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13. ABSTRACT ***(Maximum 200 Words)***

The objective of the present research is to determine whether there is a coherent body of evidence implicating oxidative damage in the pathogenesis of Parkinson's Disease and the MPTP model of Parkinsonism. We found that there are significant decreases in α-ketoglutarate dehydrogenase complex and a significant increase in tissue malondialdehyde levels in the superior frontal cortex, the Parkinsonian syndrome known as Progressive Supranuclear Palsy. We are continuing studies looking at in situ hybridization probes for free radical enzymes. We have developed a novel column-switching assay for measurement of the oxidative marker of DNA damage in human body fluids. We have recently applied this to ALS patients and have found significant increases. We have also developed a novel assay for nitrogamma tocopherol, a marker for oxidative damage mediated by peroxynitrite. We are presently collecting samples from Parkinson's Disease patients to carry out measurements. We have continued our studies showing that oxidative damage plays a critical role in MPTP toxicity. We found that mice, which were deficient in cellular glutathione peroxidase, showed increased sensitivity to MPTP toxicity, which was accompanied by increases in free radical production. We also demonstrated that administration of MPTP to primates results in increased α–synuclein in the substantia nigra. These studies have, therefore, made significant progress on the original aims of the proposal.

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4. INTRODUCTION

Oxidative stress from endogenous and exogenous oxidants has been implicated as a major cause of Parkinson's Disease. The subject of the present research is to:

1. Investigate whether there is coherent evidence of increased oxidative damage to proteins, lipids and DNA in postmortem tissue of patients with Parkinson's Disease.
2. To develop novel HPLC based assays for quantitation of products of oxidative damage in human CSF, plasma and urine samples and to apply these biomarkers to study whether they are altered in patients with Parkinson's Disease.
3. To determine whether oxidative stress plays a key role in neuronal death, which occurs in the MPTP model of Parkinson's Disease and whether the same stress may be operating in vulnerable human nigral neurons.

In particular, we were going to examine whether transgenic mice with alterations in free radical scavenging enzymes or which overexpress Bcl2 are resistant to MPTP neurotoxicity. We were also going to examine whether free radical spintraps, neuronal nitric oxide synthase inhibitors and creatine can block MPTP neurotoxicity. Finally, we were going to examining the expression of genes encoding proteins which may determine vulnerability to oxidative stress in human dopamine neurons.

5. BODY

The program has now continued in two phases centered at the Weill Medical College of Cornell University and at the Massachusetts General Hospital. Dr. Anne B. Young, Chief of Neurology at Massachusetts General Hospital is now supervising the studies, which are ongoing there.

Objective #1 – to determine whether there is coherent evidence of increased oxidative damage to the protein, lipid and/or DNA fractions in postmortem human brain tissue of patients with PD as compared to age-matched controls.

We are continuing to collect post mortem brain tissue to carry out this specific aim. It has been difficult to collect adequate numbers of control and Parkinson's Disease specimens. We carried out initial studies of 3-nitrotyrosine and protein carbonyls. However, there was a large variance in the measurements. We have continued to work on an assay for nitro-gammatocopherol. This has recently become a reliable assay for nitration in our laboratory. We intend to utilize this assay in studies of both human postmortem brain material as well as in plasma and in experimental animal models.

We have carried out further studies of postmortem brain tissue in patients with the Parkinsonian syndrome Progressive Supranuclear Palsy. We measured activities of mitochondrial enzymes as well as tissue malondialdehyde levels in post mortem superior frontal cortex from 14 pathologically confirmed cases of PSP and 13 age-matched control brains. We found significant decreases in α-ketoglutarate dehydrogenase complex and significant increases in tissue malondialdehyde levels in the superior frontal cortex of PSP samples. There were no alterations in complex I or complex IV activities.

We have also completed a comprehensive study of the activity and localization of two essential antioxidant systems (superoxide dismutase [SOD] enzymes and total glutathione) in seven brain regions (frontal cortex, caudate nucleus, globus pallidus, subthalamic nucleus, medial substantia nigra pars compacta, calcarine cortex and cerebellar cortex) of post-mortem control and Parkinsonian PSP brains. The most robust findings of this study were the striking increases in SOD1 (Cu/ZnSOD) activity and glutathione levels within most of the PSP brain regions examined, relative to controls. By contrast, only the subthalamic nucleus exhibited a significant increase (+68%) in SOD2 (MnSOD) activity. These observations were confirmed by SOD1- and SOD2-immunohistochemistry. These data demonstrate a marked increase in antioxidant activity and protein levels within the pathological PSP brain. The increased antioxidants measured are attributable, in part, to a glia reaction, and are supportive of the hypothesis that there is an underlying insidious oxidative stress. Together, these findings provide further evidence that oxidative damage may play a role in the pathogenesis of the Parkinsonian syndrome associated

with Progressive Supranuclear Palsy. It is also possible that mitochondrial dysfunction may contribute as shown by the reduction in α-ketoglutarate dehydrogenase activities.

We are continuing studies using immunocytochemical assays for oxidative damage from Parkinson's Disease postmortem tissue. This is being done on a panel of human brain tissue from normal midbrain as well as a smaller number of samples from patients with Parkinson's Disease.

Objective #2: To develop novel HPLC based assays for quantitation of products of oxidative damage in human CSF, plasma and urine samples and to apply these biomarkers to study whether they are altered in patients with Parkinson's Disease.

We have developed a novel column switching assay to measure 8-hydroxy-2-deoxyguanosine concentrations in urine, plasma and CSF as well as other biological matrices. This is an unique methodology, which was recently published in detail. We have recently utilized this technology to assess OH^8dG levels in samples from patients with ALS. We have been successful in demonstrating for the first time that OH^8dG levels are significantly elevated in the CSF, plasma and urine of ALS patients. We are continuing to collect samples from Parkinson's Disease patients to carry out similar measurements.

We have now established an assay for nitro-gammatocopherol for use in human body fluids. We have utilized this is 6 normal samples and have demonstrated a consistent ability to detect this compound. It requires a hexane extraction of plasma in which an internal standard for retinol acetate and COQ9 are added. The hexane extracted sample is then dried down and reconstituted in a mobile phase consisting of 50% methanol and 50% ethanol. Samples are then run by HPLC using a 12 electrode coularray system. Utilizing this methodology we can then measure α-tocopherol, gamma-tocopherol, nitro-gammatocopherol and CoQ10 levels. We will utilize this methodology for examining whether there is evidence of increased nitration mediated by peroxynitrite in plasma samples of Parkinson's Disease patients as compared to normal controls.

We have also carried out studies examining whether there are mitochondrial DNA mutations in DNA obtained from blood samples of Parkinson's Disease patients as compared to normal controls. It is well known that there appears to be a mitochondrial complex I deficiency in platelets of Parkinson's Disease patients. We carried out complete sequencing of all mitochondrial DNA encoded complex I and transfer RNA genes in 28 Parkinson's Disease patients and 8 control subjects. In addition, we screened up to 243 additional Parkinson's Disease patients and 209 control subjects by restriction digest for selected mutations. In the Parkinson's Disease patients, 15 complex I missense mutations and 9 transfer RNA mutations were identified.

Some Parkinson's Disease patients were found to carry complex I mutations that alter highly conserved amino acids. We were unable to confirm associations of mutations at precisions 4366, 5460 and 15927 with PD. The

findings indicate that mitochondrial DNA mutations with a high mutational burden in complex I and transfer RNA genes do not appear to play a major role in the risk for Parkinson's Disease. This finding however, required further study since there could be low level heteroplasmic mutations which have been missed by this type of DNA sequencing. They are, therefore, continuing these studies. We have recently obtained platelet samples from 19 Parkinson's Disease patients as well as 19 normal controls. The 19 Parkinson's Disease subjects all were documented to halve low complex I activity in their platelets. We will, therefore, carry out further sequencing of these samples.

Objective #3: to determine whether oxidative stress plays a role in the neuronal death in the MPTP model of Parkinson's disease, and whether the same stress may be operating in human nigral neurons.

We have carried out further studies examining the role of oxidative damage in MPTP toxicity. One interesting and novel observation, which we made, was that administration of MPTP to primates resulted in increased α-synuclein staining in substantia nigra neurons. This is the first evidence to show direct linkage between the potential findings of oxidative damage and deposition of α-synuclein, which is a major component of Lewy bodies, found in Parkinson's Disease.

We have carried out further studies of MPTP toxicity in mice, which have a deficiency in cellular glutathione peroxidase. Hydrogen peroxide is converted to H2O2 by either catalase or selenium glutathione peroxidases. Catalase is thought to be rather low in the brain and is localized to peroxisomes. Glutathione peroxidase I is known to be a major enzyme in the brain which reduces H2O2. It is found both in the cytocell as well as in mitochondria. There is also recent evidence that glutathione peroxidase may play a major role in detoxifying peroxynitrite. We found that glutathione peroxidase knockout show no evidence of neuropathological behavioral abnormalities at 2 – 3 months of age. Intrastriatal injections of malonate resulted in a significant two fold increase of lesion size in homozygote glutathione peroxidase knockout mice as compared to both heterozygote glutathione knock out and wildtype control mice.

Malonate induced increases in conversion of salicylate to 2-3 and 2-5-dihydroxybenzoic acid, an index of hydroxyl radical generation, were greater in homozygote glutathione peroxidase knockout mice as compared with both heterozygote glutathione peroxidase knockout and wildtype control mice. Administration of MPTP resulted in significantly greater depletions of dopamine, DOPAC and homovanillic acid in the glutathione peroxidase knock out mice as compared to those seen in wildtype control mice. Striatal 3-nitrotyrosine concentrations after MPTP were significantly increased in the glutathione peroxidase knockout mice as compared with wildtype control mice. This is consistent with an increased generation of peroxynitrite in these mice.

Lastly, systemic administration of 3-nitrppropionic acid resulted in significantly greater striatal damage and increase in 3-nitrotyrosine in the

glutathione peroxidase knockout mice as compared to wildtype control mice. These findings indicate that a knockout of glutathione peroxidase may be compensated at baseline under nonstress conditions that but after administration of mitochondrial toxins glutathione peroxidase plays an important role in detoxifying increases in oxygen radicals.

Recent studies have indicated that induction of inducible nitric oxide synthase may play a role in MPTP neurotoxicity. It has been demonstrated that mice with the knockout of iNOS are resistant to MPTP toxicity. We are commencing studies utilizing minocycline either alone or in combination with creatine to determine whether this will exert neuroprotective effects against MPTP toxicity. Minocylcine has recently been demonstrated to show neuroprotective effects in a transgenic mouse model of Huntington's Disease. It has been demonstrated to inhibit the expression of iNOS.

In order to determine whether the same kinds of oxidative mechanisms may be operative in human Parkinson's disease, we are examining the expression of genes encoding proteins which are involved in oxidative mechanisms in human substantia nigra. Human nigra neurons exhibit a characteristic pattern of vulnerability in Parkinson's disease (ventral tier>dorsal tier>pars lateralis>paranigral nucleus). As described in detail in our last progress report (revised, April 2000), we have adopted the laser Capture Microdissection methods to accomplish these studies. Since the last report, we have optimized the parameters for capture of human nigral neurons, and for reliable isolation of the mRNAs. We now have in hand human nigra tissue of acceptable quality for these studies, and expect to proceed to the planned array analysis shortly.

6. KEY RESEARCH ACCOMPLISHMENTS

a. The finding of increased lipid peroxidation in the subthalamic frontal cortex and enhanced expression of superoxide dismutase and glutathione in the Parkinsonian disease Progressive Supranuclear Palsy

b. The applications of measurements of 8-hydroxy2-deoxyguanosine in human body fluids to patients with neurodegenerative diseases

c. The finding that MPTP caused expression of α-synuclein in substantia nigra neurons. This directly links oxidative damage to Lewy body generation.

d. The finding that mice, which are deficient in the antioxidant enzyme glutathione peroxidase, show increased vulnerability and increased evidence of oxidative damage produced by MPTP.

7. REPORTABLE OUTCOMES

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8. CONCLUSIONS

We have made substantial progress in finding that there are increased markers of oxidative damage in postmortem tissue in a parkinsonian syndrome. We have developed two highly sensitive assays for measurement of oxidative damage to DNA as well as a marker of nitration for examination of parkinsonian patients.

We have found that a number of novel therapeutic or genetic manipulations can markedly attenuate or increase both oxidative damage and dopaminergic neurotoxicity in the MPTP model of parkinsonism.

These studies greatly strengthen the implication of oxidative damage in Parkinson's Disease pathogenesis.

9. REFERENCES

None.

10. APPENDICES

1 copy of each of the cited papers in reportable outcomes.

of the PLB–SR–calcium pump complex (Fig. 1). When β-adrenergic signaling is blocked, it is possible that the PLB and the calcium pump remain connected, calcium transport is inhibited, and DCM develops. In fact, an earlier study[5] reported that DCM in the MLP-deficient mouse did not fully develop in transgenic mice in which the β-adrenergic signaling was constitutively active. Further study into the effects of chronic activation of β-adrenergic signaling will establish whether this is a feasible therapeutic approach for treatment of human DCM. Koss and Kranias[2] and Minamisawa *et al.*[1] propose that a therapeutic approach to heart failure may involve gene therapy or small molecules that specifically interfere with the PLB–SR–calcium pump interaction.

This is an exciting proposal based on a remarkable finding. Clinicians trying to understand why hearts fail go through frequent mood swings—optimism on learning of results such as those published by Minamisawa *et al.*, followed by the sad realization that heart failure syndrome is complex and unpredictable, and that it is often difficult to translate discoveries from mouse to man. Less than two years ago, news broke that hypertrophy and even heart failure might be 'cured' by inhibition of a calcium-dependent phosphatase, calcineurin[6]. In this case the therapy would reduce the amount of calcium available to calmodulin (CaM), thereby inhibiting formation of the calcium–CaM complex and preventing calcineurin's phoshatase activity. This theory of heart failure did not fit with earlier reports associating heart failure with a reduction in the amount of calcium released to the myofilaments from the SR. However, the theory does fit with the generally held principle that increasing intracellular calcium might be harmful in treatment of heart failure. Apart from activating calcineurin, increased intracellular calcium is thought to be arrhythmogenic. Yet if increased intracellular calcium is harmful, we are faced with the conundrum of why the PLB-deficient mouse does not develop any cardiac pathologies, even though calcium is chronically increased in the myocytes. The results of Minamisawa *et al.* strongly suggest that how one increases intracellular calcium may determine whether or not calcium has detrimental effects. Somewhere in this maze of calcium mechanisms lies the truth, and the most recent findings have brought us one step closer to it.

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NOS knockouts and neuroprotection

Neuronal nitric oxide synthase (nNOS) is known to be involved in the dopaminergic neurodegeneration that occurs in the MPTP model of Parkinson disease. Recent studies indicate that different isoforms of NOS may act synergistically to cause neuronal injury in Parkinson disease and other neurodegenerative diseases (pages 1403—1409).

THOMAS GRÜNEWALD & M. FLINT BEAL

OVER THE PAST years it has become evident that neurodegenerative disorders such as Parkinson disease (PD), Alzheimer disease, amyotrophic lateral sclerosis and Huntington disease share common mechanisms of pathogenicity. Among those, free radical-induced oxidative stress, mitochondrial dysfunction and excitotoxic processes are pivotal in cell death.

The role of the free radical nitric oxide (NO) in nervous system morphogenesis and developmental synaptic plasticity is well established[1]. However in pathological conditions such as brain ischemia, NO shows a Janus face with either protective or detrimental characteristics[2]. In the nervous system, three highly homologous isoforms of NOS (endothelial, neuronal and inducible NOS) catalyze the formation of NO from L-arginine and molecular oxygen. Whereas the calcium-dependent neuronal NOS isoform is constitutively expressed in brain cells, the inducible NOS isoform is only expressed after pathological stimuli, including ischemia, viral and bacterial infections or trauma.

In this issue of *Nature Medicine*, Liberatore *et al.* establish fundamental principles for how two different isoforms of nitric oxide synthase may be involved in the development of Parkinsonian-like symptoms in mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine). This is not only an important contribution to the elucidation of the neurodegenerative process in idiopathic PD, but also suggests potential neuroprotective strategies.

In PD research, it has long been argued that in addition to genetic factors, exposure to environmental toxins such as epoxy resins, pesticides and other chemicals launches a cascade of events that lead to the destruction of dopaminergic nigrostriatal projections.

In the early 1980s, however, the hypothesis of toxins as a disease-triggering factor gained further support. After injection of MPTP, a group of drug addicts in California developed a severe neurological disorder that closely resembled the clinical symptoms of idiopathic PD (ref. 3). MPTP is structurally analogous to naturally occurring isoquinolines, which may be implicated as endogenous neurotoxins in PD. This observation led to widespread use of MPTP in experimental animal models.

The neurotoxic effects are thought to be mediated by the active metabolite of MPTP, the methylpyridinium ion (MPP^+), which is selectively taken up by dopaminergic neuronal terminals in the

Is calcium the 'cure' for dilated cardiomyopathy?

Different studies report different findings about the roles of calcium in myocyte function and in the development of dilated cardiomyopathy. But determining whether increasing intracellular calcium is helpful or harmful for heart failure patients will require further investigation.

R. JOHN SOLARO

HUMAN DILATED CARDIOMYOPATHY (DCM), a leading cause of death and debilitation, has been genetically linked to mutations in proteins in the cardiac myocyte cytoskeletal network. Deletion of essential cytoskeletal proteins disrupts this network and causes DCM-like symptoms in animal models. One such protein is muscle LIM protein (MLP). MLP-deficient mice have a phenotype similar to the clinical manifestations of DCM. A poorly understood consequence of the loss of cardiac myocyte cytoskeletal integrity is a reduction in amounts of calcium released from the sarcoplasmic reticulum (SR) for delivery to troponin C, the protein that triggers activation of the myofilaments. This reduction causes fewer myosin cross-bridge interactions with thin filaments, cellular force generation falls, and end-diastolic volume increases to maintain cardiac output.

A therapy for DCM could be simply to correct the defect in calcium delivery to the myofilaments. As reported in the 29 October issue of *Cell*, Minamisawa *et al.* have done just that[1] in experiments with phospholamban (PLB)-deficient mice[2]. PLB is a protein that inhibits the calcium pump that loads the SR with calcium. Compared with those of normal mice, myocytes from the hearts of the PLB-deficient mice show increases in contraction, resulting from an increase in the amplitude of the spike of the transient change in intracellular calcium that occurs with each beat of the heart. There is also a relative increase in rates of rise and fall of the calcium transient in heart cells from the PLB-deficient mice. Minamisawa *et al.*[1] went on to generate mice lacking both MLP and PLB by mating PLB-deficient mice with the MLP-deficient mice. The 'double-knockout' mice showed essentially no signs or symptoms of DCM.

Why do hearts lacking MLP develop DCM? The cytoskeletal network forms a scaffold that physically connects the internal elements of the cardiac cell (contractile elements of the sarcomere and the nuclear membrane) to the cell surface membrane and its array of receptors, transporters and exchangers (Fig. 1). The phenotype of the MLP-deficient mouse and genetic linkage of muscular dystrophies and cardiac myopathies to mutations of sarcomeric proteins[3] (actin, myosin, troponins T and I, myosin binding protein C and tropomyosin), as well as to proteins connecting desmin to the nuclear envelope, indicate myocyte cytoskeletal integrity is required for normal muscle function. Elements of the cytoskeleton not only provide mechanical stability to the myocytes but also couple cellular mechanics to signal transduction pathways. Flux of ions across channels, activation of second messenger signaling pathways, and release of stress-related peptides such as endothelin and angiotensin all affect the mechanical state of the cell. Myocardial stretch, an inevitable consequence of increased end-diastolic volume, places strain on the myocyte cytoskeleton, activating signaling cascades leading to cell hypertrophy.

There is also evidence for direct effects of myocardial stretch on protein synthesis[4]. Cellular strain can be transduced by signaling of integrins in focal adhesions, leading to transcriptional activation and new protein synthesis. This mechanism requires a connection between the extracellular matrix and the contractile cytoskeleton. In hearts of MLP-null mice, it is possible that disruptions in nuclear membrane connections alter cell signaling and activation of transcription, causing expansion of the extracellular matrix and subsequent fibrosis. This series of changes and myocardial remodeling may disrupt β-adrenergic signaling. Normally, β-adrenergic receptor signaling induces PLB phosphorylation and dissociation

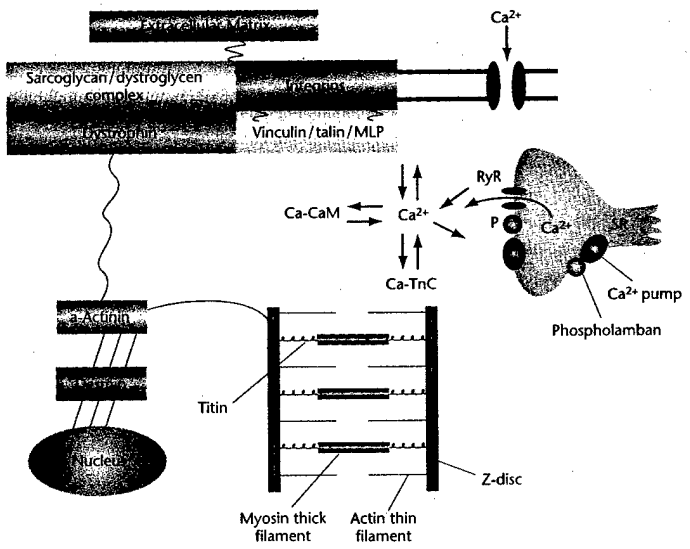

Fig. 1 The cytoskeletal network and excitation contraction coupling in a working heart cell. The cytoskeleton forms a network of struts and pillars connecting the surface membrane with its array of receptors and channels to the extracellular matrix and intracellular elements such as the nucleus and myofilaments. The thick and thin filaments of the sarcomere transmit force to the Z-disc, a node of interactions among the cytoskeletal elements actin, α-actinin, titin, and desmin. Force is activated by Ca^{2+}-binding to troponin C (TnC) on the actin-containing thin filament. Ca^{2+} delivered to the myofilaments is released from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyR) acting in concert with surface membrane Ca^{2+} channels. During relaxation Ca^{2+} is returned to the SR by the Ca^{2+} pump, which also regulates the amounts of Ca^{2+} stored in the SR. The pump is inhibited by phospholamban, which when phosphorylated (P) dissociates from the pump.

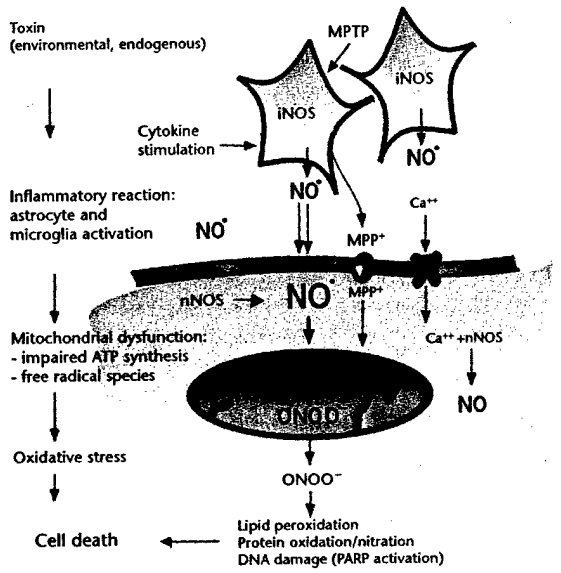

Fig. 1 Putative contribution of iNOS and nNOS to cell death in the MPTP model of Parkinson's disease. Both the constitutively expressed neuronal NOS and the inducible form of NOS collaborate to form nitric oxide ($NO^{\cdot}$). After combination of NO with superoxide ($O_2^{\cdot}$) the highly reactive peroxynitrite ($ONOO^-$) will cause oxidative damage to lipids, proteins and DNA and thus finally lead to cell death.

substantia nigra. MPP^+ accumulates in mitochondria, where it disrupts oxidative phosphorylation by inhibiting complex I of the respiratory chain, finally leading to reduced ATP synthesis. The impaired ATP supply represents an essential event in the downstream cascade leading to cell death. Failure of ATP-dependent ion pumps and channels allows ambient concentrations of glutamate to excite the N-methyl-D-aspartate (NMDA) receptor and mediate cellular calcium influx. Increased calcium levels activate the calcium and calmodulin-dependent neuronal isoform of nitric oxide synthase (nNOS). Inhibition of complex I results in increased formation of free radical species by mitochondria such as the superoxide anion ($O_2^{\cdot}$). NO can react with superoxide to produce peroxynitrite ($ONOO^-$), ultimately resulting in oxidative damage and protein nitration. NO also induces iron liberation from the cells, thus making iron readily available for free radical generation through the Fenton reaction.

As a consequence of oxidative injury to DNA by peroxynitrite, the highly energy consuming DNA repair enzyme poly (ADP-ribose) polymerase (PARP) is activated, promoting further ATP depletion. Madir and co-workers recently demonstrated that PARP activation is a determinant of MPTP-induced dopaminergic cell death[4]. PARP-deficient mice ($PARP^{-/-}$) are resistant to the toxic effects of MPTP, and show a significant lower reduction in striatal dopamine, dihydroxyphenylacetic acid and homovanillic acid.

The same authors provided a link between NO toxicity and MPTP-induced parkinsonism. Poly (ADP-ribose) formation is a marker of PARP catalytic activity. After MPTP administration, mice lacking the gene for neuronal NO synthase ($nNOS^{-/-}$) did not show nuclear protein poly(ADP-ribosyl)ation. The $nNOS^{-/-}$ mice were previously shown to be resistant to MPTP, indicating that $ONOO^-$ results in DNA damage, which then results in activation of PARP. A consistent study reported that that the nNOS inhibitor 7-nitroindazole protects against striatal dopamine depletions and loss of tyrosine hydroxylase–positive neurons in the substantia nigra in MPTP-treated mice and baboons[5,6].

Liberatore *et al.* provide new data on the role of iNOS in the MPTP model of neurodegeneration. The authors administered MPTP to wild-type littermates and iNOS-deficient mice ($iNOS^{-/-}$). Wild-type MPTP-treated mice developed robust gliosis coinciding with microglia activation and an upregulation of iNOS in the substantia nigra pars compacta. Whereas $iNOS^{-/-}$ mice showed a similar gliosis, twice as many dopaminergic neurons survived in an identical dosing regimen. This is consistent with prior work showing that iNOS is involved in delayed cell death after cerebral ischemia[7]. Striatal dopaminergic fibers, however, were equally affected and underwent cell death after treatment with MPTP in both wild-type and iNOS deficient mice, reflecting a spatial difference in iNOS production. One of their most interesting findings is that the constitutive nNOS activity in the ventral midbrain is apparently 600% higher than iNOS at its peak level.

These observations demonstrate how different isoforms of nitric oxide synthase may be collaborating to cause neuronal injury in PD and other neurodegenerative diseases. Recently, Langston *et al.* reported a detailed neuropathological study of three patients who initially injected MPTP and developed severe parkinsonism[8]. The patients developed widespread loss of nerve cells in the substantia nigra, accompanied by considerable astrocytic and microglial activation, and extraneuronal neuromelanin accumulation many years after the patients' last exposure to MPTP. These findings indicate that a limited duration of exposure to a neurotoxin may lead to a self-perpetuating, ongoing neurodegenerative process. Similar inflammatory changes are seen in idiopathic PD, and this supports the view that an inflammatory reaction may contribute to cell death[9]. Inflammatory cytokines such as interleukin-1; interferon-γ or tumor necrosis factor-α can activate both iNOS directly and nNOS by causing an increase in intracellular calcium.

Clearly the multifactorial pathogenicity of PD demands multi-modal therapeutic approaches. Thus, therapeutic strategies of the future may involve a combination of iNOS and nNOS inhibitors, PARP inhibitors, free radical scavengers, excitatory amino acid antagonists and immunomodulating agents.

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Runt domains take the lead in hematopoiesis and osteogenesis

It has been nearly a decade since AML1 was first identified as the DNA-binding protein PEBP2αB/CBFA2 in mice. Molecular, genetic and biochemical studies have converged to reveal new information about how this unique family of transcription factors functions, and the linkage between developmental processes and human disease.

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HUMAN CANCERS DEVELOP through a multistep process associated with the accumulation of mutations in proto-oncogenes and tumor suppressor genes. The inheritance of mutant alleles can 'jump-start' this process, making genetic analysis of families predisposed to the development of cancers particularly useful for the identification of genes that suppress malignant transformation. In contrast to many other types of cancer, heritable acute leukemias are rare, thwarting attempts to identify genes associated with the disease by linkage analysis of familial pedigrees. Most researchers in the field have, until recently, focused their attention on the analysis of acquired mutations that result from chromosomal translocations or inversions[1].

Song *et al.*[2] have recently reported the identification of two mutations that are associated with a rare autosomal dominant disease, familial platelet disorder (FPD), and that also predispose individuals to acute myelogenous leukemia (AML). These occur in the *AML1* gene which is commonly associated with chromosomal abnormalities[1]. This comes on the heels of a report by Osato *et al.*[3], who previously described sporadic point mutations in the *AML1* gene of six patients with myeloblastic leukemias. Unexpectedly, the FPD- and AML-associated mutations all clustered in one region of the *AML1* gene, a DNA-binding element known as the "Runt domain." Other genes having Runt domains, such as *PEBP2αA* or *CBFA1*, have been implicated in the origin of cleidocranial dysplasia (CCD), an autosomal dominant disorder affecting skeletal patterning[4]. Point mutants in the PEBP2αA/CBFA1 Runt domain abolish its DNA-binding ability and are associated with CCD in humans (refs. 5–7; H. Kanegene, personal communication).

Genes in the *AML/PEBP2/CBF* gene family encode a small but essential group of heterodimeric transcription factors, whose members act as 'master regulators' of developmental gene expression for both hematopoiesis and osteogenesis. *AML1* (also known as *PEBP2αB* or *CBFA2*) encodes the α subunit of the heterodimer, and also contains the DNA-binding Runt domain. The β subunit of this factor (the product of the single *PEBP2β/CBFB* gene) enhances α-subunit DNA binding through a mechanism that stabilizes the interaction between the α-subunit Runt domain and DNA (refs. 8–10). There is more than 90% amino-acid sequence identity between the Runt domains of all three α-subunit genes (*AML1/PEBP2αB/CBFA2*, *PEBP2αA/CBFA1* and *PEBP2αC/CBFA3*), so it is likely that these gene products use identical DNA-binding and heterodimerization mechanisms.

Recent analysis of the Runt domain's three-dimensional structure may provide insight into the functional consequences of mutations associated with either AML, FPD or CCD (refs. 8,11). The Runt domain is constructed as an immunoglobulin-like protein fold[8,10] (Fig. 1). Although a relatively uncommon DNA-binding motif, this fold is related to the DNA-binding domain of the tumor suppresser p53. Point mutations in the Runt domain associated with AML (ref. 3), FPD (ref. 2) and CCD (refs. 5–7) all occur on the DNA-binding surface of the domain[8] (Table 1 and Fig. 1). Six of nine known mutations alter essential lysine or arginine residues that should form direct contacts with the DNA (Fig. 1)(refs. 8,11), and thereby account for the reported loss of DNA-binding function *in vitro* (Table 1). Two mutations (Met124→Arg and Phe146→Ser) would be expected to destabilize the structure of the Runt domain itself and should therefore cause deficiencies in both DNA binding and heterodimerization, based on the identification of the heterodimerization interface in a ternary Runt-domain–β–DNA complex[8–10]. As the Arg139→Gln mutation is proximal to the Ser140→Asn mutation, it is believed to confer a similar phenotype, although this effect has not yet been proven (Table 1). The Lys167→Asn mutation is predicted to disrupt DNA binding only.

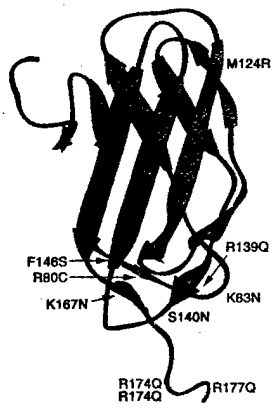

Fig. 1 Three-dimensional structure of the Runt domain and location of mutations causing deficiencies in DNA binding associated with AML, FPD and CCD. Orange, DNA-binding elements; red, AML/FPD-associated mutants; blue, CCD-associated mutants.

Nonsense mutations, missense mutations or intragenic deletion of one allele of *AML1* co-segregate with disease in FPD/AML pedigrees[2], and *AML1* haploinsufficiency is believed to cause the observed autosomal dominant congenital platelet defects in these patients[2]. Several of the missense mutations remain heterodimerization-competent, providing a clue as to how the unaffected allele may show an altered phenotype. AML1 α/β heterodimerization has been implicated in the leukemic transformation of cells having a β-subunit chimera, *PEBP2β/CBFB–MYH11*, which results from the chromosome 16 inversion[9,10]. The *PEBP2β/CBFB* gene is also required for normal hematopoiesis, with *PEBP2β/CBFB*$^{-/-}$ mice showing the complete loss in definitive hematopoiesis found in *AML1*$^{-/-}$ knockouts[12,13]. Heterodimerization competency in the mutated allele could reduce the availability of β to the unaffected gene product, altering the DNA-binding efficiency for the wild-type allele. This could alter expression of AML1-dependent genes with consequences dependent on the severity of the transcriptional defect. A detailed analysis of the relationship between heterodimerization and transcriptional activation should be made to understand the patterns of gene expression at AML1-dependent genes.

It is also unclear how altered AML1 transcriptional regulation causes a predisposition for the acquisition of additional mutations and leukemia. Two of the three AML patients with Runt-domain point mutations also had translocation-generated mutations, and both had relapsed from an earlier leukemic episode[3]. FPD patients typically show a spectrum of chro-

MPTP induces alpha-synuclein aggregation in the substantia nigra of baboons

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity reproduces many of the features of Parkinson's disease (PD). α-Synuclein has been identified as a prominent component of the Lewy body (LB), the pathological hallmark of PD. MPTP-treated primates have been reported to develop intraneuronal inclusions but not true Lewy bodies. We administered MPTP to baboons and used a monoclonal α-synuclein antibody to define the relationship between neuronal degeneration and α-synuclein immunoreactivity in the substantia nigra. MPTP-induced neuronal degeneration was associated with the redistribution of α-synuclein from its normal synaptic location to aggregates in degenerating neuronal cell bodies. α-Synuclein aggregation induced by MPTP models the early stages of Lewy body formation and may be a fundamental step in the evolution of neuronal degeneration in PD. *NeuroReport* 11: [illegible] © 2000 Lippincott Williams & Wilkins.

Key words: Baboon; MPTP; Synuclein; Substantia nigra

INTRODUCTION

α-Synuclein is a 143 amino acid protein localized to axons and synapses in the normal human brain [1]. The important role of α-synuclein in the pathogenesis of PD has been recently discovered. α-Synuclein mutations cause familial PD (A53T and A30P) [2,3] and α-synuclein is a major constituent of the LB, the pathological hallmark of idiopathic PD [1,4]. In human and non-human primates, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces clinical, biochemical and neuropathological changes similar to those found in idiopathic Parkinson's disease [5]. Aged primates exposed to MPTP develop intraneuronal inclusions similar, but not identical, to Lewy bodies [6]. In the present study we examined the distribution of α-synuclein in baboons (*Papio anubis*) treated with MPTP to determine whether neurodegeneration produced by MPTP is associated with redistribution and aggregation of α-synuclein as seen in humans with idiopathic PD.

MATERIALS AND METHODS

Tissue sections from six male *P. anubis* were used in these experiments. The three baboons in the treatment group received one or two i.m. injections of 0.4 mg/kg MPTP (MPTP-HCl, Sigma) daily at 11:00 h (and 18.00 h) for 6 days followed by an injection of 0.27 mg/kg at 11:00 h on day 7. Ten days after the first injection the three treated animals and three untreated controls were placed under chemical restraint (ketamine 10 mg/kg) and euthanized by an overdose of sodium pentobarbital (120 mg/kg). Fresh whole brains were hemisected by a midline sagittal cut. One hemisphere and brain stem half was flash frozen for neurochemical studies, while the other half was immersion fixed overnight in cold (4°C) 4% paraformaldehyde/lysine/sodium m-periodate solution for histopathological evaluation. The brain stem and cerebellum were separated by a midbrain cut at the level of the mammary bodies. The tissue specimens were cryoprotected in 15% glycerol solution made in 0.1 M phosphate buffer. The brainstem was sectioned coronally at 50 μm and subsequently stained for routine cell identification using cresyl violet and immunocytochemically for tyrosine hydroxylase (TH) activity (TH antisera; 1:1000 dilution; Protos Biotechnology, New York, NY) and α-synuclein staining using a well-characterized α-synuclein monoclonal antibody (H3C), which recognizes

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the C-terminus of α-synuclein [7] (1:1000 dilution, courtesy of Dr David Clayton). The immunocytochemical methods used have previously been reported [8]. All animals used in these procedures were in strict compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by all local Animal Care Committees.

RESULTS

Nissl-stained sections of the substantia nigra from MPTP-treated animals showed marked neuronal loss and gliosis, as reported previously (Fig. 1A,B) [8,9]. Neuronal degeneration was most severe in the central A9 region of the substantia nigra with less involvement of medial (A10) and lateral (A8) regions. The pattern of loss of tyrosine hydroxylase positive neurons was essentially identical to that seen in Nissl stained sections.

In control animals, α-synuclein immunoreactivity was confined to fine punctae throughout all regions (A8–10) of the substantia nigra (Fig. 1C). In MPTP-treated animals, synuclein immunoreactivity was depleted in the most severely affected (middle third) region of the substantia nigra, where no neurons remained. In regions with neuronal degeneration and pyknosis, there was a striking redistribution of αsynuclein from synaptic profiles into neuronal cell bodies and dendrites (Fig. 1D). Fine immunoreactive granular intraneuronal accumulations and larger aggregates were prominent in many neuronal somata.

DISCUSSION

MPTP causes α-synuclein aggregation in degenerating neurons of the primate substantia nigra. The redistribution of synuclein from its normal synaptic and axonal location to cell bodies and dendrites associated with MPTP toxicity is very similar to the redistribution of α-synuclein found in idiopathic PD and dementia with Lewy bodies [1,4] (personal observations). The impact of α-synuclein redistribution on neuronal function is unclear. α-Synuclein aggregates have been reported to induce apoptotic cell death in human neuroblastoma cell lines [10]. Expression of α-synuclein in songbirds is related to song learning [7], suggesting that it may play a role in neuronal plasticity potentially involving pre-synaptic vesicle function [11], but its normal function in the human brain is unknown.

How MPTP toxicity leads to α-synuclein aggregation is not known. Oxidative mechanisms may be involved because the active metabolite of MPTP, MPP+, inhibits the electron transport chain initiating a vicious cycle of oxidative damage that causes cellular injury and death [12]. Oxidizing conditions favor self-aggregation of the microtubule protein tau to form neurofibrillary tangles in Alzheimer's disease [13]. Oxidative injury is associated with the development of neuronal inclusions in transgenic mice expressing human superoxide dismutase-1 with the G93A mutation. These mice show evidence of increased oxidative injury [14] and develop Lewy-like bodies in degenerating

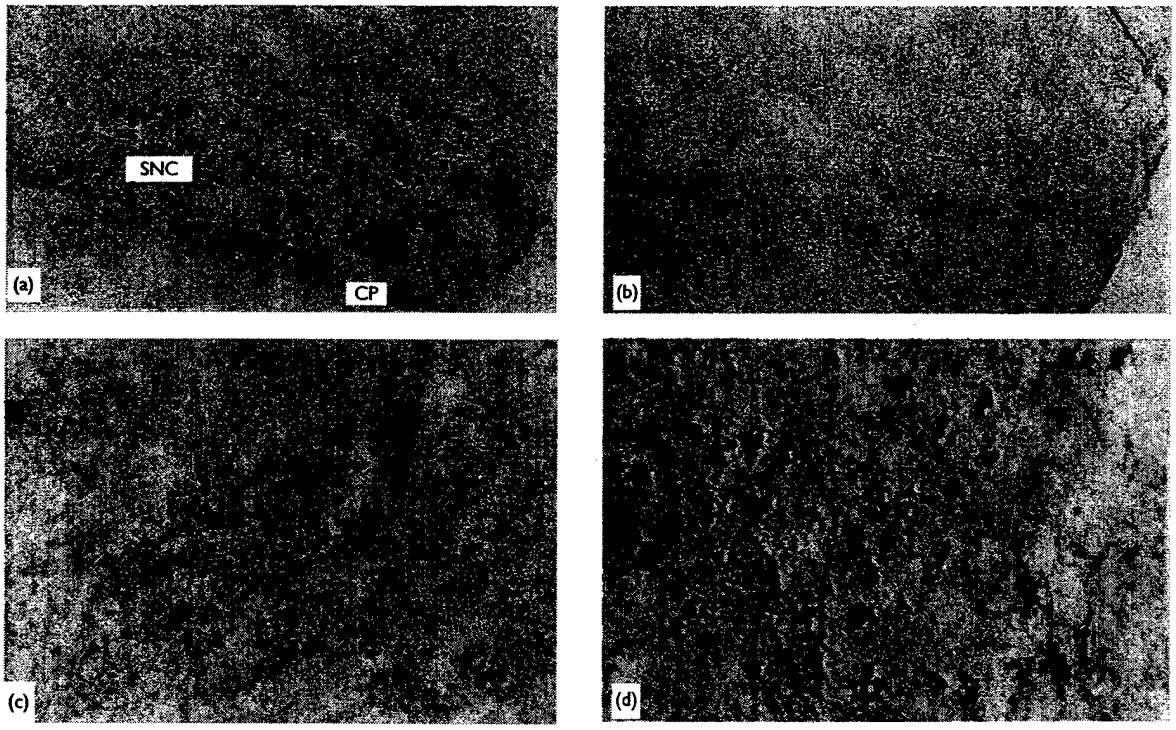

Fig. 1. α-Synuclein immunoreactivity in MPTP treated monkeys. General cell stains (**A,B**) show that systemic MPTP treatment causes marked neuronal loss within the middle third of the substantia nigra pars compacta (SNC) of baboons (B) compared with untreated controls (A) (cresyl violet stain). (α-Synuclein immunoreactivity was distributed in fine terminal-like structures within the SNC of untreated animals (**C**). Punctate staining was reduced and deposits of α-synuclein immunoreactivity were prominent in the SNC of animals treated with MPTP (**D**, arrowhead). CP: cerebral peduncle.

motor neurons [15]. Patients with amyotrophic lateral sclerosis (ALS), who show evidence of oxidative injury postmortem [16], also develop Lewy body-like intraneuronal inclusions. Oxidative injury is very prominent in PD brain [17] and in MPTP-treated primates [9]. *In vitro* studies suggest that oxidative stress can also induce α-synuclein aggregation *in vitro* [18]. Oxidative injury induced by MPTP toxicity could therefore cause α-synuclein aggregation in our experimental animals. Transglutaminase, a cross-linking enzyme involved with signal transduction and apoptosis, catalyses the formation of the covalent synuclein polymers *in vitro* [19]. This enzyme also cross-links tau [20], beta amyloid [21] and mutant huntingtin [22], and may contribute to inclusion formation in Alzheimer's and Huntington's disease. There is also evidence that synuclein mutations associated with PD accelerate synuclein aggregation [23–25]. Whatever the mechanisms involved, our results support the notion that α-synuclein aggregation is the initial event leading to Lewy body formation.

CONCLUSION

MPTP neurotoxicity causes the redistribution and aggregation of α-synuclein within degenerating neurons of the primate substantia nigra. This may reflect the initial stages in the formation of Lewy bodies, the pathological hallmark of idiopathic PD in humans. Therapies that successfully prevent this process in the MPTP animal model may suppress Lewy body formation and cell death in patients with PD.

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Mice Deficient in Cellular Glutathione Peroxidase Show Increased Vulnerability to Malonate, 3-Nitropropionic Acid, and 1-Methyl-4-Phenyl-1,2,5,6-Tetrahydropyridine

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Glutathione peroxidase (GSHPx) is a critical intracellular enzyme involved in detoxification of hydrogen peroxide (H_2O_2) to water. In the present study we examined the susceptibility of mice with a disruption of the glutathione peroxidase gene to the neurotoxic effects of malonate, 3-nitropropionic acid (3-NP), and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). Glutathione peroxidase knock-out mice showed no evidence of neuropathological or behavioral abnormalities at 2–3 months of age. Intrastriatal injections of malonate resulted in a significant twofold increase in lesion volume in homozygote GSHPx knock-out mice as compared to both heterozygote GSHPx knock-out and wild-type control mice. Malonate-induced increases in conversion of salicylate to 2,3- and 2,5-dihydroxybenzoic acid, an index of hydroxyl radical generation, were greater in homozygote GSHPx knock-out mice as compared with both heterozygote GSHPx knock-out and wild-type control mice. Administration of MPTP resulted in significantly greater depletions of dopamine, 3,4-dihydroxybenzoic acid, and homovanillic acid in GSHPx knock-out mice than those seen in wild-type control mice. Striatal 3-nitrotyrosine (3-NT) concentrations after MPTP were significantly increased in GSHPx knock-out mice as compared with wild-type control mice. Systemic 3-NP administration resulted in significantly greater striatal damage and increases in 3-NT in GSHPx knock-out mice as compared to wild-type control mice. The present results indicate that a knock-out of GSHPx may be adequately compensated under nonstressed conditions, but that after administration of mitochondrial toxins GSHPx plays an important role in detoxifying increases in oxygen radicals.

Key words: MPTP; 3-nitropropionic acid; malonate; oxidative damage; free radicals; glutathione; Parkinson's; Huntington's

The formation of hydrogen peroxide and related oxygen radicals is suspected to be involved in the mechanism of nerve cell death and in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Coyle and Puttfarcken, 1993; Beal, 1995). There is substantial evidence that the brain, which consumes large amounts of oxygen, is particularly vulnerable to oxidative damage. The relative roles of endogenous and exogenous antioxidants in protecting the brain against oxidative stress are still being clarified. The major antioxidant defenses consist of antioxidant scavengers such as glutathione, vitamin C, vitamin E, and antioxidant enzymes.

The antioxidant enzymes in the brain include Cu,Zn- and manganese superoxide dismutase, which catalyze the conversion of $O_2^{\cdot}$ to H_2O_2 (Fridovich, 1989). H_2O_2 is then converted to H_2O by either catalase or selenoglutathione peroxidases. Catalase is thought to be relatively low in the brain and is localized to peroxisomes (Gaunt and De Duve, 1976; Halliwell, 1992). The selenoglutathione peroxidases include the "classic" enzyme selenoglutathione peroxidase-I (GSHPx; GSH:H_2O_2 oxidoreductase, EC 1.11.19) and a more recently characterized phospholipid hydroperoxide glutathione peroxidase (Fisher et al., 1999). Among the brain glutathione peroxidases, only GSHPx is known to reduce H_2O_2, indicating that GSHPx may be a major protective enzyme against the action of H_2O_2 in the brain (Jain et al., 1991). Recent evidence showed that GSHPx also plays a major role in detoxifying peroxynitrite ($ONOO^-$) (Sies et al., 1997). GSHPx is present both in the cytosol and in mitochondria (Vitorica et al., 1984), which are a major intracellular source of free radicals (Boveris and Chance, 1973).

Malonate and 3-nitropropionic acid (3-NP) are inhibitors of succinate dehydrogenase, which model Huntington's disease (Beal et al., 1993a,b). 1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) has been extensively used to replicate the dopaminergic neuronal loss occurring in Parkinson's disease (Bloem et al., 1990). Its active metabolite 1-methyl-4-phenylpyridinium (MPP^+) selectively inhibits mitochondrial complex I activity (Tipton and Singer, 1993). These neurotoxins produce impaired energy metabolism and oxidative stress, which plays a direct role in neuronal injury. We and others observed an increased formation of oxygen-derived free radicals when neurons were challenged with malonate, 3-NP, MPTP, or MPP^+

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(Hasegawa et al., 1990; Chiueh et al., 1992; Schulz et al., 1995a; Sriram et al., 1997; Huang and Lee, 1998).

In the present study, we investigated the importance of the glutathione system in protecting the brain from mitochondrial toxins. Our hypothesis was that an impairment of GSHPx activity, which may be compensated when occurring in isolation, may lead to irreversible cell loss when combined with increased free radical generation caused by mitochondrial toxins. Specifically, we sought to determine if GSHPx knock-out mice would be more sensitive to malonate, 3-NP, or MPTP toxicity than control mice.

MATERIALS AND METHODS

Experimental animals. Our experiments were approved by the local Animal Care Committee and were conducted in strict accordance with the National Institutes of Health guidelines for the care and use of experimental animals. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Mice (2- to 3-months-old) were in a B6C3F1 background. The wild-type controls were obtained from Taconic (Germantown, NY), whereas those deficient in cellular GSHPx were provided by Dr. Julie Andersen, University of Southern California (Los Angeles, CA) and bred locally. We bred the GSHPx homozygote mice with the BGC3F1 mice to produce heterozygote GSHPx knock-out mice for use as a further control for genetic background effects. The GSHPx knock-out mice were generated as previously described (Ho et al., 1997) by insertion of a neomycin resistance gene cassette into the *Eco*RI site located in exon 2 of the GSHPx mouse gene. This introduces a *Bam*HI site into exon 2, which gives a 4.3 kb band on Southern blot analysis instead of the 11 kb band found in the normal controls. A herpes thymidine kinase gene cassette was placed at a second *Eco*RI site in the 3′ untranslated region for positive–negative selection with G418/gangcyclovir in embryonic stem cells. These mice show an 85% reduction in cortex GSHPx activity from 0.155 ± 0.021 to 0.024 ± 0.017, $p < 0.001$ as previously described (Lawrence and Burk, 1976).

Intrastriatal microinjections. Control (n = 10), heterozygote GSHPx knock-out mice (n = 10), and homozygous and GSHPx knock-out (n = 12) mice were anesthetized with methoxyflurane and malonate (1.4 μmol in 0.7 μl, pH 7.4) that was stereotaxically injected into the left striatum (anterior, 0.5 mm; lateral, 2 mm from bregma; ventral, 3.5 mm from dura). The injections were performed over 2 min using a 10 μl 26 gauge blunt-tipped Hamilton syringe. The needle was left in place for 5 min before being slowly withdrawn. Seven days after striatal injection animals were killed, and the brains were rapidly removed, placed in cold saline, and sectioned coronally at 1 mm intervals. Slices were stained in 2% 2,3,5-triphenyltetrazolium chloride monohydrate solution at room temperature in the dark for 30 min, and post-fixed in phosphate-buffered 4% paraformaldehyde (PFA) (Bederson et al., 1986). The lesioned area (noted by pale staining) was measured on the posterior surface of each section using Neurolucida (Microbrightfield, Colchester, VT) image analysis software. We previously showed that these measurements exhibit no significant differences from those obtained with Nissl staining (Schulz et al., 1995a). Lesion volumes (mean ± SEM) were calculated by multiplying the lesion area by the slice thickness.

Salicylate assay and 3-nitrotyrosine measurement. The salicylate hydroxyl radical trapping method was used for measuring levels of $^{\bullet}OH$ radicals in striatal tissue after injection of malonate in control (n = 13), GSHPx heterozygote knock-out (n = 13), and homozygous GSHPx knock-out (n = 11) mice (Floyd et al., 1984). Salicylate (200 mg/kg, 5 ml/kg, i.p.) was administered 30 min before striatal malonate injection. Sixty minutes after malonate injection, the animals were killed, and the left and right striata were rapidly dissected from a 2-mm-thick slice on a chilled glass plate and immediately frozen at −70°C. To examine the effects of 3-NP on 3-NT levels, control (n = 8) and GSHPx knock-out (n = 8) mice received six doses of 50 mg/kg intraperitoneally at 12 hr intervals. Mice were killed 1 hr after the last dose. The striata were rapidly dissected and placed in chilled 0.1 M perchloric acid. The samples were thawed in 0.25 ml of chilled 0.1 M perchloric acid, sonicated, and centrifuged twice. Salicylate and its metabolites 2,3- and 2,5-dihydroxybenzoic acid (DHBA), tyrosine, and 3-NT were quantified in the supernatant by HPLC with 16-electrode electrochemical detection (Beal et al., 1990). Data (mean ± SEM) were expressed as the ratio of 2,3- and 2,5-DHBA to salicylate and of 3-NT to tyrosine to normalize for varying brain concentrations of salicylate and tyrosine.

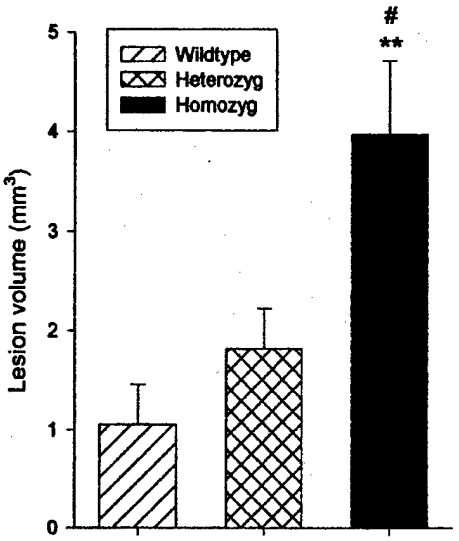

Figure 1. Malonate induced striatal lesion volumes in wild-type controls, heterozygote, and homozygote GSHPx knock-out mice. $^{**}p < 0.01$, as compared with controls; $\#p < 0.05$, as compared with heterozygote GSHPx knock-out mice.

Dopamine measurement. MPTP (15 mg/kg, 5 ml/kg, i.p.) was administered four times at 2 hr intervals to control (n = 10) and GSHPx knock-out (n = 10) mice. An additional set of animals of each type was also treated with 0.1 M PBS (5 ml/kg, i.p.) at the times of MPTP injections. The animals were killed at 1 week, and both striata were rapidly dissected on a chilled glass plate and frozen at −70°C. The samples were subsequently thawed in 0.25 ml of chilled 0.1 M perchloric acid and sonicated. Aliquots were taken for protein quantification using a fluorometric assay (Beal et al., 1990). Other aliquots were centrifuged, and dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were measured in supernatants by HPLC and electrochemical detection. Concentrations of dopamine and metabolites were expressed as nanograms per milligram of protein (mean ± SEM).

MPP^+ levels. To determine whether MPTP uptake or metabolism was altered, MPTP 20 mg/kg was administered intraperitoneally twice, 2 hr apart, and mice were killed 2 hr after the last dose (n = 8/group). Striatal tissue from this experiment was also used for 3-NT determinations. MPP^+ levels were quantified by HPLC with UV detection at 295 nm. Samples were sonicated in 0.1 M perchloric acid, and an aliquot of supernatant was injected onto a Brownlee aquapore X03–224 cation exchange column (Rainin, Woburn, MA). Samples were eluted isocratically with 90% 0.1 M acetic acid and 75 mM triethylamine HCl, pH 2.3, adjusted with formic acid and 10% acetonitrile.

Histological study. 3-NP (50 mg/kg, 5 ml/kg, i.p.) was administered eight times at 12 hr intervals to control (n = 8) and GSHPx knock-out (n = 9) mice. An additional set of animals of each type was also treated with 0.1 M PBS (5 ml/kg, i.p.) at the times of 3-NP injections. Twelve hours after the last injection, the animals were deeply anesthetized with pentobarbital and perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde. Brains were post-fixed for 1 hr, rinsed in 0.1 M PBS, and then cryoprotected in a graded series of 10% and 20% glycerol/2% DMSO solution. Frozen brains were sectioned at 50 μm using a sledge microtome and Nissl-stained as previously described (Beal et al., 1989). Bilateral striatal lesion volumes were computed in serial sections through the rostrocaudal extent of each brain by videomicroscopic capture of brain sections and subsequent volume analysis using Neurolucida (Microbrightfield) image analysis software.

Statistical analysis. Results are expressed as the mean ± SEM. Statistical comparisons were made using Student's t test (unpaired) or one-way ANOVA followed by Fisher's PLSD *post hoc* tests.

RESULTS

The lesion volumes after intrastriatal injection of malonate in wild-type controls and GSHPx knock-out mice are shown in

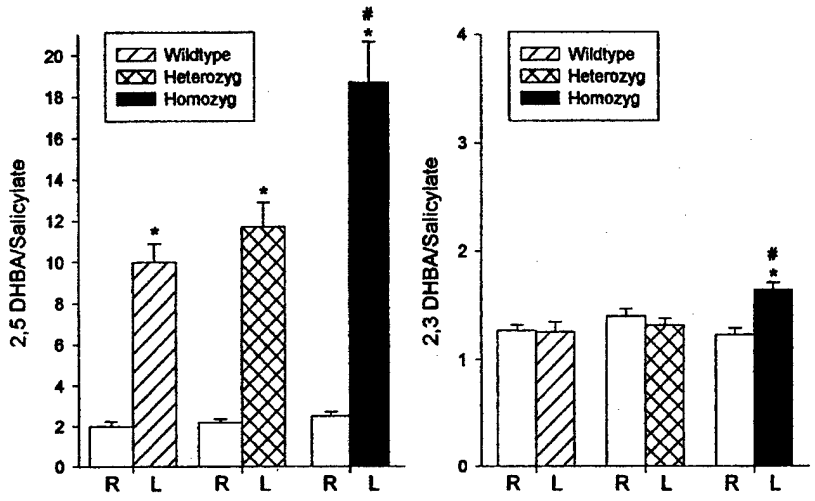

Figure 2. Malonate induced increases in the conversion of salicylate to 2,3 and 2,5-DHBA in wild-type controls, heterozygote, and homozygote GSHPx knock-out mice. $*p < 0.001$, as compared with the uninjected striatum; $\#p < 0.001$, as compared with heterozygote GSHPx knock-out and wild-type controls.

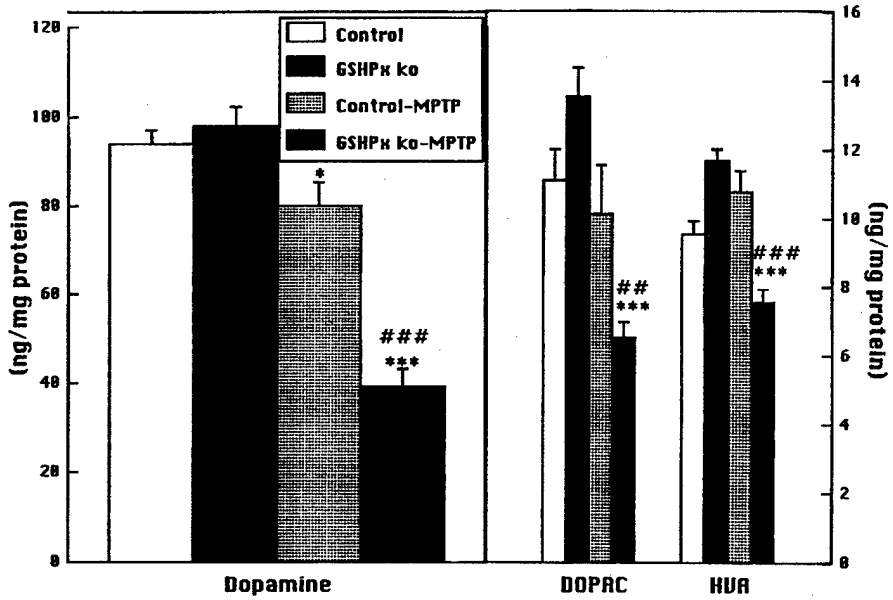

Figure 3. Effects of MPTP administered at 15 mg/kg X4 on dopamine, DOPAC, and HVA in wild-type control and GSHPx knock-out mice. $*p < 0.05$, $***p < 0.001$, as compared to PBS-treated animals; $\#\#p < 0.01$, $\#\#\#p < 0.001$, as compared to wild-type treated with MPTP.

Figure 1. Lesion volumes after malonate injections were significantly larger in homozygous GSHPx compared to both heterozygous GSHPx ($p < 0.01$) and wild types ($p < 0.001$). There was no significant difference between heterozygous GSHPx knock-out and wild-type mice. Injection of vehicle resulted in negligible lesions in both controls and GSHPx knock-out mice (0.24 ± 0.04 vs 0.34 ± 0.07 mm^3). After administration of salicylate, intrastriatal injection of malonate resulted in a significant increase in 2,3 DHBA compared to the unlesioned side only in homozygous GSHPx knock-out mice ($p < 0.001$) (Fig. 2). The level of 2,3 DHBA in the lesioned side of homozygous GSHPx knock-out was significantly higher than in the lesioned side in both heterozygous GSHPx knock-out and wild types ($p < 0.001$). A significant increase in 2,5 DHBA was seen in the lesioned striata in all groups, but the increase in homozygous GSHPx knock-out was significantly larger than the increase in both heterozygous GSHPx knock-out and the wild-type mice ($p < 0.0001$). There was no significant difference between heterozygous GSHPx knock-out and wild-type mice.

The effects of administration of MPTP in wild-type control and GSHPx knock-out mice are seen in Figure 3. We used a relatively low dose of MPTP, 4×15 mg/kg, which produced a small significant dopamine depletion of 15% in wild-type controls. In contrast, the same dose of MPTP produced a significant 61% depletion of dopamine in GSHPx knock-out mice that was significantly, $p < 0.001$, greater than that seen in controls. Depletions of DOPAC and HVA in controls did not reach significance, but they were highly significant in GSHPx knock-out mice and were significantly ($p < 0.001$) greater than those seen in wild-type controls. The increased sensitivity to MPTP was not caused by an alteration in uptake or metabolism of MPTP to MPP^+ because striatal MPP^+ levels did not significantly differ at 2 hr after MPTP administration (MPP^+ 8.4 ± 1.3 ng/mg protein in controls and 9.5 ± 1.0 ng/mg protein in GSHPx knock-out mice).

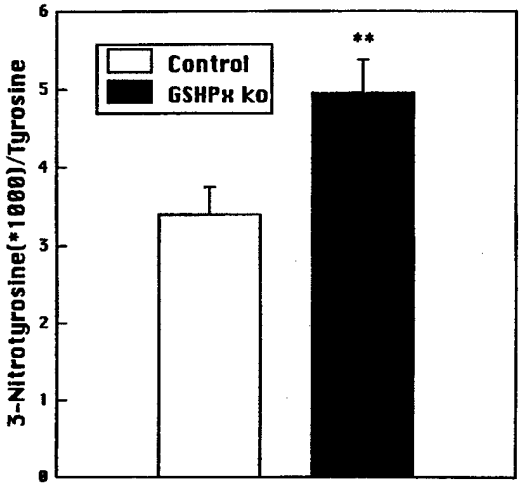

Figure 4. Effects of MPTP 20 mg/kg X2 on striatal 3-NT levels 2 hr after MPTP administration in wild-type control and GSHPx knock-out mice. $^{**}p < 0.01$, as compared with wild-type controls.

The effects of MPTP on striatal 3-NT levels are shown in Figure 4. MPTP administration in the GSHPx knock-out mice resulted in a significant increase in 3-NT levels as compared with wild-type controls ($p < 0.01$). We previously found that saline-injected controls had 3-NT levels of 1–2 3-NT/1000 tyrosines (Schulz et al., 1995b), consistent with the findings in Figure 6.

Systemic administration of 3-NP resulted in bilateral striatal lesions in both wild-type controls and GSHPx knock-out mice (Fig. 5). The areas of neuronal loss and increased gliosis within the caudate putamen were significantly (almost fourfold) greater in the GSHPx knock-out mice (Fig. 5). Striatal lesion volumes were 3.72 ± 0.26 mm^3 in controls and 14.12 ± 0.92 mm^3 in GSHPx knock-out mice, $p < 0.01$. The effects of 3-NP on 3-NT levels are shown in Figure 6. 3-Nitrotyrosine levels increased after 3-NP administration in both controls and GSHPx knock-out mice, but the increases were significantly ($p < 0.05$) greater in the GSHPx knock-out mice than those observed in the controls.

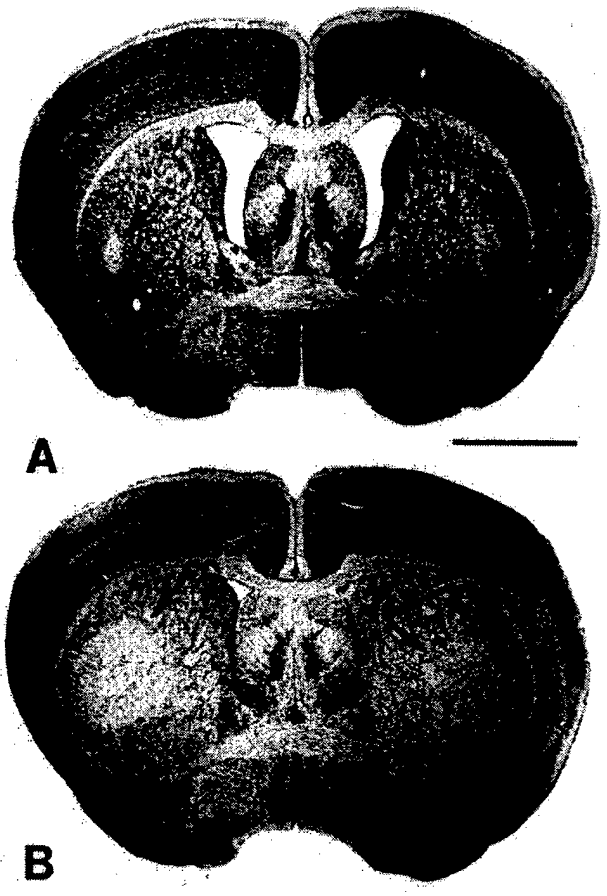

Figure 5. Photomicrographs of 3-NP lesions in Nissl-stained whole-brain sections through the striatum of wild-type (*A*) and glutathione peroxidase knock-out (*B*) mice. Bilateral striatal lesions are present in both *A* and *B* and are represented by staining pallor in the lateral aspect (*arrows*). The lesions are significantly larger in the glutathione peroxidase knock-out mouse. Scale bar, 2 mm.

DISCUSSION

The glutathione (GSH) system plays a major role in controlling cellular redox states and is a primary defense mechanism for H_2O_2 and peroxide removal in brain. Immunocytochemical studies showed localization of GSHPx to both brain astrocytes and neurons (Damier et al., 1993; Olanow, 1993; Trepanier et al., 1996). In cultured cerebellar astrocytes, cytosolic GSH and GSHPx were 57 and 245% higher than those found in granule cells (Huang and Philbert, 1995). Other studies also showed increased GSH in astrocytes as compared to neurons (Slivka et al., 1987; Raps et al., 1989). The ratio of mitochondrial to cytosolic GSH and mitochondrial GSHPx however is higher in cerebellar granule cells than astrocytes, suggesting that the GSHPx system may be particularly important in neuronal mitochondria. Depletion of GSH leads to mitochondrial damage and reductions in mitochondrial enzymes in brain (Jain et al., 1991; Martinez et al., 1995), and it causes calcium-mediated cell death in PC12 cells (Jurma et al., 1997). Further evidence implicating GSH in normal brain function are the observations that glutathione depletion *in vivo* results in dystrophic axons in dopaminergic neurons and enhances the neurotoxicity of ischemia, 6-hydroxydopamine, MPP^+, and MPTP (Pileblad et al., 1989; Mizui et al., 1992; Andersen et al., 1996; Wullner et al., 1996; Nakamura et al., 1997).

The fact that mice with a knock-out of GSHPx show no neuronal degeneration up to 3 months of age is, therefore, somewhat surprising. It is, however, consistent with a previous report that mice deficient in cellular GSHPx develop normally, are fertile, and show no increase in lung toxicity to hyperoxia (Ho et al., 1997). Histological examination at 4 and 15 months of age was normal in all tissues, including the brain, and protein carbonyls and lipid peroxidation products were unaltered from controls (Ho et al., 1997). These observations suggest an alternative means of removing H_2O_2 under baseline physiological conditions. Although catalase activity (EC 1.11.16) was reported to be low in the brain, it is widely distributed throughout the brain (Gaunt and De Duve, 1976; Brannan et al., 1981). Both catalase and GSHPx are found in cultured astrocytes (Copin et al., 1992; Huang and Philbert, 1995; Desagher et al., 1996). H_2O_2 easily crosses cell membranes and therefore could leave the cell to damage neighboring cells or be detoxified by them (Halliwell, 1992). It was recently suggested that catalase was the main hydrogen peroxi-

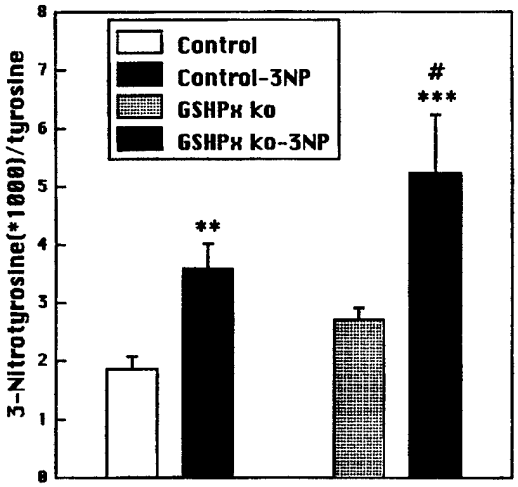

Figure 6. Effects of 3-NP on 3-NT levels in wild-type control and GSHPx knock-out mice. $^{**}p < 0.01$, $^{***}p < 0.001$, as compared with PBS; $\#p < 0.05$, as compared with wild-type control.

dase activity in astrocytes and that it protected neighboring neurons (Desagher et al., 1996). In other studies of cultured astrocytes, both GSHPx and catalase were shown to be complementary in detoxification of H_2O_2 (Dringen and Hamprecht, 1997). Inhibitors of either enzyme only marginally reduced the rate of disappearance of H_2O_2 from the incubation media, however inhibition of both enzymes strongly reduced H_2O_2 clearance. It therefore appears that both H_2O_2 detoxifying systems can increase H_2O_2 clearance sufficiently under physiological conditions to prevent toxicity. This is not the case with other free radical scavengers such as manganese superoxide dismutase, in which a deficiency leads to premature death with both cardiac and CNS damage (Li et al., 1995; Lebovitz et al., 1996).

We, however, wondered whether GSHPx may play a more critical role under conditions in which neuronal metabolism is stressed by mitochondrial toxins. Both malonate and 3-NP are succinate dehydrogenase inhibitors that produce striatal lesions *in vivo* after either local striatal or systemic administration, respectively. Studies using ^{13}C magnetic resonance spectroscopy showed that 3-NP preferentially inhibits oxidative metabolism in GABAergic neurons *in vivo,* whereas astrocyte metabolism was spared (Hassel and Sonnewald, 1995). The neurotoxicity of these compounds is associated with increases in $OH^{\bullet}$ generation as assessed by the salicylate-trapping method, as well as with increases in 3-NT, a marker of peroxynitrite (Schulz et al., 1995c). Similarly, MPTP neurotoxicity is associated with increases in $OH^{\bullet}$ generation and 3-NT (Schulz et al., 1995a). In the present study we therefore examined whether GSHPx knock-out mice would show increased susceptibility to these toxins.

The intrastriatal administration of malonate resulted in a significant twofold increase in lesion volume in GSHPx knock-out mice, as compared with both heterozygote GSHPx knock-out and wild-type control mice. Furthermore, the administration of malonate resulted in increased $OH^{\bullet}$ generation, as assessed using the salicylate-trapping method in homozygous GSHPx knock-out mice, as compared with both heterozygote GSHPx knock-out and wild-type control mice. The heterozygote GSHPx knock-out mice were produced by crossing the homozygous GSHPx knock-out mice with the background strain, which should control for any genetic variation between the GHSPx knock-out mice and the original background strain. 3-Nitropropionic acid lesions were also significantly greater in GSHPx knock-out mice. Lastly, MPTP neurotoxicity, as assessed by levels of dopamine, DOPAC, and HVA, was markedly exacerbated in the GSHPx knock-out mice.

GSHPx may therefore play an important role in initially compensating for increased generation of oxidants in these illnesses. GSHPx can detoxify reactive oxygen species by catalyzing the conversion of H_2O_2 to H_2O, but it also acts to reduce lysophospholipid hydroperoxides (Marinho et al., 1997; Fisher et al., 1999). Its role in detoxification of peroxynitrite (Sies et al., 1997) may be particularly crucial, because we and others found that inhibitors of neuronal nitric oxide synthase block malonate, 3-NP, and MPTP neurotoxicity (Schulz et al., 1995a; Hantraye et al., 1996; Przedborski et al., 1996). In the present study we found that striatal 3-NT concentrations were significantly increased after MPTP administration in GSHPx knock-out mice as compared with controls. We also found that increases in striatal 3-NT after systemic administration of 3-NP were significantly greater in GSHPx knock-out mice as compared with controls. This evidence therefore indicates that GSHPx plays an important role in the detoxification of peroxynitrite *in vivo*.

These results therefore indicate that although other free radical scavenging mechanisms are able to compensate for a loss of GSHPx under physiological conditions, they are inadequate in response to a metabolic stress. This has important implications for the pathogenesis of Huntington's disease (HD) and Parkinson's disease (PD). In both of these neurodegenerative diseases there is strong evidence implicating deficient energy production and increased free radical production (Beal, 1997). In HD there are increases in cerebral lactate *in vivo,* as assessed by magnetic resonance spectroscopy (Jenkins et al., 1993), decreases in mitochondrial complex II-III activity in postmortem tissue, and increased oxidative damage to DNA (Gu et al., 1996; Browne et al., 1997). In PD, several authors found reduced mitochondrial complex I activity in the substantia nigra and in platelets and evidence of increased oxidative damage (for review, see Beal, 1995). GSH is significantly depleted in the substantia nigra of PD patients, as well as in incidental Lewy body disease, which may be a presymptomatic stage of PD (Dexter et al., 1994). GSHPx activity is also reduced in the substantia nigra of PD patients (Ambani et al., 1975; Kish et al., 1985).

In PD it is possible that a latent genetic defect in free radical scavenging enzymes or in mitochondrial electron enzymes may be compensated under physiological conditions, but may increase susceptibility to environmental toxins. Environmental factors could also contribute to some of the variance in age of onset of HD (Gusella et al., 1997). The present results are therefore consistent with the possibility that genetic defects may interact with environmental toxins in the pathogenesis of neurodegenerative diseases.

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Rapid Communication

Frontal Lobe Dysfunction in Progressive Supranuclear Palsy: Evidence for Oxidative Stress and Mitochondrial Impairment

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**Department of Neurology and Neuroscience, Cornell University Medical College, New York; ‡Cornell University Medical College at Burke Medical Research Institute, White Plains, New York; †Neurology Service and ‖Department of Neuropathology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts; and §Mayo Clinic Jacksonville, Jacksonville, Florida, U.S.A.*

Abstract: Recent data from our laboratory have shown a regionally specific increase in lipid peroxidation in postmortem progressive supranuclear palsy (PSP) brain. To extend this finding, we measured activities of mitochondrial enzymes as well as tissue malondialdehyde (MDA) levels in postmortem superior frontal cortex (Brodmann's area 9; SFC) from 14 pathologically confirmed cases of PSP and 13 age-matched control brains. Significant decreases (−39%) in α-ketoglutarate dehydrogenase complex/glutamate dehydrogenase ratio and significant increases (+36%) in tissue MDA levels were observed in the SFC in PSP; no differences in complex I or complex IV activities were detected. Together, these results suggest that mitochondrial dysfunction and lipid peroxidation may underlie the frontal metabolic and functional deficits observed in PSP. **Key Words:** Tauopathy—Lipid peroxidation—Mitochondrial dysfunction—Malondialdehyde.
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Progressive supranuclear palsy (PSP) is a neurological disorder with rapid progression (Golbe et al., 1988) characterized by cognitive impairment, extrapyramidal symptoms, and the appearance of supranuclear gaze palsy. The hallmark pathology is the presence of straight neurofibrillary filaments (Dickson et al., 1985), extensive neuronal degeneration, and gliosis in multiple subcortical brain regions; the frontal cortex is generally unaffected using standard histochemical techniques (Steele et al., 1964; Hauw et al., 1994; Litvan et al., 1996*a*). Despite the apparent lack of cortical degeneration, neurofibrillary tangles and *tau*-containing neuronal and glial inclusions are present within the cortex, particularly in the prefrontal and precentral areas (Hauw et al., 1994). A robust association between PSP and inheritance of two copies of the H1 *tau* extended haplotype has previously been reported (Conrad et al., 1997; Baker et al., 1999).

Positron emission tomography (PET) studies have indicated that PSP is associated with marked frontal lobe glucose hypometabolism (D'Antona et al., 1985; Foster et al., 1988; Goffinet et al., 1989; Blin et al., 1990; Johnson et al., 1992). Many of the behavioral and cognitive symptoms attributable to frontal lobe dysfunction, such as apathy, depression, and bradyphrenia, are common in patients with PSP (Litvan et al., 1996*b*). However, the biochemical basis for this frontal hypometabolism remains unknown.

Substantial evidence has shown that selective defects in mitochondrial energy production lead to increased free radical production and oxidative damage to DNA (Mecocci et al., 1993) and RNA (Nunomura et al., 1999) in several neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (for review, see Beal, 1997). In AD, activities of particular mitochondrial enzymes—α-ketoglutarate dehydrogenase complex (KGDHC) and cytochrome oxidase—have been shown to be reduced, whereas oxidative damage to lipids and proteins is increased (Hensley et al., 1995; Gibson et al., 1998). Recent data from our laboratory have shown a regionally specific increase in lipid peroxidation in postmortem PSP brain (Albers et al., 1999), adding PSP to the list of diseases associated with oxidative stress. These data, coupled with a report showing defects in oxidative phosphorylation in muscle mitochondria from PSP patients (DiMonte et al., 1994) and substantial evidence for frontal lobe glucose hypometabolism in PSP, led us to search for evidence of mitochondrial dysfunction and oxidative damage in the superior frontal cortex (SFC) in PSP.

MATERIALS AND METHODS

Human brain tissue

Tissue from 14 pathologically confirmed cases of PSP [mean ± SD age, 75.21 ± 7.15 years; postmortem interval (PMI), 15.27 ± 9.17 h] and 13 control cases (mean ± SD age, 68.08 ± 9.39 years; PMI, 16.63 ± 6.44 h) was provided by the Harvard Brain Tissue Resource Center (Belmont, MA, U.S.A.). There was no significant difference in age, PMI, or gender between the PSP and control groups (Table 1). The pathological diagnosis of PSP was made by examination of the contralateral hemisphere using the NINDS pathological criteria (Hauw et al., 1994; Litvan et al., 1996*a*). None of the PSP brains examined showed any evidence of a coexisting neurological disorder. The 13 control brains examined had a distributive diagnosis of control with no pathological evidence of

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Abbreviations used: AD, Alzheimer's disease; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17; GDH, glutamate dehydrogenase; KGDHC, α-ketoglutarate dehydrogenase complex; MDA, malondialdehyde; PET, positron emission tomography; PMI, postmortem interval; PSP, progressive supranuclear palsy; SFC, superior frontal cortex.

TABLE 1. *PSP and control cases in the present study*

Diagnosis, case	Cause of death	*Tau* genotype	Age, sex	PMI (h)
PSP				
B3972	Congestive heart failure	H1/H1	73, M	4.75
B4044	Unknown	H1/H1	75, F	8.6
B3973	Pneumonia	H1/H1	87, F	11.25
B3938	Heart attack	H1/H1	81, F	21.5
B4027	Unknown	H1/H1	69, M	4.0
B4016	Pneumonia	H1/H1	81, F	5.75
B3926	Myocardial infarction	H1/H1	74, M	13.4
B3549	Heart attack	H1/H1	60, M	31.2
B4208	Congestive heart failure	H1/H1	77, M	17.3
B4361	Unknown	H1/H1	78, F	15.25
B4382	Respiratory failure	H1/H1	80, M	17.58
B4428	Pneumonia	H1/H1	65, M	34.08
B4442	Pneumonia	H1/H1	81, M	9.8
B4476	Respiratory failure	H1/H1	72, F	19.38
Control				
B3236	Heart attack	H1/H1	64, M	10.0
B3296	Unknown	H1/H2	76, F	32.5
B3626	Heart attack	H1/H2	65, F	16.6
B3688	Heart attack	H1/H2	66, M	18.7
B3700	Heart attack	H1/H2	62, F	16.0
B3806	Cardiac arrest	H1/H1	70, F	15.0
B3941	Cancer	H1/H1	63, M	23.0
B3983	Heart attack	H1/H2	71, M	8.7
B4019	Lung cancer	H1/H1	65, M	16.2
B4030	Heart attack	H1/H1	81, F	11.5
B4034	Cardiac-related	H1/H1	88, M	16.25
B4077	Myocardial infarction	H1/H1	51, M	10.2
B4252	Heart attack	H1/H2	63, M	21.5

neurological disease (Table 1). All tissue samples were stored at −80°C and processed in parallel.

Genotyping and polymorphism analysis

Genotyping of a single nucleotide polymorphism in exon 2 and a deletion polymorphism in the intron 5′ of exon 10 was performed in all PSP and control cases to determine tau haplotypes. PCR conditions and primer sequences have been described previously (Baker et al., 1999). All samples were also genotyped for the intronic dinucleotide repeat polymorphism by PCR. All polymorphisms tested have been shown previously to be in complete disequilibrium with each other (Baker et al., 1999). Extended *tau* haplotypes (H1/H1) were therefore assigned based on the genotypes at each polymorphism.

HPLC determination of tissue malondialdehyde (MDA) levels

Thiobarbituric acid-reactive substances were prepared from blinded samples and assayed using sensitive HPLC with fluorometric detection as described previously (Halliwell and Chirico, 1993; Albers et al., 1999). Quantitation of MDA levels (μmol/ml) was based on integration of peak area and compared with MDA standards.

KGDHC and glutamate dehydrogenase (GDH) enzyme activity measurements

Tissue homogenates prepared from blinded samples were assayed for KGDHC and GDH activities as previously described (Gibson et al., 1998). In brief, KGDHC activities (in mU, i.e., nmol/min/mg of protein) were measured in the presence of saturating concentrations of thiamine pyrophosphate in a 96-well plate reader so that assays could be performed on all samples simultaneously. GDH activities (in mU, i.e., nmol/min/mg of protein) were measured as the KGDHC, NH_4-dependent, and ADP-dependent oxidation of NADH.

Mitochondrial enzyme activity assays

Activities of the electron transport chain enzymes complex I (NADH:ubiquinone oxireductase) and complex IV (ferrocytochrome *c*:oxygen oxidoreductase or cytochrome *c* oxidase) from blinded samples were determined spectrophotometrically in triplicate, using established methods (Browne et al., 1998). Mitochondrial concentrations were estimated by measuring activity of the mitochondrial matrix enzyme citrate synthase (Browne et al., 1998).

Statistics

As equal group variances could not be assumed, statistical comparisons between PSP and control values were made using the two-tailed Mann–Whitney *U* test (InStat; GraphPad, San Diego, CA, U.S.A.)

RESULTS AND DISCUSSION

All PSP cases used in these studies were shown to have the *tau* H1/H1 genotype (Table 1), consistent with previous reports (Conrad et al., 1997; Baker et al., 1999).

Tissue MDA levels in SFC in PSP and control cases are presented in Fig. 1A. A significant increase in tissue MDA levels was observed in the PSP group (0.53 ± 0.05 μmol/ml, n = 14) compared with the control group (0.39 ± 0.02 μmol/ml, n = 13). In the same cases, significant reductions in the KGDHC/GDH ratio were observed (Fig. 1B). No correlation was observed between the increase in tissue MDA levels and the decrease in KGDHC/GDH ratios, suggesting these biochemical changes may be occurring within different cell populations. The GDH values of the control and PSP cases were similar (Table 2) and consistent with values reported previously (Gibson et al., 1998). No differences were observed in complex I or IV activities in any of these same PSP cases (Table 2); these values are consistent with values reported previously in parietal cortex (Browne et al., 1998). Together, these complex data reinforce the GDH data, demonstrating that the significant decrement in KGDHC activity in PSP SFC does not reflect nonspecific loss of mitochondria.

Our evidence for mitochondrial impairment and oxidative stress in SFC in PSP is particularly interesting in light of the absence of marked cell loss. Our data suggest that the significant defect in particular mitochondrial enzymes, such as KGDHC, may underlie the marked deterioration of frontal lobe glucose metabolism in PSP. As a similar decrement (55–57%) in KGDHC activity has recently been reported in the frontal cortex in AD (Gibson et al., 1998), together these studies suggest that KGDHC may be a sensitive early marker of dysfunctional neurons.

Two PET studies have suggested that the SFC may be more affected than other frontal cortical regions in PSP (Foster et al., 1988; Blin et al., 1990). Clinically, frontal lobe dysfunction is considered a typical feature of PSP. Indeed, significant correlations have been reported between frontal glucose hypometabolism and performance on neuropsychological tests of verbal fluency and card sorting ability (Blin et al., 1990; Robbins et al., 1994). Future studies are required to investigate if the biochemical changes in SFC reported herein are causative of these metabolic and neuropsychological deficits or merely a secondary phenomenon, like deafferentation from degenerating subcortical structures.

Although the etiological basis of PSP is unknown, genetic studies have identified the *tau* locus in PSP patients as a potential risk factor for developing the disease (Conrad et al., 1997; Baker et al., 1999). Baker et al. (1999) showed that an extended tau haplotype (H1) is significantly overrepresented in PSP patients, extending an earlier report of an association between an intronic dinucleotide polymorphism (between exons 9 and 10) and PSP. The association of PSP with the intronic polymorphism is a reflection of the inheritance of the broader

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Mitochondrial DNA mutations in complex I and tRNA genes in Parkinson's disease

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Article abstract—*Objective:* To identify mitochondrial DNA (mtDNA) mutations that predispose to PD. *Background:* Mitochondrial complex I activity is deficient in PD. mtDNA mutations may account for the defect, but the specific mutations have not been identified. *Methods:* Complete sequencing was performed of all mtDNA-encoded complex I and transfer RNA (tRNA) genes in 28 PD patients and 8 control subjects, as well as screening of up to 243 additional PD patients and up to 209 control subjects by restriction digests for selected mutations. *Results:* In the PD patients, 15 complex I missense mutations and 9 tRNA mutations were identified. After screening additional subjects, rare PD patients were found to carry complex I mutations that altered highly conserved amino acids. However, no significant differences were found in the frequencies of any mutations in PD versus control groups. The authors were unable to confirm previously reported associations of mutations at nucleotide positions (np) 4336, 5460, and 15927/8 with PD. Complex I mutations previously linked to Leber's hereditary optic neuropathy, one of which has been linked to atypical parkinsonism, were not associated with PD. *Conclusions:* mtDNA mutations with a high mutational burden (present in a high percentage of mtDNA molecules in an individual) in complex I or tRNA genes do not play a major role in the risk of PD in most PD patients. Further investigations are necessary to determine if any of the rare mtDNA mutations identified in PD patients play a role in the pathogenesis of PD in those few cases. **Key words:** PD—Leber's hereditary optic neuropathy—Mitochondrial DNA—NAD(P)H dehydrogenase (quinone)—Transfer RNA—Polymorphism.

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The recent identification of genetic mutations causing rare forms of familial PD provides the opportunity to better understand the mechanisms that lead to PD.[1-3] However, genetic factors that influence the risk of developing PD remain uncertain for most late-onset PD patients. Several studies have focused on the pathogenetic basis of complex I deficiency in PD. PD patients have decreased activity of complex I of the mitochondrial electron transport chain in the substantia nigra and in platelets.[4-7] The mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) inhibits complex I and reproduces many of the clinical and pathologic features of PD.[8] Although nuclear genetic factors also may play a role in the complex I deficiency in PD,[9] indirect evidence indicates a key role for mitochondrial DNA (mtDNA) mutations. Cybrid cell lines expressing mtDNA from PD patients exhibit complex I deficiency, suggesting that the complex I defect results from mtDNA mutations.[10,11] There appears to be a maternal bias in the inheritance of at least some familial cases of PD,[12,13] which is consistent with an influence of mitochondrial genetic factors. Although the specific mtDNA mutations that account for the complex I deficiency have yet to be identified in PD,[5-7] extrapyramidal features, particularly dystonia, have been reported in association with several mtDNA mutations.[14-16] Furthermore, a family with atypical parkinsonism associated with neuronal loss in the substantia nigra was found to harbor the most common primary mutation of Leber's hereditary optic neuropathy (LHON) (at nucleotide position [np] 11778), demonstrating an association between an inherited mtDNA mutation and parkinsonism with nigral cell loss.[17]

To identify mtDNA mutations that might influence the risk of developing PD, we sequenced the mtDNA-encoded complex I genes and transfer RNA (tRNA) molecules in 28 PD patients and 8 control

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subjects, and screened for selected mtDNA mutations in up to 243 additional PD patients and up to 209 additional control subjects.

Methods. *Experimental design.* Mutations were identified initially by complete sequencing of all mitochondrial complex I and tRNA genes in 28 PD patients and in 8 control subjects, all of which were Caucasian and from the Boston area. Next, selected mutations were screened by restriction digests in additional PD patients and control subjects from the Boston area (group 1, which also includes the sequenced patients). Further testing of hypotheses generated from these initial data was conducted on an independent group of PD patients and control subjects (group 2) in a blinded manner, and in DNA samples derived from postmortem brain tissue from PD patients and control subjects. Approximately half of the postmortem samples also were analyzed in a blinded manner.

PD patients and control subjects. A total of 271 PD patients and 218 control subjects were included in these studies. Variability in the numbers of patients screened by restriction digests for each mutation was caused by differences in the numbers of samples available at the time of screening because of ongoing collection of DNA samples during the study. There were three sources for the DNA samples. The first group included PD patients (n = 97) and spousal control subjects (n = 25) recruited from the movement disorders clinics at Boston University Medical Center, Brigham and Women's Hospital, and Massachusetts General Hospital in Boston (group 1). The second group consisted of PD patients (n = 128) and control subjects (n = 136) from a previously described case–control study in a local community in New York City (group 2). All patients (1) were diagnosed with PD by a neurologist with expertise in movement disorders, (2) had a good clinical response to levodopa, and (3) had either a rest tremor or a history of asymmetric onset. Patients with atypical features, including supranuclear gaze palsy, cerebellar ataxia, or early and prominent autonomic dysfunction, were excluded.[18,19] All control subjects had a negative family history for PD. The postmortem group included PD patients (n = 46) and control subjects (n = 57) identified in postmortem brain samples using standard pathologic criteria. Subjects for sequencing were the first 28 PD patients and the first 8 control subjects collected from group 1.

DNA extraction. DNA was isolated from platelets (group 1), whole blood (group 2) or frontal cortex (postmortem group) using a standard protocol including proteinase K and sodium dodecyl sulfate digestion followed by phenol and chloroform extractions. In some subjects from group 1, DNA was isolated and sequenced separately from platelets and from leukocytes. Results were identical from these two DNA sources in each case.

Sequencing. The PCR amplification of mtDNA was performed with a GeneAmp System 9600 thermal cycler (Perkin-Elmer, Foster City, CA). Each of 40 cycles included 1 minute at 94, 50, and 72 °C. PCR products were purified with the Microcon 100 system (Amicon, Bedford, MA). Big Dye sequencing reaction premix (Perkin-Elmer, Norwalk, CT) was used for sequencing reactions followed by purification on a Sephadex column and sequencing on a polyacrylamide gel using an ABI 377 automated sequencer (Perkin-Elmer). Nuclear pseudogenes are unlikely to be a confounding factor in these analyses because we used standard DNA isolation procedures, screened only for homoplasmic or high mutational burden mutations, and found identical sequencing results using DNA isolated from platelets (which contain only mtDNA) and leukocytes from the same individuals. Mixing of known ratios of mutant and wild-type DNA followed by sequencing reveals the ability to consistently detect mtDNA mutations present at 75% mutational burden or higher. A subset of mtDNA mutations with mutational burdens of 60% to 70% can be identified. PCR and sequencing primers are available on the *Neurology* website.

Restriction digests. Selected mutations were studied based on restriction endonuclease polymorphisms. Restriction digests were analyzed by ultraviolet illumination of a 2% agarose gel permeated with ethidium bromide. PCR primers and restriction endonucleases are available on the *Neurology* website.

Statistical analyses. Chi-square analyses were used to compare frequencies of mutations in patients and control subjects.

Terminology. For convenience, variations in sequence from the normal human sequence are referred to as *mutations* when thought to be pathogenic or when their pathogenic significance is unknown. Sequence variants not suspected of having pathogenic significance are referred to as *polymorphisms* (for example, base pair substitutions in protein-coding genes that do not result in alteration of an amino acid). Mutations are identified by the normal base pair followed by the nucleotide position of the mutation followed by the mutant base pair. For example, T4216C represents the presence of a C at np 4216 where a T normally is found. Nucleotide positions are listed according to the conventions of the Cambridge sequence.[20] References to numbers of mutations in the text and tables do not include those known to be errors or consensus changes in the Cambridge sequence (see Results).

Results. Sequencing of all mtDNA-encoded complex I and tRNA genes in 28 PD patients and 8 control subjects revealed 20 missense mutations in complex I genes and 9 tRNA mutations. A summary of these mutations is shown in table 1. Mutations present in multiple PD patients and in none of the eight control subjects, and mutations that altered evolutionarily conserved amino acids, were selected for screening by restriction digests in additional subjects. In addition, complex I mutations previously linked to LHON underwent additional screening.

Complex I mutations. Of the 20 complex I missense mutations identified, 15 were found in PD patients, 7 in control subjects, and 2 in both PD patients and control subjects when considering the sequencing data alone (see table 1). When data from screening of additional subjects by restriction digests also are considered, the number of mutations seen in control subjects increases to 13, including 8 seen in both PD patients and control subjects (table 2). A missense mutation at np 5460 in the gene encoding the ND2 subunit of complex I that has been reported to be associated with PD[21] was found among our sequenced cases in only 1 of 28 PD patients and in 2 of 8 control subjects. A meta-analysis of previously published reports of this mutation in PD patients[21-24] reveals it to be present in 10 of 130 PD patients and in 12 of 181 control subjects. When data from the current study also are included, this

Table 1 tRNA mutations and complex I missense mutations identified by sequencing

Mutation	Gene	Disease association	PD (n = 28)	Control (n = 8)
A4024G	ND1		0	1
T4216C*	ND1	LHON	6	0
A4336C*	$tRNA^{Gln}$	PD; AD	2	1
G4491A	ND2		2	0
T4561C	ND2		1	0
C4654T*	ND2		1	0
A4884G	ND2		1	0
A4917G*	ND2	LHON	4	0
G5046A	ND2		1	0
G5460A	ND2	PD; AD	1	2
A5558G	$tRNA^{Trp}$		1	0
T5664C	$tRNA^{Ala}$		1	0
A7559G	$tRNA^{Asp}$		1	0
T10034C*	$tRNA^{Gly}$		3	0
A10398G*	ND3		8	1
T10463C*	$tRNA^{Arg}$		3	0
A10750G*	ND4L		1	0
G10775A	ND3		1	0
G11150A	ND4		0	1
A12308G	$tRNA^{Leu-2}$		7	2
A13117G*	ND5		1	0
G13708A*	ND5	LHON	3	0
A13780G*	ND5		3	0
A13966G	ND5		1	0
A14002G	ND5		0	1
G14433A	ND6		0	1
T14582C	ND6		0	1
A15924G*	$tRNA^{Thr}$	Infantile my	4	0
G15928A*	$tRNA^{Thr}$	PD, MS	3	0

Nucleotide position of complex I missense mutations and tRNA mutations identified by complete sequencing of these mitochondrial genes in 28 PD patients and eight controls. The nos. of patients testing positive for the mutation in each subgroup are shown. None of the differences in mutation frequencies between PD and controls was significant. This sequencing data comes from the first 28 PD patients and the first eight controls from group one.

* Mutations selected for further analyses by restriction digests.

Infantile my = fatal infantile myopathy with respiratory chain deficiency; LHON = Leber's hereditary optic neuropathy; tRNA = transfer RNA.

becomes 11 of 158 PD patients and 14 of 189 control subjects ($p = 0.87$). A missense mutation at np 13780 in the ND5 gene was found by sequencing in three PD patients and in none of the eight control subjects. However, after screening additional subjects for this mutation by restriction digests, the trend toward an increased frequency of this mutation in PD compared with control subjects was not significant.

A mutation in the ND2 gene that alters an evolutionarily highly conserved amino acid was identified by sequencing at np 4654 in a single PD subject (see table 1). No additional PD patients with this mutation were identified by restriction digests, and none of the control subjects harbored this mutation (see table 2). A mutation at np 13117 that alters a moderately conserved amino acid in the ND5 gene also was identified in a single PD subject by sequencing. Further screening by restriction digests again revealed no additional PD patients and no control subjects with this mutation. A mutation at np 10750 in the ND4L gene that alters a highly conserved amino acid was identified by sequencing in one PD subject. Screening additional subjects by restriction digests revealed 1 additional PD patient as well as 1 control with this mutation, for a combined total of 2 PD patients (of 196) and 1 of 107 control subjects with this mutation.

LHON-associated mutations. The most common primary mutations in complex I genes previously linked to LHON (np 3460, 11778, and 14484)[14] were not found in any subjects, including both sequencing and restriction digest data. Secondary LHON mutations (np 4216, 4917, and 13708),[25] which increase the penetrance of a primary LHON mutation but alone are insufficient to cause disease, were found in similar frequencies in PD patients and control subjects after accounting for ethnicity. There were no significant differences in the frequencies of any of these mutations in PD versus control patients within any ethnic group, although there were relatively few subjects in the Hispanic, black, and Asian groups. When data from ethnic groups are combined, a nonsignificant trend toward increased frequencies of the secondary LHON mutations in PD patients appears. Among the 18 PD patients with a history of a mother or sibling with PD, 3 were positive for the 4216 mutation, 3 for the 4917 mutation, and none for the 13708 mutation. All subjects had negative test results for the primary LHON mutations at np 3460 (n = 140 PD patients, 113 control subjects), 11778 (n = 173 PD patients, 152 control subjects), and 14484 (n = 135 PD patients, 91 control subjects). Further details are available on the *Neurology* website.

A common polymorphism at np 10398 has not been linked directly to LHON but sometimes is associated with the 4216[26] and 13708[27] mutations and so was analyzed along with the secondary LHON mutations here. The 10398 polymorphism was found at increased frequency in both Hispanics and blacks compared with Caucasians. No differences between PD and control groups in the frequency of this mutation was noted after accounting for ethnicity.

Synonymous polymorphisms. Fifty-nine synonymous polymorphisms (not associated with an amino acid change) were identified by sequencing in complex I genes. A list of these polymorphisms and their frequencies is available on the *Neurology* website. Of these, 31 were previously unidentified polymorphisms by comparison with the Mitomap database.[28] All but 2 of these 31 new polymorphisms were found in a only a single subject. The A12612G polymorphism was found in three PD patients, and the T3020C polymorphism was found in one PD patient and one control subject. Several additional differences from the Cambridge sequence,[20] which are known errors or consensus changes,[26,29,30] were identified in each subject as follows:

Table 2 Complex I gene missense mutations selected for additional screening

		PD					Control				
Mutation	Gene	Group 1 sequencing	Group 1 other	Group 2	Brain	Total	Group 1 sequencing	Group 1 other	Group 2	Brain	Total
A3505G	ND1	0 (28)	3 (59)	4 (104)		7 (191)	0 (8)	0 (14)	1 (81)		1 (103)
A3547G	ND1	0 (28)	2 (59)	0 (109)		2 (196)	0 (8)	0 (14)	2 (83)		2 (105)
T4216C	ND1	6 (28)	7 (54)	16 (124)	9 (46)	38 (252)	0 (8)	4 (14)	11 (136)	8 (41)	23 (199)
C4654T	ND2	1 (28)	0 (55)	0 (110)		1 (193)	0 (8)	0 (14)	0 (81)		0 (103)
A4917G	ND2	4 (28)	6 (51)	10 (125)	5 (44)	25 (248)	0 (8)	4 (13)	7 (136)	4 (42)	15 (199)
A10398G	ND3	8 (28)	11 (68)	48 (125)	10 (46)	77 (267)	1 (8)	1 (17)	80 (135)	17 (57)	99 (217)
A10750G	ND4L	1 (28)	0 (61)	1 (107)		2 (196)	0 (8)	1 (15)	0 (84)		1 (107)
A13117G	ND5	1 (28)	0 (27)	0 (109)		1 (164)	0 (8)		0 (83)		0 (91)
G13708A	ND5	3 (28)	1 (55)	8 (125)	6 (45)	18 (253)	0 (8)	1 (13)	6 (135)	3 (42)	10 (198)
A13780G	ND5	3 (28)	0 (61)	2 (108)	0 (27)	5 (224)	0 (8)	0 (15)	1 (82)	0 (34)	1 (139)

Mutations in complex I genes selected for screening by restriction digests. The nos. of patients testing positive for the mutation in each subgroup are shown. Nos. in parentheses indicate the total no. of subjects analyzed for the mutation in that subgroup (see Methods for explanation of variable numbers of subjects screened). Mutations at np 3505 and 3547 were not identified from sequencing of the complex I genes but were further analyzed by restriction digests because of their possible association with LHON (Johns DR, unpublished data, 1999). For group 1, data obtained by sequencing (PD: group 1 sequencing) is shown separately from data obtained from screening by restriction digests (PD: group 1 other). None of the differences in mutation frequencies between PD and controls was significant, with the exception of the 10398 mutation (more common in controls, $p < 0.001$). However, this difference could be accounted for by differences in ethnic frequencies between PD and controls in group two (see text and *Neurology* website).

G3243T, G4985A, A4769G, T11335C, G13702C, C14199A, C14272G, C14365G, and C14368G. The single exception was a control subject who harbored a synonymous C to A transition at np 14365.

tRNA mutations. Nine single base pair variants were identified by sequencing in tRNA genes (see table 1). Each of these nine were identified in PD patients, and two also were identified in control subjects. Of these, three were selected for restriction digest screening of additional subjects based on previous reports of their association with PD (at np 4336,[31,32] 15927,[33] and 15928[33]) (table 3; see later). In addition, mutations at 10034, 10463, and 15924 each were found by sequencing in at least 3 of 28 PD patients and none of the 8 control subjects. These also were screened by restriction digests in additional subjects.

4336 $tRNA^{Gln}$ mutation. A mutation at np 4336 in the $tRNA^{Gln}$ gene has been reported to be associated with PD.[31,32] Combining sequencing and restriction digest data, we identified this mutation in 6 of 252 PD patients and in 4 of 200 control subjects ($p = 0.78$). A meta-analysis of previously published reports of this mutation in PD patients[21-24, 31-33] revealed it to be present in 10 of 291 PD patients and in 5 of 434 control subjects ($p = 0.03$). When data from the current study are included in the meta-analysis, the result is that 16 of 543 PD patients and 9 of 634 control subjects ($p = 0.07$) have the 4336 mutation, indicating a strong trend toward an increased frequency of this mutation in PD, but the trend does not reach statistical significance in the combined analysis.

15924 $tRNA^{Thr}$ mutation. The np 15924 mutation previously has been linked to lethal infantile mitochondrial myopathy.[34] We identified this mutation in 4 of 28 PD patients and in none of the 8 control subjects by sequencing (see table 1). To determine if this mutation was associated with PD, we screened additional subjects for this mutation with restriction digests (see table 3). A nonsignif-

Table 3 tRNA gene mutations selected for additional screening

		PD					Control				
Mutation site	Gene	Group 1 sequencing	Group 1 other	Group 2	Brain	Total	Group 1 sequencing	Group 1 other	Group 2	Brain	Total
A4336C	$tRNA^{Gln}$	2 (28)	2 (55)	2 (124)	0 (45)	6 (252)	1 (8)	4 (14)	0 (136)	0 (42)	4 (200)
T10034C	$tRNA^{Gly}$	3 (28)	1 (68)	2 (127)	1 (46)	7 (269)	0 (8)	0 (17)	2 (134)	1 (57)	3 (216)
T10463C	$tRNA^{Arg}$	3 (28)	7 (60)			10 (88)	0 (8)	3 (16)			3 (24)
A15924G	$tRNA^{Thr}$	4 (28)	5 (69)	9 (128)	3 (46)	21 (271)	0 (8)	1 (17)	13 (131)	1 (57)	15 (213)
G15927A	$tRNA^{Thr}$	0 (28)	0 (69)	0 (128)	1 (46)	1 (271)	0 (8)	0 (17)	0 (131)	0 (57)	0 (213)
G15928A	$tRNA^{Thr}$	3 (28)	7 (69)	8 (128)	4 (46)	22 (271)	0 (8)	3 (17)	5 (131)	8 (57)	16 (213)

Nucleotide position of mitochondrial DNA mutations identified in transfer RNA (tRNA) genes of PD and control patients. In every case in which both mutations were analyzed, the presence or absence of the 10463 mutation corresponded with that of the 15928 mutation. See table 2 legend for explanation of nos.

icant trend toward an increased frequency of this mutation also was seen in the postmortem group, in which 3 of 46 PD patients harbored this mutation versus 1 of 57 control subjects ($p = 0.21$). However, combining sequencing and restriction digest data from all groups, it was found in 21 of 271 PD patients and in 15 of 213 control subjects ($p = 0.77$), indicating no significant association of this mutation with PD.

15927 and 15928 $tRNA^{Thr}$ mutation. Mayr-Wohlfart et al. report an increased frequency of loss of an HpaII restriction site, which occurs in the presence of either the np 15927 or the np 15928 mutation, in patients with PD[33] or MS.[35] Combining sequencing and restriction digest data from all groups, we found the 15928 mutation in 21 of 268 PD patients and in 15 of 209 control subjects ($p = 0.79$; see table 3). The 15927 mutation was present in only 1 of 268 PD patients and in none of 209 control subjects.

Other mutations. A mutation at np 10034 in the $tRNA^{Gly}$ gene was analyzed by restriction digests in additional subjects based on its identification in three PD patients and no control subjects by sequencing. This additional screening revealed no difference in the frequency of the 10034 mutation in PD compared with control subjects ($p = 0.35$, including sequencing and restriction digest data from all groups; see table 3). A trend toward increased frequency of the 10034 mutation in PD versus control subjects is seen when analysis is restricted to Caucasians (7 of 237 PD versus 1 of 125 control subjects; $p = 0.18$). In the case of the 10463 mutation, combined sequencing and restriction digest data from group 1 revealed it to be present in approximately equal frequencies in PD and control subjects (10 of 88 PD patients and in 3 of 24 control subjects). This mutation was not analyzed further in group 2 or in the postmortem group. However, in every case in which both mutations were analyzed, the presence or absence of the 10463 mutation corresponded with that of the 15928 mutation.

Data regarding the evolutionary conservation of amino acids altered by complex I mutations and of base pairs and binding in tRNA mutations are available on the *Neurology* website and from reference numbers 36 and 37.

Discussion. These data demonstrate that homoplasmic mtDNA mutations (those present in 100% of the mtDNA molecules in an individual) or mutations present at high mutational burdens (see Abstract) in complex I and tRNA genes are not likely to influence the risk of PD in most PD patients. However, a few PD patients were identified with mutations that alter conserved amino acids in complex I genes. Interpretation of the significance of these mutations is complicated by the highly polymorphic nature of mtDNA. Complete sequencing of mitochondrial genes has been reported for relatively few normal subjects, and it is likely that many uncommon nonpathogenic polymorphisms have yet to be identified. Simply demonstrating that a base pair variant is found in a patient or family with a disease and is not found in numerous control subjects is insufficient to prove pathogenicity. On the other hand, the identification of a mutation in a control subject does not rule it out as a potentially pathogenic mutation because of the incomplete penetrance of many mtDNA mutations and the possibility that some control subjects could later develop PD. Furthermore, the development of symptoms caused by a mtDNA mutation can depend on exposure to particular environmental factors.[38,39] Mutations at np 4654 and np 10750 altered moderately to highly conserved amino acids. The 4654 mutation was identified in only a single PD patient. The 10750 mutation was seen in two PD patients but also in one control subject. Whereas these mutations may have played a role in the development of PD in these three PD patients, for the reasons stated earlier, this conclusion remains uncertain. Although fewer missense mutations were identified by sequencing in control subjects compared with PD patients, this is consistent with the fewer control subjects that were analyzed by sequencing.

LHON-associated complex I mutations. Parkinson's disease and LHON, which share the biochemical feature of complex I dysfunction, also might have common genetic risk factors.[5] This possibility is supported by the finding of complex I deficiency in cybrid cell lines expressing mtDNA from PD patients[10,11] and by the identification of a LHON-associated mutation in a family with atypical parkinsonism.[17] However, we find no association between any of the primary or secondary LHON-associated complex I mutations and PD. The trends toward increased frequencies of the 4216, 4917, and 13708 mutations in PD patients can be accounted for by the increased frequency of Hispanics and blacks—who are less likely to carry these secondary LHON mutations—among the control population compared with the PD patients in group 2 rather than by a true association of these mutations with PD. A similar argument also applies to the common 10398 polymorphism, which has not been linked directly to LHON but sometimes is associated with the 4216[26] or the 13708[27] mutations. The 10398 polymorphism is more common in blacks and Hispanics, resulting in an elevated frequency of this mutation in the control group. No association of any of these mutations was noted in the subgroup analysis of PD patients with an affected mother or sibling. Two additional missense mutations, at np 3505 and 3547, that may be associated with LHON (Johns DR, unpublished data, 1999) also were analyzed. The 3505 mutation was identified in 7 of 191 PD patients and in only 1 of 103 control subjects ($p = 0.18$; see table 2). Although this difference does not reach significance, these data raise the possibility of a role for this mutation in PD. No difference was noted in the frequencies of the 3547 mutation in PD and control subjects. These data indicate that these primary and secondary LHON-associated mutations are not significant risk factors in most cases of PD.

Two (of 26) black control subjects harbored the 4216 mutation, 1 in association with the 4917 mutation and the other with the 13708 mutation (data available on the *Neurology* website). Among the 66 Hispanic subjects, 1 carried the 4216 mutation and 2 the 4917 mutation. These mutations are associated

with mtDNA haplotype group J, which has been considered Caucasoid specific.[40] However, our data demonstrate that these mutations are not exclusively associated with Caucasians.

5460 complex I (ND2) mutation. The 5460 mutation is reported to be heteroplasmic with regional variations of the mutational burden in the brains of PD patients[41] and has been reported to be present at an increased frequency in PD patients.[21] However, our data, as well as a meta-analysis of the literature, do not support a role for this mutation in PD.

4336 $tRNA^{Gln}$ mutation. A meta-analysis of previously published data on the 4336 mutation in PD patients revealed this mutation to be present at an increased frequency in PD patients ($p = 0.03$). However, the inability to properly control for ethnicity across studies in this meta-analysis, and the possibility of a bias toward the publication of positive results, cast doubt on the validity of this analysis. When data from the current study are included in the meta-analysis, the 4336 mutation is found in 16 of 543 PD patients and 9 of 634 control subjects ($p = 0.07$), indicating a trend that no longer reaches significance despite the numerous patients screened and the lack of correction for multiple comparisons. The presence of this mutation may be associated with a slightly increased risk of PD, but clearly the mutation is uncommon and is not a factor in most PD patients.

$tRNA^{Thr}$ mutations. Mayr-Wohlfart et al. used a single restriction digest to screen for the 15927 or 15928 $tRNA^{Thr}$ mutations in PD patients and control subjects.[33] The presence of either of these two mutations eliminates an HpaII site. This site was lost in 15 of 100 PD patients and in only 3 of 100 control subjects. Binding of the 15928 base pair in the lower stem of the $tRNA^{Thr}$ is highly conserved, and thus the G to A mutation at this site, which eliminates binding, is a reasonable candidate for a pathogenic mutation. However, in contrast to this prior study, we found only a single PD subject with the 15927 mutation and similar frequencies in PD and control groups for the 15928 mutation. A mutation at np 15924 previously linked to fatal infantile mitochondrial myopathy[34] also was present at similar frequencies in the PD and control groups ($p = 0.77$). These data indicate that the 15924 and 15928 $tRNA^{Thr}$ mutations are not likely to be major risk factors for PD. As with other mutations found in only a single patient, further studies are needed to determine if the 15927 mutation is relevant to PD.

Do mtDNA mutations influence the risk of developing PD? The inability to identify mutations in association with most PD patients in the current study is not necessarily in conflict with the data from cybrids, which indicates a role for mtDNA mutations in PD. A possible explanation is that the relevant mutations may lie in ribosomal RNA (rRNA) genes or in the noncoding D-loop. The relatively specific defect in complex I activity suggests that complex I genes are the most likely sites for mutations, but a nonsignificant trend toward decreased complex IV activity in cybrids[10] raises the possibility of a more general defect, as would be predicted from a tRNA or rRNA mutation.

Other possible explanations relate to the sensitivity of the techniques used here for detecting heteroplasmic mutations. Heteroplasmic mutations at low mutational burdens (less than 60%) are unlikely to be detected by direct sequencing (see Methods). Pathogenic mtDNA mutations identified in other neurologic disorders manifest symptoms only at higher mutational burdens (usually 70% to 100%).[14,42,43] However, it is possible that development of a late-onset neurodegenerative disorder such as PD could be influenced by mutations at lower mutational burdens. Screening of blood-derived mtDNA from PD patients allows for the subsequent in vitro analyses of mutations in cybrids but has the disadvantage that mutations may be present at a lower mutational burden in blood compared with brain. This limitation is less critical for the data derived primarily from restriction digests because this technique allows the detection of mutations present at low mutational burdens (less than 5%). Another potential explanation for the failure to identify mtDNA mutations in most PD patients is that the mutations are not necessarily inherited. Randomly or semi-randomly positioned mutations that are acquired secondary to oxidative damage to mtDNA may not reach the threshold for detection at any specific site, although cumulatively they may be sufficient to cause mitochondrial dysfunction. The development of convenient techniques for screening large populations for low mutational burden mutations may facilitate the search for acquired or inherited low mutational burden mutations in the future.[44]

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Inhibition of neuronal nitric oxide synthase protects against MPTP toxicity

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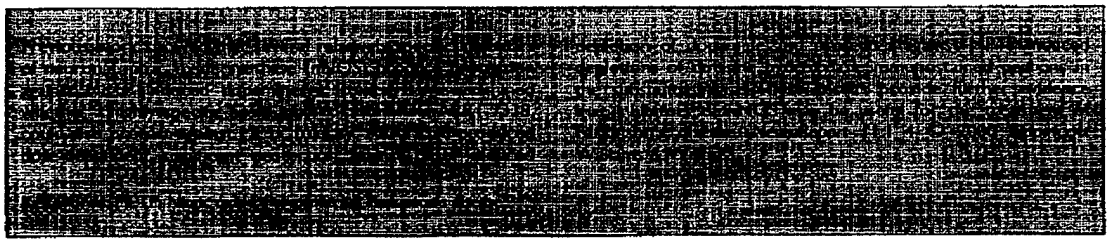

INTRODUCTION

We previously showed that co-administration of the relatively selective neuronal nitric oxide synthase (nNOS) inhibitor 7-nitroindazole (7-NI) produces dose-dependent almost complete neuroprotection against MPTP induced dopamine depletion [1]. Furthermore, 7-NI produced striking protection against MPTP-induced motor deficits, dopamine depletion and loss of substantia nigra neurons in baboons [2]. This work was confirmed and extended by showing that 7-NI protected against both MPTP induced depletion of dopamine and neuronal loss within the substantia [3]. In addition, it was shown that mice deficient in nNOS were partially resistant to MPTP. We found that another nNOS inhibitor S-methyl-thiocitrulline also protects against MPTP-induced dopamine depletion in mice [4]. These findings were recently called into question by the observation that 7-NI is a competitive inhibitor of monoamine oxidase B *in vitro* [5]. Furthermore, it was shown that high levels of 7-NI given i.p. can inhibit the conversion of MPTP to MPP^+ *in vivo* [6]. It was suggested that a major part of the neuroprotective effects of 7-NI on MPTP were due to its ability to block monoamine oxidase B. In the present experiments, we examined the ability of a novel highly selective nNOS inhibitor, ARR17338, which does not inhibit monoamine oxidase B, on MPTP induced neurotoxicity.

MATERIALS AND METHODS

Male Swiss–Webster mice weighing 30–35 g were obtained from Taconic Farms (Germantown, NY, USA). MPTP (Research Biochemicals, Natick, MA, USA) was dissolved in phosphate buffered saline (pH 7.4). MPTP (15 mg/kg, 5 ml/kg, i.p.) was administered five times at 2 h intervals to mice receiving normal drinking water or water containing 5 μg/ml ($n=8$), 10 μg/ml ($n=16$), 40 μg/ml ($n=9$) or 120 μg/ml ($n=13$) ARR17338 (imino-thiophen-2yl-methyl)-(1,2,3,4-tetrahydro-isoquinolin-7-yl)-amine). An additional set of animals was treated with 0.1 M phosphate buffered saline (PBS, 5 mg/kg, i.p.) at the times of the MPTP injections ($n=17$). ARR17338 inhibits NOS with an IC_{50} of 40 nM *vs* rat nNOS, 10 μM *vs* human eNOS and 5 μM *vs* mouse iNOS, and possesses an $IC_{50} > 100$ μM *vs* monoamine oxidase B *in vitro* [7].

Animals were sacrificed at 1 week and the striata were rapidly dissected and placed in chilled 0.1 M perchloric acid. Tissue was subsequently sonicated, and aliquots were taken for protein quantification using a fluorometric assay [8]. Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC)

and homovanillic acid (HVA) were quantified by HPLC with 16-electrode electrochemical detection [8]. Concentrations of dopamine and metabolites are expressed as ng/mg protein (mean ± s.e.m.).

To determine whether MPTP uptake or metabolism was altered, MPTP was administered at a dose of 30 mg/kg every 2 h for two doses and mice were then sacrificed at 2 h ($n=9$/group). The striata were dissected. MPP^+ levels were quantified by HPLC with u.v. detection at 295 nm. Samples were sonicated in 0.1 M perchloric acid and an aliquot of supernatant was injected onto a Brownlee aquapore X03-224 cation exchange column (Rainin, Woburn, MA, USA) and diluted with 90% 0.1 M acetic acid, 75 mM triethylamine HCl (pH 2.3) and 10% acetonitrile.

The midbrains from the above animals were immersion-fixed in 4% buffered paraformaldehyde, cryoprotected in 10% and 20% glycerol solution in 0.1 M PO_4, and frozen sectioned at 50 µm. Tissue sections were subsequently immunostained for tyrosine hydroxylase (TH; TH antisera; 1:1,000 dilution; Eugene Tech International, Inc.) Immunocytochemistry was performed using a conjugated second antibody method [9]. The procedure was as follows: tissue sections were preincubated in absolute methanol/0.3% hydrogen peroxide solution for 30 min, washed in phosphate buffered saline (PBS; pH 7.4), 3× 10 min each, placed in 10% normal goat serum (Gibco Labs) for 1 h, incubated free floating in primary antiserum at room temperature for 12–18 h (all dilutions of primary antisera above included 0.3% Triton X-100 and 10% normal goat serum), washed in PBS 3× for 10 min each, placed in periodate-conjugated goat anti-rabbit IgG (1:300 in PBS) (Boehringer-Mannheim), washed in PBS 3× for 10 min each, and reacted with 3,3'-diaminobenzidine HCl (1 mg/ml) in Tris–HCl buffer with 0.005% hydrogen peroxide.

Serial-cut, midbrain sections containing the substantia nigra (Bregma levels −2.92 mm to −3.08 mm; intra-aural levels 0.88 mm to 0.72 mm) [10] in PBS-treated control, MPTP-treated, and MPTP-treated/ARR17338-treated (120 µg/ml) mice were scanned by microscopic video-capture and analyzed. Analysis of TH-positive neurons within the substantia nigra pars compacta (area A9) was completed in each serial section using Neurolucida software (Microbrightfield). Total TH-positive neuron counts in the circumscribed substantia nigra compacta were made in each section. All computer identified cell profiles were manually verified as neurons and exported to Microsoft Excel software. The data are expressed as mean ± s.e.m.

The statistical significance of differences was determined by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference *post hoc* test to compare group means. All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

RESULTS

As shown in Fig. 1, administration of ARR17338 produced dose-dependent protection against MPTP induced depletion of striatal dopamine concentrations. There was also significant protection against depletion of DOPAC at the highest dose of ARR1 7338. There was no significant difference in MPP^+ levels in the control and mice treated with 40 µg/ml ARR1 7338 (18.7 ± 3.0 *vs* 25.5 ± 4.5 ng/mg protein).

Histological examination of serially cut TH-positive immunostained sections through the midbrains of MPTP-treated, MPTP-treated/ARR17338-treated, and PBS-treated control mice showed that in comparison to PBS-treated control and MPTP-treated/ARR17338-treated mice, there was a significant loss of TH-positive neurons (16%) within

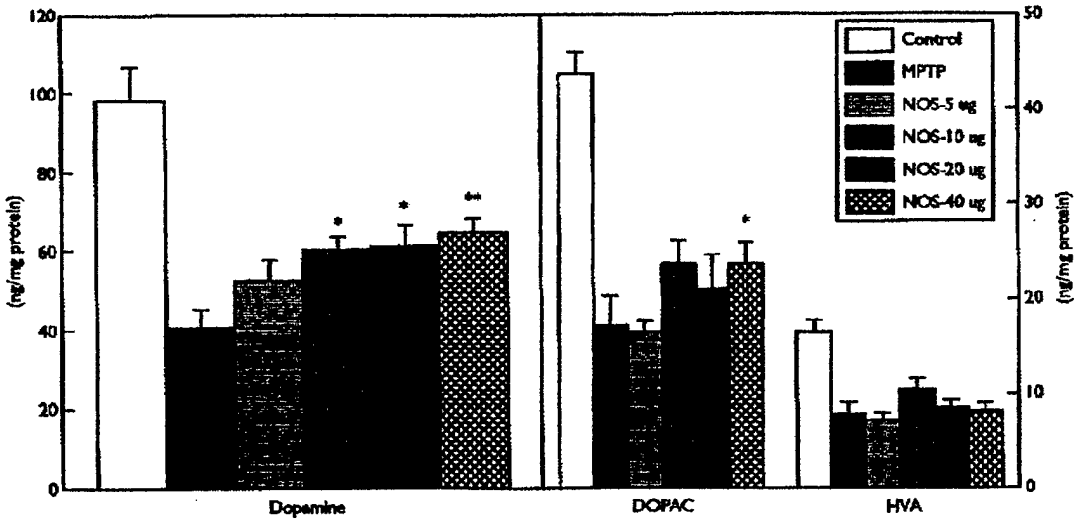

Fig. 1. Effects of increasing doses of ARR17338 on MPTP induced depletion of dopamine, DOPAC and HVA. * $p < 0.05$, ** $p < 0.01$ compared with MPTP alone.

the substantia nigra in the MPTP-treated group of animals (Fig. 2). There was significant protection against loss of nigral neurons in MPTP-treated/ARR17338-treated animals in comparison to MPTP-treated littermates control mice (PBS-treated control mice: 136 ± 5.3; MPTP-treated/ARR17338-treated mice: 129 ± 5.5; MPTP-treated littermate controls: 114 ± 9.2, $p < 0.01$).

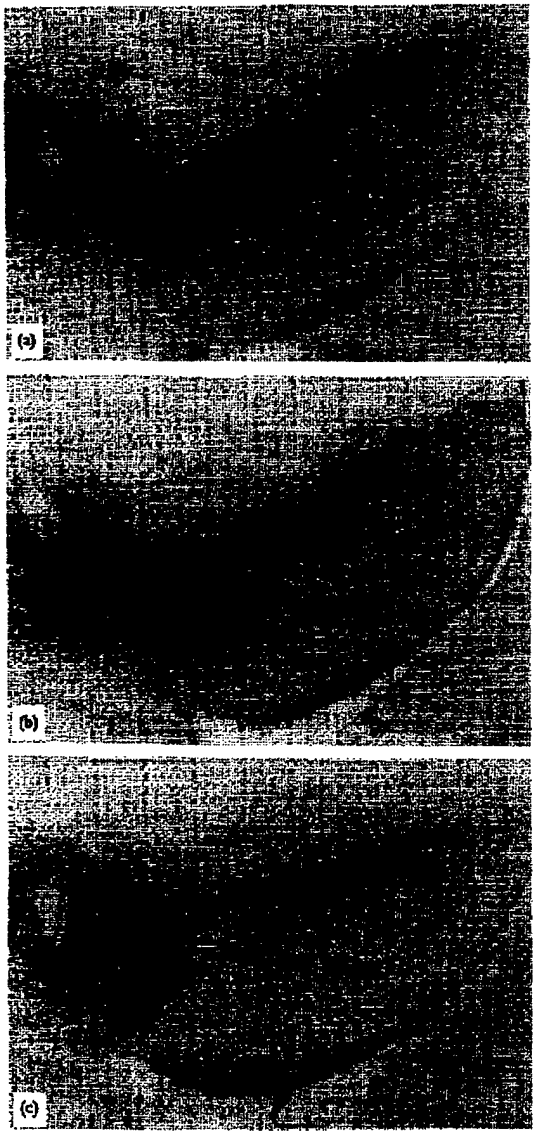

Fig. 2. Photomicrographs of tyrosine hydroxylase-immunostained sections of the substantia nigra from PBS treated (a), MPTP-treated/ARR17338-treated (b), and littermate control MPTP-treated (c) mice. Littermate control MPTP-treated mice show neuronal loss within the medial segment of the substantia nigra pars compacta (c) compared with PBS treated and MPTP-treated/ARR17338-treated animals. Bar in (c) = 300 μm.

DISCUSSION

A potential role for nitric oxide and peroxynitrite in MPTP neurotoxicity is strongly supported by prior studies. MPTP administration results in mitochondrial dysfunction that can cause increased cellular calcium levels, leading to activation of nitric oxide synthase and to the generation of free radicals. The reaction of superoxide ($O_2^{\cdot}$) and nitric oxide ($NO^{\cdot}$) leads to the production of peroxynitrite ($ONOO^-$) which can mediate both oxidative damage and nitration of tyrosines [11–13]. The potential importance of peroxynitrite in MPTP neurotoxicity has been strengthened by findings that 3-nitrotyrosine (3-NT) levels in the striatum are increased following MPTP and that MPTP administration leads to nitration and inactivation of tyrosine hydroxylase [1,14].

If $ONOO^-$ plays a role in MPTP induced dopaminergic toxicity, then inhibitors of nitric oxide synthase should be neuroprotective. We initially showed that the relatively selective nNOS inhibitor 7-NI produced dose-dependent neuroprotection against the neurotoxicity of MPTP [1]. Furthermore it blocked MPTP induced increases in 3-NT levels, which are a relatively specific marker of $ONOO^-$-mediated toxicity. The finding that 7-NI protects against MPTP was confirmed and extended by Przedborski and colleagues [3]. These authors showed that 7-NI protected against both MPTP induced dopamine depletion as well as loss of substantia nigra neurons. They also showed that mice deficient in nNOS were resistant to MPTP toxicity. We subsequently found that S-methyl-thiocitrulline, another relatively selective nNOS inhibitor, also protected against both MPTP and malonate toxicity [4]. We also found striking neuroprotection against MPTP neurotoxicity in baboons [2]. However, administration of the non-specific NOS inhibitor L-nitroarginine methyl ester did not protect against MPTP toxicity in marmosets [15].

A recent study showed that 7-NI is a competitive MAO-B antagonist *in vitro* [16]. In addition, MPP^+ levels following MPTP administration were reduced when 7-NI was co-administered i.p. at a dose of 50 mg/kg [17]. It was suggested that this might account for a major portion of its neuroprotective effects. This dose administered i.p., however, is likely to have effects of cerebral blood flow, which could contribute to the observed decreases in MPP^+ concentrations [16]. Furthermore, we found that 7-NI protects against MPP^+ induced substantia nigra degeneration, and that MPP^+ neurotoxicity is attenuated in nNOS knockout mice [16]. These findings indicate that 7-NI has neuroprotective effects independent of its effects on MPTP conversion to MPP^+. Finally, 7-NI protects against methamphetamine-induced dopaminergic toxicity [6,19].

In the present experiments, we examined whether another highly potent nNOS inhibitor, ARR17338 protects against MPTP neurotoxicity. A related compound, ARR17477 is neuroprotective against transient middle cerebral artery occlusion in rats [20]. ARR17338 shows an ~200-fold selectivity for nNOS, as compared with endothelial NOS and 100-fold *vs* inducible NOS. It does not inhibit MAO-B *in vitro*. In the current experiment, we found that

ARR17338 produced significant neuroprotection against MPTP neurotoxicity. It protected against both a depletion of dopamine levels, as well as a loss of tyrosine hydroxylase immunoreactive neurons in the substantia nigra. Interestingly the degree of protection was similar to that seen with S-methyl-thiocitrulline and was not as profound as the protection seen with 7-NI. This would suggest that part of the protective effects mediated by 7-NI could be due to either MAO-B inhibition or that 7-NI may have effects on other forms of NOS, such as inducible NOS, at the doses utilized *in vivo*.

CONCLUSIONS

The present results show that highly selective nNOS inhibitor, ARR17338, produces dose-dependent neuroprotection against the dopaminergic neurotoxicity of MPTP. These results therefore provide further evidence that nNOS inhibitors may be useful for the treatment of PD.

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Energetics in the pathogenesis of neurodegenerative diseases

M. Flint Beal

Mitochondria have been linked to both necrotic and apoptotic cell death, which are thought to have a major role in the pathogenesis of neurodegenerative diseases. Recent evidence shows that nuclear gene defects affecting mitochondrial function have a role in the pathogenesis of Friedreich's ataxia, Wilson's disease and hereditary spastic paraplegia. There is also accumulating evidence that mitochondrial dysfunction might have a role in the pathogenesis of amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease and Alzheimer's disease. If this is so, a number of therapeutic targets are implicated that might result in novel treatments for neurodegenerative diseases.

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EVIDENCE that implies mitochondria have a crucial role in both necrotic and apoptotic cell death is accumulating rapidly. Both these conditions are distinct forms of cell death (as defined morphologically); however, in neuronal populations they can either coexist or be sequential events, depending on the severity of the initiating insult[1]. Cellular energy reserves appear to have an important role in these two forms of cell death, with apoptosis favored under conditions with mild insults and preserved ATP levels. Necrosis is frequently induced by toxic insults, such as glutamate excitotoxicity, in which ATP is depleted. In response to glutamate and NMDA there is usually a prominent and persistent depolarization of the mitochondrial membrane potential[2,3], followed by a depletion of energy reserves that results in necrosis[4]. A requirement for mitochondrial Ca^{2+} uptake in glutamate-mediated excitotoxicity has been recently demonstrated[5,6]. Activation of NMDA receptors leads to a more-rapid increase in mitochondrial Ca^{2+} levels and a greater increase in free-radical production and $NO^{\bullet}$ production than does activation of non-NMDA receptors[7–9] (see Fig. 1).

Mitochondria are essential in controlling specific apoptosis pathways[10]. The mechanisms by which they exert this function include release of caspase activators, such as cytochrome c, caspase 9 and apoptosis-inducing factor (AIF), and disruption of oxidative phosphorylation. The redistribution of cytochrome c during apoptosis can be prevented by overproduction of the anti-apoptotic protein Bcl2, which is localized to the outer mitochondrial membrane. Oversynthesis of the pro-apoptotic protein Bax triggers cytochrome c efflux from mitochondria[11].

The mitochondrial permeability transition pore (PTP) could be crucial in both necrotic and apoptotic cell death. Its activation increases the inner mitochondrial membrane permeability to solutes with a molecular mass of up to 1.5 kDa. Proposed components of the PTP include the inner mitochondrial membrane adenine nucleotide transporter that interacts with cyclophilin D, the voltage-dependent anion channel in the outer membrane, the peripheral GABA receptor in the outer membrane and mitochondrial creatine kinase. Bax interacts with the voltage-dependent anion channel to accelerate opening of the PTP, contributing to cytochrome c release. The opening of the channel is favored by Ca^{2+} and oxidizing agents, whereas closure is favored by protons (low matrix pH) and adenine nucleotides (ADP). Cyclosporin A is an effective blocker of the channel that appears to prevent an interaction of cyclophilin with the adenine nucleotide transporter. It also reduces excitotoxicity *in vitro*[2,3] and reduces both hypoglycemic and ischemic cell death *in vivo*[12].

A consequence of mitochondrial dysfunction is increased generation of free radicals and oxidative damage, which are strongly implicated in the pathogenesis of neurodegenerative diseases. Mitochondria, the most important physiological source of $O_2^{\bullet}$ in animal cells, are estimated to produce 2–3 nanomoles of $O_2^{\bullet}$/min/mg protein[13]. Mitochondrial dysfunction in neurodegenerative diseases might be a consequence of either nuclear DNA or mitochondrial DNA (mtDNA) (Box 1). A useful technique for investigating the role of mtDNA defects in neurodegenerative diseases is the production of cybrid cell lines in which a patient's mitochondria are fused with mitochondria depleted (rho^0) cells (Fig. 2).

Neurodegenerative diseases with nuclear gene defects that affect mitochondria

Friedreich's ataxia

Friedreich's ataxia is an autosomal recessive disease characterized by the association of progressive gait and limb ataxia, cardiomyopathy and diabetes. It is the most-common inherited ataxia with an incidence of about 1 in 50 000. In most individuals, a GAA trinucleotide repeat expansion in the first intron of a gene on chromosome 9 interferes with transcription and results in reduced levels of the protein, frataxin. The regions of degeneration observed in the disease correlate with sites of frataxin transcription, which are highest in the heart, spinal cord and dorsal-root ganglia[19]. Insights into disease pathogenesis have come from the study of the yeast frataxin homolog[20]. Disruption of the yeast gene results in respiratory insufficiency with an inability to carry out oxidative phosphorylation, a loss of

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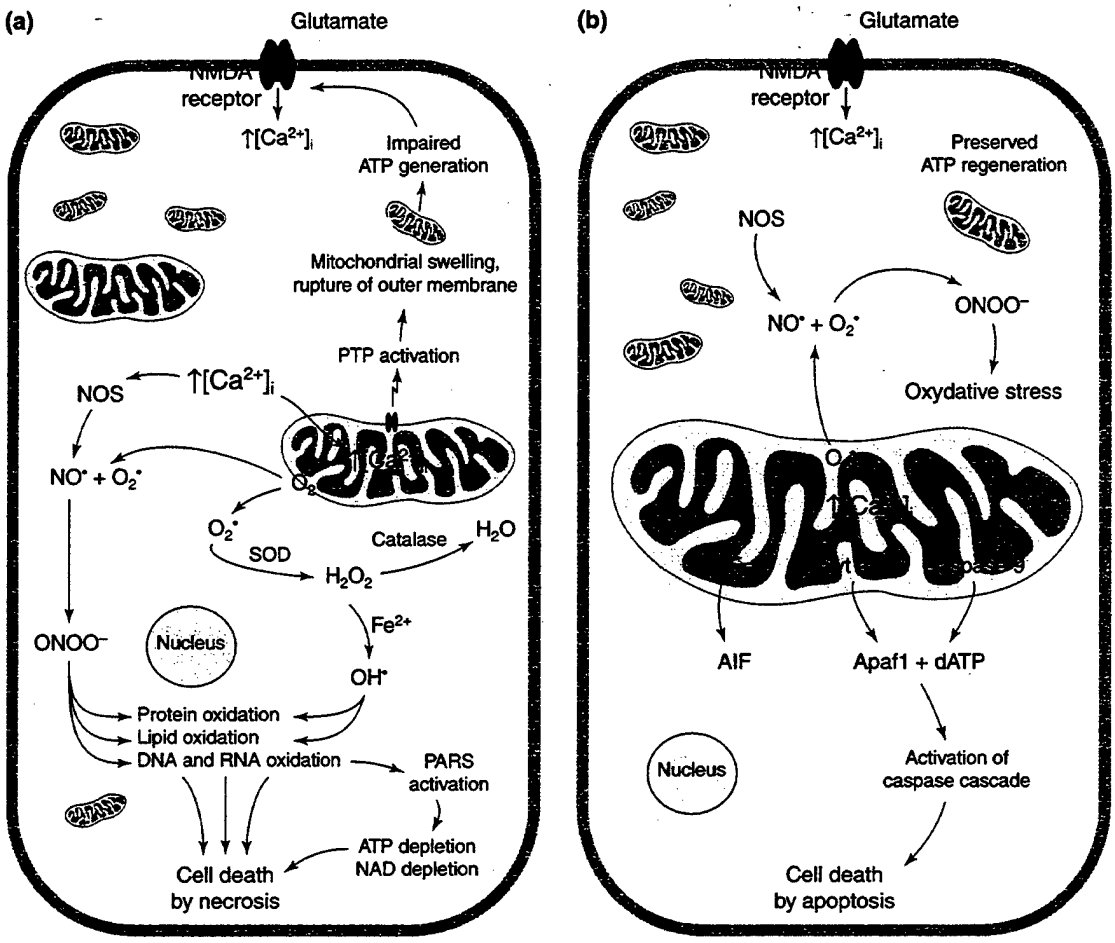

Fig. 1. ***Involvement of mitochondria in cell death.*** **(a)** *depicts the effects of a severe excitotoxic insult that results in cell death by necrosis, whereas* **(b)** *depicts the results of a mild excitotoxic insult that results in apoptosis. After a severe insult (such as ischemia) there is a large increase in glutamate-mediated activation of NMDA receptors, an increase in intracellular Ca^{2+} concentration, activation of NOS, increased mitochondrial Ca^{2+} and superoxide generation, followed by the formation of $ONOO^-$. An increase in superoxide generation can also lead to increased H_2O_2 production, which can then react with transition metal ions (Fe^{2+} or Cu^+) to generate the highly reactive $OH^•$ radical. This results in damage to cellular macromolecules, including DNA, which leads to activation of poly-ADP ribose polymerase (PARS). Activation of PARS results in ATP and NAD depletion, which directly contributes to cell death. Both mitochondrial accumulation of Ca^{2+} and oxidative damage lead to activation of the permeability transition pore (PTP) that is linked to excitotoxic cell death. A mild excitotoxic insult* **(b)** *can occur because of impaired functioning of excitotoxicity-amino-acid receptors, which allows more Ca^{2+} influx, impaired functioning of other ionic channels or impaired energy production, which can allow the voltage-dependent NMDA receptor to be activated by resting levels of glutamate. This can then lead to increased mitochondrial Ca^{2+} and free-radical production (by mitochondria and via activation of neuronal NOS, which generates $ONOO^-$), yet relatively preserved ATP generation. The mitochondria can then release cytochrome c, caspase 9, apoptosis inducing factor (AIF) and perhaps other mediators that cause apoptosis. The interaction of dATP and Apaf1 converts pro-caspase 9 to an active caspase, which initiates a caspase cascade that leads to apoptotic cell death. The precise role of the PTP in this mode of cell death is still being clarified but there does appear to be involvement of the adenine nucleotide transporter, which is a key component of the PTP.*

mtDNA, an increase in iron content and hypersensitivity to oxidative stress mediated by H_2O_2 (Refs 19–21). The mitochondrial damage is proportional to the concentration and duration of exposure to extracellular iron, establishing iron accumulation as causal to mitochondrial damage[22].

Human frataxin is also localized to mitochondria[19,23]. Endomyocardial biopsies from individuals with Friedreich's ataxia show a marked reduction in the activities of enzymes such as aconitase, which contains iron–sulfur clusters and is particularly susceptible to oxidative stress[24]. There is increased iron content in the dentate nucleus and in fibroblasts of individuals with Friedreich's ataxia[25,26], and fibroblasts show an increased sensitivity to H_2O_2 when compared with controls[27]. A study using phosphorus magnetic resonance spectroscopy demonstrated that *in vivo* ATP production was impaired in the muscle of individuals with Friedreich's ataxia, and that this is correlated with numbers of GAA repeats in the gene for frataxin[28]. There are increased concentrations of 8-hydroxy-2-deoxyguanosine in the urine of these individuals, consistent with oxidative damage (J. Schulz, M. Bogdanov and M.F. Beal, unpublished observations). It is also of interest that mutations in the α-tocopherol (vitamin E)-binding protein, which reduces concentrations of vitamin E, result in a clinical phenotype identical to Friedreich's ataxia[29]. The data therefore support a role of mitochondrial dysfunction and oxidative damage in disease pathogenesis.

Box 1. Genetic causes of bioenergetic defects in neurodegenerative diseases

Diseases with nuclear DNA-encoded mitochondrial defects
Friedreich's ataxia (GAA expansions or point mutations in frataxin)
Wilson's disease (point mutations in a P-type ATPase)
Hereditary spastic paraplegia (point mutations in a mitochondrial protein)
Deafness–dystonia (mutations that affect the import of mitochondrial proteins)
Leigh's disease (mutations that affect cytochrome-oxidase assembly)

Diseases with nuclear DNA mutations with secondary mitochondrial dysfunction
Huntington's disease (CAG repeat expansions in huntingtin)
Cerebellar degenerations (CAG repeat expansions in ataxins)

Diseases with mitochondrial DNA (mtDNA)-encoded mitochondrial defects
Leber's disease with dystonia (mtDNA mutation in *ND6*)
Kearns–Sayre syndrome (mtDNA deletions)
MELAS (mitochondrial encephalopathy, lactic acidosis and strokes) (mtDNA tRNA mutations)
MERRF (myoclonus epilepsy and ragged red fibers) (mtDNA tRNA mutations)
Leigh's disease (mtDNA mutations in ATP synthase)

Diseases with possible mtDNA-encoded mitochondrial defects
Amyotrophic lateral sclerosis
Parkinson's disease
Alzheimer's disease
Progressive supranuclear palsy

Wilson's disease

Wilson's disease is an autosomal recessive neurological disorder that is characterized by abnormalities in copper homeostasis. The illness results in accumulation of copper in the liver and basal ganglia, which results in cirrhosis and progressive dystonia, tremor, spasticity and dysarthria. The illness is caused by mutations in a copper P-ATPase. Two isoforms of the protein have been identified: one in the cytoplasm and the other localized to mitochondria[30]. The mutations cause increases in intracellular copper levels by impairing interaction with the copper chaperone Atox1 (also known as Hah1; Refs 31,32). Wilson's disease might therefore lead to an accumulation of mitochondrial copper concentrations, resulting in oxidative damage that is similar to that seen in individuals with Friedreich's ataxia. Several studies have shown increased oxidative damage in the liver and peripheral tissues of individuals with Wilson's disease[33].

Hereditary spastic paraplegia

Familial spastic paraparesis is a disorder that results in progressive stiffness of the legs with hyperreflexia and gait impairment, and a selective degeneration of upper motoneurons. Recently, a gene for an autosomal recessive variant was identified and termed 'paraplegin'[34]. It encodes a nuclear-encoded mitochondrial metalloprotease, that could have a role in protein folding or the import of protein into mitochondria. Individuals with a defect in this gene show impaired oxidative phosphorylation and ragged red fibers on muscle biopsies. Environmental spastic paraparesis caused by ingestion of chickling peas is known as lathyrism and it also is linked to mitochondrial dysfunction[35].

Dystonia

The etiology of most sporadic cases of dystonia remains unknown. Some families with Leber's optic atrophy and dystonia, have mutations in the mtDNA-encoded ND6 subunit of complex I of the electron-transport chain[36]. The deafness dystonia syndrome is caused by mutations in a protein encoded by nuclear DNA (TIM8), which is involved in the import of mitochondrial proteins[37,38]. Leigh's disease, which produces dystonia, can be caused by mutations of the *SURF1*, a nuclear gene that regulates the assembly of cytochrome oxidase[39,40]. The evidence to date therefore shows that nuclear gene defects resulting in mitochondrial metal accumulation, defects in protein import or in defective assembly of respiratory complexes can result in neurodegenerative diseases with distinct phenotypes.

Huntington's disease

The gene mutation in Huntington's disease (HD) is an expansion of a trinucleotide repeat in the coding region of a protein called huntingtin, the function of which is unknown[41]. The means by which it leads to neuronal degeneration also remain unknown, but a defect in energy metabolism could have a role and could contribute to neuronal cell death[42]. Consistent with this hypothesis, it has been found that: (1) lactate levels are elevated in the occipital cortex and basal ganglia in individuals with HD (Ref. 43); (2) there is a reduced phosphocreatine to inorganic phosphate ratio (PCr–Pi) in resting muscle of individuals with HD (Ref. 44); (3) mitochondrial toxins cause selective degeneration of the striatum of animals, which closely resembles the pathology seen in HD (Refs 45,46); and (4) this experimental degeneration can be blocked by prefeeding animals the same agents that lower brain lactate levels in individuals with HD (Refs 34,47). Reductions in both *N*-acetylaspartate (NAA), and creatine and phosphocreatine concentrations in the basal ganglia of individuals with HD correlate with both clinical disability and CAG-repeat expansion[48].

Biochemical studies of HD postmortem tissue show a decrease in complex-II and complex-III activity in HD caudate with a smaller decrease in complex-IV activity[49,50]. The finding of a complex-II and complex-III defect in HD basal ganglia is of interest as inherited defects in complex II are associated with basal-ganglia degeneration[51]. Lymphoblast mitochondria of individuals with HD show increased stress-induced mitochondrial depolarization that correlates with CAG-repeat number[52]. Ultrastructural studies of cortical biopsies obtained from individuals with both juvenile- and adult-onset HD show abnormal mitochondria[53]. A further piece of evidence that implicates a metabolic defect in HD is the progressive weight loss exhibited by these individuals, despite high calorific intake[54].

A breakthrough in HD research was the development of transgenic animal models. Transgenic mice (R6/2) with 150 CAG repeats in an N-terminal fragment of exon 1 of huntingtin produce a phenotype that consists of choreiform movements, involuntary stereotypic movements, tremor and epileptic seizures. Using magnetic resonance spectroscopy profound decreases in NAA concentrations were found by six to seven weeks of age, yet no cell loss, which might reflect impaired mitochondrial energy production, as NAA is synthesized within mitochondria[55,56]. The evidence

therefore strongly supports secondary mitochondrial dysfunction as a consequence of the gene mutation responsible for HD. The means by which this occurs is speculative; however it could be due to binding of mutant huntingtin to transcription factors involved in regulation of nuclear genes that are responsible for the production of mitochondrial proteins. Only 22 mitochondrial proteins are encoded by mtDNA; the remainder are encoded by nuclear DNA. Recent evidence shows an early downregulation of expression of neuronal genes well before both phenotypical and pathological changes in an animal model of spinocerebellar ataxia which has a CAG-repeat expansion[57].

Diseases with possible mtDNA-encoded mitochondrial defects

Amyotrophic lateral sclerosis

There is substantial evidence that mitochondrial dysfunction might have a role in the pathogenesis of sporadic amyotrophic lateral sclerosis (SALS). There are mitochondrial abnormalities in liver biopsies and anterior horn cells of individuals with SALS (Refs 58–60). Studies of skeletal muscle biopsies of individuals with SALS show impairment of mitochondrial function[61,62] with a 50% reduction specific activity of complex I in comparison with age-matched controls. In addition, functional imaging of mitochondria using the ratios of NADPH and flavoprotein autofluorescence of permeabilized muscle fibers show defective mitochondria at the single-fiber level. Muscle biopsies of individuals with SALS also show increased mitochondrial volume and Ca^{2+} levels[63]. There is a decrease in cytochrome-oxidase activity of individual motoneurons of individuals with SALS (Ref. 64). Peripheral blood lymphocytes from these individuals show increased cytosolic Ca^{2+} and impaired responses to uncouplers of oxidative phosphorylation[65]. A recent study of ALS cybrids showed a significant decrease in complex-I activity, trends towards reduced complex-III and complex-IV activities, and increases in activities of free-radical-scavenging enzymes[15]. An out-of-frame mutation of mtDNA-encoded subunit 1 of cytochrome c oxidase was reported in an individual with Motor Neuron disease[66]. Substantial evidence therefore implicates mitochondrial dysfunction in individuals with SALS and there is also evidence for increased oxidative damage.

A major discovery in ALS research was the finding of mutations in copper–zinc superoxide dismutase (SOD1) in autosomal dominant inherited familial ALS (Ref. 67). Synthesis of the protein that results from the G93A *Sod1* mutation *in vitro* causes a loss of mitochondrial membrane potential as well as an elevation in cytosolic Ca^{2+} concentrations[68]. Neuropathological studies of transgenic ALS mice with *Sod1* mutations have demonstrated that vacuolization of mitochondria is an early pathological feature[69–71] that precedes immediately a rapid phase of motor weakness and loss of motoneurons[72]. The evidence therefore implicates mitochondrial dysfunction in both SALS as well as familial ALS associated with *Sod1* mutations.

Parkinson's disease

An association between neurodegeneration and mitochondrial dysfunction or oxidative damage, or both, stems from studies of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism. MPTP first came to light as a contaminant of synthetic opiates that led to an outbreak of parkinsonism in young individuals. MPTP is metabolized to MPP^+ (1-methyl-4-phenylpyridinium), which inhibits complex I of the electron-transport chain, leading to reductions in mitochondrial ATP production[73].

In idiopathic Parkinson's disease (PD), there is a 30–40% decrease in complex-I activity in the substantia nigra[74–76], and reduced staining for complex-I subunits in the substantia nigra, although preserved staining for subunits of the other electron-transport complexes, as demonstrated immunohistochemically[77]. Strong support for a mtDNA-encoded defect comes from two studies showing that cybrids made from individuals with PD show reductions in complex-I activity[16,78]. These defects are associated with increased free-radical production, increased susceptibility to the MPTP metabolite MPP^+ and impaired mitochondrial Ca^{2+} buffering[79]. A family with multisystem degeneration with parkinsonism has been reported with the 11778 mtDNA mutation, which produces a complex-I defect[80]. Direct sequencing of mtDNA complex-I and tRNA genes, however, failed to show homoplasmic mutations, suggesting that the etiology of the observed complex-I defects is due to heteroplasmic mutations or might involve genetic and environmental interactions[81].

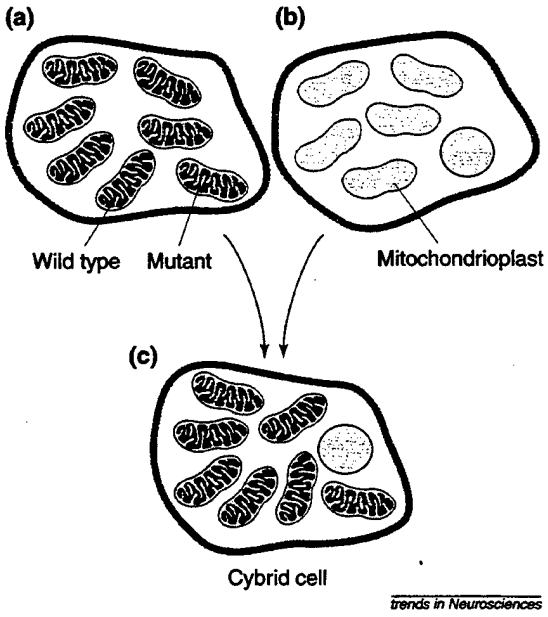

Fig. 2. ***Cybrids for investigating mitochondrial DNA (mtDNA) defects.*** *Although mitochondria are strongly implicated in neurodegenerative diseases, new techniques are needed to investigate their role. A novel technique pioneered by King and Attardi is to transfer mitochondria from living cells* **(a)** *to mtDNA-deficient cell lines [rho^0 cells;* **(b)***][14]. Cell lines can be depleted of mtDNA by exposing them to low concentrations of ethidium bromide, which inhibits replication of mitochondrial but not nuclear DNA. Exposed cells lose their mtDNA and assume an anaerobic phenotype. Cybrids are cells formed by fusing mitochondria from a patient's platelets or other tissues with the rho^0 cells* **(c)**. *If defects in oxidative phosphorylation are found in the cybrids they are attributable to alterations in a patient's mtDNA, as the patient's mitochondria now function in the presence of a different nuclear genome. Abnormal mitochondrial function has been reported in cybrids made from individuals with amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease[15–18].*

Box 2. Possible therapeutics for energy dysfunction

Coenzyme Q_{10} (antioxidant and component of electron transport)
Creatine (substrate for creatine kinase)
Lipoic acid (antioxidant and possible mitochondrial co-factor)
Carnitine (involved in mitochondrial fatty acid import)
Ginkgo biloba (antioxidant and mitochondrial enhancing activities)
Dichloroacetate (maintains pyruvate dehydrogenase activation)
Nicotinamide (supplies electrons for complex I)

Alzheimer's disease

There are decreases in cytochrome oxidase activity in Alzheimer's disease (AD) postmortem tissue[82–84]. Immunostaining for both cytochrome oxidase subunits II and IV is reduced in Purkinje cells in individuals with AD, but the decrease is much more marked for the mtDNA-encoded cytochrome oxidase subunit II than that of subunit IV, which is nuclear DNA encoded[85]. Cybrids made with platelets from individuals with AD are deficient in cytochrome oxidase[17,18]. A recent study, however, was unable to demonstrate electron-transport-chain deficits in cybrid cell lines made from a synaptosomal fraction of autopsied brain tissue or platelets of these individuals[86].

Owing to reduced cytochrome oxidase activity in both postmortem tissue of individuals with AD and cybrid cell lines, attempts were made to determine whether mtDNA mutations in cytochrome-oxidase subunits were associated with AD. An initial report suggested this was the case, but subsequent work established that the putative disease associated polymorphisms were due to a nuclear pseudogene[87–90]. Nuclear pseudogenes are fragments of mtDNA that were incorporated into the nuclear genome long ago. Other reports that suggested specific mtDNA polymorphisms could be associated with AD or AD with PD have been controversial[91–97]. A report of a mutation in the mitochondrial *Nd2* gene associated with AD was not confirmed by two groups[98–100]. However, one individual with a mutation in the gene for the amyloid precursor protein had a G to C mutation at position 5705 in the gene encoding $tRNA^{Asn}$, which might have contributed to an early age of disease onset[101]. Two novel polymorphisms in the 12S rRNA were identified in Japanese individuals with AD (Ref. 94).

If mitochondrial dysfunction has a central role in the pathogenesis of AD, an increase in oxidative damage might be expected to occur as a consequence. Consistent with this possibility, there is a threefold increase in oxidative damage to mtDNA in postmortem tissue from individuals with AD compared with age-matched controls[102], and the levels of numerous other markers of oxidative damage are also increased. The role of mitochondrial dysfunction in AD therefore remains circumstantial, but it is increasingly plausible.

Therapeutic approaches

Recent studies showed that vitamin E and vitamin C reduced the risk for AD (Ref. 103). An additional study showed that individuals with lower levels of β-carotene and vitamin E had reduced performance on the Mattis Dementia Rating Scale[104]. An interventional trial showed that administration of vitamin E to individuals with AD appeared to slow the progression of the disease[105].

There has been considerable interest in the use of coenzyme Q_{10} for treatment of mitochondrial disorders. This compound has been reported to improve ATP generation *in vitro*[106]; it also serves as an important antioxidant in both mitochondrial and lipid membranes[107,108]. Oral administration of coenzyme Q_{10} significantly attenuated lesions produced by intrastriatal administration of malonate and 3-nitropropionic acid (3-NP), and it significantly extended survival in a transgenic mouse model of ALS (Refs 47,109). In addition, a coenzyme Q_{10} analog significantly improved cardiac-mass measurements in individuals with Friedreich's ataxia[110].

Another potential therapeutic strategy for disease with mitochondrial dysfunction is to use creatine to increase brain energy stores and to compensate for an energetic defect. Creatine and phosphocreatine have an important role in brain energy metabolism. PCr shuttles energy from mitochondria to the cytoplasm where it regenerates ATP, which is used by the Na^+–K^+ ATPase to maintain membrane potential[111]. Creatine can also exert neuroprotective effects by stabilizing the mitochondrial transition pore and by increasing glutamate reuptake[112]. It has been shown that creatine supplementation attenuates 3-NP- and MPTP-induced neurotoxicity[113,114]. Finally, administration of creatine extends survival in a dose-dependent manner and significantly protects against loss of anterior horn motoneurons in a transgenic mouse model of ALS (Ref. 115); it also increases survival in transgenic mouse models of HD in a dose-dependent manner[116]. Other agents that can improve energy metabolism are shown in Box 2.

Concluding remarks

There is substantial evidence to implicate impaired energetics in the pathogenesis of neurodegenerative diseases, such as Friedreich's ataxia, Wilson's disease, Hereditary Spastic paraplegia and some forms of dystonia. The evidence in favor of energetic defects in the major neurodegenerative diseases including ALS, HD, PD and AD is more circumstantial. In these diseases, definitive proof will require the identification of genetic defects that can be causally linked to mitochondrial dysfunction and disease pathogenesis. If mitochondrial dysfunction does have a causative role in disease pathogenesis, then a number of therapeutic targets are implicated, including the PTP, cytochrome c release from mitochondria and free-radical scavengers. It might also be possible to buffer energy levels in the brain using coenzyme Q_{10} or creatine. These approaches could result in novel treatments for neurodegenerative diseases.

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