Severe nigrostriatal degeneration without clinical parkinsonism in patients with polymerase gamma mutations

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The role of mitochondria in the pathogenesis of neurodegeneration is an area of intense study. It is known that defects in proteins involved in mitochondrial quality control can cause Parkinson’s disease, and there is increasing evidence linking mitochondrial dysfunction, and particularly mitochondrial DNA abnormalities, to neuronal loss in the substantia nigra. Mutations in the catalytic subunit of polymerase gamma are among the most common causes of mitochondrial disease and owing to its role in mitochondrial DNA homeostasis, polymerase gamma defects are often considered a paradigm for mitochondrial diseases generally. Yet, despite this, parkinsonism is uncommon with polymerase gamma defects. In this study, we investigated structural and functional changes in the substantia nigra of 11 patients with polymerase gamma encephalopathy. We characterized the mitochondrial DNA abnormalities and examined the respiratory chain in neurons of the substantia nigra. We also investigated nigrostriatal integrity and function using a combination of post-mortem and in vivo functional studies with dopamine transporter imaging and positron emission tomography. At the cellular level, dopaminergic nigral neurons of patients with polymerase gamma encephalopathy contained a significantly lower copy number of mitochondrial DNA (depletion) and higher levels of deletions than normal control subjects. A selective and progressive complex I deficiency was seen and this was associated with a severe and progressive loss of the dopaminergic neurons of the pars compacta. Dopamine transporter imaging and positron emission tomography showed that the degree of nigral neuronal loss and nigrostriatal depletion were severe and appeared greater even than that seen in idiopathic Parkinson’s disease. Despite this, however, none of our patients showed any signs of parkinsonism. The additional presence of both thalamic and cerebellar dysfunction in our patients suggested that these may play a role in counteracting the effects of basal ganglia dysfunction and prevent the development of clinical parkinsonism.
Introduction

Parkinsonism is defined clinically by the combination of two or more of the following features: rigidity, bradykinesia, tremor and postural instability. Syndromes in which parkinsonism occurs are aetiologically heterogeneous, but the one feature they all share is dysfunction of the nigrostriatal pathway, most commonly due to loss of the substantia nigra dopaminergic neurons, and this is widely accepted as the cause of the cardinal clinical features (Dickson, 2012).

Parkinson’s disease is the most common and best characterized parkinsonian disorder. While the pathophysiology of Parkinson’s disease is not completely understood, it is thought that loss of the dopaminergic input to the striatum results in secondary changes that ultimately lead to reduced frontal cortical activation and inhibition of volitional movement, increased muscle tone producing rigidity and involuntary movements such as tremor and dystonia. Additional pathological involvement of the nuclei of the noradrenergic, serotonergic and cholinergic systems are believed to contribute to the pathogenesis of the various motor and non-motor manifestations of Parkinson’s disease (Pifl et al., 2012).

The role of mitochondria in the pathogenesis of nigral degeneration and parkinsonism is an area of intense study. Inhibitors of the mitochondrial respiratory chain complex I such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone cause parkinsonism in humans and primates (Singer and Ramsay, 1990; Greenamyre et al., 1999); complex I deficiency has also been described in the substantia nigra of patients with Parkinson’s disease (Schapira et al., 1989) and multiple respiratory chain defects were found in muscle (Bindoff et al., 1991). Familial forms of Parkinson’s disease have been linked to genes encoding mitochondrial proteins such as PINK1 (Valente et al., 2004), DJ1 (Bonifati et al., 2003; now known as PARK7) and parkin (Kitada et al., 1998; now known as PARK2) that play a role in mitochondrial quality control. Moreover, high levels of mitochondrial DNA deletions have been shown in nigral neurons of sporadic Parkinson’s disease (Gu et al., 2002; Bender et al., 2006; Reeve et al., 2008).

Polymerase gamma (POLG) is the enzyme that replicates and repairs mitochondrial DNA (Ropp and Copeland, 1996). Mutations in POLG, the gene encoding the catalytic POLG subunit, are known to cause mitochondrial DNA deletions in neurons (Reeve et al., 2008) and respiratory complex I deficiency (Lax et al., 2012a). Clinically, POLG mutations cause a wide spectrum of disease including myopathy, ataxia, epileptic encephalopathy and liver disease (Van Goethem et al., 2001; Ferrari et al., 2005; Hakonen et al., 2005; Tzoulis et al., 2006). Parkinsonism, including levodopa-responsive Parkinson’s disease, has been described (Cottrell et al., 2000; Luoma et al., 2004; Davidson et al., 2006; Reeve et al., 2012) but is an uncommon manifestation. The substantia nigra of patients with POLG mutations has otherwise not been systematically studied.

In this study, we used histological and in vivo functional imaging techniques to study nigral integrity and nigrostriatal function in 11 patients with mitochondrial encephalopathy caused by POLG mutations. Our findings reveal a severe nigrostriatal degeneration in patients with POLG encephalopathy, but surprisingly, this is not accompanied by clinical parkinsonism.

Materials and methods

Patients

Eleven patients with POLG encephalopathy, five living and six deceased, were included in this study. All patients underwent detailed clinical examination and the clinical and genetic features are summarized in Table 1. The five living patients were investigated with MRI, PET and dopamine transporter (DAT) imaging. Post-mortem material was available from the other six patients.

Ethical considerations

The clinical investigations were deemed quality control by our ethical committee. Informed consent for the autopsy was obtained from the families of patients and control subjects. The tissue study was approved by the Regional Ethical Committee (REK) of Western Norway (no: 2010/23).

Post-mortem material

Formalin fixed, paraffin embedded tissue from patients was available from the mesencephalon (n = 6), pons (n = 3), thalamus (n = 4) and striatum (n = 4). Mesencephalon and pons were examined from two control subjects, two patients with Parkinson’s disease (Patients PD-1 and PD-2) and one patient with advanced Alzheimer’s disease. The demographic information of patients and control subjects is summarized in Tables 1 and 2. Fresh frozen material was available from the substantia nigra of two patients (Patients WS-10A and WS-3A) aged 24 and 43 years, with a disease duration of 8 and 26 years, respectively, and four healthy control subjects with a mean age of 58 ± 11.3 years.

There were no statistically significant differences in post-mortem interval or length of fixation between patient and control tissue. Samples were dissected at autopsy and either snap-frozen immediately in isopentane, which had been cooled in liquid nitrogen, and stored at −80°C, or fixed in formaldehyde and later embedded in paraffin blocks according to standard procedures.

Histology and immunohistochemistry

Routine investigation of formalin-fixed paraffin embedded sections included haematoxylin and eosin, cresyl violet and glial fibrillary acidic protein (GFAP) immunohistochemistry. After deparaffinization, sections were boiled in citrate buffer, pH 6 (Dako) or EDTA buffer, pH 9 (Dako), for 15 min, or treated with proteinase K (Dako) at room temperature for 5 min. For tau immunostaining, the sections were not pretreated. Sections were immunostained on a Ventana or Dako.

Keywords: substantia nigra; POLG; mitochondria; neurodegenerative disease; Parkinson’s disease

Abbreviations: DAT = dopamine transporter; FDG = fluorodeoxyglucose; POLG = polymerase gamma
immunostainer using the Dako EnVision™ kit according to the manufacturer’s instructions. Sections were developed with 3,3′-diaminobenzidine for 1–5 min and then counterstained with haematoxylin.

The following primary antibodies were used: anti-GFAP diluted 1:2000 (Dako), anti-HLA-DR diluted 1:100 (Dako), anti-alpha-synuclein diluted 1:20 (Leica Novocastra), anti-tau diluted 1:1000 (Dako) and anti-cleaved caspase-3 diluted 1:100 (Cell Signaling Technology). The haematoxylin and eosin and immunohistochemical stainings were analysed on a Zeiss light microscope using Zeiss imaging software.

Table 1 Genetic and clinical features of the patients with POLG mutations

<table>
<thead>
<tr>
<th>Subject</th>
<th>Mutation</th>
<th>Age of onset</th>
<th>Age now/at death*</th>
<th>Epilepsy</th>
<th>SLE</th>
<th>Ataxia</th>
<th>PN</th>
<th>PEO</th>
<th>DAT</th>
<th>PET</th>
<th>Pathology</th>
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<tbody>
<tr>
<td>AT-2A</td>
<td>A467T/A467T</td>
<td>16</td>
<td>53*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<td>42</td>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>WS-1A</td>
<td>W748S/W748S</td>
<td>6</td>
<td>41*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>WS-3A</td>
<td>W748S/W748S</td>
<td>17</td>
<td>43*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<td>12</td>
<td>45</td>
<td>−</td>
<td>−</td>
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<td>34</td>
<td>+</td>
<td>+</td>
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<td>−</td>
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<tr>
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<td>28*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>W748S/W748S</td>
<td>16</td>
<td>24*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<td>58</td>
<td>−</td>
<td>−</td>
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<td>+</td>
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<tr>
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<td>A467T/W748S</td>
<td>24</td>
<td>50</td>
<td>−</td>
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<td>+</td>
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<tr>
<td>AL-2A</td>
<td>A467T/G848S</td>
<td>0.6</td>
<td>0.6*</td>
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<td>−</td>
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<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Mean/total 15.6 ± 9.1 38.1 ± 16.1 8/11 7/11 10/11 10/11 9/11 5 4 6

All ages are given in years.
+ = present or investigation performed.
− = feature not present or investigation not performed.
P = peripheral neuropathy; SLE = stroke-like episode; PEO = progressive external ophthalmoplegia.

Table 2 Pathology and immunohistochemistry findings

| Patient | AOD | Substantia nigra Neuronal loss % Complex I deficient neurons % Complex IV deficient neurons Red nucleus Neuronal loss % Complex I deficient neurons Locus coeruleus neuronal loss Thalamus Pathology MRI Striatum Pathology MRI |
|---------|-----|----------------------|-----------------------|-----------------|---------------------|------------------|-------------------|--------------------|-----------------------|---------------------|-------------------|--------------------|
| AT-2A   | 53  | + + +                | 92.1                   | 0                | + + +               | 45.2             | NA                | NA                 | –                     | NA                   | –                 |
| WS-1A   | 41  | + + (+)              | 91.9                   | 1                | + + +               | 100              | NA                | NA                 | +                     | +                   | −                 |
| WS-3A   | 43  | + + +                | 93.2                   | 2.1              | + + +               | 76.3             | NA                | NA                 | +                     | +                   | +                 |
| WS-8A   | 28  | + + +                | 66.7                   | NA               | NA                  | NA               | + + +             | + + +               | −                     | −                   | +                 |
| WS-10A  | 24  | + + +                | 50                     | −                | −                   | −                | +                 | +                  | −                     | −                   | +                 |
| AL-2A   | 0.6 | +                    | 56.9                   | NA               | NA                  | NA               | +                 | +                  | −                     | −                   | −                 |

Mean/total 31.6 6/6 80.2 ± 17.1 0.8 ± 1 3/3 73.8 ± 27.5 3/3 3/4 4/6 2/4 0/6

Mean/total 71.5 2/2 5.3 ± 0.8 2.4 ± 2 0/2 0 2/2

Mean/total 46.5 0/2 0.6 ± 0.8 0 0/2 0 0/2 0/2 0/2 0/2 0/2

The patients are coded as in Table 1.
AOD = age of death.
MRI = presence of lesions detectable by MRI.
Complex deficiencies are given in percentages of cells completely lacking cytoplasmic staining. Mean values given with standard deviation. All ages are given in years.
Changes are graded as severe: + + + , moderate: + + , mild: + or normal: −.
Disease duration was 8 and 15 years for Patients PD-1 and PD-2, respectively and at least 10 years for Patient AD.
Mitochondrial respiratory chain immunohistochemistry

Formalin-fixed paraffin embedded sections (4 μm) of tissue were cut, deparaffinized in Histoclear (Gentaer) and rehydrated in a series of descending ethanol concentrations (100%, 90%, 70%). Antigen retrieval was performed by incubating the sections in 1mM EDTA pH 8 for 15 min. Sections were permeabilized in Tris-buffered saline (TBS) with Tween-20 (TBST) and incubated in primary antibody diluted in TBST at optimal concentration for 1 h at room temperature. Sections were then washed in three changes of TBS and detection was performed with horseradish peroxidase-coupled secondary antibody utilizing a commercial kit (MACH4 Universal AP Polymer Kit, Biocare Medical) according to the manufacturer’s protocol. Finally the sections were counterstained in Mayer haematoxylin solution, and differentiated in Scott tap water. Primary antibodies were used against respiratory complex I 20 kDa subunit (NDUF88) and 30 kDa subunit (NDUFS3), complex II 70 kDa subunit (SDHA), complex III core-2 protein subunit (UQCRCC2), complex-IV subunit CO1 and porin subunit VDAC1. All antibodies were purchased from Abcam.

DNA extraction

Genomic DNA was extracted from homogenate of frozen nigral tissue using a commercial kit (DNA Mini Kit, Qiagen).

Neuron microdissection

Neurons were dissected from frozen post-mortem material and used for mitochondrial DNA analysis. Frozen sections (20 μm) were cut on PEN 1 mm membrane slides (Zeiss) that had been pretreated with UV light (254 nm) for 30 min to lyophilize the membrane for better tissue adhesion, air-dried for 1 h and stained with cresyl violet (Sigma Aldrich) using a standard protocol. Laser microdissection was carried out on a PALM microdissection microscope (Zeiss). The substantia nigra was identified and neurons were collected from the pars compacta. Only cells that could be positively identified as neurons, with a visible nucleus and normal morphological characteristics, were microdissected avoiding glial carry-over. A total of 70 neurons were collected from each patient or control: 60 were collected in four pools of 15 for quantitative mitochondrial DNA studies and 10 were collected individually (a single neuron per sample) for deletion analysis by long-range PCR. Each pool of neurons was collected in 25 μl and each individual neuron in 15 μl lysis buffer (50 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 1% Tween-20, 20 mg/ml proteinase K) and lysed overnight at 56°C. The lysate was used directly for quantitative PCR.

Molecular mitochondrial DNA studies

In substantia nigra homogenate, mitochondrial DNA deletions were detected using long-range PCR to amplify an ~8 kb fragment using primers 8232–8263 and 16496–16465 and conditions: one cycle at 92°C for 2 min and 35 cycles of 92°C for 10 s, 63°C for 30 s and 68°C for 8 min. The PCR products were electrophoresed through a 0.7% agarose gel at 40V for 4 h.

In single neurons, deletions were detected by a nested PCR approach. First we amplified an ~11 kb fragment using primers 5367–5386 and 129–110 and conditions: one cycle at 93°C for 2 min, 10 cycles of 93°C for 10 s, 58°C for 15 s and 68°C for 11 min, followed by 20 cycles at 93°C for 10 s, 58°C for 15 s and 68°C for 11 min plus 5 s per additional cycle and a final extension of 7 min at 68°C. We then used 1 μl of PCR product as template for a second round of amplification with the primers used for the 8 kb fragment amplification and the following conditions: one cycle at 93°C for 2 min, 10 cycles of 93°C for 10 s, 63°C for 15 s and 68°C for 9 min, followed by 20 cycles of 93°C for 10 s, 63°C for 15 s and 68°C for 9 min plus 5 s per additional cycle and a final extension of 7 min at 68°C. The PCR products were electrophoresed through a 0.7% agarose gel at 30 V for 4 h.

Quantification of total and deleted mitochondrial DNA was performed in microdissected neurons by real-time PCR, using TaqMan® fluorogenic probes and a 7500 fast sequence detection system (ABI). Two regions of mitochondrial DNA were amplified, one that is commonly deleted (MT-ND4) and one that is rarely deleted (MT-ND1), and compared with amplification of a single-copy nuclear gene (APP). The sequences of probes and primers are available upon request. The percentage of deletion was calculated using the ND4/ND1 amplification ratio and depletion using the ND1/APP amplification ratio of the patients compared with four control subjects. Amplification of ND1, ND4 and APP was performed in the same well in a triplex reaction and all samples were run in triplicate. Thermal cycling consisted of one

Figure 1 DAT imaging findings. DAT scan showing comparison of tracer uptake in a patient with POLG encephalopathy (A), a patient with Parkinson’s disease (B) and (C) a control subject.
cycle at 95°C for 20 s and 40 cycles at 95°C for 3 s and 60°C for 30 s. Statistical comparison was performed by Mann-Whitney U test.

**Dopamine transporter imaging**

DAT-scan was performed on five patients with POLG encephalopathy and compared with scans from six healthy subjects and six patients with essential tremor and no evidence of parkinsonism (normal control subjects) and 19 patients with clinical Parkinson’s disease. The patients with Parkinson’s disease were chosen randomly based on clinical diagnosis and without reference to results of their DAT-scan. The demographic information of the groups can be found in the Supplementary material.

DAT scintigraphy was performed with 185 MBq 123I ioflupane (DATScan) intravenously after blocking thyroid update with perchlorate. Images were acquired 3 h post-injection on a double-headed Siemens e.cam (LEHR collimator, dynamic SPECT over 3 × 10 min, fixed circle, 120 projections, matrix 128 × 128). For reading, images were reconstructed on the Segami Oasis platform (Segami Corporation, Inc., http://www.segamicorp.com) using the Ordered Subset Expectation maximization algorithm with resolution recovery (OSEM 3D) and Chang’s attenuation correction (coefficient 0.12/cm), re-angulated and summed up into a single 5 cm thick slice through the basal ganglia. For quantification, images were processed on Xeleris 3 (GE Healthcare, http://www.gehealthcare.com). Images were reconstructed by filtered back projection using a 10th order Butterworth filter with a critical frequency of 0.5 and Chang’s attenuation correction and thereafter processed with the fully automatic quantification software ‘DATQuant’. This software performs automatic registration using a template based on ~150 patients from the ENC2DAT trial (Dickson et al., 2012) and calculates specific binding ratios for the caudate nuclei, anterior and posterior putaminal and the entire striatum on each side based on occipital reference regions (Supplementary material). Statistical comparison was performed by Mann-Whitney U test.

**18F-fluorodeoxyglucose positron emission tomography**

PET was performed on four POLG patients using 180 MBq 18F-fluorodeoxyglucose (FDG) produced according to Good Manufacturing Practice at Bergen PET-centre. Patients rested with eyes open in a dimly lit room 30 min before and after the injection. Images were acquired in list mode on a Siemens Biograph 40 PET-CT from 30–60 min post-injection and OSEM-reconstructed using a 336 × 336 matrix with CT-based attenuation correction. Images were processed and visualized on Segami Oasis with automatic coregistrations with volumetric magnetic resonance images of the same patient taken on a General Electric Signa Excite 3 T HDX scanner with 40 mT/m gradients. PET examinations were assessed for areas of abnormal metabolism using the software Neurostat to compare each examination against a normal database of the Department of Nuclear Medicine at Austin Hospital, Melbourne, Australia (Minoshima et al., 1995; Lim et al., 2009).

Furthermore, to detect consistent patterns of altered glucose metabolism, we compared the patients with 10 control subjects investigated for neoplasia (n = 9) and epilepsy (n = 1) as part of clinical routine and who had normal PET scans. Controls comprised six males and four females and had a mean age of 54.4 ± 18.9 years (range 22–78). The analysis was performed using the statistical parametric mapping software (SPM8) (www.fil.ion.ucl.ac.uk/spm) running under MATLAB R2012a (www.mathworks.com). After converting the data from DICOM to NiTi, the data were spatially normalized into the MNI anatomical reference space, provided by a PET template, included in the SPM software, and resliced to cubic voxel size of 2 mm³, and finally smoothed with a 3D Gaussian smoothing kernel with a filter width of 8 mm.

Due to the small number of patients, a non-parametric analysis was performed, using the SnPM8 software (http://go.warwick.ac.uk/tenichols/spm) (Nichols and Holmes, 2002). The analysis was based on 1001 permutations, a variance smoothing (12 mm), and proportional scaling. Further, the analysis was restricted to voxels within the grey matter, as provided by a grey matter probability mask, including all voxels having at least 50% probability of being grey matter. The resulting pseudo t-statistic was explored with an uncorrected threshold of T(pseudo) > 2.33 (Supplementary material).

**Statistical analyses**

Statistical analyses were performed in SPSS (v.20.0.0.1) and Graph Pad Prism (v.6).

**Results**

**Clinical features**

The clinical and genetic features of all patients, living and deceased, are summarized in Table 1. Ten patients had a juvenile or adult onset syndrome of mitochondrial spinocerebellar ataxia and epilepsy. These were either homozygous for the c.1399G > A, p.A467T or c.2243G > C, p.W748S POLG mutations or compound heterozygous in trans (p.A467T/W748S). The patients with mitochondrial spinocerebellar ataxia and epilepsy had a progressive spinocerebellar ataxia with midline and appendicular features, posterior spinal cord dysfunction with loss of proprioception and vibratory sensation and axonal sensorimotor peripheral neuropathy. Eight of the patients had epilepsy with focal and secondary generalized seizures and seven of these had had at least one stroke-like episode with cortical stroke-like lesions showing a predilection for the occipital lobe. Nine patients had ocular myopathy in the form of ptosis and progressive external ophthalmoplegia. One patient (Patient AL-2A) had infantile onset encephalopathy with severe epilepsy and liver involvement (Alper’s disease). He was compound heterozygous for the c.1399G > A, p.A467T and c.2542G > A, p.G848S mutations on different alleles.

All patients were repeatedly evaluated and no features of parkinsonism or other movement disorder were identified; muscle tone was normal and there were no signs of resting tremor, bradykinesia or dystonia. All adult patients had postural instability due to spinocerebellar ataxia.

**Dopamine transporter imaging**

DAT imaging showed severely reduced mean striatal tracer binding in all POLG patients compared with the 12 control subjects (P < 0.0003) with no significant difference between right and left side (data shown in Supplementary material). Patients with Parkinson’s disease had asymmetrically reduced striatal tracer binding compared with control subjects (P < 0.0001). Striatal...
tracer binding in POLG patients was 62% lower than in control subjects in the entire striatum, 65% lower in the anterior putamen, 71% lower in the posterior putamen and 66% lower in the caudate (Table 3). This reduction was even more pronounced than that seen in the patients with Parkinson’s disease, also when compared to their most severely affected side (Table 3). The decrease in striatal tracer binding was significantly more pronounced in the putamen than in the caudate in both the patients with POLG ($P < 0.015$) and Parkinson’s disease ($P < 0.0001$), although this difference was most prominent in Parkinson’s disease. The putamen/caudate ratio was highest in the older POLG patients with the longest disease duration. Patients with Parkinson’s disease showed—in addition—a putaminal gradient with more severe findings in the posterior putamen ($P < 0.0001$), but this difference was not significant in the POLG group ($P = 0.15$).

**18**F-fluorodeoxyglucose-positron emission tomography and magnetic resonance imaging

Individual assessment of the images by visual inspection and analysis in Neurostat software revealed hypometabolism in the thalami, caudate heads, cerebellum and upper brainstem. The statistical non-parametric mapping analysis, which compares all four subjects in the control group ($n = 10$), only confirmed significant bilateral hypometabolism in the thalamus (Supplementary material and Fig. 2).

MRI performed within 1 week of the PET examination showed mild to moderate cerebellar atrophy in all patients. There were no abnormalities of the thalamus, striatum or upper brainstem. Two patients showed symmetrical high T₂ signal and hypertrophy of the inferior olives. One patient had mild, unilateral occipital atrophy after a stroke-like episode several years before.

**Pathology**

The mesencephalon was examined in six patients (five adult patients and one infant), the pons at the level of the locus coeruleus in three, the thalamus and striatum in four. Histological findings were consistently severe in the five adults, but mild in the infant. Severe loss of pigmentation and yellowish discoloration of the nigra was noted on gross inspection. Microscopically, there was severe neuronal loss in all parts of the substantia nigra, including pars compacta, and in the red nucleus. In the pars compacta, neuronal loss appeared more pronounced in the ventrolateral (A9) than the ventromedial tier (Fig. 3). Moderate neuronal loss was seen in the locus coeruleus of the adults whereas it was normal in the infant. Neuronal loss was accompanied by heavy astroglisis and microglial activation, especially within and around the substantia nigra. Remaining neurons in the substantia nigra and locus coeruleus exhibited partial loss of neuromelanin pigmentation. There were no Lewy bodies and no synuclein or tau positivity.

Neuronal loss in the substantia nigra was more pronounced in the adult POLG patients than in the two patients with clinical Parkinson’s disease or the patient with Alzheimer disease (Fig. 3). With the exception of the locus coeruleus, there was normal neuronal density in the pontine nuclei.

In three of the four patients in whom the thalamus was examined, there was neuronal loss accompanied by astro- and microgliosis. The striatum was unremarkable in two of four patients, showed mild neuronal loss and gliosis in one and was normal with the exception of a circumscribed area of neuronal loss and gliosis in the caudate of Patient WS-8A.

Caspase 3 staining of the substantia nigra and pons was negative with the exception of a few scattered caspase 3-positive nuclei, mostly around the substantia nigra, in Patient WS-8A. No definite positivity was seen in the nigral neurons.

**Immunohistochemistry** (Table 2 and Fig. 4) revealed a severe and selective loss of complex I in surviving neurons both in the substantia nigra (80.2 ± 17.1% complex I deficient neurons) and the red nucleus (73.8 ± 27.5%). This was substantially more than in patients with Parkinson’s disease (5.3 ± 0.8%) and Alzheimer’s disease (12.5%), and the healthy control subjects (0.6 ± 0.8%). Of the two control subjects, only the oldest (60 years) had any complex I negative neurons in the nigra (1.1%), whereas none were seen in the youngest (33 years) individual. Complex I deficiency was equally severe in all parts of the substantia nigra including the retrorubral zone and in the neurons of the red nucleus. The percentage of complex I deficient neurons was lowest in the youngest patient and appeared to increase with age and disease duration (Fig. 4). In contrast, only a small number of complex IV deficient neurons were present (1.0 ± 1.1%) in the substantia nigra of the POLG patients. Interestingly, the number of complex IV deficient neurons appeared to be higher in the patients with Parkinson’s disease (2.4 ± 2.4%), but this difference was not statistically significant. Complexes II, III and porin stained all cells.

**Mitochondrial DNA studies**

Studies of mitochondrial DNA in microdissected substantia nigra pars compacta neurons from both patients showed both
Figure 2  FDG-PET findings. (A and B) FDG-PET (left), axial $T_1$ weighted MRI (middle) and fusion images (right). Visual scan inspection showed varying degrees of glucose hypometabolism in the thalamus, head of caudate (A), cerebellum and brainstem (B). (C) Statistical non-parametric mapping results from the group comparison of the four patients against the control group; a threshold of pseudo-$T > 2.33$ was applied. The figure displays the results as maximum intensity projection (left), coronal view (middle), and axial view (right). Patients have consistently and significantly decreased thalamic metabolism compared with control subjects.
quantitative loss (mitochondrial DNA depletion) and evidence of mitochondrial DNA deletions. Pooled patient neurons contained \( \sim 50-60\% \) less mitochondrial DNA than control neurons and the difference was highly significant \( (P = 0.0006) \). The comparison of relative mitochondrial DNA copy number in patient and control neurons is shown in Fig. 5.

Deletions of mitochondrial DNA were detectable by long-range PCR in nigral homogenate and single nigral neurons in both patients and control subjects (Fig. 6). Deletions appeared as smears on homogenate PCR. Single nigral neurons contained one or several deleted species. Quantification of deleted mitochondrial DNA by quantitative PCR in pools of 10 microdissected nigral neurons showed that the patients had on average \( \sim 40\% \) more deleted mitochondrial DNA than the control subjects. Quantification in single microdissected neurons showed that the level of deletion in individual patient neurons varied between 10–60\% higher than in control subjects (data not shown).

Discussion

Our findings show that POLG mutations cause damage to mitochondrial DNA, which in turn leads to a respiratory chain dysfunction that primarily affects complex I. At the cellular and organism level, these changes are associated with severe neuronal degeneration of the substantia nigra and significant loss of the nigrostriatal projections. The degree of substantia nigra neuronal loss is severe and appears greater even than that seen in idiopathic Parkinson’s disease. Despite this, however, none of our patients showed any of the clinical signs usually expected with such a degree of substantia nigra involvement.

Similar to Parkinson’s disease, nigral neuronal loss in our patients appears greater in the ventrolateral tier of the pars compacta projecting to the posterior putamen and this is reflected in the pattern of nigrostriatal depletion, being more severe initially in the putamen than in the caudate nucleus, but becoming global later. Nigral loss also appears progressive in POLG disease with the least pronounced histological and imaging findings seen in the youngest patients. Unlike degenerative parkinsonism, however, there is no deposition of \( \alpha \)-synuclein or tau, suggesting a different pathogenic process.

The absence of apoptotic changes and of caspase immunoreactivity (with the exception of mild, extranigral findings in one case) suggests that the principal mechanism of nigral neuronal death in POLG disease is non-apoptotic. Apoptosis is an energy requiring process and thus not favoured in states of severe ATP deficiency (Greijer and van der Wall, 2004). In addition, it appears that cells in different parts of the nervous system respond to energy failure differently. Cyanide intoxication in mice was shown to give predominantly apoptosis in the cortex and necrosis in the substantia nigra (Mills et al., 1999). This propensity for nigral neurons to undergo necrosis compared with other neuronal populations could explain the lack of apoptotic markers in our patients.

Our studies in microdissected nigral neurons show that both depletion and multiple deletions of mitochondrial DNA are present and that deletions are found at significantly higher levels than in control substantia nigra neurons. Multiple mitochondrial DNA deletions have been reported in brain and muscle of patients with POLG encephalopathy (Hakonen et al., 2008) and in nigral

Figure 3  Substantia nigra histology. Haematoxylin and eosin staining of the ventrolateral tier of the substantia nigra in POLG encephalopathy (A), Parkinson’s disease (B) and control subjects (C). The POLG patient shows the most severe neuronal depletion. Lewy bodies can be seen in some of the surviving neurons in the Parkinson patient (arrows), but not in POLG. Magnification is \( \times 100 \) in the upper panel and \( \times 400 \) in the lower panel.
neurons of a POLG patient with clinical features of parkinsonism (Reeve et al., 2008). Interestingly, mitochondrial DNA deletions have also been found at similar levels in the substantia nigra of patients with Parkinson’s disease (Bender et al., 2006; Reeve et al., 2008) and appear to accumulate in the substantia nigra with age (Bender et al., 2006; Kraytsberg et al., 2006; Reeve et al., 2008). Mitochondrial DNA depletion of the same order as we describe was found in microdissected dorsal root ganglia neurons from a patient with POLG disease (Lax et al., 2012b). Our study is, however, the first and most comprehensive description of the molecular findings in the substantia nigra.

Significant numbers of complex I deficient neurons were found in the substantia nigra and red nucleus in all of our patients. The degree of complex I deficiency was less pronounced in the younger patients, but still present in high levels (~60%) even in the substantia nigra of the infant who had only mild changes in neuronal density. These findings suggest that complex I loss is an early and progressive event that precedes neuronal loss. The reason for the selective complex I defect is unclear. This complex contains the highest number (seven) of mitochondrially encoded subunits and five of these are located within the part of mitochondrial DNA that is most commonly affected by deletion. Other studies have shown that neuronal metabolism is highly dependent on complex I activity and that this complex is functioning close to its maximum capacity in the nervous system (Kann et al., 2011). Complex I may be, therefore, the most vulnerable site in the CNS to defects interfering with mitochondrial DNA homeostasis.

Although we show that parkinsonism is not a necessary corollary of substantia nigra neuronal loss, L-DOPA responsive extrapyramidal disease, with pathological changes in the substantia nigra...
nigra and/or nigrostriatal depletion measured in vivo by imaging techniques, has been described in a small number of patients with POLG (Cottrell et al., 2000; Luoma et al., 2004; Davidson et al., 2006; Martinez and Greenamyre, 2012; Reeve et al., 2012) and twinkle (now known as C10orf2) mutations (Vandenberghe et al., 2009). Paradoxically, POLG-related disease is perhaps the most common mitochondrial disease currently known, yet clinical parkinsonism is an uncommon manifestation. In patients with clinical parkinsonism, substantia nigra neuronal loss is expected. In contrast, our studies are the first to look at nigral structure and function in POLG encephalopathy in an unselected group of patients with no clinical parkinsonism and all 11 of them showed severe nigral depletion. These data show that nigral degeneration is a common, possibly universal phenomenon in POLG encephalopathy. How then can we explain the lack of clinical correlate?

One possible explanation is compensation by concurrent alterations or lesions in other neuroanatomical structures and pathways. Degeneration of the cerebellar dentato-olivary system is common in patients with POLG encephalopathy (Tzoulis et al., 2010; Lax et al., 2012a). Nigral degeneration without parkinsonism has been described in other disorders in which cerebellar involvement occurs including the dominant spinocerebellar ataxias SCA3 and SCA2 (especially the latter), (Bouchard et al., 1979; Varrone et al., 2004; Kozlov et al., 2011) and multiple system atrophy type C (MSA-C) (Purcell et al., 2007). Moreover, ipsilateral improvement of rigidity has been reported in a patient with parkinsonism after a cerebellar stroke (Erichsen et al., 2009) and surgical dentatectomy has been reported to produce similar results (Toth, 1961). Our findings, together with these observations, suggest that dysfunction of the cerebellum and/or its connections can modulate the function of the basal ganglia and may improve clinical parkinsonism; especially rigidity.

Thalamic inhibition, either lesional or by deep brain stimulation, has been shown to improve tremor in Parkinson’s disease (Machado et al., 2006). Thalamic involvement is common in POLG encephalopathy (Tzoulis et al., 2010) and our current FDG-PET findings show that thalamic pathology and hypometabolism are present even in the absence of detectable lesions on MRI. Thus, it is possible that thalamic inhibition also modulates the effect of nigrostriatal depletion to the cortex. Based on current knowledge this could potentially explain the absence of tremor, but is not sufficient to explain the complete absence of parkinsonism. Other potential sites include the subthalamic nucleus and globus pallidus internus. Inhibition of these structures can ameliorate positive and negative parkinsonian manifestations. The globus pallidus was normal or only mildly affected in our patients and the subthalamic nucleus was not available for study.

Although it is not possible to provide a comprehensive explanation for the lack of parkinsonian features in our patients, our findings suggest that disruption of other motor circuits can moderate the effects of nigrostriatal depletion. What is nevertheless clear from our findings is that severe nigrostriatal degeneration can occur without the clinical correlate of parkinsonism. This
raises fundamental questions about our current understanding of the pathophysiological model of parkinsonism and suggests that other and yet unknown mechanisms may contribute to the generation of the parkinsonian syndrome.

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Supplementary material

Supplementary material is available at Brain online.

References


