Mesencephalic complex I deficiency does not correlate with parkinsonism in mitochondrial DNA maintenance disorders

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Genetic evidence from recessively inherited Parkinson’s disease has indicated a clear causative role for mitochondrial dysfunction in Parkinson’s disease. This role has long been discussed based on findings that toxic inhibition of mitochondrial respiratory complex I caused parkinsonism and that tissues of patients with Parkinson’s disease show complex I deficiency. Disorders of mitochondrial DNA maintenance are a common cause of inherited neurodegenerative disorders, and lead to mitochondrial DNA deletions or depletion and respiratory chain defect, including complex I deficiency. However, parkinsonism associates typically with defects of catalytic domain of mitochondrial DNA polymerase gamma. Surprisingly, however, not all mutations affecting DNA polymerase gamma manifest as parkinsonism, but, for example, spacer region mutations lead to spinocerebellar ataxia and/or severe epilepsy. Furthermore, defective Twinkle helicase, a close functional companion of DNA polymerase gamma in mitochondrial DNA replication, results in infantile-onset spinocerebellar ataxia, epilepsy or adult-onset mitochondrial myopathy, but not typically parkinsonism. Here we sought for clues for this specificity in the neurological manifestations of mitochondrial DNA maintenance disorders by studying mesencephalic neuropathology of patients with DNA polymerase gamma or Twinkle defects, with or without parkinsonism. We show here that all patients with mitochondrial DNA maintenance disorders had neuronopathy in substantia nigra, most severe in DNA polymerase gamma-associated parkinsonism. The oculomotor nucleus was also affected, but less severely. In substantia nigra, all patients had a considerable decrease of respiratory chain complex I, but other respiratory chain enzymes were not affected. Complex I deficiency did not correlate with parkinsonism, age, affected gene or inheritance. We conclude that the cell number in substantia nigra correlated well with parkinsonism in DNA polymerase gamma and Twinkle defects. However, complex I defect is a general consequence of mitochondrial DNA maintenance defects, and does not explain manifestation of parkinsonism or degree of mesencephalic cell death in patients with mitochondrial DNA maintenance disorders.

Keywords: parkinsonism; mitochondrial disorders; mitochondrial dysfunction; mitochondrial DNA; ataxia

Abbreviations: CI/II/III/IV = respiratory chain complex I/II/III/IV; IOSCA = infantile onset spinocerebellar ataxia; MIRAS = mitochondrial recessive ataxia syndrome

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Introduction

Parkinson’s disease is a common neurodegenerative disease affecting ~0.3% of the population in Western countries. In idiopathic Parkinson’s disease the typical symptoms include tremor, rigidity and bradykinesia, which form the clinical triad required for the diagnosis. The age of onset is in the 60s and symptoms are typically unilateral. The neuropathological hallmarks of the disease are the loss of dopaminergic neurons, as well as Lewy bodies, protein aggregates, which accumulate first in locus coeruleus, Raphe nucleus and dorsal motor nucleus of vagus, then substantia nigra and later pervade most regions of the brain (Braak et al., 2004). The genetic background of Parkinson’s disease is still mostly unknown, although recent breakthroughs have revealed multiple disease and risk loci (Simon-Sanchez et al., 2009; Saad et al., 2011; Lil et al., 2012).

A minor group of Parkinson’s disease is hereditary, first identified in a large Italian pedigree with SNCA mutations (Polymeropoulos et al., 1997). At present 18 different loci, with different phenotypes ranging from early onset recessive to dominant Parkinson’s disease, have been identified (Gasser et al., 2011). In autosomal recessive juvenile Parkinson’s disease the disease onset is typically in the early 30s to 40s; the symptoms are bilateral with resting and postural tremor, rigidity in all limbs and the disease progresses slower than in idiopathic Parkinson’s disease (Lücking et al., 2000; Hague et al., 2003; Khan et al., 2003; Ibañez et al., 2006; Guo et al., 2008). The neuropathology also differs from the idiopathic disease: Lewy bodies are rarely found but the pigment neuron loss and gliosis is clear in substantia nigra (Mori et al., 1998; van de Warrenburg et al., 2001; Samaranch et al., 2010). Understanding disease mechanisms in these rare monogenic Parkinson’s disease forms has provided valuable insight into the overall pathogenesis of Parkinson’s disease.

The majority of defective proteins underlying hereditary Parkinson’s disease participate in mitochondrial functions or quality control, making mitochondrial dysfunction a common denominator for recessive Parkinson’s disease (parkin, DJ1, PINK1, POLG) (Lücking et al., 2000; Bonifati et al., 2003; Hague et al., 2003; Khan et al., 2003; Luoma et al., 2004; Ibañez et al., 2006; Guo et al., 2008). For decades, the mitochondrial contribution has been attributed to mitochondrial respiratory complex I (CI) deficiency. Toxic inhibition of CI results in parkinsonism and has been used as a classic model for experimental-induced dopaminergic neuronal death (MPTP, rotenone) (Langston and Ballard, 1983; Langston et al., 1983; Betarbet et al., 2000). Furthermore, CI deficiency in substantia nigra as well as in platelets, has been reported in patients suffering from idiopathic Parkinson’s disease (Bindoff et al., 1989; Schapira et al., 1989; Krige et al., 1992; Haas et al., 1995). Most mitochondrial disorders with CI deficiency, however, do not result in parkinsonism, leaving the mechanistic link of CI deficiency and Parkinson’s disease open.

The minimal mitochondrial DNA replisome consists of DNA polymerase (POLG), helicase Twinkle (encoded by C10orf2) and the mitochondrial single stranded DNA binding protein (encoded by SSBP1) (Korhonen et al., 2004). Mutations in POLG and C10orf2 cause autosomal recessive and dominant progressive external ophthalmoplegia, but the clinical phenotype of the patients shows specificity depending on the nature of the mutation in a protein. Dominant POLG and Twinkle mutations both result in ptosis, progressive weakness of extraocular muscles, and mitochondrial myopathy, but additionally the dominant POLG mutations also result in parkinsonism, starting after 30 years of age, and premature menopause (Suomalainen et al., 1992, 1997; Speibrink et al., 2001; Van Goethem et al., 2001; Luoma et al., 2004). Clinically POLG–Parkinson’s disease resembles autosomal recessive juvenile Parkinson’s disease; the onset of parkinsonism is earlier than in idiopathic Parkinson’s disease. The patients exhibit bradykinesia, rigidity, resting tremor and they respond well to levodopa treatment (Luoma et al., 2004). The neuropathology of POLG–Parkinson’s disease also resembles autosomal recessive juvenile Parkinson’s disease; Lewy bodies are rarely seen, but substantia nigra is severely affected (Moslemi et al., 1999; Betts-Henderson et al., 2009). Twenty per cent of surviving neurons in substantia nigra were reported to be COX-deficient (Betts-Henderson et al., 2009). Recessive POLG and Twinkle mutations lead to spinocerebellar ataxia, mitochondrial recessive ataxia syndrome (MIRAS), infantile-onset spinocerebellar ataxia (IOSCA) and sensory polyneuropathy, associated with severe epilepsy, but not to parkinsonism (Koskinen et al., 1994a; Rantamäki et al., 2001; Hakonen et al., 2005; Nikali et al., 2005). For an unknown reason, therefore, parkinsonism seems to be a specific feature of POLG disease.

We searched for the cause of the variability in disease manifestation of mitochondrial DNA maintenance disorders, with special focus on the question why some disorders manifest with parkinsonism and some not. We report here neuropathological findings of mesencephalon of six patients with dominant or recessive POLG and Twinkle disorders manifesting as mitochondrial myopathy and/or ataxia, with or without parkinsonism.

Materials and methods

The study was approved by Helsinki University Central Hospital institutional ethics review board. Written consent was acquired from all patients or their relatives and the study was carried out according to the Declaration of Helsinki.

Patients

Table 1 summarizes the clinical findings of the patients.

Patient P1: The clinical features of this patient have been summarized previously in brief [Family V, II-6 in Luoma et al. (2004)]. Patient P1 is the sixth child of 11. Two of her brothers had progressive external ophthalmoplegia and parkinsonism, resembling Patient P1’s symptoms. Bilateral ptosis and progressive external ophthalmoplegia were diagnosed when she was 26. At the age of 31 she started to experience weakness of lower limbs. Her symptoms developed into hypomimia and sensoric neuropathy. At the age of 46 her parkinsonism was diagnosed, based on rigidity and lack of associated movements. Combination of levodopa and carbidopa was started, with some clinical improvement. At the age of 52, percutaneous endoscopic gastrostomy was performed because of difficulty in swallowing. She also had difficulty of initiation of movements, and started to experience occasional off-phases. Knee-heel test was abnormal and...
| Patient | Disease | Amino acid change | Age of onset | Presenting symptom | Age and cause of death | Parkinsonism age of onset | Parkinsonism | Ophthalmoplegia | Ataxia and epilepsy with seizures | Serum copper | Urine copper | Creatine kinase | Lactate dehydrogenase | Dopamine transporter activity in nucleus caudatus | Duodopa | derby | Hypothyroidism |
|---------|---------|------------------|-------------|-------------------|-----------------------|-------------------------|----------------|----------------|---------------------------------|-------------|-------------|----------------|-----------------|--------------------------------|
| P1      | POLG-PD | POLG recessive compound heterozygote p.3468D | 26          | Ptosis            | 61, pneumonia         | 26                      | Y.26          | Y.26           | Y.26                            | 0.72         | 0.58         | 336 U/l        | 0.58             | c.1402A→G and c.3316G→A mutations in POLG, resulting in p.Asn468Asp and p.Ala1105Thr amino acid changes in POLG, respectively. The same mutation combination was also found in other affected members of the family. |
| P2      | POLG-PD | POLG recessive homozygote p.961G to A | 33          | Ptosis, ataxia, dysarthria, dysphagia | 62, cardiac arrest     | Y.26                     | Y.26          | Y.26           | Y.26                            | 0.72         | 0.58         | 336 U/l        | 0.58             | c.1402A→G and c.3316G→A mutations in POLG, resulting in p.Asn468Asp and p.Ala1105Thr amino acid changes in POLG, respectively. The same mutation combination was also found in other affected members of the family. |
| P3      | MIRAS   | POLG recessive homozygote p.1105G to A | 35          | Ophthalmoplegia, ptosis, ptosis | 56, pneumonia         | Y.26                     | Y.26          | Y.26           | Y.26                            | 0.72         | 0.58         | 336 U/l        | 0.58             | c.1402A→G and c.3316G→A mutations in POLG, resulting in p.Asn468Asp and p.Ala1105Thr amino acid changes in POLG, respectively. The same mutation combination was also found in other affected members of the family. |
| P4      | MIRAS   | POLG recessive homozygote p.1105G to A | 31          | Ptosis, ataxia, dysarthria, dysphagia | 52, ptosis, dementia | Y.26                     | Y.26          | Y.26           | Y.26                            | 0.72         | 0.58         | 336 U/l        | 0.58             | c.1402A→G and c.3316G→A mutations in POLG, resulting in p.Asn468Asp and p.Ala1105Thr amino acid changes in POLG, respectively. The same mutation combination was also found in other affected members of the family. |
| P5      | adPEO   | POLG recessive homozygote p.1105G to A | 19          | Ptosis, ataxia, dysarthria, dysphagia | 60, vestibular fibrillation | Y.26                     | Y.26          | Y.26           | Y.26                            | 0.72         | 0.58         | 336 U/l        | 0.58             | c.1402A→G and c.3316G→A mutations in POLG, resulting in p.Asn468Asp and p.Ala1105Thr amino acid changes in POLG, respectively. The same mutation combination was also found in other affected members of the family. |
| P6      | ICSSCA  | POLG recessive homozygote p.1105G to A | 20 months | Ptosis, ataxia, dysarthria, dysphagia | 26, epileptic seizure | Y.26                     | Y.26          | Y.26           | Y.26                            | 0.72         | 0.58         | 336 U/l        | 0.58             | c.1402A→G and c.3316G→A mutations in POLG, resulting in p.Asn468Asp and p.Ala1105Thr amino acid changes in POLG, respectively. The same mutation combination was also found in other affected members of the family. |
resulting in p.Trp748Ser + p.Glu1143Gly amino acid changes, diagnostic for MIRAS.

Patient P5: The clinical features and the autopsy findings of this woman have been described previously [Patient II-6 in Suomalainen et al. (1992, 1997)] and are summarized in Tables 1 and 2. The patient suffered from autosomal dominant progressive external ophthalmoplegia, caused by heterozygous in-frame duplication of 39 base pairs (c.1053_1092dup) mutation in C70orf2, resulting in duplication of 13 amino acids in Twinkle linker region (p.Asn352_Trp364dup). She showed no signs of parkinsonism.

Patient P6: The clinical features and general neuropathology of the patient have been described previously [Patient 1 in Lönnqvist et al. (1998)], and are summarized in Tables 1 and 2. She had typical infantile onset spinocerebellar ataxia (IOSCA). No signs of parkinsonism was noticed at any point. She was homozygous for c.1523 A>G mutation in C70orf2, leading to p.Tyr508Cys amino acid change in Twinkle.

As a healthy control we used samples from a 65-year-old female and a 41-year-old male who had no history of neurological diseases and who had died in an accident.

Morphological studies and neuropathological examination

The autopsy examinations were performed 4–72 h post-mortem, according to standard procedures, including neuropathological examination by one neuropathologist (A.P.). The neuropathological samples were studied after fixation in formalin. After macroscopic dissection, paraffin-embedded sections were sectioned at 4–8 μm and stained using haematoxylin and eosin. To study the mesencephalon regarding substantia nigra and the oculomotor nucleus complex, sections from the level of the upper corpora quadrigemina were chosen. Immunohistochemistry was performed for the 4 subunits of respiratory chain complexes using sensitive polymer-based detection system, using Lab Vision 480 Autostainer™ (Thermo Fisher Scientific). To unmask the antigens we used citrate buffer (pH 6.0) for p62, citrate buffer combined with formic acid for alpha-synuclein and enzymatic trypsin digestion for tau. Endogenous peroxidase activity was blocked by REAL™ Peroxidase-Blocking Solution (Dako, S2023). Primary antibody dilutions, alpha synuclein 1:2000 (clone 42, BD Biosciences), p62 1:1000 (clone D-3) and Tau 1:800 (clone AT8, Innogenetics), were incubated for 30 min at room temperature. Incubation time for polymer-based, horseradish-labelled, secondary antibody (Dako, K5007) was 30 min at room temperature. After incubation with secondary antibody, the reaction was visualized with DAB (3,3′-diaminobenzidine tetrahydrochloride). The sections were counterstained with Mayer’s haematoxylin (Dako, S3301) and mounted with Mountex™ (Histolab). Immunohistochemistry against respiratory chain components were done as previously described (Hakonen et al., 2008), using the following monoclonal antibodies from MitoSciences: Complex I: NDUF53 (#MS110) and NDUF9 (#MS111). Complex II: 70 kDa Fp subunit (#MS204). Complex III: UQRC2 (#MS304). Complex IV: COXII (#MS405). We also used polyclonal antibodies against tyrosine hydroxylase (NB300-109, Novus Biologicals), ND1 and AFG3L2 (kind gifts from Drs Anne Lombes and Thomas Langer, respectively). All antibodies were diluted to 10 μg/ml using DAKO REAL™ antibody diluent (Dako S3022), except 7 μg/ml for Complex III, 1:1000 for ND1 and AFG3L2 and 1:2500 for tyrosine hydroxylase.

We counted the pigment neurons of substantia nigra, pars compacta, from patients and age-matched controls, using an ocular square grid. Cells with intact nucleus, including pyknotic cells, were counted, excluding so-called ghost cells, which consist mainly of extra-neuronal pigment. In addition to the age-matched controls, we counted the pigment neurons of substantia nigra, pars compacta, from a 13-year-old control subject, to account for possible age-associated changes in the total neuronal amounts. The total number of pigment neurons in our controls was comparable with the current knowledge in the literature (~650 for adults, ~750 for the 13-year-old control subject per mesencephalic section) (Feamley and Lees, 1991).

Results

Neuropathological findings in mesencephalon and nigrostriatal pathway

Table 2 summarizes the general neuropathological findings in CNS of the patients.

First we established whether mesencephalic findings were consistent with clinical manifestations of the patients. Patients P1 and P2 both exhibited levodopa-responsive parkinsonism (POLG-Parkinson’s disease), not present in other patients (Table 1). Both of these patients with POLG-Parkinson’s disease had strong neurodegeneration of mesencephalon with subtotal neuronal loss in substantia nigra (Figs 1, 2A, 3A and B). No alpha-synuclein- or p62-positive Lewy bodies or tau-positive neurites, typical for Parkinson’s disease or Alzheimer’s disease, were present in any of the patients, including those with POLG-Parkinson’s disease (Fig. 2B). Patients with MIRAS, Twinkle-progressive external ophthalmoplegia and IOSCA had a moderate loss of pigmented neurons in substantia nigra, especially in its lateral and dorsal area (Figs 1, 2C–E and 3C–E). In all patients, we quantified preferentially neuronal nuclei, i.e. grey matter. The white matter changes in the patients showed some secondary axonal loss and mild gliosis.

The oculomotor nucleus complex showed considerable neuronal loss in patients with POLG-Parkinson’s disease, but only slightly decreased neuronal number was present in dominant Twinkle-progressive external ophthalmoplegia, and no decrease was seen in patients with MIRAS or IOSCA (Fig. 4A–E). We examined also other components of the nigrostriatal pathway to rule out other possible causes for parkinsonism. In patients with POLG-Parkinson’s disease, the lenticular and anterior striatal samples had only a minor loss and pyknosis of larger neurons in corpus striatum, most likely representing secondary trans-synaptic degeneration.

We found corpora amylacea, spherical structures developing upon neurodegeneration and during ageing, in all samples, but this change was especially pronounced in POLG Patients P1, P2 and P3. In Patients P1 and P2, the largest concentration of corpora amylacea was in the vicinity of the substantia nigra and aqueduct, and under the ependyma close to dorsal CSF space. Patient P3 had a very large amount of corpora amylacea in the rostral linear nucleus (Supplementary Fig. 1A and B). Corpora amylacea were detected by their likely unspecific reactivity to polyclonal antibodies (ND1, AFG3L2).
Complex I immunoreactivity is severely reduced in all patients with POLG and Twinkle defects

Mitochondrial respiratory complex I deficiency has been specifically associated with Parkinson’s disease, therefore, we used our patient material with a known genetic defect in mitochondrial DNA maintenance proteins POLG or Twinkle, with or without parkinsonism, to study whether the type of respiratory complex deficiency explained the differential clinical manifestations of the patients. POLG and Twinkle both are required for mitochondrial DNA maintenance and have been shown to lead to either multiple mitochondrial DNA deletions or mitochondrial DNA depletion in the brain, which in turn could affect mitochondrial DNA-encoded complex I, III and IV (CI, III and IV, respectively), but not nuclear-encoded complex II (CII) (Suomalainen et al., 1992, 1997; Spelbrink et al., 2001; Van Goethem et al., 2001). We examined the CI, CII, CIII and CIV immunoreactivity in the six autopsy-derived mesencephalic samples of our dominant and recessive POLG and Twinkle mutant patients and an age-matched control. All the POLG and Twinkle-mutant patients had almost no immunoreactivity for CI in the pigment neurons of substantia nigra (Fig. 5A, E, I, M, Q, U and Table 3). This change was apparent and uniform with all three independent CI antibodies, indicating almost complete absence of the protein in pigment neurons of substantia nigra of our patients. In addition, the substantia nigra neuropil and mesencephalic neuropil in general had minor loss of immunoreactivity against CI. Both patients with POLG-Parkinson’s disease had intense positive CII immunoreactivity, suggesting accumulation of mitochondria or upregulation of CII protein amount in these neurons (Fig. 5B, F and Table 3), but CIII and CIV amounts were similar to controls (CIV: Fig. 5 and Table 3). To study whether CI deficiency was associated with active proteolysis by mitochondrial matrix-facing AAA-protease, we analysed the amount of this protease in all patient samples (Hornig-Do et al., 2012). AFG3L2 immunoreactivity was present in the neurons of substantia nigra and oculomotor nucleus of all patients, being somewhat more intense in patients with MIRAS and POLG-Parkinson’s disease, but the significance of this finding remains to be studied (Supplementary Fig. 2).
The respiratory chain complex deficiency in oculomotor nucleus

Whereas CI deficiency was a clear uniform finding in substantia nigra of all our patients, this was not the case in the oculomotor nucleus, which was analysed from the same sections as substantia nigra. Only three of six patients had a clear reduction in the CI immunoreactivity in the oculomotor nucleus (Patient P1, POLG-Parkinson’s disease; Patient P5, Twinkle-autosomal dominant progressive external ophthalmoplegia; Patient P6, IOSCA) (Fig. 6A, U and Table 3). A small increase of CII in the oculomotor nucleus was present in one patient with POLG-Parkinson’s disease, Patient P1 (Fig. 6B and Table 3). Other patients did not have any changes in CII immunoreactivity (Fig. 6F, J, N, R and V). CIII and CIV amounts were not changed (Fig. 6 and Table 3). AFG3L2 immunoreactivity was present also in the oculomotor nucleus, with no apparent differences between samples (Supplementary Fig. 3).
Multiple causes of mitochondrial dysfunction have been linked to Parkinson’s disease, the strongest evidence currently available from identification of genetic causes of Parkinson’s disease. These include defects of DJ1, PINK1 and Parkin, which are suggested to contribute to mitochondrial quality control, as well as POLG, the mitochondrial replicative polymerase (Kitada et al., 1998; Mori et al., 1998; Lücking et al., 2000; van de Warrenburg et al., 2001; Bonifati et al., 2003; Dekker et al., 2003; Luoma et al., 2004; Valente et al., 2004; Albanese et al., 2005; Ibáñez et al., 2006). Furthermore, substantia nigra of patients

**Discussion**

Multiple causes of mitochondrial dysfunction have been linked to Parkinson’s disease, the strongest evidence currently available from identification of genetic causes of Parkinson’s disease. These include defects of DJ1, PINK1 and Parkin, which are
with Parkinson’s disease has been suggested to accumulate mitochondrial DNA deletions more than controls of the same age (Bender et al., 2006; Kraytsberg et al., 2006), and an extensive literature from the last 40 years has discussed the contribution of respiratory chain complex I inhibitors and CI deficiency as an underlying mechanistic cause for Parkinson’s disease. In this study, we used a unique autopsy material, with mitochondrial disease patients with or without parkinsonism, who suffered from mitochondrial DNA maintenance disorders due to POLG or Twinkle deficiencies, two necessary proteins at the mitochondrial DNA replication fork. These nuclear gene defects cause secondary accumulation of mitochondrial DNA mutations, and sometimes mitochondrial DNA depletion, in post-mitotic tissues of the patients, including neurons (Suomalainen et al., 1992; Hakonen et al., 2008). However, only patients with catalytic defects of POLG developed parkinsonism, raising the question whether

Figure 4  Oculomotor nucleus shows neurodegenerative changes in patients with progressive external ophthalmoplegia. Thin arrows point to the region of oculomotor nucleus. (A) Patient P2, POLG-Parkinson’s disease. The amount of neurons is reduced in oculomotor nucleus and their appearance is damaged (thick arrow). The Edinger-Westphal nucleus shows spongiform degeneration and lacks neurons (asterisk). (B) Patient P3, POLG MIRAS. No degenerative changes in neurons in oculomotor nucleus (asterisk). (C) Patient P4, POLG MIRAS. No degenerative changes in neurons (thick arrow). (D) Patient P5, Twinkle-autosomal dominant progressive external ophthalmoplegia. A slight to moderate loss of neurons in the oculomotor complex. (E) Patient P6, Twinkle IOSCA. The amount of neurons in oculomotor nucleus is not decisively reduced (arrow). (F) Control subject with normal oculomotor nucleus. (A, D and F) haematoxylin and eosin × 40. (B) SMI32 immunostaining × 40. (C and E) Haematoxylin and eosin × 100. Scale bar = 500 μm (A, B, D and F) and 200 μm (C and E).
Respiratory chain immunoreactivity in substantia nigra shows widespread Complex I deficiency in all patients with mitochondrial DNA maintenance disorders. Patients P1 and P2, POLG-Parkinson's disease; Patients P3 and P4, MIRAS; Patient P5, Twinkle autosomal dominant progressive external ophthalmoplegia; Patient P6, Twinkle IOSCA; C = Healthy control. All patients (A, E, I, M, Q and U) have a clear lack of immunoreactivity against Complex I (CI; brown colour indicates positive reactivity, blue counterstaining reveals absence of complex; the area of cytoplasm with mitochondria circled with dotted line; pigment indicated with asterisk), when compared with negative IgG controls (D, H, L, P, T and X) and healthy control (Y). Complex II (CII) immunoreactivity is mildly increased in Patient P1 and Patient P2 (B and F). CIV enzyme complex is well preserved in all patients (C, G, K, O, S and W). Antibodies used: CI, NDUFS3; CII 70 kDa subunit; CIV, COX2 subunit; 0, IgG replacing primary antibody as negative control. Magnification × 40; scale bar = 50 μm.
differences in respiratory chain deficiency could explain the variable CNS manifestations. We examined the mesencephalic morphology and respiratory chain phenotype in our patient material with recessive and dominant POLG and Twinkle defects. Despite differences in clinical features, all of our patients, with or without parkinsonism, exhibited a drastic lack of CI immunoreactivity, in the pigment neurons of substantia nigra. This finding strongly suggests that neuronal CI deficiency is not a primary driving cause of parkinsonism.

The respiratory chain enzyme defect in our patients was specific to CI. An important note is that our patient with autosomal dominant progressive external ophthalmoplegia showed similar CI deficiency as patients with POLG-Parkinson’s disease, although her disease was myopathy, with the only prominent symptom from CNS being major depression (Suomalainen et al. 1992). Her basal ganglia have been shown to harbour a major amount of deleted mitochondrial DNA, up to 60% of total mitochondrial DNA (Suomalainen et al. 1992, 1997). These observations strongly suggest that CI deficiency or accumulation of mitochondrial DNA deletions cannot explain Parkinson’s disease. Respiratory chain complexes have been previously analysed in the cerebellum and frontal cortex of MIRAS and IOSCA, and both showed lack of CI immunoreactivity in the Purkinje cells of cerebellum and large neurons of frontal cortex (Hakonen et al., 2008). In our patients, CII hyperreactivity was found in both substantia nigra and oculomotor nucleus of patients with POLG-Parkinson’s disease, raising a question whether substrates entering respiratory chain at CII are favoured by these neurons. Based on previous reports and our current results, we propose that CI enzyme deficiency is a general feature in the CNS of patients with mitochondrial DNA maintenance disorders and probably also in other mitochondrial disorders affecting the CNS, and does not correlate with or directly cause parkinsonism. Furthermore, the severity of CI defect does not explain the variability of Twinkle and POLG disease manifestations (myopathy, spinocerebellar ataxia, Parkinson’s disease) in the different brain regions of the patients.

The widespread CI deficiency in different mitochondrial DNA maintenance disorders is a likely consequence of mitochondrial DNA depletion or deletions. However, the lack of correlation of CI deficiency to disease manifestations raised the question of whether CI deficiency is pathogenic or a physiological response.

Flow of nutrient-derived reducing equivalents to poorly functional respiratory chain has potential to result in over-reduction of CI, escape of electrons to prematurely react with oxygen, increasing reactive oxygen species production from CI. Therefore, active downregulation of CI upon respiratory chain dysfunction could reduce cellular reactive oxygen species production. Neuronal cells are known to tolerate well respiratory chain deficiency, as mice with TFAM knockout, and thus with loss of mitochondrial DNA, developed a neurodegenerative disease manifestation only at 6 months of age (Sorensen et al., 2001; Ekstrand et al., 2007). If CI was actively downregulated, a protease could be involved. The mitochondrial matrix-facing AAA-protease AFG3L2 is a good candidate for this as it has been reported to act on mitochondrial translation products upon mitochondrial dysfunction, and we studied its abundance in the patient neurons (Hornig-Do et al., 2012). A slight increase of the protease was seen in POLG patient neurons, but not in the others, and it remains to be determined whether CI deficiency and the protease activity are linked.

Our patients with POLG-Parkinson’s disease developed their first signs of parkinsonism at the ages of 46 and 57. Their presenting symptoms for parkinsonism were typical, the progression of parkinsonism slow, and response to levodopa was well preserved, mimicking autosomal recessive juvenile Parkinson’s disease closely, but being different from idiopathic Parkinson’s disease (Mori et al., 1998; Lücking et al., 2000; Luoma et al., 2004; Lees et al., 2009). However, autosomal recessive juvenile Parkinson’s disease usually shows bilateral symptoms rather than unilateral as described for POLG-Parkinson’s disease. The neuropathological hallmark of Parkinson’s disease is substantia nigra degeneration, leading to the death of the pigmented dopaminergic neurons, and in idiopathic Parkinson’s disease also the occurrence of Lewy bodies (Braak et al., 2004). Correlating with clinical manifestation, our patients with POLG-Parkinson’s disease showed subtotal loss of the substantia nigra pigment neurons, whereas other patients had some degree of substantia nigra neuron loss, but not as severe. None of the patients had Lewy body-like structures. In autosomal recessive juvenile Parkinson’s disease, perturbed mitochondrial quality control is suggested to contribute to pathology (Greene et al., 2003; Clark et al., 2006; Park et al., 2006; Ekstrand et al., 2007; Narendra et al., 2008, 2010; Vives-Bauza et al., 2010), but this mechanism has been

Table 3  Immunoreactivity of mitochondrial respiratory chain complexes in mesencephalic nuclei of patients with mitochondrial DNA maintenance disorders

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<th>Patient</th>
<th>Disease</th>
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<th>Complex II</th>
<th>Complex IV</th>
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<td></td>
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<td>Substantia nigra</td>
<td>Oculomotor nucleus</td>
<td>Substantia nigra</td>
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<tr>
<td>P1</td>
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<tr>
<td>P2</td>
<td>POLG-Parkinson’s disease</td>
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<tr>
<td>P3</td>
<td>MIRAS</td>
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<td>P5</td>
<td>Autosomal dominant progressive external ophthalmoplegia</td>
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<td>P6</td>
<td>IOSCA</td>
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*Increase in immunoreactivity.
++ + severe lack of immunoreactivity; ++ moderate lack; + slight lack; - no changes.
Respiratory chain immunoreactivity in oculomotor nucleus shows variable levels of Complex I in patients with mitochondrial DNA maintenance disorders. Patients P1 and P2, POLG-Parkinson’s disease; Patients P3 and P4, MIRAS; Patient P5, Twinkle autosomal dominant progressive external ophthalmoplegia; Patient P6, Twinkle IOSCA; C = healthy control. (A) POLG-Parkinson’s disease (Patient P1) had almost complete lack of Complex I immunoreactivity (brown colour indicates positive reactivity, blue counterstaining reveals absence of complex), Patient P2 (POLG-Parkinson’s disease), Patient P3 (MIRAS) and Patient P4 (MIRAS) showed partially reduced CI immunoreactivity; Patient P5 (Twinkle autosomal dominant progressive external ophthalmoplegia) shows moderately reduced reactivity and Patient P6 (Twinkle IOSCA) did not have any CI immunoreactivity (U), when compared with IgG controls (D, H, L, P, T and X) and healthy control C (Y). Complexes II and IV are well preserved in all samples (CII: B, F, J, N, R and V and CIV: C, G, K, O, S and W). Antibodies used: CI, NDUFS3; CII 70 kDa subunit; CIV, COX2 subunit; 0, IgG replacing primary antibody as negative control. Magnification × 40, scale bar = 50 μm.
chondrial myopathy and atrophy of extraocular muscles (Suomala et al., 1992, 1997), and the vacular degeneration of oculomotor nucleus in these patients with progressive external ophthalmoplegia suggested trans-synaptic deafferentation and dying-back axonal degeneration, after primary muscle atrophy and lack of motor stimulus. These patients typically require recurrent blepharoplastia, because of total ptosis. The patients with POLG or Twinkle-associated spinocerebellar ataxia (MIRAS or IOSCA), however, had no or mild degeneration in the oculomotor nucleus complex, despite early-onset total ophthalmoplegia of patients with IOSCA, and different types of ocular symptoms (restricted eye movements, mild ptosis, nystagmus, saccadic eye movements) in MIRAS (Lönnqvist et al., 1998; Rantamäki et al., 2001; Hakonen et al., 2005). Patients with IOSCA do not have ptosis, and patients with MIRAS either have no or mild ptosis, and do not require blepharoplasty (Koskinen et al., 1994b; Van Goethem et al., 2004; Hakonen et al., 2005). Their symptoms cannot be explained by oculomotor nucleus neuronal death, but may be contributed to by damage in nucleus trochlearis and abducens, which, however, were not available for our study. It is clear that the origin of ophthalmoplegia in the recessive POLG and Twinkle spinocerebellar ataxias has different basis from external ophthalmoplegia in POLG-Parkinson’s disease and autosomal dominant progressive external ophthalmoplegia. Based on the patients’ clinical manifestations we propose that ophthalmoplegia in mitochondrial spinocerebellar ataxias is of CNS origin, and primarily of muscle origin in POLG-Parkinson’s disease and autosomal dominant progressive external ophthalmoplegia. The basis, why different types of mutations in the same or functionally closely linked proteins affect different brain regions and tissue types, remains to be clarified.

In conclusion, our results strongly suggest that POLG-Parkinson’s disease forms with autosomal recessive juvenile Parkinson’s disease a distinct clinical entity, mitochondrial early-onset parkinsonism, lacking Lewy bodies. All patients with mitochondrial DNA maintenance defects had mesencephalic neuronopathy, suggesting that these disorders form a continuum, which eventually, if the patient survived the other symptoms, could also manifest as parkinsonism. Indeed, some patients with MIRAS and Twinkle mutation patients with parkinsonism have been described in the literature (Baloh et al., 2007; Remes et al., 2008; Vandenbergh et al., 2009). Dopaminergic neurons of substantia nigra have some specific energetic requirements: they connect with an enormous network of neurons and pulse calcium waves in a pace-maker manner. Their axons have only thin or no myelin sheaths, resulting in increased energy requirement upon excitation (Braak et al., 2004; Surmeier et al., 2011). However, our data indicate that the role of CI deficiency as a primary cause of parkinsonism should be revisited.

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**Supplementary material**

Supplementary material is available at *Brain* online.

**References**


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