis. The observation that complex I function appears unaffected in substantia nigra - a site of major pathological change in MSA - casts doubt on the relevance of any defect observed in other tissues (*ie.* muscle) in this disorder.

The results presented here are useful in the interpretation of the complex I defect observed in PD substantia nigra. As argued previously (Schapira *et al.* 1990a), normal respiratory chain function in MSA nigra, despite severe degeneration, suggests that the complex I defect in PD is not simply a reflection of neuronal loss and co-existing gliosis, and must have some alternate cause.

There is concern that L-dopa could contribute to the nigral complex I deficiency in PD. *In vitro* studies were conflicting with some showing reversible 25% complex I defect in rat nigra and striatum induced by L-dopa administration (Przedborski *et al.* 1993) while others showed no effect on the complex I activity of cells (Pardo *et al.* 1995; Morikara *et al.* 1996). The absence of a respiratory chain defect in the striatum from PD patients that had been treated with L-dopa (Cooper *et al.* 1995) contrasts with that expected from the animal model (Przedborski *et al.* 1993).

Fourteen of the 15 MSA patients whose substantia nigra were analysed post-mortem in this study were known to be on L-dopa in doses comparable to PD patients, although the mean period of treatment (3.4 years) in the MSA group was less than in the group of 16 PD patients (5 years). Normal activity in the MSA samples would suggest that, in human substantia nigra, L-dopa does not induce a complex I deficiency, at least in the context of MSA pathology. Furthermore, there was no correlation between individual L-dopa dose and substantia nigra complex I activity in MSA and PD patients. Thus, it would suggest that in MSA at least, L-dopa treatment does not have an effect on complex I activity. Recently, Shults *et al.* (1995) reported that treatment of early onset PD with carbidopa/L-dopa alone and carbidopa/L-dopa plus selegiline did not affect the activities of complex I, II+III and IV and CS in platelets. Platelet respiratory chain function was also not significantly different from control in the 4 MSA patients taking L-dopa at the time of analysis (performed by Mrs. M. Gash, personal communication). These observations also support the suggestion that impaired complex I activity in PD patients is a characteristic of the disease and not due to medication (Shults *et al.* 1995).

3.5.2 MRC function and oxidative stress in PD

Mitochondrial complex I deficiency (Schapira *et al.* 1989; Mann *et al.* 1992a; Janetzky *et al.* 1994), low GSH levels (Riederer *et al.* 1989; Sian *et al.* 1994a) and increased iron concentrations (Riederer *et al.* 1989; Dexter *et al.* 1991) have been described in PD substantia nigra. The cumulative data from this laboratory on complex I activity in PD substantia nigra homogenates confirms, at a significant level, a specific complex I defect. The present study was undertaken to address the relationship of these abnormalities to neuronal loss and Lewy body formation.

There were no significant alterations from controls in PD substantia innominata for mitochondrial function, iron levels or GSH values. These results emphasise the apparent specificity for the decrease in complex I activity and GSH levels for the PD substantia nigra within the brain and imply that these biochemical abnormalities are not involved in the cholinergic neuronal degeneration and Lewy body formation of the PD substantia innominata. In

this study, the normal levels of complex I and GSH in DLB cingulate cortex further support the lack of association between Lewy body formation and decreases in these parameters.

It is important to understand the role of mitochondrial dysfunction and oxidative stress in the pathogenesis of dopaminergic cell death in PD substantia nigra. The anatomical selectivity of neuronal loss that underlies the clinical features of PD and distinguishes it from other parkinsonian syndromes such as MSA. The PD substantia nigra, and the pars compacta in particular, have high concentrations of iron, an imbalance of iron:ferritin ratio (Dexter *et al.* 1990; Mann *et al.* 1994), increased superoxide dismutase activity (Saggu *et al.* 1989), low complex I activity and decreased GSH levels. The results would suggest that an alternative mechanism underlines cell death in the PD substantia innominata. The specificity of the combined mitochondrial and GSH abnormalities for the nigra in PD suggests that they are a consequence of some particular properties of the substantia nigra. A 'two-hit' hypothesis for the cell death in PD could be used to explain these observations (Gu *et al.* 1998b).

It has been argued that the aetiology of PD may be genetic or environmental, although as has been noted in the Introduction (section 1.1), no conclusive data are available to support either of these as dominant. However, we suggest that one or other of these may be involved as a background effect upon which the other is superimposed to induce severe dopaminergic cell loss in the substantia nigra. For instance, a genetic or environmental insult could induce cell loss and Lewy body formation in the nigra, innominata and locus ceruleus. The profound cell loss of the substantia nigra pars compacta is then induced by an additional but alternate cause, genetic or environmental (endogenous or exogenous) to produce the clinical features of PD. This hypothesis would suggest that some individuals may be exposed to only one of the two 'hits' and therefore not develop PD, but would bear the pathological consequences of their respective exposure.

3.5.2 MRC function and oxidative stress in AD

There is increasing evidence that oxidative stress and damage and mitochondrial dysfunction may play a role in the pathogenesis of AD. Increased levels of 8-hydroxy-2-deoxyguanosine have been found in AD parietal cortex, although not in cerebellum, frontal or temporal cortex (Mecocci *et al.* 1994). Elevated levels of thiobarbituric acid-reactive substances, malondialdehyde and protein carbonyls, and enhanced activities of glutathione peroxidase, glutathione reductase and catalase all suggest free radical production and damage (Balazs and Leon, 1994; Lovell *et al.* 1995). These changes are not present uniformly throughout the AD brain (Schapira and Reichmann, 1995). The findings of depressed levels of GSH in AD cingulate cortex and AD substantia innominata but not temporal cortex support these observations (Gu *et al.* 1998b).

Reduced complex II+III activities in AD temporal cortex support the suggestion of a mitochondrial abnormality in AD although its relationship to pathogenesis is unknown (Cooper *et al.* 1993). The normal activities of complexes II and III when assayed separately might suggest that the decrease in the combined II+III activity may be the result of an abnormality of membrane function, although there were no data to confirm this. Since then, there have been several reports of mitochondrial abnormalities in AD brain involving complexes II+III and IV (Mutisya *et al.* 1994; Parker *et al.* 1994a), although the complex IV deficiency at least appears to be restricted to certain areas (Kish *et al.* 1992). These finding were generally confirmed by a further study looking at frontal and parietal cortex in AD patients (Chagnon *et al.* 1995). Cytochrome oxidase staining has been found to be decreased in AD hippocampus (Simonian and Hyman, 1993). Furthermore, a reduction in mRNA levels of mitochondrial encoded subunit 2 (COX II) of cytochrome oxidase was also found (Simonian and Hyman, 1994). Recently, Davis *et al.* (1997) found that AD can be associated with specific, maternally inherited mtDNA mutations in genes that encoded COX I and COX II. These mutations can be detected in the blood of AD patients. They suggested that these specific point mutations in COX genes may cause the COX defect in AD blood. However, more recently there was a report provides compelling evidence that the DNA isolation method used by

Davis *et al.* (1997) employed results in the co-amplication of authentic mtDNA-encoded COX genes together with highly similar COX-like sequences embedded in nuclear DNA ("mtDNA pseudogene"), therefore the observed heteroplasmy by Davis *et al.* (1997) is an artefact (Hirano *et al.* 1997).

Thus there is a consensus for a mitochondrial deficiency in AD temporal cortex. It is interesting to contrast the specific complex I deficiency, anatomically selective for the substantia nigra in PD, with the more comprehensive respiratory chain defect present in several areas of the AD brain and peripheral tissue.

The normal mitochondrial function and GSH levels in the PD substantia innominata suggest that complex I deficiency and oxidative stress do not contribute to either cholinergic cell death or Lewy body formation in this area (Gu *et al.* 1998b). The latter proposition is further supported by normal mitochondrial function and GSH levels in DLB cingulate cortex, an area rich in Lewy bodies. Selective complex I deficiency is specific for the neurodegeneration of the PD substantia nigra, normal complex I activities in HD caudate nucleus (Gu *et al.* 1996) and AD cortex, implying that it is not simply a by-product of cell death. Thus the complex I defect and GSH reduction in PD may either be a consequence of the biochemistry and pharmacology of the degenerating substantia nigra or the result of a pathogenetic mechanism separate from that involving the substantia innominata.

3.5.3 ApoE genotype frequencies

In this study, the findings of the apoE genotype frequencies in AD subjects were similar to those found in other studies (Strittmatter *et al.* 1993; Galasko *et al.* 1994) and confirmed that the ε4 allele shows a strong association with AD. The ε4 allele frequencies were also significantly increased to a similar extent in the DLB group but were not significantly different from controls in either of the PD groups, with or without dementia. These data were in agreement with other recent reports (Hardy *et al.* 1994; Lippa *et al.* 1995). Thus, the simplest inference from these results was that DLB shares risk factors with AD and the

aetiology and pathology of DLB were more closely related to AD than to PD. The apparent increase in ε4 allele frequency in cases of both AD and DLB, which was essentially without tangles, did not support the view that the key role of apoE was in promoting tangle formation and would appear to point to the plaques as a common pathological feature of apoE-related dementia. There was supporting evidence that in AD brain, apoE genotype correlated with plaque load rather than with tangle density, which is more consistent with an apoE-β-amyloid interaction than an apoE-tau protein interaction *in vivo* (Levy-Lahad and Bird, 1996). These results were supported by the observations that the mean density of β-amyloid plaques was lower in apoE ε3/3 cases than in the groups with the apoE ε4 alleles. Equally, from this study the apoE genotype would not appear to play a role in Lewy body formation, at least according to the results presented here. However, the apoE ε4 allele frequency was reported as 40.9 (59/144) in AD patients with intracortical Lewy bodies but only 5.5 (1/18) in DLB (Galasko *et al.* 1994). This appears to conflict with data from our studies or from other groups (Pickering-Brown *et al.* 1994). This may be due to the small number of individuals analysed (Galasko *et al.* 1994).

Patients with PD have a high incidence of dementia. Neuropathological explanations for dementia in PD include coexisting AD, Lewy bodies and neuronal loss. In this study, the apoE ε4 allele frequency was similar in PD with dementia and controls, suggesting the biological basis for dementia in PD is likely to differ from that of AD. The lack of an association between an increased frequency of ε4 allele and dementia in PD implied that, while the dementia of AD and PD shares some clinical and biological features, genetic susceptibility as measured by the apoE genotype was distinct in these two disorders. This finding was supported by other studies (Arai *et al.* 1994b; Marder *et al.* 1994). However, it has also been suggested that the apoE ε4 allele may be a risk factor for dementia in PD and that the dementia might have a genetic background that is closely related to that of AD (Arai *et al.* 1994a). The data presented here, however, suggests that ε4 is not be a major risk factor for dementia in PD.

CHAPTER 4 MITOCHONDRIAL FUNCTION IN HUNTINGTON'S DISEASE

4.1 Aim

To extend the analysis of mitochondrial function in caudate nucleus from Huntington's disease (HD) patients to determine the reproducibility of the respiratory chain defect in this disorder.

4.2 Introduction

HD is an autosomal dominant, fully penetrant disorder caused by an unstable CAG trinucleotide repeat on chromosome 4 which encodes a protein (huntingtin) of approximately 348kDa with unknown function. The CAG repeat within the IT-15 gene encodes a polyglutamine stretch proportional to the number of triplets. The polyglutamine tract is therefore the obvious candidate to mediate the gain-of-function toxicity of mutant huntingtin.

Although the gene defect responsible for HD has been identified, the mechanism by which CAG repeat length induces neuronal death is not yet known, and the biochemical basis of HD pathogenesis remains obscure. Several lines of evidence have suggested that an abnormality of energy metabolism may be involved. Striatal and cerebral cortex glucose hypometabolism has been observed early in the course of HD and appears to precede bulk tissue loss (Kuwert *et al.* 1990 and 1993). Elevated lactate levels were found in the occipital cortex of 16 HD patients and the basal ganglia of 8 HD patients examined by magnetic resonance spectroscopy (MRS) (Jenkins *et al.* 1993). These results were confirmed by the same group (Koroshetz *et al.* 1997) who provided evidence more recently to support a link between the expression of the CAG repeat and a defect in energy metabolism. They demonstrated a significant decrease in the phosphocreatine:inorganic phosphate ratios in resting muscles in HD patients and similarly elevated lactate:pyruvate ratios have also been seen in HD cerebrospinal fluid (Koroshetz *et al.* 1994 and 1997).

However, a more recent report suggested that there were no increase in cerebral lactate or decrease in phosphocreatine and ATP, therefore the energy metabolism was normal in the 4 cerebral locations measured by MRS (Hoang *et al.* 1998).

The polyglutamine tract of huntingtin has also been shown to bind GAPDH amongst other proteins which is an important enzyme in glycolysis (Burke *et al.* 1996).

Few reports have described the results of direct respiratory chain enzyme analysis of HD brain tissue. A decreased succinate linked oxidation and complex IV in HD caudate nucleus were observed (Brennan *et al.* 1985). A preliminary report from this laboratory described a decrease in complex II+III activity in caudate nucleus from 4 HD patients (Mann *et al.* 1990). The results presented here extend this previous study and provide more detailed data on the individual respiratory chain enzyme activities in caudate nucleus from HD patients.

4.3 Methods

The diagnosis of HD was made on clinical grounds and confirmed post-mortem by pathological examination of the brains from which samples of caudate nucleus were obtained (Brain Bank). Brains were matched with controls for age and post-mortem delay.

Frozen samples were homogenized and MRC enzyme activities were determined as described in chapter 2, section 2.4.

4.4 Results

MRC enzyme activities were determined in 10 HD and 10 matched controls.

The complex I activity in the caudate nucleus (CN) from HD was not significantly different from the control values when enzyme results were expressed as CS ratios (Table 4.1, group B). However, the CS ratios of complex II and complex III separately and complex II+III were significant

decreased (all p<0.01), and complex IV showed a significantly decrease in HD compared to the control (p<0.05) (Table 4.1, group B).

Table 4.1 Mitochondrial respiratory chain activity in caudate nucleus from age and PM-delay matched controls and HD patients

	Control		HD	
	A	B	A	B
n	5	5	4	6
Cx I/CS (x100)	3.45±0.92	2.55±0.44	3.31±2.06	2.62±0.77
CxII/CS (x100)	1.00±0.23	0.90±0.16	0.55±0.17	0.36±0.15
Cx III/CS (x100)	0.40±0.12	0.41±0.12	0.20±0.06	0.17±0.09
Cx II+III/CS (x100)	1.72±0.16	1.40±0.35	0.71±0.47	0.76±0.23
Cx IV/CS (x100)	0.69±0.09	0.84±0.29	0.48±0.19	0.55±0.19

Enzyme activities expressed as CS ratio. Values are mean±SD. Cx I: complex I, Cx II: complex II, Cx III: complex III, Cx II+III: complex II+III, CxIV; complex IV.
A: previous study (Mann *et al.* 1990); B: this study.

The complex II and III activities in CN from HD (n=4, group A) previously showed a significant defect compared with matched controls (n=5) (Mann *et al.* 1990). There were no significant differences between the data from group-A and the data from group-B, as judged by Mann-Whitney U test (Table 4.1). Therefore, the cumulative data were presented for MRC activities in age and PM-delay matched CN samples from controls and HD (Table 4.2).

Complex I activities in CN from HD patients were not significantly different from control when expressed per unit protein or when corrected for any variation in mitochondrial mass between samples by relating enzyme activities to CS levels (Table 4.2).

Mean complex II+III activities were significantly reduced: 57% (p<0.0001) per unit protein, and 53% (p<0.0001) when corrected for CS activity. This enzyme reflects electron transport between succinate and cytochrome c via co-enzyme Q and includes complex II and complex III activities. These enzymes may be

assayed separately and thereby potentially allow the dissection of the complex II+III defect into one or other component. In fact both complex II and complex III activities were each found to be decreased: 53% ($p<0.0001$) for complex II per unit protein and 56% ($p<0.001$) when CS corrected; 60% ($p<0.001$) for complex III per unit protein and 55% ($p<0.0005$) when CS corrected.

Complex IV activities in HD caudate were also decreased: 38% ($p<0.005$) per unit protein and 33% ($p<0.01$) when CS corrected.

Table 4.2 The cumulative data on mitochondrial respiratory chain activities in caudate nucleus from age and PM-delay matched controls and HD patients

	Control	HD
n	10	10
Age	63.4±17.4	61.6±14.1
PM-delay (hrs)	29.4±13.3	34.7±15.6
Complex I	4.89±0.98	4.19±1.28
Complex II	15.6±2.87	7.43±2.03****
Complex III	6.64±1.78	2.67±1.04****
Complex II+III	25.9±5.91	11.2±4.82****
Complex IV (k)	1.31±0.39	0.81±0.39**
CS	169±29	153±43
Complex I/CS (x100)	3.00±0.83	2.90±1.37
Complex II/CS(x100)	9.52±1.53	4.24±1.92****
Complex III/CS(x100)	4.01±1.10	1.82±0.90***
Complex II+III/CS(x100)	15.6±3.12	7.42±3.22****
Complex IV/CS(x100)	0.77±0.22	0.52±0.18*

Enzyme activities expressed as nmol/min/mg protein or CS ratios, except for complex IV which is k/min/mg where k is the rate constant. Values mean ± SD.
*: $p<0.01$; **: $p<0.005$; ***: $p<0.0005$; ****: $p<0.0001$, as judged by Mann-Whitley U test.

4.5 Discussion

The molecular genetic basis for HD is now understood. The HD gene is expressed in peripheral tissues as well as within the central nervous system (Ambrose *et al.* 1994; Trottier *et al.* 1995). Both the normal and mutant HD gene alleles are expressed at the mRNA and protein levels in most tissues. Interestingly, the normal and mutant huntingtin are expressed in lymphoblast and fibroblast lines but no clear evidence of expression in HD striatum or cortex was seen, although a faint smear on the Western blot suggested the presence of mutant huntingtin (Trottier *et al.* 1995). Immunohistochemistry with monoclonal antibodies to huntingtin demonstrated intense punctuate staining in nerve fibres and varicosities, with light staining in some neuronal perikarya. Staining was particularly strong in the zona reticulata of the substantia nigra especially in the melanised dopaminergic neurons. There was little staining of the striatum.

As a dominant disease, mutant huntingtin probably has its effect through either a deleterious gain-of-function or a negative influence on the normal gene product. It has been suggested that the increased polyglutamine stretch encoded by the mutant gene may render the HD protein more sensitive to transglutaminase and inactivation perhaps through conformational change (Perutz *et al.* 1994). Whatever the mechanism by which the abnormal gene product acts, there is substantial evidence for an abnormality of energy production in HD.

PET and MRS studies have identified abnormalities of glucose utilisation and lactate production in HD (Beal, 1992). It has been argued that deficient energy metabolism may cause neuronal death through excitotoxic mechanisms (Beal, 1992 and 1994). Impaired respiratory chain function can induce glutamate toxicity through partial depolarisation, release of the Mg^{2+} dependent inhibition of N-methyl-D-aspartate acid (NMDA) receptors and consequent activation by glutamate (Novelli *et al.* 1988; Simpson and Isacson, 1993). The suggestion that glutamate toxicity may be important in HD was first proposed some years ago (Olney and de Gubareff, 1978) and has subsequently been supported by

several lines of evidence including the recent demonstration by MRS of increased glutamate levels in HD caudate (Taylor-Robinson *et al.* 1994).

The findings that lactate concentrations are increased in HD cerebral cortex and treatment with coenzyme Q_{10}, an essential cofactor of electron transport chain which shuttles electrons between complexes I, II and III, and functions as an antioxidant (Beyer, 1992), resulting in significant decreases in cortical lactate concentrations in 18 HD patients, provide supporting evidence for a generalized energy defect in HD.

The observations of disordered energy metabolism and potential glutamate toxicity in vivo in HD have stimulated the search for a toxin model of HD. Slow intrastriatal injections of quinolinic acid, a tryptophan metabolite and an NMDA agonist, can produce an approximate pathological model of HD in primates (Ferrante *et al.* 1993).

Much interest has recently been focused on the use of 3-NP, an irreversible inhibitor of succinate dehydrogenase (SDH, complex II), as a toxin model for HD. Accidental human exposure to 3-NP through eating mildewed sugar cane induced nausea, vomiting, encephalopathy and coma followed, in survivors, by dystonia and choreiform movements (Ludolph *et al.* 1991). 3-NP has been shown to decrease ATP formation and cause neuronal damage by excitotoxic mechanisms in cerebral cortex (Ludolph *et al.* 1992). Intrastriatal injections of 3-NP into rats produced dose-dependent ATP depletion, increased lactate concentrations and neuronal loss that was reduced by prior decorticating and removal of striatal glutaminergic input (Beal *et al.* 1993). Subacute systemic administration of 3-NP to rats produced elevated lactate levels in basal ganglia which preceded magnetic resonance imaging changes in the striatum. Chronic administration of 3-NP to primates can replicate many of the characteristic motor and histological features of HD which further supports the possibility that subtle impairment of energy metabolism may play a role in its pathogenesis (Brouillet *et al.* 1995).

The results of this analysis of respiratory chain function in HD caudate nucleus provide a significant biochemical parallel to the HD animal model as induced by 3-NP or malonate, another inhibitor of succinate dehydrogenase (Greene *et al.* 1993). We have found marked reductions in the activities of both complexes II and III and more mild changes in complex IV. These results confirmed the original observations of Brennan and co-workers (Brennan *et al.* 1985). The degree of complexes II and III defects as assessed individually was comparable and this raises the possibility that both may be secondary to a deficiency of ubiquinone which is used as an electron carrier by both complexes. The relationship of the complex IV defect to that of complexes II and III is uncertain; this could not be caused by ubiquinone deficiency but might imply a more general dysfunction of these membrane bound proteins.

The relationship of the mitochondrial functional deficiency to the pathogenesis of HD is unknown at present. The identification of this abnormality, however, fits well with the current hypothesis of glutamate induced excitatory damage resulting from a mitochondrial defect and ATP depletion. The observation that complex II inhibition itself induces a pathological model of HD supports this hypothesis. The anatomical specificity of the mitochondrial deficiency has not as yet been clearly defined. Direct measurement of activities of HD putamen (n=4) and HD cerebral cortex (n=4) showed no abnormality but these data need expanding (Mann *et al.* 1990). MRS data, however, have shown increased lactate concentrations in occipital cortex in HD, suggesting that defective energy metabolism may be present in areas of HD brain beyond the caudate (Jenkins *et al.* 1993; Koroshetz *et al.* 1997). The mitochondrial defect in HD caudate is clearly biochemically and anatomically distinct from the specific complex I deficiency identified in PD substantia nigra (Schapira *et al.* 1990a) and the severity of the selective complex IV deficiency in Alzheimer's disease (Parker *et al.* 1990a; Mutisya *et al.* 1994). It would be reasonable to assume that each has a separate cause. It appears, therefore, that certain neurodegenerative diseases have different respiratory chain defects and this may give some clues as to their pathogenesis. It is also important to note that not all neurodegenerative diseases are associated with a mitochondrial defect. We have analysed the brains of 15 patients with MSA and found no

respiratory chain deficiency when corrected for citrate synthase activity (Gu *et al.* 1997). As always with biochemical analysis of post mortem tissue, the question of artificial changes must be addressed. To some extent this is achieved with control tissue appropriately matched for age and post-mortem delay. In addition, the pattern of mitochondrial defect in HD caudate has not been observed in caudate or any other brain areas from in excess of 100 control and disease brains examined by this laboratory. Thus the defects observed in HD are not simply due to post mortem delay artefact.

The tissue distribution of the mitochondrial deficiency in HD will be important in determining its relationship to the molecular genetic defect already identified. The HD gene is expressed widely although it is not known whether the resulting biochemical defect is also expressed in all tissues. In terms of platelet mitochondrial function Parker *et al.* (1990b) found a 72% deficiency of complex I in 5 HD patients but no change in the activities of complexes II-IV. However, we could not identify any respiratory chain abnormality in the platelets of 11 genetically proven HD patients (Gu *et al.* 1996). The specific complex I deficiency in blood in contrast to the normal complex I activity in HD caudate is difficult to explain. The converse situation of defects of complexes II-IV in HD caudate, but no decrease in activity in blood, parallels the site of pathological damage in HD. Differential tissue biochemical expression of this abnormality may parallel the influence of the HD gene product as well as other local biochemical and neurochemical influences in caudate nucleus.

The relationship of the mitochondrial deficiency to the CAG triplet repeat on chromosome 4 is not known. Mammalian complex II has four subunits all encoded by nuclear DNA; complex III has 11 subunits, all except one (cytochrome b) encoded by nuclear DNA; complex IV comprises 13 subunits, three of which are encoded by mtDNA. The predicted molecular mass of the HD gene products is greater than any of the respiratory chain subunits and does not conform to any of the known sequence data on nuclear encoded respiratory subunits. It is possible that the HD product could affect complexes II-IV or ubiquinone function through an indirect effect, perhaps through an

abnormality of mitochondrial protein import or mitochondrial membrane structure or function.

The mechanisms by which mutant huntingtin precipitate this cascade remain unknown but may increase neuronal sensitivity to excitotoxicity stimuli. Aconitase, an Fe-S containing tricarboxylic acid cycle enzyme, has been shown to be extremely sensitive to inhibition by peroxynitrite ($ONOO^-$) and superoxide. This action has been used as an indirect indicator of free radical generation (Hausladen and Fridovich, 1994). There is evidence for oxidative damage in HD (Browne *et al.* 1997). Experiments demonstrated that complexes II and III were those segments of the MRC most sensitive to $NO^\bullet$. However, in cultured SKNMC cells, aconitase proved to be more sensitive to $NO^\bullet$ than complexes II and III (Tabrizi, S. *et al.*, in press). Thus laboratory has reported that aconitase activity was severely decreased in HD caudate, putamen and cortex, but similar to control values in the cerebellum and fibroblasts (Tabrizi, S. *et al.*, in press). The severe deficiencies of aconitase activities in affected HD brain regions suggest $NO^\bullet$/ $ONOO^-$ and free radical production is increased in these brain areas. This finding provides further support for a MRC defect in HD which primarily involved complex II/III and parallels the severity of neuronal loss in HD brain (Tabrizi, S. *et al.*, in press).

CHAPTER 5 A PILOT STUDY FOR FUSION IN THE MELAS-A3243G PATIENT PLATELETS

5.1 Aim

To analyse the feasibility of detecting a biochemical defect in A549ρ°-platelet cybrids if the defect is caused by a mtDNA mutation.

To determine if it is possible to detect the 3243A→G mutation (A3243G) of the $tRNA^{Leu(UUR)}$ by functional analysis of A549ρ°-A3243G cybrids and to analyse the correlation between mutation load and MRC function in a control nuclear background. This fusion study serves as a pilot study for the experiment described in chapter 6, where the possible contribution of mtDNA to the complex I defect observed in PD platelets was investigated.

5.2 Introduction

Several pathogenic mutations in the $tRNA^{Leu(UUR)}$ gene have been described (Goto *et al.* 1990). An A→G transition at nucleotide 3243 has been seen most frequently in patients with the MELAS syndrome. This mutation is nearly always heteroplasmic, *i.e.* mutant type mtDNA co-exists with wild-type mtDNA.

Studies in which mitochondria from patients with MRC defects were transferred to ρ° cells have allowed analysis of the disease-associated mtDNA mutation in a control nuclear background. These experiments have helped to determine whether the MRC defect is solely due to the mutated mtDNA or whether the nuclear DNA is involved. The MELAS-A3243G mtDNA genotype has been extensively studied in an osteosarcoma ρ° cell line (206ρ°). Two studies showed that the transfer of patient mitochondria from fibroblasts or myoblasts to ρ° cells failed to restore full respiratory capacity when the proportion of mutated mtDNA was over 90% of the total amount of mtDNA (Yoneda *et al.* 1992; Chomyn *et al.* 1992). A third study claimed a threshold level of 60% for a complex I defect (Dunbar *et al.* 1996). All three studies implied a causal role for the A3243G mutation in MELAS. A similar fusion study performed in 206ρ° for the MERRF-A8344G $tRNA^{Lys}$ gene mutation also revealed a threshold level of at least 90% mutant mtDNA (Chomyn *et al.*

1994). On the other hand, introduction of mtDNA with large-scale deletions into HeLaρ° cells showed that a relatively low proportion of deleted mtDNA (67%) markedly reduced the translation of mtDNA encoded proteins (Hayashi *et al.* 1991) and exhibited biochemical dysfunction.

Chomyn *et al.* (1994) generated cybrids generated from the fusion between 206ρ° cells and platelets from a MERRF patient, thus avoiding the necessity to have the mutation in cultured cells or to enucleate the donor cells before fusion with ρ° cells. In the present study, A549ρ° cells were fused with platelets from a MELAS patient and MRC function was analysed in clonal cybrids which contained various proportions of A3243G mutant and wild-type mtDNA.

5.3 Methods

The following methods were used: Analysis of A3243G mtDNA using ApaI digestion and confirmation of complete enzyme digestion (chapter 2, section 2.6.1); Determination of a A549 mtDNA polymorphism using the restriction enzyme AluI (section 2.6.2); Cell fusion, cybrid cloning and cybrid MRC function analysis (section 2.7.1-2.7.7); Quantification of mutant mtDNA using image analysis (section 2.7.8); Recloning of a cybrid (section 2.7.9); Detection of COX proteins (Western blot) (section 2.7.10); and cytochemical and immunocytochemical staining of cybrids for COX activity and COX I expression (section 2.7.11).

The platelets used in this study were isolated from a 32-years old female patient with ophthalmoplegia, proximal myopathy, deafness and stroke-like episodes and were known to harbour the A3243G mtDNA mutation.

5.4 Results

Platelets from a patient with the A3243G mtDNA mutation were fused with A549ρ° cells and 41 clonal lines were analysed.

5.4.1 A549 mtDNA polymorphism and the determination of A3243G mutant loading

The PCR product generated to detect the presence of 4 **CA**-repeats in the D-loop region failed to be digested with AluI in any of the 41 cloned cybrids. These results confirmed that the mtDNA in all cybrids was transmitted from mitochondria isolated from the platelets of this MELAS patient and that none of the cybrids contained detectable levels of A549 mtDNA (Fig. 5.1A).

5.4.2 Distribution of mutant load between cybrids

The proportion of the MELAS-A3243G mutant mtDNA was about 15% in whole blood and in isolated platelets of the MELAS patient (data not shown). The MRC enzyme functions (complexes I, II+III and IV) in the patient's platelets were normal (Table 5.1). After fusion, 41 clonal cybrids were selected. Quantification of the mutant load was determined by image analysis and corrected with a factor which reflected the efficiency of ApaI digestion (Fig. 5.1).The distribution of the mutant load in the selected cybrids lines is showed in Fig. 5.2. Platelet-mediated transformation had expanded the proportion of mutant mtDNA from the original 15% to a maximum of 89% with the majority of mutant cybrids containing 26-45% mutant mtDNA. After recloning of the cybrid line with the highest mutant load (89%), other 30 subclones were selected. Two of these 30 clones had shifted to wild-type where the remaining 28 clones had a mutant load ranging between 50 - 95% (Fig. 5.2). Five subclonal lines with high mutant load (92, 93, 94, 95 and 95%) were selected for MRC function assays.

Fig 5.3 shows that cybrids with a mutant load of 0, 30, 55, 80 and 89% did not shift significantly during cell proliferation which suggested that proportion of mutant mtDNA in the cloned cybrids was stable. Although recloning revealed that there was a drift of mutant levels in daughter clones, the overall level of mutation remained constant within individual clones.

Table 5.1. Mitochondrial respiratory chain functions in platelet mitochondria from controls and a MELAS patient

	Cx I /CS(x100)	CxII+III/CS	Cx IV/CS(x100)	CS
Control (n=5)	9.79±2.74	0.27±0.06	0.90±0.45	210±45
MELAS (n=1)	9.00	0.25	1.01	195

Cx : complex, CS: citrate synthase

5.4.3 MRC function

The activities of complex I and complex II+III in cybrids with a mutant-load of 30, 55, 80 and 89% were not significantly different from those in control cybrids when expressed per unit of protein or when corrected for any variation in mitochondrial mass between samples by relating enzyme activities to citrate synthase activities (CS ratio, Fig. 5.4, data not shown for complex II+III). Complex IV activity was decreased by 23% in the cybrid with a mutant mtDNA load of 89% compared to the cybrid with a mutant mtDNA load of 0%, but this decrease was not statistically significant (Fig. 5.5).

After subcloning of the cybrid with a mutant mtDNA load of 89%, cybrids with mutant load higher than 90% were isolated. Complex I and IV activities were determined in cybrids with 92, 93, 94, 95 and 95% mutant mtDNA. The complex I activities in these cybrids were significantly decreased compared to the activities in control cybrids when expressed per unit of protein (37%, $p<0.05$) or corrected to citrate synthase levels (38%, $p<0.05$, Fig. 5.4). Compared to control cybrids, the mean complex IV activity in the subclones with a high mutant load (>90%) was significantly reduced per unit of protein (42%, $p<0.05$) or when corrected for citrate synthase (44%, $p<0.01$, Fig. 5.5).

There was a positive correlation between the CS ratios for complex I and complex IV in the cloned cybrids (0-95% mutant-load) (Fig. 5.6). Figs 5.7.-5.8. suggested there are thresholds between percentage of mutant load and MRC

functions; If cybrids have above 90% of mutant mtDNA load, there are functional defects in complexes I and IV activities.

5.4.4 Cytochemistry and immunocytochemistry

The cybrid with a mutant mtDNA load of 95% had no detectable COX activity staining in approximately 50% of the cells (Fig. 5.9B). A qualitative assessment of negatively staining COX cells in cybrids with different amounts of mutant mtDNA is listed in Table 5.2.

Table 5.2 The relationship between % of mutant mtDNA load in cybrids and semi-quantification of negative staining for COX activity in these cybrids

% mutant mtDNA load	negative staining for COX activity in these cybrids
0	(-)
30	(-)
50	(-)
70	(+)
90	(+)
95	(++)

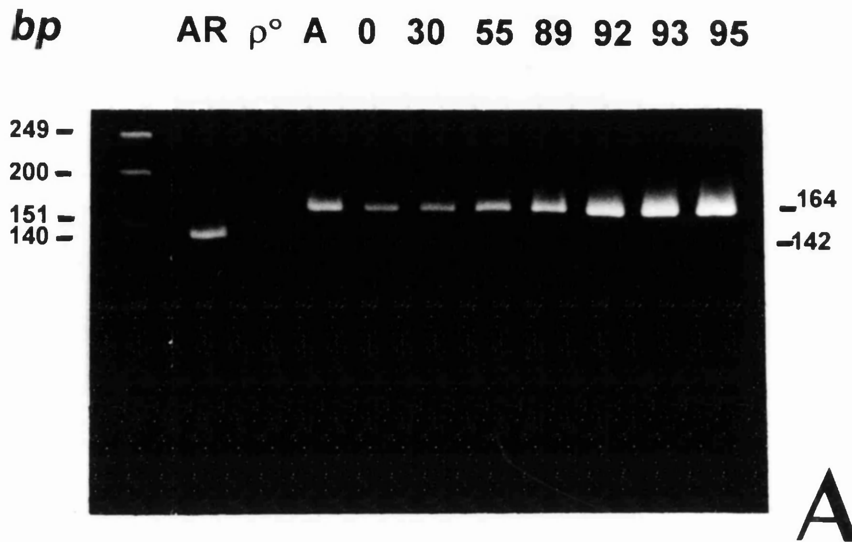
(-):all stained, no COX negative cells; (+): ~25% no staining; (++): ~50% no staining

There were no COX activity negative cells observed in cybrids with a mutant mtDNA load below 50%. There was a progressive increase in number of COX negative cells which harbouring mutant mtDNA between 70% and 95%, with approximately 50% of COX negative cells at 95% mutant mtDNA.

Cells from cybrids with 0% and 95% mutant (A3243G) mtDNA were stained immunocytochemically with antibodies to COX I. The results showed a uniform expression of COX I in mitochondria of control cells (Fig. 5.9E). However, in the 95% mutant-load cybrid clone, some cells showed normal levels of COX I, whereas other cells had decreased levels or completely lacked staining with this antibody (Fig. 5.9F). The triple exposure of cells clearly showed that in the cybrid with mutant mtDNA of 95%, COX I antibody binds heterogeneously for COX I protein (Fig. 5.9H).

5.4.5 Western-blot: COX I, II, IV and porin

Immunoblots of mitochondrial enriched fractions isolated from cybrids with mutant mtDNA loads ranging from 0 - 95% and also A549ρ° cells using antibodies to COX I, COX II and COX IV, demonstrated that only mitochondrially enriched fractions from the cybrid with mutant mtDNA load of 95% had decreased COX I. However, COX II was absent in A549ρ° cells and decreased in cybrids with mutant mtDNA loads greater than 89%, While the COX IV subunit was decreased in A549ρ° cells and in cybrid with 95% of mutant load. Similar staining intensities with the anti-porin antibody indicated that all mitochondrial loading were equal (Fig. 5.10).



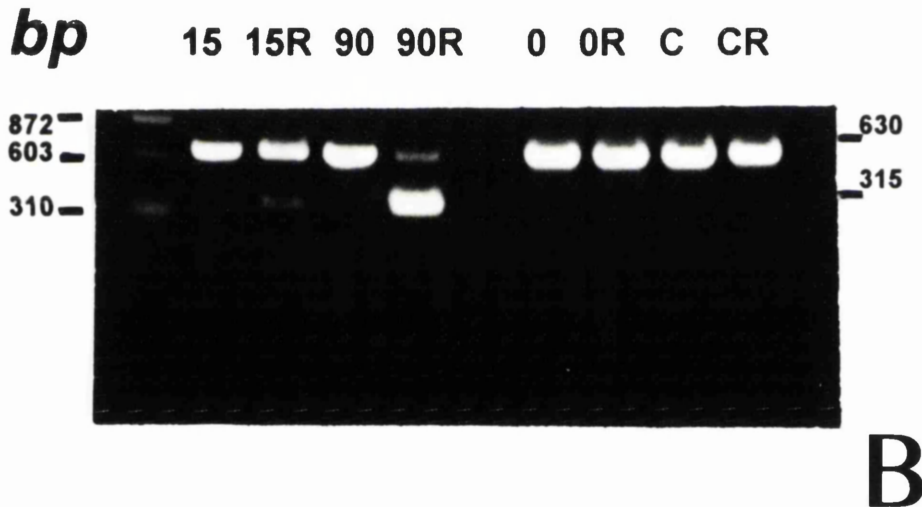


Fig. 5.1 (A) Restriction fragment length analysis for the A549 cell mtDNA polymorphism. PCR products from A549 cells (AR) and A549ρ° (ρ°) restricted with AluI and unrestricted A549 cells (A). The numbers relate to AluI-restricted PCR products generated from cybrid clones with a given % of mutant (A3243G) mtDNA. (B) Restriction fragment length analysis for some of cloned cybrids with different mutant (A3243G) mtDNA loads. The numbers represent the level of mutant mtDNA present, as determined from this analysis. Those with an "R" represent those products restricted with ApaI, 0: wild-type; C: control cells. The first lanes of Fig. (A) and (B) are molecular markers.

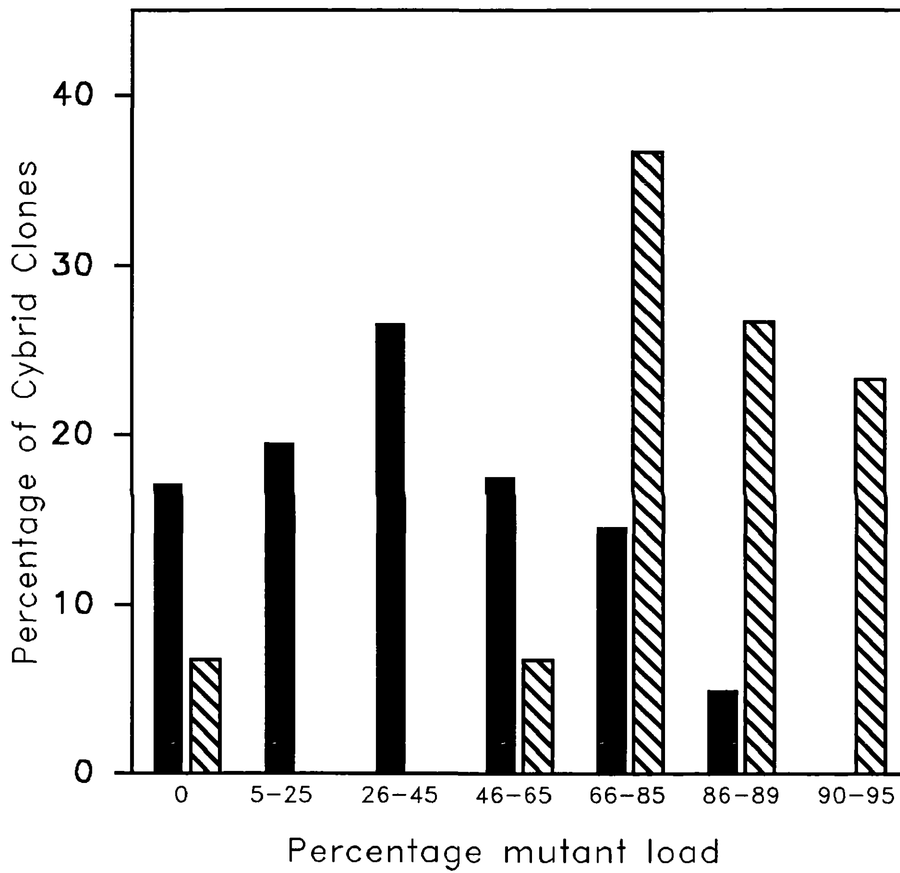


Fig. 5.2 Distribution of mutant mtDNA in A549ρ°-A3243G clonal cybrids. Filled bars: original clonal lines (n=41); hatched bars: clonal lines after subcloning an original cybrid line with 89% mutant mtDNA (n=30).

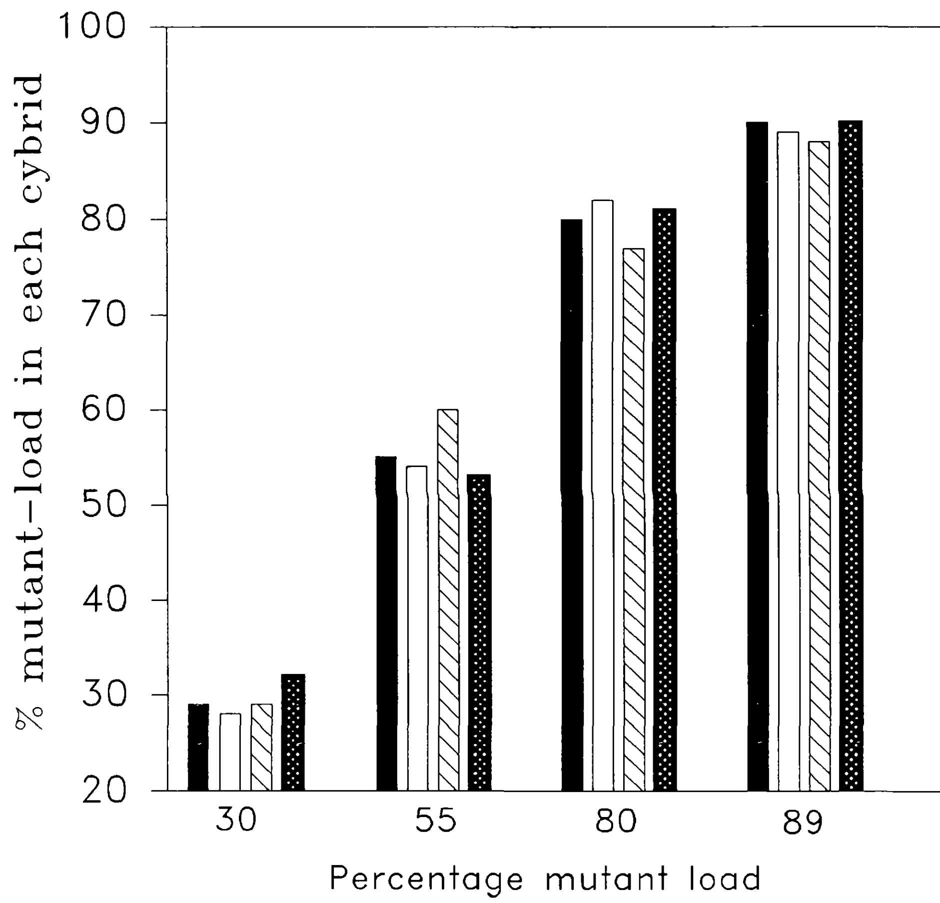


Fig. 5.3 Proportion of the mutant mtDNA in clonal cybrids over 15 cell doublings. Cell doubling: 2.5, filled bars; 5, empty bars; 10, hatched bars and 15, crossed bars.

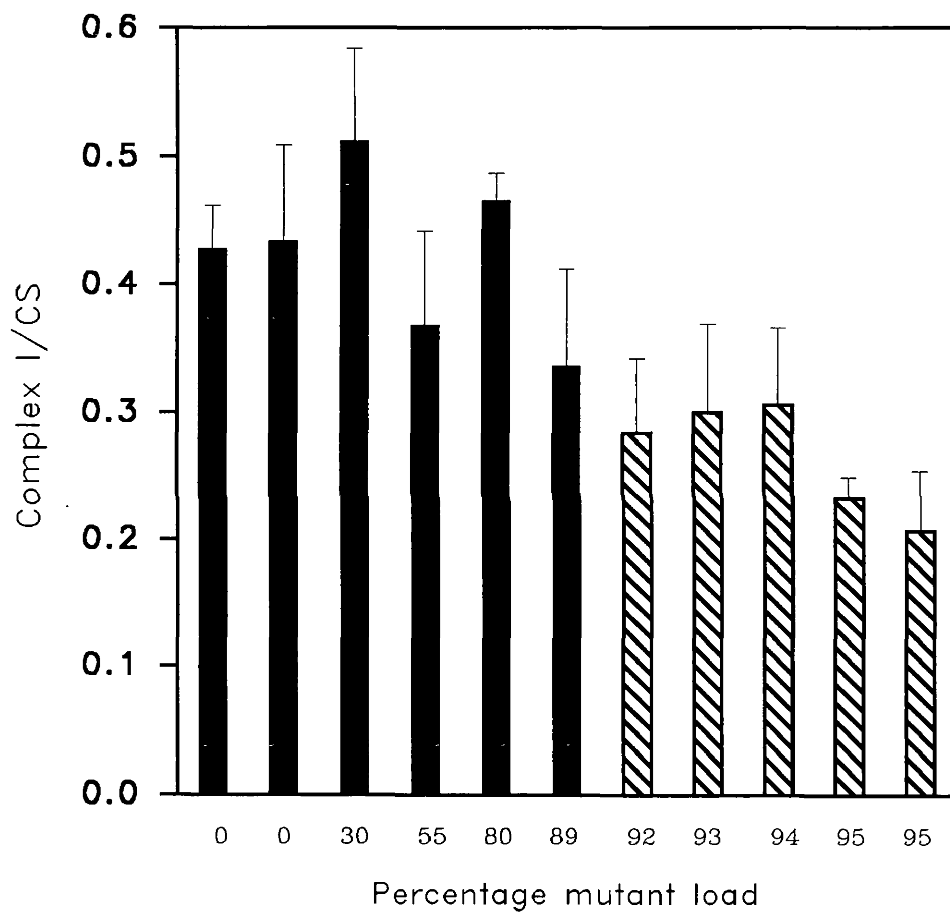


Fig. 5.4 Relationship between complex I/CS ratio and A3243G mutant load. Original cloned cybrids (filled bars) and subcloned cybrids (hatched bars). Each bar represents a different clonal line and values are mean ± SD of 3 independent mitochondrial preparations.

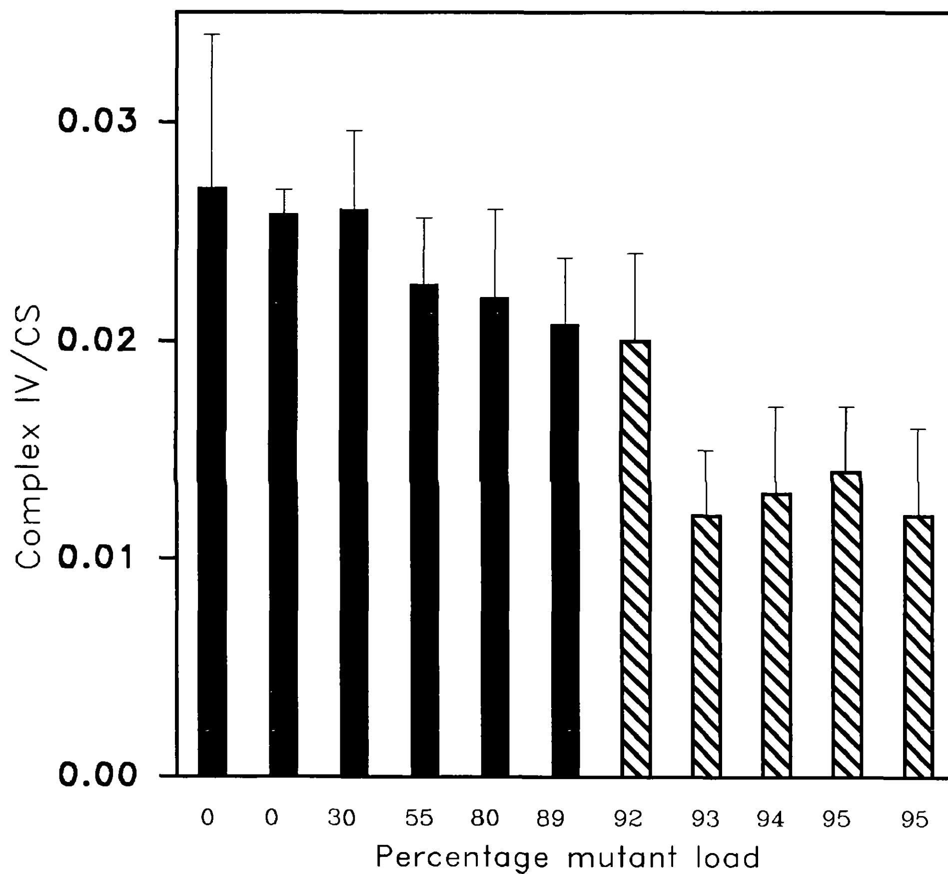


Fig. 5.5 Relationship between complex IV/CS ratio and A3243G mutant load. Original cloned cybrids (filled bars) and subcloned cybrids (hatched bars). Each bar represents a different clonal line and values are mean ± SD of 3 independent mitochondrial preparations.

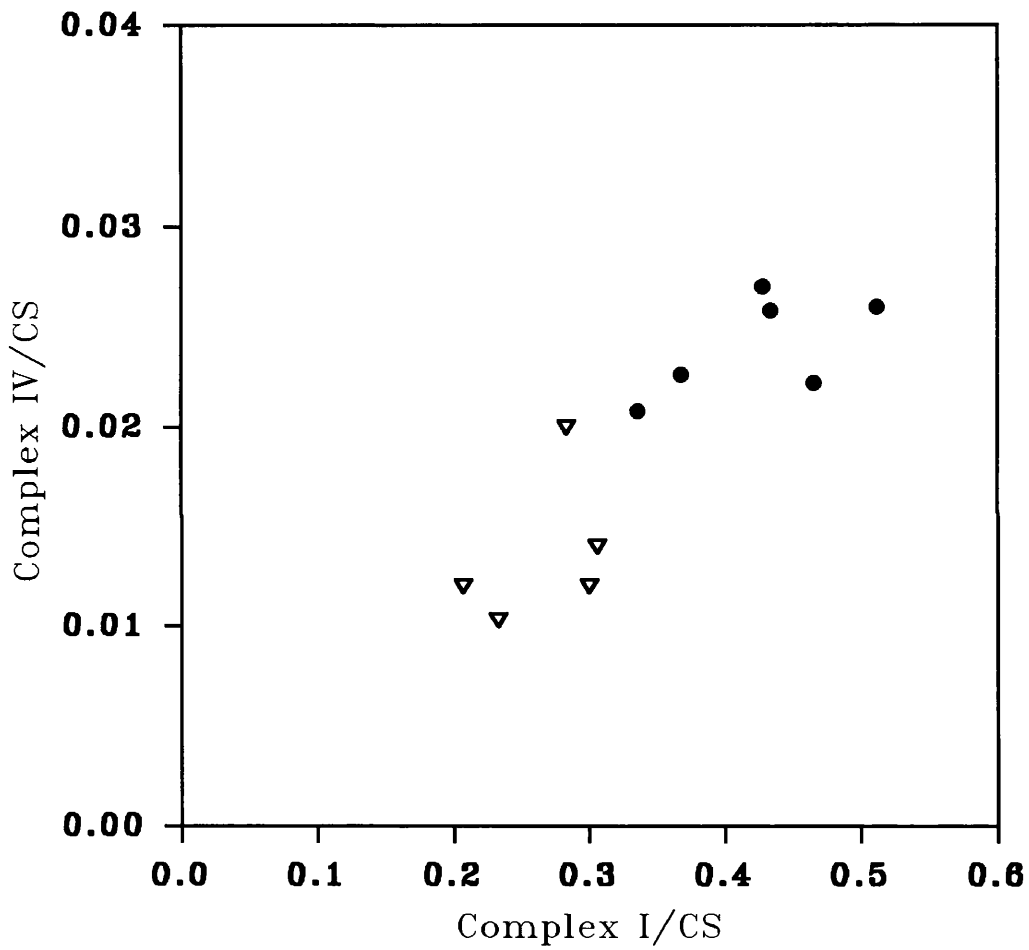


Fig. 5.6 Correlation between complex I/CS and complex IV/CS ratios. Original clonal cybrids (filled circles) and subcloned cybrids (open triangles). Spearman: r=0.86, p<0.001.

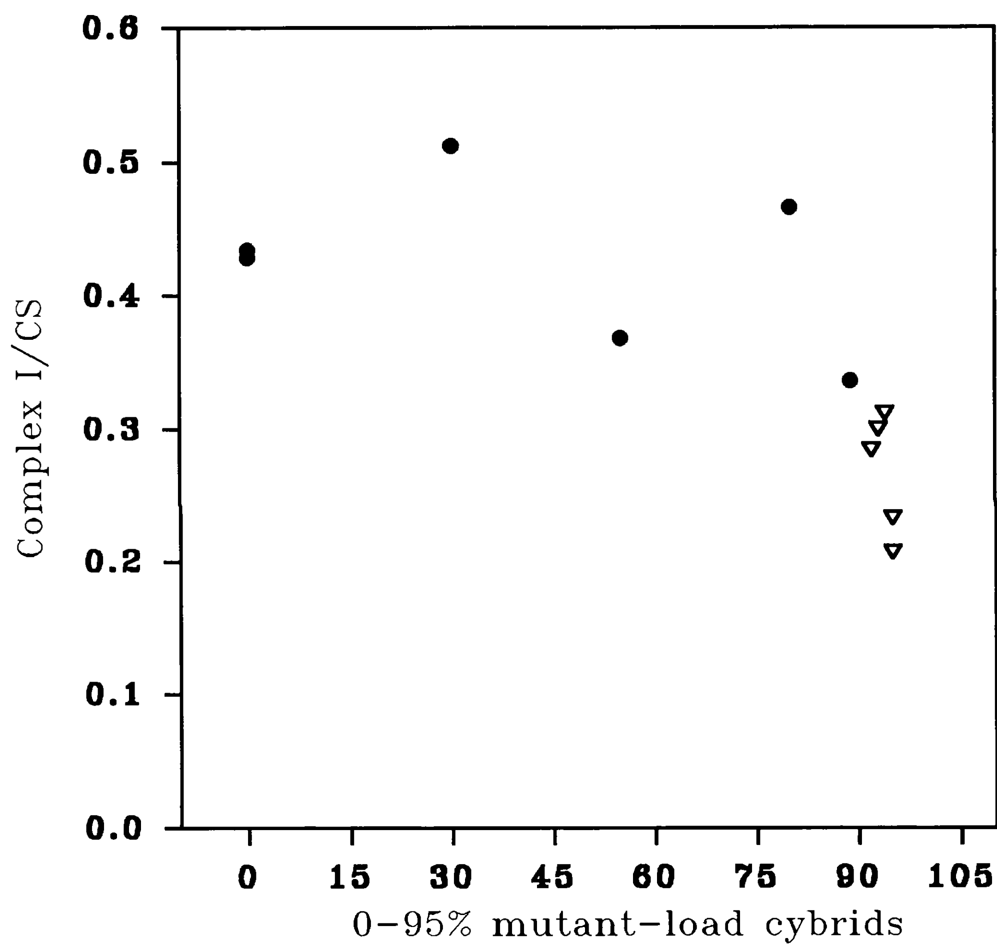


Fig. 5.7 Relationship between mean complex I/CS ratios and the percentage of A3243G mutant mtDNA load. Original clonal cybrids (filled circles) and subcloned cybrids (open triangles). Approximately 90% of A3243G mutant mtDNA load was the threshold which significantly affect complex I function.

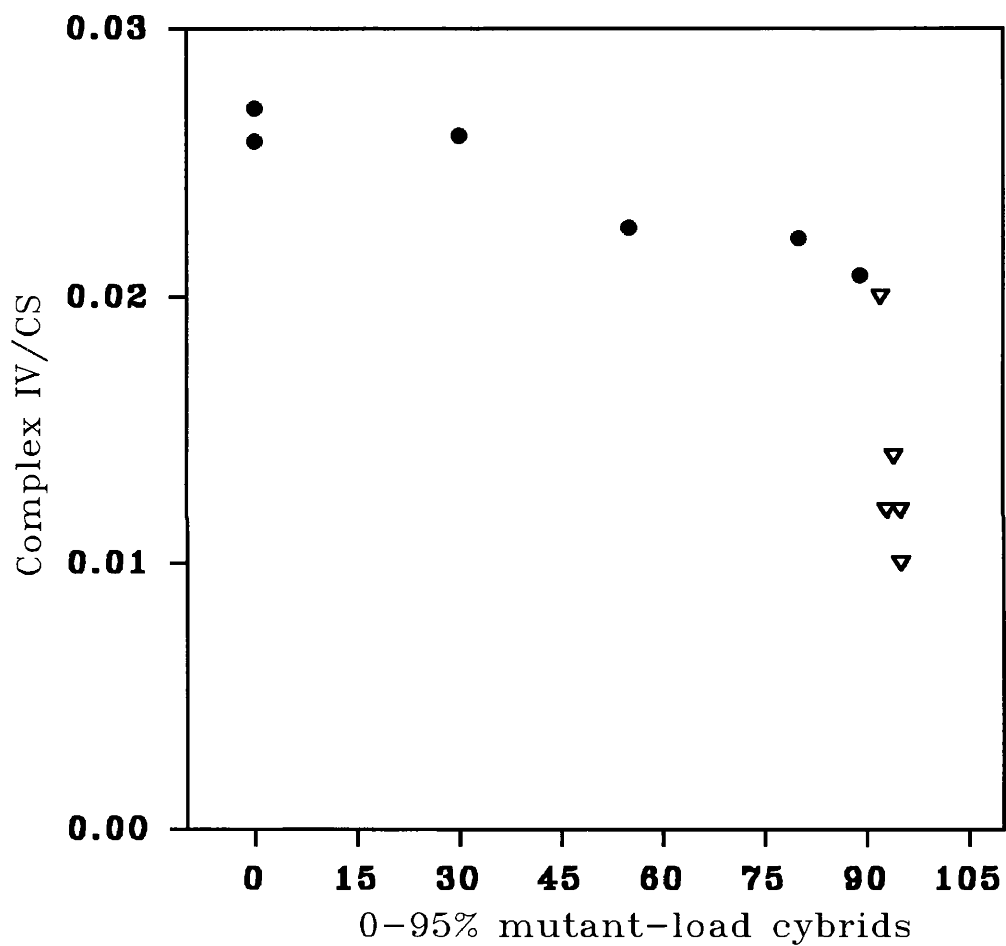


Fig. 5.8 Relationship between mean complex IV/CS ratios and the percentage of A3243G mutant mtDNA load. Original clonal cybrids (filled circles) and subcloned cybrids (open triangles). Approximately 90% of A3243G mutant mtDNA load was the threshold which significantly affect complex IV function.

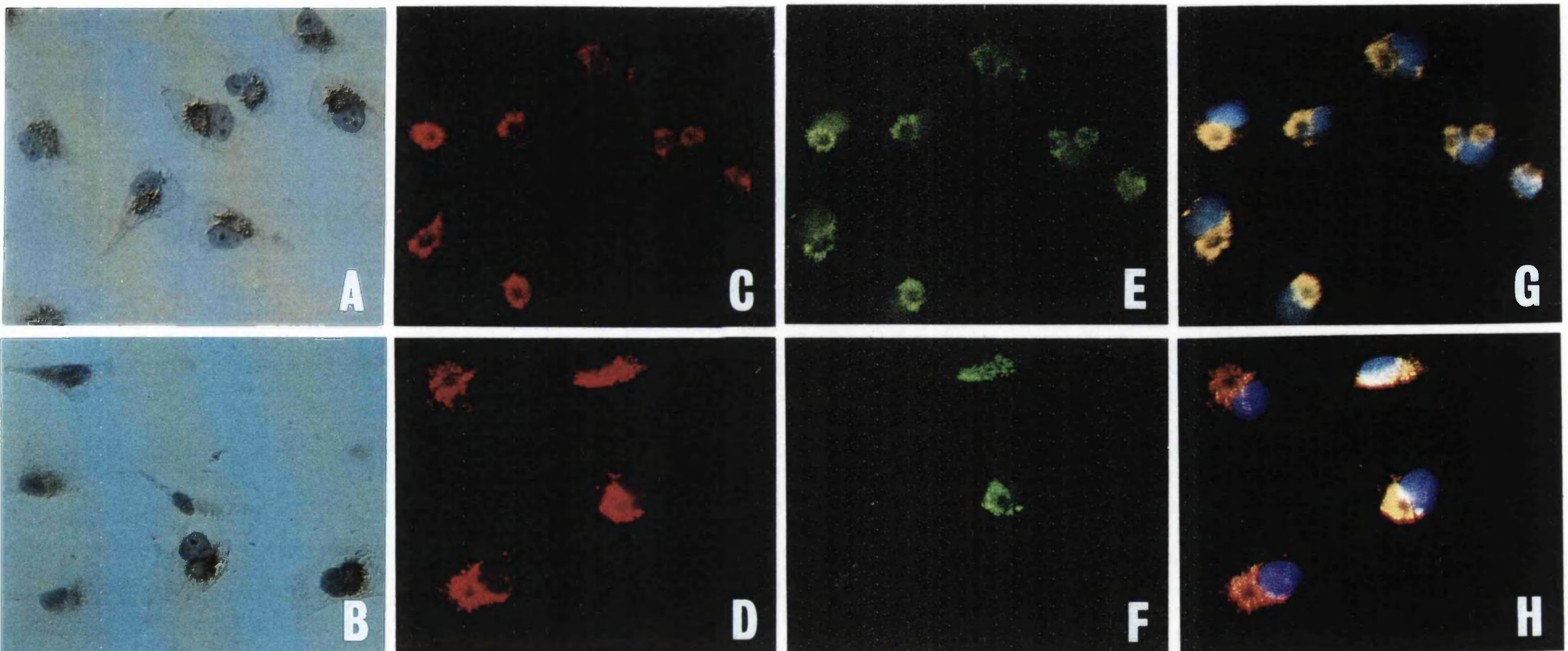


Fig. 5.9 Cytochemical and immunocytochemical staining of A549ρ° platelet cybrids. A549ρ°-control cybrids (A, C, E, G), A549ρ°-A3243G cybrids with 95% mutant mtDNA (B, D, F, H). Cells stained for; cytochrome oxidase activity (A, B); MitoTracker (C, D), and immunocytochemically using an anti-COX I antibody (E, F). Cells with decreased staining are indicated with arrows. Combined images of MitoTracker, anti-COX I antibody and Hoechst 33258 are shown in G and H. Different exposures of the same cells are shown in C, E and G and also D, F and H. Magnification x 430.

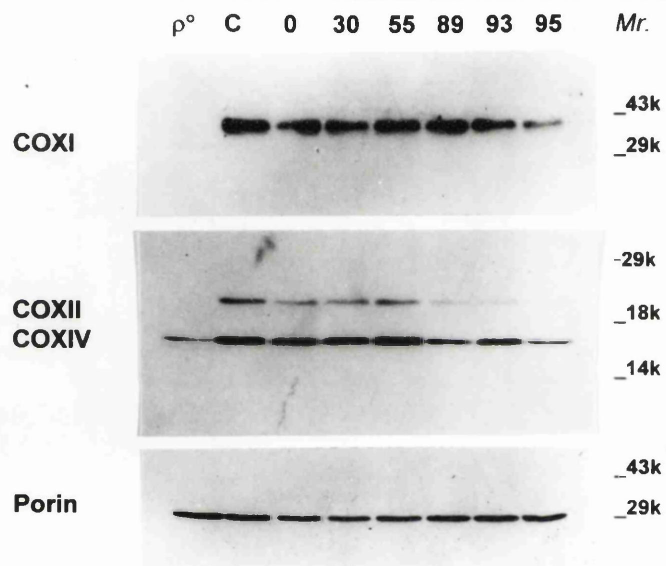


Fig. 5.10 Immunoblot analysis of mitochondrial enriched fractions from A549ρ° (ρ°); A549ρ°-control (C) and cybrids from varying percentages of mutant A3243G mtDNA, as indicated by the number. The blots were probed with antibodies against COX I, COX II, COX IV and porin. Migration of *Mr.* standards is indicated.

5.5 Discussion

The A3243G mutant mtDNA levels varied in the 41 selected clonal lines. Many cybrids (n=26) had more than 30% A3243G mtDNA. However only two clones reached more than 80% mutant mtDNA. Thus, the transformation of platelet mitochondria into human ρ° cells amplified mutant mtDNA levels from 15% up to approximately 90%. These results clearly show amplification of mutant mtDNA in this system. If there were a biochemical defect in the platelets due to mutant mtDNA, then this defect would remain or increase in a percentage of the cybrids. Using this as a model mutation it would suggest if sufficient cybrid clones are analysed then the fusion of platelets with ρ° cells is an effective method to identify whether mtDNA point mutations are present even in patients with mtDNA point mutation levels as low as 15% and normal MRC function in platelets .

The mutant load of individual clones was relatively stable up to 15 cell doublings for all mutant-load cybrids analysed. Similar results were reported by Dunbar *et al.* (1996), who also observed stable A3243G mtDNA levels in cybrids. These observations might be explained by there being no advantage of the replication rates of either mutant or wild type mtDNA. However, changes in the levels of A3243G mtDNA have been detected previously in other reports (Yoneda *et al.* 1992; Dunbar *et al.* 1995). It is difficult to explain why similar A3243G mutant-load cybrids showed a different behaviour. Possibly, different nuclear backgrounds or growth conditions gave either mutant or wild type mtDNA a replicative advantage, or cells containing either higher mutant or higher wild type mtDNA a replicative advantage.

A marked threshold effect, *i.e.* the complementation effect of A3243G mtDNA by wild-type mtDNA in 206ρ°-A3243G cybrids, was detected by measuring O_2 consumption (Yoneda *et al.* 1992; Chomyn *et al.* 1992). The positive correlation between the complex I and complex IV activities (CS corrected) in the clones was consistent with the mutation resulting in an impairment of mitochondrial translation and a multiple biochemical defect. The decreased activities of complexes I and IV only occurred at high mutation load in the

cybrids, indicating a high threshold level. The results presented here support this threshold with approximately 10% of wild-type mtDNA being enough to allow the MRC to function normally. The wild type $tRNA^{Leu}$ presumably serve the demands of the translation machinery to synthesise sufficient mtDNA-encoded proteins to give normal MRC function.

The results of cytochemical and immunocytochemical analyses of each cybrid line clearly showed that the cells within each clone were heterogeneous, with respect to COX activity and COX subunit I staining. With A3243G mutant loads of more than 70% COX staining showed an increasing percentage of COX-negative cells with increasing mutant load. This heterogeneity was confirmed by re-cloning the clonal cell line in the majority of the re-clones with a mutant load between 66-85%, but with 5 clones more than 92% and 2 clones with no detectable mutant mtDNA. This observation suggested that mtDNA segregation into daughter cells might account for the shifting of a cybrid with 89% mutant mtDNA load to cybrids with either wild type mtDNA or cybrids with more than 90% mutant mtDNA. Taken together with the heterogeneity observed in the original clonal lines with respect to COX activity and immunostaining, this suggested that although the overall mutant load within a clone remains fairly stable with passage number, the distribution of mutant and wild type mtDNA between cells in a cell line can vary quite dramatically. Under the growth conditions used however, the cells with different mutant loads were able to grow at similar rates.

It has been observed that the re-cloning technique can enrich the mutant load in some fibroblast clones of a KSS patient with a large-scale deletion of mtDNA (Moraes *et al.* 1989). They reported that of the 14 initial fibroblast clones, 4 clones showed wild-type mtDNA, 9 clones containing about 10% of deleted genomes, and 1 clone containing about 60% of deleted mtDNA. This clone (60%) was subcloned, and gave rise to clones with a large variations (33%-80% of total mtDNA) in the relative proportion of deleted mtDNA. However, the average mutant-load had not altered (~67%) (Moraes *et al.* 1989).

The immunocytochemical staining demonstrated that the mtDNA-encoded COX I was decreased in cybrids with 95% mutant mtDNA load, unlike A549ρ° cells which was completely absent.

The effect of the level of A3243G mutation on the mtDNA-encoded COX subunits was further confirmed by the Western-blot studies. The results showed that the mtDNA-encoded COX II steady-state levels were normal in cybrids with 55% mutant mtDNA, but decreased in cybrids with 89% or more mutant mtDNA. On the other hand, COX I only showed a clear decrease in steady-state levels in the cybrids with a mutant level of 95%. As for COX I, the steady-state levels of the nuclear-encoded COX subunit IV were only clearly decreased in the cybrid line with a mutant load of 95%. This might be due to the high level of the mutant $tRNA^{Leu(UUR)}$ A3243G mtDNA which is required to affects the assembly of COX.

The pathogenesis of MELAS-A3243G mtDNA mutation is not clear, although several possible mechanisms have been proposed. One hypothesis is based on the fact that the A3243G mutation, which is located on a tridecamer sequence, acts as a transcription termination signal (mtTERM). The mutation has been shown to decrease the binding affinity of purified mtTERM on a mutated DNA template and therefore affects transcription termination *in vitro* (Chomyn *et al.* 1992). Such an effect may have pathological relevance to the disease. Another theory is based on the observation of increased levels of a precursor RNA (RNA 19), a polycistronic transcript corresponding to the 16S rRNA, $tRNA^{Leu(UUR)}$ and ND1 genes (King *et al.* 1992; Kaufmann *et al.* 1996), with the mutation affecting RNA processing. The mtDNA is transcribed as large polycistronic RNA molecules that are processed into the mature RNA species. The tRNAs that flank most rRNAs and mRNAs are thought to act as recognition signals for the enzymes involved in post-transcriptional processing of the primary transcripts. Therefore, the MELAS-A3243G mutation may impair the recognition and cleavage of the polycistronic transcript at the boundaries of $tRNA^{Leu(UUR)}$ (Kaufmann *et al.* 1996).

The analysis of mitochondrial translation in cybrids (206ρ°-3243) revealed reduced synthesis, especially in high molecular mass subunits (COX II, COX III and ND2). This suggested that the decrease in mtDNA encoded polypeptide synthesis may result in a greater decrease in complexes I and IV activities (Chomyn *et al.* 1992).

Another uncommon mtDNA mutation at position 3271bp in a patient with MELAS has been demonstrated to affect $tRNA^{Leu(UUR)}$ gene function. By using intercellular transfer of T3271C mutant mtDNA into Helaρ° cells, Hayashi *et al.* demonstrated that cybrids harbouring 87% mutant had both a low complex I activity and abnormal mtDNA-encoded polypeptide synthesis including complex I subunit ND6 (Hayashi *et al.* 1993). The decreased ND6 synthesis has also been observed in cybrids harbouring the MELAS A3243G mutant (Dunbar *et al.* 1996). Based on ND6 which has a relatively high number of leucine residues specified by $tRNA^{Leu(UUR)}$ gene (~8%), it could be suggested that the mutant $RNA^{Leu(UUR)}$ gene can influence the number of specific amino acid residues which therefore affects the protein synthesis. However, the data reported by Dunbar and co-workers (1996) did not support this idea, as ND3, which contained a high proportion of leucine residues specified by $tRNA^{Leu(UUR)}$ gene (~9%), did not show a significant decrease in translation. Consequently, it is unlikely that a dysfunctional $tRNA^{Leu(UUR)}$ gene mutant plays a major role in the failure of translation, unlike the failure of translation due to the MERRF-A8344G $tRNA^{Lys}$ gene mutation (Enriquez *et al.* 1995).

CHAPTER 6 MITOCHONDRIAL DNA TRANSMISSION OF THE MITOCHONDRIAL DEFECT IN PD

6.1 Aim

To fuse platelets from PD patients with mtDNA-less A549ρ° cells to determine whether the complex I deficiency in platelets from PD patients is caused by abnormal mtDNA.

6.2 Introduction

The pathological cause of the major clinical features in PD is death of dopaminergic neurones in the substantia nigra pars compacta. Genetic and environmental factors have been proposed as aetiological agents but no specific factor appears directly applicable to the majority of patients with idiopathic PD. However, the recent linkage of a gene for autosomal dominant parkinsonism to 4q21-23 (Polymeropoulos *et al.* 1996) promises valuable insight into mechanisms of dopaminergic cell death. A single base pair change at position 209 from G to A in the α-synuclein gene, which results in an Ala to Thr substitution at position 53, was identified by the same group to be the mutation in this Italian family and three unrelated families of Greek origin with PD. This gene, which codes for a presynaptic nerve terminal protein, was thought to be involved in neuronal plasticity (Polymeropoulos *et al.* 1997). However, the relationship of the α-synuclein gene defect on chromosome 4 to the majority of patients with PD remains uncertain.

Several biochemical abnormalities have been identified in post-mortem PD substantia nigra, including oxidative damage (Dexter *et al.* 1989; Olanow, 1992), excess iron (Dexter *et al.* 1989) and decreased mitochondrial complex I activity (Schapira *et al.* 1989; Janetzky *et al.* 1994). The part that these play in pathogenesis and their connection with aetiology is not known. Nevertheless identifying the cause(s) and sequence of these abnormalities may provide important clues to aetiology. Several studies have identified a selective complex I deficiency in PD platelet mitochondria although other studies have failed to show this (see chapter 1, section 1.3.3). A defect in platelets could represent a genetically determined deficiency. However, as platelets do share

some pharmacological features with dopaminergic neurones, *e.g.* monoamine oxidase, and MPP^+ uptake (Da Prada *et al.* 1988), this may be enhanced by a toxin, either endogenous or exogenous.

Several groups have sought mutations of mtDNA as a possible cause of the complex I deficiency in PD (Schapira *et al.* 1990b; Ozawa *et al.* 1991; Shoffner *et al.* 1993). However these have involved small numbers of unselected PD patients and no clear mutation has emerged (see chapter 1, section 1.4.4.2). An alternative strategy is to take advantage of a system whereby mtDNA from a PD patient can be placed in a novel nuclear environment of $\rho°$ cells (King and Attardi, 1989). The transmission of a mitochondrial defect from the mtDNA donor cell to the resulting cybrid cells implies that the defect is determined by mtDNA. Conversely, abolition (complementation) of the defect could indicate nuclear control of the biochemical abnormality, a toxic influence on the original donor cells, or a drift of mtDNA from mutant to wild type.

The cybrid strategy has been used to investigate the pathogenesis of certain inborn errors of the respiratory chain causing the mitochondrial encephalomyopathies. The MELAS phenotype is most frequently associated with the A3243G point mutation in the $tRNA^{Leu(UUR)}$ gene. Transfer of mtDNA bearing this mutation from patient cells to $\rho°$ cells has demonstrated the passage of the concomitant biochemical defect to the recipient cells (see chapter 5) (Chomyn *et al.* 1992; Dunbar *et al.* 1996). Swerdlow *et al.* (1997) used a similar method to show that the complex I deficiency in PD platelets appears to be determined by mtDNA. Based on the model of a mtDNA mutation producing an archetypal mitochondrial disease, this study focused on PD patients selected by their low platelet complex I activity, on the hypothesis that a mtDNA defect may be relevant to only a proportion of PD patients.

6.3 Methods

6.3.1 Patients

Eight PD patients and 8 age and sex matched controls were selected for simultaneous analysis of platelet mitochondrial respiratory chain function. From this group we selected 4 PD patients with the lowest complex I activity

and controls and analysed platelet complex I activities 3 times over a period of 2 months. All patients satisfied the PD Brain Bank (London) criteria for idiopathic PD *i.e.* akinetic rigid syndrome with asymmetric onset, resting tremor and a good response to L-dopa. None of the four patients chosen had any family history of PD or any documented toxic exposure and none had any evidence of other neurological or systemic disease. All were on L-dopa therapy (Table 6.1). Hoehn and Yahr stagings were II-III in all patients.

Table 6.1 The medical details of PD patients and controls

	PD	C
Platelet MRC function		
n	8	8
Mean age (years old)	71.0±5.23	65.4±8.25
Range (years)	61-76	50-75
Platelet fusion		
n*	7	7
Mean age (years old)	71.1±5.59	64.0±7.85
Range (years)	61-76	50-70
Mean disease duration	6.9±5.2	
Range (years)	3.0-15.0	
Mean doses of L-dopa	171±123 (mg/day)	
Range	50-400 (mg/day)	

*: One of 8 PD patients did not want to participate in further fusion studies.

6.3.2 Methods

The following protocols were used; preparation of platelet mitochondrial fractions (section 2.4.9); MRC functions and DNA analysis (sections 2.4-2.5); ring-cloning and mix-cloning procedures (sections 2.7.1-2.7.5); and cytochemical and immunocytochemical stains (section 2.7.11).

6.4 Results

6.4.1 Platelet MRC function in PD

The results of platelet mitochondrial respiratory chain function analysis for 8 PD patients and 8 age-matched controls are shown in Table 6.2. The PD patients showed an overall 24% deficiency in complex I/CS ratio ($p<0.05$), whilst on a group to group analysis there was no statistical difference in complex II+III or complex IV activities. Those 4 PD patients with the lowest complex I/CS ratios were chosen for further study. This involved confirming the

consistency of their complex I defect by analysing freshly obtained samples after one and two months. These showed no significant variation for the first set of results (Fig. 6.1). One of the 4 PD patients (PD-1) with low complex I activity was chosen at random for fusion with A549ρ° cells and generation of clonal cell lines. This patient had CS corrected platelet respiratory chain activities of 6.30, 0.24 and 0.74 for complexes I, II+III and IV respectively. Thus this patient had both a 34% decrease in complex I and a 24% decrease in complex IV activities (Table 6.2).

Table 6.2. Mitochondrial respiratory chain function in platelet mitochondria from age and sex-matched controls and PD patients

	Cx I /CS(x100)	CxII+III/CS	Cx IV/CS(x100)	CS
Control (n=8)	9.59±2.12	0.25±0.052	0.98±0.11	195±32
PD (n=8)	7.28±1.09*	0.24±0.048	0.98±0.16	181±34
PD-1	6.30	0.24	0.74	210

Cx: complex; CS: citrate synthase; *$p<0.05$

6.4.2 MRC function in PD clonal cybrids

Platelets from PD-1 and a control were fused with A549ρ° cells. Following change to selection medium, to eradicate unfused A549ρ° cells, fused cells were clonally expanded to provide material for respiratory chain studies. Sixteen control and 16 PD clonal fusion lines were selected at random. The growth rate in standard medium of the A549ρ°-PD clonal cybrids (PD-1) was slower than that of the control fusion lines (C-1) or the parent A549 cells (Fig. 6.2). Mitochondrial enriched fractions were assayed for complex I, complex II+III, complex IV and CS activity (Fig. 6.3, 6.4, data not shown for complex II+III). Three of the 16 PD fusion clones had complex I activities below the control range whilst four of the clones had complex IV activities below this range. Overall there was a 25% reduction in complex I and a 20% in complex IV activities when the mean of the PD clones was compared to the respective means of the controls. There was no direct correlation between the complex I and IV activities in individual clones (Fig. 6.5). The PD clones are grouped separately from the controls although there was a slight overlap between them. The CS ratios for complex I, II+III and IV for 16 PD clonal cybrids and 16 control clonal cybrids are summarised in Fig. 6.6.

6.4.3 A549 polymorphism

None of cloned cybrids contained the 4 **CA**-repeat polymorphism of mtDNA present in A549 cells. Thus all mtDNA in these cybrids are derived from the platelets of this PD patient or the control (Fig. 6.7).

6.4.4 Cytochemistry and immunocytochemistry: COX activities and COX I staining

COX cytochemistry of control fusion clones showed activity in all cells (Fig. 6.8A). PD fusion clone No 11 was selected for COX staining as this clone had the lowest complex IV activity (see Fig. 6.4). Results showed that, whilst some cells of this clone had normal COX staining, others showed little or no detectable COX activity (Fig. 6.8B). MitoTracker was taken up by all cells indicating that they were all capable of generating the minimum membrane potential required to concentrate this dye (Fig. 6.8C-D). Immunostaining with the monoclonal anti-complex IV mitochondrially encoded subunit-I antibody, however, showed that as with the COX activity stain, there was heterogeneity (Fig. 6.8F). Some cells stained normally whilst others had little visible cross-reactivity. Superimposition of MitoTracker staining and COX I antibody staining with 33258 Hoechst staining of the nucleus demonstrated clear heterogeneity in this PD fusion clone (Fig. 6.8H).

6.4.5 MRC function in PD mixed cybrids

Platelets from the other 7 PD and 7 age and sex-matched controls were fused with A549ρ° cells. The fused cells from each individual were grown without cloning to give 7 PD and 7 control mixed cybrid lines. The MRC functions in 7 PD and 7 controls mixed cybrid lines were analysed. Comparison of the PD and control cell lines revealed a significant decrease in complex I/CS ratio (25% decrease, $p<0.01$, Fig. 6.9), but complex II+III and complex IV CS ratios were normal. The decrease in complex I/CS ratios obtained for the PD patients using both the clonal and mixed cybrid methods were identical, but complex IV/CS ratio was only significantly decreased in PD-1 using the clonal method (Table 6.3, Fig. 6.9).

Table 6.3 Mitochondrial respiratory chain functions in mixed cybrids of 7 controls and 7 PD patients and combined data from 16 clonal cybrids from C-1 and PD-1

	Cx I /CS	Cx II+III/CS	Cx IV/CS	CS (nmol/min/mg)
Mixed cybrids				
Control (n=7)	0.48±0.08	0.49±0.036	0.025±0.0052	125±21
PD (n=7)	0.36±0.06*	0.48±0.035	0.024±0.0061	142±17
Clonal cybrids				
C-1(n=16)^	0.41±0.08	0.49±0.04	0.024±0.004	145±55
PD-1 (n=16)#	0.31±0.04**	0.49±0.05	0.019±0.003***	134±52

Cx: complex, CS: citrate synthase. Values are mean ± SD.
^ : The data from 16 clonal cybrids from 1 control (C-1)
#: The data from 16 clonal cybrids from 1 PD (PD-1)
* $p<0.01$; 7 PD verses 7 control; ** $p<0.001$; *** $p<0.05$; PD-1 verses C-1.

There was a positive correlation between the ratios of complex I/CS in the platelets and in the mixed cybrids (Fig. 6.10).

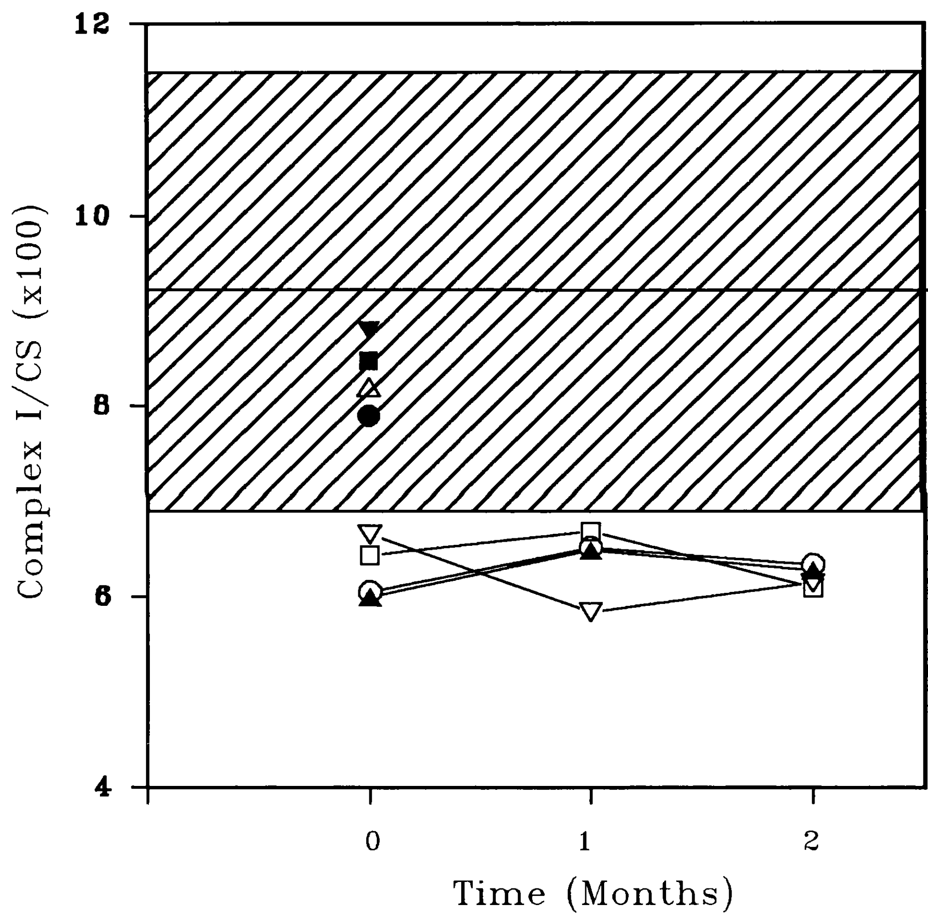


Fig. 6.1. Complex I/CS ratios in platelet mitochondrial fractions from 8 PD patients. 4 of 8 patients were studied over time. The shaded area represents the control mean ± SD (n=8). The patient studied further by clonal cybrid analysis is indicated with filled triangle.

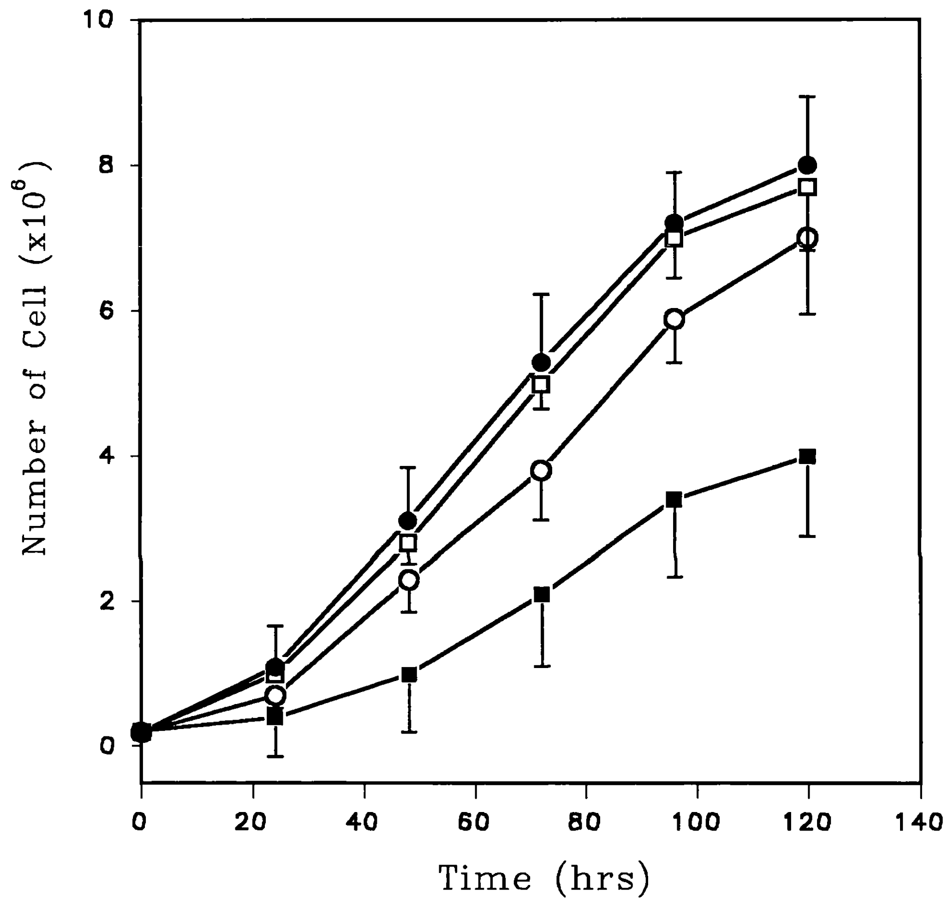


Fig. 6.2. Growth curves of A549 (filled circles), A549ρ° (filled squares), A549ρ°-control (C-1) clonal cybrid (open squares) and A549ρ°-PD (PD-1) clonal cybrid (open circles) are shown. Cells were grown in standard medium and values are mean ± SD (n=3).

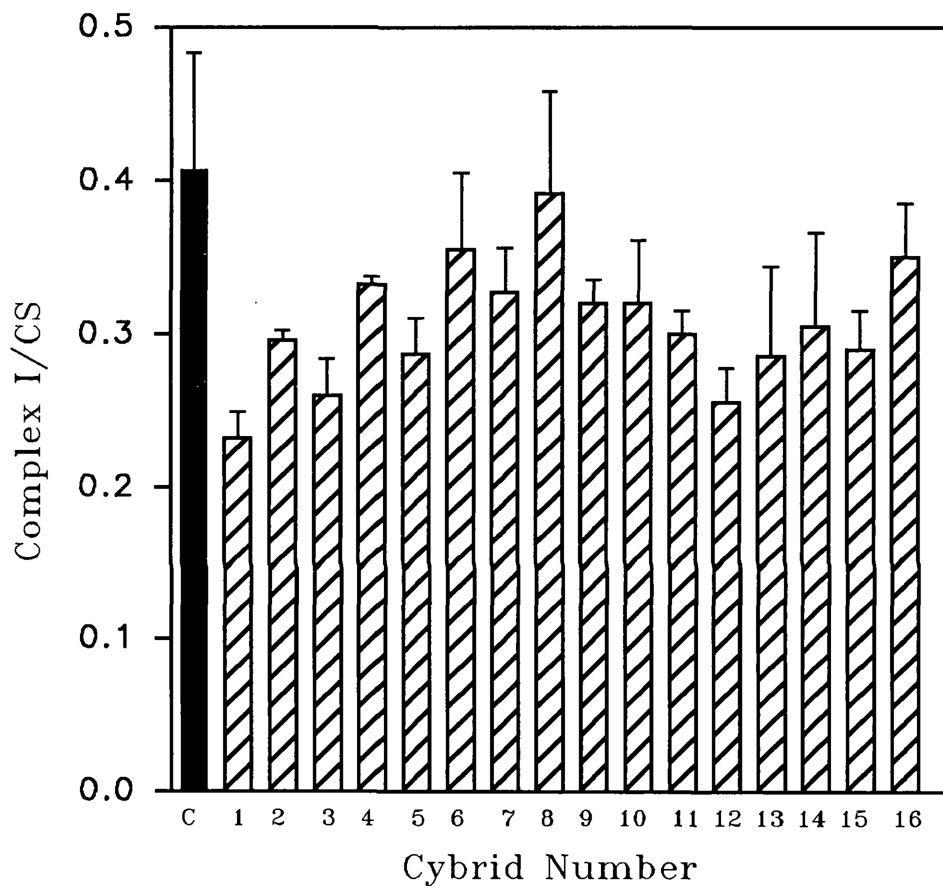


Fig. 6.3. Complex I/CS ratios in A549ρ°-control clonal cybrids (C, filled bars) and A549ρ°-PD clonal cybrids (1-16 hatched bars). Values are mean ± SD of 3 separate mitochondrial preparations for each cybrid and mean ± SD for 16 different control cybrids.

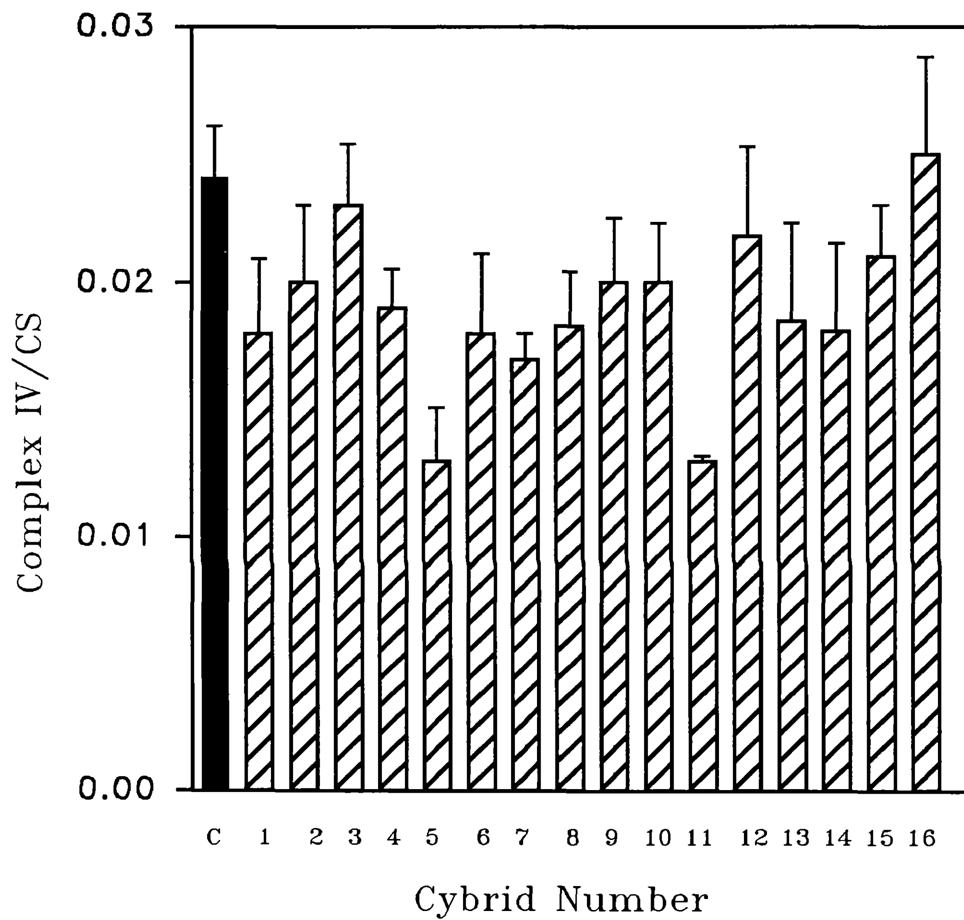


Fig. 6.4. Complex IV/CS ratios in A549ρ°-control clonal cybrids (C, filled bars) and A549ρ°-PD clonal cybrids (1-16 hatched bars). Values are mean ± SD of 3 separated mitochondrial preparations for each cybrid and mean ± SD for 16 different control cybrids.

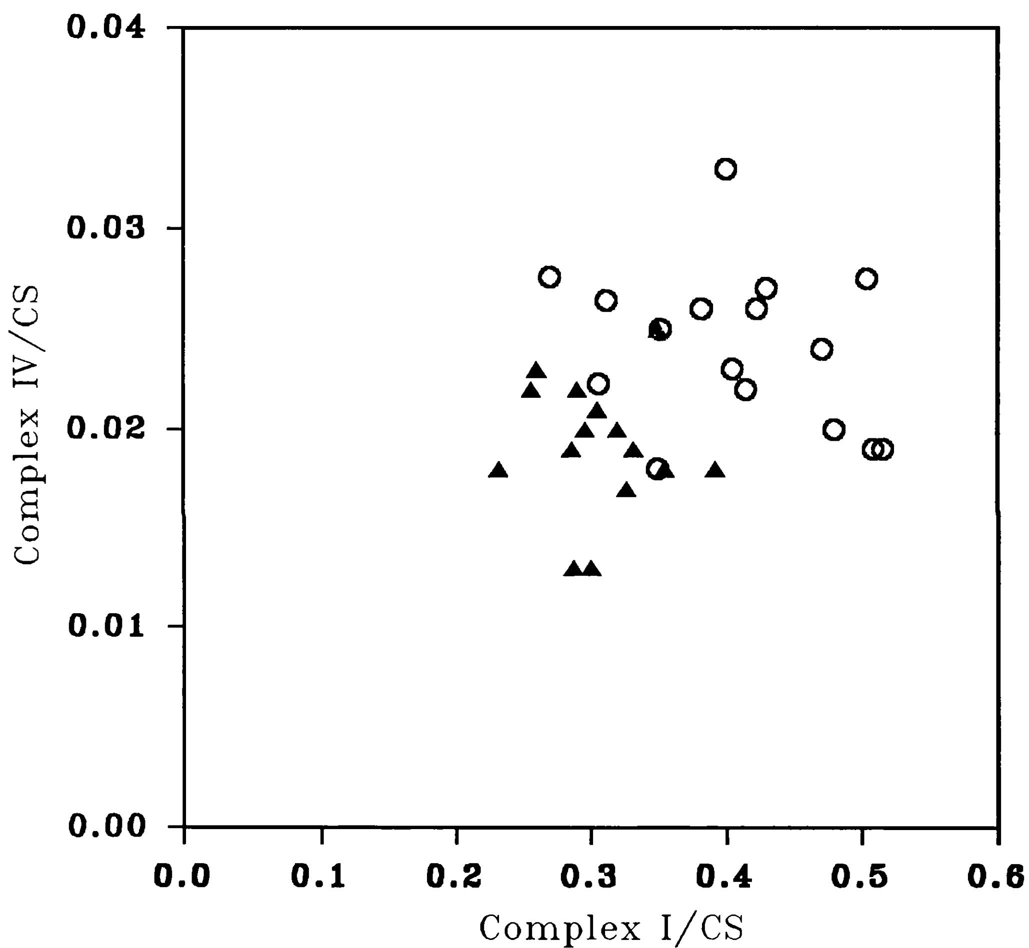


Fig. 6.5. Relationship between complex I/CS and complex IV/CS ratios in A549ρ°-control clonal cybrids (n=16, open circles) and in A549ρ°-PD clonal cybrids (n=16, filled triangles). There was no correlation between complex I/CS and complex IV/CS ratios in individual clones.

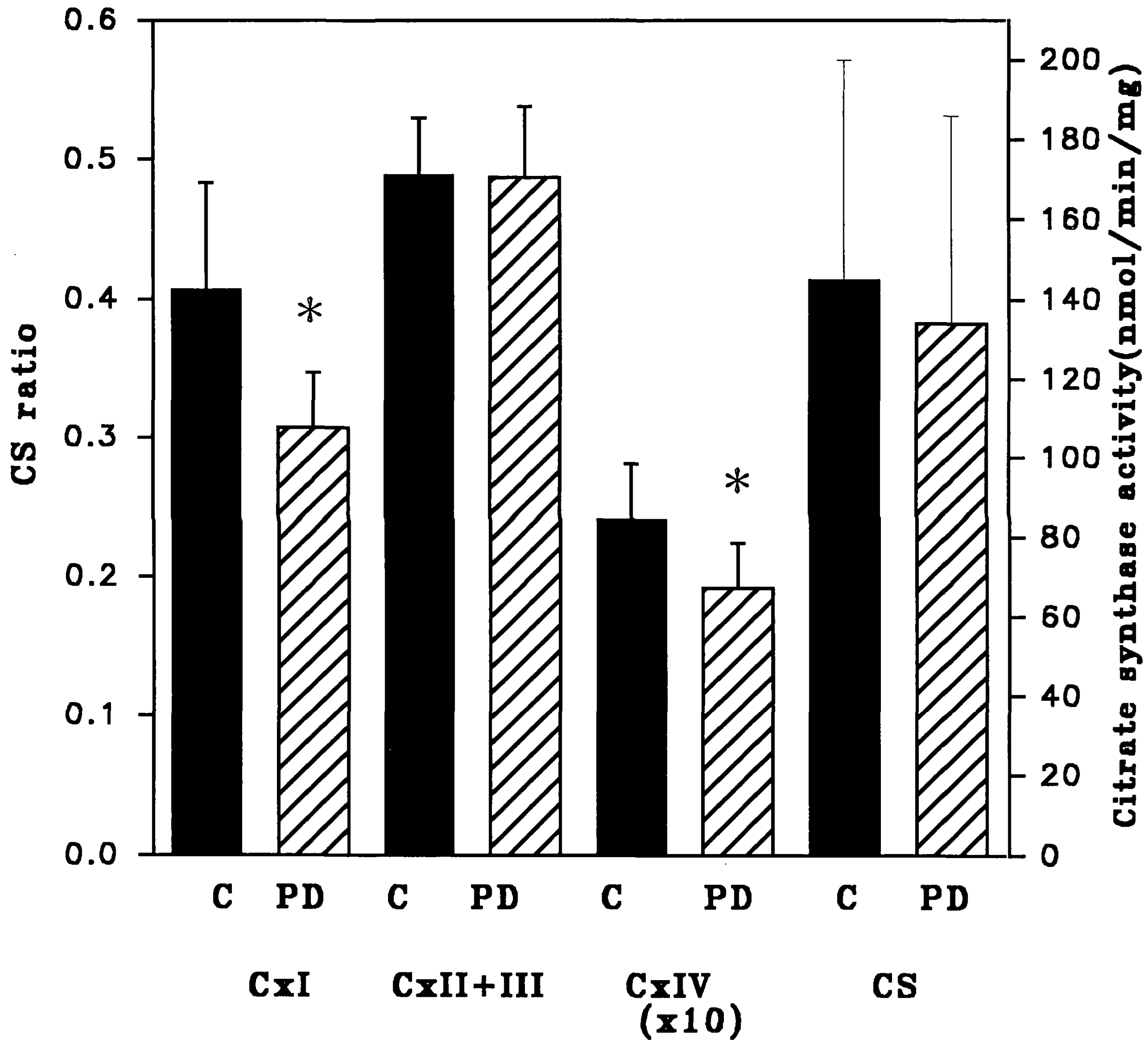


Fig. 6.6. Mitochondrial activities in A549ρ°-control clonal cybrids (C, n=16, mean ± SD, filled bars) and in A549ρ°-PD clonal cybrids (PD, n=16, mean ± SD, hatched bars). Complex I/CS (CxI/CS), complex II+III/CS (CxII+III/CS), complex IV/CS (CxIV/CS) and CS activity. * $p<0.005$, Mann-Whitney U test.

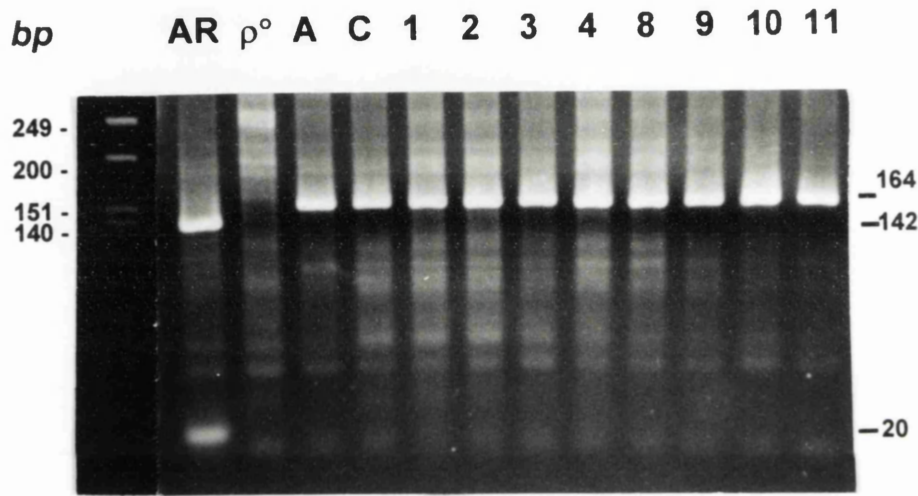


Fig. 6.7. Analysis of the A549 mtDNA polymorphism in A549ρ°-platelet cybrids. PCR products from A549 cells (A) and following AluI digestion (AR) and A549ρ° (ρ°); and AluI digestion of PCR products from a A549ρ°-control cybrid (C) and A549ρ°-PD cybrids number 1, 2, 3, 4, 8, 9, 10, and 11.

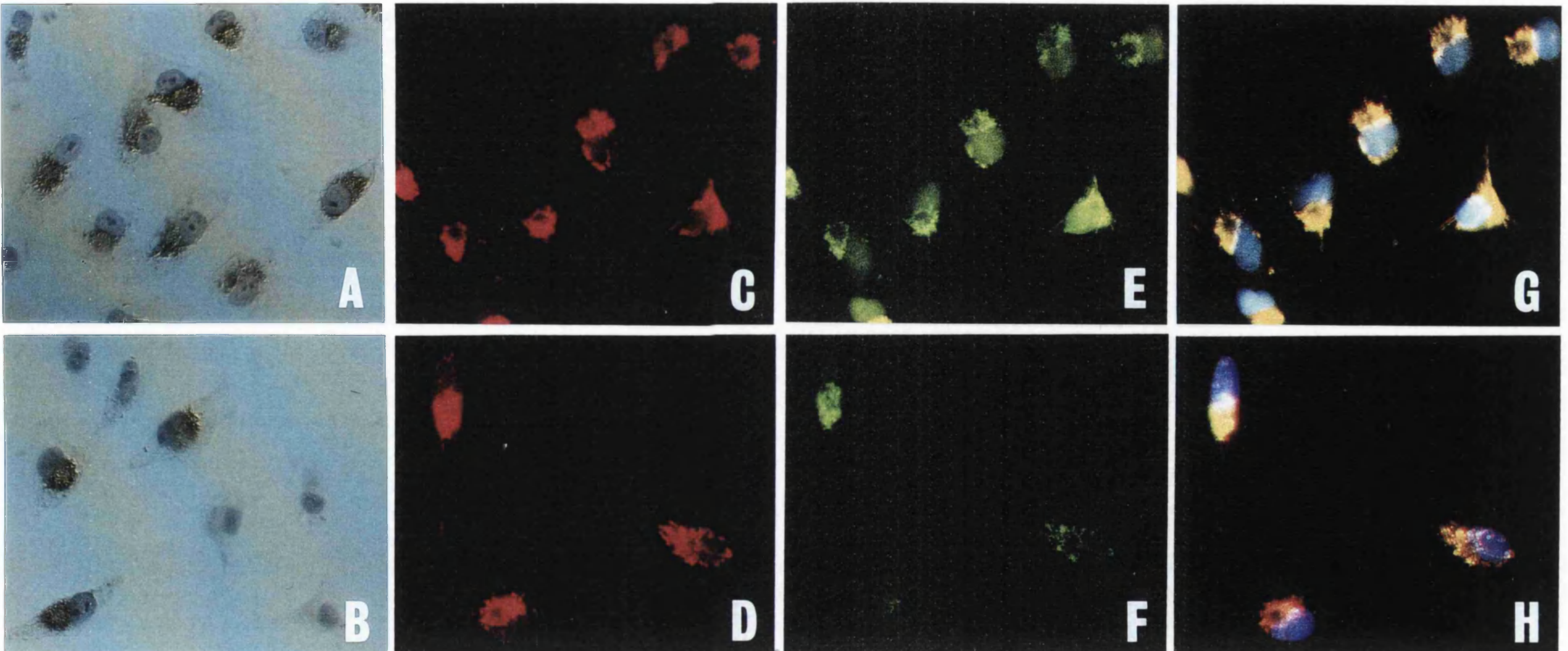


Fig. 6.8 Cytochemical and immunocytochemical staining of A549ρ°-control (A, C, E, G; clone 11), and A549ρ°-PD clonal cybrids (B, D, F, H; clone 11). Cells were stained for cytochrome oxidase activity (A, B), with MitoTracker (C, D), and immunocytochemically using anti-COX I antibody (E, F). Typical cells with decreased staining are indicated with an arrow. Combined images of MitoTracker, anti-COX I antibody and Hoechst 33258 are shown in G and H. Scale x 430.

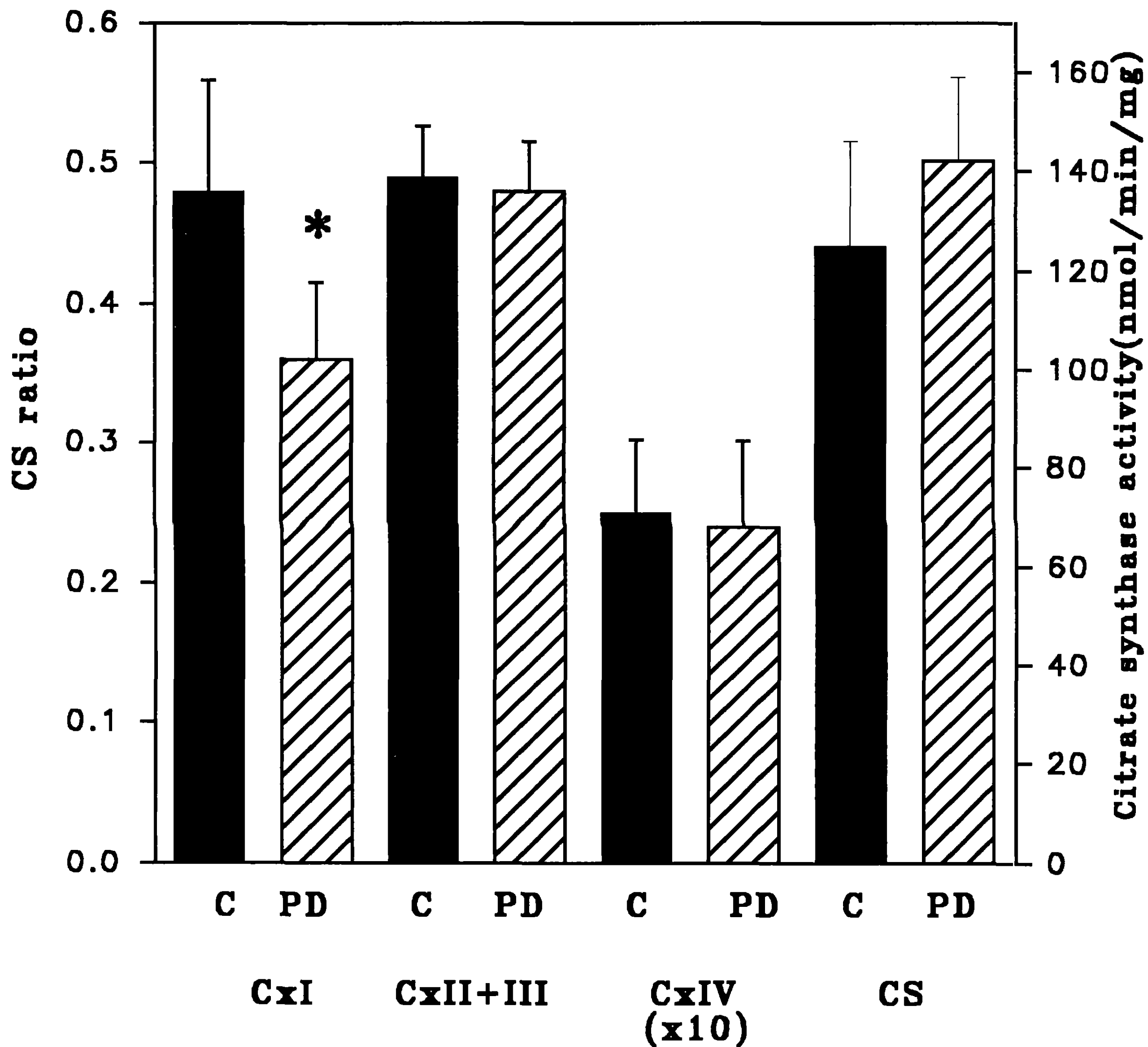


Fig. 6.9. Mitochondrial activities in A549ρ°-control mixed cybrids (C, n=7, mean ± SD, filled bars) and in A549ρ°-PD mixed cybrids (PD, n=7, mean ± SD, hatched bars). Complex I/CS (CxI/CS), complex II+III/CS (CxII+III/CS), complex IV/CS (CxIV/CS) and CS activity. * $p<0.01$, Mann-Whitney U test.

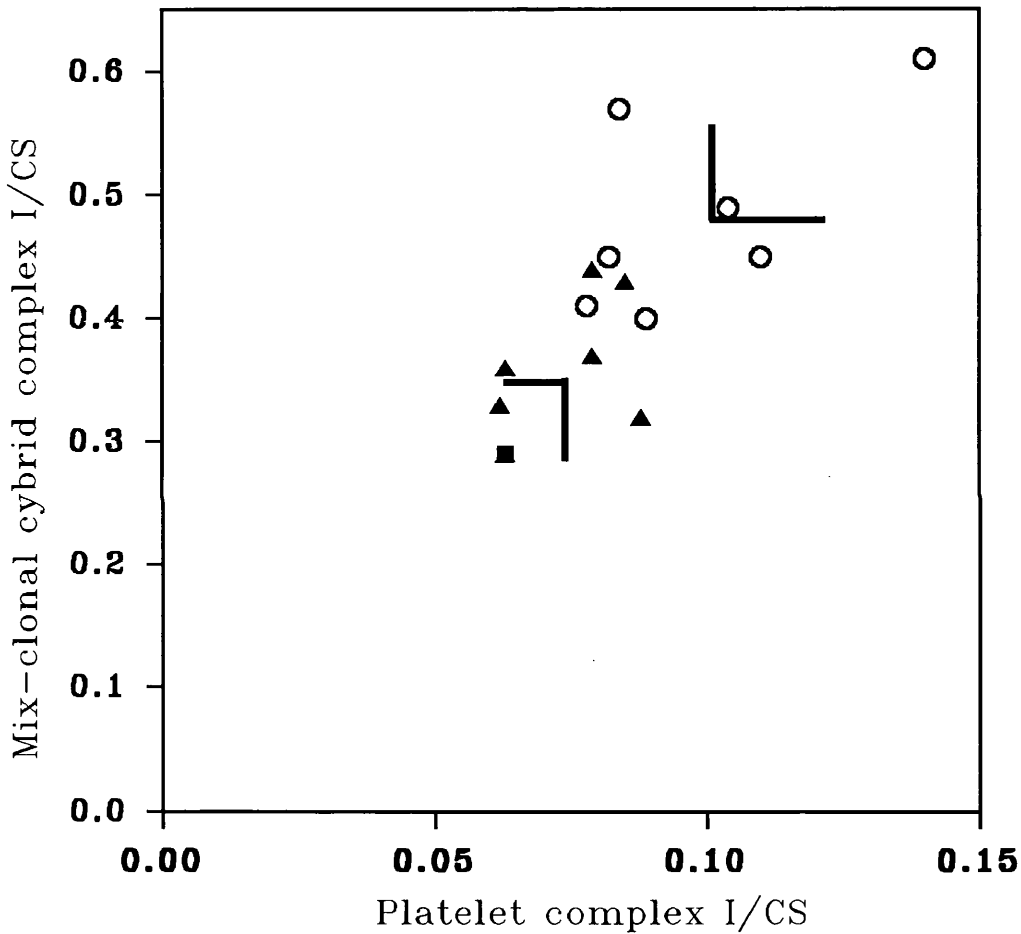


Fig. 6.10. Relationship between the complex I/CS ratios in the control (n=7, open circles) and PD patients (n=7, filled triangles) platelet mitochondrial fractions and their respective complex I/CS ratios in A549ρ° mixed cybrid lines. The patient studied further by clonal cybrid analysis is indicated with filled square. The mean ± SD for the control and PD group are indicated.

6.5 Discussion

Several groups have identified mitochondrial complex I deficiency in Parkinson's disease (PD) substantia nigra and in platelets (Parker *et al.* 1989; Schapira *et al.* 1989; Krige *et al.* 1992; Janetzky *et al.* 1994). A search for mtDNA mutations underlying this defect has not as yet produced any consistent result (Schapira *et al.* 1990b; Ozawa *et al.* 1991; Shoffner *et al.* 1993; Ikebe *et al.* 1995) (see chapter 1, section 1.4.4.2). In this study, two fusion procedures were used: firstly, the fusion of a PD patient (PD-1) with low complex I and IV activities in platelets with the analysis of clonal cybrids and secondly, the fusion of 7 PD patients and the analysis of mixed cybrids.

When the data from the clonal cybrids generated from PD-1 were combined, they demonstrated significant deficiencies in the activities of complexes I and IV, the same deficiency was seen in the platelet mitochondria of this PD patient (Table 6.3, PD-1). The other 7 PD patients, however, showed a more selective complex I defect both in their platelets and in the mixed cybrids generated from their platelets (Table 6.3). Consequently, the decreased complex I activity was a common feature in all the cybrids. However, unlike the decreased complex IV activity in the clonal cybrids, complex IV activity in 7 PD patients was normal. The results for the mixed cybrids were in remarkable agreement with the recently published study of Swerdlow *et al.* (1996). This group used the SHSY5Y neuroblastoma derived ρ° cell line, but without clonal expansion, to investigate 24 PD patients. They found a statistically significant decrease in mean complex I activity of 20% in the PD group compared to controls. Complex IV activity was decreased by 11% in the PD group but failed to reach significance. This may reflect either differing proportions of mutant mtDNA in the platelets from different PD patients or other molecular genetic aetiologies for the mitochondrial defect. It is possible that the absence of a complex IV defect in the mixed cybrids from other patients may reflect the methodology used.

The COX staining of the clonal lines showed cellular heterogeneity in the PD clones. This pattern of abnormality was the same as that seen in the A3243G

mtDNA fusion model (see chapter 5). The abnormal MRC function in the clonal cybrids generated from A3243G mtDNA fusion that implied mutant mtDNA is the cause, not nuclear DNA or a toxin. These observations are compatible with mtDNA heteroplasmy and the apparently random segregation of mtDNA mutations between cells. As the clonal lines have all been expanded from a single cell, their nuclear background should be identical and not responsible for the mtDNA heteroplasmy. Exogenous toxic influences affecting platelet mitochondrial function can also be excluded as contributory factors, as these will be diluted out by clonal expansion. Thus, a defect of mtDNA is the only explanation for the results obtained in the PD clonal line.

The pattern of respiratory chain dysfunction in the A549ρ°-PD platelet fusion clones was the same as that seen in the A3243G mtDNA ($tRNA^{Leu(UUR)}$) mutant clones (see chapter 5). This contrasts with known mtDNA mutations involving complex I genes such as the 3460 base pair mutation in ND1 in Leber's hereditary optic neuropathy, where platelet mitochondrial abnormalities were confined to complex I activity (Howell *et al.* 1991; Smith *et al.* 1994). Thus, based on the data from our PD-1 clonal cybrids, a tRNA mutation could be responsible for the mitochondrial defect, at least in the patient studied (PD-1). At present this cannot exclude this being the case in all PD patients as in the mixed cybrids of other 7 PD patients, only decreased complex I activity was observed.

The positive correlation between platelet mitochondrial complex I ratios and mixed cybrid complex I ratios suggested that the mtDNA encoded subunits were likely to be important in determination of the activity of complex I.

The apparent sporadic nature of idiopathic PD is compatible with mtDNA involvement in a proportion of patients. A mtDNA mutation may be aetiologically relevant to only a proportion of PD patients in which it may arise spontaneously or occur in a pedigree where other members are oligosymptomatic or have different clinical phenotypes of the same mutation - situations commonly found in mitochondrial disorders.

An additional important question is why PD substantia nigra shows a selective complex I defect whilst platelets and clones (at least in the selective PD-1 case studied here) showed complex I and IV defects. Explanations for this may involve both methodological and biochemical factors. Assays of respiratory chain activity in substantia nigra use either tissue homogenates or fractions. Their sensitivity is therefore less than in the mitochondrial preparations used in the analysis of our clones. Thus a 20% complex IV deficiency may appear in PD-1 fusion clones but be concealed in PD substantia nigra data.

Recently, Tabrizi *et al.* (1998) have studied the possible contribution of mtDNA to the complex I deficiency in patients with dystonia using the fusion technology. However, the results contrast with similar studies in MELAS and PD patients in this thesis, in which the mitochondrial complex I defect was maintained in at least a proportion of A549 cybrids, and suggest that the complex I defect in dystonia is not caused by an mtDNA mutation (Tabrizi *et al.* 1998).

To summarise chapters 5 and 6, clonal analysis of A549ρ^{o}-A3243G and A549ρ^{o}-PD clonal cybrids from one PD patient expressed combined complex I and IV deficiencies with 25% and 20% decreased activities in the PD clones respectively. Histocytochemical and immunocytochemical studies showed a remarkable similarity between the A3243G 'MELAS' and PD clonal cybrid. These results are in agreement with a previous study (Swerdlow *et al.* 1996) and support the proposition that a mtDNA defect may underlie the mitochondrial defect in at least a proportion of PD patients. This type of analysis may serve as a means to identify the subgroup of PD patients in whom a mtDNA defect may contribute to aetiology. This study provides support for the hypothesis that a mtDNA defect may be involved in producing the mitochondrial deficiency in a selected group of patients with PD (Gu *et al.* 1998a)

CHAPTER 7 MITOCHONDRIAL RESPIRATORY CHAIN INHIBITION AND APOPTOSIS

7.1 Aim

To investigate whether mitochondrial respiratory chain inhibition causes apoptotic cell death, and to study the mechanism of rotenone induced mitosis, endoreduplication and apoptosis.

7.2 Introduction

A variety of toxins have been identified that inhibit the NADH ubiquinone reductase (complex I) activity of the mitochondrial respiratory chain (MRC). The plant toxin rotenone is probably the most widely used inhibitor of complex I and its selective inhibition is employed to identify the *in vitro* enzymatic activity of this complex. The antibiotic piericidin A and the neurotoxin MPP^+ both appear to interact at the same site within the complex as rotenone (Ramsay *et al.* 1991). Whilst these inhibitors decrease the oxidative phosphorylation of ADP to ATP, reduce cell viability and precipitate necrosis, there is increasing evidence that apoptosis plays an important role in cell death initiated by inhibition of the MRC (Hartley *et al.* 1994; Wolvetang *et al.* 1994). This is of particular relevance to certain neurodegenerative disorders, including PD and HD, in which defects of the respiratory chain have been identified (Schapira *et al.* 1989; Gu *et al.* 1996).

Apoptotic cell death can be differentiated from necrosis by several characteristic biochemical and morphological features (Ellis *et al.* 1991). During the early stage of apoptosis, there is condensation and fragmentation of nuclear chromatin, and cleavage of nuclear DNA into oligonucleosomal fragments by the action of a calcium activated endonuclease (Williams, 1991). These processes are normally accompanied by cell shrinkage, dilation of the endoplasmic reticulum and compaction of cellular organelles. During the later stages of apoptosis, small membrane-bound vesicles known as apoptotic bodies are formed. The induction of apoptosis has been described as either rapid (primed) or slow (unprimed) (Willingham and Bhalla, 1994). Primed

apoptosis proceeds rapidly and involves the arrest of cells in the G1 phase of the cell cycle; for example staurosporine induces apoptosis within 24hrs (Jacobson and Raff, 1995). Unprimed apoptosis has a longer time course and may involve passage through the cell cycle before apoptotic death, *e.g.* taxol treatment leads to apoptotic cell death only after several days (Willingham and Bhalla, 1994). The possible relationship between apoptosis and the events that normally occur during progression through the cell cycle, especially at G2/M transition, has been described previously (Davidoff and Mendelow, 1992).

The mechanism by which MRC inhibition leads to apoptosis is not known. Inhibition of the respiratory chain not only reduces ATP production but may also increase free radical generation (Boveris and Chance, 1973; Cadenas *et al.* 1977), which have themselves been implicated in the initiation of apoptosis (Hockenbery *et al.* 1993).

The expression of the oncoprotein Bcl-2 can protect cells from apoptotic death induced by a variety of stimuli (Reed, 1994). The Bcl-2 oncoprotein has been localised to the nuclear envelope, endoplasmic reticulum, and mitochondrial membranes (Hockenbery *et al.* 1990; Jacobson *et al.* 1993), but how it prevents apoptosis is not known. Two possible mechanisms have been proposed by Hockenbery *et al.*, (1993); the first suggests that Bcl-2 acts as a signalling mechanism that might inactivate genes or enzymes which lead to damage, while the second suggests that Bcl-2 may act as an anti-oxidant.

Rotenone treatment of cells has previously been shown to induce endoreduplication, a specific type of polyploidization that arises from endomitotic chromosomal duplication (Matsumoto and Ohta, 1993) and apoptosis (Wolvetang *et al.* 1994). In order to investigate the mechanism of rotenone induced endoreduplication and apoptosis and to determine whether or not they are induced by all mitochondrial inhibitors, the effects of several inhibitors (rotenone, piericidin A and antimycin A) upon a variety of cell lines including a human lung carcinoma cell line A549, the derived A549ρ° cells, normal human skin fibroblasts and rat dorsal root ganglion neurons (DRG).

7.3 Methods

A549, A549ρ°, fibroblast cells and DRG neuronal cultures are as described in chapter 2, section 2.8.1, and treatment of cells with MRC inhibitors (rotenone, piericidin A and antimycin A) as in section 2.8.2. Immunofluorescent analysis using antibodies to α-actin, β-tubulin and Bcl-2 on cells are as described in section 2.8.3 and staining cells with PI and Tunel as in section 2.8.3. The techniques for counting cells are in section 2.8.4.

7.4 Results

7.4.1 Dose and time dependent effects of rotenone

Under standard growth conditions between 1% and 5% of the cells could be seen to be undergoing mitosis depending upon the cell type. Cells with more than one nucleus or showing morphological signs of apoptosis were uncommon (<1%). Incubation of A549 cells with rotenone (up to 1μM) for 24hrs caused a dose dependent increase in the number of cells in metaphase (Fig. 7.1B), with two or more nuclei (Fig. 7.1B,E), or showing typical morphological features of apoptosis (Fig. 7.2C, typical apoptosis induced by staurosporine). However, at 10μM rotenone, while the number of mitotic cells increased, there was a dramatic fall in the number of endoreduplicated and apoptotic cells. A549ρ° cells incubated with all concentrations of rotenone exhibited the same changes as the A549 cells (Table 7.1).

Table 7.1. The percentage of cells showing characteristic signs of mitosis, endoreduplication and apoptosis after 24hrs treatment with rotenone

Rotenone	Mitosis		Endoreduplication		Apoptosis*	
μM	A549	ρ°	A549	ρ°	A549	ρ°
0	3.0±0.3	1.0±0.4	0 ± 0	0 ± 0	0.2±0.1	0.3±0.1
0.25	5.0±0.5	5.0±0.6	2.6±0.4	1.7±0.3	1.8±0.4	2.1±0.4
0.50	8.3±0.9	7.8±1.1	15.2±1.8	16.3±1.6	3.0±0.7	3.1±0.9
0.75	10.1±1.2	9.5±1.3	20.0±1.5	19.0±2.0	8.2±1.4	7.6±1.8
1.00	13.3±2.0	11.9±1.9	22.0±1.8	20.0±2.0	15.0±1.8	14.0±1.7
10.0	17.0±2.2	13.0±1.8	2.2±1.1	8.0±0.7	3.0±0.6	2.2±0.4

Data are the percentage of cells, presented as mean ± SEM (n=3). The untreated control values have been subtracted from these values. * analysis of cells floating in the medium.

At 1µM rotenone the number of cells in metaphase increased with time peaking after 24-36hrs treatment to between 10-38% in all cell lines tested (A549, A549ρ° and fibroblasts, Fig. 7.5) although fibroblasts appeared to be affected to a lesser extent. After 48hrs incubation the number of metaphase cells was similar to control values (<5%) in all cell lines. This decrease in the number of mitotic cells was accompanied by a large increase in the percentage of both endoreduplicated live cells and apoptotic cells floating in the medium. The peak time for appearance of apoptosis and endoreduplication was between 48 and 72hrs of treatment (Fig. 7.5).

7.4.2 The effects of rotenone on the cytoskeleton

Rotenone did not significantly affect α-actin (data not shown) but mildly affected ß-tubulin (Fig. 7.1B,E) structures in A549 cells. These observations are similar to those obtained with colchicine (0.1µM) for 24hrs, which also induced mitosis and endoreduplication by causing disruption of ß-tubulin assembly (Fig. 7.1C,F) without obviously affecting α-actin (data not shown).

7.4.3 Bcl-2 expression

Staining for Bcl-2 was detectable in A549 parent cells, A549ρ° cells and fibroblasts only during metaphase (Fig. 7.3A), where staining was restricted to the chromosomes. After treatment of the cells with rotenone and colchicine for 24 hrs there were significant increases in the numbers of cells which stained positively for Bcl-2 (Fig. 7.3B,C, respectively). However this staining was also restricted to the chromosomes of cells in metaphase and therefore reflected the higher numbers of cells in metaphase after treatment.

7.4.4 Primary neurons

DRG cultures stained positively with the anti-ß-tubulin antibody TuJ1 which confirmed the cells to be neurones (Fig. 7.4A,D,G). Tubulin structures in the neurites appeared disrupted and cell apoptosis in neuronal cultures incubated with rotenone (1µM, Fig. 7.4E,F) or with colchicine (0.1µM, Fig. 7.4H,I) for 24hrs. Fig. 7.4C, F and I are combined images of TuJ1 anti-ß-tubulin antibody and PI staining and indicated that piericidin A and antimycin A caused

apoptosis in some DRG neurons after 24hrs incubation, but did not disrupt ß-tubulin structures (data not shown).

7.4.5 The effects of other MRC inhibitors on A549 and A549ρ° cells

The complex I inhibitor piericidin A (1μM) and the complex III inhibitor antimycin A (20μM) induced greater cell death in both A549 (9.1%±3.0 and 8.6%±2.4 cell death, respectively at 48hrs) compared to A549ρ° cells (2.5%±1.6 and 2.0%±1.2 cell death, respectively at 48hrs). The number of cells showing morphological signs of apoptosis in the dead A549 cells increased with incubation time with piericidin A or antimycin A. However, following piericidin A and antimycin A treatment, fewer A549ρ° cells showed signs of apoptosis than those observed with the A549 cell line (Table 7.2). To determine if the A549ρ° cells were in general more resistant to apoptosis than the control A549 cells, both cell lines were treated with staurosporine, a broad spectrum protein-kinase C inhibitor and inducer of apoptosis. The percentage of apoptotic cells for both A549 and A549ρ° cells were similar indicating that they were equally susceptible to apoptosis induced by staurosporine (Table 7.3). Treatment with either piericidin A or antimycin A (at concentrations between 0.1-1μM or 1-20μM, respectively) for 24-92hrs did not increase the number of mitotic or endoreduplicated cells in either A549 or A549ρ° cultures (data not shown).

Table 7.2. The percentage of apoptotic cells (A549) after treatment with piericidin A (1μM) and antimycin A (20μM)

	Control		Piericidin A		Antimycin A	
	A549	ρ°	A549	ρ°	A549	ρ°
24h	0.0±0.0	0.0±0.0	2.0±1.2	1.0±0.6	2.0±1.2	1.0±0.6
48h	0.0±0.0	0.0±0.0	6.3±1.2	2.5±1.1	5.8±1.5	3.0±1.7
72h	1.5±0.6	2.0±0.6	10 ±1.0	3.0±1.7	7.8±1.2	4.0±1.7
96h	3.9±1.2	3.7±1.6	23 ±2.5	7.0±1.2	19 ±2.8	4.9±1.8

Data are the percentage of cells and presented as mean ± SEM (n=3).

Table 7.3. The percentage of apoptotic cells after treatment with staurosporine

μM	A549	A549ρ°
0.00	0.4±0.1	0.0±0.2
0.25	33.3±2.4	31.3±2.4
0.50	54.7±2.9	53.7±0.9
0.75	71.3±0.8	76.3±3.2
1.00	80.3±3.8	79.3±5.0

Data are the percentage of cells and presented as mean ± SEM (n=3).

The untreated control values have been subtracted from these values.

7.4.6 The effect of lower glucose concentrations

To make the cells more dependent upon oxidative phosphorylation for ATP supply, a lower glucose concentration (5.6mM) was used in the medium. The A549 cells exhibited a similar response to rotenone (1μM) over the first 48hrs (Fig. 7.5D). However with longer incubation there was a dramatic rise in the level of cell death (Fig. 7.6) and the number of apoptotic cells decreased equally dramatically to background levels after 72hrs incubation (Fig. 7.5D), the cells showing increasing evidence of necrosis (data not shown). The number of endoreduplicated cells also decreased after 48hrs incubation with 1μM rotenone (Fig. 7.5D).

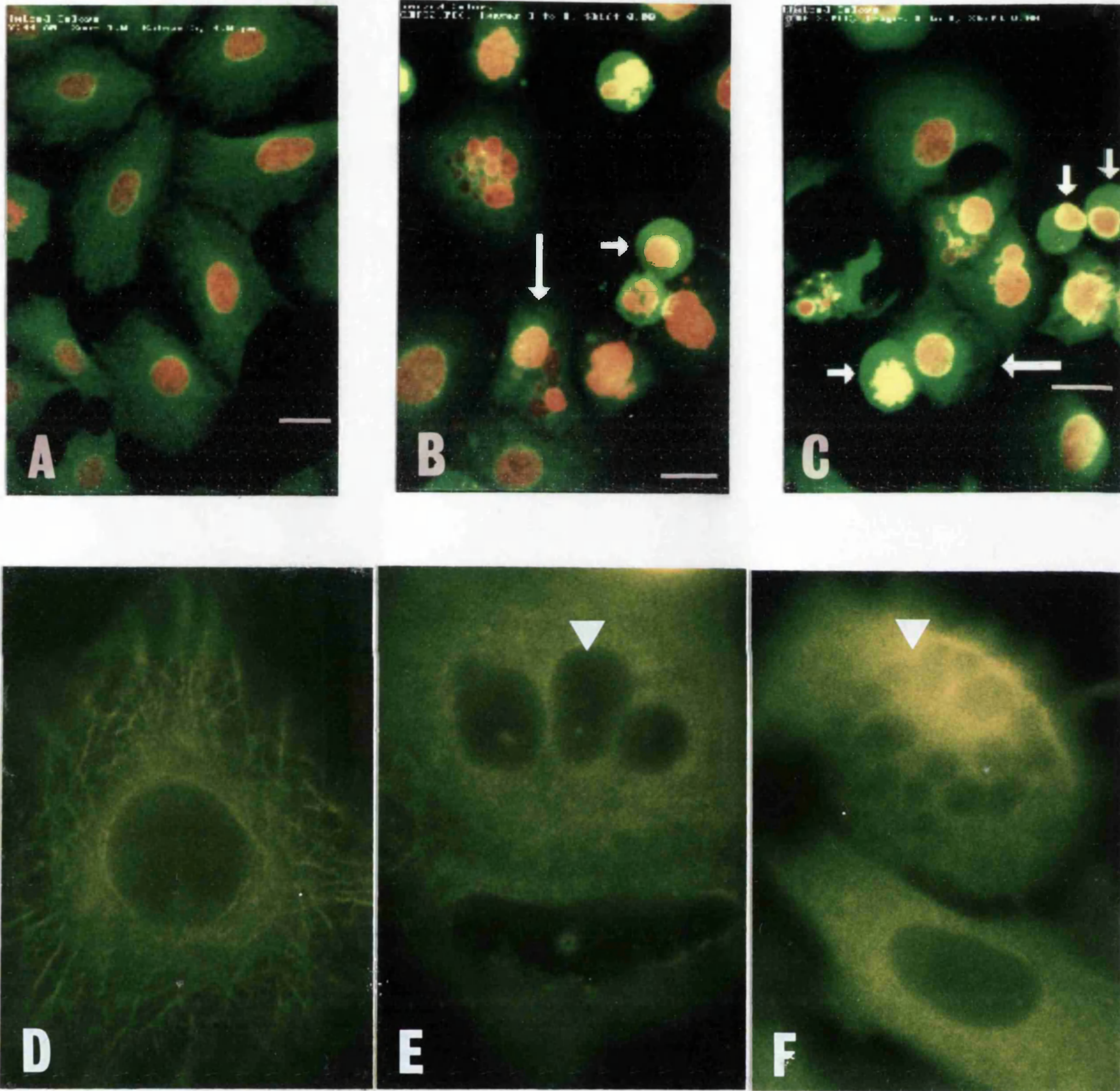


Fig. 7.1. Fluorescence immunostaining (green) with a monoclonal antibody to ß-tubulin in A549 cells. In Figs A-C the orange appearance represents the PI stained nuclei on a background of green fluorescence. Untreated A549 cells (A and D) showing normal ß-tubulin structures; 1µM rotenone (B and E) and 0.1µM colchicine (C and F) treated A549 cells showing disruption of ß-tubulin (large arrows); endoreduplication (filled triangles, E and F); and increased numbers of mitotic cells (small arrows, B and C). Bar = 20µm in A, B and C. Amplification x1070 in D, E and F.

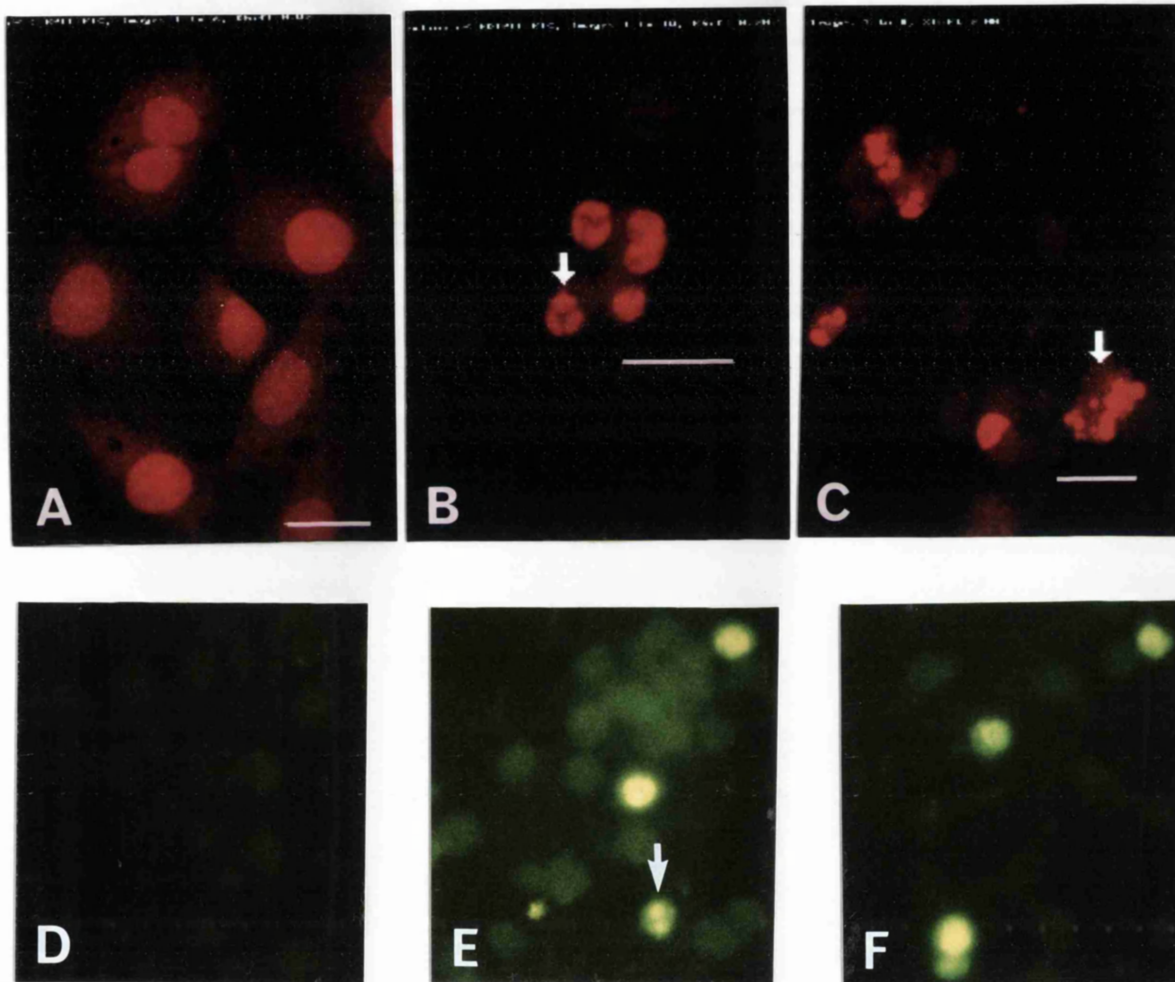


Fig. 7.2. PI (A, B and C) and Tunel (D, E and F) staining to show apoptosis in A549 cells. Untreated A549 cells with normal nuclear staining (A and D); with apoptotic nuclei (arrows) following exposure to 1μM rotenone (B and E) and 1μM staurosporine (C and F). Bar = 20μm in A, B and C. Magnification x 430 in D, E and F.

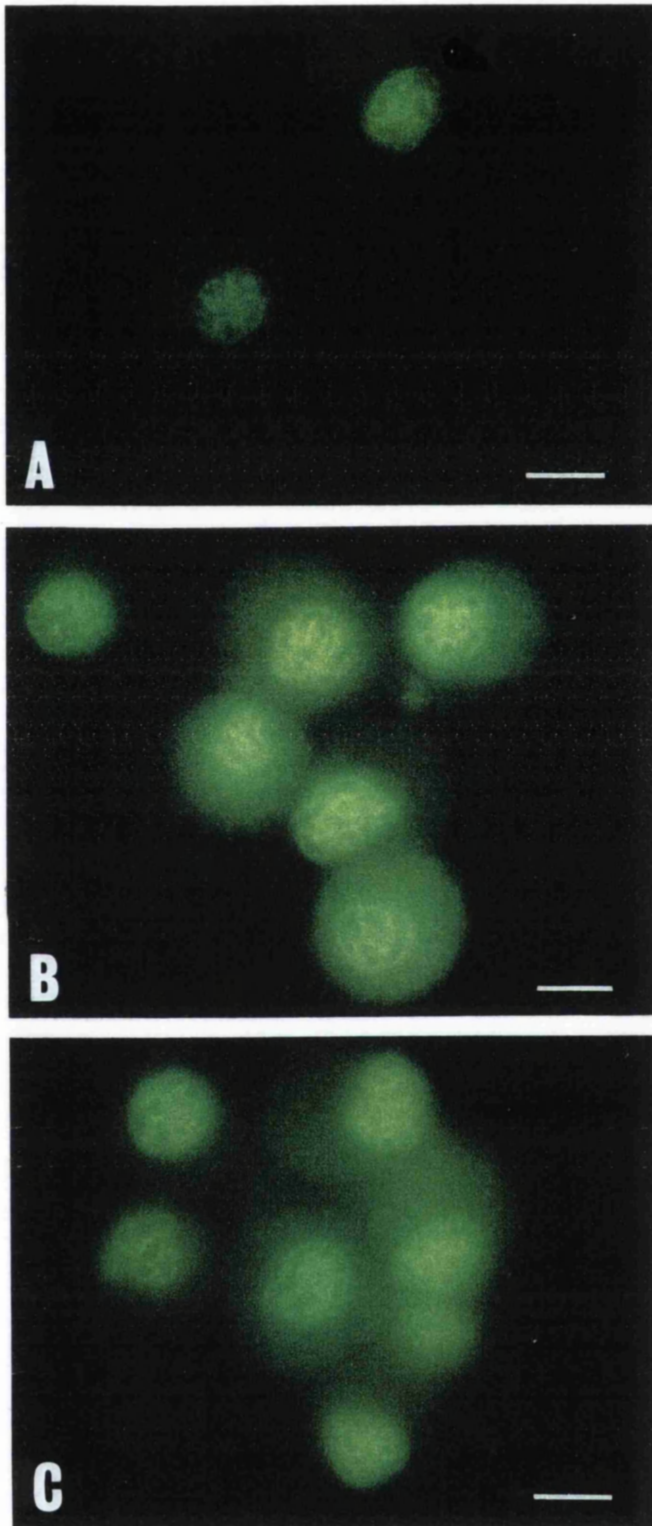


Fig. 7.3. Fluorescence immunostaining with a monoclonal antibody to Bcl-2 in A549 cells. BCL-2 staining in untreated A549 cells (A) and following treatment with 1μM rotenone (B) and 0.1μM colchicine (C). Bar = 20μm.

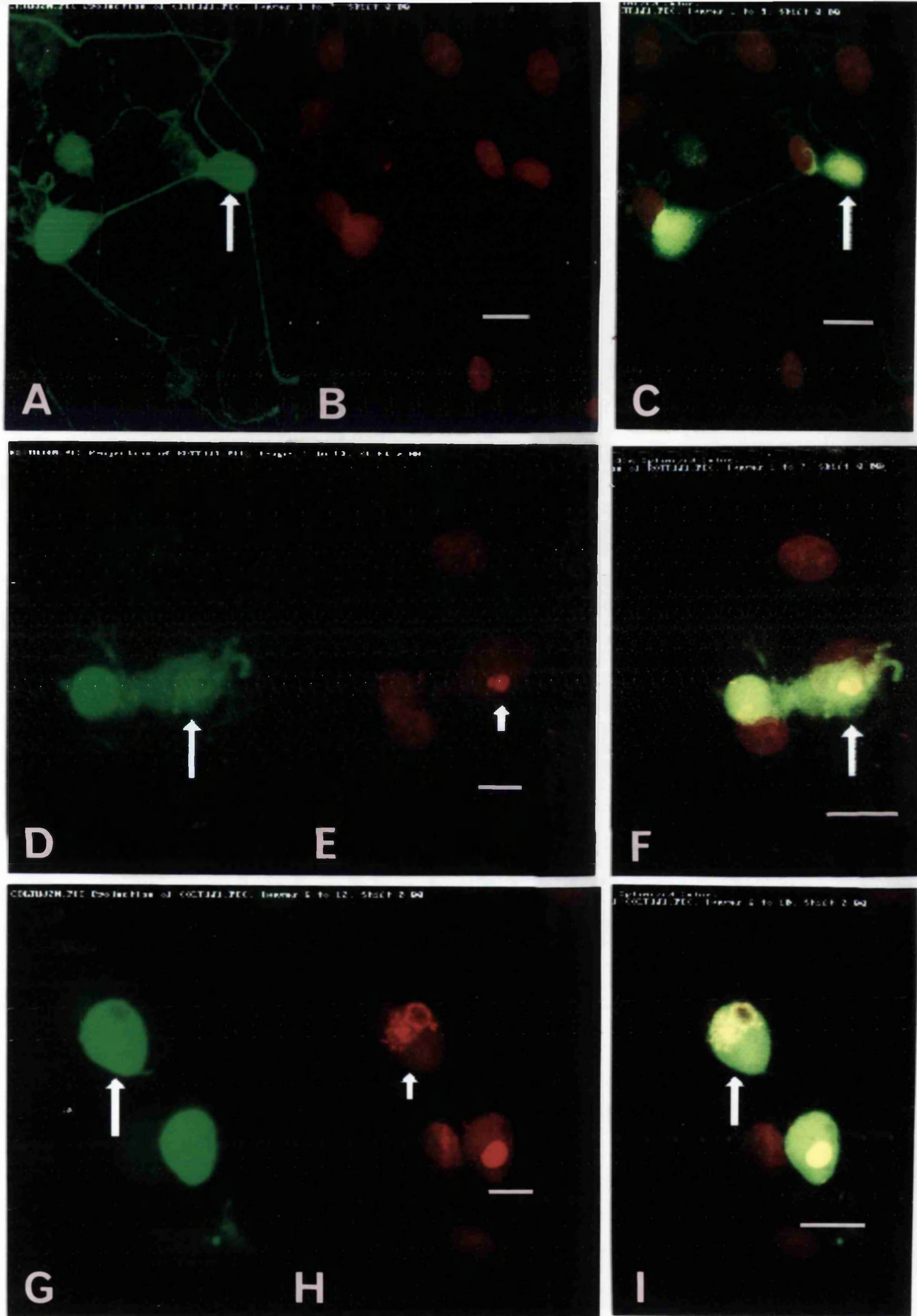


Fig. 7.4. Confocal images to show ß-tubulin and PI staining of DRG neurons with projections visible in A (arrow). Following exposure of DRG cells to 1μM rotenone (D, E, and F) or 0.1μM colchicine (G, H, and I), there is evidence of disruption of ß-tubulin (D, large arrow) or loss of neurites (G, large arrow) together with apoptosis (E and F, H and I, small arrows). C, F and I are the combined images of A and B, D and E, and G and H, respectively, and the orange appearance represents the PI stained nuclei on a background of monoclonal antibody to TuJ1 green fluorescence. Bar = 20μm.

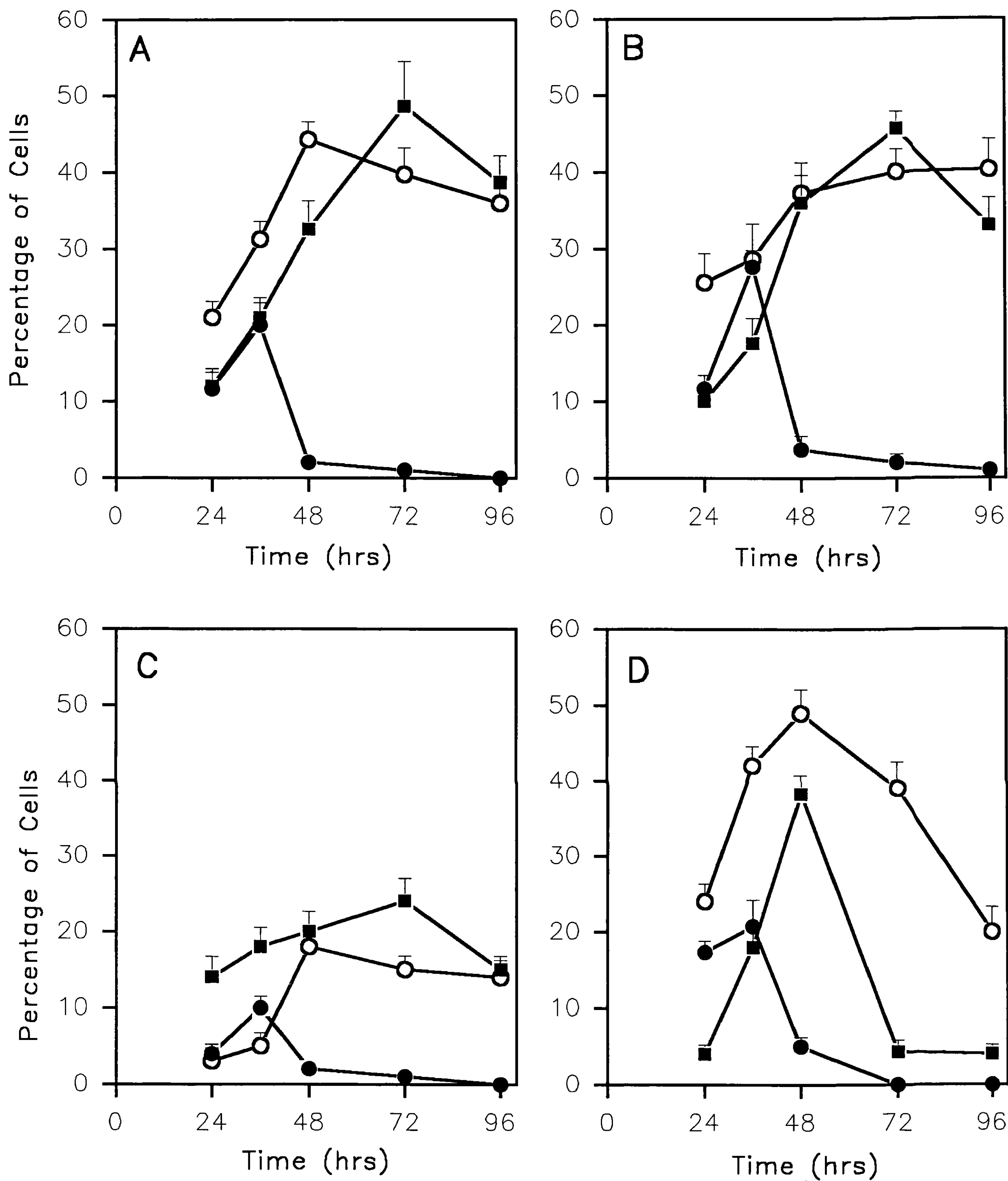


Fig. 7.5. The effect of rotenone treatment on A549 cells. The percentage of live cells in metaphase (●); showing endoreduplication (○); or the percentage of floating cells showing signs of apoptosis (■) after treatment with rotenone (1μM). The cells were grown either in 25mM glucose: A, A549 cells; B, A549ρ° cells; C, fibroblasts; or in 5.6mM glucose: D. A549 cells. Values are mean ± SEM (n=3).

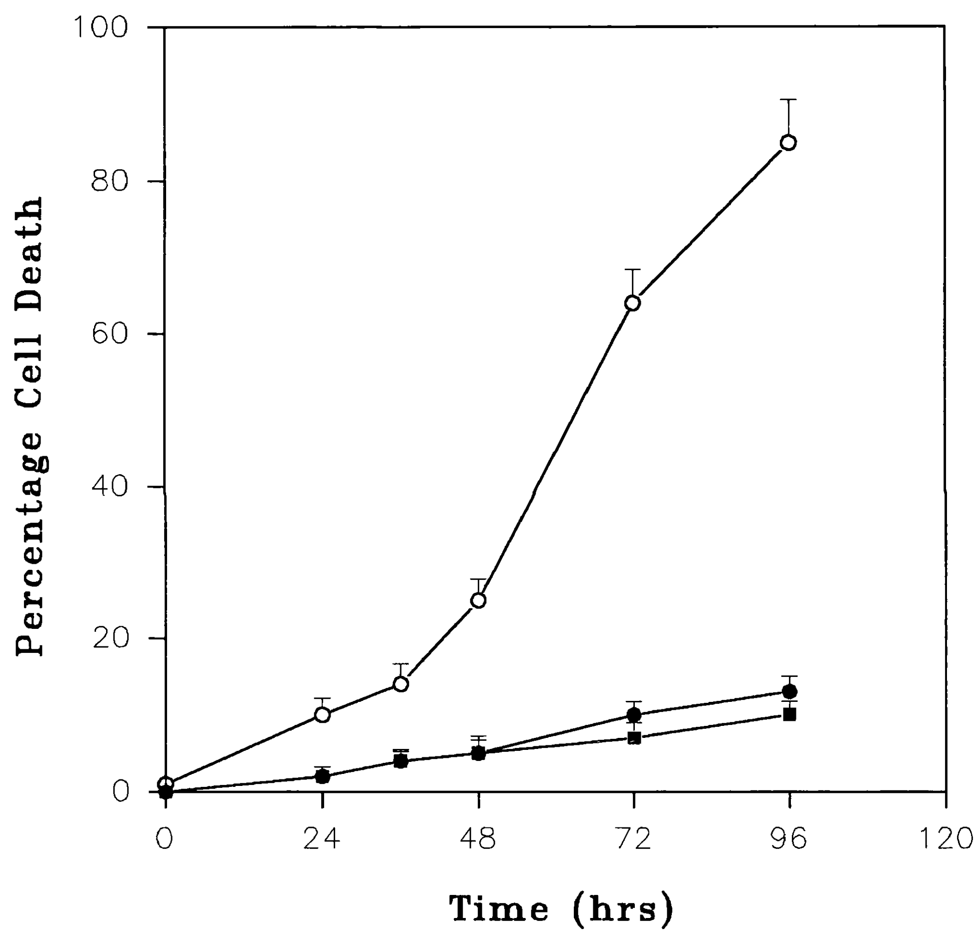


Fig. 7.6. The effect of rotenone upon the percentage of cell death. A549 cells cultured in 25mM glucose (●); in 5.6 mM glucose (○); or A549ρ° cells in 25mM glucose (■) after treatment with rotenone (1μM). Values are mean ± SEM (n=3).

7.5 Discussion

7.5.1 Dose and time dependent effects of rotenone

Rotenone has been used as a classical inhibitor of mitochondrial NADH ubiquinone reductase for many years. Inhibition of the MRC by, for example, rotenone, piericidin A and antimycin A, would be expected to result in a dramatic decrease in ATP synthesis and subsequent cell death resulting from failing energy supply. There are several reports, however, suggesting that apoptosis plays a role in cell death induced by MRC inhibition with MPP^+ (Hartley *et al.* 1994), rotenone or antimycin A (Wolvetang *et al.* 1994). Rotenone and antimycin A have been reported to induce apoptotic death in cultured human lymphoblastoid cells but not in cells depleted of mitochondrial DNA (ρ° cells) (Wolvetang *et al.* 1994) indicating apoptosis resulted specifically from their effects upon MRC function. However, while the findings in this study clearly showed that antimycin A and piericidin A preferentially induce apoptosis in A549 cells rather than A549ρ° cells, rotenone induces apoptosis equally in cells devoid of a functional MRC. This suggests that the actions of rotenone are not mediated solely via the inhibition of mitochondrial complex I activity. Rotenone not only induced endoreduplication, confirming previous observations (Matsumoto and Ohta, 1993), but also transiently increased the number of cells in metaphase. It is not clear whether this latter effect is due to a stimulation of cell division or prolonged metaphase. The failure to note increased mitosis and endoreduplication associated with rotenone treatment by some authors may suggest a cell specific response, although in this study these phenomena were observed in both A549 cells and fibroblasts, in addition to which rotenone induced endoreduplication has also been observed in Chinese hamster cells (Matsumoto and Ohta, 1993).

7.5.2 The effect of rotenone on the cytoskeleton

It has been proposed that rotenone treated cells proceed from metaphase directly to interphase, omitting anaphase and telophase, thereby resulting in endoreduplication due to the absence of cytokinesis (Matsumoto and Ohta, 1993). These endoreduplicated G1 phase cells replicate their chromosomal

DNA again in a second S phase (endoreduplicated S phase), resulting in a polyploid chromosomal appearance in the next metaphase. These studies indicated that rotenone affected microtubule morphology which may also affect spindle function. Consequently the mechanism of rotenone action may resemble that of colchicine, which extends mitosis by disrupting microtubule assembly and spindle formation (Bonfoco *et al.* 1995). Although a previous report has suggested that rotenone did not affect the spindle morphology in cells undergoing endoreduplication (Matsumoto and Ohta, 1993), there was no evidence that the spindles were functioning normally.

Recent evidence suggests that both microtubule stabilizing and destabilising agents can phosphorylate the Bcl-2 protein leading to G2/M arrest and therefore apoptosis (Haldar *et al.* 1997; Blagosklonny *et al.* 1997). The results present here are consistent with a normal physiological role of Bcl-2 as a 'guardian of microtubule integrity'.

In mammalian cells, cytokinesis is initiated by the formation of a 'furrow' in the cell surface, which gradually cleaves the cytoplasm into two compartments. The furrow is thought to be produced by localised contraction within the actin-rich cortical layer of the cytoplasm. However significant effect of rotenone treatment on α-actin in A549 cells was not detected and therefore it is suggested that the effect of rotenone on microtubule assembly is the likely cause of cytokinetic failure.

7.5.3 The effect of rotenone on Bcl-2 expression

Immunostaining for Bcl-2 revealed transient localisation to the nucleus during mitosis in A549, A549ρ° and fibroblast cells. It has been established that cells expressing Bcl-2 are protected from undergoing apoptosis by many different stimuli (Tsujimoto *et al.* 1984; Tsujimoto and Croce, 1986). The mechanism by which Bcl-2 prevents apoptotic cell death is still not clear, but it has been proposed that Bcl-2 regulates an antioxidant pathway at sites of free radical generation (Hockenbery *et al.* 1993).

There was evidence indicating that mitochondrial permeability transition (PT) constitutes a critical early event of the apoptotic process (Zamzami *et al.* 1996; Marchetti *et al.* 1996). Before cells exhibit common signs of apoptosis, they undergo a reduction of the mitochondrial transmembrane potential ($\Delta\psi_m$) that may be due to the opening of mitochondrial PT pores. Several pieces of evidence suggest that Bcl-2 inhibits apoptosis by binding to mitochondrial PT pore (Marchetti *et al.* 1996; Zamzami *et al.* 1996).

The localisation of Bcl-2 probably plays an important role in its function. Bcl-2 has been reported to be localised to the inner mitochondrial membrane (Hockenbery *et al.* 1993), but other reports favoured a wider distribution including the perinuclear endoplasmic reticulum and the outer mitochondrial membrane as well as other membrane structures (Alnemri *et al.* 1992; Monaghan *et al.* 1992). However, changes in the location of Bcl-2 appear to be important especially during metaphase where Bcl-2 has been shown to localise with the chromosomes; this feature has been observed in a variety of transformed cell lines (Willingham and Bhalla, 1994; Lu *et al.* 1994) and also in the normal skin fibroblasts used in this study, indicating that the expression of Bcl-2 during mitosis is a common event in cultured cells. Willingham and Bhalla (1994) suggested that the specific expression of Bcl-2 at the beginning of mitosis and its disappearance in late metaphase indicates that Bcl-2 acts as a 'guardian' while the chromosomes are accessible to cytoplasmic endonucleases. Consequently the nuclear DNA from a cell, unprepared for mitosis and lacking the specific localisation of Bcl-2 during G2/M phase of the cell cycle, would be vulnerable to cleavage by nucleases leading to apoptosis. While normal mitosis is a rapid event that takes in less than 1hr, it is possible that a prolonged metaphase might not be fully protected by Bcl-2 if it is turned over and not resynthesised, thus resulting in DNA cleavage and apoptosis. This has been proposed as the mechanism by which taxol induces apoptosis (Willingham and Bhalla, 1994). Although taxol and rotenone are thought to act in different ways, taxol stabilising microtubules (Bonfoco *et al.* 1995) while rotenone appears to disrupt microtubule assembly, they both involve mitotic arrest leading to apoptosis and therefore apoptosis could be explained by the failure of Bcl-2 to maintain protection during this prolonged metaphase.

7.5.4 Rotenone induced neuronal death

The fact that rotenone exposure resulted in apoptosis in DRG neurones, which are post mitotic cells, suggests that neither prolonged metaphase nor endoreduplication are important in the pathway to rotenone induced apoptosis. This would imply that whatever the mechanism for rotenone induced apoptosis these phenomena do not appeared to be important. Recently colchicine, which disrupts microtubule assembly and spindle formation resulting in mitotic arrest, has been shown to induce apoptosis in cerebellar granule cells (Bonfoco *et al.* 1995) suggesting that disruption of the cytoskeleton itself is sufficient to induce cell death in the absence of altered mitosis. Rotenone also disrupted microtubules in DRG neurones and therefore may be acting in a similar way. However it is also possible that in post-mitotic cells the inhibition of the MRC by rotenone is contributing to the apoptosis as observed for piericidin A and antimycin A.

7.5.5 The effect of other MRC inhibitors on A549 and A549ρ° cells

It was observed that piericidin A, as well as antimycin A (complex I and III inhibitors respectively), can cause apoptosis in A549 cells within 72hrs incubation; this time scale suggests that the process is unprimed apoptosis. The much lower level of apoptosis induced by these agents in A549ρ° cells, which are depleted of MRC function, suggests that the induction of apoptosis was due primarily to their inhibition of MRC function. This clearly contrasted with the situation observed with rotenone which equally caused apoptosis in A549 and A549ρ° cells. The mechanism by which inhibitors of the MRC trigger apoptosis is not known but could involve decreased ATP supply, increased free radical generation which may be blocked by variety of free radical scavengers (Seaton *et al.* 1997), altered calcium homeostasis or decreased DNA repair, although the lack of a functional MRC per se does not appear to be sufficient in itself to initiate apoptosis because ρ° cells do not spontaneously undergo apoptosis.

Although the complex I binding site for piericidin A is similar to that of rotenone (Ramsay *et al.* 1991), piericidin A induced much less apoptosis than rotenone.

This observation may be due to the fact that rotenone can induce apoptosis by an alternative mechanism.

7.5.6 Energy status

At higher glucose concentrations (25mM), cells in culture generally become less dependent upon oxidative phosphorylation and can rely predominantly upon glycolysis for their energy supply. At lower glucose concentrations (5.6mM) the cells are required to synthesise ATP from the more efficient oxidative phosphorylation system and are therefore more sensitive to MRC inhibitors.

At lower glucose concentrations rotenone had similar effects upon A549 cells for up to 48hrs of treatment. However after 48hrs the level of cell death rose dramatically and the number of apoptotic cells decreased dramatically while the number of endoreduplicated cells declined more slowly. This suggests that while the energy supply is sufficient, rotenone results in endoreduplication and apoptosis, but when the energy supply becomes limited the cells die by necrosis. At high concentrations of rotenone (10μM), the level of apoptosis and endoreduplication was dramatically decreased. This may suggest that at these concentrations the inhibition of the MRC is most important and the cells die by necrosis before endoreduplication and apoptosis occur.

These results demonstrate that whilst apoptosis induced by agents such as antimycin A or piericidin A may be mediated via the MRC, rotenone may bypass this mechanism by disrupting microtubule formation. Whether the actions of rotenone involve an alteration in PT as well as microtubule formation is unknown. The ability of rotenone to induce apoptosis in a manner independent of the MRC must be borne in mind when using this agent in models of complex I deficiency-associated neurodegeneration.

The MRC function in PD

Within the brain at least, complex I deficiency in PD is confined anatomically to the substantia nigra. The absence of any mitochondrial defect in PD substantia innominata or cingulate cortex in the present study further supports this conclusion. The reduction in levels of GSH found in PD substantia nigra also appears selective for the substantia nigra, again with unaltered GSH concentrations in PD substantia innominata and cingulate cortex (Gu *et al.* 1998b). The complex I deficiency and decrease in GSH levels are linked, at least in anatomical distribution, which implies that one may cause the other, or that both are the result of a separate primary event.

The absence of any detectable mitochondrial defect in MSA brain provides a biochemical dimension to the clinical and pathological features which distinguish this disorder from PD. These results suggested that, in human substantia nigra, L-dopa does not induce the complex I deficiency, at least in the context of MSA pathology. Furthermore, there was no relationship between individual L-dopa dose and substantia nigra complex I activity in MSA and PD patients. This suggests that L-dopa treatment does not have an effect on complex I activity. The underlying aetiology and pathogenesis of MSA, however, remain unknown.

The MRC function in PD, AD and DLB

It has been argued that the aetiology of PD may be genetic or environmental or both, but no conclusive data are available to support either of these as dominant. It may be true that one or other of these factors is involved as a background effect upon which the other factor is superimposed to induce severe neuronal cell loss in the substantia nigra of PD. For example, a genetic or environmental insult could induce cell loss and Lewy body formation in the nigra and innominata. The profound cell loss of the substantia nigra pars compacta would then be induced by an additional but alternative cause, genetic or environmental (endogenous or exogenous) to produce the clinical features of PD. This hypothesis would suggest that some individuals may be

exposed to only one of the two 'hits' and therefore not develop PD, but would bear the pathological consequence of their respective exposure (Gu *et al.* 1998b). Thus 'one-hit' brains would show either mild but diffuse neuronal cell loss (substantia nigra, substantia innominata, locus ceruleus) with Lewy body formation or, at the other extreme, selective substantia nigral cell loss. Patients with DLB might have just 'one-hit', giving rise to Lewy bodies and the 'second-hit' was missing.

The findings that apoE genotype frequencies in AD and DLB patients were significantly increased but normal in PD with or without dementia were similar to those found in other studies (Strittmatter *et al.* 1993; Galasko *et al.* 1994). The risk factors for dementia in both AD and DLB may be similar (at least for apoE) but the presence of Lewy bodies in DLB suggested that DLB and PD may share other risk factors.

The MRC function in HD

Marked reductions in the activities of both complexes II and III and more mild changes in complex IV were found in the caudate nucleus from HD brains. These results confirm the previous observations (Brennan *et al.* 1985; Mann *et al.* 1990) and have subsequently been reproduced by others (Browne *et al.* 1997). The degree of complex II and III defects as assessed individually was comparable and this raises the possibility that both may be secondary to a deficiency of ubiquinone which is used as an electron carrier by both complexes. The relationship of the complex IV defect to that of complexes II and III is uncertain; it could not be caused by ubiquinone deficiency but might imply a more general dysfunction of these membrane bound proteins.

Evidence is accruing that the biochemical and neurochemical abnormalities in HD involve a defect in energy metabolism and glutamate excitotoxicity. The underlying molecular genetic defect has been identified as an expanded CAG triplet in IT15 gene, but the function of mutant protein product remains unknown. There is now direct evidence for a mitochondrial respiratory chain defect in HD to support the MRS data showing elevated lactate levels. Further work is now required to identify the relationship of the biochemical changes to

the molecular defect of HD and the sequence of events which terminates in neuronal death in HD striatum.

MtDNA transmission of the MRC defect in PD

As described previously, a deficiency of the mitochondrial respiratory chain complex I activity has been found in the substantia nigra and platelets from patients with PD. It has been suggested that mtDNA may play an important role in the origin of this defect. To pursue this hypothesis, fusion of anucleated cells with experimental cells lacking mtDNA (ρ°) has been used to determine whether or not the defect is caused by abnormal mtDNA in this study. a positive control was selected to analyse the potential of this technique to identify mutant mtDNA at the biochemical level.

The studies with the A3243G cybrids demonstrated that deficiencies of complexes I and IV activities required that >90% of the clone's mtDNA was mutant. Clonal lines were themselves heteroplasmic indicating random drift of mutant mtDNA during cell division. This is also shown by the variation in mutant loads between clones derived from the initial platelet fusion, although total mutant mtDNA levels remained stable with a clonal passage. The results with the A3243G mutation are in accord with a previous study which assessed mitochondrial function by oxygen utilisation and again showed that >90% mutant mtDNA was required for biochemical expression (Chomyn *et al.* 1992). These data show for the first time that cells within clonal lines are heterogeneous in terms of their COX activity and COX I staining and therefore, by implication, either carry different mutant mtDNA loads or express the mutation differently.

This method clearly demonstrated the feasibility of this approach in using biochemical analyses to identify the presence of abnormal mtDNA.

We hypotheses that these patients with the lowest platelet complex I activity were those most likely to have a defect of mtDNA determining their deficiency. Therefore a group of PD patients were pre-selected for cybrids studies according to this criterion.

The analysis of A549ρ°-PD clonal cybrids from one patient revealed defects of complexes I and IV in the cybrid lines as a whole. The clones showed variable staining with cytochrome c oxidase activity and COX I antibody. This pattern of MRC deficiency with histochemical and immunohistochemical staining in A549ρ°-PD cybrid bears a striking resemblance to the results obtained with the A549ρ°-A3243G cybrids. Thus, based on the data from the PD cybrid clones, a tRNA mutation may be responsible for the mitochondrial defect, at least in this patient.

The analysis of A549ρ°-PD mixed cybrids in 7 PD patients showed a complex I defect in the PD cybrids in this study is in remarkable agreement with the results of a recently published study (Swerdlow *et al.* 1996), which are comparable to the 25% statistically significant decreases in complex I ratios but not in complex IV ratios from mixed cybrids in this study. The absence of a complex IV defect in other patients and/or tissues may be explained by both methodological and biochemical factors. Assays of respiratory chain activity in substantia nigra use either tissue homogenates or fractions. Their sensitivity is therefore less than in the mitochondrial preparations used in the analysis of the recloned cybrids. Thus a 20% of complex IV deficiency may appear in the clones but be concealed in the substantia nigra.

These results suggested that complex I deficiency in PD has a genetic component arising from mtDNA and indicates that the complex I defect may be a primary phenomenon in PD.

As seen with the A3243G mutation, clonal cybrids have the potential to amplify the level of mutant mtDNA load, and this may prove an important tool to investigate further those PD patients with apparently normal platelet mitochondrial function possibly due to a sub-threshold level of mutant mtDNA.

Apoptosis

Exposure of cells to the mitochondrial complex I inhibitor rotenone, caused an increase in the proportion of cells in metaphase and induced

endoreduplication and apoptosis. Normal human fibroblasts, a human lung carcinoma cell line (A549) and its derivative cell line which lacked mitochondrial DNA (A549ρ^{o}) were equally sensitive to the actions of rotenone indicating that these effects are neither cell specific nor the result of rotenone acting via the mitochondrial respiratory chain. Prolonged incubation with rotenone resulted in a decrease in the number of mitotic cells while the number of endoreduplicated and apoptotic cells increased. Rotenone induced effects on the cell cycle may be caused by disruption of microtubule formation. The levels of Bcl-2 were markedly increased after rotenone treatment, but the Bcl-2 was restricted to cells in metaphase and correlated with the increased number of mitotic cells. Rotenone induced apoptosis in primary dorsal root ganglion neurones suggesting that prolonged metaphase and endoreduplication were not intermediates in the pathway to apoptosis. Other mitochondrial respiratory chain inhibitors (piericidin A and antimycin A) did not cause endoreduplication or increase the number of cells in metaphase but did induce apoptosis which was more pronounced in A549 than in A549ρ^{o} cells. These results imply that, although the mitochondrial respiratory chain inhibitors piericidin A and antimycin A induce apoptosis via a mechanism that involves inhibition of the mitochondrial respiratory chain, the predominant toxic effects of rotenone are mediated via a pathway independent of the mitochondrial respiratory chain.

Although the complex I binding site for piericidin A was the same as rotenone, piericidin A included much less apoptotic cell death than rotenone within the same period of incubation. This observation may be due to the fact that rotenone has an effect on the microtubule structures but piericidin A does not. Inhibition of MRC activity by certain types of mitochondrial toxins can interfere with normal mitochondrial energy metabolism, leading to an apoptotic response.

Future work

These experiments establish a potential role for mtDNA in PD. Further work needs to be undertaken to understand the place of mtDNA mutations/polymorphisms in PD aetiology. It has been predicted that low

platelet complex I activity (possibly combined with low complex IV activity) may be a marker for those patients in whom a mtDNA sequence change is relevant. The mtDNA of these patients needs to be sequenced to define a haplotype that may be common to this group. The theory also predicts that PD patients with 'normal' platelet complex I activity may not show any abnormality on clonal cybrid studies, unless their 'normal' complex I activity is the result of a low level of mutant mtDNA in platelets.

REFERENCES

Adams, J.D., Mukherjee, S.K., Klaidman, L.K., Chang, M.L. and Yasharel, R. (1996) Apoptosis and oxidative stress in the aging brain. *Annals of the New York Academy of Sciences* **786**, 135-151.

Allsopp, T.E., Wyatt, S., Paterson, H.F. and Davies, A.M. (1993) The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. *Cell* **73,** 295-307.

Alnemri, E.S., Robertson, N.M., Fernandes, T.F., Croce, C.M. and Litwack, G. (1992) Overexpressed full-length human BCL2 extends the survival of baculovirus-infected Sf9 insect cells. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 7295-7299.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. and Yuan, J. (1996) Human ICE/CED-3 protease nomenclature. *Cell* **87**, 171.

Ambrose, C.M., Duyao, M.P., Barnes, G., Bates, G.P., Lin, C.S., Srinidhi, J., Baxendale, S., Hummerich, H., Lehrach, H., Altherr, M., *et al* (1994) Structure and expression of the Huntington's disease gene: evidence against simple inactivation due to an expanded CAG repeat. *Somatic Cell and Molecular Genetics* **20**, 27-38.

Ames, B.N., Shigenaga, M.K. and Hagen, T.M. (1995) Mitochondrial decay in aging. *Biochimica et Biophysica Acta* **1271**, 165-170.

Andersen, J.K., Frim, D.M., Isacson, O., Beal, M.F. and Breakefield, X.O. (1994) Elevation of neuronal MAO-B activity in a transgenic mouse model does not increase sensitivity to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Brain Research* **656**, 108-114.

Andrew, S.E., Goldberg, Y.P., Kremer, B., Telenius, H., Theilmann, J., Adam, S., Starr, E., Squitieri, F., Lin, B., Kalchman, M.A., *et al* (1993) The relationship between trinucleotide (CAG) repeat length and clinical features of Huntington's disease. *Nature Genetics* **4**, 398-403.

Arai, H., Higuchi, S., Muramatsu, T., Iwatsubo, T., Sasaki, H. and Trojanowski, J.Q. (1994a) Apolipoprotein E gene in diffuse Lewy body disease with or without co-existing Alzheimer's disease. *Lancet* **344**, 1307.

Arai, H., Muramatsu, T., Higuchi, S., Sasaki, H. and Trojanowski, J.Q. (1994b) Apolipoprotein E gene in Parkinson's disease with or without dementia. *Lancet* **344**, 889.

Armstrong, M., Daly, A.K., Cholerton, S., Bateman, D.N. and Idle, J.R. (1992) Mutant debrisoquine hydroxylation genes in Parkinsons disease. *Lancet* **339,** 1017-1018.

Attardi, G., Chomyn, A., King, M.P., kruse, B., Polosa, P.L. and Murdter, N.N. (1989) Regulation of mitochondrial gene expression in mammalian cells. *Biochemical Society Transactions* **18,** 509-513.

Balazs, L. and Leon, M. (1994) Evidence of an oxidative challenge in the Alzheimer's brain. *Neurochemical Research* **19**, 1131-1137.

Barbeau, A., Roy, M., Paris, S., Cloutier, T., Plasse, L. and Poirier, J. (1985) Ecogenetics of Parkinsons disease: 4-hydroxylation of debrisoquine. *Lancet* **ii,** 1213-1215.

Bates, T.E., Heales, S.J., Davis, S.E., Boakye, P. and Clarke, J.B. (1994) Effects of 1-methyl-4-phenylpyridium on isolated rat brain mitochondria; evidence for a primary involvment of energy depletion. *Journal of Neurochemistry* **63,** 640-648.

Beal, M.F. (1992) Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Annals of Neurology* **31**, 119-130.

Beal, M.F., Brouillet, E., Jenkins, B., Henshaw, R., Rosen, B. and Hyman, B.T. (1993) Age-dependent striatal excitotoxic lesions produced by the endogenous mitochondrial inhibitor malonate. *Journal of Neurochemistry* **61**, 1147-1150.

Beal, M.F. (1994) Neurochemistry and toxin models in Huntington's disease. *Current Opinion in Neurology* **7**, 542-547.

Ben-Shachar, D., Riederer, P. and Youdim, M.B.H. (1991) Iron-melanin interaction and lipid peroxidation: implications for Parkinsons disease. *Journal of Neurochemistry* **57,** 1609-1614.

Benecke, R., Strumper, P. and Weiss, H. (1992) Electron transfer complex I defect in idiopathic dystonia. *Annals of Neurology* **32,** 683-686.

Benecke, R., Strumper, P. and Weiss, H. (1993) Electron transfer complexes I and IV of platelets are abnormal in Parkinsons disease but normal in Parkinson-plus syndromes. *Brain* **116,** 1451-1463.

Benjamin, R., Leake, A., Edwardson, J.A., Mckeith, I.G., Ince, P.G., Perry, R.H. and Morris, C.M. (1994) Apolipoprotein E genes in Lewy body and Parkinson's disease. *Lancet* **343**, 1565.

Bernardi, P., Broekemeier, K.M. and Pfeiffer, D.R. (1994) Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. *Journal of Bioenergetics and Biomembranes* **26**, 509-517.

Beyer, R.E. (1992) An analysis of the role of coenzyme Q in free radical generation and as an antioxidant. *Biochemistry and Cell Biology* **70**, 390-403.

Bindoff, L.A., Birch-Machin, M.A., Carrlidge, N.E.F., Parker, W.D. and Turnbull, D.M. (1991) Respiratory chain abnormalities in skeletal muscle from patients with Parkinsons disease. *Journal of the Neurological Sciences* **104,** 203-208.

Birch-Machin, M.A., Shepherd, I.M., Watmough, N.J., Sherratt, H.S., Bartlett, K., Darley-Usmar, V.M., Milligan, D.W., Welch, R.J., Aynsley-Green, A. and Turnbull, D.M. (1989) Fatal lactic acidosis in infancy with a defect of complex III of the respiratory chain. *Pediatric Research* **25**, 553-559.

Blagosklonny, M.V., Giannakakou, P., el-Deiry, W.S., Kingston, D.G., Higgs, P.I., Neckers, L. and Fojo, T. (1997) Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Research* **57**, 130-135.

Blake, C.I., Spitz, E., Leehey, M., Hoffer, B.J. and Boyson, S.J. (1997) Platelet mitochondrial respiratory chain function in Parkinson's disease. *Movement Disorders* **12**, 3-8.

Blin, O., Desnuelle, C., Rascol, O., Borg, M., Peyro Saint Paul, H., Azulay, J.P., Bille, F., Figarella, D., Coulom, F., Pellissier, J.F., *et al* (1994) Mitochondrial respiratory failure in skeletal muscle from patients with Parkinson's disease and multiple system atrophy. *Journal of the Neurological Sciences* **125**, 95-101.

Bodnar, A.G., Cooper, J.M., Holt, I.J., Leonard, J.V. and Schapira, A.H. (1993) Nuclear complementation restores mtDNA levels in cultured cells from a patient with mtDNA depletion. *American Journal of Human Genetics* **53**, 663-669.

Bonfoco, E., Ceccatelli, S., Manzo, L. and Nicotera, P. (1995) Colchicine induces apoptosis in cerebellar granule cells. *Experimental Cell Research* **218**, 189-200.

Boveris, A. and Chance, B. (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochemical Journal* **134**, 707-716.

Bowling, A.C., Mutisya, E.M., Walker, L.C., Price, D.L., Cork, L.C. and Beal M.F. (1993) Age-dependent impairment of mitochondrial function in primate brain. *Journal of Neurochemistry* **60,** 1964-1967.

Brennan, W.A., Jr., Bird, E.D. and Aprille, J.R. (1985) Regional mitochondrial respiratory activity in Huntington's disease brain. *Journal of Neurochemistry* **44**, 1948-1950.

Brouillet, E., Hantraye, P., Ferrante, R.J., Dolan, R., Leroy-Willig, A., Kowall, N.W. and Beal, M.F. (1995) Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 7105-7109.

Browne, S.E., Bowling, A.C., MacGarvey, U., Baik, M.J., Berger, S.C., Muqit, M.M., Bird, E.D. and Beal, M.F. (1997) Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. *Annals of Neurology* **41**, 646-653.

Buja, L.M., Eigenbrodt, M.L. and Eigenbrodt, E.H. (1993) Apoptosis and necrosis. Basic types and mechanisms of cell death. *Archives of Pathology and Laboratory Medicine* **117**, 1208-1214.

Burke, J.R., Enghild, J.J., Martin, M.E., Jou, Y.S., Myers, R.M., Roses, A.D., Vance, J.M. and Strittmatter, W.J. (1996) Huntingtin and DRPLA proteins selectively interact with the enzyme GAPDH. *Nature Medicine* **2**, 347-350.

Burns, A. (1991) Clinical diagnosis of Alzheimers disease. *Dementia* **2** 186-194.

Byrne, E., Trounce, I., Dennett, X., Gilligan, B., Morley, J.B. and Marzuki, S. (1988) Progression from MERRF to MELAS phenotype in a patient with combined respiratory complex I and IV deficiencies. *Journal of the Neurological Sciences* **88**, 327-337.

Cadenas, E., Boveris, A., Ragan, C.I. and Stoppani, A.O. (1977) Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. *Archives of Biochemistry and Biophysics* **180**, 248-257.

Cardellach, F., Galofre, J., Cusso, R. and Urbano-Marquez, A. (1989) Decline in skeletal muscle mitochondrial respiratory chain function with ageing. *Lancet* **ii,** 44-45.

Cardellach, F., Marti, M.J., Fernandez-Sola, J., Martin, C., Hoek, J.B., Tolosa, E. and Urbano-Marquez, A. (1993) Mitochondrial respiratory chain activity in skeletal muscle from patients with Parkinson's disease. *Neurology* **43**, 2258-2262.

Chagnon, P., Betard, C., Robitaille, Y., Cholette, A. and Gauvreau, D. (1995) Distribution of brain cytochrome oxidase activity in various neurodegenerative diseases. *Neuroreport* **6**, 711-715.

Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide metablism in mammalian organs. *Physiological Reviews* **59,** 527-605.

Chen, K., Wu, H.F. and Shih, J.C. (1993) The deduced amino acid sequences of human platelet and frontal cortex monoamine oxidase B are identical. *Journal of Neurochemistry* **61,** 187-190.

Chiba, K., Trevor, A.J. and Castagnoli, N.J. (1984) Metabolism of the neurotoxic tertiary amine, MPTP by brain monoamine oxidase. *Biochemical and Biophysical Research Communications* **120,** 574-578.

Chomyn, A., Cleeter, M.W.J., Ragan, C.I., Riley, M., Doolittle, R.F. and Attardi, G. (1986) ORF6, the last unidentified reading frame of human mtDNA, codes for an NADH dehydrogenase subunit. *Science* **234,** 614-618.

Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., Lai, S.T., Nonaka, I., Angelini, C. and Attardi, G. (1992) MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no changes in levels of upstream and downstream mature transcripts. *Proceedings of the National Academy of Sciences of the United States of America* **89,** 4221-4225.

Chomyn, A., Lai, S.T., Shakeley, R., Bresolin, N., Scarlato, G. and Attardi, G. (1994) Platelet-mediated transformation of mtDNA-less human cells: analysis of phenotypic variability among clones from normal individuals--and complementation behavior of the $tRNA^{Lys}$ mutation causing myoclonic epilepsy and ragged red fibers. *American Journal of Human Genetics* **54**, 966-974.

Ciacci, F., Moraes, C.T., Silvestri, G., Shanske, S., Schon, E.A. and di Mauro, S. (1992) The 'MELA-3,243' mutation in mtDNA is found in many patients with Progressive External Ophthalmoplegia (PEO). *Neurology* **42 (suppl 3),** 417.

Clayton, D.A. (1982) Replication of animal mitochondrial DNA. *Cell* **28**, 693-705.

Cleeter, M.W., Cooper, J.M. and Schapira, A.H.V. (1992) Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: evidence for free radical involvement. *Journal of Neurochemistry* **58**, 786-789.

Cock, H.R., Tabrizi, S.J., Cooper, J.M. and Schapira, A.H.V. (1998) The influence of nuclear background on the biochemical expression of 3460 Leber's hereditary optic neuropathy. *Annals of Neurology* **44,** 187-193.

Cohen, G. (1990) Monoamine oxidase and oxidative stress at dopaminergic synapses. *Journal of Neural Transmission* **32 [Suppl],** 229-238.

Collins, M.A., Neafsey, E.J., Matsubara, K., Cobuzzi, R.J. and Rollema, H. (1992) Indole-N-methylated -carbolinium ions as potential brain-bioactivated neurotoxins. *Brain Research* **570,** 154-160.

Cooper, J.M., Mann, V.M. and Schapira, A.H.V. (1992) Analyses of mitochondrial respiratory chain function and mitochondrial DNA deletion in human skeletal muscle: effect of ageing. *Journal of the Neurological Sciences* **113**, 91-98.

Cooper, J.M., Wischik, C. and Schapira, A.H.V. (1993) Mitochondrial function in Alzheimer's disease. *Lancet* **341**, 969-970.

Cooper, J.M., Daniel, S.E., Marsden, C.D. and Schapira, A.H.V. (1995) L-dihydroxyphenylalanine and complex I deficiency in Parkinson's disease brain. *Movement Disorders* **10**, 295-297.

Coore, H.G., Denton, R.M., Martin, B.R. and Randle, P.J. (1971) Effects of insulin and adrenaline on rat epididymal-fat-pad pyruvate dehydrogenase. *Biochemical Journal* **123**, 38-39.

Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L. and Pericak-Vance, M.A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261**, 921-923.

Cotter, T.G., Lennon, S.V., Glynn, J.M. and Green, D.R. (1992) Microfilament-disrupting agents prevent the formation of apoptotic bodies in tumor cells undergoing apoptosis. *Cancer Research* **52**, 997-1005.

Crews, S., Ojala, D., Posakony, J., Nishiguchi, J. and Attardi, G. (1979) Nucleotide sequence of a region of human mitochondrial DNA containing the precisely identified origin of replication. *Nature* **277**, 192-198.

Da Prada, M., Cesura, A.M., Launay, J.M. and Richards, J.G. (1988) Platelets as a model for neurones? *Experimentia* **44,** 115-126.

Das, H.K., McPherson, J., Bruns, G.A., Karathanasis, S.K. and Breslow, J.L. (1985) Isolation, characterization, and mapping to chromosome 19 of the human apolipoprotein E gene. *Journal of Biological Chemistry* **260**, 6240-6247.

Davidoff, A.N. and Mendelow, B.V. (1992) Unexpected cytokinetic effects induced by puromycin include a G2-arrest, a metaphase-mitotic-arrest, and apoptosis. *Leukemia Research* **16**, 1077-1085.

Desjardins, P., Frost, E. and Morais, R. (1985) Ethidium bromide-induced loss of mitochondrial DNA from primary chicken embryo fibroblasts. *Molecular and Cellular Biology* **5**, 1163-1169.

Dexter, D.T., Carter, C., Javoy-Agid, F., Agid, Y., Lees, A.J., Jenner, P. and Marsden, C.D. (1986) Lipid peroxidation as a cause of nigral cell death in Parkinsons disease. *Lancet* **ii,** 639-640.

Dexter, D.T., Carter, C., Well, F.R., Javoy-Agid, F., Agid, Y., Lees, A., Jenner, P. and Marsden, C.D. (1989) Basal lipid peroxidation in substantia nigra is increased in parkinsons disease. *Journal of Neurochemistry* **52,** 381-389.

Dexter, D.T., Carayon, A., Vidaihet, M., Ruberg, M., Agid, F., Agid, Y., Lees, A.J., Well, F.R., Jenner, P. and Marsden, C.D. (1990) Decreased ferritin levels in brain in Parkinsons disease. *Journal of Neurochemistry* **55,** 16-20.

Dexter, D.T., Carayon, A., Javoy-Agid, F., Agid, Y., Wells, F.R., Daniel, S.E., Lees, A.S., Senner, P.S. and Marsden, C.D. (1991) Alterations in the levels of iron, ferritin and other trace metals in Parkinsons disease and other neurodegenerative diseases affecting the basal ganglia. *Brain* **114,** 1953-1957.

Dexter, D.T., Ward, R.J., Well, F.R., Daniel, S.E., Lees, A.J. and Peters, T.J. (1992) α-tocopherol levels in brain are not altered in Parkinsons disease. *Annals of Neurology* **32,** 591-593.

Dexter, D.T., Sian, J., Rose, S., Hindmarsh, J.G., Mann, V.M., Cooper, J.M., Wells, F.R., Daniel, S.E., Lees, A.J., Schapira, A.H., *et al* (1994) Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. *Annals of Neurology* **35**, 38-44.

Di Donato, S., Zeviani, M., Giovannini, P., Savarese, N., Rimoldi, M., Mariotti, C. and et al. (1993) Respiratory chain and mitochondrial DNA in muscle and brain in Parkinsons disease patients. *Neurology* **43,** 2262-2268.

Di Monte, D., Jewell, S.A., Ekstrom, G., Sandy, M.S. and Smith, M.T. (1986) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium (MPP^+) cause rapid ATP depletion in isolated hepatocytes. *Biochemical and Biophysical Research Communications* **137,** 310-315.

Di Monte, D.A., Royland, J.E., Anderson, A., Castagnoli, K., Castagnoli, N.J. and Langston, J.W. (1997) Inhibition of monoamine oxidase contributes to the protective effect of 7-nitroindazole against MPTP neurotoxicity. *Journal of Neurochemistry* **69,** 1771-1773.

Drucker, G.N., Neafsey, E.J. and Collins, M.A. (1990) Dopamine uptake inhibitory capacities of β-carbolin and 3,4-dihydro-carbolin analogs of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) oxidation products. *Brain Research* **509,** 125-133.

Dunbar, D.R., Moonie, P.A., Swingler, R.J., Davidson, D., Roberts, R. and Holt, I.J. (1993) Maternally transmitted partial direct tandem duplication of mitochondrial DNA associated with diabetes mellitus. *Human Molecular Genetics* **2**, 1619-1624.

Dunbar, D.R., Moonie, P.A., Jacobs, H.T. and Holt, I.J. (1995) Different cellular backgrounds confer a marked advantage to either mutant or wild-type mitochondrial genomes. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 6562-6566.

Dunbar, D.R., Moonie, P.A., Zeviani, M. and Holt, I.J. (1996) Complex I deficiency is associated with 3243G:C mitochondrial DNA in osteosarcoma cell cybrids. *Human Molecular Genetics* **5**, 123-129.

Duvoisin, R.C. and Jonhson, W.G. (1992) Hereditary Lewy-body parkinsonism and evidence for a genetic aetiology of Parkinsons disease. *Brain Pathology* **2,** 309-320.

Duyao, M., Ambrose, C., Myers, R., Novelletto, A., Persichetti, F., Frontali, M., Folstein, S., Ross, C., Franz, M., Abbott, M., *et al* (1993) Trinucleotide repeat length instability and age of onset in Huntington's disease. *Nature Genetics* **4**, 387-392.

Earley, F.G.P., Patel, S.D., Ragan, C.I. and Attarsi, G. (1987) Photolabelling of a mitochondrially encoded subunit of NADH dehydrogenase with [^{3}H]dihydrorotenone. *FEBS Letters* **219,** 108-113.

Egensperger, R., Kosel, S., Schnopp, N.M., Mehraein, P. and Graeber, M.B. (1997) Association of the mitochondrial tRNA(A4336G) mutation with Alzheimer's and Parkinson's diseases. *Neuropathology and Applied Neurobiology* **23**, 315-321.

Ehringer, H. and Hornykiewicz, O. (1960) Vertilung von noradrenalin und dopamin (3-hydroxytyramin) im gehrin des menschen und ihr verhalten bei erkrankungen des extrapyramidalen system. *Klin Wocheschr* **38**, 1236-1239.

Ellis, R.E., Yuan, J.Y. and Horvitz, H.R. (1991) Mechanisms and functions of cell death. *Annual Review of Cell Biology* **7**, 663-698.

Enriquez, J.A., Chomyn, A. and Attardi, G. (1995) MtDNA mutation in MERRF syndrome causes defective aminoacylation of tRNA(Lys) and premature translation termination. *Nature Genetics* **10**, 47-55.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. and Hancock, D.C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**, 119-128.

Ferrante, R.J., Kowall, N.W., Cipolloni, P.B., Storey, E. and Beal, M.F. (1993) Excitotoxin lesions in primates as a model for Huntington's disease: histopathologic and neurochemical characterization. *Experimental Neurology* **119**, 46-71.

Fields, J.Z., Albores, R.R., Neafsey, E.J. and Collins, M.A. (1992) Inhibition of mitochondrial succinate oxidation-similarities and differences between N-methylated beta-carbolines and MPP^+. *Archives of Biochemistry and Biophysics* **294**, 539-543.

Finel, M., Skehel, J.M., Albracht, S.P., Fearnley, I.M. and Walker, J.E. (1992) Resolution of NADH:ubiquinone oxidoreductase from bovine heart mitochondria into two subcomplexes, one of which contains the redox centers of the enzyme. *Biochemistry* **31**, 11425-11434.

Forno, L.S., Langston, J.W., DeLanney, L.E., Irwin, I. and Ricaurte, G.A. (1986) Locus ceruleus lesion and eosinophilic inclusions in MPTP-treated monkeys. *Annals of Neurology* **20,** 449-455.

Galasko, D., Saitoh, T., Xia, Y., Thal, L.J., Katzman, R., Hill, L.R. and Hansen, L. (1994) The apolipoprotein E allele epsilon 4 is overrepresented in patients with the Lewy body variant of Alzheimer's disease. *Neurology* **44**, 1950-1951.

Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., *et al* (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature* **373**, 523-527.

Gasser, T., Muller-Myhsok, B., Wszolek, Z.K., Oehlmann, R., Calne, D.B., Bonifati, V., Bereznai, B., Fabrizio, E., Vieregge, P. and Horstmann, R.D. (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nature Genetics* **18,** 262-265.

Gentleman, S.M., Williams, B., Royston, M.C., Jagoe, R., Clinton, J., Perry, R.H., Ince, P.G., Allsop, D., Polak, J.M. and Roberts, G.W. (1992) Quantification of beta A4 protein deposition in the medial temporal lobe: a comparison of Alzheimer's disease and senile dementia of the Lewy body type. *Neuroscience Letters* **142**, 9-12.

Gerbitz, K.-D., Obermaier-krusser, B., Zierz, S., Pongratz, D., Muller-Hocker, J. and Lestienne, P. (1990) Mitochondrial myopathies: divergence of genetic deletions, biochemical defects and the clinical syndromes. *Journal of Neurology* **237,** 5-10.

Giles, R.E., Blanc, H., Cann, H.M. and Wallace, D.C. (1980) Maternal inheritance of human mitochondrial DNA. *Proceedings of the National Academy of Sciences of the United States of America* **77,** 6715-6719.

Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., *et al* (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* **349**, 704-706.

Golbe, L.I., Di Iorio, G., Bonavita, V., Miller, D.C. and Duvoisin, R.C. (1990) A large kindred with autosomal dominant Parkinsons disease. *Annals of Neurology* **27,** 276-282.

Goto, Y.I., Nonaka, I. and Horai, S. (1990) A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature* **348**, 651-653.

Goto, Y.I., Nonaka, I. and Horai, S. (1991) A new mtDNA mutation associated with mitochondrial myopathy, encephalopathy,lactic acidosis and stroke-like episodes (MELAS). *Biochimica et Biophysica Acta* **1097,** 238-240.

Greene, J.G., Porter, R.H., Eller, R.V. and Greenamyre, J.T. (1993) Inhibition of succinate dehydrogenase by malonic acid produces an "excitotoxic" lesion in rat striatum. *Journal of Neurochemistry* **61**, 1151-1154.

Gsell, W., Conrad, R., Hickethier, M., Sofic, E., Frolich, L., Wichart, I., Jellinger, K., Moll, G., Ransmayr, G., Beckmann, H., *et al* (1995) Decreased catalase activity but unchanged superoxide dismutase activity in brains of patients with dementia of Alzheimer type. *Journal of Neurochemistry* **64**, 1216-1223.

Gu, M., Gash, M.T., Mann, V.M., Javoy-Agid, F., Cooper, J.M. and Schapira, A.H.V. (1996) Mitochondrial defect in Huntington's disease caudate nucleus. *Annals of Neurology* **39**, 385-389.

Gu, M., Gash, M.T., Cooper, J.M., Wenning, G.K., Daniel, S.E., Quinn, N.P., Marsden, C.D. and Schapira, A.H. (1997) Mitochondrial respiratory chain function in multiple system atrophy. *Movement Disorders* **12**, 418-422.

Gu, M., Cooper, J.M., Taanman, J.W. and Schapira, A.H.V. (1998a) Mitochondrial DNA transmission of the mitochondrial defect in Parkinson's disease. *Annals of Neurology* **44,** 177-186.

Gu, M., Owen, A.D., Toffa, S.E.K., Cooper, J.M., Dexter, D.T., Jenner, P., Marsden, C.D. and Schapira, A.H.V. (1998b) Mitochondrial function, GSH and iron in neurodegeneration and Lewy body diseases: evidence for a two-hit hypothesis in Parkinson's disease. *Journal of the Neurological Sciences* **158,** 24-29.

Gusella, J.F., Wexler, N.S., Conneally, P.M., Naylor, S.L., Anderson, M.A., Tanzi, R.E., Watkins, P.C., Ottina, K., Wallace, M.R., Sakaguchi, A.Y., *et al* (1983) A polymorphic DNA marker genetically linked to Huntington's disease. *Nature* **306**, 234-238.

Gyllensten, U., Wharton, D., Josefsson, A. and Wilson, A.C. (1991) Paternal inheritance of mitochondrial DNA in mice. *Nature* **352,** 255-257.

Haas, R.H., Nasirian, F., Nakano, K., Ward, D., Pay, M., Hill, R. and Shults, C.W. (1995) Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. *Annals of Neurology* **37**, 714-722.

Haldar, S., Basu, A. and Croce, C.M. (1997) Bcl-2 is the guardian of microtubule integrity. *Cancer Research* **57**, 229-233.

Hammans, S.R., Sweeney, M.G., Brockington, M., Morgan-Hughes, J.A. and Harding, A.E. (1991) Mitochondrial encephalopathies: molecular genetic diagnosis from blood samples. *Lancet* **337**, 1311-1313.

Hammans, S.R., Sweeney, M.G., Holt, I.J., Cooper, J.M., Toscano, A., Clark, J.B., Morgan-Hughes, J.A. and Harding, A.E. (1992) Evidence for intramitochondrial complementation between deleted and normal mitochondrial DNA in some patients with mitochondrial myopathy. *Journal of the Neurological Sciences* **107**, 87-92.

Hansen, L., Salmon, D. and Galasko, D. (1990) The Lewy body variant of Alzheimers disease: a clinical and pathologic entity. *Neurology* **40,** 1-7.

Hantraye, P., Brouillet, E., Ferrante, R., Palfi, S., Dolan, R., Matthews, R.T. and Beal, M.F. (1996) Inhibition of neuronal nitric oxide synthase prevents MPTP- induced parkinsonism in baboons. *Nature Medicine* **2**, 1017-1021.

Hardy, J., Crook, R., Prihar, G., Roberts, G., Raghavan, R. and Perry, R. (1994) Senile dementia of the Lewy body type has an apolipoprotein E epsilon 4 allele frequency intermediate between controls and Alzheimer's disease. *Neuroscience Letters* **182**, 1-2.

Hardy, J. and Allsop, D. (1991) Amyloid deposition as the central event in the aetiology of Alzheimers disease. *Trends in Pharmacological Sciences* **12,** 383-388.

Hartley, A., Stone, J.M., Heron, C., Cooper, J.M. and Schapira, A.H. (1994) Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: relevance to Parkinson's disease. *Journal of Neurochemistry* **63**, 1987-1990.

Hartwell, L. (1992) Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cell. *Cell* **71,** 543-546.

Hasegawa, E., Takeshige, K., Oishi, T., Murai, Y. and Minakami, S. (1990) 1-Methyl-4-phenylpyridinium (MPP^+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochemical and Biophysical Research Communications* **170**, 1049-1055.

Hatefi, Y., Haavik, A.G. and Griffiths, D.E. (1962) Studies on the electron transfer system; preparation and properties of mitochondrial DPNH-coenzyme Q reductase. *Journal of Biological Chemistry* **237,** 1676-1685.

Hatefi, Y. (1985) The mitochondrial electron-transport and oxidative-phosphorylation system. *Annual Review of Biochemistry* **54,** 1015-1069.

Hattori, N., Tanaka, M., Ozawa, T. and Mizuno, Y. (1991) Immunohistochemical studies on complexes I, II, III, and IV of mitochondria in Parkinson's disease. *Annals of Neurology* **30**, 563-571.

Hausladen, A. and Fridovich, I. (1994) Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. *Journal of Biological Chemistry* **269**, 29405-29408.

Hayakawa, M., Torii, K., Sugiyama, S., Tanaka, M. and Ozawa, T. (1991) Age-associated accumulation of 8-hydroxydeoxyguanosine in mitochondrial DNA of human diaphragm. *Biochemical and Biophysical Research Communications* **179**, 1023-1029.

Hayakawa, M., Hattori, K., Sugiyama, S. and Ozawa, T. (1992) Age-associated oxygen damage and mutations in mitochondrial DNA in human hearts. *Biochemical and Biophysical Research Communications* **189**, 979-985.

Hayashi, J., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y.-I. and Nonaka, I. (1991) Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 10614-10618.

Hayashi, J., Ohta, S., Takai, D., Miyabayashi, S., Sakuta, R., Goto, Y. and Nonaka, I. (1993) Accumulation of mtDNA with a mutation at position 3271 in tRNA(Leu)(UUR) gene introduced from a MELAS patient to HeLa cells lacking mtDNA results in progressive inhibition of mitochondrial respiratory function. *Biochemical and Biophysical Research Communications* **197**, 1049-1055.

Heikkila, R.E., Nicklas, W.J., Vyas, I. and Duvoisin, R.C. (1985) Dopaminergic toxicity of rotenone and the 1-methyl-4-phenylpyridinium ion after their stereotaxic administration to rats: implication for the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity. *Neuroscience Letters* **62,** 389-394.

Hirano, M., Shtilbans, A., Mayeux, R., Davidson, M.M., DiMauro, S., Knowles, J.A. and Schon, E.A. (1997) Apparent mtDNA heteroplasmy in Alzheimer's disease patients and in normals due to PCR amplification of nucleus-embedded mtDNA pseudogenes. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 14894-14899.

Hixson, J.E. and Vernier, D.T. (1990) Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *Journal of Lipid Research* **31**, 545-548.

Ho, S.L., Kapadi, A.L., Ramsden, D.B. and Williams, A.C. (1995) An allelic association study of monoamine oxidase B in Parkinson's disease. *Annals of Neurology* **37**, 403-405.

Hoang, T.Q., Bluml, S., Dubowitz, D.J., Moats, R., Kopyov, O., Jacques, D. and Ross, B.D. (1998) Quantitative proton-decoupled ^{31}P MRS and ^{1}H MRS in the evaluation of Huntington's and Parkinson's diseases. *Neurology* **50,** 1033-1040.

Hockenbery, D.M., Nunez, G., Milliman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**, 334-336.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.I. and Korsmeyer, S.J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241-251.

Hockenbery, D.M. (1995) bcl-2, a novel regulator of cell death. *Bioessays* **17**, 631-638.

Hofhaus, G., Johns, D.R., Hurko, O., Attardi, G. and Chomyn, A. (1996) Respiration and growth defects in transmitochondrial cell lines carrying the 11778 mutation associated with Leber's hereditary optic neuropathy. *Journal of Biological Chemistry* **271**, 13155-13161.

Holt, I.J., Harding, A.E. and Morgan-Hughes, J.A. (1988) Deletions of mitochondrial DNA in patients with mitochondrial myopathies. *Nature* **331,** 717-719.

Holt, I.J., Harding, A.E. and Morgan-Hughes, J.A. (1989) Deletions of muscle mitochondrial DNA in myopathies:sequence analysis and possible mechanisms. *Nucleic Acids Research* **17,** 4465-4469.

Holt, I.J., Harding, A.E., Petty, R.K.H. and Morgan-Hughes, J.A. (1990) A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. *American Journal of Human Genetics* **46,** 428-433.

Hotamisligil, G.S., Girmen, A.S., Fink, J.S., Tivol, E., Shalish, C., Trofatter, J., Baenziger, J., Diamond, S., Markham, C., Sullivan, J., *et al* (1994) Hereditary variations in monoamine oxidase as a risk factor for Parkinson's disease. *Movement Disorders* **9**, 305-310.

Howell, N., Bindoff, L.A., McCullough, D.A., Kubacka, I., Poulton, J., Mackey, D., Taylor, L. and Turnbull, D.M. (1991) Leber hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees. *American Journal of Human Genetics* **49**, 939-950.

Huang, C.C., Lu, C.S., Chu, N.S., Hochberg, F., Lilienfeld, D., Olanow, W. and Calne, D.B. (1993) Progression after chronic manganese exposure. *Neurology* **43**, 1479-1483.

Huoponen, K., Vilkki, J., Aula, P., Nikoskelainen, E.K. and Savontaus, M.L. (1991) A new mtDNA mutation associated with Leber hereditary optic neuroretinopathy. *American Journal of Human Genitics* **48,** 1147-1153.

Ichiki, T., Tanaka, M., Nishikimi, M., Suzuki, H., Ozawa, T., Kobayashi, M. and Wada, Y. (1988) Deficiency of subunits of Complex I and mitochondrial encephalomyopathy. *Annals of Neurology* **23**, 287-294.

Ihara, Y., Hayabara, T., Sasaki, K., Fujisawa, Y., Kawada, R., Yamamoto, T., Nakashima, Y., Yoshimune, S., Kawai, M., Kibata, M., *et al* (1997) Free radicals and superoxide dismutase in blood of patients with Alzheimer's disease and vascular dementia. *Journal of Neurological Sciences* **153,** 76-81.

Ikebe, S., Tanaka, M., Ohno, K., Sato, W., Hattori, K., Kondo, T., Mizuno, Y. and Ozawa, T. (1990) Increase of deleted mitochondrial DNA in the striatum in Parkinsons disease and senescence. *Biochemical and Biophysical Research Communications* **170,** 1044-1048.

Ikebe, S., Tanaka, M. and Ozawa, T. (1995) Point mutations of mitochondrial genome in Parkinson's disease. *Brain Research* **28**, 281-295.

Irwin, I. and Langston, J.W. (1985) Selective accumulation of MPP^+ in the substantia nigra: A key to neurotoxicity? *Life Sciences* **36,** 207-212.

Irwin, I. and Langston, J.W. (1993) MPTP and Parkinson's disease. In: Harvey, A.L. (Ed.) *Natural and Synthetic Neurotoxins*, pp. 225-256. London: Academic Press

Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C. and Raff, M.C. (1993) Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature* **361**, 365-369.

Jacobson, M.D., Burne, J.F. and Raff, M.C. (1994) Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO Journal* **13,** 1899-1910.

Jacobson, M.D. and Raff, M.C. (1995) Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* **374**, 814-816.

Janetzky, B., Hauck, S., Youdim, M.B., Riederer, P., Jellinger, K., Pantucek, F., Zochling, R., Boissl, K.W. and Reichmann, H. (1994) Unaltered aconitase activity, but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. *Neuroscience Letters* **169**, 126-128.

Javitch, J.A., DAmato, R.J., Strittmatter, S.M. and Synder, S.H. (1985) Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proceedings of the National Academy of Sciences of the United States of America* **82,** 2173-2177.

Jenkins, B.G., Koroshetz, W.J., Beal, M.F. and Rosen, B.R. (1993) Evidence for impairment of energy metabolism in vivo in Huntington's disease using localized ^1H NMR spectroscopy. *Neurology* **43**, 2689-2695.

Jenner, P., Schapira, A.H. and Marsden, C.D. (1992) New insights into the cause of Parkinson's disease. *Neurology* **42**, 2241-2250.

Jenner, P. and Olanow, C.W. (1996) Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology* **47**, S161-70.

Johns, D.R. and Neufeld, M.J. (1991) Cytochrome b mutation in Leber's hereditary optic neuropathy. *Biochemical and Biophysical Research Communications* **181,** 1358-1364.

Kapsa, R.M., Jean-Francois, M.J., Lertrit, P., Weng, S., Siregar, N., Ojaimi, J., Donnan, G., Masters, C. and Byrne, E. (1996) Mitochondrial DNA polymorphism in substantia nigra. *Journal of the Neurological Sciences* **144**, 204-211.

Kaufmann, P., Koga, Y., Shanske, S., Hirano, M., DiMauro, S., King, M.P. and Schon, E.A. (1996) Mitochondrial DNA and RNA processing in MELAS. *Annals of Neurology* **40**, 172-180.

King, M.P., Koga, Y., Davidson, M. and Schon, E.A. (1992) Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the $tRNA^{leu(UUR)}$ mutation associated with MELAS. *Molecular and Cellular Biology* **12,** 480-490.

King, M.P. and Attardi, G. (1988) Injection of mitochondria into human cells leads to rapid replacement of the endogenous mitochondrial DNA. *Cell* **52,** 811-819.

King, M.P. and Attardi, G. (1989) Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* **246,** 500-503.

King, M.P. and Attardi, G. (1993) Post-transcriptional regulation of the steady-state levels of mitochondrial tRNAs in HeLa cells. *Journal of Biological Chemistry* **268**, 10228-10237.

King, T.E. (1967) Preparations of succinate-cytochrome c reductase and the cytochrome b-c1 particle, and reconstitution of succinate-cytochrome c reductase. *Methods in Enzymology* **10**, 275-296.

Kish, S.J., Bergeron, C., Rajput, A., Dozic, S., Mastrogiacomo, F., Chang, L.J., Wilson, J.M., DiStefano, L.M. and Nobrega, J.N. (1992) Brain cytochrome oxidase in Alzheimer's disease. *Journal of Neurochemistry* **59**, 776-779.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**, 605-608.

Koga, Y., Nonaka, I., Kobayashi, M., Tojyo, M. and Nihei, K. (1988) Findings in muscle in complex I (NADH coenzyme Q reductase) deficiency. *Annals of Neurology* **24**, 749-756.

Kokmen, E., Chandra, V. and Schoenberg, B.S. (1988) Trends in incidence of dementing illness in Rochester, Minnesota, in three quinquennial periods, 1960-1974. *Neurology* **38**, 975-980.

Koller, W., Vetere-Overfield, B., Gray, C., Alexander, C., Chin, T., Dolezal, J., Hassanein, R. and Tanner, C. (1990) Environmental risk factors in Parkinson's disease. *Neurology* **40**, 1218-1221.

Koroshetz, W.J., Jenkins, B., Rosen, B. and Beal, M.F. (1994) Evidence for a metabolic disorder in Huntinton's disease. *Neurology* **44**, A338.

Koroshetz, W.J., Jenkins, B.G., Rosen, B.R. and Beal, M.F. (1997) Energy metabolism defects in Huntington's disease and effects of coenzyme Q_{10}. *Annals of Neurology* **41**, 160-165.

Korsmeyer, S.J. (1992) Bcl-2 initiated a new category of oncogenes: regulators of cell death. *Blood* **80,** 879-886.

Kremer, B., Goldberg, P., Andrew, S.E., Theilmann, J., Telenius, H., Zeisler, J., Squitieri, F., Lin, B., Bassett, A., Almqvist, E., *et al* (1994) A worldwide study of the Huntington's disease mutation. The sensitivity and specificity of measuring CAG repeats. *New England Journal of Medicine* **330**, 1401-1406.

Krige, D., Carroll, M.T., Cooper, J.M., Marsden, C.D. and Schapira, A.H. (1992) Platelet mitochondrial function in Parkinson's disease. The Royal Kings and Queens Parkinson Disease Research Group. *Annals of Neurology* **32**, 782-788.

Krueger, M.J., Tan, A.K., Ackrell, B.A. and Singer, T.P. (1993) Is complex II involved in the inhibition of mitochondrial respiration by N-methyl-4-phenylpyridinium cation (MPP^+) and N-methyl-beta-carbolines? *Biochemical Journal* **291**, 673-676.

kruse, B., Narasimhan, N. and Attardi, G. (1989) Termination of transcription in human mitochondria: identification and purification of a DNA binding protein factor that promotes termination. *Cell* **58,** 391-397.

Kuwert, T., Lange, H.W., Langen, K.J., Herzog, H., Aulich, A. and Feinendegen, L.E. (1990) Cortical and subcortical glucose consumption measured by PET in patients with Huntington's disease. *Brain* **113**, 1405-1423.

Kuwert, T., Lange, H.W., Boecker, H., Titz, H., Herzog, H., Aulich, A., Wang, B.C., Nayak, U. and Feinendegen, L.E. (1993) Striatal glucose consumption in chorea-free subjects at risk of Huntington's disease. *Journal of Neurology* **241**, 31-36.

Lane, D.P. (1992) Cancer. p53, guardian of the genome. *Nature* **358,** 15-16.

Langston, J.W., Ballard, P.A., Tetrud, J.W. and Irwin, I. (1983) Chroinic parkinsonism in human due to a product of meperidine-analog synthesis. *Science* **219,** 979-980.

Langston, J.W., Irwin, I., Langston, E.B. and Forno, L.S. (1984) Pargyline prevents MPTP-induced parkinsonism in primates. *Science* **225,** 1480-1482.

Lazzarini, A.M., Myers, R.H., Zimmerman, T.R., Mark, M.H., Golbe, L.I., Sage, J.I., Johnson, W.G. and Duvoisin, R.C. (1994) A clinical genetic study of Parkinson's disease: evidence for dominant transmission. *Neurology* **44**, 499-506.

Lestienne, P. and Ponsot, G. (1988) Kearns-Sayre syndrome with muscle mitochondrial DNA deletion. *Lancet* **i,** 885.

LeVine, S.M. (1997) Iron deposits in multiple sclerosis and Alzheimer's disease brains. *Brain Research* **760**, 298-303.

Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C.E., Jondro, P.D., Schmidt, S.D. and Wang, K. (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* **269**, 973-977.

Levy-Lahad, E. and Bird, T.D. (1996) Genetic factors in Alzheimer's disease: a review of recent advances. *Annals of Neurology* **40**, 829-840.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479-489.

Li, X.J., Li, S.H., Sharp, A.H., Nucifora, F.C., Schilling, G., Lanahan, A., Worley, P., Snyder, S.H. and Ross, C.A. (1995) A huntingtin-associated protein enriched in brain with implications for pathology. *Nature* **378**, 398-402.

Lima, B., Reino, A., Goncalves, A., Machano, C., Leite, I., Cunha, L. and Regateiro, F. (1997) Allele and genotypes frequencies of Debrisoquine hydroxylase gene polymorphism (CYP2D6) in early onset Parkinson's disease (PD). *Movement Disorders* **12**, 27.

Lindenboim, L., Haviv, R. and Stein, R. (1995) Inhibition of drug-induced apoptosis by survival factors in PC12 cells. *Journal of Neurochemistry* **64**, 1054-1063.

Lippa, C.F., Smith, T.W., Saunders, A.M., Crook, R., Pulaski-Salo, D., Davies, P., Hardy, J., Roses, A.D. and Dickson, D. (1995) Apolipoprotein E genotype and Lewy body disease. *Neurology* **45**, 97-103.

Lovell, M.A., Ehmann, W.D., Butler, S.M. and Markesbery, W.R. (1995) Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. *Neurology* **45**, 1594-1601.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin reagent. *Journal of Biological Chemistry* **193**, 265-275.

Lu, Q.L., Hanby, A.M., Nasser Hajibagheri, M.A., Gschmeissner, S.E., Lu, P.J., Taylor-Papadimitriou, J., Krajewski, S., Reed, J.C. and Wright, N.A. (1994) Bcl-2 protein localizes to the chromosomes of mitotic nuclei and is correlated with the cell cycle in cultured epithelial cell lines. *Journal of Cell Science* **107**, 363-371.

Ludolph, A.C., He, F., Spencer, P.S., Hammerstad, J. and Sabri, M. (1991) 3-Nitropropionic acid-exogenous animal neurotoxin and possible human striatal toxin. *Canadian Journal of Neurological Sciences* **18**, 492-498.

Ludolph, A.C., Seelig, M., Ludolph, A.G., Sabri, M.I. and Spencer, P.S. (1992) ATP deficits and neuronal degeneration induced by 3-nitropropionic acid. *Annals of the New York Academy of Sciences* **648**, 300-302.

Lyras, L., Cairns, N.J., Jenner, A., Jenner, P. and Halliwell, B. (1997) An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. *Journal of Neurochemistry* **68**, 2061-2069.

Maeda, M., Watanabe, Y., Yong, J., Adachi, Y., Urakami, K., Harada, H. and Nakashima, K. (1997) The study of apolipoprotein E polymorphism and CYP2D6 mutant gene in patients with Parkinson's disease in San-in, Japan. *Movement Disorders* **12**, 28.

Mann, V.M., Cooper, J.M., Javoy-Agid, F., Agid, Y., Jenner, P. and Schapira, A.H.V. (1990) Mitochondrial function and parental sex effect in Huntingtons disease. *Lancet* **336,** 749.

Mann, V.M., Cooper, J.M., Krige, D., Daniel, S.E., Schapira, A.H.V. and Marsden, C.D. (1992a) Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. *Brain* **115**, 333-342.

Mann, V.M., Cooper, J.M. and Schapira, A.H.V. (1992b) Quantitation of a mitochondrial DNA deletion in Parkinson's disease. *FEBS Letters* **299**, 218-222.

Mann, V.M., Cooper, J.M., Daniel, S.E., Srai, K., Jenner, P., Marsden, C.D. and Schapira, A.H.V. (1994) Complex I, iron, and ferritin in Parkinson's disease substantia nigra. *Annals of Neurology* **36**, 876-881.

Maraganore, D.M., Harding, A.E. and Marsden, C.D. (1991) A clinical and genetic study of familial Parkinson's disease. *Movement Disorders* **6**, 205-211.

Marchetti, P., Susin, S.A., Decaudin, D., Gamen, S., Castedo, M., Hirsch, T., Zamzami, N., Naval, J., Senik, A. and Kroemer, G. (1996) Apoptosis-associated derangement of mitochondrial function in cells lacking mitochondrial DNA. *Cancer Research* **56**, 2033-2038.

Marder, K., Maestre, G., Cote, L., Mejia, H., Alfaro, B., Halim, A., Tang, M., Tycko, B. and Mayeux, R. (1994) The apolipoprotein epsilon 4 allele in Parkinson's disease with and without dementia. *Neurology* **44**, 1330-1331.

Markesbery, W.R. (1997) Oxidative stress hypothesis in Alzheimer's disease. *Free Radical Biology and Medicine* **23**, 134-147.

Markey, S.P., Johannessen, J.N., Chiueh, C.C., Burns, R.S. and Herkenham, M.A. (1984) Intraneuronal generation of pyridinium metablite may cause drug-induced parkisonism. *Nature* **311,** 464-467.

Marsden, C.D. (1987) Parkinson's disease in twins. *Journal of Neurology, Neurosurgery and Psychiatry* **50**, 105-106.

Marsden, C.D. (1990) Parkinson's disease. *Lancet* **335,** 948-952.

Marttila, R.J., Kaprio, J., Koskenvuo, M. and Rinne, U.K. (1988) Parkinson's disease in a nationwide twin cohort. *Neurology* **38**, 1217-1219.

Matsubara, K.C.M., Collins, M.A., Akane, A., Ikebuchi, J., Neafsey, E.J., Kagawa, M. and Shiono, H. (1993) Potential bioactivated neurotoxicants, N-methylated - carbolinium ions, are present in human brain. *Brain Research* **610,** 90-96.

Matsumoto, K. and Ohta, T. (1993) Mitosis of rotenone-induced endoreduplication in Chinese hamster cells. *Japanese Journal of Genetics* **68**, 185-194.

Mattson, M.P., Guo, Q., Furukawa, K. and Pedersen, W.A. (1998) Presenilins, the endoplasmic reticulum, and neuronal apoptosis in Alzheimer's disease. *Journal of Neurochemistry* **70**, 1-14.

Mattson, M.P. and Guo, Q. (1997) Cell and molecular neurobiology of presenilins: a role for the endoplasmic reticulum in the pathogenesis of Alzheimer's disease?. *Journal of Neuroscience Research* **50**, 505-513.

McNaught, K.S., Thull, U., Carrupt, P.A., Altomare, C., Cellamare, S., Carotti, A., Testa, B., Jenner, P. and Marsden, C.D. (1996) Toxicity to PC12 cells of isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Neuroscience Letters* **206**, 37-40.

McNaught, P.T.U., Thull, U., Carrupt, P.-A., Altomare, C., Cellamare, S., Carotti, A., Testa, B., Jenner, P.M.C. and Marsden, C.D. (1995) Inhibation of complex I by isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Biochemical Pharmacology* **50,** 1903-1911.

Mecocci, P., MacGarvey, U. and Beal, M.F. (1994) Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Annals of Neurology* **36**, 747-751.

Mecocci, P.M.U., MacGarvey, U., Kaufman, A.E., Koontz, D., Shoffner, J.M., Wallace, D.C. and Beal, M.F. (1993) Oxidative damage to mitochondrial DNA shows marked age-dependent increses in human brain. *Annals of Neurology* **34,** 609-616.

Mitchell, I.J., Cross, A.J., Sambrook, M.A. and Crossman, A.R. (1985) Sites of neurotoxic action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the macaque monkey include the ventral tegmental area and the locus ceruleus. *Neuroscience Letters* **61,** 195-200.

Mizuno, Y., Ohto, S., Tanaka, M., Takamiya, S., Suzuki, K., Sato, T., Oya, H., Ozawa, T. and Kagawa, T. (1989) Deficiencies in complex I subunits of the respiratory chain in Parkinsons disease. *Biochemical and Biophysical Research Communications* **163,** 1450-1455.

Mizuno, Y., Suzuki, K. and Ohto, S. (1990) Postortem changes in mitochondrial respiratory enzymes in brain and a preliminary obseration in Parkinsons disease. *Journal of the Neurological Sciences* **96,** 49-57.

Monaghan, P., Robertson, D., Amos, T.A., Dyer, M.J., Mason, D.Y. and Greaves, M.F. (1992) Ultrastructural localization of bcl-2 protein. *Journal of Histochemistry and Cytochemistry* **40**, 1819-1825.

Moraes, C.T., Schon, E.A., DiMauro, S. and Miranda, A.F. (1989) Heteroplasmy of mitochondrial genomes in clonal cultures from patients with Kearns-Sayre syndrome. *Biochemical and Biophysical Research Communications* **160**, 765-771.

Morikara, N., Nakagawa-Hattori, Y. and Mizuno, Y. (1996) Effect of dopamine, dimethoxyphenylethylamine, papaverine, and related compounds on mitochondrial respiration and complex I activity. *Journal of Neurochemistry* **66**, 1174-1181.

Mutisya, E.M., Bowling, A.C. and Beal, M.F. (1994) Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. *Journal of Neurochemistry* **63**, 2179-2184.

Nagata, S. (1997) Apoptosis by death factor. *Cell* **88**, 355-365.

Nagatsu, T. and Yoshida, M. (1988) An endogenous substance of the brain, tetrahydroisoquinoline, produces parkinsonism in primates with decreased dopamine, tyrosine hydroxylase and biopterin in the nigrostriatal regions. *Neuroscience Letters* **87,** 178-182.

Nakase, H., Moraes, C.T., Rizzuto, R., Lombes, A., Mauro, S. and Schon, E.A. (1990) Transcription and translation of deleted mitochondrial genomes in Kearns-Sayer syndrome: implications for pathogenesis. *American Journal of Human Genetics* **46,** 418-427.

Naoi, M., Maruyama, W., Dostert, P., Hashizume, Y., Nakahara, D., Takahashi, T. and Ota, M. (1996) Dopamine-derived endogenous 1(R),2(N)-dimethyl-6,7-dihydroxy-1,2, 3,4-tetrahydroisoquinoline, N-methyl-(R)-salsolinol, induced parkisonism in rat: biochemical, pathological and behavioral studies. *Brain Research* **709,** 285-295.

Nicholl, D.J., Bennett, P., Ramsden, D.B., Ho, S.L. and Williams, A.C. (1995) The Hha1 polymorphism in the CYP2D6 gene is not associated with Parkinson's disease in a Caucasian population. *European Journal of Neurology* **2,** 580-582.

Nicklas, W.J., Vyas, I. and Heikkila, R.E. (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Life Sciences* **36,** 2503-2508.

Niwa, T., Takeda, N., Kaneda, N., Hashizume, Y. and Nagatsu, T. (1987) Presence of tetrahydroisoquinoline and 2-methyl-tetrahydroquinoline in parkinsonian and norml human brains. *Biochemical and Biophysical Research Communications* **144,** 1084-1089.

Novelli, A., Reilly, J.A., Lysko, P.G. and Henneberry, R.C. (1988) Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. *Brain Research* **451**, 205-212.

Olanow, C.W. (1992) An introduction to the free radical hypothesis in Parkinsons disease. *Annals of Neurology* **32,** S2-S9.

Olney, J.W. and de Gubareff, T. (1978) Glutamate neurotoxicity and Huntington's chorea. *Nature* **271**, 557-559.

Ozawa, T., Tanaka, M., Ino, H., Ohno, K., Sano, T., Wada, Y., Yoneda, M., Tanno, Y., Miyatake, T., Tanaka, T., *et al* (1991) Distinct clustering of point mutations in mitochondrial DNA among patients with mitochondrial encephalomyopathies and with Parkinson's disease. *Biochemical and Biophysical Research Communications* **176**, 938-946.

Ozelius, L., Kramer, P.L., Moskowitz, C.B., Kwiatkowski, D.J., Brin, M.F., Bressman, S.B., Schuback, D.E., Falk, C.T., Risch, N., de Leon, D., *et al* (1989) Human gene for torsion dystonia located on chromosome 9q32-q34. *Neuron* **2**, 1427-1434.

Palmer, A.M. and Burns, M.A. (1994) Selective increase in lipid peroxidation in the inferior temporal cortex in Alzheimer's disease. *Brain Research* **645**, 338-342.

Papp, M.I., Kahn, J.E. and Lantos, P.L. (1989) Glial cytoplasmic inclusions in the CNS of patients with multiple system atrophy (striatonigral degeneration, olivopontocerebellar atrophy and Shy-Drager syndrome). *Journal of the Neurological Sciences* **94**, 79-100.

Pardo, B., Mena, M.A., Fahn, S. and de Yebenes, J.G. (1993) Ascorbic acid protects against levadopa-induced neurotoxicity on a catecholamine-rich human neuroblastoma cell line. *Movement Disorders* **8,** 278-284.

Pardo, B., Mena, M.A. and de Yebenes, J.G. (1995) L-dopa inhibits complex IV of the electron transport chain in catecholamine-rich human neuroblastoma NB69 cells. *Journal of Neurochemistry* **64**, 576-582.

Parker, W.D., Boyson, S.J. and Parks, J.K. (1989) Abnormalities of the electron transport chain in idiopathic Parkinsons disease. *Annals of Neurology* **26,** 719-723.

Parker, W.D., Filley, C.M. and and Parks, J.K. (1990a) Cytochrome oxidase deficiency in Alzheimer's disease. *Neurology* **40**, 1302-1303.

Parker, W.D., Boyson, S.J., Luder, A.S. and Parks, J.K. (1990b) Evidence for a defect in NADH:ubiquinone oxidoreductase (complex I) in Huntington's disease. *Neurology* **40**, 1231-1234.

Parker, W.D., Mahr, N.J., Filley, C.M., Parks, J.K., Hughes, D., Young, D.A. and Cullum, C.M. (1994a) Reduced platelet cytochrome c oxidase activity in Alzheimer's disease. *Neurology* **44**, 1086-1090.

Parker, W.D., Parks, J., Filley, C.M. and Kleinschmidt-DeMasters, B.K. (1994b) Electron transport chain defects in Alzheimer's disease brain. *Neurology* **44**, 1090-1096.

Parkinson study group (1993) Effects of tocopherol and deprenyl on the progression of disability in early Parkinsons disease. *New England Journal of Medicine* **328,** 176-183.

Payami, H., Bernard, S., Larsen, K., Kaye, J. and Nutt, J. (1995) Genetic anticipation in Parkinson's disease. *Neurology* **45**, 135-138.

Perry, T.L. and Yong, V.W. (1986) Idiopathic Parkinsons disease, progressive suprannuclear palsy and glutathione metabolism in the substantia nigra of patients. *Neuroscience Letters* **67,** 269-274.

Perutz, M.F., Johnson, T., Suzuki, M. and Finch, J.T. (1994) Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 5355-5358.

Petit, P.X., LeCouer, H., Zorn, E., Dauguet, C., Mignotte, B. and Gougeon, M.L. (1995) Alterations of mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *Journal of Cell Biology* **130,** 157-167.

Pickering-Brown, S.M., Mann, D.M., Bourke, J.P., Roberts, D.A., Balderson, D., Burns, A., Byrne, J. and Owen, F. (1994) Apolipoprotein E4 and Alzheimer's disease pathology in Lewy body disease and in other beta-amyloid-forming diseases. *Lancet* **343**, 1155.

Plaitakis, A., Berl, S. and Yahr, M.D. (1982) Abnormal glutamate metabolism in an adult onset degerative neurological disorder. *Science* **41,** 193-196.

Plaitakis, A., Berl, S. and Yahr, M.D. (1984) Neurological disorders associated with deficiency of glutamate dehydrogenase. *Annals of Neurology* **15,** 144-153.

Poirier, J. (1994) Apolipoprotein E in animal models of CNS injury and in Alzheimer's disease. *Trends in Neurosciences* **17**, 525-530.

Polymeropoulos, M.H., Higgins, J.J., Golbe, L.I., Johnson, W.G., Ide, S.E., Di Iorio, G., Sanges, G., Stenroos, E.S., Pho, L.T., Schaffer, A.A., *et al* (1996) Mapping of gene for Parkinson's disease to chromosome 4q21-q23. *Science* **274,** 1197-1199.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., *et al* (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* **276**, 2045-2047.

Poulton, J., Deadman, M.E. and Gardiner, R.M. (1989) Duplications of mitochondrial DNA in mitochondrial myopathy. *Lancet* **1**, 236-240.

Przedborski, S., Kostic, V., Jackson-Lewis, V., Naini, A.B., Simonetti, S., Fahn, S., Carlson, E., Epstein, C.J. and Cadet, J.L. (1992) Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *Journal of Neuroscience* **12,** 1658-1667.

Przedborski, S., Jackson-Lewis, V., Muthane, U., Jiang, H., Ferreira, M., Naini, A.B. and Fahn, S. (1993) Chronic levodopa administration alters cerebral mitochondrial respiratory chain activity. *Annals of Neurology* **34**, 715-723.

Przedborski, S., Jackson-Lewis, V., Yokoyama, R., Shibata, T., Dawson, V.L. and Dawson, T.M. (1996) Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 4565-4571.

Quinn, N.P. (1995) Multiple system atrophy. In: Marsden, C.D. and Fahn, S. (Eds.) *Movement disorders*, pp. 262-281. Butterworth-Heinemann

Ragan, C.I. (1987) Structure of NADH-ubiquinone reductase (Complex I). *Current Topics in Bioenergetics* **15,** 1-36.

Ragan, C.I., Wilson, M.T., Darley-Usmar, V.M. and Lowe, P.N. (1987) Subfractionation of mitochondria and isolation of oxidative phosphorylation. In: Darley-Usmar, V.M., Rickwood, D. and Wilson, M.T. (Eds.) *Mitochondria, a practical approach*, pp. 79-112. London: IRL press

Ragan, C.I. and Bloxham, D.P. (1977) Specific labelling of a constituent polypeptide of bovine heart mitochondrial reduced nicotinamide-adenine dinucleotide-ubiquinone reductase by the inhibitor diphenyleneiodonium. *Biochemical Journal* **163,** 605-615.

Ramsay, R.R., Dadgar, J., Trevo, A. and Singer, T.P. (1986) Energy-driven uptake of N-methyl-4-phenylpyridine by brain mitochondria mediates the neurotoxicity of MPTP. *Life Sciences* **39,** 581-588.

Ramsay, R.R., Kowal, A.T., Johnson, M.K., Salach, J.I. and Singer, T.P. (1987) The inhibition site of MPP^+, the neurotoxic bioactivation product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine is near the Q-binding site of NADH dehydrogenase. *Archives of Biochemistry and Biophysics* **259**, 645-649.

Ramsay, R.R., Krueger, M.J., Youngster, S.K., Gluck, M.R., Casida, J.E. and Singer, T.P. (1991) Interaction of 1-methyl-4-phenylpyridinium ion (MPP^+) and its analogs with the rotenone/piercidin binding site of NADH dehydrogenase. *Journal of Neurochemistry* **56,** 1184-1190.

Ramsay, R.R. and Singer, T.P. (1992) Relation of superoxide generation and lipid peroxidation to the inhibition of NADH-Q oxidoreductase by rotenone, piericidin A, and MPP^+. *Biochemical & Biophysical Research Communications* **189**, 47-52.

Redfearn, E.R. (1967) Isolation and determination of ubiqinone. *Methods in Enzymology* **10**, 381-384.

Reed, J.C. (1994) Bcl-2 and the regulation of programmed cell death. *Journal of Cell Biology* **124**, 1-6.

Reed, J.C. (1997) Cytochrome c: can't live with it - can't live without it. *Cell* **91**, 559-562.

Reichman, H., Florke, S., Hebenstreit, G., Schrubar, H. and Riederer, P. (1993) Analyses of energy metablism and mitochondrial genome in post-mortem brain from patients with Alzheimer's disease. *Journal of Neurology* **240,** 377-380.

Richey, P.L., Siedlak, S.L., Smith, M.A. and Perry, G. (1995) Apolipoprotein E interaction with the neurofibrillary tangles and senile plaques in Alzheimer disease: implications for disease pathogenesis. *Biochemical and Biophysical Research Communications* **208**, 657-663.

Riederer, P., Sofic, E., Rausch, W.D., Schmidt, B., Reynolds, G.P., Jellinger, K. and Youdim, M.B.H. (1989) Transition metals, ferritin, glutathione and ascorbic acid in parkinsonian brains. *Journal of Neurochemistry* **52,** 515-520.

Rinne, J.O., Roytta, M., Paljarvi, L., Rummukainen, J. and Rinne, U.K. (1991) Selegiline (deprenyl) treatment and death of nigral neurones in Parkinsons disease. *Neurology* **41,** 859-861.

Roberts, G.W., Leigh, P.N. and Weinberger, D.R. (1993a) Alzheimer's disease. In: *Neuropsychiatric disorders*, pp. 2.1-2.20. London: Wolfe

Roberts, G.W., Leigh, P.N. and Weinberger, D.R. (1993b) Cortical Lewy body disease. In: *Neuropsychiatric disorders*, pp. 4.1-4.8. London: Wolfe

Roberts, G.W., Leigh, P.N. and Weinberger, D.R. (1993c) Parkinson's disease. In: *Neuropsychiatric disorders*, pp. 9.1-9.22. London: Wolfe

Roberts, G.W., Leigh, P.N. and Weinberger, D.R. (1993d) Huntington's disease. In: *Neuropsychiatric disorders*, pp. 10.1-10.6. London: Wolfe

Roberts, H.S.S., Smith, S.C., Marzuki, S. and Linnane, A.W. (1980) Evidence that cytochrome b is the antimycin binding component of the yeast mitochondrial cytochrome bc1 complex. *Archives of Biochemistry and Biophysics* **200,** 387-395.

Ross, C.A. (1995) When more is less: pathogenesis of glutamine repeat neurodegenerative diseases. *Neuron* **15**, 493-496.

Rossetti, Z.L., Sotgiu, A., Sharp, D.E., Hadjiconstantinou, M. and Neff, N.H. (1988) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and free radicals in vitro. *Biochemical Pharmacology* **37**, 4573-4574.

Saggu, H., Cooksey, J., Dexter, D., Wells, F.R., Lees, A.J., Jenner, P. and Marsden, C.D. (1989) A selective increase in particulate superoxide dismutase activity in parkinsonian substantia nigra. *Journal of Neurochemistry* **53,** 692-697.

Sandy, M.S., Langston, J.W., Smith, M.T. and Di Monte, D.A. (1993) PCR analysis of platelet mtDNA: Lack of specific changes in Parkinson's disease. *Movement Disorders* **8,** 74-82.

Schapira, A.H.V., Cooper, J.M., Dexter, D., Jenner, P., Clark, J.B. and Marsden, C.D. (1989) Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* **1**, 1269.

Schapira, A.H.V., Mann, V.M., Cooper, J.M., Dexter, D., Jenner, P., Clark, J.B. and Marsden, C.D. (1990a) Anatomic and disease specificity of NADH CoQ1 reductase (Complex I) deficiency in Parkinson's disease. *Journal of Neurochemistry* **55,** 2142-2145.

Schapira, A.H.V., Holt, I.J., Sweeney, M., Harding, A.E., Jenner, P. and Marsden, C.D. (1990b) Mitochondrial DNA analysis in Parkinson's disease. *Movement Disorders* **5,** 294-297.

Schapira, A.H.V. (1994) Respiratory chain abnormalities in human disease. In: Darley-Usmer, V. and Schapira, A.H.V. (Eds.) *Mitochondria: DNA, Proteins and Disease*, pp. 241-278. London: Portland Press

Schapira, A.H.V. (1995) Oxidative stress in Parkinson's disease. *Neuropathology and Applied Neurobiology* **21**, 3-9.

Schapira, A.H.V. and Reichmann, H. (1995) Electron transport chain defects in Alzheimer's disease. *Neurology* **45,** 599-600.

Schapira, A.H.V., Warner, T., Gash, M.T., Cleeter, M.W., Marinho, C.F. and Cooper, J.M. (1997) Complex I function in familial and sporadic dystonia. *Annals of Neurology* **41**, 556-559.

Schon, E.A., Rizzuto, R., Moraes, C.T., Nakase, H., Zeviani, M. and di Mauro, S. (1989) A direct repeat is a hotpot for large-scale deletions of human mitochondrial DNA. *Science* **244,** 346-349.

Schulz, J.B., Matthews, R.T., Muqit, M.M., Browne, S.E. and Beal, M.F. (1995) Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. *Journal of Neurochemistry* **64**, 936-939.

Seaton, T.A., Cooper, J.M. and Schapira, A.H. (1997) Free radical scavengers protect dopaminergic cell lines from apoptosis induced by complex I inhibitors. *Brain Research* **777**, 110-118.

Shimizu, S., Eguchi, Y., Kosaka, H. Kamiike, W., Matsuda, H. and Tsujimoto, Y. (1995) Provention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. *Nature* **374,** 811-813.

Shoffner, J.M., Lott, M.T., Lezza, A.M.S., Seibel, P., Ballinger, S.W. and Wallace, D.C. (1990) Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA lys mutation. *Cell* **61,** 931-937.

Shoffner, J.M., Watt, R.L., Juncos, J.L., Torroi, A. and Wallace, D.C. (1991) Mitochondrial oxidative phosphorylation defects in Parkinson's disease. *Annals of Neurology* **30**, 332-339.

Shoffner, J.M., Brown, M.D., Torroni, A., Lott, M.T., Cabell, M.F., Mirra, S.S., Beal, M.F., Yang, C.C., Gearing, M. and Salvo, R. (1993) Mitochondrial DNA variants observed in Alzheimer disease and Parkinson disease patients. *Genomics* **17**, 171-184.

Shoubridge, E.A., Karpati, G. and Hastings, K.E.M. (1990) Deletion mutants are functionally dominant over wild-type mitochondrial genomes in skeletal muscle fiber segments in mitochondrial disease. *Cell* **62,** 43-49.

Shults, C.W., Nasirian, F., Ward, D.M., Nakano, K., Pay, M., Hill, L.R. and Haas, R.H. (1995) Carbidopa/levodopa and selegiline do not affect platelet mitochondrial function in early parkinsonism. *Neurology* **45**, 344-348.

Shuster, R.C., Rubenstein, A.J. and Wallace, D.C. (1988) Mitochondrial DNA in anucleate human blood cells. *Biochemical and Biophysical Research Communications* **155,** 1360-1365.

Sian, J., Dexter, D.T., Lees, A.J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P. and Marsden, C.D. (1994a) Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Annals of Neurology* **36**, 348-355.

Sian, J., Dexter, D.T., Lees, A.J., Daniel, S., Jenner, P. and Marsden, C.D. (1994b) Glutathine-related enzymes in brain in Parkinson's disease. *Annals of Neurology* **36,** 356-361.

Sies, H. (1985) Sies, H. (Ed.) *Oxidative stress*, Lodon: Academic Press

Simonetti, S., Chen, X., DiMauro, S. and Schon, E.A. (1992) Accumulation of deletions in human mitochondrial DNA during normal aging: analysis by quantitative PCR. *Biochimica et Biophysica Acta* **1180,** 113-122.

Simonian, N.A. and Hyman, B.T. (1993) Functional alterations in Alzheimer's disease: diminution of cytochrome oxidase in the hippocampal formation. *Journal of Neuropathology and Experimental Neurology* **52,** 580-585.

Simonian, N.A. and Hyman, B.T. (1994) Functional alterations in Alzheimer's disease: selective loss of mitochondrial-encoded cytochrome oxidase mRNA in the hippocampal formation. *Journal of Neuropathology and Experimental Neurology* **53,** 508-512.

Simpson, J.R. and Isacson, O. (1993) Mitochondrial impairment reduces the threshold for in vivo NMDA-mediated neuronal death in the striatum. *Experimental Neurology* **121,** 57-64.

Smeyne, R.J., Vendrell, M., Hayward, M., Baker, S.J., Miao, G.G., Schilling, K., Robertson, L.M., Curran, T. and Morgan, J.I. (1993) Continuous c-fos expression precedes programmed cell death in vivo. *Nature* **363,** 166-169.

Smith, C.A., Gough, A.C., Leigh, P.N., Summers, B.A., Harding, A.E., Maraganore, D.M., Sturman, S.G., Schapira, A.H.V., Williams, A.C. and et al. (1992) Debrisoquine hydroxylase gene polymorphism and susceptibility to Parkinson's disease. *Lancet* **339,** 1375-1377.

Smith, P.R., Cooper, J.M., Govan, G.G., Harding, A.E. and Schapira, A.H.V. (1994) Platelet mitochondrial function in Leber's hereditary optic neuropathy. *Journal of the Neurological Sciences* **122,** 80-83.

Steinman, H.M. (1995) The Bcl-2 oncoprotein functions as a pro-oxidant. *Journal of Biological Chemistry* **270,** 3487-3490.

Strittmatter, W.J., Saunders, A.M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G.S. and Roses, A.D. (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 1977-1981.

Strong, T.V., Tagle, D.A., Valdes, J.M., Elmer, L.W., Boehm, K., Swaroop, M., Kaatz, K.W., Collins, F.S. and Albin, R.L. (1993) Widespread expression of the human and rat Huntington's disease gene in brain and nonneural tissues. *Nature Genetics* **5,** 259-265.

Sturman, S.G., Steventon, G.B., Waring, R.H. and Williams, A.C. (1990) MAO-B and Parkinson's disease. *Movement Disorders* **5,** 338-339.

Sweeney, M.G., Davis, M.B., Lashwood, A., Brockington, M., Toscano, A., Harding, A.E. and . (1992) Evidence against an X-linked locus close to DXS7 determining visual loss susceptibility in British and Italian families with Leber hereditary optic neuropathy. *American Journal of Human Genetics* **51,** 741-748.

Swerdlow, R.H., Parks, J.K., Miller, S.W., Tuttle, J.B., Trimmer, P.A., Sheehan, J.P., Bennett, J.P., Davis, R.E. and Parker, W.D. (1996) Origin and functional consequences of the complex I defect in Parkinson's disease. *Annals of Neurology* **40,** 663-671.

Taanman, J.W., Burton, M.D., Marusich, M.F., Kennaway, N.G. and Capaldi, R.A. (1996) Subunit specific monoclonal antibodies show different steady-state levels of various cytochrome-c oxidase subunits in chronic progressive external ophthalmoplegia. *Biochimica et Biophysica Acta* **1315,** 199-207.

Taanman, J.W., Bodnar, A.G., Cooper, J.M., Morris, A.A.M., Clayton, P.T., Leonard, J.V. and Schapira, A.H.V. (1997) Molecular mechanisms in mitochondrial DNA deletion syndrome. *Human Molecular Genetics* **6,** 935-942.

Tabrizi, S., Cooper, J.M. and Schapira, A.H.V. (1998) Mitochondrial DNA in focal dystonia: a cybrid analysis. *Annals of Neurology* **44,** 258-261.

Takeshige, K. and Minakami, S. (1979) NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. *Biochemical Journal* **180,** 129-135.

Tatuch, Y., Christodoulou, J., Feigenbaum, A., Clarke, J.T., Wherret, J., Smith, C., Rudd, N., Petrova-Benedict, R. and Robinson, B.H. (1992) Heteroplasmic mtDNA mutation (T-G) at 8993 can cause Leigh disease when the percentage of abnormal mtDNA is high. *American Journal of Human Genetics* **50,** 852-858.

Taylor-Robinson, S.D., Weeks, R.A., Sargentoni, J., Marcus, C.D., Bryant, D.J., Harding, A.E. and Brooks, D.J. (1994) Evidence for glutamate excitotoxicity in Huntington's disease with proton magnetic resonance spectroscopy. *Lancet* **343,** 1170.

Telenius, H., Kremer, H.P., Theilmann, J., Andrew, S.E., Almqvist, E., Anvret, M., Greenberg, C., Greenberg, J., Lucotte, G., Squitieri, F., *et al* (1993) Molecular analysis of juvenile Huntington disease: the major influence on (CAG)n repeat length is the sex of the affected parent. *Human Molecular Genetics* **2,** 1535-1540.

Thierbach, G. and Michaelis, G. (1982) Mitochondrial and nuclear myxothiazol resistance in Saccharomyces cerevisiae. *Molecular and General Genetics* **186,** 501-506.

Thompson, C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267,** 1456-1462.

Trottier, Y., Devys, D., Imbert, G., Saudou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E.C. and Mandel, J.L. (1995) Cellular localization of the Huntington's disease protein and discrimination of the normal and mutated form. *Nature Genetics* **10,** 104-110.

Trounce, I., Byrne, E. and Marzuki, S. (1989) Deline in skeletal muscle mitochondrial respiratory chain function: possible factor in aging. *Lancet* **i,** 637-639.

Tsujimoto, Y., Finger, L.R., Yunis, J., Nowell, P.C. and Croce, C.M. (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* **226,** 1097-1099.

Tsujimoto, Y. and Croce, C.M. (1986) Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proceedings of the National Academy of Sciences of the United States of America* **83,** 5214-5218.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1995) Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 A. *Science* **269,** 1069-1074.

Turers, J.F. and Boveris, A. (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochemical Journal* **191,** 421-427.

Turrens, F., Alexandre, A. and Leminger, A.L. (1985) Ubisemiquinone is the electron donor for superoxide anion by complex III of heart mitochondria. *Archives of Biochemistry and Biophysics* **273,** 408-414.

Utermann, G., Langenbeck, U., Beisiegel, U. and Weber, W. (1980) Genetics of the apolipoprotein E system in man. *American Journal of Human Genetics* **32,** 339-347.

van Gelder, B.F. and Muijers, A.O. (1966) On cytochrome c oxidase II. The ratio of cytochrome a to cytochrone a3. *Biochimica et Biophysica Acta* **118,** 47-57.

Vaux, D., Cory, S. and Adams, J. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335,** 440-442.

Vayssiere, J.-L., Petit, P.X., Risler, Y. and Mignotte, B. (1994) Commitment to apoptosis is associated with changes in mitochondrial biogenesis and activity in cell lines conditionally immortalized with simian virus 40. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 11752-11756.

Wahl, A.F., Donaldson, K.L., Fairchild, C., Lee, F.Y., Foster, S.A., Demers, G.W. and Galloway, D.A. (1996) Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis. *Nature Medicine* **2,** 72-79.

Walker, J.E. (1992) The NADH-ubiquinone reductase (complex I) of respiratory chains. *Quarterly Reviews of Biophysics* **25,** 253-324.

Wallace, D.C. (1987) Maternal genes: mitochondrial diseases. *Birth Defects* **23,** 137-190.

Wallace, D.C., Singh, G., Lott, M.T., Hodge, J.A., Schurr, T.G., Lezza, A.M.S., ElsasII, L.J. and Nikoskelainen, E.K. (1988) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science* **242,** 1427-1430.

Ward, C.D., Duvfoisin, R.C., Ince, S.E., Nutt, J.D., Elbridge, R. and Calne, D.B. (1983) Parkinson's disease in 65 pairs of twins and in a set of quadruplets. *Neurology* **33,** 815-824.

Warner, T.T., Fletcher, N.A., Davis, M.B., Ahmad, F., Conway, D., Feve, A., Rondot, P., Marsden, C.D. and Harding, A.E. (1993) Linkage analysis in British and French families with idiopathic torsion dystonia. *Brain* **116,** 739-744.

Warner, T.T. and Schapira, A.H.V. (1998) The role of the α-synuclein gene mutation in patients with sporadic Parkinson's disease in the United Kingdom. *Journal of Neurology, Neurosurgery and Psychiatry* **65,** 378-379.

Wenham, P.R., Price, W.H. and Blandell, G. (1991) Apolipoprotein E genotyping by one-stage PCR. *Lancet* **337**, 1158-1159.

Wexler, N.S., Young, A.B., Tanzi, R.E., Travers, H., Starosta-Rubinstein, S., Penney, J.B., Snodgrass, S.R., Shoulson, I. and Gomez, F. (1987) Homozygotes for Huntington's disease. *Nature* **326,** 194-197.

Wharton, D.C. and Tzagoloff, A. (1967) Cytochrome oxidase from beef heart mitochondria. *Methods in Enzymology* **10,** 245-250.

Williams, G.T. (1991) Programmed cell death: apoptosis and oncogenesis. *Cell* **65,** 1097-1098.

Willingham, M.C. and Bhalla, K. (1994) Transient mitotic phase localization of bcl-2 oncoprotein in human carcinoma cells and its possible role in prevention of apoptosis. *Journal of Histochemistry and Cytochemistry* **42,** 441-450.

Wiseman, A. and Attardi, G. (1978) Reversible tenfod reduction in mitochondria DNA content of human cells treated with ethidium bromide. *Molecular and General Genetics* **167,** 51-63.

Wisniewski, T. and Frangione, B. (1992) Apolipoprotein E: a pathological chaperone protein in patients with cerebral and systemic amyloid. *Neuroscience Letters* **135,** 235-238.

Wolvetang, E.J., Johnson, K.L., Krauer, K., Ralph, S.J. and Linnane, A.W. (1994) Mitochondrial respiratory chain inhibitors induce apoptosis. *FEBS Letters* **339,** 40-44.

Wooten, G.F., Currie, L.J., Bennett, J.P., Harrison, M.B., Trugman, J.M. and Parker, W.D. (1997) Maternal inheritance in Parkinson's disease. *Annals of Neurology* **41**, 265-268.

Wylie, A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endonuclease activition. *Nature* **284,** 555-556.

Yen, T.C., Chen, Y.S., King, K.L., Yeh, S.H. and Wei, Y.H. (1989) Liver mitochondrial respiratory functions decline with age. *Biochemical and Biophysical Research Communications* **165,** 994-1003.

Yen, T.Z., Su, J.H., King, K.L. and Wei, Y.H. (1991) Ageing-associated 5kb deletion in human liver mitochondrial DNA. *Biochemical and Biophysical Research Communications* **178,** 124-131.

Yoneda, M., Chomyn, A., Martinuzzi, A., Hurko, O. and Attardi, G. (1992) Marked replicative advantage of human mtDNA carrying a point mutation that causes the MELAS encephalomyopathy. *Proceedings of the National Academy of Sciences of the United States of America* **89,** 11164-11168.

Yoshida, M., Niwa, T. and Nagatsu, T. (1990) Parkinsonism in monkeys produced by chronic administration of an endogenous substance of the brain, tetrahydroisoquinoline: the behavioral and biochemical changes. *Neuroscience Letters* **119,** 109-113.

Yoshino, H., Nakagawa-Hatori, Y., Kondo, T. and Mizuno, Y. (1992) Mitochondrial complex I and II activity of lymphocytes and platelets in Parkinson's disease. *Journal of Neural Transmission* **4,** 27-34.

Zamzami, N., Susin, S.A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M. and Guido Kroemer. (1996) Mitochondrial control of nuclear apoptosis. *Journal of Experimental Medicine* **183,** 1533-1544.

Zeviani, M., Servidei, S., Gellera, C., Bertini, E., DiMauro, S. and DiDonato, S. (1989) An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region. *Nature* **339,** 309-311.

APPENDIX

Appendix 1: Buffers for mitochondrial preparation

1.1 Brain homogenisation buffer

Sucrose	0.25M
Tris-HCl	10mM
EDTA-K_2	1mM
pH 7.4	

1.2 Modified Tyrodes Hepes Buffer

NaCl	150mM
HEPES	5mM
NaH_2PO_4	0.55mM
$NaHCO_3$	7mM
KCl	2.7mM
Glucose	5.6mM
EDTA-K_2	1mM
pH 7.4	

Appendix 2: Buffers for DNA Extraction

2.1 DNA extraction buffer

NaCl	75mM
EDTA-K_2	50mM

2.2 TE buffer

Tris-HCl	10mM
EDTA-K_2	1mM
pH 7.4	

2.3 Cell lysis buffer

KCl	50mM
Tris-base	20mM
$MgCl_2$	2.5mM
Tween-20	0.45% (v/v)
Nonidate P40	0.45% (v/v)
pH 8.3	

2.4 TBE bufffer

Tris-HCl	90mM
Boric acid	90mM
EDTA-K_2	2.5mM

2.5 Praparation of 8% Acrylamide gel for DNA

30% Acrylagel	5.15ml
2% bis-Acylagel	2.65ml
5xTBE	4.0ml
10% Ammonium persulphate	0.2ml
ddH_2O	8.0

	20ml

Polymerisation was initiated by the addition of 20µl of TEMED.

Appendix 3: Western blot

3.1 Extraction buffer

Lauryl maltoside	1.5%
PMSF (in aceton)	2mM
Leupeptin	1μg/μl
pepstatin A (in methanol)	1μg/μl

make the mixture solution in PBS

3.2 Denature buffer

Tris-HCl	50mM
SDS	4%
Glycerol	12%
Bromphenol blue	0.01%

2-mercaptoethanol (2%, v/v) was added at last.

3.3 12.5% separatting SDS-PAGE gel

Stock concentrations	volume added
30% Acrylagel	5.2ml
2% bis-Acrylagel	2.0ml
3.75M Tris-HCl pH 8.6	1.25ml
20% SDS	62.5μl
10% APS	105μl
ddH_2O	3.9ml

	12.5ml

Polymerisation was initiated by the addition of 8μl of TEMED.

3.4 5% Stacking gel

Stock concentrations	volume added
30% Acrylagel	1ml
2% bis-Acrylagel	0.4ml
0.5M Tris-HCl pH 6.8	1.25ml
20% SDS	50μl
10% APS	52μl
ddH_2O	7.1ml

	10ml

Polymerisation was initiated by the addition of 8μl of TEMED.

3.5 Running buffer

Tris-HCl	25mM
Glycine	194mM
SDS	0.1% (w/v)

3.6 Transfer buffer (Towbin)

Tris-HCl	25mM
Glycine	194mM
Methanol	20% (v/v)

pH 9.0

3.7 Primary antibodies for COX I, II and porin

products	code	dilution
COX I	1D6-E1-A8	1:1,000
COX II	12C4-F12	1:30,000
COX IV	10G8-D12-C12	1:3,000
Porin	31HL	1:150,000

Appendix 4: Cell staining solution

541 COX activity staining dye
Prepared fresh every time:

0.5M PBS, pH 7.4	2ml
3',3-diaminobenzine	10mg
Oxidised cytochrome c	20mg
2mg/ml Catalase	20μl
ddH_2O	18ml

	20ml

PUBLICATIONS

appears to be a general marker for cerebellar involvement in a variety of neurological conditions.

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References

1. Courchesne E, Yeung-Courchesne R, Press GA, et al. Hypoplasia of cerebellar vermal lobules VI and VII in autism. N Engl J Med 1988;318:1349–1354
2. Mirakami JW, Courchesne E, Press GA, et al. Reduced cerebellar hemisphere size and its relationship to neural hypoplasia in autism. Arch Neurol 1989;46:689–694
3. Courchesne E. Neuroanatomic imaging in autism. Pediatrics 1991;87:781–790
4. Courchesne E, Saitoh O, Yeung-Courchesne R, et al. Abnormality of cerebellar vermian lobules VI and VII in patients with infantile autism: identification of hypoplastic and hyperplastic subgroups with MR imaging. Am J Radiol 1994;162:123–130
5. Ritvo ER, Garber HJ. Cerebellar hypoplasia and autism. N Engl J Med 1988;319:1152
6. Kleiman MD, Neff S, Rosman NP. The brain in infantile autism: are posterior fossa structures abnormal? Neurology 1992:42:753–760
7. Piven J, Nehme E, Simon J, et al. Magnetic resonance imaging in autism: measurement of the cerebellum, pons, and fourth ventricle. Biol Psychiatry 1992;31:491–504
8. Schaefer GB, Thompson JN Jr, Bodensteiner JB, et al. Age-related changes in the relative growth of the posterior fossa. J Child Neurol 1991;6:15–19
9. Reiss AL, Freund L, Tseng JE, et al. Neuroanatomy in fragile-X females: the posterior fossa. Am J Hum Genet 1991;49:279–288
10. American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 3rd ed. Washington, DC: American Psychiatric Association, 1980
11. Schaefer GB, Thompson JN Jr, Bodensteiner JB, et al. Quantitative morphometric analysis of brain growth using magnetic resonance imaging. J Child Neurol 1990;5:127–130
12. Norusis MJ. SPSS/PC+ advanced statistics 4.0. Chicago: SPSS, 1990
13. Sokal RR, Rohlf FJ. Biometry. 2nd ed. San Francisco: WH Freeman, 1981

Mitochondrial Defect in Huntington's Disease Caudate Nucleus

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Although the Huntington's disease (HD) gene defect has been identified, the structure and function of the abnormal gene product and the pathogenetic mechanisms involved in producing death of selective neuronal populations are not understood. Indirect evidence from several sources indicates that a defect of energy metabolism and consequent excitotoxicity are involved in HD. Toxin models of HD may be induced by 3-nitropropionic acid or malonate, both inhibitors of succinate dehydrogenase, complex II of the mitochondrial respiratory chain. We analyzed mitochondrial respiratory chain function in the caudate nucleus (n = 10) and platelets (n = 11) from patients with HD. In the caudate nucleus, severe defects of complexes II and III (53–59%, $p < 0.0005$) and a 32–38% ($p < 0.01$) deficiency of complex IV activity were demonstrated. No deficiencies were found in platelet mitochondrial function. The mitochondrial defect identified in HD caudate parallels that induced by HD neurotoxin models and further supports the role of abnormal energy metabolism in HD. The relationship of the mitochondrial defect to the role of huntingtin is not known.

Gu M, Gash MT, Mann VM, Javoy-Agid F, Cooper JM, Schapira AHV. Mitochondrial defect in Huntington's disease caudate nucleus. Ann Neurol 1996;39:385–389

Huntington's disease (HD) is an autosomal dominant, fully penetrant disorder caused by an unstable CAG trinucleotide repeat on chromosome 4 that encodes an unknown protein (huntingtin) of approximately 340 kd (see [1] for review). HD is associated with a characteristic combination of pathological features: loss of striatal projection neurons with sparing of the patch-matrix compartmentation of the striatum and the NAPDH-diaphorase–positive large aspiny neurons together with proliferative changes in spiny neuron dendrites.

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Although the biochemical basis of HD pathogenesis is not understood, several lines of evidence suggest that an abnormality of energy metabolism may be involved. In 1985 Brennan and colleagues [2] examined respiratory activity in the caudate and cerebral cortex of 10 HD patients and compared results to 7 matched control subjects. Results expressed per unit of protein showed a mean decrease of 39% in succinate-linked oxidation and a 30% decrease in cytochrome oxidase (complex IV) activity in HD caudate nucleus. Striatal and cerebral cortex glucose hypometabolism was observed early in the course of HD and appears to precede bulk tissue loss [3, 4]. Elevated lactate levels were found in the occipital cortex of 16 HD patients and the basal ganglia of 8 HD patients examined by magnetic resonance spectroscopy (MRS) [5]. Similarly elevated lactate-pyruvate ratios were also seen in HD cerebrospinal fluid [6].

Few reports have described the results of direct respiratory chain enzyme analysis of HD brain tissue. Apart from the report of Brennan and colleagues [2] a preliminary report from our own group described a decrease in complex II/III activity in the caudate nucleus from 4 HD patients [7]. The results presented here extend our previous study and provide more detailed data on the individual respiratory chain enzyme activities.

In 1990 Parker and coauthors [8] described a 72% decrease in complex I activity in platelet mitochondria from 5 HD patients. We analyzed platelet mitochondrial function in 11 HD patients and 17 matched control subjects to assess the possible tissue distribution of any respiratory chain defect and to compare any such peripheral abnormality with caudate nucleus.

Materials and Methods

The diagnosis of HD was made on clinical grounds and confirmed postmortem by pathological examination of the brains from which samples of caudate nucleus were obtained. Brains were matched with control brains for age and postmortem delay. Tissue samples were coded and homogenized and complex I, II/III, and IV and citrate synthase activities were assayed blind and in triplicate as previously described [9]. Complex II activity was assayed by measuring the reduction of 2,6-dichlorophenol indophenol (74 μM) at 600 nm in potassium phosphate (50 mm), pH 7.4, ethylenediaminetetraacetic acid (EDTA) (100 μM), KCN (1 mM), rotenone (10 μM), and sodium succinate (20 mM). The sample was preincubated at 25°C for 10 minutes before initiation with coenzyme Q_2 (CoQ_2) (50 μM). The rate sensitive to 2-thenoyltrifluoroacetone (1 mM) was taken as complex II activity. Complex III was assayed by monitoring the ubiquinol-2 reduction of cytochrome *c* at 550 nm. The reaction contained potassium phosphate (35 mM), pH 7.2, EDTA (1 mM), magnesium chloride (5 mM), KCN (2 mM), rotenone (5 μM), cytochrome *c* (15 μM), and sample (5–10 μg of protein). The reaction was initiated by ubiquinol-2 (15 μM) and the first-order rate constant calculated after first subtracting the blank rate determined in the absence of sample. Ubiquinol-2 was prepared as described by Ragan and coauthors [10].

The diagnosis of HD for the patients undergoing platelet mitochondrial function analysis was made on clinical grounds and confirmed by the presence of the chromosome 4 CAG triplet repeat characteristic of this disorder. Platelet mitochondrial fractions were prepared from patients and matched control subjects and coded, and respiratory chain enzymes were assayed blind and in triplicate as previously described [11]. Two of the HD patients were taking neuroleptics; 6, tetrabenazine (a dopamine-depleting agent); and 1, amantadine (a dopaminergic/anticholinergic drug). Two HD patients were untreated.

Statistical analysis was performed using the Mann-Whitney *U* test.

Results

Complex I activities in HD caudate were not significantly different from those in control caudate when expressed per unit of protein or when corrected for any variation in mitochondrial mass between samples by relating enzyme activities to citrate synthase levels (see Table).

Mean complex II/III (succinate cytochrome *c* reductase) activities were significantly reduced: 57% ($p < 0.0001$) per unit of protein and 53% ($p < 0.0001$) when corrected for citrate synthase activity. This enzyme reflects electron transport between succinate and cytochrome *c* via CoQ (ubiquinone) and this includes complex II (succinate ubiquinone reductase) and complex III (ubiquinol cytochrome *c* reductase) activities. These enzymes may be assayed separately and thereby potentially allow the dissection of the complex II/III defect into one or other component. In fact, both complex II and complex III activities were found to be decreased: 53% ($p < 0.0001$) for complex II per unit of protein and 56% ($p < 0.0001$) when corrected for citrate synthase activity; 59% ($p < 0.0001$) for complex III per unit of protein and 55% ($p < 0.0005$) when corrected for citrate synthase activity.

Complex IV activities in HD caudate were also decreased: 38% ($p < 0.005$) per unit of protein and 32% ($p < 0.01$) when corrected for citrate synthase activity.

For platelet mitochondrial function analysis, control subjects and HD patients were matched for age, sex, and cigarette smoking status, the latter because smoking cigarettes depresses complex I activity in platelets [12]. In HD platelets, we found no significant change in NADH CoQ_1 reductase (complex I) or succinate cytochrome *c* reductase (complex II/III) activities but observed a 64% ($p = 0.001$) and 37% ($p = 0.049$) increase in cytochrome oxidase activities expressed per unit of protein or as a citrate synthase ratio, respec

Mitochondrial Respiratory Chain Activities in Control and Huntington's Disease Caudate Nucleus and Platelets[a]

	Caudate Nucleus				Platelets			
	Specific Activity		Citrate Synthase Ratio		Specific Activity		Citrate Synthase Ratio	
	Control	HD	Control	HD	Control	HD	Control	HD
n	10	10	10	10	17	11	17	11
Age (yr)	63.4 ± 17.4	61.6 ± 14.1			57.2 ± 10.1	49.6 ± 11.3		
Postmortem delay (hr)	29.4 ± 13.3	34.7 ± 15.6						
Complex I	4.9 ± 1.0	4.2 ± 1.3	3.0 ± 0.8	2.9 ± 1.4	18.0 ± 4.2	19.0 ± 4.2	7.6 ± 1.4	7.1 ± 1.7
Complex II	15.6 ± 2.9	7.4 ± 2.0 (n = 9, $p < 0.0001$)	9.5 ± 1.9	4.2 ± 1.9 (n = 9, $p < 0.0001$)				
Complex III	6.6 ± 1.8	2.7 ± 1.0 (n = 9, $p < 0.0001$)	4.0 ± 1.1	1.8 ± 0.9 (n = 9, $p < 0.0005$)				
Complex II/III	25.9 ± 5.9	11.2 ± 4.8 ($p < 0.0001$)	15.6 ± 3.1	7.4 ± 3.2 ($p < 0.0001$)	47.8 ± 16.4	50.1 ± 7.6	20.1 ± 6.3	18.7 ± 3.3
Complex IV (K)	1.31 ± 0.39	0.81 ± 0.39 ($p < 0.005$)	0.77 ± 0.22	0.52 ± 0.18 ($p < 0.01$)	2.08 ± 0.64 (n = 15)	3.42 ± 1.07 (n = 10, $p = 0.001$)	0.92 ± 0.25 (n = 15)	1.26 ± 0.49 (n = 10, $p = 0.049$)
Citrate synthase	168 ± 30	153 ± 43			244 ± 58	273 ± 45		

[a]Results are expressed as mean ± SD (standard deviation) in nmol/min/mg total protein (specific activity) or a ratio with citrate synthase activity (ratio × 100). Complex I represents NADH CoQ_1 reductase (rotenone sensitive); complex II, succinate ubiquinone reductase; complex III, ubiquinol cytochrome *c* reductase; complex II/III, succinate cytochrome *c* reductase (antimycin A sensitive); complex IV, cytochrome oxidase. K is the first-order rate constant (min^{-1} mg^{-1}). Significance was determined using Mann-Whitney U test. Caudate nucleus data from 4 HD and 5 controls have been reported previously [7].

tively. Drug treatment at the routine therapeutic doses used had no observable effect on the function of complexes I to III.

Discussion

Although the molecular genetic basis for HD is now understood, identity of the gene product and the biochemical explanation for disease pathogenesis are not known. The HD gene is expressed in peripheral tissues as well as within the central nervous system [13]. Both the normal and mutant HD gene alleles are expressed at the messenger RNA (mRNA) and protein levels in some tissues. Interestingly, the normal and mutant huntingtin are expressed in lymphoblast and fibroblast lines but no clear evidence of expression in HD striatum or cortex was seen, although a faint smear on the Western blot suggested the presence of mutant huntingtin.

Positron emission tomography (PET) and MRS studies have identified abnormalities of glucose utilization and lactate production in HD. It has been argued that deficient energy metabolism may cause neuronal death through excitotoxic mechanisms [14]. Impaired respiratory chain function can induce glutamate toxicity through partial depolarization, release of the Mg^{2+}-dependent inhibition of *N*-methyl-D-aspartate (NMDA) receptors, and consequent activation by glutamate [15]. The suggestion that glutamate toxicity may be important in HD was supported recently by the demonstration by MRS of increased glutamate levels in HD caudate [16].

These observations of disordered energy metabolism and potential glutamate toxicity in vivo in HD have stimulated the search for a toxin model of HD. Much interest has recently focused on the use of 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase (SDH, complex II), as a toxin model for HD. Accidental human exposure to 3-NP through eating mildewed sugar cane induced nausea, vomiting, encephalopathy, and coma followed, in survivors, by dystonia and choreiform movements [17]. 3-NP decreases ATP formation and causes neuronal damage by excitotoxic mechanisms in cerebral cortex [18]. Intrastriatal injections of 3-NP into rats produced dose-dependent ATP depletion, increased lactate concentrations, and neuronal loss that was reduced by prior decortication and removal of striatal glutaminergic input [19]. Subacute systemic administration of 3-NP to rats produced elevated lactate levels in basal ganglia which preceded magnetic resonance imaging changes in the striatum.

The results of our analysis of respiratory chain function in HD caudate nucleus provide a significant biochemical parallel to the HD model as induced by 3-NP or malonate, another inhibitor of SDH [19, 20]. We found marked reductions in the activities of complexes II and III and more mild changes in complex IV. These results confirm the original observations of

Brennan and colleagues [2]. The degree of complex II and III defects as assessed individually was comparable and this raises the possibility that both may be secondary to a deficiency of ubiquinone which is used as an electron carrier by both complexes. The relationship of the complex IV defect to that of complexes II and III is uncertain; it could not be caused by ubiquinone deficiency but might imply a more general dysfunction of these membrane-bound proteins.

The relationship of the mitochondrial deficiency to the pathogenesis of HD is, of course, unknown at present. The identification of this abnormality, however, fits well with the current hypothesis of glutamate-induced excitatory damage resulting from a mitochondrial defect and ATP depletion. The observation that complex II inhibition itself induces a pathological model of HD supports this hypothesis. The anatomical specificity of the mitochondrial deficiency has not as yet been clearly defined. Direct measurement of activities of HD putamen (n = 4) and HD cerebral cortex (n = 4) showed no abnormality but these data need expanding [7]. MRS data, however, show increased lactate concentrations in occipital cortex in HD, suggesting that defective energy metabolism may be present in areas of HD brain beyond the caudate [5].

The tissue distribution of the mitochondrial deficiency in HD will be important in determining its relationship to the molecular genetic defect already identified. The HD gene is expressed widely although it is not known whether the resulting biochemical defect caused by the abnormal protein product is also expressed in all tissues. In terms of platelet mitochondrial function, Parker and coworkers [8] found a 72% deficiency of complex I in 5 HD patients but no change in the activities of complexes II to IV. We could not identify any respiratory chain abnormality in the platelets of our 11 genetically proved HD patients although complex IV was increased in HD. Why complex IV should be increased in HD platelets, but decreased in HD caudate is unclear. We cannot discount that this effect may be the result of drug treatment. The presence of a previously reported complex I deficiency in blood [8] in conjunction with the normal complex I activity in HD caudate in our study is difficult to explain. The converse situation of defects of complexes II to IV in HD caudate, but no decrease in activity in blood, parallels the site of pathological damage in HD. Differential tissue biochemical expression of the complex II/III deficiency may parallel the influence of the HD gene product as well as other local biochemical and neurochemical influences in the caudate nucleus.

Finally, the relationship of the mitochondrial deficiency to the CAG triplet repeat on chromosome 4 is not known. It is possible that the HD gene product could alter complexes II to IV or ubiquinone function through an indirect effect, perhaps through an abnormality of mitochondrial protein import or mitochondrial membrane structure or function.

The therapeutic implications of identifying biochemical abnormalities in HD are already being explored. CoQ_{10} (ubiquinone) at a dose of 360 mg/day for 1 month attenuated the development of cortical lactic acidosis in 13 of 15 HD patients [6].

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References

1. Albin RL, Tagle DA. Genetics and molecular biology of Huntington's disease. Trends Neurosci 1995;18:11–14
2. Brennan WA, Bird ED, Aprille JR. Regional mitochondrial respiratory activity in Huntington's disease brain. J Neurochem 1985;44:1948–1950
3. Kuwert T, Lange HW, Boecker H, et al. Striatal glucose consumption in chorea-free subjects at risk of Huntington's disease. J Neurol 1993;241:31–36
4. Martin WRW, Clark C, Ammann W, et al. Cortical glucose metabolism in Huntington's disease. Neurology 1992;42:223–229
5. Jenkins B, Koroshetz W, Beal MF, Rosen B. Evidence for an energy metabolism defect in Huntington's disease using localized proton spectroscopy. Neurology 1993;43:2689–2695.
6. Koroshetz WJ, Jenkins B, Rosen B, Beal MF. Evidence for a metabolic disorder in Huntington's disease. Neurology 1994; 44:A338
7. Mann VM, Cooper JM, Javoy-Agid F, et al. Mitochondrial function and parental sex effect in Huntington's disease. Lancet 1990;336:749
8. Parker WD, Boyson SJ, Luder AS, Parks JK. Evidence for a defect in NADH: ubiquinone oxidoreductase (complex I) in Huntington's disease. Neurology 1990;40:1231–1234
9. Schapira AHV, Mann VM, Cooper JM, et al. Anatomic and disease specificity of NADH CoQ_1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 1990;55:2142–2145
10. Ragan CI, Wilson MT, Darley-Usmar VM, Lowe PN. Subfractionation of mitochondria, and isolation of the proteins of oxidative phosphorylation. In: Darley-Usmar VM, Rickwood D, Wilson MT, eds. Mitochondria, a practical approach. London: IRL Press, 1987:79–112
11. Krige D, Carroll MT, Cooper JM, et al. Platelet mitochondrial function in Parkinson's disease. Ann Neurol 1992;32:782–788
12. Smith PR, Cooper JM, Govan GG, et al. Smoking and mitochondrial function: a model for environmental toxins. Q J Med 1993;86:657–660
13. Trottier Y, Devys D, Imbert G, et al. Cellular localization of the Huntington's disease protein and discrimination of the normal and mutated form. Nature Genet 1995;10:104–110
14. Beal MF. Neurochemistry and toxin models in Huntington's disease. Curr Opin Neurol 1994;7:542–547
15. Simpson JR, Isacson O. Mitochondrial impairment reduces the threshold for *in vivo* NMDA-mediated neuronal death in the striatum. Exp Neurol 1993;121:57–64
16. Taylor-Robinson SD, Weeks RA, Sargentoni J, et al. Evidence

for glutamate excitotoxicity in Huntington's disease with proton magnetic resonance spectroscopy. Lancet 1994;343:1170
17. Ludolph AC, He F, Spencer PS, et al. 3-Nitropropionic acid: exogenous animal neurotoxin and possible human striatal toxin. Can J Neurol Sci 1991;18:492–498
18. Ludolph AC, Seelig M, Ludolph A, et al. 3-Nitropropionic acid decreases cellular energy levels and causes neuronal degeneration in cortical explants. Neurodegeneration 1992;1:155–161
19. Beal MR, Brouillet E, Jenkins B, et al. Age-dependent striatal excitotoxic lesions produced by the endogenous mitochondrial inhibitor malonate. J Neurochem 1993;61:1147–1150
20. Greene JG, Porter RHP, Eller RV, Greenamyre JT. Inhibition of succinate dehydrogenase by malonic acid produces an "excitotoxic" lesion in rat striatum. J Neurochem 1993;61:1151–1154

Use of Anti-Neurofilament Antibody to Identify Paired-Helical Filaments in Inclusion-Body Myositis

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Paired-helical filaments (PHFs) are an important diagnostic criterion of the inclusion-body myositis (IBM) muscle biopsy; but, until now, their presence could be identified only by electronmicroscopy. In this report, we describe an easy immunocytochemical procedure, utilizing commercially available antibody, that enables reliable identification of muscle PHFs by light microscopy. This procedure greatly facilitates diagnosis of IBM.

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Sporadic inclusion-body myositis (s-IBM) is the most common muscle disease in patients age 50 years and older [1]. Pathologic diagnostic criteria include vacuolated muscle fibers [1], congo-red positivity [2, 3], various degrees of inflammation [1], and cytoplasmic inclusions of paired-helical filaments (PHFs) 15 to 21 nm in diameter (also referred to as "tubulofilaments") [1, 4]. Because identification of PHFs requires both electronmicroscopy and considerable experience, the diagnosis of IBM is usually made in highly specialized centers.

IBM-muscle PHFs closely resemble Alzheimer's disease (AD) brain PHFs [4]. Since AD PHFs are composed of hyperphosophorylated τ [5], we studied the composition of IBM PHFs with a variety of antibodies directed against phosphorylated τ [4, 6]. We have found that the commercially available monoclonal antibody SMI-31, directed against phosphorylated neurofilament heavy chain [7] but cross-reacting with phosphorylated τ ([8, 9]; Mirabella M, et al, unpublished data), strongly and exclusively stains PHFs in IBM muscle. This, for the first time, enables their easy and highly reliable light-microscopic identification.

Even though cytoplasmic PHFs are most abundant in s-IBM and hereditary inclusion-body myopathies (h-IBMs), they can also occur in the cytoplasm of the vacuolated muscle fibers of oculopharyngeal muscular dystrophy (OPMD) [1], where they have the same characteristics as those of IBM PHFs.

Materials and Methods

We evaluated the specificity of SMI-31 for PHFs in muscle biopsies (obtained with informed consent) of 12 s-IBM, 8 h-IBM, 3 OPMD, and 16 disease- and 4 normal-control patients, including 5 with polymyositis, 4 with amyotrophic lateral sclerosis, 1 with Duchenne's muscular dystrophy, 1 with mild neuropathy with abundant tubular aggregates, 2 with morphologically nonspecific myopathies, and 3 with other vacuolar myopathies (namely, 2 with acid maltase deficiency and 1 with vacuolar myopathy of unknown cause without cytoplasmic PHFs). The light-microscopic peroxidase–antiperoxidase (PAP) staining was performed as follows: (1) Freshly cut 10- to 12-μm frozen sections were placed on Fisher Superfrost/Plus slides, dried with a room temperature (RT) hair dryer for 1 hour, and incubated with 1:10 diluted normal rabbit serum (DAKO) for 1 hour; (2) the sections were then incubated in 200 μl of 1:1,000 diluted SMI-31 antibody (Lot 10, Sternberger Monoclonals Inc, Baltimore, MD), overnight at 4°C in a humid chamber; (3) after three 5-minute phosphate-buffered-saline (PBS) rinses, sections were incubated in 1:35 dilution rabbit antiserum against mouse IgG (DAKO), for 1 hour at RT, which was followed by three 5-minute PBS rinses; (4) sections were then incubated for 1 hour at RT in monoclonal mouse PAP complex (DAKO), diluted 1:50, followed by (a) three 5-minute PBS rinses, (b) 5- to 7-minute incubation in diaminobenzidene (DAB) solution (5 mg DAB/10 ml PBS + 70 ml 3% H_2O_2), (c) three 5-minute rinses in PBS, and (d) mounting.

Results and Discussion

Strong SMI-31–immunoreactive deposits were present within the vacuoles and in the vacuole-free cytoplasm

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Mitochondrial Respiratory Chain Function in Multiple System Atrophy

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Summary: Multiple system atrophy (MSA) is a clinicopathological entity distinct from idiopathic Parkinson's disease (PD) that is responsible for 5–10% of cases of parkinsonism. Degeneration of nigral neurones is a feature of both diseases. A specific deficiency of mitochondrial complex I activity has been found in PD substantia nigra. We have analysed mitochondrial function in substantia nigra and platelets from MSA patients to identify any respiratory chain defect in this disorder and to determine its tissue specificity. As our MSA patients had been on L-DOPA, we also sought to establish whether this treatment could cause the complex I defect as seen in PD. We found no significant difference in respiratory chain activity corrected for mitochondrial mass between control and MSA patients in either of the tissues studied. These results provide a biochemical dimension to the differences between MSA and idiopathic PD. In addition, the fact that L-DOPA failed to induce a complex I defect in MSA substantia nigra suggests that this treatment is unlikely to cause the complex I deficiency in PD, without additional factors that may operate in PD. **Key Words:** Multiple system atrophy—Mitochondria—L-DOPA.

Multiple system atrophy (MSA) is a distinct sporadic adult-onset neurodegenerative disorder characterized clinically by autonomic failure, parkinsonism, cerebellar ataxia, and pyramidal signs in any combination and pathologically by cell loss and gliosis in a selection of the following structures: substantia nigra, locus ceruleus, striatum, olives, pons, cerebellum, intermediolateral cell columns, and Onuf's nucleus of the spinal cord. A characteristic cytological marker for MSA (the glial cytoplasmic inclusion) was described in 1989 (1). MSA accounts for ~10% of patients with parkinsonism in specialized brain banks and represents the most common cause of parkinsonism after Parkinson's disease (PD) (2).

The cause of neuronal death in MSA is unknown. MSA and PD share some important clinical and pathological features (Table 1), in particular, progressive nigral dopaminergic cell loss, suggesting the possibility of similar mechanisms of cell death. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to destroy substantia nigral neurones probably through its ability to inhibit mitochondrial complex I activity and generate free radicals. The discovery of complex I deficiency in the substantia nigra in PD (3,4) suggested a direct biochemical link between the idiopathic disease and the MPTP model. The relevance of this biochemical defect to the cause of dopaminergic cell death in PD is enhanced by the finding that the complex I deficiency is confined to the substantia nigra within the brain and is not present in the caudate nucleus, putamen, globus pallidus, cerebral cortex, or cerebellum (see ref. 5 for review). Activities of the other respiratory chain complexes (II/III and IV) were not significantly different from those of age-matched controls in any of the brain areas examined, including the substantia nigra. Mitochondrial respiratory chain

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TABLE 1. *Comparison of clinicopathological features of MSA and PD*

Feature	MSA	PD
Sporadic	Always	85–90%
Parkinsonism	90%	Always
Autonomic dysfunction	95%	50%[a]
Ataxia	50%	Never[b]
Extensor planters	50%	Never[b]
Good or excellent LD response	25%	Always
Nigral cell loss	95%	Always
Striatal cell loss	90%	Never[c]
OPCA	80%	Never[c]
Lewy bodies	10%[c]	Always
GCIs	Always	Never[c]

MSA, multiple system atrophy; PD, Parkinson's disease; LD, best L-DOPA response at any stage; GCIs, glial cytoplasmic inclusions; OPCA, olivopontocerebellar atrophy.

[a] Usually milder and later than in MSA.

[b] Unless due to additional pathology, e.g., stroke, cervical spondylosis, etc.

[c] A small number of MSA cases have incidental Lewy bodies and a very small number have both MSA and PD (2).

dysfunction has also been considered as a potential cause for the neuronal loss in MSA. To investigate the potential contribution of mitochondrial dysfunction to the pathogenesis of MSA and determine its tissue specificity, we investigated substantia nigra and platelet mitochondrial respiratory chain function in MSA patients and matched controls. In addition, most MSA patients are given L-DOPA, and so this disease can provide information on whether this drug can induce the complex I defect observed in PD.

METHODS

Brain Studies

At postmortem brains were divided midsagittally; half were formalin fixed for neuropathological examination, while the remaining half were frozen, initially at −20°C and subsequently at −70°C, for biochemical assay. Mitochondrial respiratory chain function was assayed in homogenates of substantia nigra from 8 control subjects and 8 MSA patients, matched for refrigeration and postmortem delay, and added to our cumulative data on 33 controls and 7 MSA patients to produce substantia nigra data on a total of 41 controls and 15 MSA patients. The cumulative control data were collected using identical assay conditions and techniques.

A pathological diagnosis of MSA was based on an appropriate clinical history (2) with the finding of cell loss and gliosis in the absence of Lewy bodies in substantia nigra and striatum with widespread oligodendroglial cytoplasmic inclusions on Bielschowsky silver impregnation or anti-ubiquitin staining.

Brain homogenates were prepared and mitochondrial respiratory chain activity and protein content determined as described elsewhere (6). NADH CoQ_1 reductase (complex I), succinate cytochrome *c* reductase (complexes II and III), cytochrome oxidase (complex IV), and citrate synthase activities were assayed in triplicate by standard techniques (6,7). The values for control (n = 22 and 19) and MSA (n = 7) complex II/III and IV activities in substantia nigra have been published previously (8,9).

Details of pharmacological treatment were available from the MSA patients whose brains were examined postmortem. Fourteen patients were known to have taken L-DOPA during their lifetime for a mean period of 4.2 years (range 1–12 years) with a mean disease duration of 6.9 years. Ten were taking L-DOPA up to the time of death, with a mean daily dose of 550 mg (range 50–1,200 mg).

Platelet Studies

Seven patients with MSA were identified according to the clinical criteria of Quinn (2). Four patients were taking L-DOPA (mean dose 400 mg/day, range 250–600 mg). MSA patients were compared with age- and sex-matched control subjects with no evidence of neurological disease. Platelet mitochondria were prepared and assays of respiratory chain enzyme activity were performed as described (10). Statistical analysis was performed using the Mann–Whitney *U* test.

RESULTS

Brain Studies

In MSA substantia nigra, there was a significant decrease in the activities of all respiratory chain enzymes, NADH CoQ_1 reductase (23%), succinate cytochrome *c* reductase (25%), and cytochrome oxidase (35%) as well as citrate synthase (28%), when enzyme results were expressed per unit total protein (Table 2).

There was severe neuronal loss and degeneration in MSA substantia nigra, which was probably reflected in a decrease in mitochondrial numbers and in the activities of all the mitochondrial enzymes measured. Thus, respiratory chain activities expressed per unit protein will be less in tissues with fewer mitochondria even though the enzymes themselves may be functioning normally. To correct for any variations

TABLE 2. *Mitochondrial respiratory chain activity in substantia nigra and platelets from control and multiple system atrophy (MSA) patients*

	n	Age (yrs)	Postmortem delay (h)	Complex I	Complex II/III	Complex IV (k/min/mg)	Citrate synthase
Substantia nigra							
Control	41	72.2 ± 14.9	18.3 ± 8.9	3.50 ± 1.27	11.9 ± 2.6 (n = 22)	1.09 ± 0.38 (n = 19)	111.9 ± 29.5
MSA	15	65.0 ± 10.3 (p = 0.015)	21.2 ± 9.4	2.69 ± 0.85 (p = 0.029)	8.9 ± 1.6 (p = 0.022) (n = 7)	0.71 ± 0.18 (p = 0.004) (n = 7)	91.5 ± 19.0 (p = 0.014)
Platelets							
Controls	22	62.1 = 13.0	—	17.8 ± 4.0	49.1 ± 16.3	2.15 ± 0.61 (n = 19)	248 ± 62
MSA	7	61.4 ± 5.4	—	16.0 ± 2.5	42.8 ± 17.4	2.39 ± 0.54 (n = 5)	232 ± 45

Results are means ± SD. Enzyme activities are in nmol/min/mg total protein ulcss otherwise stated. *k* is the first-order rate constant. Complex I is rotenone-sensitive NADH CoQ_1 reductase; complex II/III is antimycin A-sensitive succinate cytochrome *c* reductase; complex IV is cytochrome oxidase. Age refers to age at death for substantia nigra and age at venesection for platelets. Data for complex II/III and IV activities in control substantia nigra have been reported previously (9). Statistical analysis by Mann-Whitney *U* test.

in mitochondrial mass between samples, we have expressed activities as a ratio with citrate synthase (Table 3). These results do not show any significant difference from control for any of the respiratory chain enzyme activities in MSA substantia nigra.

For the substantia nigra samples, the mean age of the MSA group was significantly different from the cumulative control group age (7.2 years; $p < 0.02$). Thus, we have taken data from controls (n = 22) who match the age range of the MSA group and used these for an additional comparison (Table 4). These data show that with correction for age, there is again no statistically significant difference between the citrate synthase–corrected results for complex I in MSA brain when compared with control brain. This fits with our observation that within the age groups analysed, there is no decline of complex I activity in substantia nigra.

Figure 1 shows the relationship of substantia nigra complex I activity to the mean L-DOPA dose taken by the MSA patients (n = 14) during the last 3 months of life. There is no significant correlation between these two parameters, supporting our general observation that L-DOPA does not cause a decrease in complex I function in MSA. A similar correlation between L-DOPA dose and substantia nigra complex I activity in PD (n = 38) also failed to show any relationship (data not shown).

Platelet Studies

Mitochondrial respiratory chain function in MSA platelets did not differ significantly from control whether results were expressed per unit protein or corrected for citrate synthase concentration.

DISCUSSION

These studies have failed to identify any citrate synthase–corrected mitochondrial abnormality in MSA substantia nigra or platelets.

To our knowledge, respiratory chain function in MSA substantia nigra has not been reported by another group. Our previous smaller study also failed to show any citrate synthase–corrected mitochondrial defect in MSA substantia nigra (8). Our results in platelets are in agreement with those of Benecke et

TABLE 3. *Mitochondrial respiratory chain activity in substantia nigra homogenates and platelet mitochondrial fractions from control and multiple system atrophy (MSA) patients: corrected for citrate synthase (CS) activity*

	n	Complex I/CS (×100)	Complex II/III/CS (×10)	Complex IV/CS (×100)
Substantia nigra				
Control	41	3.21 ± 1.21	1.06 ± 0.28 (n = 22)	1.02 ± 0.56 (n = 19)
MSA	15	3.00 ± 1.01	1.09 ± 0.30 (n = 7)	0.87 ± 0.31 (n = 7)
Platelets				
Control	22	7.44 ± 1.65	2.02 ± 0.57	0.90 ± 0.24 (n = 19)
MSA	7	7.05 ± 1.00	1.82 ± 0.49	1.12 ± 0.18 (n = 5)

Values are means ± SD. Statistical analysis by Mann-Whitney *U* test.

TABLE 4. *Substantia nigra complex I activity in MSA and age-matched control subgroup*

	Control	MSA
n	22	15
Age (yrs)	62.6 ± 14.4	65.0 ± 10.3
PM delay (hrs)	16.7 ± 7.6	21.2 ± 9.4
Complex I/mg protein	3.33 ± 1.33	2.69 ± 0.85
CS	105.2 ± 30.5	91.5 ± 19.0
Complex I/CS (×100)	3.24 ± 1.34	3.00 ± 1.01

Values are means ± SD. MSA, multiple system atrophy; PM, postmortem; CS, citrate synthase. No significant differences were detected as calculated by Mann-Whitney *U* test.

al. (11) who also found no change in MSA. However, Blin et al. (12) found an ~30% complex I deficiency in skeletal muscle from their MSA patients (n = 5). We have not studied mitochondrial function in MSA skeletal muscle. The observation that complex I–IV function appears unaffected in substantia nigra—a site of major pathological change in MSA—in pathologically proven cases casts doubt on the relevance of any defect observed in other tissues in patients with a clinical diagnosis of MSA.

The results presented here are useful in the interpretation of the complex I defect observed in PD substantia nigra. As argued previously (8), normal respiratory chain function in MSA nigra, despite severe degeneration, suggests that the complex I defect in PD is not simply a reflection of neuronal loss and coexisting gliosis and must have some alternative cause.

There is debate concerning the possible contribution of L-DOPA to the nigral complex I deficiency in PD. Przedborski et al. (13) have described a reversible ~25% complex I defect in rat substantia nigra and striatum induced by L-DOPA administration. We have not found any respiratory chain deficiency in striatum from PD patients who had been treated with L-DOPA, but we and others have observed an ~37% citrate synthase-corrected complex I defect in PD nigra (8,14–16). Ten of the 15 MSA patients in whom substantia nigra was analysed postmortem in this study were known to be on L-DOPA at the time of death in quantities comparable with those used in PD patients, although the mean period of treatment in this MSA group (4.2 years) was less than might be seen in a group of PD patients. Nevertheless, with use of identical assay conditions and techniques, no complex I defect was seen in MSA. In addition, there was no statistically significant correlation between actual L-DOPA dose at the time of death and the individual's substantia nigra complex I activity. These results would suggest that in human substantia nigra, L-DOPA does not induce a complex I deficiency, at least in the context of MSA pathology.

Platelet respiratory chain function was also not significantly different from control in the four MSA patients taking L-DOPA at the time of analysis (data not shown). The failure of L-DOPA to induce a mitochondrial deficiency in platelets has been noted previously (16,17).

The absence of any detectable mitochondrial defect in MSA substantia nigra provides an additional and biochemical dimension to the clinical and pathological features that distinguish this disorder from PD. The underlying aetiology and pathogenesis of MSA, however, remain unknown.

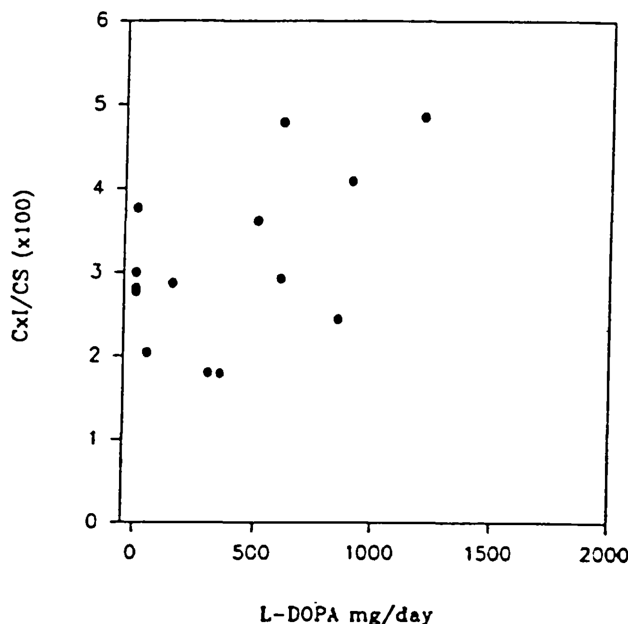

FIG. 1. Relationship between complex I (cxI)/citrate synthase (cs) ratio in the substantia nigra of patients dying with MSA and the level of L-DOPA therapy at death (Spearman r = 0.408, p = 0.148).

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REFERENCES

1. Papp MI, Kahn JE, Lantos PL. Glial cytoplasmic inclusions in the CNS of patients with multiple system atrophy (striatonigral degeneration, olivopontocerebellar atrophy and Shy Drager syndrome). *J Neurol Sci* 1989;94:79–100.
2. Quinn N. Multiple system atrophy. In: Marsden CD, Fahn

S, eds. *Movement disorders 3.* London: Butterworth-Heinemann, 1995:262–81.
3. Schapira AHV, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* 1989;1:1269.
4. Janetzky B, Hauck S, Youdim MBH, et al. Unaltered aconitase activity but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. *Neurosci Lett* 1994;169:126–8.
5. Schapira AHV. Evidence for mitochondrial dysfunction in Parkinson's disease: a critical appraisal. *Mov Disord* 1994;9:125–38.
6. Schapira AHV, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem* 1990;54:823–7.
7. Wharton DC, Tzagoloff A. Cytochrome oxidase from bovine heart mitochondria. *Methods Enzymol* 1967;10:245–57.
8. Schapira AHV, Mann VM, Cooper JM, et al. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. *J Neurochem* 1990;55:2142–5.
9. Mann VM, Cooper JM, Krige D, Daniel SE, Schapira AHV, Marsden CD. Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. *Brain* 1992;115:333–42.
10. Krige D, Carroll MT, Cooper JM, Marsden CD, Schapira AHV. Platelet mitochondrial function in Parkinson's disease. *Ann Neurol* 1992;32:782–8.
11. Benecke R, Strümper P, Weiss H. Electron transfer complexes I and IV of platelets are abnormal in Parkinson's disease but normal in Parkinson-plus syndrome. *Brain* 1993;116:1451–63.
12. Blin O, Desnuelle C, Rascol O, et al. Mitochondria respiratory failure in skeletal muscle from patients with Parkinson's disease and multiple system atrophy. *J Neurol Sci* 1994;125:95–101.
13. Przedborski S, Jackson-Lewis V, Muthane U, et al. Chronic levodopa administration alters cerebral mitochondrial respiratory chain activity. *Ann Neurol* 1993;34:715–23.
14. Mann VM, Cooper JM, Daniel SE, Jenner P, Marsden CD, Schapira AHV. Complex I, iron and ferritin in Parkinson's disease substantia nigra. *Ann Neurol* 1994;36:876–81.
15. Cooper JM, Daniel SE, Marsden CD, Schapira AHV. L-DOPA and complex I deficiency in Parkinson's disease brain. *Mov Disord* 1995;10:295–7.
16. Bravi D, Anderson JJ, Dagani F, et al. Effect of aging and dopaminomimetic therapy on mitochondrial respiratory function in Parkinson's disease. *Mov Disord* 1992;7:228–31.
17. Shults CW, Nasirian F, Ward DM, et al. Carbidopa/levodopa and selegiline do not affect platelet mitochondrial function in early parkinsonism. *Neurology* 1995;45:344–8.

Mitochondrial DNA Transmission of the Mitochondrial Defect in Parkinson's Disease

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Several groups have identified mitochondrial complex I deficiency in Parkinson's disease (PD) substantia nigra and in platelets. A search for any mitochondrial DNA (mtDNA) mutation underlying this defect has not yet produced any consistent result. We have made use of a mtDNA-less (ρ^0) cell line to determine if the complex I deficiency follows the genomic transplantation of platelet mtDNA. From a preselected group of PD patients with low platelet complex I activity, 7 patients were used for detailed study. All 7 patients were used for mixed cybrid analysis and demonstrated a selective 25% deficiency of complex I activity. Individual clonal analysis of A549 ρ^0/PD platelet fusion cybrids from 1 of the patients expressed combined complex I and IV deficiencies with 25% and 20% decreased activities in the PD clones, respectively. Histocytochemical, immunocytochemical, and cellular functional imaging studies of these clones showed the cells within the clones were heterogeneous with respect to cytochrome *c* oxidase (COX) function, COX I content, and mitochondrial respiratory chain activity. These results are in agreement with a previous study and support the proposition that an mtDNA abnormality may underlie the mitochondrial defect in at least a proportion of PD patients. This ρ^0 technology may serve as a means to identify the subgroup of PD patients in whom an mtDNA defect may contribute to development of the disease.

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Parkinson's disease (PD) is a common neurodegenerative disease, with a prevalence in the West of approximately 1 in 350.[1] The pathological substrate of the major clinical features in PD is death of dopaminergic neurons in the substantia nigra pars compacta. Genetic and environmental factors have been invoked as causal agents, but none appear directly applicable to most patients with idiopathic PD. The recent identification of a mutation in the α-synuclein gene in autosomal dominant parkinsonism[2] promises to provide valuable insight into the biochemical mechanisms that may be involved in selective dopaminergic cell death. However, the relationship of the α-synuclein defect to most patients with PD remains uncertain.

Several biochemical abnormalities have been identified in post mortem PD substantia nigra, including oxidative damage,[3–5] excess iron[6,7] and decreased mitochondrial complex I activity.[8–10] The part that these play in pathogenesis and their connection with etiology are not known. Nevertheless, identifying the cause(s) and sequence of these abnormalities may provide important clues to etiology. We and others have shown that the complex I defect in PD appears to be specific for the substantia nigra within the PD brain and is not present in multiple system atrophy,[9,11–13] another disease in which there is severe dopaminergic cell death. Several studies have identified a selective complex I deficiency in PD platelet mitochondria,[14–16] although others have failed to show this defect.[17,18] Although platelets do share some pharmacological features with dopaminergic neurons, such as monoamine oxidase activity and MPP^+ uptake,[19] a complex I defect in platelets could also represent a genetically determined deficiency that may or may not be enhanced by a toxin, endogenous or exogenous.

Several groups have sought mutations of mitochondrial DNA as a possible cause of the complex I deficiency in PD.[10,20,21] However, these have involved small numbers of unselected PD patients and no clear mutation has emerged as being relevant. An alternative strategy is to take advantage of a system whereby mtDNA from a PD patient can be placed in the novel nuclear environment of a mtDNA-less ρ^0 cell line.[22] The transmission of a mitochondrial respiratory chain defect from the donor to the resulting cybrid fusions would imply that the defect was determined by mtDNA. Conversely, abolition (complementation) of the defect would indicate nuclear control of the biochemical abnormality, a toxic influence on the original

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donor cells, or drift of mtDNA from mutant to wild type during clonal expansion.

The cybrid strategy has been used to investigate the pathogenesis of certain inborn errors of the respiratory chain causing the mitochondrial encephalomyopathies. These have included the transfer of mtDNA bearing the A3243G point mutation in the $tRNA^{Leu(UUR)}$ gene, most frequently associated with the MELAS (myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) phenotype, and the A8344G $tRNA^{Lys}$ mutation associated with MERRF (myoclonic epilepsy with ragged-red fibers). In all cases reported there was a transfer of the biochemical defect to the recipient cells.[23–25] MtDNA mutations usually occur in heteroplasmic form; ie, mutant and wild type coexist within a cell and probably within a mitochondrion. The proportion of mutant/wild-type molecules may vary both between tissues and between affected individuals. It is believed that a biochemical defect may result only when a threshold proportion of mutant mtDNA is crossed. This phenomenon, together with random segregation of mutant and wild-type molecules during embryogenesis, is thought to contribute to the variable tissue expression that characterizes the mitochondrial encephalomyopathies. We have recently taken advantage of the ρ^0 system by using A549 ρ^0 cells to demonstrate both nuclear control in mtDNA-depletion syndrome and nuclear influences on the biochemical expression of the complex I defect in cells with the 3,460–base pair (3,460 bp) mtDNA mutation of Leber's hereditary optic neuropathy.[26,27] Swerdlow and colleagues[28] have used a similar principle to show that the complex I deficiency in PD platelets appears to be determined by mtDNA. We have now used this technique together with a modified approach to study PD patients, and the proposition that mtDNA abnormalities may be responsible for the complex I defect. We have also focused our study on PD patients selected by their low platelet complex I activity, because it is our belief that an mtDNA defect may be relevant to only a proportion of PD patients. Our results support the belief that mtDNA is responsible for the complex I deficiency in at least a subgroup of PD patients.

Materials and Methods

Patient Samples

Eight PD patients and 8 age-matched controls were used for simultaneous analysis of platelet mitochondrial respiratory chain function. For the time course study, we selected 4 PD patients from this group with mean platelet complex I activities of more than one standard deviation below the control mean. Seven of the 8 patients were available for the fusion studies; age-matched and sex-matched controls were studied in parallel. One of the PD patients was selected for cybrid clonal studies. All patients satisfied the PD Brain Bank (London) criteria for idiopathic PD—akinetic rigid syndrome with asymmetric onset, resting tremor, and a good response to L-dopa.[29] None of the PD patients chosen had any family history of PD or of any documented toxin exposure, and none had any evidence of any other neurological or systemic disease. All were receiving L-dopa therapy. Hoehn and Yahr staging was II–III in all those studied.

Platelet Mitochondrial Preparation

Platelets were isolated essentially as described previously,[15] except that prostacyclin I_2 (0.07 nM final concentration) was added to the platelet-rich plasma. Platelet mitochondrial-enriched fractions were prepared by using nitrogen cavitation and mitochondrial enzyme activities, determined as described by Krige and associates,[15] at 28°C.

Cell Culture

A human lung carcinoma cell line (A549) depleted of mitochondrial DNA (ρ^0 cells) was kindly supplied by Dr I. Holt (Ninewells Hospital, Dundee, UK). A549 (ρ^0 cells were cultured in standard medium (ie, Dulbecco's modified Eagle medium) supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 30 μg/ml geneticin, 0.2 mM uridine, 2 mM glutamine, and 1 mM sodium pyruvate at 37°C in a humidified gas mixture containing 8% CO_2 for 48 hours.

A549 ρ^0–Platelet Fusions

1×10^5 to 1×10^6 A549 ρ^0 cells were centrifuged in a 20-ml conical tube (200 *g* for 5 minutes). The medium was removed from above the pellet and 2 ml of freshly isolated washed platelets (1×10^7 to 4×10^7) resuspended in 2 ml Ca^{2+}-free Dulbecco's modified Eagle medium was carefully added on top of the A549 ρ^0 cells. Cell fusions were undertaken as described by Chomyn and co-workers.[25]

Cybrids Selection and Cloning

After fusion, the cells were plated onto a 10-cm plate and allowed to recover for 3 days in standard medium. After this period, the cells were harvested and plated onto ten 10-cm plates to give a low cell density in selection medium (identical to standard medium, except 5% dialyzed fetal calf serum was used and uridine and pyruvate were omitted). In this medium, only fused cybrids survived and divided. After 3 to 4 weeks in selection medium, individual growing clones were ring-cloned and grown in standard medium.

For mixed cybrids, the procedure was identical except that the ring-cloning steps were omitted. In addition, the cybrids were cultured in selection medium until 10 plates of confluent cells were obtained and A549 ρ^0 cells were cultured in parallel for comparison.

DNA Analysis

Cells were harvested by trypsinization and centrifuged at 200 *g* for 10 minutes. The supernatant was removed and the pellet resuspended in 600 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). After centrifugation (12,000 *g* for 5 minutes), the pellet was resuspended in TE buffer and recentrifuged. The resulting pellet was resuspended in 200 μl of lysis buffer (50 mM KCl, 20 mM Tris, 2.5 mM $MgCl_2$, 0.45% [vol/vol] Tween 20, and 0.45% Nonidet P-40, pH

8.3) and 0.4 mg/ml proteinase K. This was incubated at 55°C for 20 minutes followed by addition of 100 μl of double-distilled water and a further incubation at 90°C for 10 minutes.

Polymerase Chain Reaction Amplification

A pair of primers (forward, 5′-CTA ACA CCA GCC TAA CCA GA-3′; and reverse, 5′-GGT TAG CAG CGG TGT GTG AG-3′, mismatch in bold) was used to differentiate between A549 mtDNA and the patient's mtDNA. This relies on the fact that the A549 cells have only 4 CA repeat sequences in the D-loop region, compared with the more common 5 CA repeat sequences. Polymerase chain reaction was conducted in a final volume of 100 μl as described by Mann and collaborators.[30] The reaction conditions consisted of an initial denaturation of 94°C for 4 minutes, followed by 30 cycles of 59°C for 1 minute, 72°C for 1 minute, and 92°C for 1 minute. The reaction was followed by a final extension of 72°C for 10 minutes. Polymerase chain reaction products were digested with *AluI* and electrophoresed on 1.2% agarose gels containing 1 μg/ml ethidium bromide and visualized by using an ultraviolet transilluminator. A product of 164 bp represents the undigested fragment from mtDNA with 5 CA repeats, but when only 4 CA repeats were present (A549 mtDNA) the fragment was cleaved to 142-bp and 20-bp fragments.

Cybrid Mitochondrial Function Analysis

Mitochondrial enriched fractions were prepared from 10 confluent 10-cm plates essentially as described by Bodnar and colleagues.[26] Mitochondrial fractions were stored at −70°C for 2 to 4 days before the spectrophotometric analysis of mitochondrial function, as described,[15] at 28°C.

Cytochemistry and Immunocytochemistry

For cytochrome *c* oxidase (COX) staining, cells (2×10^4) were plated onto coverslips and grown for 24 to 48 hours. The coverslips were washed in phosphate-buffered saline (PBS), briefly air-dried, and then incubated with COX dye solution (0.04 M sodium phosphate buffer, pH 7.4, 0.5 mg/ml 3′,3-diaminobenzine, 1 mg/ml oxidized cytochrome *c*, and 2 μg/ml catalase) at 37°C for 1.5 hours. After a rinse in PBS, the cells were incubated in hematoxylin solution (Mayers) for 2.5 minutes, washed in water, and dehydrated in ethanol and xylene at room temperature before being mounted in DPX (Merck, Dagenham, UK).

For immunocytochemistry, cells seeded on glass coverslips were first cultured for 45 minutes in medium containing 2 μM MitoTracker CMXRos-H_2 (Molecular Probes, Eugene, OR), followed by culturing in standard medium for 30 minutes. Cells were subsequently washed in PBS, fixed with 4% paraformaldehyde in PBS for 20 minutes, washed, permeabilized for 15 minutes in methanol at −20°C, and washed again. Protein binding sites were blocked for 30 minutes with 10% normal goat serum in PBS at 37°C in a humidified atmosphere, followed by incubation for 45 minutes at 37°C with 4 μg of monoclonal antibody against subunit I of COX[31] (COXI) and 10 μg Hoechst 33258 dye/100 μl of PBS, and 10% normal goat serum. After washing in PBS, the coverslips were incubated with a fluorescein isothiocyanate–conjugated goat anti-mouse IgG antibody at 37°C for 45 minutes, and after further washes in PBS, coverslips were mounted onto glass slides in Citifluor–glycerol–PBS solution and analyzed by using a fluorescence microscope (Zeiss Axiophot).

To assess the mitochondrial membrane potential ($\Delta\psi_m$), the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-nidazolocarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR) was added to living cells essentially as described.[32] In brief, cells seeded on glass coverslips were cultured for 15 minutes in medium containing 3 μg/ml JC-1. Coverslips were then rinsed in standard medium, mounted onto glass slides, and fluorescence was inspected with a Zeiss Axiophot microscope.

Results

Platelet Mitochondrial Function in PD

The results of platelet mitochondrial respiratory chain function analysis for the 8 PD patients and 8 matched controls are shown in the Table and in Figure 1. The PD patients showed an overall 24% deficiency in complex I/citrate synthase (CS) activity ($p = 0.038$), whereas on a group-to-group analysis there was no statistical difference in complex II + III/CS or complex IV/CS ratios. Those 4 PD patients with the lowest complex I/CS ratios were chosen for further study. Further analyses on freshly obtained samples after 1 and 2 months showed the ratios remained at less than 1 SD of the control mean, confirming the consistency of their complex I defect with time (see Fig 1). Platelets from 7 of these patients were used for ρ^0 fusions and mixed cybrid analysis, and 1 of the 4 PD patients with the lowest complex I activities was chosen at random for extensive ρ^0 fusion clonal analysis. This pa-

Table. Respiratory Chain Function in Platelet Mitochondria Isolated from 8 Parkinson's Disease Patients and 8 Controls

	Complex I/CS (× 100)	Complex II + III/CS (× 10)	Complex IV/CS (× 100)	CS
Control (n = 8)	9.59 ± 2.12	2.50 ± 0.52	0.98 ± 0.11	195 ± 32
PD (n = 8)	7.28 ± 1.09[a]	2.40 ± 0.48	0.98 ± 0.16	181 ± 34

Data (mean ± SD values) are expressed as ratios with citrate synthase (CS), which is expressed as nanomoles per minute per milligram of protein. Statistical analysis by Mann–Whitney *U* test.

[a]$p = 0.038$.

PD = Parkinson's disease.

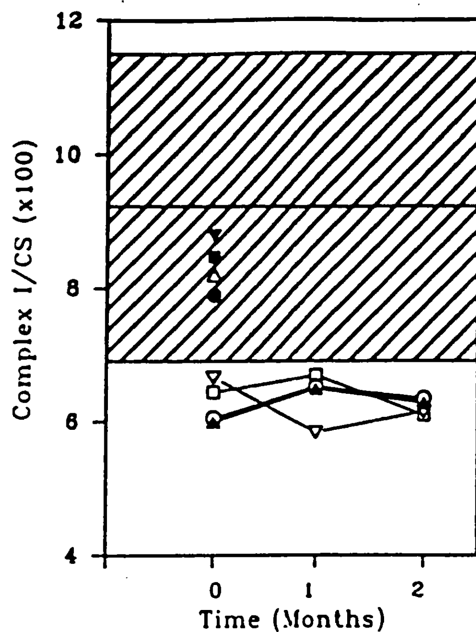


Fig 1. Complex I/citrate synthase (CS) ratio in platelet mitochondrial fractions from 8 Parkinson's disease patients, 4 of whom were studied over time. The shaded area represents control mean ± SD values; n = 8.

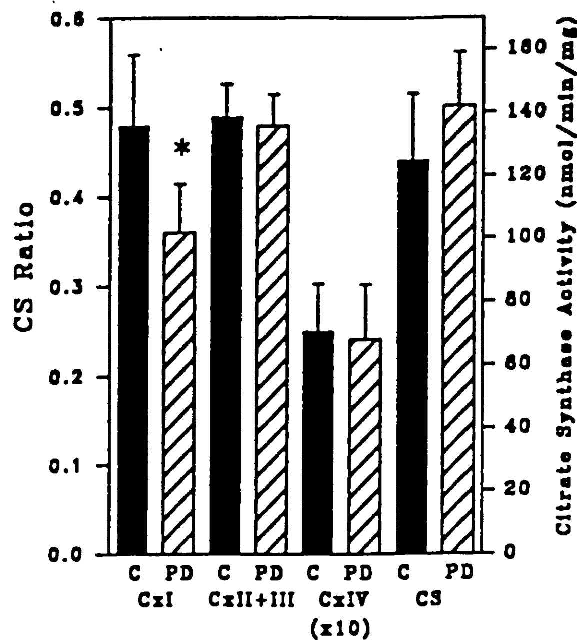


*Fig 2. Mitochondrial activities in control and Parkinson's disease (PD) platelet A549 ρ⁰ mixed cybrid lines. Complex I (CxI), complex II/III (Cx II+III), and complex IV (CxIV) activities are expressed as ratios with citrate synthase (CS) activity. Data are mean ± SD values for mixed cybrid lines from control (n = 7) and PD patients (n = 7). Mann–Whitney statistical analysis. *p = 0.007.*

tient had platelet respiratory chain CS-corrected ratios of 6.30, 2.40, and 0.74 for complexes I, II + III, and IV, respectively. Thus, both complex I and complex IV functions were decreased in this patient by 34% and 24% of the control group mean (see Table).

Mitochondrial Function in PD Mixed Cybrids

The results of respiratory chain analysis in the mixed cybrid lines from the 7 PD patients and 7 matched controls are given in Figure 2, with CS-corrected complex I results portrayed graphically in Figure 3. There was a statistically significant 25% reduction in mean complex I/CS ratios in the PD group; ratios for complexes II to IV were comparable with control values. Figure 3 shows that there was a significant correlation between platelet and mixed cybrid complex I/CS ratios (Spearman correlation, $r = 0.86$; $p < 0.001$). This suggests that the mtDNA-encoded subunits are important in determining the activity of complex I. In the cybrid studies, 5 of the 7 PD patients fell below the control range, whereas in the platelet studies, only 3 of the 7 PD patients were below the respective control range.

Mitochondrial Function in PD Cybrid Clones

Sixteen control and 16 PD clonal cybrid lines were selected from the A549 ρ^0–platelet fusion cells from 1 control and 1 PD patient and grown to provide material for respiratory chain studies. Mitochondrial enriched fractions were assayed for complex I, complex IV, and CS activity. Eight of the 16 PD cybrid clones had complex I/CS ratios of less than 1 SD below the control mean, and 10 of the clones had complex IV/CS ratios of less than 1 SD below the control mean (Fig 4). Overall there was a 25% ($p < 0.005$) reduction in complex I/CS and 20% ($p < 0.005$) in complex IV/CS ratios when the mean values of the PD clones were compared with the respective mean values of the controls.

COX cytochemistry of control cybrid clones showed activity in all cells (Fig 5A). PD cybrid clone 11 was selected for COX staining, because this clone had the lowest complex IV/CS ratio. Results showed that although some cells of this clone had normal COX staining, others showed little or no detectable activity (see Fig 5B, arrow). Mitotracker was taken up by all cells, indicating that they were all capable of generating the minimum membrane potential required to concentrate this dye (see Fig 5C and D). Immunostaining with the monoclonal antibody to the mitochondrially encoded subunit COXI however, showed that as with the COX activity stain, the control cells all stained (see Fig 5E) but there was heterogeneity in the PD clones. Some cells stained normally and others had little visible cross-reactivity (see Fig 5F, arrow). Superimposition of Mitotracker and COX I antibody stains, together with Hoechst 33258 staining of the nucleus, demonstrated a

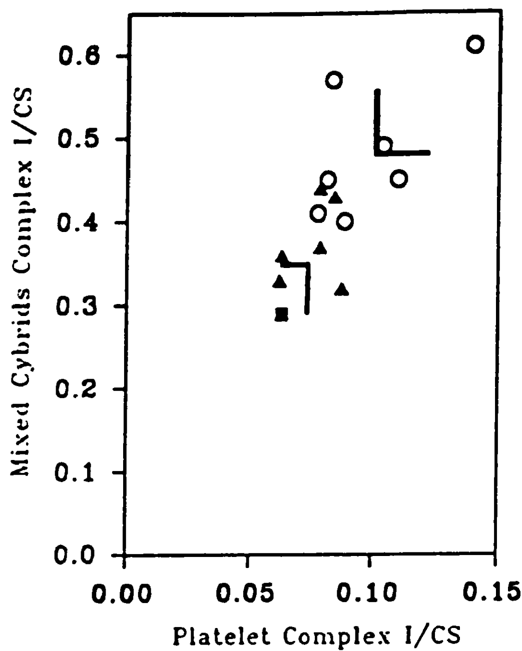
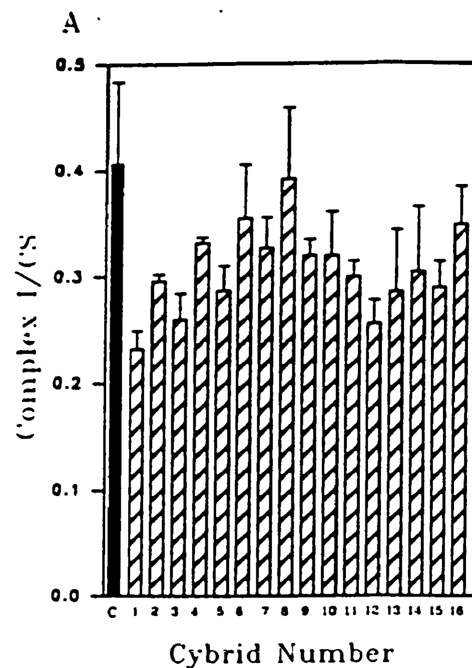


Fig 3. Relationship between the complex I (CxI)/citrate synthase (CS) ratios in the control (○) and Parkinson's disease (PD) patient (▲) platelet mitochondrial fractions and their respective CxI/CS ratios in the platelet A549 ρ^0 mixed cybrid lines. The patient studied further by clonal cybrid analysis is indicated (■). Mean ± SD values for the control and PD groups are indicated. Spearman correlation, r = 0.86; p < 0.001.

uniform staining in the control cells (see Fig 5G) but the clear heterogeneity of COXI distribution in the PD clones (see Fig 5H).

The dye JC-1 exists as a green fluorescent monomer at low concentrations. However, at higher concentrations, JC-1 forms red fluorescent "J-aggregates".[32] The accumulation of JC-1 in mitochondria depends on the membrane potential $\Delta\psi_m$. Thus, the wavelength of the fluorescence emission reflects energy state of the mitochondria and JC-1 can be considered an indirect indicator of oxidative phosphorylation capacity. Figure 6A shows that A549 parent cells accumulate JC-1 at a high level, as demonstrated by the yellow fluorescent staining of the mitochondria (green + red = yellow), indicating normal oxidative phosphorylation. The A549 ρ^0 cells accumulate JC-1 at a lower level as shown by the green fluorescent staining, reflecting the absence of a respiratory chain and oxidative phosphorylation in these cells (see Fig 6B). The A549 ρ^0/A3243G fusion cells are shown for comparison and demonstrated a heterogeneous picture with some cells fluorescing yellow and others green (see Fig 6C). The A549 ρ^0/PD platelet cybrid cells staining with JC-1 (see Fig 6D) exhibited a pattern similar to that of the A549 ρ^0/ A3243G (MELAS) fusion cells, with heterogeneity of

A



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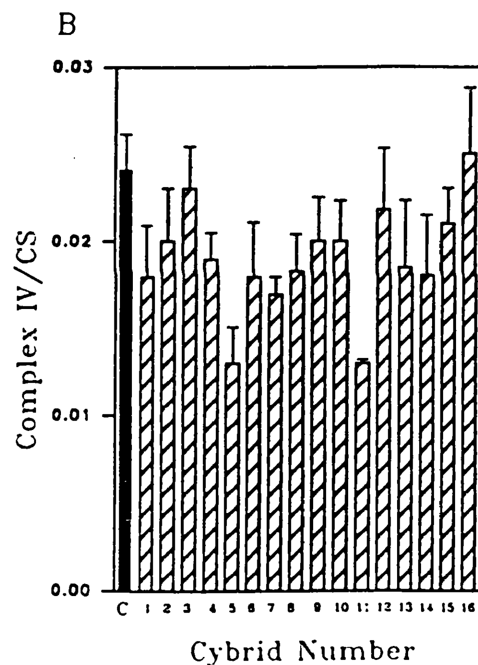

Fig 4. (A) Complex I/citrate synthase (CS) ratios. (B) Complex IV/CS ratios in A549 ρ^0/control platelet cybrid clones (C, filled column) and A549 ρ^0/Parkinson's disease (PD) platelet cybrid clones (1–16, hatched columns). Data are mean ± SD values of three separate mitochondrial preparations for each PD cybrid and mean ± SD values for 16 different control cybrids.

$\Delta\psi_m$ and, by implication, different respiratory capacity between cells. Approximately 5% of the A549 ρ^0/PD platelet cybrids were consistently seen to have very low $\Delta\psi_m$, with a greater proportion having a less severe decrease in $\Delta\psi_m$.

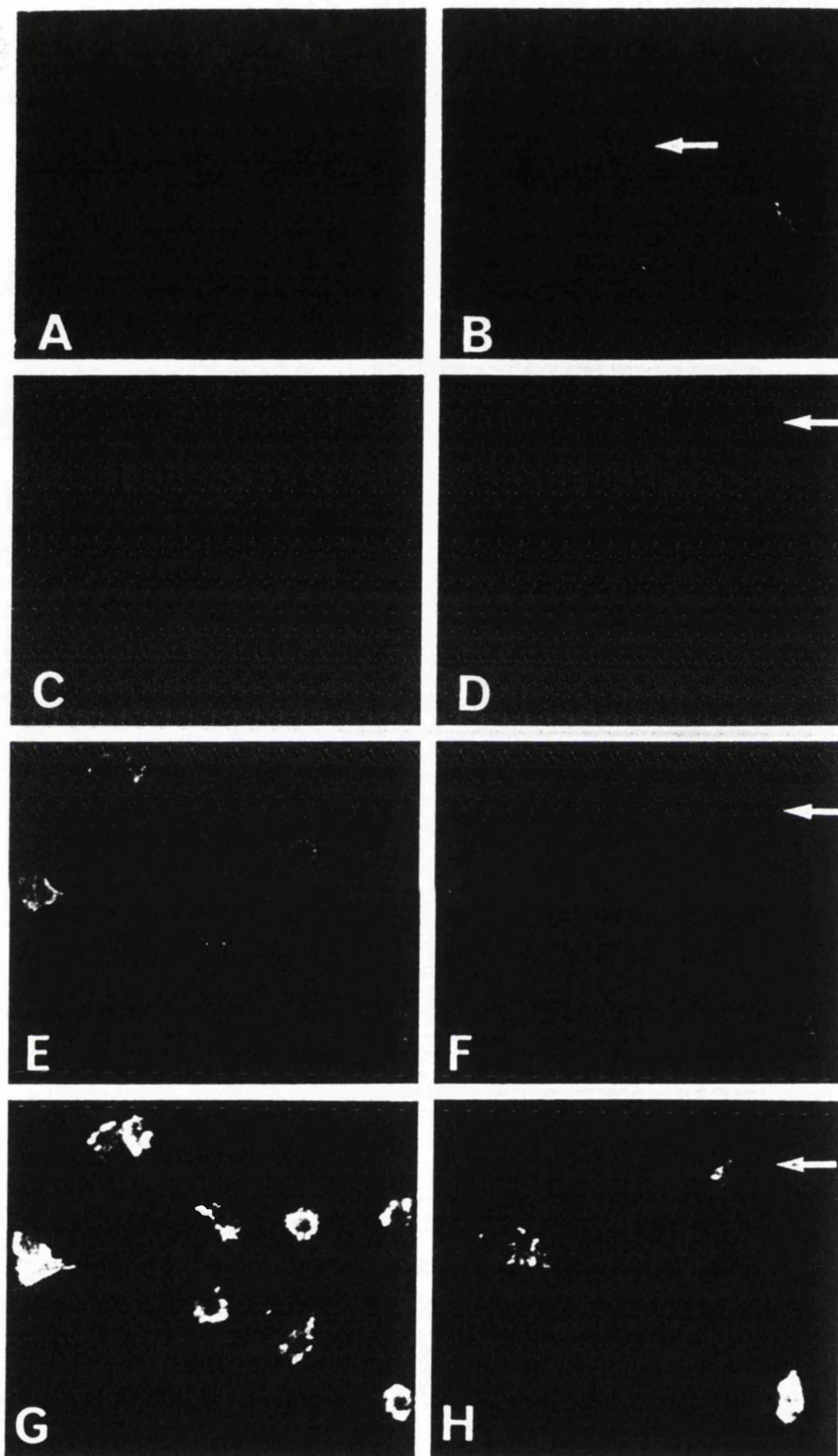

Fig 5. A549 ρ^0 control platelet cybrids (A, C, E, and G) and A549 ρ^0/Parkinson's disease platelet cybrids (B, D, F, and H) stained for cytochrome c *oxidase activity (A and B), with Mitotracker (C and D), and immunocytochemically by using anti-COXI antibody (E and F). Typical cells with decreased staining are indicated with an* arrow. *Combined images of Mitotracker, anti-COXI antibody, and Hoechst 33258 are shown in G and H. (Magnification, × 100.)*

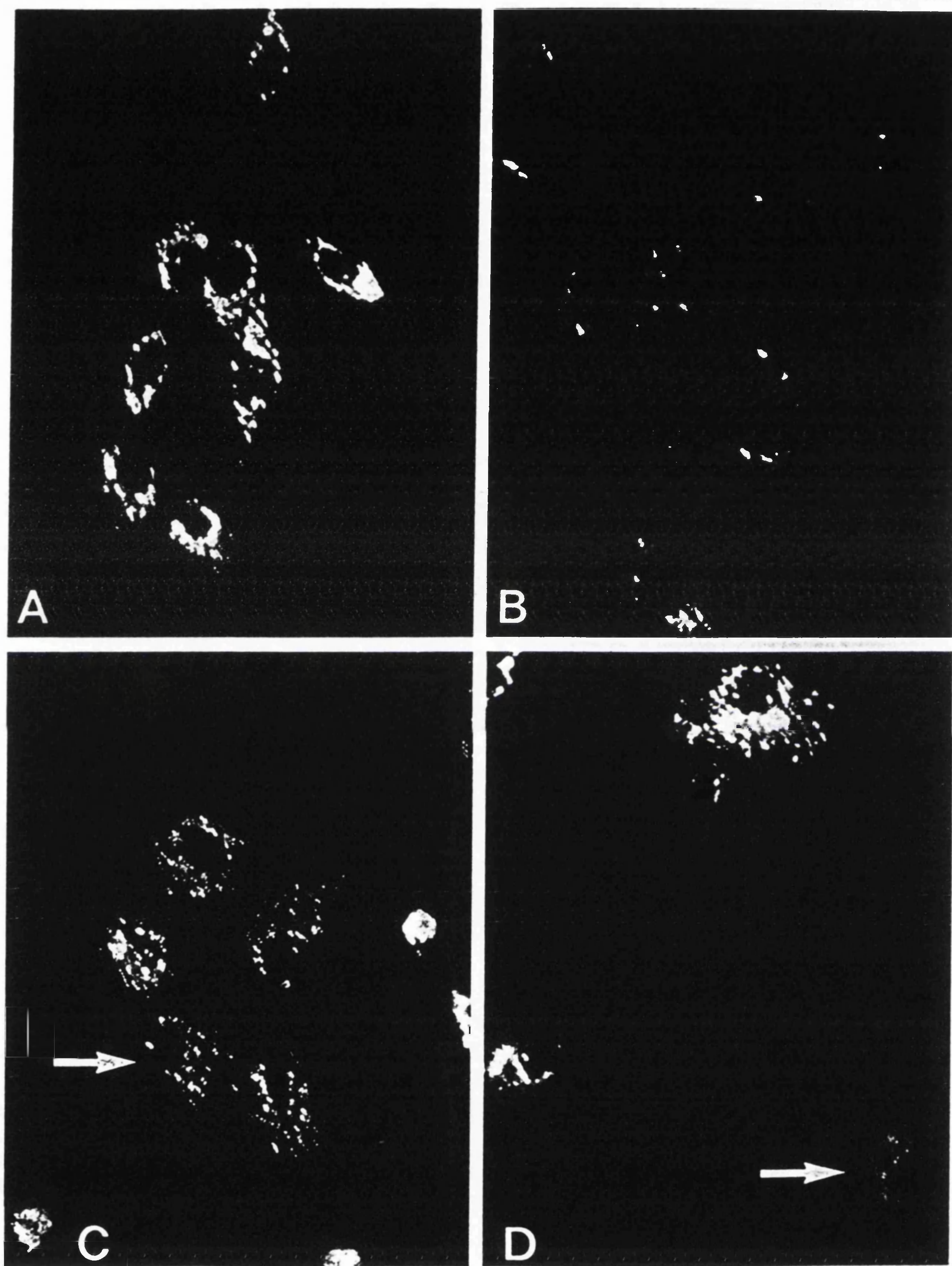

Fig 6. Assessment of mitochondrial membrane potential in A549 cells (A), A549 ρ^0 cells (B), A549 ρ^0/A3243G platelet cybrids (C), and A549 ρ^0/Parkinson's disease platelet cybrids (D) with the dye JC-1. Cell nuclei were stained blue with 2 µg/ml diaminophenylindole. Cells with low membrane potential in C and D are indicated by arrows. (Magnification, × 167.) Results are representative of three separate experiments in each case.

Discussion

The results of our PD mixed cybrids showed a 25% selective reduction of mean complex I/CS ratios. This study reproduces and extends the recently published findings of Swerdlow and colleagues[28] who, in 24 PD patients, reported a 20% selective decrease in mean complex I activities.

The PD cybrid clones generated in our experiments demonstrated significant deficiencies in the activities of complexes I and IV when compared with control cybrid clonal lines. This pattern of abnormality is the same as that seen in a A549 ρ^0/A3243G cybrid model (Gu M, Cooper JM, Taanman JW, Schapira AHV, unpublished observations). Likewise, COX and JC-1 staining of the clonal lines showed cellular heterogeneity in the PD clones, as was also demonstrated in the A549 ρ^0 cybrids containing A3243G mutant mtDNA. These observations reflect mtDNA heteroplasmy and the apparently random segregation of mtDNA mutations between cells. Each clonal line has been expanded from a single cell, and thus the same nuclear background. Exogenous toxic influences affecting platelet mitochondrial function can also be excluded as contributory factors, as these will be diluted out by clonal expansion. Thus, although we cannot exclude the transmission of an infective protein, such as is implicated in prion diseases, a defect of mtDNA is the most likely explanation for the results obtained in the PD clonal line.

The same combined deficiency (complex I and IV) was seen in the platelet mitochondria of the PD patient from whom the clonal lines were developed. Other PD patients, however, showed a more selective complex I defect. This may reflect either differing proportions of mutant mtDNA in the mixed cybrid samples or alternate molecular genetic etiologies for the mitochondrial defect in these patients.

Thus, in both the mixed cybrids and the clonal cybrid studies, a deficiency of complex I was identified. Two of the PD patients with low platelet complex I/CS ratios had complex I/CS ratios within the control range on mixed cybrid analysis. This might be because either they had a non-mtDNA–related complex I defect or the mixed cybrids did not contain sufficient mutant mtDNA to cause a biochemical defect. This question could be addressed by clonal analysis. The complex IV defect was only evident on clonal expansion in our experiments. For the A3243G mutation, we have observed that clonal lines have the potential to amplify the level of mutant mtDNA load (unpublished observations), and this may explain the emergence of the complex IV defect with this technique. This may prove an important phenomenon to investigate further those PD patients with apparently normal platelet mitochondrial function.

The pattern of respiratory chain defect in the PD cybrid clonal analysis involved a deficiency of complexes I and IV. This contrasts with known mtDNA mutations involving complex I genes such as the 3,460-bp mutation in ND1 in Leber's hereditary optic neuropathy, where platelet mitochondrial abnormalities are confined to complex I activity.[33,34] Thus, based on the data from the PD patient used for this clonal analysis, a tRNA mutation may be responsible for the mitochondrial defect. Further clonal, as opposed to mixed, cybrid analysis will determine whether this is the case for other PD patients. If so, the absence of a complex IV defect in postmortem tissues from PD patients may be explained by both methodological and biochemical factors. Assays of respiratory chain activity in substantia nigra use either tissue homogenates or mitochondrial enriched fractions. Their sensitivity is therefore less than in the mitochondrial preparations used in the analysis of our clones. Thus, a 20% complex IV deficiency may appear in the clones but be concealed in the scatter of control and PD substantia nigra data. In addition, or perhaps instead, a mild complex I defect may be amplified by biochemical and pharmacological properties local to the substantia nigra. For instance, L-dopa has been shown to produce a mild complex I defect in control rat nigra,[35] and L-dopa treatment for PD may exacerbate an underlying complex I abnormality.

The apparent sporadic nature of idiopathic PD is compatible with mtDNA involvement in a proportion of patients. History of a maternal inheritance pattern is not a sine qua non for mtDNA mutations; most patients with chronic progressive external ophthalmoplegia, those with Kearns-Sayre syndrome, and even 40% of those with the 11778 LHON mutation have no family history.[36–38] Also, a mtDNA mutation may be etiologically relevant to only a proportion of PD patients in which it may arise spontaneously or occur in a pedigree where other members are oligosymptomatic or have different clinical phenotypes of the same mutation (ie, situations commonly found in mitochondrial disorders).[14]

By using their neuroblastoma-derived ρ^0 cell line, Swerdlow and others were the first to report platelet-fusion studies to assess of the possible contribution of mtDNA to a respiratory chain defect in PD, Alzheimer's disease, and multiple system atrophy.[28,39,40] In all of these disorders, mixed cybrids have demonstrated a deficiency of respiratory chain activity, implying an mtDNA component to etiology or pathogenesis. The use of a different ρ^0 cell line in the studies described here has provided confirmation of the results in PD of Swerdlow and associates[28,39,40] and also shows that this is not a peculiarity of the neuroblastoma cell line they have used in all their studies. We have also published a study[41] on complex I activity in patients with sporadic

dystonia showing a comparable defect to that seen in PD platelets. We have also used sporadic dystonia patients in mixed fusion and clonal studies with A549 ρ^0 cells but found no transmission of the complex I deficiency under these circumstances.[42] These results provide a useful negative disease control for A549 ρ^0 cell fusions in PD. Furthermore, we have also transferred the respiratory chain defect associated with the A3243G mtDNA mutation to A549 ρ^0 cells, indicating that this nuclear background can discriminate between mtDNA and non-mtDNA causes of complex I deficiency.

These experiments establish further a potential role for mtDNA in PD. Additional work must be undertaken to understand the place of mtDNA mutations (inherited or somatic) or mtDNA polymorphisms in PD etiology. We have predicted that low platelet complex I activity (possibly combined with low complex IV activity) may be a marker for those patients in whom an mtDNA sequence change may be relevant. The mtDNA of these patients must be sequenced to define a haplotype that may be common to this group. Our theory also predicts that PD patients with platelet complex I activity in the high normal range may not show any abnormality on clonal or mixed cybrid studies, unless their "normal" complex I activity is the result of a low level of mutant mtDNA in platelets.

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References

1. Román GC, Zhang Z-X, Ellenberg JH. The neuroepidemiology of Parkinson's disease. In: Ellenberg JH, Koller WC, Langston JW, eds. Etiology of Parkinson's disease. New York: Marcel Dekker, 1995:203–343
2. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease. Science 1997;276:2045–2047
3. Dexter DT, Carter CJ, Wells FR, et al. Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. J Neurochem 1989;52:381–389
4. Sanchez-Ramos J, Övervik E, Ames BN. A marker of oxyradical-mediated DNA damage (8-hydroxy-2'deoxyguanosine) is increased in nigro-striatum of Parkinson's disease brain. Neurodegeneration 1994;3:197–204
5. Olanow CW. A radical hypothesis for neurodegeneration. Trends Neurosci 1993;16:439–444
6. Riederer P, Sofic E, Rausch WD, et al. Transition metals, ferritin, glutathione and ascorbic acid in parkinsonian brains. J Neurochem 1989;52:515–520
7. Dexter DT, Wells FR, Lees AJ, et al. Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. J Neurochem 1989;52:1830–1836
8. Schapira AHV, Cooper JM, Dexter D, et al. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 1990; 54:823–827
9. Janetzky B, Hauck S, Youdim MBH, et al. Unaltered aconitase activity but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. Neurosci Lett 1994;169:126–128
10. Schapira AHV. Evidence for mitochondrial dysfunction in Parkinson's disease: a critical appraisal. Mov Disord 1994;9:125–138
11. Schapira AHV, Mann VM, Cooper JM, et al. Anatomic and disease specificity of NADH CoQ_1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 1990;55:2142–2145
12. Cooper JM, Daniel SE, Marsden CD, Schapira AHV. L-Dihydroxyphenylalanine and complex I deficiency in Parkinson's disease brain. Mov Disord 1995;10:295–297
13. Gu M, Gash MT, Cooper JM, et al. Mitochondrial respiratory chain function in multiple system atrophy. Mov Disord 1997; 12:418–422
14. Parker WD, Boyson SJ, Parks JK. Abnormalities of the electron transport chain in idiopathic Parkinson's disease. Ann Neurol 1989;26:719–723
15. Krige D, Carroll MT, Cooper JM, et al. Platelet mitochondrial function in Parkinson's disease. Ann Neurol 1992;32:782–788
16. Haas RH, Nasirian F, Nakano K, et al. Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. Ann Neurol 1995;37:714–722
17. Bravi D, Anderson JJ, Dagani F, et al. Effect of aging and dopaminomimetic therapy on mitochondrial respiratory function in Parkinson's disease. Mov Disord 1992;7:228–231
18. Blake CI, Spitz E, Leehey M, et al. Platelet mitochondrial respiratory chain function in Parkinson's disease. Mov Disord 1997;12:3–8
19. Da Prada M, Cesura AM, Launay JM, Richards JG. Platelets as a model for neurones? Experientia 1988;44:115–126
20. Shoffner JM, Brown MD, Torrino A, et al. Mitochondrial DNA variants observed in Alzheimer's disease and Parkinson's disease patients. Genomics 1993;17:171–184
21. Ozawa T, Tanaka M, Ino H, et al. Distinct clustering of point mutations in mitochondrial DNA among patients with mitochondrial encephalomyopathies and with Parkinson's disease. Biochem Biophys Res Commun 1991;176:938–946
22. King MP, Attardi G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science 1989;246:500–503
23. Chomyn A, Martinuzzi A, Yoneda M, et al. MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. Proc Natl Acad Sci USA 1992;89:4221–4225
24. Dunbar DR, Moonie PA, Zeviani M, Holt IJ. Complex I deficiency is associated with 3243G:C mitochondrial DNA in osteosarcoma cell cybrids. Hum Mol Genet 1996;5:123–129
25. Chomyn A, Lai ST, Shake R, et al. Platelet-mediated transformation of mtDNA-less human cells: analysis of phenotypic variability among clones from normal individuals and complementation behaviour of the $tRNA^{Lys}$ mutation causing myoclonic epilepsy and ragged red fibers. Am J Hum Genet 1994; 54:966–974
26. Bodnar AG, Cooper JM, Holt IJ, et al. Nuclear complementation restores mtDNA levels in cultured cells from a patient with mtDNA depletion. Am J Hum Genet 1993;53:663–669
27. Cock HR, Tabrizi SJ, Cooper JM, Schapira AHV. The influence of nuclear background on the biochemical expression of 3460 Leber's hereditary optic neuropathy. Ann Neurol 1998; 44:187–193
28. Swerdlow RH, Parks JK, Miller SW, et al. Origin and functional consequences of the complex I defect in Parkinson's disease. Ann Neurol 1996;40:663–671

29. Hughes AJ, Daniel SE, Blankson S, Lees AJ. A clinicopathologic study of 100 cases of Parkinson's disease. Arch Neurol 1993;50:140–148
30. Mann VM, Cooper JM, Schapira AHV. Quantitation of a mitochondrial DNA deletion in Parkinson's disease. FEBS Lett 1992;299:218–222
31. Taanman J-W, Burton MD, Marusich MF, et al. Subunit specific monoclonal antibodies show different steady-state levels at various cytochrome-c oxidase subunits in chronic progressive external ophthalmoplegia. Biochim Biophys Acta 1996:1315:199–207
32. Reers M, Smiley ST, Mottola-Hartshorn C, et al. Mitochondrial membrane potential monitored by JC-1 dye. In: Attardi GM, Chomyn A, eds. Methods in enzymology, vol 260: mitochondrial biogenesis and genetics. San Diego: Academic Press, 1995:406–417
33. Howell N, Bindoff LA, McCullough DA, et al. Leber's hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees. Am J Hum Genet 1991;49:939–950
34. Smith PR, Cooper JM, Govan GG, et al. Platelet mitochondrial function in Leber's hereditary optic neuropathy. J Neurol Sci 1994;122:80–83
35. Przedborski S, Jackson-Lewis V, Muthane U, et al. Chronic levodopa administration alters cerebral mitochondrial respiratory chain activity. Ann Neurol 1993;34:715–723
36. Petty RKH, Morgan-Hughes JA. Mitochondrial myopathy: a genetic study of 71 cases. J Med Genet 1988;25:528–535
37. Zeviani M, Moraes CT, DiMauro S, et al. Deletions of mitochondrial DNA in Kearns-Sayre syndrome. Neurology 1988;38:1339–1346
38. Harding AE, Sweeney MG, Govan GG, Riordan-Eva P. Pedigree analysis in Leber hereditary optic neuropathy families with a pathogenic mtDNA mutation. Am J Hum Genet 1995;57:77–86
39. Davis RE, Miller S, Herrnstadt C, et al. Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. Proc Natl Acad Sci USA 1997;94:4526–4531
40. Swerdlow RH, Parks JK, Wooten GF, et al. As in Parkinson's disease, a bioenergetic defect transfers with mitochondrial DNA of patients with multisystem atrophy. Mov Disord 1997;12(Suppl 1):3
41. Schapira AHV, Warner T, Gash MT, et al. Complex I function in familial and sporadic dystonia. Ann Neurol 1997;41:556–559
42. Tabrizi SJ, Cooper JM, Schapira AHV. Mitochondrial DNA in focal dystonia: a cybrid analysis. Ann Neurol 1998;44:258–259

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Mitochondrial function, GSH and iron in neurodegeneration and Lewy body diseases

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Abstract

The cause of neuronal loss in patients with idiopathic Parkinson's disease is unknown. Oxidative stress and complex I deficiency have both been identified in the substantia nigra in Parkinson's disease but their place in the sequence of events resulting in dopaminergic cell death is uncertain. We have analysed respiratory chain activity, iron and reduced glutathione concentrations in Parkinson's disease substantia innominata and in the cingulate cortex of patients with Parkinson's disease, Alzheimer's disease and dementia with Lewy bodies to investigate their association with neuronal death and Lewy body formation. No abnormalities of mitochondrial function, iron or reduced glutathione levels were identified in Parkinson's disease substantia innominata or cingulate cortex. Mitochondrial function also appeared to be unchanged in cingulate cortex from patients with Alzheimer's disease and from patients with dementia with Lewy bodies, however, iron concentrations were mildly increased in both, and reduced glutathione decreased only in Alzheimer's disease. These results confirm the anatomic specificity of the complex I deficiency and decreased levels of reduced glutathione within the Parkinson's disease brain and suggest that these parameters are not associated with cholinergic cell loss in Parkinson's disease nor with Lewy body formation in this or other diseases. We propose that our data support a 'two-hit' hypothesis for the cause of neuronal death in Parkinson's disease.
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Keywords: Mitochondria; Parkinson's disease; Free radicals; Iron; Lewy body

1. Introduction

The aetiology of Parkinson's disease (PD) remains undefined. The recent identification of a mutation in the α-synuclein gene in certain families with autosomal dominant parkinsonism provides the basis for identifying the biochemical mechanisms that may be involved in selective dopaminergic cell death, at least in these families [27]. No environmental agent has been associated with idiopathic PD, although rare events, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity have provided some clues as to potential nigral toxins. Several biochemical abnormalities have been identified in PD brain which may be important in pathogenesis. These include mitochondrial complex I deficiency, oxidative stress and damage, and excess iron. The cause(s) of these abnormalities, their inter-relationship, sequence of development and precise role in the cause of dopaminergic cell death in PD are at present uncertain and are the subject of much research. To date, biochemical studies have focused on the substantia nigra, as this is a site of severe neuronal loss in PD and its degeneration is thought to be the cause of the major clinical features of this disorder. However, other areas of the central nervous system are also affected in the pathology of PD, and any hypothesis of aetiology and pathogenesis must encompass this observation. For instance, the substantia innominata is the site of cholinergic

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cell loss and Lewy body formation in PD. If the cause of the cell loss and Lewy body formation in the innominata and the nigra were the same, and if mitochondrial dysfunction and oxidative stress are involved, then it would be expected that the same biochemical abnormalities should exist in both tissues. Lewy body formation also occurs in the cingulate cortex of Alzheimer's disease (AD) patients with dementia with Lewy bodies type (DLB), although without the profound cell loss seen in the innominata or nigra of PD. Thus, the cingulate cortex provides a useful internal 'control' to judge if Lewy body formation per se is associated with complex I deficiency and oxidative stress. In contrast, the cingulate cortex of AD patients shows neuronal loss but few or no Lewy bodies, thereby providing the means to dissect further the relationship between the pathology of neuronal degeneration, Lewy body formation and the biochemistry of PD. We have therefore undertaken comprehensive mitochondrial respiratory chain analysis and measurement of reduced glutathione (GSH) and iron levels in substantia innominata from patients with PD to provide some insight into the link between these parameters and the nigral pathology of PD. These results are compared with those from cinculate (PD, AD, DLB) and temporal (AD) cortex.

We also present our cumulative data on complex I activity in PD and control substantia nigra and its relationship to L-dopa intake to investigate further its possible role in pathogenesis.

2. Methods

2.1. Tissue selection

Patients and control samples were matched for age and post-mortem delay. Brains were divided post-mortem; one hemibrain was fixed, the other stored at −70°C until used for biochemical analysis. Pathological diagnoses were made on the fixed hemibrains and were based on established criteria [13].

2.2. Mitochondrial function

Samples from controls and patients were homogenised and assays were undertaken in triplicate and in parallel, as previously described [9,32]. Protein was measured by the technique described by Lowry et al. [18].

2.3. Determination of total iron levels

Total iron was measured by inductively coupled plasma spectroscopy in solubilized brain tissues according to the technique described by Dexter et al. [5].

2.4. Measurement of reduced glutathione

GSH was measured in all tissues except cingulate cortex using the derivatization reaction between GSH and iodoacetic acid to produce *S*-carboxymethyl derivatives, which are subsequently detected by high-performance liquid chromatography with ultraviolet detection, according to the method of Sian et al. [34]. Cingulate cortex tissues were assayed using electrochemical detection according to the method of Stein et al. [36]. Briefly, tissues were homogenized in 0.25 M perchloric acid and separated using a thoroughly degassed mobile phase containing 96.5% monochloroacetic acid (MCA) buffer (0.1 M MCA; 3.3 mM heptane sulfonic acid, adjusted to pH 2.6 with sodium hydroxide), 3% methanol and 0.5% *N,N*-dimethylformamide and a 50×4.6 mm ODS analytical column. Oxygen was thoroughly excluded from the system, as this leads to oxidation of thiols. Detection was by a dual gold/mercury amalgam electrode.

3. Results

These are given in Tables 1–3. Respiratory chain activities are expressed per unit protein and per unit citrate synthase (CS) to correct for any variation in mitochondrial mass.

PD substantia innominata (Table 1) showed no significant difference from controls in mitochondrial function, iron or GSH values.

In a separate analysis, GSH levels were measured in substantia innominata from AD brains (n=8; age,

Table 1
Substantia innomominata: Mitochondrial activity, iron and GSH levels in PD and matched controls

	Control	PD
n	11	12
Age (years)	71.6±10.5	78.6±7.40
Post-mortem delay (h)	17.4±12.4	20.2±11.7
Complex I	3.41±0.67	3.38±1.22
Complex II/III	15.1±7.08	14.9±5.40
Complex IV	0.93±0.38	0.86±0.19
Citrate synthase (CS)	150±40.7	126±30.4
Complex I/CS (×100)	2.46±0.91	2.72±0.80
Complex II–III/CS (×10)	1.00±0.28	1.19±0.31
Complex IV/CS (×100)	0.62±0.13	0.70±0.16
Iron	6.99±1.63 (n=10)	8.96±2.95 (n=11)
GSH	0.51±0.19 (n=8)	0.66±0.24 (n=8)

Enzyme activities are expressed as nmol/min/mg protein, except for cytochrome oxidase (complex IV), which is k/min/mg, where k is the first order rate constant.
Values are expressed as mean±S.D.
Figures in parenthesis are sample numbers where they differ from the rest of the column.
Iron and GSH results were measured as µmol/g dry weight and wet weight, respectively.
No significant difference from controls was found for any values, as judged by the Mann-Whitney U test.

Table 2
Cingulate cortex: Mitochondrial activity, iron, and GSH levels in PD, AD, DLB and matched controls

	Control	PD	AD	DLB
n	10	10	10	10
Age (years)	82.2±4.9	76.2±7.4	80.9±6.0	78.9±6.6
Post-mortem delay (h)	46.1±17.0	39.2±19.7	34.1±17.6	35.1±22.6
Complex I	4.34±1.89	3.41±1.15	3.31±1.17	3.66±1.54
Complex II/III	11.3±4.98	13.7±6.38	11.0±5.43	12.0±6.43
Complex IV	0.93±0.50	1.29±0.78	0.80±0.37	0.84±0.42
Citrate synthase (CS)	186±70.6	158±61.1	139±48.6	150±49.5
Complex I/CS (×100)	2.47±0.86	2.42±0.89	2.51±0.70	2.44±0.55
Complex II–III/CS (×10)	0.72±0.32	0.80±0.39	0.81±0.27	0.81±0.31
Complex IV/CS (×100)	0.48±0.18	0.67±0.24	0.55±0.15	0.53±0.21
Iron	3.35±0.62 (*n*=11)	2.93±0.84 (*n*=13)	4.72±1.35[a] (*n*=12)	4.51±1.38[a] (*n*=11)
GSH	0.97±0.35 (*n*=11)	0.93±0.50 (*n*=11)	0.49±0.25[a]	0.75±0.29 (*n*=12)

Enzyme activities are expressed as nmol/min/mg protein, except for cytochrome oxidase (complex IV) which is k/min/mg, where k is the first order rate constant.
Iron and GSH results were measured as μmol/g dry weight and wet weight, respectively.
Values are expressed as mean±S.D.
Figures in parentheses are sample numbers when they differ from $n=10$.
[a] $P<0.05$ Krushal-Wallis non-parametric ANOVA test.

79.1±8.7 years; post-mortem delay, 27.6±16.0 h) and compared with matched controls ($n=5$; age, 74.6±10.8 years; post-mortem delay, 31.4±10.5 h). The GSH levels were significantly decreased in the AD samples (control, 0.73±0.18 μmol/g; AD, 0.43±0.20 μmol/g; $P<0.05$).

Mitochondrial function, GSH and iron levels in PD cingulate cortex (Table 2) were not significantly different from those of controls. In AD cingulate cortex, mitochondrial function was not significantly different from that of controls, but mean iron levels were increased (41%, $P<0.05$) and mean GSH levels were decreased (49%, $P<0.05$). In DLB cingulate cortex, mitochondrial function again appeared to be unaffected, whilst iron concentration was significantly elevated (35%, $P<0.05$), but GSH levels remained comparable to those of controls. The additional samples studied for iron and GSH levels did not result in any significant change in age or post-mortem (PM) delay with the control group (data not shown). AD temporal cortex (Table 3) showed a significant decrease in the activities of complexes II/III, with a mean reduction of 25%, whether expressed per unit protein or corrected for CS activity. Individual analyses of the activities of complex II and III, however, were not significantly different from those of controls.

Table 3
Temporal cortex: Mitochondrial activity in AD and matched controls

	Control	AD
n	9	10
Age (years)	75.9±12.7	76.1±11.9
Post-mortem delay (h)	41.0±4.3	52.0±27.0
Complex I	11.27±2.08 (8)	10.05±2.67
Complex II	18.57±3.45	17.50±5.70
Complex III	6.33±1.55	5.16±1.62
Complex II/III	23.40±5.10 (8)	17.60±5.10 (9)[a,b]
Complex IV	0.84±0.26 (8)[b]	0.77±0.36 (9)[b]
Citrate synthase (CS)	156.0±24.2[b]	140.0±19.8[b]
Complex I/CS (×100)	7.67±1.68 (8)	6.82±2.02
Complex II/CS (×100)	12.5±3.4	11.8±4.7
Complex III/CS (×100)	4.2±0.8	3.5±1.3
Complex II–III/CS (×10)	15.9±3.4 (8)	11.9±3.9 (9)[a]
Complex IV/CS (×100)	0.56±0.16 (8)[b]	0.52±0.22 (9)[b]

Enzyme activities are expressed as nmol/min/mg protein, except for cytochrome oxidase (complex IV), which is k/min/mg, where k is the rate constant. Values are expressed as mean±S.D.
[a] $P<0.05$ by Mann-Whitney U test.
[b] see Ref. [3]. Figures in parentheses are sample numbers when they differ from $n=9$ and $n=10$ for controls and AD, respectively.

4. Discussion

Mitochondrial complex I deficiency [14,19,32,33], low GSH levels [26,34,35] and increased iron concentrations [7,12,30] have been described in PD substantia nigra. Table 4 shows our cumulative data to date on complex I activity in PD substantia nigra homogenates and affirms, at a significant level, the specific complex I defect we have observed previously. The present study was undertaken to address the relationship of these abnormalities to neuronal loss and Lewy body formation.

Table 4
Substantia nigra: Mitochondrial activity in PD and matched controls

	Control	PD
n	42	43
Age (years)	72.2±14.9	76.6±7.2
Post-mortem delay (h)	18.5±8.9	15.4±9.1
Complex I	3.50±1.26	2.45±0.78[b]
Citrate synthase (CS)	113.0±30.6	110.0±39.1
Complex I/CS (×100)	3.19±1.22	2.40±0.95[a]

Enzyme activities are expressed as nmol/min/mg protein or as the citrate synthase ratio.
[a] $P<0.005$, [b] $P<0.0001$. Values are expressed as mean±S.D.
Statistical significance was determined by the Mann-Whitney U test.

There were no significant alterations from controls in PD substantia innominata for mitochondrial function, iron levels or GSH values. These results emphasise the apparent specificity for the decrease in complex I activity and GSH levels for the PD substantia nigra within the brain and imply that these biochemical abnormalities are not involved in the cholinergic neuronal degeneration and Lewy body formation of the PD substantia innominata. The normal levels of complex I and GSH in DLB cingulate cortex further support the lack of association between Lewy body formation and decreases in these parameters.

The data presented here have implications for our understanding of the role of mitochondrial dysfunction and oxidative stress in the pathogenesis of dopaminergic cell death in PD substantia nigra. The anatomical selectivity of neuronal loss underlies the clinical features of PD and distinguishes it from other parkinsonian syndromes, such as multiple system atrophy. The PD substantia nigra, and the pars compacta in particular, have high concentrations of iron, an imbalance of the iron–ferritin ratio [6,20], increased superoxide dismutase activity [31], low complex I activity and decreased GSH levels. Our results would suggest that additional, as yet unidentified, biochemical abnormalities must be responsible for cell death in the PD substantia innominata and, presumably, for the intense Lewy body formation in the substantia nigra. The specificity of the combined mitochondrial and GSH abnormalities for the nigra in PD suggests that they are a consequence of some particular properties of this area. We propose that these observations may support a 'two-hit' hypothesis for PD.

It has been argued that the aetiology of PD may be genetic or environmental, although, as has been noted in the Section 1, no conclusive data are yet available to support either of these as being dominant in the majority of sporadic PD patients. However, we suggest that one or other of these may be involved as a background effect upon which the other is superimposed, to induce severe dopaminergic cell loss in the substantia nigra. For instance, a genetic or environmental insult could induce cell loss and Lewy body formation in the nigra, innominata and locus ceruleus. The profound cell loss of the substantia nigra pars compacta is then induced by an additional, but alternate, cause, either genetic or environmental (endogenous or exogenous), to produce the clinical features of PD. This hypothesis would suggest that some individuals may be exposed to only one of the two 'hits' and, therefore, not develop PD, but would bear the pathological consequences of their respective exposure. Thus 'one-hit' brains would show, at one end of the spectrum, either mild but diffuse neuronal cell loss (substantia nigra, substantia innominata, locus ceruleus) with Lewy body formation or, at the other extreme, selective substantia nigral cell loss.

We have recently identified a group of brains from individuals with no history of PD during life, but which exhibited dopaminergic neuronal loss in the substantia nigra zona compacta, substantia innominata and locus ceruleus, in addition to Lewy bodies in surviving neurones. We suggested that this group may represent either presymptomatic PD or the brains of patients exposed to one or other, but not to both, components of the two-hit hypothesis [15]. These brains showed a mean 35% decrease ($P<0.05$) in nigral GSH levels; although complex I activity was decreased by 29%, this failed to reach statistical significance [8]. Iron and ferritin levels in these brains were not significantly different from those of controls. At present, we are still unable to distinguish whether these brains are presymptomatic or 'one-hit', but they should form an important test bed for our hypothesis.

There is increasing evidence that oxidative stress and damage and mitochondrial dysfunction may play a role in the pathogenesis of AD. Increased levels of 8-hydroxy-2-deoxyguanosine have been found in AD parietal cortex, but not cerebellum, frontal or temporal cortex [21]. Elevated levels of thiobarbituric acid-reactive substances, malondialdehyde and protein carbonyls, and enhanced activities of glutathione peroxidase, glutathione reductase and catalase all suggest free radical production and damage [1,11,17]. These changes are not present uniformly throughout the AD brain. Our findings of depressed levels of GSH in AD cingulate cortex and AD substantia innominata support these observations.

There have also been several reports of mitochondrial abnormalities in AD brain involving complexes II/III and IV [2,23,25,29], although the complex IV deficiency at least appears to be restricted to certain areas [16]. Our results showing reduced complex II/III activities in AD temporal cortex support the suggestion of a mitochondrial abnormality in AD, although its relationship to pathogenesis is unknown. The normal activities of complexes II and III, when assayed separately, might suggest that the decrease in the combined II/III activity may be the result of an abnormality of membrane function, although we have no data to confirm this. It is interesting to contrast the specific complex I deficiency, anatomically selective for the substantia nigra in PD, with the more comprehensive respiratory chain defect present in several areas of the AD brain. In this context, it is worthy to note that, whereas oxidative stress might induce multi-enzyme complex failure of the respiratory chain, it has not been shown to be capable of producing a selective complex I deficiency [10,37].

In summary, our cumulative data on PD substantia nigra show a highly significant defect in complex I activity. Previous studies in our own and other laboratories have indicated that, within the brain at least, this deficiency is confined anatomically to the substantia nigra. The absence of any mitochondrial defect in PD substantia innominata or cingulate cortex in the present study further supports this conclusion. The reduction in levels of GSH found in PD substantia nigra also appears to be selective for this brain area, again with unaltered GSH concentrations in PD

substantia innominata and cingulate cortex. Thus, the complex I deficiency and decrease in GSH levels are linked, at least in anatomical distribution. The implication is that one may cause the other, or both are the result of a more proximate event.

We have argued that the normal mitochondrial function and GSH levels of PD substantia innominata suggest that complex I deficiency and oxidative stress do not contribute to either cholinergic cell death or Lewy body formation in this area. The latter proposition is further supported by normal mitochondrial function and GSH levels in DLB cingulate cortex, an area rich in Lewy bodies. Selective complex I deficiency is specific to the neurodegeneration of the PD substantia nigra and is not found in Huntington's disease (HD) caudate nucleus [9] or AD cortex, implying that it is not simply a by-product of cell death. Thus, the complex I defect and GSH reduction in PD may either be a consequence of the biochemistry and pharmacology of the substantia nigra or the result of a pathogenetic mechanism that is separate from that involving the substantia innominata. Therefore, the mitochondrial defect and oxidative stress may be insufficient to explain the aetiology of PD, but they constitute the 'second hit' which focuses the attack of the aetiology of the disease on the nigra.

Several studies have looked at the potential effect of L-dopa therapy on complex I activity. In vitro studies are conflicting, with some showing an inhibitory action of dopamine on complex I [28], whilst others show no effect [4,22,24]. L-Dopa has been noted to cause a reversible inhibition of striatal and nigral complex I activity in rats [28]. In humans, however, we observed no effect of L-dopa on complex I activity in patients with multiple system atrophy (MSA) [33]. Furthermore, we have correlated individual L-dopa dose and substantia nigral complex I activity in PD patients and found no relationship (Fig. 1).

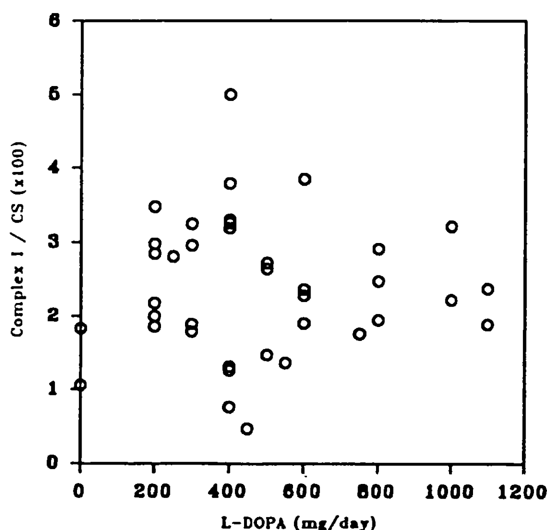

Fig. 1. Plot of mean L-dopa dose during the last three months of life against post-mortem citrate synthase-corrected complex I activity in 39 patients with PD.

Thus, we would suggest that, in PD and MSA patients at least, L-dopa does not have any deleterious effect on complex I activity.

The MPTP model produces nigral cell loss and complex I deficiency without significantly affecting other areas outside the substantia nigra, e.g. substantia innominata. An alternative toxin with similar uptake and conversion pathways (e.g. isoquinolines, β-carbolines) targeting complex I of nigral neurones would also result in selective loss in this area. If such a toxin were to be involved in the aetiology of idiopathic PD, then its effect would have to be superimposed upon the background pathology of neuronal loss and Lewy body formation seen in other areas of the PD brain. As argued above, this background pathology may itself be genetically or environmentally determined.

We suggest that the differential distribution of the pathological and biochemical abnormalities identified to date in PD brain support a 'two-hit' hypothesis for aetiology.

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References

[1] Balazs L, Leon M. Evidence of an oxidative challenge in the Alzheimer's brain. Neurochem Res 1994;19:1131–7.
[2] Chagnon P, Bétard C, Robitaille Y, et al. Distribution of brain cytochrome oxidase activity in various neurodegenerative diseases. Mol Neurosci 1995;6:711–5.
[3] Cooper JM, Wischik C, Schapira AHV. Mitochondrial function in Alzheimer's disease. Lancet 1993;341:969–70.
[4] Dagani F, Ferrari R, Anderson JJ, et al. L-Dopa does not affect electron transfer chain enzymes and respiration of rat muscle mitochondria. Mov Disord 1991;6:315–9.
[5] Dexter DT, Wells FR, Lees AJ, et al. Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. J Neurochem 1989;52:1830–6.
[6] Dexter DT, Carayon A, Vidailhet M, et al. Decreased ferritin levels in brain in Parkinson's disease. J Neurochem 1990;55:16–20.
[7] Dexter DT, Carayon A, Javoy-Agid F, et al. Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neurodegenerative diseases affecting the basal ganglia. Brain 1991;114:1953–75.
[8] Dexter DT, Sian J, Rose S, et al. Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. Ann Neurol 1994;35:38–44.
[9] Gu M, Cooper JM, Gash M, et al. Mitochondrial defect in Huntington's disease caudate nucleus. Ann Neurol 1996;39:385–9.
[10] Hartley A, Cooper JM, Schapira AHV. Iron induced oxidative stress and mitochondrial dysfunction: relevance to Parkinson's disease. Brain Res 1993;627:349–53.

[11] Hensley K, Hall N, Sabramaniam R, et al. Brain regional correspondence between Alzheimer's disease histopathology and biomarkers for protein oxidation. J Neurochem 1995;65:2146–56.
[12] Hirsch EC, Brandel J-P, Galle P, et al. Iron and aluminium increase in the substantia nigra of patients with Parkinson's disease: an X-ray microanalysis. J Neurochem 1991;56:446–51.
[13] Hughes AJ, Daniel SE, Blankson S, et al. A clinicopathologic study of 100 cases of Parkinson's disease. Arch Neurol 1993;50:140–8.
[14] Janetzky B, Hauck S, Youdim MBH, et al. Unaltered aconitase activity but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. Neurosci Lett 1994;169:126–8.
[15] Jenner P, Schapira AHV, Marsden CD. New insights into the cause of Parkinson's disease. Neurology 1992;42:2241–50.
[16] Kish SJ, Bergeron C, Rajput A, et al. Brain cytochrome oxidase in Alzheimer's disease. J Neurochem 1992;59:776–9.
[17] Lovell MA, Ehmann WD, Butler SM, et al. Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain of Alzheimer's disease. Neurology 1995;45:1594–601.
[18] Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the folin reagent. J Biol Chem 1951;193:265–75.
[19] Mann VM, Cooper JM, Krige D, et al. Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. Brain 1992;115:333–42.
[20] Mann VM, Cooper JM, Daniel SE, et al. Complex I, iron and ferritin in Parkinson's disease substantia nigra. Ann Neurol 1994;36:876–81.
[21] Mecocci P, MacGarvey U, Beal MF. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. Ann Neurol 1994;36:747–51.
[22] Morikawa N, Nakagawa-Hattori Y, Mizuno Y. Effect of dopamine, dimethoxyphenylethylamine, papaverine, and related compounds on mitochondrial respiration and complex I activity. J Neurochem 1996;66:1174–81.
[23] Mutisya EM, Bowling AC, Beal MF. Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. J Neurochem 1994;3:2179–84.
[24] Pardo B, Mena MA, de Yebenes JG. L-Dopa inhibits complex IV of the electron transport chain in catecholamine-rich human neuroblastoma N869 cells. J Neurochem 1995;64:576–82.
[25] Parker WD, Parks J, Filley CM, et al. Electron transport chain defects in Alzheimer's disease. Neurology 1994;44:1090–6.
[26] Perry TL, Godin DV, Hansen S. Parkinson's disease: a disorder due to nigral glutathione deficiency. Neurosci Lett 1982;33:305–10.
[27] Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease Science 1997;276:2045–7.
[28] Przedborski S, Jackson-Lewis V, Muthane U, et al. Chronic levodopa administration alters cerebral mitochondrial respiratory chain activity. Ann Neurol 1993;34:715–23.
[29] Reichmann H, Flörke S, Hebenstreit G, et al. Analyses of energy metabolism and mitochondrial genome in post-mortem brain from patients with Alzheimer's disease. J Neurol 1993;240:377–80.
[30] Riederer P, Sofic E, Rausch WD, et al. Transition metals, ferritin, glutathione and ascorbic acid in parkinsonian brains. J Neurochem 1989;52:515–20.
[31] Saggu H, Cooksey J, Dexter D, et al. A selective increase in particulate superoxide dismutase activity in Parkinson's substantia nigra. J Neurochem 1989;53:692–7.
[32] Schapira V AH, Cooper JM, Dexter D, et al. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 1990;54:823–7.
[33] Schapira V AH, Mann VM, Cooper JM, et al. Anatomic and disease specificity of NADH CoQ_1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 1990;55:2142–5.
[34] Sian J, Dexter DT, Lees AJ, et al. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Ann Neurol 1994;36:348–55.
[35] Sofic E, Lange KW, Jellinger K, et al. Reduced and oxidised glutathione in the substantia nigra of patients with Parkinson's disease. Neurosci Lett 1992;142:128–30.
[36] Stein AF, Dills RL, Klaassen CD. High-performance liquid chromatographic analysis of glutathione and its thiol and disulfide degradation products. J Chromatogr 1986;381:359–70.
[37] Thomas PK, Cooper JM, King RHM, et al. Myopathy in vitamin E deficient rats: muscle fibre necrosis associated with disturbances of mitochondrial function. J Anat 1993;183:451–61.

Mitochondria in the Etiology and Pathogenesis of Parkinson's Disease

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Mitochondria play a critical role in cellular energy metabolism. The identification of a respiratory chain defect in Parkinson's disease (PD) provides not only a direct link with toxin models of parkinsonism but also insight into the mechanisms involved in etiology and pathogenesis. The presence of the complex I deficiency in PD substantia nigra and platelets suggests the involvement of a systemic cause. Genomic transplantation studies have been undertaken that involve the transfer to a novel nuclear background of mitochondrial DNA (mtDNA) from PD patients with a complex I defect, followed by both mixed and clonal expansion of the resulting cybrids. The mixed cybrids with the PD mtDNA expressed the complex I defect present in the original PD donor platelets. Clonal expansion of one such mixed cybrid culture produced a spectrum of clones with complex I and complex IV activities, ranging from severe deficiency to normal range, a pattern typical of a heteroplasmic mtDNA mutation. Histochemical, immunohistochemical, and functional assessments of $\Delta\psi_m$ all showed a pattern in the PD clones typical of that produced by a mtDNA mutation. Patients with focal dystonia and a platelet complex I defect were used as disease controls for the cybrid studies. The mitochondrial abnormality was eradicated by transfer of dystonia mtDNA to a control nuclear background in both mixed and clonal cybrids, with no evidence of clonal heterogeneity. These results help to validate our findings in the PD patients and suggest that the complex I deficiency in dystonia is not due to an abnormality of mtDNA. We hypothesize that the mtDNA defect alone may be the cause of PD in a proportion of patients and may contribute to pathogenesis in others. Identification of the mtDNA genotype responsible for PD may allow the testing of neuroprotective strategies in appropriate patients.

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The clinical features ascribed to Parkinson's disease (PD) cover a relatively narrow spectrum involving bradykinesia, rigidity, and tremor. Inevitably there is some overlap with other neurodegenerative disorders, particularly those now included in the multiple system atrophies. The neuropathology of PD is characterized by severe degeneration of the dopaminergic cells of the substantia nigra pars compacta and their projections to the striatum, and by the presence of Lewy bodies in a proportion of surviving neurons. Although other neuronal populations and neurotransmitter systems are involved, it is the loss of nigral neurons that underlies the majority of the core features of PD. A central question in any attempt to understand the etiology of PD is whether the clinical and pathologic features are the result of a single cause or the product of any one of a number of potential causes, each of which may share the same biochemical pathways that result in neuronal death.

Two observations support the proposition that PD is a symptom–pathology complex resulting from different etiologic factors. 1-Methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) is known to induce parkinsonism in humans and other primates.[1,2] Although not typical, MPTP parkinsonism shares many of the clinical and pharmacologic features of idiopathic PD. In addition, MPTP induces relatively selective neuronal loss in the substantia nigra and simulates, but does not exactly reproduce, the pathology of PD.[3] Autosomal dominant familial parkinsonism, such as has been documented in a large American–Italian kindred,[4] is similar to idiopathic PD but is characterized by some atypical features, such as early onset and lack of tremor, in the family referenced here. Pathology on the limited number of brains available for study from such families has demonstrated predominant nigrostriatal dopaminergic cell death and Lewy bodies. The identification of a mutation in the α-synuclein gene in certain parkinsonian families has provided insight into the molecular mechanisms that may be involved.[5] That two such widely different etiologic factors as MPTP toxicity and an α-synuclein mutation should be capable of producing such similar clinical and pathologic outcomes is

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strongly supportive of the contention that idiopathic PD itself may well have multiple etiologies. It appears inevitable that both genetic and environmental factors are likely to be involved in PD. A proportion of cases may be due solely to one or the other, but a higher proportion is probably the result of a combination of both.

It is against this background of multiple etiologies but common pathology that the contribution of mitochondria and mitochondrial dysfunction to both the etiology and the pathogenesis of PD must be considered.

Mitochondria: Molecular Biology and Bioenergetics

Mitochondria are ubiquitous and play a pivotal role in cell metabolism, particularly in energy provision by ATP synthesis. Cellular mitochondrial density reflects metabolic activity. Therefore, neurons, as well as skeletal and myocardial muscles have high mitochondrial mass. Each mitochondrion contains two to 10 molecules of DNA. Mitochondrial DNA (mtDNA) is a circular double-stranded molecule of 16.5 kilobases comprising a heavy (H) and a light (L) chain but without any histone coat. MtDNA encodes a full complement of 22 transfer RNAs (tRNAs) and 12S and 16S ribosomal RNAs in addition to 13 proteins, all of which are part of the respiratory chain and oxidative phosphorylation (OXPHOS) system. MtDNA is dependent on the cell nucleus for encoding its replication, transcription, translation, repair, and regulatory factors.

The OXPHOS system is located on the mitochondrial inner membrane and comprises five multimeric proteins termed complexes I–V, NADH ubiquinone oxidoreductase, succinate ubiquinone oxidoreductase, ubiquinol cytochrome c reductase, cytochrome oxidase, and ATP synthase, respectively. Seventy (84%) of the 83 subunits of the OXPHOS system are encoded by nuclear DNA and the remainder by mtDNA. Complex I is the largest of all the OXPHOS proteins with 41 subunits, seven of which (ND1 to 6 and ND4L) are coded for by mtDNA. Complex II is exclusively encoded by nuclear DNA; complex III has 11 subunits, of which cytochrome b is encoded by mtDNA. Complex IV (COX) has 13 subunits with COI, COII, and COIII encoded by mtDNA, and ATPase has 14 subunits of which proteins 6 and 8 are coded for by mtDNA.

Many mutations of mtDNA have now been associated with human disease. Because mtDNA is inherited exclusively through the maternal line, mtDNA-related defects would be expected to exhibit maternal inheritance. This is indeed seen, although only in a minority of cases. Patients with mtDNA deletions, the most common mtDNA mutation, appear as sporadic cases; even 40% of patients with the 11,778-bp Leber's hereditary optic neuropathy (LHON) mutation have no family history.[6] Therefore, although maternal inheritance may alert the physician to an mtDNA defect, such a pattern will not necessarily be apparent in the majority of those with mtDNA mutations.

Mitochondrial Function in PD

Analysis of OXPHOS activity in PD postmortem brain has demonstrated an approximate 37% decrease in complex I activity in substantia nigra pars compacta,[7–10] although other reports with fewer samples have also suggested additional defects in complexes II–III and in the immunoreactivity of the citric acid cycle enzyme α-ketoglutarate dehydrogenase.[11,12] The complex I defect in PD exactly mimics the biochemical lesion induced by MPTP[13] and provides a direct parallel between the toxin model and the idiopathic disease. Similar assays of other brain areas in PD have not identified any deficiency of OXPHOS.[9,10,14–16] Likewise, no OXPHOS defect has been identified in the brains, including substantia nigra, of patients with multiple system atrophy.[17] Therefore, the OXPHOS abnormality in PD appears to be selective for, or at least most severe in, the substantia nigra. Furthermore, the results from the multiple system atrophy brains indicate that the complex I deficiency is not simply the result of neuronal degeneration.

OXPHOS function has also been studied in skeletal muscle and platelets of PD patients. In vivo ^{31}phosphorus magnetic resonance spectroscopy of muscle in PD has given conflicting results. The first study showed no defect,[18] whereas the second suggested deficient OXPHOS.[19] Likewise, analysis by polarography and enzyme activities has given normal results or multiple OXPHOS defects, sometimes with exceedingly low values (see ref. 20 for review). In contrast, results from analysis of PD platelets show more consistency. Whereas platelet homogenates in PD show no deficiency,[21] the great majority of studies using purified platelet mitochondria show a complex I defect isolated or in conjunction with a less severe deficiency of other complexes (see ref. 20 for review).[22]

A systemically expressed mitochondrial abnormality in PD implies either a genetic or toxic cause. When a genetic etiology is under consideration, several confounding factors must be taken into account:

1. Although some PD families with autosomal dominant or maternal inheritance[23] have been described, the vast majority of patients with idiopathic PD appear as sporadic cases. This observation would be compatible with a mtDNA defect (see above), autosomal dominant inheritance with low penetrance, or genetic (nuclear or mitochondrial) environmental interaction.
2. If the complex I defect is genetically determined, this may be caused by a mutation of nuclear- or mtDNA-encoded subunits.

3. The apparent variability of tissue expression of a systemically distributed mutation may be the result of (a) differential expression of a nuclear encoded protein, (b) variations in load of a mtDNA mutation, or (c) sampling error, eg, a heterogeneous population of PD patients with varying etiologies, only some of which lead to a systemic defect.

To circumvent some of these difficulties and investigate the role of others, we have sought to target a subpopulation of PD patients for detailed study. Mitochondrial studies in vivo are limited by the types of tissue that are readily available for analysis. Platelets were selected because they are easily obtainable and have consistently been shown to have a complex I deficiency in PD. We have studied 25 PD patients for platelet OXPHOS activity and have shown a 16% defect specific for complex I.[24] However, there was significant overlap between the PD patients and controls (Fig 1) such that the assay was too insensitive to serve as a diagnostic test. Nevertheless, there was a significant proportion of PD patients with complex I activities >1 SD below control mean. We have hypothesized that the PD patients with the lowest platelet complex I activity are those most likely to carry any putative mtDNA mutation. There are other implications from this hypothesis, and these are discussed below.

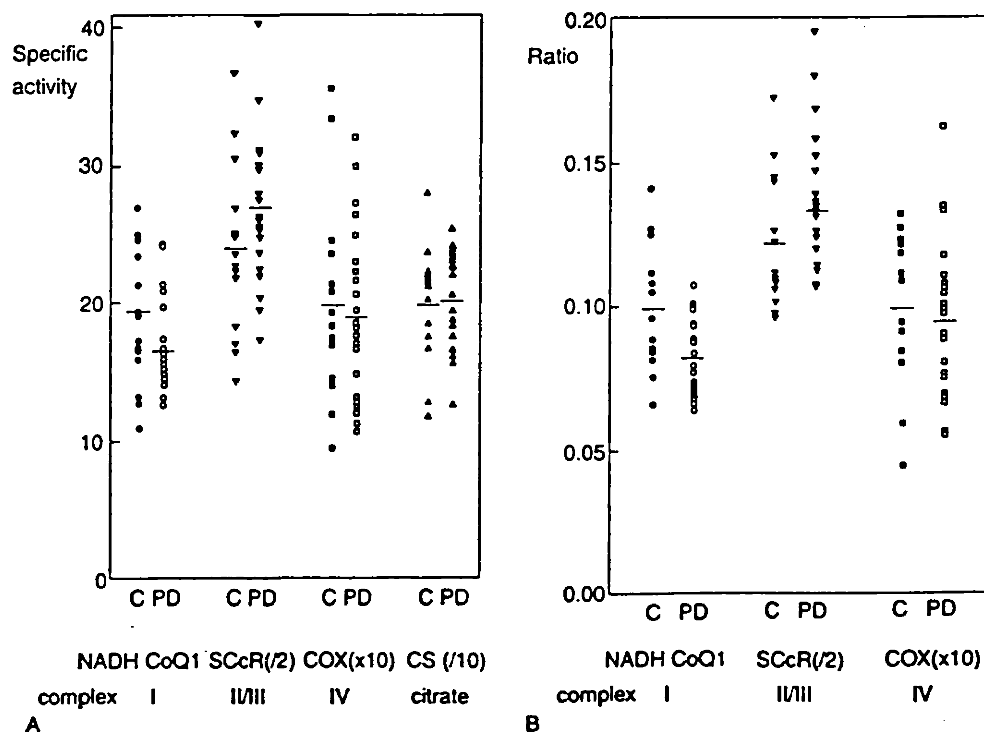
Eight patients with idiopathic PD were selected from those with low platelet complex I activities. Four of these patients underwent three serial complex I measurements, each 1 month apart, to assess the consistency of the defect with time.[25] The reproducibility of the complex I defect was such that activity consistently fell >1 SD below control mean over time.

The Genetic Basis of Complex I Deficiency in PD

King and Attardi[26] pioneered a cell cybrid system for the investigation of mtDNA mutations. This involves the use of ρ^0 (mtDNA-less) cells that have been developed from a parent cell line by exposure to ethidium bromide through serial passage. The ρ^0 cells can survive and grow in medium enriched with pyruvate and uridine. ρ^0 cells serve as recipients for donor mtDNA, thereby placing the donor mtDNA in a novel nuclear environment. This enables the structural and functional consequences of an mtDNA mutation to be investigated in isolation, ie, without the compounding effects of any nuclear influence.[27–29]

We have used this system to investigate the genetic origins of the complex I defect in PD (Fig 2). We have taken platelets from PD patients with low complex I activity and fused them with ρ^0 cells, in this case an A549 lung-derived cell line. Platelets have no nucleus and therefore can be fused directly. The resulting cy-

Fig 1. (A) Specific activities of mitochondrial enzymes in platelet mitochondrial fraction. C = control (n = 15); PD = Parkinson's disease (n = 25). Bars represent mean activities. (B) Ratio of mitochondrial respiratory chain enzyme activities to citrate synthase activities in platelet mitochondrial fractions. Bars represent mean activities. From ref. 24.



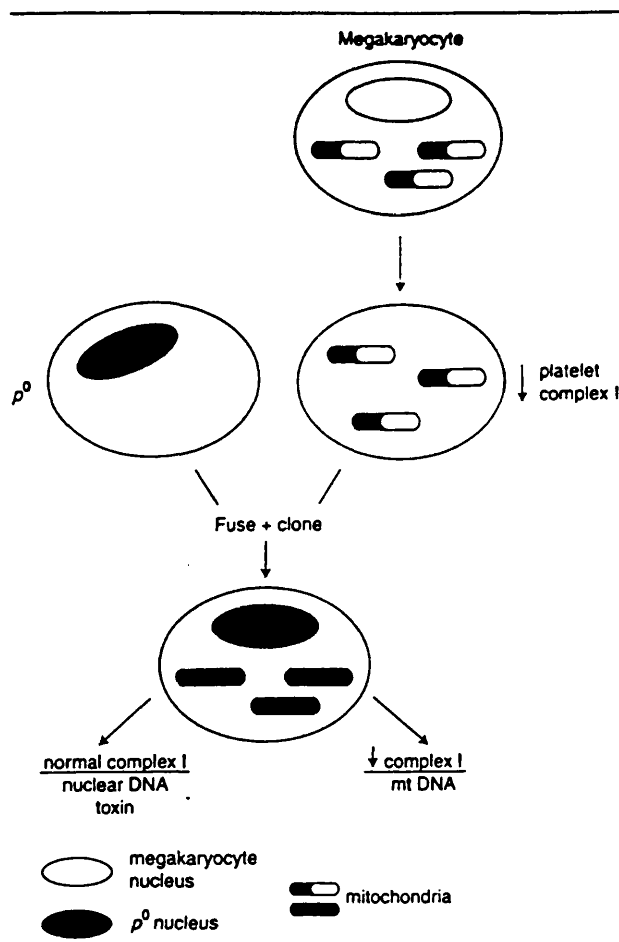


Fig 2. Schematic illustration of the principle of the ρ⁰ cybrid studies used in the identification of an mtDNA contribution to the complex I defect in PD.

brids from fusion of A549 nuclei with PD-derived mtDNA were grown for each patient. As Fig 2 predicts in this system, if the complex I defect is still expressed in the cybrids, the only cause could be the PD mtDNA derived from the platelets with the complex I deficiency. If the complex I function is normalized, then the original platelet defect must have been determined either by a nuclear defect in the PD patients or by an endogenous toxin circulating in the blood or sequestered in the bone marrow from which the platelets were derived.

The results of OXPHOS analysis in the cybrids produced from the PD patients produced two intriguing results. The first was that the PD cybrids had a specific 25% ($p = 0.007$) complex I deficiency. Therefore, the complex I defect present in the original donor platelets had been transferred with the transfer of mitochondria and had been maintained through multiple passages through which the PD mtDNA was the only remnant of the donor mitochondria. The donor PD mtDNA must therefore determine the complex I defect in both the platelets and the cybrids. The second result was that there was a high correlation ($r = 0.86$; $p < 0.001$) between the complex I activity in a PD patient's platelets and in his/her fusion cybrids. The combination of platelet and cybrid complex I activities provides a more specific discriminator between PD patients and controls. In addition, these results also imply that the mitochondrially encoded subunits of complex I are important in determining the activity of the enzyme.

The experiments described above implicate mtDNA in the causation of the complex I defect in our preselected group of patients. Interestingly, another study, using an unselected group of PD patients, found that the complex I defect also transfers from platelets to cybrids using a neuroblastoma ρ^0 cell.[30] However, it is not clear from this study whether the results applied to all PD patients or only to a subgroup and whether there was any correlation between complex I activity in platelets and cybrids. Furthermore, complex I activity is known to be influenced by nuclear background and this same group, using the same ρ^0 nuclear background, have described OXPHOS defects due to mtDNA in Alzheimer's disease and multiple system atrophy.[31,32] Therefore, to address whether the complex I deficiency in our study may be influenced by the nuclear background of the A549 cells, we undertook a similar study using patients with sporadic focal dystonia.[33] These patients had also been shown to have a complex I defect in platelets on the same order of magnitude as that seen in PD patients.[34] As in PD, the cause of the complex I deficiency in focal dystonia is unknown. After fusion with A549 ρ^0 cells and both mixed and clonal cybrid growth, analysis of OXPHOS function was normal in the dystonia-derived cells. This investigation not only serves as an important "disease control" for the PD studies but also indicates that the complex I defect in dystonia is not caused by mtDNA. Therefore, it is clear that the results obtained from the PD study using the A549 cells are not due to any influence of the host ρ^0 cell nucleus. It will be important to undertake similar disease control studies using the neuroblastoma cell line.

mtDNA mutations are heteroplasmic, ie, mutant and wild-type molecules coexist in the same cell and probably in the same mitochondrion. The level of mutant mtDNA may therefore vary from one cell to another and therefore from one tissue to another. As indicated above, the threshold for biochemical expression depends on the particular mtDNA mutation, the mutant load, and the nuclear background. This heteroplasmic pattern may be expressed in a tissue culture by intercellular heterogeneity of mtDNA expression and function. This can be seen in A549/A3243G (the common "MELAS" mutation) cybrids with COX histochemistry as variation in cell staining, some cells having strong uniform activity whereas others fail to stain at all (Fig 3B). Staining with a monoclonal antibody to

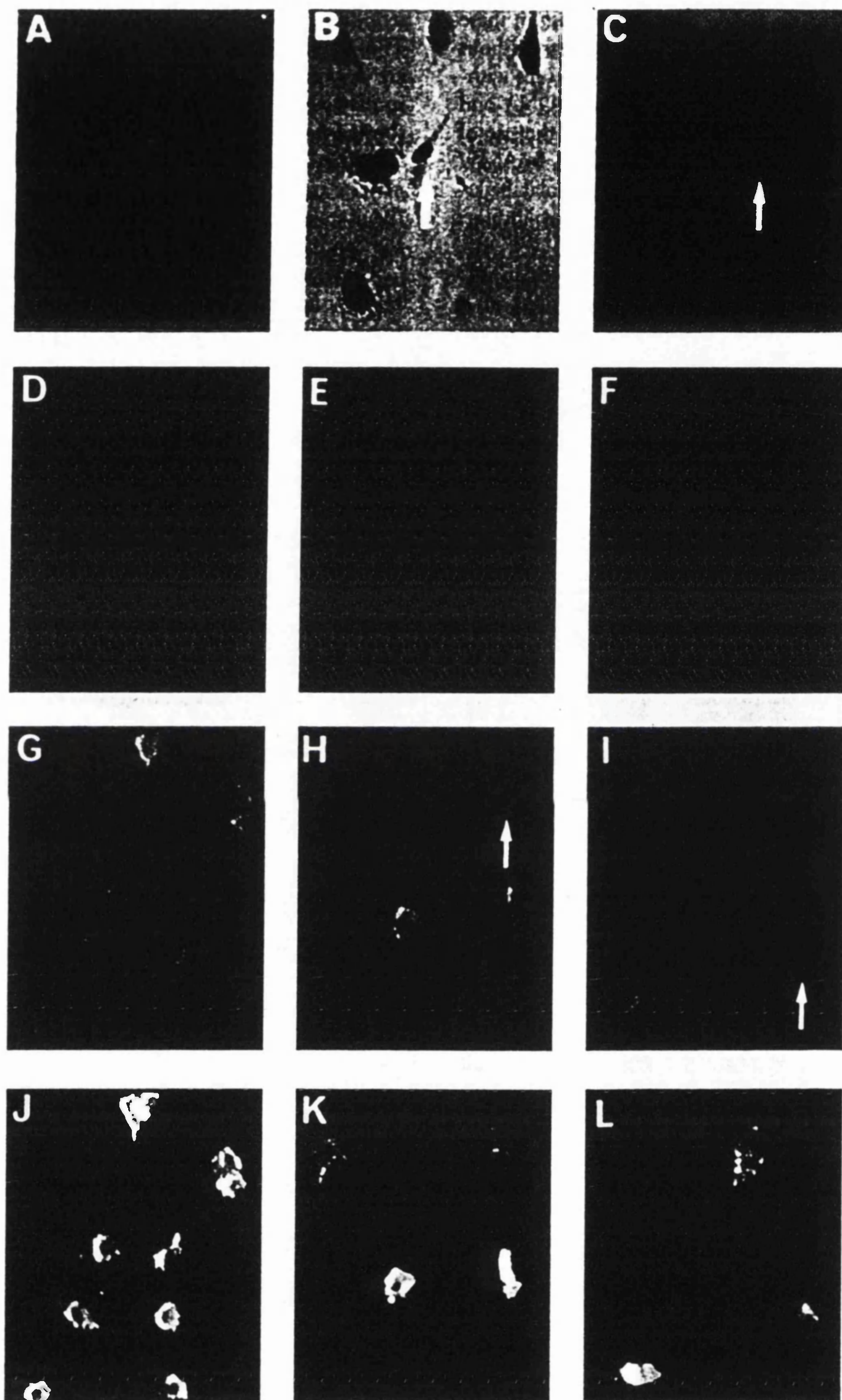


Fig 3. A549p^0 control platelet cybrids (A,D,G,J), A549p^0/A3243G platelet cybrids (B,E,H,K), and A549p^0/PD platelet cybrids (C,F,I,L) stained for cytochrome oxidase activity (A–C), with Mitotracker (D–F), and immunocytochemically using anti-COI antibody (G–I). Typical cells with decreased staining are indicated by arrows. Combined images of Mitotracker, anti-COI antibody, and Hoechst 33258 are shown in J, K, and L. Magnification ×365. See ref. 25.

the mitochondrially encoded COI subunit of COX parallels the COX histochemical function stain in these cells, with some staining positively and others without crossreaction (Fig 3H). This implies that the cells without staining have little or no translated mtDNA and therefore have a high mutant load. Finally, the use of the membrane potential-dependent dye JC-1 in A549/A3243G cybrids also demonstrated intercellular heterogeneity of membrane potential ($\Delta\psi_m$), an indirect measure of cellular respiration (Fig 4C).

After the transfer of the complex I defect in PD platelets with mtDNA and the implication of a mtDNA mutation as the cause, we undertook investigations to determine whether the mtDNA mutation responsible was heteroplasmic. Our hypothesis, based on other mtDNA mutation models, was that cells cloned from one of the PD mixed cybrids would demonstrate a spectrum of complex I activity and intercellular heterogeneity in JC-1 staining, reflecting the range of mutation load in different cells. Therefore, expanded clonal analysis of the cybrids obtained from one of the PD patients with platelet complex I deficiency was undertaken. Interestingly, OXPHOS assays of individual clones showed a range of complex I and complex IV activities that varied from those within to those significantly below the control range. The mean of all PD clones (n = 16) studied showed significant reductions of 25% ($p < 0.005$) and 20% ($p < 0.005$), respectively, for complex I and IV activities compared to controls. Likewise, COX histochemistry, COI immunostaining, and JC-1 functional staining all showed intercellular heterogeneity (Figs 3C, 3I, 3L, and 4D). The A549/A3243G ρ^0 clonal cybrids in Figs 3 and 4C are shown as a positive control for comparison. Similar studies were also undertaken on dystonia cybrids and showed no difference from control clones. In addition, fibroblasts from a patient with focal dystonia and a platelet complex I defect were stained with JC-1. All the dystonia fibroblasts stained uniformly bright, as did the controls, indicating normal $\Delta\psi_m$ (data not shown).

These results support the platelet fusion results in implicating mtDNA in the cause of the OXPHOS defect in PD. The pattern of staining in the PD clones for all the methods used was identical to that seen in the A3243G mtDNA mutation clones. This supports the hypothesis that a heteroplasmic mtDNA mutation is present in the PD clones. Furthermore, clonal analysis revealed a complex IV (COX) defect in some. The combination of complex I and IV deficiency is typical of a mtDNA tRNA mutation and is seen, for example, with the A3243G mutation. This suggests that, at least in the PD patient studied by clonal analysis, a mutation in an mtDNA tRNA gene might be responsible for the OXPHOS defect.

Mitochondria in the Pathogenesis of PD

The data presented thus far demonstrate a complex I defect in PD substantia nigra and platelets and the involvement of mtDNA in determining this in at least a proportion of patients. It is not known whether those

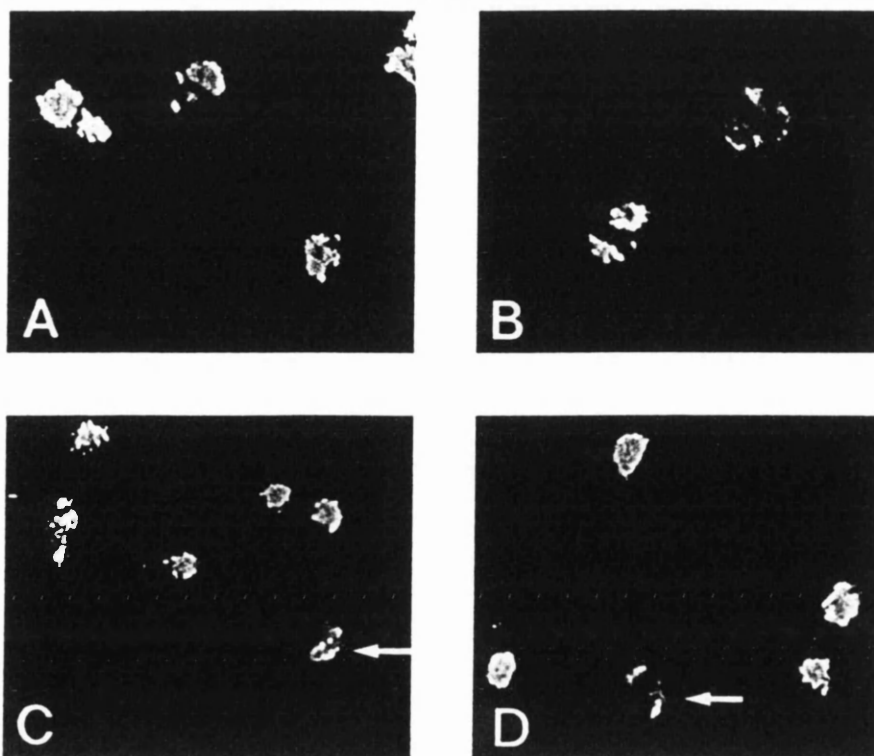


Fig 4. In vivo assessment of mitochondrial membrane potential in A549 cells (A), A549ρ^0 cells (B), A549ρ^0/A3243G platelet cybrids (C), and A548ρ^0/PD platelet cybrids (D). Cells were cultured in the presence of 3 μg/ml JC-1. Cells with low membrane potential fluoresce green, whereas cells with high membrane potential fluoresce yellow. Cybrids with low membrane potential are indicated by arrows.

patients with a defect in substantia nigra also express the same abnormality in platelets, although it is reasonable at this stage to assume that the converse is true, ie, those patients with a significant defect of complex I in platelets will also have such a deficiency in substantia nigra. Other factors operating at the level of the nigra might selectively enhance, or possibly even produce, a complex I deficiency and, in such cases, platelet complex I function may be normal. For example, levodopa causes a complex I defect in rat substantia nigra,[35] and auto-oxidation of dopamine results in free radical generation, which may also impair complex I function.[36] Additional observations also suggest that complex I may be the target of both exogenous and endogenous toxins.

MPTP

Much has been written on the mechanism of action of MPTP (see ref. 37 for review). It is increasingly clear that MPTP, via its active metabolite 1-methyl-4-phenylpyridinium (MPP^+), is not only an inhibitor of complex I but also a cause of increased free radical generation. MPP^+ is a specific reversible inhibitor of complex I and causes a decrease in ATP levels. MPP^+ probably interacts with complex I at the same site as rotenone and piericidin A.[38] MPP^+ induces more severe and irreversible inhibition of complex I if cytochrome oxidase (complex IV) is inhibited.[39] This inhibition was prevented by free radical scavengers, indicating oxidative damage of complex I under these conditions. Complex I inhibition results in increased free radical generation from the respiratory chain and therefore the MPP^+ model suggests that a self-amplifying cycle of complex I deficiency and damage may result in progressive cell damage. Such a situation would fit well with the progressive striatal lesion in MPP^+-exposed patients as determined by ^{18}fluorodopa PET.[40] MPP^+ may also generate free radicals directly.[41-43] In addition, the recent observation that in primates[44] and rats[45] the nitric oxide ($NO^\cdot$) synthase inhibitor 7-nitroindazole can protect against MPTP toxicity implicates $NO^\cdot$ production in the mediation of this toxin's effects. However, another $NO^\cdot$ synthase inhibitor, L-N^G-nitroarginine methylester,[46] had no protective effect. Indeed 7-nitroindazole has recently been shown to be an inhibitor of monoamine oxidase (MAO),[47] which is required for the conversion of MPTP to MPP^+. $NO^\cdot$ has been shown to directly inhibit complex I, II, and III, although the specificity of action is dependent on the conditions used.[48] We have recently shown that $NO^\cdot$ enhances the inhibitory effect of MPP^+ on complex I from 30 to 60% under conditions in which $NO^\cdot$ itself had no direct effect on respiratory chain function (Cleeter et al, unpublished observations). Furthermore, the combined effects of $NO^\cdot$ and MPP^+ on complex I could be blocked by catalase, reduced oxyhemoglobin (HbO_2), and reduced glutathione (GSH). The decrease in ATP production resulting from the direct inhibition of complex I by MPP^+ could, inter alia, enhance glutamate toxicity through release of the Mg^{2+} blockade of *N*-methyl-D-aspartate (NMDA) receptors. This could cause $NO^\cdot$ synthase activation and $NO^\cdot$ production which, in turn, would lead to a further reduction in OXPHOS activity by the mechanisms outlined above. The striatum and substantia nigra are supplied with $NO^\cdot$ synthase-positive neurons,[49,50] and therefore the elements for such $NO^\cdot$ involvement in MPTP toxicity are in place and could contribute to toxicity, notwithstanding the demonstration of 7-nitroindazole inhibition of MAO-B.

Complex I Inhibitors and Apoptosis

The demonstration that MPTP could induce parkinsonism focused attention on the possible role(s) of more common environmental agents in the etiology of PD. The structural properties of MPTP and its uptake and conversion characteristics led to the investigation of structurally related compounds, eg, isoquinoline and its tetrahydroisoquinoline (TIQ) derivatives, as potential nigral toxins. Isoquinolines inhibit complex I and α-ketoglutarate dehydrogenase,[51-53] induce free radical generation,[54,55] and induce parkinsonism in primates[56] and rats.[57] We have recently shown that the complex I inhibitors rotenone, MPP^+, isoquinoline, and TIQ all reduced cell survival in PC12 cells.[58] Cell survival appeared inversely related to the inhibition of ATP synthesis by these compounds, with as little as a 20% decrease in ATP synthesis by TIQ causing marked cell death. Investigation of the mode of cell death by these agents using both propidium iodide and FITC–TUNEL assessments demonstrated that all caused apoptosis. Apoptotic cell death induced in both PC12 and SKNMC cells by these toxins was reduced by 20 to 79% by a variety of antioxidants, including *N*-acetylcysteine, dihydrolipoic acid, and pyrrolidine dithiocarbamate. This implies the involvement of free radicals in mediating the proapoptotic action of these complex I inhibitors.

Mitochondria and Apoptosis

The inner mitochondrial membrane potential is produced and maintained by the activity of the respiratory chain and the pumping of proteins out of the mitochondrial matrix. However, ρ^0 cells can maintain $\Delta\psi_m$, as evidenced by their uptake of JC-1, although the potential is low.[59] Both ρ^0 and ρ^+ (with mtDNA) cells lose their $\Delta\psi_m$ and generate free radicals in response to agents that stimulate apoptosis. The loss of $\Delta\psi_m$ precedes nuclear fragmentation and results in the opening of permeability transition pores (megachannels).[60] Permeability transition and $\Delta\psi_m$ collapse allow the escape

of 10 to 15-kDa molecules, which can induce apoptotic changes in isolated nuclei.[61]

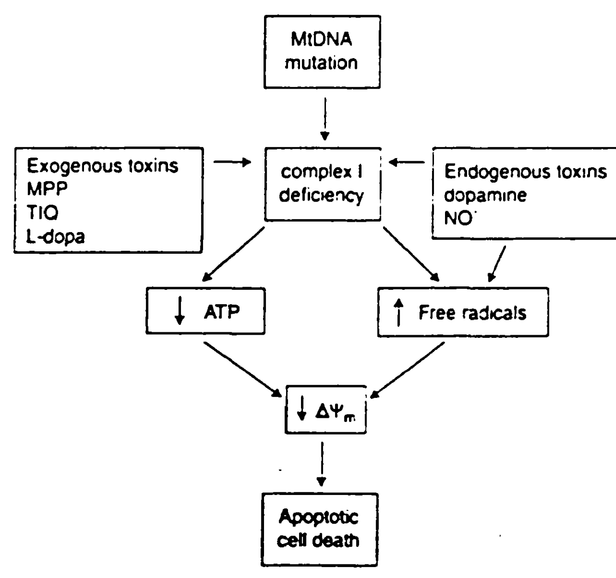
Hypothesis

We and others have provided evidence that mitochondrial dysfunction, and complex I deficiency in particular, is present in PD. In addition, evidence is now emerging that a defect of mtDNA function determines the complex I deficiency in at least a proportion of cases. We suggest that the mitochondrion may be the common target for inborn and induced metabolic abnormalities that cause dopaminergic cell death in PD (Fig 5).

The transfer of complex I deficiency with mtDNA from PD patients clearly implicates mtDNA mutations as the cause of the defect. The consequent decrease in $\Delta\psi_m$ is demonstrated in our studies. In some instances this may be sufficient to induce cell death, and in others it may lower the threshold for apoptotic cell death. Additional factors, eg, exogenous toxins, including levodopa, may enhance the complex I deficiency, cause a further fall in $\Delta\psi_m$, and precipitate cell death. A similar scenario could explain the potential contribution of other factors such as endogenous toxins, eg, dopamine, NO˙, and free radicals.

Therefore, the etiology and the pathogenesis of dopaminergic cell death in PD may be multifactorial. The processes outlined above may be relevant only to a proportion of PD patients. The mechanisms by which a mutation in the α-synuclein gene might cause neuronal death are unknown and may involve entirely different pathways. Alternatively, the α-synuclein mutation may also affect energy or free radical metabolism

Fig 5. A hypothesis for the role of mitochondrial dysfunction in the etiology and pathogenesis of Parkinson's disease.



and follow similar pathways to cell death as a mtDNA defect.

Identification of the mtDNA defect responsible for the complex I deficiency may not be straightforward. We advocate a focus on those PD patients with a demonstrable mitochondrial abnormality and the capacity to transfer the deficiency with mtDNA, ie, cybrid studies. Although the pattern of respiratory chain deficiency might provide some clues to the nature of the mutation, it is likely that the entire mtDNA genome will require sequencing. The complex I deficiency may be determined not by a single heteroplasmic mutation but by a collection of "polymorphisms" which, as a haplotype, result in defective complex I activity. PD patients with such a haplotype might suffer sufficient dopaminergic cell death and develop the disease only if they are exposed to one of the other factors in Fig 5, eg, an external toxin. Aminoglycoside-induced deafness is a model for this hypothesis. Individuals carrying the 1,555-bp mtDNA polymorphism suffer no ill effects unless they are exposed to aminoglycosides, which even at normal therapeutic concentrations may induce deafness.[62] The corollary of this is that there is a group of individuals with the PD-associated mtDNA defect but no disease. They have not been exposed to the additional exogenous or endogenous toxin required to precipitate severe dopaminergic cell death. We have previously hypothesized that Lewy body-positive controls may fall into this group.[63] These brains, obtained from patients without any clinical evidence of PD during life, had a significant decrease in GSH and an intermediate decrease in complex I activity, in the context of a depletion of dopaminergic neurons and the presence of Lewy bodies beyond those expected for age-matched brains.[64] These brains should provide a useful source by which to test this element of the mitochondrial hypothesis. Those controls with low platelet complex I activity may be the equivalent of these Lewy body-positive controls and may likewise serve as an experimentally important group to identify individuals with the PD-associated mtDNA genotype but no appropriate environmental exposure or additional genetic defect required to induce the disease.

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References

1. Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic parkinsonism in humans due to a product of meperidine analog synthesis. Science 1983;219:979–980
2. Langston JW, Forno LS, Robert CS, Irwin I. MPTP causes selective damage to the zona compacta of the substantia nigra in the squirrel monkey. Brain Res 1984;292:390–394

3. Forno LS, Langston JW, DeLanney LE, Irwin I. An electron microscopic study of MPTP-induced inclusion bodies in an old monkey. Brain Res 1988;448:150–157
4. Golbe LI, Di Iorio G, Bonavita V, Miller DC, Duvoisin RC. A large kindred with autosomal dominant Parkinson's disease. Ann Neurol 1990;27:276–282
5. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease. Science 1997;276:2045–2047
6. Harding AE, Sweeney MG, Govan GG, Riordan-Eva P. Pedigree analysis in Leber hereditary optic neuropathy families with a pathogenic mtDNA mutation. Am J Hum Genet 1995;57:77–86
7. Schapira AHV, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1989;1:1269
8. Schapira AHV, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 1990;54:823–827
9. Janetzky B, Hauck S, Youdim MBH et al. Unaltered aconitase activity but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. Neurosci Lett 1994;169:126–128
10. Gu M, Owen AD, Toffa SEK, et al. Mitochondrial function, GSH and iron in neurodegeneration and Lewy body diseases. J Neurol Sci 1998 [in press]
11. Mizuno Y, Suzuki K, Ohta S. Postmortem changes in mitochondrial respiratory enzymes in brain and a preliminary observation in Parkinson's disease. J Neurol Sci 1990;96:49–57
12. Mizuno Y, Matuda S, Yoshino H, Moro H, Hattori N, Ikebe S. An immunohistochemical study on α-ketoglutarate dehydrogenase complex in Parkinson's disease. Ann Neurol 1994;35:201–210
13. Nicklas WJ, Vyas I, Heikkila RE. Inhibition of NADH-linked oxidation in brain mitochondria by MPP^+, a metabolite of the neurotoxin MPTP. Life Sci 1985;36:2503–2508
14. Schapira AHV, Mann VM, Cooper JM, et al. Anatomic and disease specificity of NADH CoQ_1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 1990;55:2142–2145
15. Mann VM, Cooper JM, Daniel SE, Jenner P, Marsden CD, Schapira AHV. Complex I, iron and ferritin in Parkinson's disease substantia nigra. Ann Neurol 1994;36:876–881
16. Cooper JM, Daniel SE, Marsden CD, Schapira AHV. L-dihydroxyphenylalanine and complex I deficiency in Parkinson's disease brain. Mov Disord 1995;10:295–297
17. Gu M, Gash MT, Cooper JM, et al. Mitochondrial respiratory chain function in multiple system atrophy. Mov Disord 1997;12:418–422
18. Taylor DJ, Krige D, Barnes PRJ, et al. A ^{31}P magnetic resonance spectroscopy study of mitochondrial function in skeletal muscle of patients with Parkinson's disease. J Neurol Sci 1994;125:77–81
19. Penn AMW, Roberts T, Hodder J, Allen PS, Zhu G, Martin WRW. Generalized mitochondrial dysfunction in Parkinson's disease detected by magnetic resonance spectroscopy of muscle. Neurology 1995;45:2097–2099
20. Schapira AHV. Evidence for mitochondrial dysfunction in Parkinson's disease: a critical appraisal. Mov Disord 1994;9:125–138
21. Mann VM, Cooper JM, Krige D, Daniel SE, Schapira AHV, Marsden CD. Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. Brain 1992;115:333–342
22. Haas RH, Nasirian F, Nakano K, et al. Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. Ann Neurol 1995;37:714–722
23. Wooten GF, Currie LJ, Bennett JP, Harrison MB, Trugman JM, Parker WD. Maternal inheritance in Parkinson's disease. Ann Neurol 1997;41:265–268
24. Krige D, Carroll MT, Cooper JM, Marsden CD, Schapira AHV. Platelet mitochondrial function in Parkinson's disease. Ann Neurol 1992;32:782–788
25. Gu M, Cooper JM, Taanman JW, Schapira AHV. Mitochondrial DNA transmission of the mitochondrial defect in Parkinson's disease. Ann Neurol 1998 [in press]
26. King MP, Attardi G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science 1989;246:500–503
27. Chomyn A, Martinuzzi A, Yoneda M, et al. MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. Proc Natl Acad Sci USA 1992;89:4221–4225
28. Chomyn A, Lai ST, Shake R, Bresolin N, Scarlato G, Attardi G. Platelet-mediated transformation of mtDNA-less human cells: analysis of phenotypic variability among clones from normal individuals—and complementation behaviour of the $tRNA^{Lys}$ mutation causing myoclonic epilepsy and ragged red fibers. Am J Hum Genet 1994;34:966–974
29. Dunbar DR, Moonie PA, Zeviani M, Holt IJ. Complex I deficiency is associated with 3243G:C mitochondrial DNA in osteosarcoma cell cybrids. Hum Mol Genet 1996;5:123–129
30. Swerdlow RH, Parks JK, Miller SW, et al. Origin and functional consequences of the complex I defect in Parkinson's disease. Ann Neurol 1996;40:663–671
31. Swerdlow RH, Parks JK, Wooten GF, Miller SW, Davis RE, Parker WD. As in Parkinson's disease, a bioenergetic defect transfers with mitochondrial DNA of patients with multisystem atrophy [abstract]. Mov Disord 1997;12(Suppl 1):P3
32. Davis RE, Miller S, Herrnstadt C, et al. Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. Proc Natl Acad Sci USA 1997;94:4526–4531
33. Tabrizi SJ, Cooper JM, Schapira AHV. Mitochondrial DNA in focal dystonia: a cybrid analysis. Ann Neurol 1998 [in press]
34. Schapira AHV, Warner T, Gash MT, Cleeter MJW, Marinho CFM, Cooper JM. Complex I function in familial and sporadic dystonia. Ann Neurol 1997;41:556–559
35. Przedborski S, Jackson-Lewis V, Muthane U, et al. Chronic levodopa administration alters cerebral mitochondrial respiratory chain activity. Ann Neurol 1993;34:715–723
36. Zhang Y, Marcillat O, Giulivi C, Ernster I, Davies KJ. The oxidative inactivaton of mitochondrial electron transport chain components and ATP. J Biol Chem 1990;265:16330–16336
37. Tipton KF, Singer TP. Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. J Neurochem 1993:61:1191–1206
38. Ramsay RR, Krueger MJ, Youngster SK, Gluck MR, Casida JE, Singer TP. Interaction of MPP^+ and its analogs with the rotenone/piericidin binding site of NADH dehydrogenase. J Neurochem 1991;51:1184–1190
39. Cleeter MJW, Cooper JM, Schapira AHV. Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: evidence for free radical involvement. J Neurochem 1992;58:786–789
40. Vingerhoets FJG, Snow BJ, Tetrud JJ, Langston JW, Schulzer M, Calne DB. Positron emission tomographic evidence for progression of human MPTP-induced dopaminergic lesions. Ann Neurol 1994;36:765–770
41. Adams JD, Johannessen JN, Schuller HM, Bacon JP, Markey SP. The role of oxidative stress in the systemic toxicity of MPTP, and MPP^+, in MPTP. In: Markey SP, Castagnoli N,

Trevor AJ, Kopin IJ, eds. A neurotoxin producing a parkinsonian syndrome. New York: Academic Press, 1989:571–574
42. Hasegawa E, Takeshige K, Oishi T, Murai Y, Minikami S. 1-methyl-4-phenylpyridinium (MPP+) induces NADH dependent superoxide formation, and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. Biochem Biophys Res Commun 1990;170:1049–1055
43. Chiueh CC, Huang S-J, Murphy DL. Enhanced hydroxyl radical generation by 2'methyl analogue of MPTP: suppression by clorgyline and deovenyl. Synapse 1992;11:346–348
44. Hantraye P, Brouillet E, Ferrante R, et al. Inhibition of neuronal nitric oxide synthase prevents MPTP-induced parkinsonism in baboons. Nature Med 1996;2:1017–1021
45. Przedborski S, Donaldson D, Murphy PL, et al. Role of neuronal nitric oxide in 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. Proc Natl Acad Sci USA 1996;93:4565–4571
46. Mackenzie GM, Jackson MJ, Jenner P, Marsden CD. Nitric oxide synthase inhibition and MPTP toxicity in the common marmoset. Synapse 1997;26:301–306
47. Di Monte DA, Royland JE, Anderson A, Castagnoli K, Castagnoli N, Langston JW. Inhibition of monoamine oxidase contributes to the protective effect of 7-nitroindazole against MPTP neurotoxicity. J Neurochem 1997;69:1771–1773
48. Bolaños JP, Almeida A, Stewart V, et al. Nitric oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases. J Neurochem 1997;68: 2227–2240
49. Bredt DS, Glatt CE, Hwang PM, Fotuhi M, Dawson TM, Snyder SH. Nitric oxide synthase protein and messenger RNA are discretely localised in neuronal populations of the mammalian CNS together with NAPDH and diaphorase. Neuron 1991;7:615–624
50. McGeer PL, Itagaki S, Akiyama H, McGeer EG. Rate of cell death in parkinsonism indicates active neuropathological process. Ann Neurol 1988;24:574–576
51. McNaught KStP, Thull U, Carrupt PA, et al. Inhibition of complex I by isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Biochem Pharmacol 1995;50:1903–1911
52. McNaught KStP, Altomare C, Cellamare S, et al. Inhibition of α-ketoglutarate dehydrogenase by isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Neuroreport 1995;6:1105–1108
53. Suzuki K, Mizuno Y, Yoshida M. Inhibition of mitochondrial NADH-ubiquinone oxidoreductase activity and ATP synthesis by tetrahydroisoquinoline. Neurosci Lett 1988;86:105–108
54. Maruyama W, Dosert W, Naoi M. Dopamine-derived 1-methyl-6,7-dihydroxyisoquinoline as hydroxyl promotors and scavengers: *in vivo* and *in vitro* studies. J Neurochem 1995;64: 2635–2643
55. Maruyama W, Dosert W, Matsubara K, Naoi M. N-methyl (R) salsolinol produces hydroxyl radicals: involvement to neurotoxicity. Free Rad Biol Med 1995;19:67–75
56. Nagatsu T, Yoshida M. An endogenous substance of the brain, tetrahydroisoquinoline, produces parkinsonism in primates with decreased dopamine, tyrosine hydroxylase and biopterin in the nigrostriatal regions. Neurosci Lett 1988;87:178–182
57. Naoi M, Maruyama W, Dosert P, et al. Dopamine-derived endogenous 1(R),2(N)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, N-methyl (R) salsolinol, induced parkinsonism in rat: biochemical, pathological and behavioural studies. Brain Res 1966;709:285–295
58. Seaton TA, Cooper JM, Schapira AHV. Free radical scavengers protect dopaminergic cell lines from apoptosis induced by complex I inhibitors. Brain Res 1997;777:110–118
59. Marchetti P, Susin SA, Decaudin D, et al. Apoptosis-associated derangement of mitochondrial function in cells lacking mitochondrial DNA. Cancer Res 1996;56:2033–2038
60. Bernardi P, Broeckemeier KM, Pfeiffer DR. Recent progress on regulation of the mitochondrial permeability transition pore: a cyclosporin-sensitive pore in the inner mitochondrial membrane. J Bioenerg Biomembr 1994;26:509–517
61. Zamzami N, Susin SA, Marchetti P, et al. Mitochondrial control of nuclear apoptosis. J Exp Med 1996;83:1533–1544
62. Prezant TR, Agapian JV, Bohlman C, et al. Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndrome deafness. Nature Genet 1993;4:289–294
63. Jenner P, Schapira AHV, Marsden CD. New insights into the cause of Parkinson's disease. Neurology 1992;42:2241–2250
64. Dexter DT, Sian J, Rose S, et al. Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. Ann Neurol 1994;35:38–44

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