Secondary abnormalities of mitochondrial DNA associated with neurodegeneration

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Abstract

The central nervous system has a particularly high energy requirement, thus making it very susceptible to defects in mitochondrial function. A number of neurodegenerative diseases, in particular Parkinson's disease (PD), Huntington's disease (HD) and Friedreich's ataxia (FRDA), are associated with mitochondrial dysfunction. The identification of a mitochondrial complex-I defect in PD provides a link between toxin models of the disease, and clues to the pathogenesis of idiopathic PD. We have undertaken genomic transplantation studies involving the transfer of mitochondrial DNA (mtDNA) from PD patients with a complex-I defect to a novel nuclear background. Histochemical, immunohistochemical and functional analysis of the resulting cybrids all showed a pattern in the PD clones indicative of a mtDNA mutation. There is good evidence for the involvement of defective energy metabolism and excitotoxicity in the aetiology of HD. We, and others, have shown a severe deficiency of complex II/III confined to the striatum that mimics the toxin-induced animal models of HD. There is also a milder defect in complex IV in the caudate. The tricarboxylic acid cycle enzyme aconitase is particularly sensitive to inhibition by peroxynitrite and superoxide radicals. We have found this enzyme to be severely decreased in HD caudate, putamen and cortex in a pattern that parallels the severity of neuronal loss seen. We propose a scheme for the role of nitric oxide, free radicals and excitotoxicity in the pathogenesis of HD. FRDA is caused by an expanded GAA repeat in intron 1 of the X25 gene encoding a protein called frataxin. Frataxin is widely expressed and is a mitochondrial protein, although its function is unknown. We have found abnormal magnetic
resonance spectroscopy in the skeletal muscle of FRDA patients, which parallels our biochemical findings of reduced complexes I–III in patients’ heart and skeletal muscle. There is also reduced aconitase activity in these areas. Increased iron deposition was seen in patients’ tissues in a pattern consistent with a mitochondrial location. The mitochondrial iron accumulation, defective respiratory chain activity and aconitase dysfunction suggest that frataxin may be involved in mitochondrial iron regulation. There is also evidence that oxidative stress contributes to cellular toxicity.

Introduction

There is evidence for mitochondrial dysfunction in a number of neurodegenerative diseases, in particular Parkinson’s disease (PD), Huntington’s disease (HD) and Friedreich’s ataxia (FRDA). In PD and HD the respective defects parallel those induced by toxin models of the disease. In FRDA we have found similar abnormalities to yeast knock-out models of the disease. In this chapter we will discuss biochemical models of these diseases and demonstrate the evidence for mitochondrial dysfunction in humans with these conditions.

Parkinson’s disease

PD is one of the commonest neurodegenerative diseases and is characterized by the triad of tremor, rigidity and bradykinesia. Onset is usually at age 60–80 years, the prevalence in the West being 1 in 150–350, with a lifetime risk of 1 in 40. Pathologically there is degeneration of the substantia nigra, locus coeruleus and substantia innominata, and appearance of Lewy bodies in the cytoplasm of surviving neurons in these and other areas. The substantia nigra projects dopaminergic fibres to the striatum and consequently dopamine levels and tyrosine hydroxylase activity in the striatum are markedly decreased. The pathogenesis of PD is unknown but much information has come from toxin models of the disease, which have been shown to cause similar (but not identical) patterns of selective neuronal loss in humans, primates and rodents.

In the early 1980s 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) was found to cause a Parkinsonian syndrome in drug addicts who took a synthetic heroin substitute contaminated with this compound [1]. MPTP induces Parkinsonism in humans and other primates through specific uptake and conversion pathways which preferentially target the dopaminergic neurons of the substantia nigra. MPTP is oxidized to its toxic metabolite 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B, found in high concentrations in glia. MPP+ is taken up via the dopamine transporter and is consequently concentrated within nigrostriatal dopaminergic neurons [2,3]. MPP+ is further concentrated in the mitochondria where it specifically inhibits complex I of the mitochondrial respiratory chain [4,5] with a consequent reduction in ATP synthesis. Several studies, both in vivo and in vitro, also implicate the involvement of free radicals in MPTP toxicity. In addition, there is evidence that nitric oxide (NO) plays a role in MPTP-induced neuronal death, probably via the produc-
tion of peroxynitrite, an extremely toxic and reactive species which is formed by the combination of NO and the superoxide radical ($O_2^-\cdot$). 7-Nitroindazole, a selective neuronal NO synthase (nNOS) inhibitor, protects against MPTP-induced dopamine depletion in mice [6] and dopamine depletion and loss of tyrosine hydroxylase-positive neurons in baboons [7]. Also, nNOS knock-out mice are resistant to MPTP-induced neuronal death [8]. Although 7-nitroindazole also inhibits monoamine oxidase B, this is thought not to be the mechanism by which it protects against MPTP toxicity.

Isoquinolines are structurally related to MPTP and MPP$^+$ and occur naturally in the human brain [9,10], being formed by a condensation of biogenic amines with aldehydes. A number of animal models of PD have been studied using isoquinoline derivatives. N-Methyl-$p$-salsinol injected into rat striatum resulted in behavioural, biochemical and pathological changes very similar to those seen in PD, with selective toxicity to dopaminergic neurons [11,12]. This compound has been found in human brains [10] and in the cerebrospinal fluid of patients with newly diagnosed and untreated PD [13], suggesting that N-methyl-$p$-salsinol may be an endogenous neurotoxin involved in the pathogenesis of PD. Intraventricular administration of 1,2,3,4-tetrahydro-2-methyl-6,7-isoquinolinetriol into rats caused reduced striatal dopamine and reduced noradrenaline in the locus coeruleus, with a similar potency to MPTP [14]. It has been shown that isoquinoline derivatives are potent inhibitors of complex I of the mitochondrial respiratory chain [15] and $α$-ketoglutarate dehydrogenase [16]. Studies with PC12 cells, however, suggest that the cytotoxicity of these compounds is related to their affinity for the dopamine re-uptake system and their ability to concentrate in neurons rather than inhibition of the mitochondrial respiratory chain [17].

A number of biochemical studies of PD post-mortem tissue have been performed. Complex-I defects have been detected in the substantia nigra of PD brains but not in other brain regions [18,19]. These findings are supported by immunohistochemical evidence of a decrease in complex-I subunits in some dopaminergic neurons, whereas subunits of other complexes appear unaffected [20]. As discussed above, MPTP inhibits complex I via its toxic metabolite MPP$^+$, though the exact mechanism is not known. Post-mortem analysis of patients with multiple system atrophy, with similar substantia nigra degeneration and l-dopa therapy, demonstrated normal mitochondrial respiratory chain function in the substantia nigra [19,21], thus supporting the specificity of the complex-I defect in PD and making it unlikely that it is related purely to neurodegeneration itself. Complex-I abnormalities have also been observed in both platelets and muscle from PD patients (see [22] for review). Much work has been carried out in order to determine whether the defect in mitochondrial respiratory chain function is primary or secondary. As complex I is composed of 41 subunits and seven are coded for by mitochondrial DNA (mtDNA), a number of groups have looked for mtDNA mutations in PD patients. No conclusive results have emerged from sequencing studies so far. However, fusion studies from both our laboratory [23] and that of Swerdlow et al. [24] support the role of mtDNA in PD. These studies use cells that are experimentally depleted of mtDNA ($ρ^0$ cells) and therefore devoid of mitochondrial respira-
tory chain function. ρ⁰ cells are used extensively in mitochondrial research to investigate, inter alia, nuclear–mitochondrial interactions. Mitochondria from donor tissue-culture cells or platelets can be introduced into ρ⁰ cells by fusion to form cybrids. The effect of a new nuclear background on the donor mitochondria can be determined by subsequent biochemical and genetic analysis. If clonal lines are used, this technique also allows analysis of cybrid lines with varying proportions of wild-type and mutant mtDNA. 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. Alternatively, abolition (termed complementation) of the defect would indicate nuclear control of the biochemical abnormality, a toxic influence of the original donor cells, or drift of mtDNA from mutant to wild type during clonal expansion.

This system has been used to investigate the defects in mitochondrial myopathy encephalopathy, lactic acidosis and stroke-like episodes (MELAS) [25] and myoclonic epilepsy with ragged red fibres (MERRF) [26] syndromes in addition to our own work with mtDNA depletion syndrome [27], PD [23], focal dystonia [28] and Leber hereditary optic neuropathy (LHON) [29].

Eight preselected patients with a mean 24% deficiency of complex I were studied. Both mixed cybrid analysis (all fusion clones from each patient pooled and analysed together) and individual cybrid analysis (many single clones from one patient analysed) were performed. Analysis of the PD fusions provided

\[ \text{Figure 1 Mitochondrial activities in control and PD platelet A549 ρ⁰ mixed cybrid lines. Complex-I (CxI), complex-II/III (CxII + III) and complex-IV (CxIV) activities are expressed as ratios with CS activity. Values are means ± S.D. for mixed cybrid lines from control (C) and PD patients (both n = 7); Mann–Whitney U statistical analysis was used; *, } P = 0.007. Reproduced from [23] with permission. ©1998, Lippincott, Williams & Wilkins. \]
Secondary abnormalities of mitochondrial DNA

Evidence for a mtDNA defect underlying the complex-I defect in these patients. The mixed PD fusion clones had a 25% decrease in citrate synthase (CS)-corrected complex-I activity (Figure 1). There was a significant correlation between an individual control or patient’s complex-I activity and their cybrid complex-I activity, implying that the donor mtDNA-encoded subunits were major determining factors in complex-I activity. The analysis of the individual clonal lines from one patient produced a mean decrease of 25% in complex I and 20% in complex IV (Figure 2). This same combined deficiency had been seen in the original PD patient from whom the individual clones were derived. These experiments establish a role for mtDNA in PD and further work is ongoing to elucidate the role of mtDNA mutations/polymorphisms in PD.

Of great interest in PD research recently is the finding of a mutation in the α-synuclein gene in a large Italian kindred and three unrelated Greek families, all with autosomal-dominant familial Parkinsonism [30]. Another mutation in α-synuclein has since been found in a PD family of German extraction [31]. No mutations have been found in sporadic PD cases [32]. The exact function of α-synuclein is not known but it is a presynaptic protein which may be involved in neuronal plasticity. Although this mutation appears to be rare and confined to familial PD, an understanding of its biochemical consequences should provide valuable insight into the pathogenetic mechanisms that may be involved in primary idiopathic, sporadic PD.
Huntington’s disease

HD results from a mutation leading to an expanded CAG repeat in the coding region of the IT15 gene on chromosome 4. The gene is widely expressed throughout the body but it causes selective neuronal death predominantly within the striatum. The function of huntingtin protein and its role in the pathophysiology of the disease is unknown. There is good evidence that defective energy metabolism and consequent excitotoxicity are involved in the pathogenesis of HD.

Toxin models of HD

Lesions with N-methyl-D-aspartate (NMDA) agonists, such as quinolinic acid, result in animal models that closely mimic the neurochemical and histological changes seen in HD striatum [33–35]. In rats, motor hyperactivity, learning deficits and reduction in striatal glucose metabolism are seen [36,37]. In primates, excitotoxin lesions result in chorea inducible by dopamine agonists [33,38].

A number of mitochondrial toxins have been used to replicate the neurochemical, histological and clinical abnormalities seen in HD. Most work has concentrated on malonate and 3-nitropropionic acid (3-NPA), which are reversible and irreversible inhibitors, respectively, of succinate dehydrogenase (complex II of the mitochondrial respiratory chain). Intrastriatal injection of malonate in rats produced dose-dependent striatal excitotoxic lesions that were attenuated by NMDA antagonists [39,40]. The glutamate-release inhibitor lamotrigine also attenuated the lesions [41]. Co-injection of malonate with succinate blocked the lesion formation, consistent with its effect on complex II [40]. 3-NPA is a naturally occurring plant and fungal toxin that causes neurological illness in animals and humans. Ingestion in livestock resulted in hind-limb weakness, goose stepping and knocking together of the hind limbs while walking [42]. In China, children exposed to 3-NPA from a fungal contaminant of sugar cane developed a gastrointestinal disturbance followed by encephalopathy and coma, and by dystonia upon recovery. Computerized tomography scans showed bilateral hypodensities in the putamen and globus pallidus. Chronic low-grade systemic administration of 3-NPA to rats produces age-dependent lesions that closely mimic HD [43,44]. The lesions appear to occur by an excitotoxic mechanism as prior decortication, which removes the striatal glutaminergic input, significantly attenuates the lesions. Similar chronic administration of 3-NPA to primates produces selective striatal degeneration and abnormal choreiform movements [45].

Studies in vitro on cultured cerebellar granule neurons showed that 3-NPA treatment resulted in concentration- and time-dependent neurotoxicity, and its toxicity was increased when the cells were exposed to subtoxic doses of NMDA [46]. Both MK-801 and 2-amino-5-phosphonovaleric acid delayed but did not prevent the 3-NPA toxicity, suggesting that excitotoxicity is involved in 3-NPA toxicity but that it is not the only mechanism.
### Table 1  HD brain results

Statistical significance by Mann–Whitney U test. C, control; BA, Brodmann area; ND, not determined; NS, not significant.

<table>
<thead>
<tr>
<th></th>
<th>Caudate (n = 10)</th>
<th>Putamen (n = 15)</th>
<th>Cortex (BA 10 and 38, n = 10)</th>
<th>Cerebellum (n = 5)</th>
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<tbody>
<tr>
<td>Complex II/III (nmol/min per mg of protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C</td>
<td>25.9 ± 5.9</td>
<td>20.8 ± 8.46</td>
<td>22.62 ± 6.4</td>
<td>15.05 ± 5.35</td>
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<tr>
<td>HD</td>
<td>11.2 ± 4.8</td>
<td>8.30 ± 2.98</td>
<td>16.48 ± 6.5</td>
<td>16.91 ± 2.46</td>
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<tr>
<td>P</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>0.105</td>
<td>NS</td>
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<tr>
<td>CS (µmol/min per mg of protein)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>168 ± 30</td>
<td>222.4 ± 70.0</td>
<td>259.1 ± 59.5</td>
<td>244.3 ± 41.5</td>
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<tr>
<td>HD</td>
<td>154 ± 43</td>
<td>131.2 ± 48.9</td>
<td>188.8 ± 45.2</td>
<td>215.6 ± 63.6</td>
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<tr>
<td>P</td>
<td>ND</td>
<td>0.0016</td>
<td>0.0104</td>
<td>NS</td>
</tr>
<tr>
<td>Complex II/III/CS ratio</td>
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<tr>
<td>C</td>
<td>0.156 ± 0.031</td>
<td>0.098 ± 0.03</td>
<td>0.088 ± 0.001</td>
<td>0.063 ± 0.02</td>
</tr>
<tr>
<td>HD</td>
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<td>0.089 ± 0.032</td>
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<tr>
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<td>0.8</td>
<td>NS</td>
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<td>GAPDH (µmol/min per mg of protein)</td>
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<td>C</td>
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<td>1.87 ± 0.28</td>
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<tr>
<td>P</td>
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<td>NS</td>
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<td>NS</td>
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<td>Aconitase (nmol/min per mg of protein)</td>
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<td>C</td>
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<td>13.4 ± 4.21</td>
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<tr>
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<td>12.8 ± 5.42</td>
</tr>
<tr>
<td>P</td>
<td>0.0003</td>
<td>0.0009</td>
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</table>
**Evidence for abnormal energy metabolism in human HD**

In HD brain positron emission tomography has demonstrated glucose hypometabolism in the striatum and cerebral cortex [47,48]. Using magnetic resonance spectroscopy (MRS), increased lactate levels have been found in the occipital cortex and basal ganglia of HD patients in some [49] but not all studies. Analysis of HD cerebrospinal fluid has shown increased lactate/pyruvate ratios [50].

Defects in mitochondrial respiratory chain function have been found in HD post-mortem brain tissue. We and others [51–53] have found a severe deficiency in the activities of complexes II and III with a milder decrease in complex-IV activity in the caudate but not in cerebellum or cortex (Table 1). Complex-II/III activity but not complex-IV activity was reduced in the putamen. These biochemical defects mimic very closely the HD animal models induced by the complex-II inhibitors 3-NPA and malonic acid, discussed above, and parallel in anatomical terms the site of the main pathology in HD. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been found to bind to the expanded polyglutamine stretch of mutant huntingtin [54], but we and others have found the activity of this enzyme to be normal throughout HD brain [52,53]. However, GAPDH has many other functions, e.g. in translational regulation, and it is possible that these activities may be influenced by mutant huntingtin. Aconitase is a tricarboxylic acid cycle enzyme that is particularly sensitive to inhibition by peroxynitrite and $O_2^•^{-}$ [55,56] and thus has been suggested to be a good marker of excitotoxic cell damage [57]. We have also shown that aconitase is inhibited

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**Figure 3 Schematic representation of the relationship between excitotoxicity and the biochemical abnormalities in HD brain and their effect on energy metabolism.** Reversible inhibition of complex IV by NO will contribute to mitochondrial respiratory chain inhibition and $O_2^•^{-}$ production. The $O_2^•^{-}$ and $ONOO^-\,$ generated will primarily target aconitase activity (A), followed by complex II/III (B), and also irreversible inhibition of complex-IV activity. Decreased ATP levels will, in turn, enhance glutamate sensitivity and amplify the cycle. Reproduced from ref [53] with permission. ©1999, Lippincott, Williams and Wilkins.
by NO in intact SKNMC cells [53], and both complex II/III and aconitase are inhibited in submitochondrial particle preparations from rat brain [53]. We measured aconitase activity in HD brain regions and, interestingly, found activity to be severely decreased in HD caudate, putamen and cortex but normal in cerebellum (Table 1). This pattern of aconitase inhibition parallels the severity of neuronal loss seen in the disease. We propose, based on our findings and those of others, a scheme for the role of NO, free radicals and excitotoxicity in HD (Figure 3). Impaired energy metabolism together with increased oxidative stress and free-radical damage would form a self-amplifying cycle of cell toxicity, determined and initiated by excitotoxicity and NO production. The mechanism(s) by which mutant huntingtin precipitates this chain of events is unknown. It may for instance increase neuronal sensitivity to excitotoxic stimuli. We are currently investigating a transgenic model of HD [58] to help determine the effect of mutant huntingtin expression on mitochondrial function.

**Friedreich's ataxia**

FRDA is an autosomal-recessive disease with a frequency of 1 in 50,000 live births; it is the commonest cause of progressive ataxia. Patients usually present in adolescence with ataxia, but may have come to medical attention earlier because of skeletal deformities. FRDA patients develop spasticity, areflexia and cardiomyopathy, the last being the usual cause of death.

FRDA is caused by an expanded GAA repeat in intron 1 of the X25 gene encoding a protein now termed frataxin [59]. The intronic repeat appears to reduce mRNA levels for frataxin, with a consequent reduction in the concentration of this protein [60]. The concentration of frataxin is inversely proportional to the length of the GAA repeat on the smaller allele — this representing the remnant expression of the X25 gene. Although widely expressed, this protein is of unknown function. Its homology to a yeast protein (YFH1) led to the suggestion that frataxin may be a mitochondrial protein [61]. The identification of a processed N-terminal amino acid sequence and co-localization with mitochondria confirmed that frataxin is indeed a mitochondrial protein [59,62,63].

Deletion of the *YFH1* gene in yeast results in accumulation of iron within mitochondria, loss of mtDNA, a severe growth deficit on fermentable carbon sources, and an inability to grow on glycerol and ethanol [64]. Studies in our laboratory (J. Bradley, J.C. Blake, S. Chamberlain, P.K. Thomas, J.M. Cooper and A.H.V. Schapira, unpublished work) have demonstrated severe deficiencies of the activities of complexes I and II/III in cardiac muscle from nine FRDA patients. Analysis of respiratory chain function in cardiac muscle from two FRDA patients showed activities which fell at the low end of the laboratory’s control range, but had abnormal inter-complex ratios [65]. We also found severe reductions in aconitase activities in cardiac muscle. Furthermore, reduction in the activities of complexes I–III and aconitase were seen in FRDA skeletal muscle. This last observation fits with our data using 31P MRS in FRDA skeletal muscle *in vivo* (R. Loë, J.M. Cooper, J.L. Bradley, D.
Manners, P. Styles, D.J. Taylor and A.H.V. Schapira, unpublished work). There was a highly significant decrease in $V_{\text{max}}$ in the FRDA patients and this decrease was correlated inversely to the length of the patient’s GAA repeat — indicating a direct link between the mutation and the MRS result.

These observations have led to the hypothesis that abnormal iron metabolism, respiratory chain inhibition and free-radical generation constitute the pathogenetic mechanisms that underlie FRDA. If proven, there would be a rationale for effective anti-oxidant therapy at least, in an attempt to modify disease progression in FRDA.

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References
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