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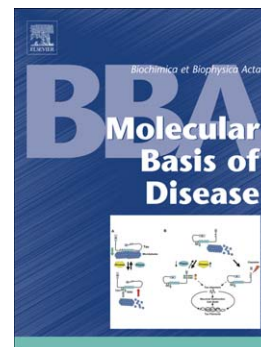
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The impact of mitochondrial tRNA mutations on the amount of ATP synthase differs in the brain compared to other tissues.

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Summary

The impact of point mutations in mitochondrial tRNA genes on the amount and stability of respiratory chain complexes and ATP synthase (OXPHOS) has been broadly characterized in cultured skin fibroblasts, skeletal muscle samples, and mitochondrial cybrids. However, less is known about how these mutations affect other tissues, especially the brain. We have compared OXPHOS protein deficiency patterns in skeletal muscle mitochondria of patients with Leigh (8363G>A), MERRF (8344A>G), and MELAS (3243A>G) syndromes. Both mutations that affect mt-tRNA^{Lys} (8363G>A, 8344A>G) resulted in severe combined deficiency of complexes I and IV, compared to an isolated severe defect of complex I in the 3243A>G sample (mt-tRNA^{Leu(UUR)}). Furthermore, we compared obtained patterns with those found in the heart, frontal cortex, and liver of 8363G>A and 3243A>G patients. In the frontal cortex mitochondria of both patients, the patterns of OXPHOS deficiencies differed substantially from those observed in other tissues, and this difference was particularly striking for ATP synthase. Surprisingly, in the frontal cortex of the 3243A>G patient, whose ATP synthase level was below the detection limit, the assembly of complex IV, as inferred from 2D-PAGE immunoblotting, appeared to be hindered by some factor other than the availability of mtDNA-encoded subunits.

Abbreviations: **BN-PAGE** = Blue-Native PAGE; **COX** = cytochrome *c* oxidase, complex IV; **CS** = citrate synthase; **mt-tRNA** = mitochondrial tRNA; **mtDNA** = mitochondrial DNA; **OXPHOS** = oxidative phosphorylation system; **SQR** = succinate:coenzyme Q10 reductase, complex II.

1. Introduction

The mammalian organism fully depends on the oxidative phosphorylation system (OXPHOS) as the major energy (ATP) producer of the cell. Disturbances of OXPHOS may be caused by mutations in either mitochondrial DNA (mtDNA) or nuclear DNA, and environmental factors have also been shown to have important effects on OXPHOS. In human mitochondria, a small circular DNA molecule (16 659bp) codes for 13 polypeptides that form, together with nuclear-encoded subunits, five inner-membrane OXPHOS complexes. For the translation of these 11 mRNAs (nine monocistronic and two bicistronic), mitochondria contain a separate translational system made of protein components, encoded exclusively by nuclear genes and RNA components encoded by the mitochondrial genome (two ribosomal RNA genes and 22 transfer RNA (tRNA) genes). Mitochondrial translation is precisely controlled to meet tissue-specific demands for mtDNA-encoded structural subunits of the OXPHOS complexes [1]. Although the basal components of the mitochondrial expression system are known, the mechanism of regulation of the system in response to the metabolic needs of the cell is poorly understood [2, 3].

Since the late 1980s, large-scale deletions and point mutations in mtDNA (approximately 120 in tRNAs and 11 in rRNAs) have been identified to cause disorders of mitochondrial protein synthesis, which is associated with defects of mitochondrial bioenergetics in tissues that most depend on OXPHOS. Despite numerous clinical studies on patients with mt-tRNA mutations, which usually involve morphological and functional characterization of OXPHOS insufficiency in skin fibroblast cultures or skeletal muscle, little is known regarding tissue specificity of OXPHOS deficiencies.

The aim of this study was to determine the steady-state levels of OXPHOS protein complexes in the mitochondria of various tissues (skeletal muscle, heart, frontal cortex and liver) of patients with mt-tRNA mutations. We chose (i) a patient with an 8363G>A mutation in mt-tRNA^{Lys}, who died of Leigh syndrome; (ii) a patient with an 8344A>G mutation in the same tRNA, who suffers from MERRF syndrome (myoclonic epilepsy with ragged-red fibres); and (iii) a patient with a 3243A>G mutation in mt-tRNA^{Leu(UUR)}, who died of MELAS syndrome (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes). In the skeletal muscle of these patients, the mutations manifested themselves as described in previous studies [4-11]; however, these results disclose new aspects of OXPHOS deficiencies in the brain, particularly in the case of ATP synthase. Furthermore, the 3243A>G frontal cortex mitochondria showed a marked loss of the complex IV holoenzyme, accompanied by accumulation of assembly intermediates, which might be caused by the virtual absence of complex V in this sample. A similar phenomenon was described in yeast ATP synthase mutants [12-15].

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2. Patients, materials and methods

2.1. Ethics

This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and was approved by the Committee of Medical Ethics of the Faculty of Medicine and General Faculty Hospital. Informed parental consent, in accordance with the guidelines of the Faculty of Medicine, Charles University, was obtained for all biopsies and autopsies.

2.2. Case Reports

2.2.1. Patient 1, with an 8363G>A mtDNA mutation

The boy was born at term with a birth weight of 2590 g (5th percentile) and a length of 48 cm. The early postnatal adaptation was uneventful, but a failure to thrive and progressive hypotony developed beginning in infancy, and he was wheelchair-dependent from the age of 8 years. Psychological investigations revealed moderate mental retardation, which later became severe. At the age of 8 he had his first epileptic paroxysm, and at the age of 11, an acute stroke-like encephalopathy developed, resulting in generalized weakness and respiratory failure requiring ventilatory support. The liver function was unaffected and aminotransferases were always within the reference range. The EEG pattern was abnormal; an EMG demonstrated peripheral neuropathy, echocardiography showed hypertrophic cardiomyopathy, and MRI revealed bilateral necrotic basal ganglia lesions typical of Leigh syndrome. The boy died at the age of twelve. Metabolic analyses revealed hyperlactacidemia (2.5-5.5 mmol.l⁻¹, controls <2.1 mmol.l⁻¹) with an increased lactate/pyruvate ratio (L/P 28).

2.2.2. Patient 2, with a 3243A>G mtDNA mutation

The girl was born at term with a birth weight of 3140 g and a length of 50 cm. Her early postnatal adaptation was uneventful, but a failure to thrive and growth retardation were observed beginning in infancy. At the age of 16 years, her weight and height were 30 kg and 140 cm (<3rd percentile). Cardiac evaluation revealed dilated cardiomyopathy and Wolf-Parkinson-White syndrome. At the age of 18 years, blurred vision, emesis and tiredness developed, and subsequently, seizures and coma. An MRI study showed symmetrical lesions within the basal ganglia, compatible with Leigh syndrome, in addition to several hypodensities in the subcortical regions of the frontal and occipital parts of the brain, which are consistent with a diagnosis of acute ischemia. Metabolic analyses revealed metabolic acidosis (BE -10 to -25 mmol.l⁻¹, controls \pm 2 mmol.l⁻¹), as well as increased levels of lactate in the blood (4-10 mmol.l⁻¹, controls <2.1 mmol.l⁻¹), cerebrospinal fluid (12 mmol.l⁻¹, controls <2.1 mmol.l⁻¹), and urine (4976 mM/mol creatinine, controls <60 mM/mol creatinine). Despite intensive treatment, the girl died of progressive encephalomyopathy and respiratory failure due to acquired infection during the terminal phase of the disease, at the age of 18 years.

2.2.3. Patient 3, with an 8344A>G mtDNA mutation

The boy was born at term with a birth weight of 3500 g and length of 51 cm, with normal postnatal adaptation. Beginning at the age of 11 years, he developed progressive muscle weakness and paresthesia. Now, at the age of 15, he has generalized muscle hypotrophy and hyporeflexia. No mental deterioration has been observed. An EMG disclosed diffuse myopathic abnormalities and normal nerve conduction velocities. The EEG and cardiac evaluation were normal. Metabolic analyses revealed an increased level

of lactate in the blood (5.6-7.88 mmol.l⁻¹, controls <2.1 mmol.l⁻¹) and lactate in the urine (5148 mM/mol creatinine, controls <60 mM/mol creatinine).

2.3. Tissues

All studied tissues were obtained from three patients harbouring one of two mutations in mt-tRNA^{Lys} (8363G>A and 8344A>G) or one mutation in mt-tRNA^{Leu(UUR)} (3243A>G), as well as from age-related controls. Open muscle biopsies from the tibialis anterior muscle were frozen at -80 °C. Post-mortem tissue specimens obtained at autopsy of 8363G>A and 3243A>G patients, and controls were frozen less than 2 hours after death. The studies were performed in available stored material from the muscle (tibialis anterior), heart, liver, and brain (frontal cortex).

2.4. mtDNA analysis

Total genomic DNA was isolated by phenol extraction from available tissues. The DNA sample from the muscle biopsy of patient 1 was used for the sequencing of the whole mtDNA molecule on an AbiPrism 3100 Avant Genetic Analyser (Applied Biosystems). To determine the amount of mtDNA containing the mutation, PCR/RFLP analysis was performed. PCR products (8279 – 8485) were radioactively labelled with [α -³²P]dCTP in the final cycle of PCR, and run on a non-denaturing 10% (w/v) polyacrylamide gel after complete digestion with *TspRI* (NEB). The mutation abolishes one of two *TspRI* restriction sites on the fragment. The proportions of wild-type to mutant mtDNA were measured using a PhosphorImager and ImageQuant software (Molecular Dynamics). The levels of heteroplasmy of the 3243A>G and 8344A>G mutations were determined as described elsewhere [16].

2.5. Electrophoresis

BN-PAGE (Blue Native PAGE) [17] was used for the separation of mitochondrial membrane protein complexes on polyacrylamide 6–15% (w/v) gradient gels using a MiniProtean® 3 System (Bio-Rad Laboratories). 5 - 50 µg of protein, which was prepared as described previously [18], was loaded in each lane. Two-dimensional BN/SDS/PAGE [17] was performed as described previously [18]. The protein content was measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories), using BSA as a standard.

2.6. Immunoblot analysis

Proteins were electroblotted from the gels onto Immobilon™-P PVDF membranes (Millipore) using semi-dry transfer for 90 min at a constant current of 0.8 mA/cm². Membranes were air-dried overnight, rinsed twice with 100% (v/v) methanol, and blocked in TBS and 10% (w/v) non-fat dried milk for 1 – 2 h. Primary detection of BN/PAGE-blot was performed with mouse monoclonal antibodies raised against the complex I subunit NDUFA9 (2 µg/ml), ATP-synthase subunit alpha (2-3 µg/ml), complex III subunit Core 2 (0.5 µg/ml), complex IV subunit COX2 (0.5-1 µg/ml), and complex II subunit 70kDa protein (1 µg/ml) (Mitosciences), at indicated dilutions. Primary detection of two-dimensional BN/SDS/PAGE-blot for the COX assembly was performed according to Stiburek et al. [18]. Blots were incubated with primary antibodies in TBS, 0.3% (v/v) Tween 20, and 2% non-fat dried milk for 2 h. Secondary detection was carried out with a goat anti-mouse IgG–horseradish peroxidase conjugate (1:1000 – 1:4000) (Sigma–Aldrich) in TBS, 0.1% Tween 20, and 2% non-fat dried milk, for 1 h. The immunoblots were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). The immunoblot-images of chemiluminescence signals were captured using the VersaDoc Imaging System, Model 4000 (Bio-Rad Laboratories), and analysed by the Quantity One

application (Bio-Rad Laboratories). All blotting experiments were repeated with independently isolated mitochondrial samples. Duplicate experiments yielded consistent results.

2.7. Spectrophotometric assays

The activities of respiratory chain complexes in isolated muscle mitochondria were measured spectrophotometrically by standard methods at 37 °C. NADH:coenzyme Q10 reductase (NQR, complex I), succinate:coenzyme Q10 reductase (SQR, complex II), succinate:cytochrome *c* reductase (SCCR, complex II+III), NADH:cytochrome *c* reductase (NCCR, complex I+III), coenzyme Q10:cytochrome *c* reductase (QCCR, complex III), and cytochrome *c* oxidase (COX, complex IV) were measured according to Rustin et al [19], and citrate synthase (CS) according to [20]. Total protein amount was determined by the method of Lowry [21].

2.8. High resolution oxygraphy in muscle fibers

Muscle fibers were separated mechanically according to [22], and oxygen consumption by saponin-skinned muscle fibers was determined using multiple substrate inhibitor titrations as described previously [23].

3. Results

3.1. Heteroplasmy of mt-tRNA mutations in investigated tissues

Analyses of heteroplasmy by radioactive PCR-RFLP showed 87 – 97% heteroplasmy of mt-tRNA mutations in the tissues of all patients (Tab. 1). Despite the narrow range of the mutation load found in patient tissues, the severity of the clinical/functional phenotype did not correlate with the level of heteroplasmy.

3.2. Steady-state levels of OXPHOS complexes in 8363G>A, 8344A>G and 3243A>G skeletal muscle

To investigate the impact of mtDNA mutations on the system of oxidative phosphorylation, immunoblots of mitochondrial fractions resolved by BN-PAGE were prepared. Dilutions of samples from control mitochondria were loaded on the same gels in order to express the residual steady-state levels of OXPHOS complexes in the patient tissues as a percentage of control values.

In the 8363G>A skeletal muscle sample obtained at autopsy, profoundly decreased levels of complex I (5% of control) and IV (< 10% of control) were detected. The sample also revealed a diminished amount of complex V holoenzyme (35% of control), along with accumulated sub-complexes, most likely V* (F1-ATPase with several c-subunits) and F1-ATPase (Fig. 1A). The same sub-complexes were also detected in a bioptic muscle sample from the 8363G>A patient using BN-PAGE with Coomassie staining, followed by the second denaturing electrophoretic dimension with silver staining (Fig. 2).

A very similar pattern, although less severe, was found in the 8344A>G skeletal muscle sample. The amount of complex I was reduced to approximately 25% of control, the level

of complex IV holoenzyme was < 15% of control, and the holoenzyme level of complex V was decreased to 60% of the control value. Immunodetection of complex V further showed sub-complexes similar to those observed in the 8363G>A sample (Fig. 1A, 2A).

In 3243A>G skeletal muscle, relative levels of OXPHOS holoenzymes were normal, except for a decrease in the content of complex I to 30% of control and complex IV to 60% of control (Fig. 1A).

3.3. Steady-state levels of OXPHOS complexes in other 8363G>A and 3243A>G tissues

Only the 8363G>A and 3243A>G samples of heart, frontal cortex, and liver tissue were available for comparison of the impact of mtDNA mutations on the system of oxidative phosphorylation in skeletal muscle and other tissues (Tab. 1).

The 8363G>A heart sample showed the same considerable reduction in complexes I (5% of control) and IV (< 10% of control) content as skeletal muscle, but despite the greater reduction of complex V holoenzyme (15% of control), there was a significantly lower accumulation of F1-ATPase. The 3243A>G heart sample had a pronounced reduction of complex I (20% of control), similar to skeletal muscle (Fig. 1B).

In the 8363G>A frontal cortex, the assembly of complexes I and IV were less affected than in the above-mentioned tissues. The amount of complex I was decreased to 40% of control, and the holoenzyme level of complex IV was reduced to 50% of control. Conversely, complex V (<20% of control) appeared to be the most severely affected member of the OXPHOS system. The decrease was more substantial than in skeletal muscle; nevertheless, no detectable sub-complexes could be found. The 3243A>G frontal cortex sample showed a dramatic reduction of complex V to below the detection limits of the method, and of complex I to 10% of the control value. Probing of the immunoblots

with an anti-COX2 antibody showed a reduction of complex IV to 20% of control, as well as the presence of a high molecular weight sub-complex (Fig. 1C). Due to the unexpected OXPHOS deficiency pattern observed in brain tissue, particularly in the 3243A>G patient, the immunoblotting was also performed on the frontal cortex sample from another 3243A>G patient. In the only available sample with a 65% level of heteroplasmy, the immunoblotting analysis revealed normal levels of OXPHOS complexes in comparison to control (data not shown).

Despite the highest level of heteroplasmy (Tab. 1), the 8363G>A liver sample only showed an isolated deficiency of complex I (40% of control) (Fig. 1D). Steady-state levels of OXPHOS complexes in the 3243A>G liver mitochondria were comparable to control (data not shown).

3.4. Activities of respiratory chain complexes in 8363G>A, 8344A>G and 3243A>G skeletal muscle

To characterize the impact of the 8363G>A, 8344A>G and 3243A>G mutations on the function of respiratory chain complexes, the specific activities of the respective enzymes and citrate synthase (CS, control enzyme) were measured spectrophotometrically. The values expressed as relative ratios of the activities of complexes I, III and IV normalized to complex II (II, SQR) or CS were compared to the relative ratios of steady-state protein levels normalized to complex II (Fig. 3). Consistent with immunoblotting results, the spectrophotometry revealed lower activity ratios for complex I (I/II and I/CS) in the isolated muscle mitochondria of all three patients, and a severe deficiency of complex IV in 8363G>A and 8344A>G isolated muscle mitochondria. The COX/SQR (IV/II) and COX/CS (IV/CS) ratios in the 3243A>G patient were just below the control range.

3.5. Oxygraphic analysis in 8363G>A and 8344A>G skeletal muscle fibers

The functional consequences of the 8363G>A mutation in comparison to the 8344A>G mutation were analysed by high-resolution oxygraphy of the patients' skeletal muscle fibers permeabilized by a low concentration saponin treatment (Tab. 2). In both samples, a decrease of ADP-stimulated oxygen consumption compared to control fibers was observed using pyruvate as a substrate, and an increase was found after succinate addition. A pronounced reduction in ADP-stimulated respiration was found after pyruvate (33% of the mean control value) as well as after glutamate (32% of the mean control value) additions in 8363G>A muscle fibers. Indeed, ADP-stimulated respiration after succinate treatment increased to 158% of the mean control value. In 8344A>G muscle fibers, ADP-stimulated respiration after pyruvate addition was 70% of the mean control value, but after glutamate, it was within the reference range. Similarly to 8363G>A muscle fibers, the ADP-stimulated respiration after succinate addition increased to 222% of the mean control value in the 8344A>G sample. The absolute oxygen consumption after ascorbate + TMPD treatment was, in both samples, within control levels; however, normalization of the data to the level of respiration after succinate addition revealed decreased ratios after treatment with all of the substrates (Tab. 2).

3.6. Mitochondrial content in skeletal muscles with mt-tRNA^{Lys} mutations

In isolated muscle mitochondria, Western blotting showed an increased level of complex II holoenzyme, which is entirely encoded by nuclear DNA, up to 150% and 160% of control in 8363G>A and 8344A>G patient samples, respectively (Fig. 1A). After normalization, this leads to artificially decreased amounts of complex III in the skeletal muscle mitochondria of both patients (Fig. 3, Complex III). Accordingly, the II/CS activity

ratio remained on the upper border of the reference range (Fig. 3, Complex II), indicating a higher mitochondrial membrane content (altered mitochondrial morphology).

In tissue homogenates, the specific activity of CS was increased to 380% and 285% of the mean control value in the 8363G>A and 8344A>G patient samples, respectively. Histochemistry revealed increased amounts of SDH product in 8363G>A skeletal muscle and ragged red fibers in 8344A>G skeletal muscle.

Based on the above-mentioned observations and on the increased ADP-stimulated oxygen consumption after succinate treatment (antimycin A-sensitive) of patients' skeletal muscle fibers (Chapter 3.5), a proliferation of mitochondria with altered morphology could be expected as a result of cellular energetic imbalance.

3.7. Immunoblots of Two-dimensional Native/Denaturing Gels

Immunoblotting of BN/SDS/PAGE (native in the first dimension and denaturing in the second dimension) with COX1, COX2, COX4 and COX5A antibodies was used to analyze the assembly of complex IV in 8363G>A skeletal muscle, and to confirm the alignment of the high molecular weight band identified in the 3243A>G frontal cortex after immunoblotting of BN/PAGE with COX2 antibody.

In 8363G>A skeletal muscle, the level of free S1 sub-complex was found to be below the detection limit of the method (Fig. 4). This probably reflects the limiting character of the COX1 subunit in the holoenzyme assembly.

In 3243A>G frontal cortex, significantly increased levels of all known complex IV assembly intermediates were observed, including free apoCOX1 (S1 sub-complex), apoCOX2, and apoCOX5A (Fig. 5). The high molecular weight band just below the COX holoenzyme, which was detected with anti-COX2 antibody (Fig. 1C), very likely represents the S3 assembly intermediate (Fig. 5). Long exposure of the same immunoblot

also revealed the band representing free apoCOX2 subunit (data not shown). This finding further supports the assumption that the final reduction in the content of COX holoenzyme in the 3243A>G frontal cortex mitochondria is not caused solely by the limiting character of mtDNA-encoded subunits of COX (translational defect) (Fig. 4).

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4. Discussion

This paper has presented a detailed analysis disclosing the tissue-specific impact of the 8363G>A mtDNA mutation on the amount, stability, and function of the OXPHOS complexes in a patient who died of Leigh syndrome. Furthermore, these data were compared with that from two other mtDNA mutations; the first one in the same tRNA (8344A>G) and the other one in mt-tRNA^{Leu(UUR)} (3243A>G), both of which are at least twice as common as the total number of other mtDNA point mutations known to cause disorders affecting the central nervous system [24, 25]. Although just one patient with a comparable level of heteroplasmy was available per studied mutation, the observed data demonstrate intriguing tissue-specific patterns of OXPHOS protein deficiencies, with the most unexpected findings in the 3243A>G frontal cortex.

In the skeletal muscle of patient 1, the lowest level of heteroplasmy but the most severe OXPHOS defect suggests a more profound impact of the 8363G>A mutation on the translational system than that of the 8344A>G mutation. Concerning the 3243A>G mutation, the relatively proportional levels of heteroplasmy in 8344A>G and 3243A>G skeletal muscles, the more significant decrease in the content of complex I, the severe decrease in the amount of complex IV, and the slightly lower level of complex V in patient 2 all indicate a less pronounced impact of the 3243A>G mutation on mitochondrial translation in this tissue. However, the different nuclear backgrounds [26-30], distributions of heteroplasmy levels in cells and mitochondria [25, 31, 32], and environmental factors [33, 34] which have been shown to influence expression of mitochondrial respiratory insufficiency, prevent the reduction of these results to any simple quantitative trait.

The frequency of UUR (Leu) codons in mitochondrially-translated subunits of OXPHOS implies decreased steady-state levels of complex I subunits, namely ND6, ND3, ND2 and ND5 (14 – 9 UURs) in 3243A>G mitochondria. In 8363G>A and 8344A>G mitochondria, the distribution of AAR (Lys) codons anticipates diminished levels of ND5, ND2, ND4 and COX1 (21 - 10 AARs). Moreover, all these subunits contain two X/Lys/Lys/X motifs, or one X/Lys/Lys/X and one Lys/X/Lys motif (<http://www.mitomap.org>), which are apparently strong stalling points for the ribosome. The particular abundance and distribution of codons in mitochondrially-translated subunits of respiratory chain complexes appears to be a plausible explanation for the isolated defect of complex I in the patient with a mutation in mt-tRNA^{Leu(UUR)}, along with the combined deficiency of complexes I and IV in patients with mutations in mt-tRNA^{Lys}. Furthermore, the complete absence of unassembled apoCOX1 in the skeletal muscle of the 8363G>A patient, as revealed by BN/SDS/PAGE immunoblotting, conforms to the limiting character of the COX1 subunit in holoenzyme assembly. On the contrary, the OXPHOS deficiency patterns found in the frontal cortex mitochondria of the 8363G>A and 3243A>G patients could suggest, similarly to [18], a specific character for brain OXPHOS. First, in both frontal cortex samples, the decrease in the content of complex V was more profound than in that of complexes I and IV. Second, despite such a pronounced defect of complex V, no sub-complexes similar to those observed in skeletal muscle and heart could be detected, even when the immunoblot exposure was prolonged (data not shown). Unfortunately, no 8344A>G frontal cortex specimen was available for the analysis. However, a selectively decreased expression of COX2 subunit was previously reported in frontal cortex and cerebellum of a MERRF patient [35]. F1-ATPase, which was observed in heart, was found along with a sub-complex denoted V* in skeletal muscle. The sub-complex V* is likely composed of F1-ATPase and several c-F_O subunits.

These sub-complexes were described previously in 8993T>G mitochondria [36, 37], ρ^0 cells [37], and in cells with inhibited mitochondrial translation [38]. The observed steady-state levels of OXPHOS complexes suggest that the brain ATP synthase is most sensitive to disturbances of the mitochondrial translational system caused by the studied mt-tRNA mutations. Such a tissue-specific impact of mt-tRNA mutations with comparable tissue heteroplasmy is likely to result from tissue-specific variations in the nature of mitochondria. Indeed, it was shown that the brain, liver and kidney OXPHOS system is mainly controlled at the phosphorylation level by ATP synthase and a phosphate carrier, in contrast to the muscle and heart, where it is essentially controlled at the level of the respiratory chain [39]. On the other hand, instead of diminished energy provisions, an insufficient discharge of mitochondrial membrane potential leading to reactive oxygen species (ROS) production was proposed as the underlying pathogenic mechanism of ATP synthase deficiency [40]. Accordingly, the complete lack of complex V from 3243A>G frontal cortex may be, apart from the translational defect, responsible for the unusual assembly pattern of COX in this sample. Indeed, it was shown in yeast that (i) cells deficient in ATP synthase have a severe reduction of COX holoenzyme [12-15] and (ii) no decrease in COX synthesis is observed in uncoupled ATP synthase mutants, where the maintenance of mitochondrial potential is severely compromised by a massive proton leak through the F_0 sector [41, 42]. Although the COX1 subunit was shown to be a key regulatory target for COX reduction in yeast cells [12], some other mechanism is likely to be involved in the hindered assembly of COX in the 3243A>G frontal cortex mitochondria, since this sample had high accumulated levels of all three mitochondrially encoded subunits, either free or partially assembled.

Although it is necessary to analyze considerably more samples with high levels of heteroplasmy (such samples are difficult to obtain), these data show new effects of mt-

tRNA mutations on the brain which differ substantially from those described for skeletal muscle, heart, and liver tissues.

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Figure legends

Fig. 1 Analysis of the assembly of OXPHOS complexes by immunoblotting of BN-PAGE. BN-PAGE of lauryl maltoside-solubilised mitochondria isolated from autoptic (8363G>A, 3243A>G) and bioptic (8344A>G) muscle (A) and from autoptic (8363G>A, 3243A>G) heart (B), frontal cortex (C) and liver (D) was electroblotted onto PVDF membranes and probed with monoclonal antibodies that detect the native forms of the OXPHOS complexes. Three aliquots of control mitochondria corresponding to the indicated dilutions of control samples were loaded on the same gels. The migration of holoenzymes (I – V) and molecular mass standards (kDa) are indicated; the actual position of sub-complex V* and/or F₁ of complex V (*) in skeletal muscle (A) and heart (B) is directly below complex III (around 370 – 470 kDa).

Fig. 2 Complex V (V) subassemblies V* and F₁-ATPase (F₁), compatible with those described previously for T8993G mitochondria, ρ^0 cells, or where mitochondrial translation has been inhibited.

Long exposure of complex V immuno-detection with the monoclonal antibody against the ATP-alpha subunit from Fig. 1A (A). Coomassie staining of BN-PAGE (above) and silver staining of two-dimensional BN/SDS/PAGE (below) of the 8363G>A sample obtained from skeletal muscle (bioptic sample) (B).

Fig. 3 Relative steady-state protein levels and activities of respiratory chain complexes normalized to complex II (II) or citrate synthase (CS) in isolated muscle mitochondria.

The ratios of specific activities are expressed as the percentage of the mean of the control values (reference ranges are shown).

Fractions by the x-axis indicate which ratios are concerned; I - complex I, III - complex III, IV - complex IV, (wb) – the ratio of holoenzyme levels obtained from a Western blot, (act.) – the ratio of specific activities obtained using spectrophotometry.

Fig. 4 Subunit composition of complex IV subassemblies in skeletal muscle obtained from the 8363G>A patient (bioptic sample).

Two-dimensional BN/SDS/PAGE of lauryl maltoside-solubilised mitochondria isolated from the skeletal muscle of a control or the 8363G>A patient, obtained at biopsy, were electroblotted onto PVDF membranes and probed with monoclonal antibodies specific for the subunits COX1, COX2, COX4 and COX5A. The positions of the holoenzyme (S4) and subassemblies (S1 – S3) are indicated, along with the migration of the molecular mass standard (kDa).

Fig. 5 Subunit composition of complex IV subassemblies in the frontal cortex obtained at autopsy of the 3243A>G patient.

Two-dimensional BN/SDS/PAGE of lauryl maltoside-solubilised mitochondria isolated from the frontal cortex of a control or the 3243A>G patient, obtained at autopsy, were electroblotted onto PVDF membranes and probed with monoclonal antibodies specific for the subunits COX1, COX2, COX4 and COX5A. The positions of holoenzyme (S4) and subassemblies (S1 – S3, apoCOX2) are indicated, along with the migration of the molecular mass standard (kDa).

Figure 1

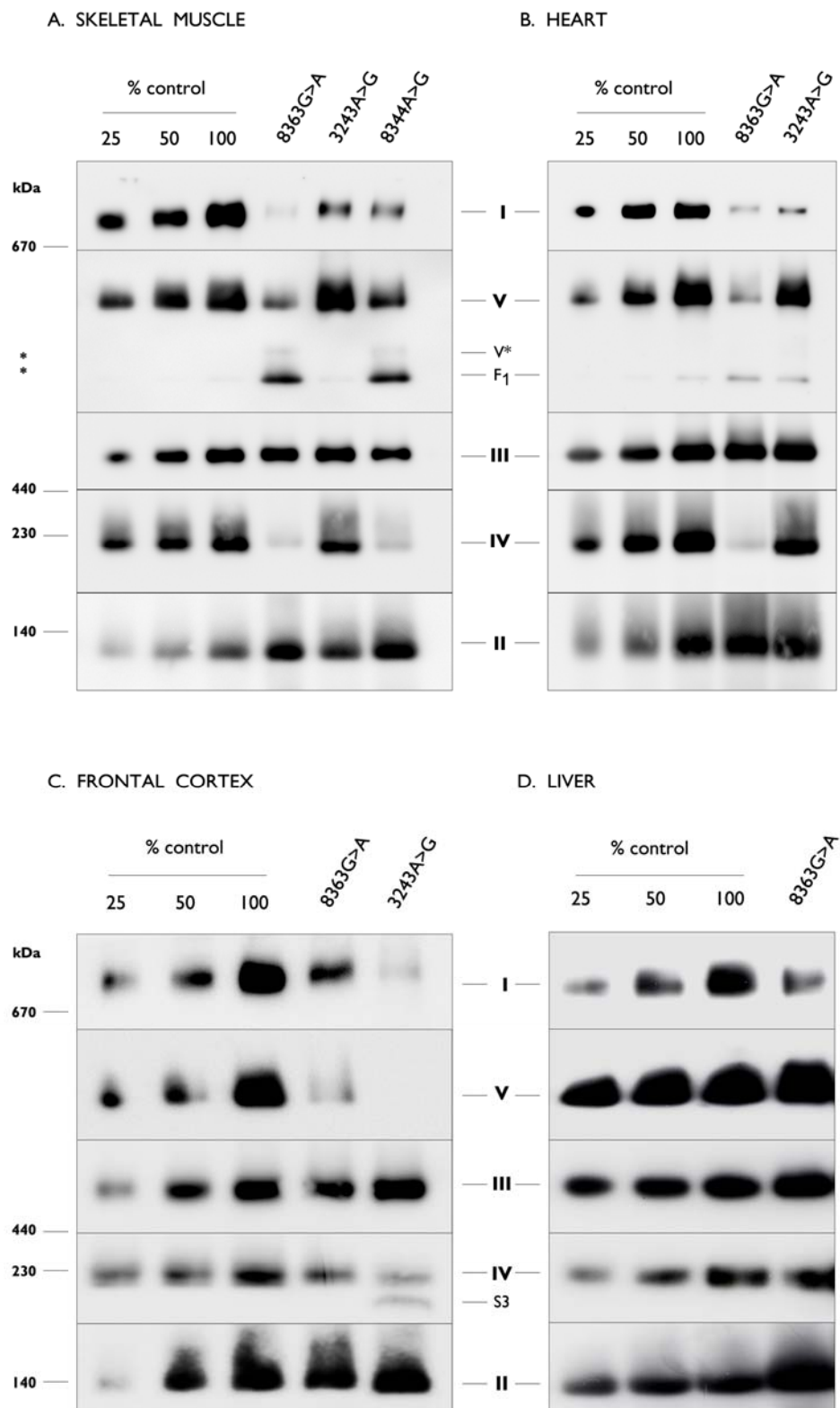


Figure 2

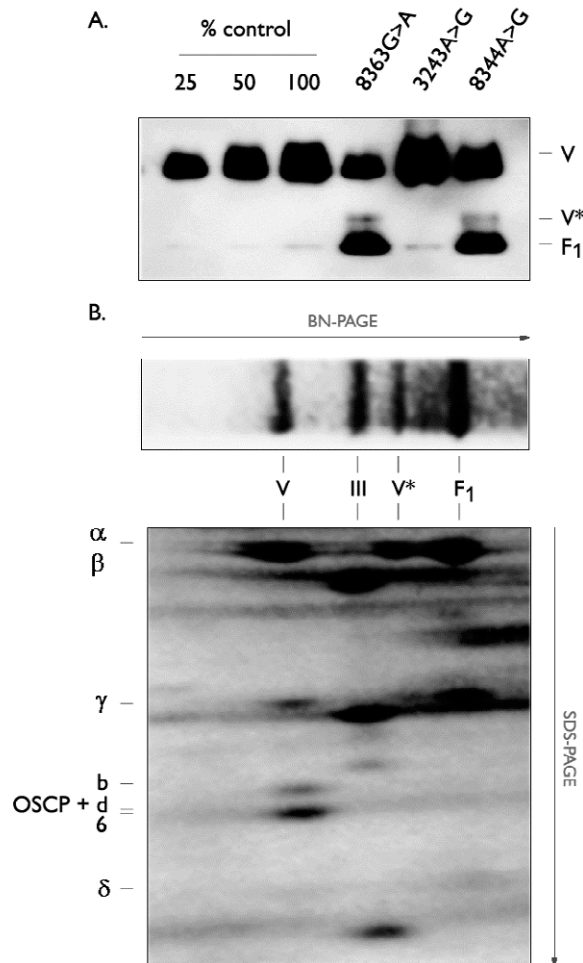


Figure 3

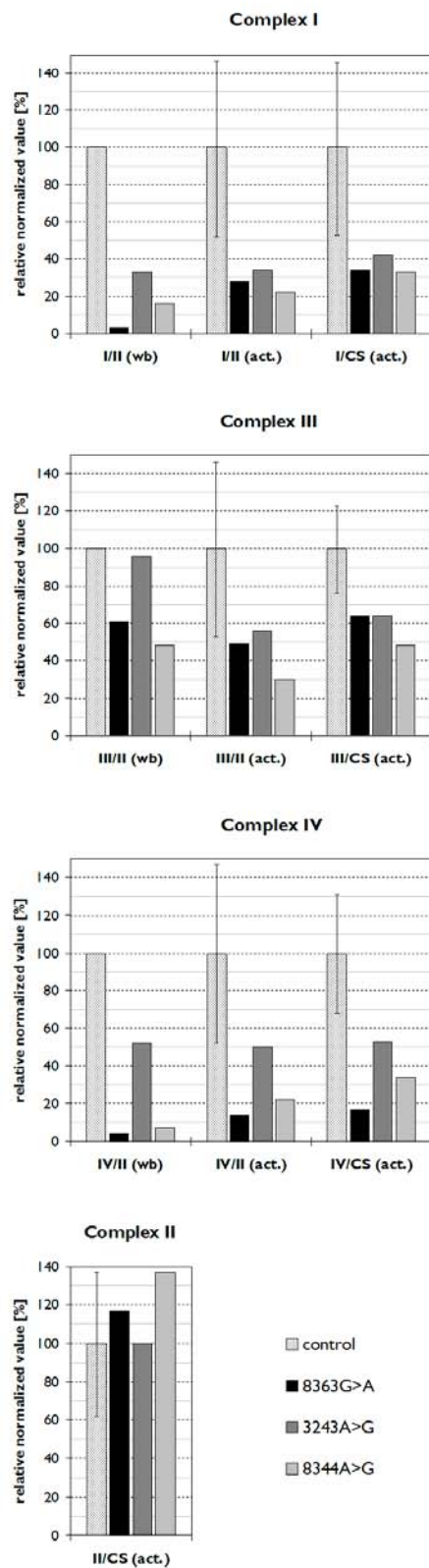


Figure 4

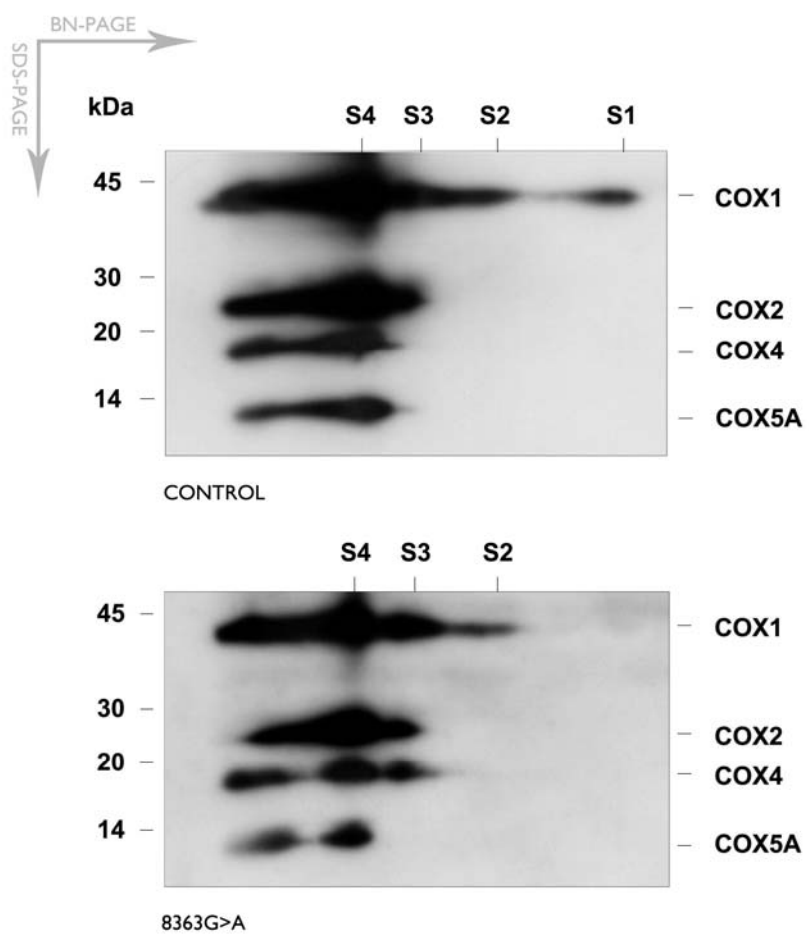
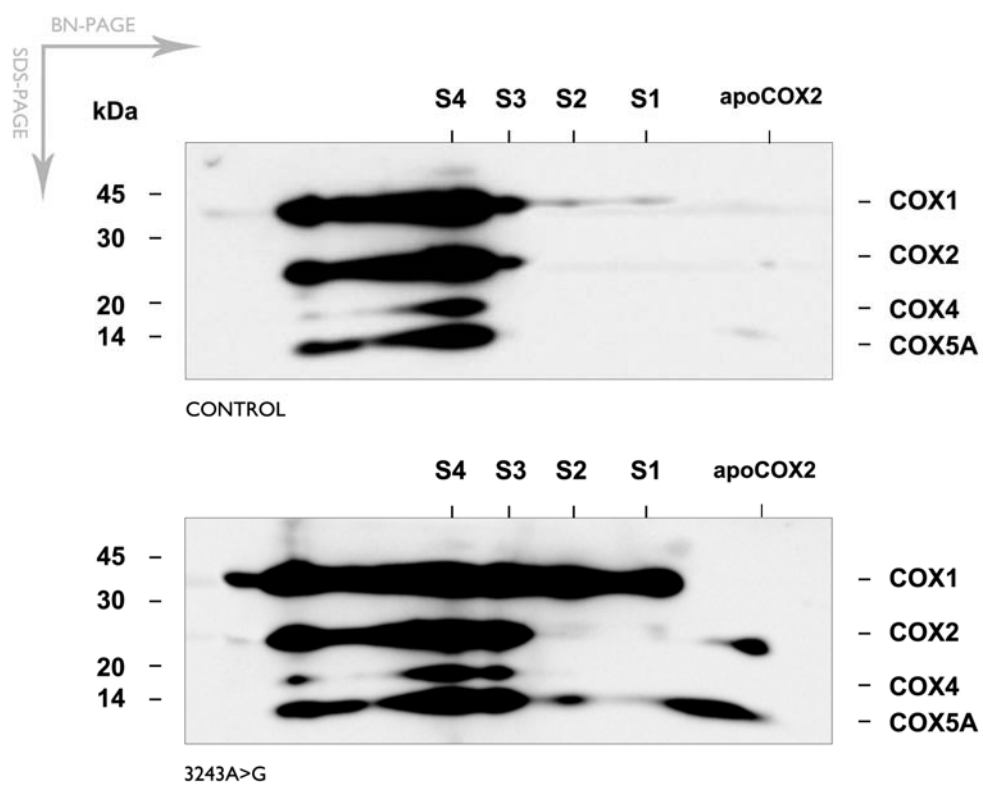


Figure 5



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Table 1

Summary of clinical consequences, mt-DNA mutation loads and character of OXPHOS deficiencies in investigated tissues of the patients.

<i>Skeletal muscle</i>	8363G>A	3243A>G	8344A>G
<i>clinical phenotype</i>	myopathy (+++)	myopathy (++)	myopathy (++)
<i>heteroplasmy</i>	80%	90%	89%
<i>OXPHOS protein deficiencies*</i>	I&IV (↓↓↓), V (↓↓), V ^{sub}	I (↓↓)	I&IV (↓↓↓), V (↓), V ^{sub}
<i>ADP-stimulated respiration after pyruvate/glutamate/succinate</i>	↓↓ / ↓↓ / ↑↑	nd	↓ / N / ↑↑↑
<i>heart</i>	8363G>A	3243A>G	8344A>G
<i>clinical phenotype</i>	cardiomyopathy ^H (++)	cardiomyopathy ^D (++)	-
<i>heteroplasmy</i>	89%	89%	nd
<i>OXPHOS protein deficiencies</i>	I&IV&V (↓↓↓), V ^{sub}	I (↓↓↓)	nd
<i>frontal cortex</i>	8363G>A	3243A>G	8344A>G
<i>clinical phenotype</i>	encephalopathy (++)	encephalopathy (++)	-
<i>heteroplasmy</i>	87%	89%	nd
<i>OXPHOS protein deficiencies</i>	V (↓↓↓), I&IV (↓↓)	V (↓↓↓), I&IV (↓↓↓), IV ^{sub}	nd
<i>liver</i>	8363G>A	3243A>G	8344A>G
<i>clinical phenotype</i>	-	-	-
<i>heteroplasmy</i>	97%	87%	nd
<i>OXPHOS protein deficiencies</i>	I (↓↓)	N	nd

-, absent; ++, moderate; +++, severe; ↓↓↓, < 30% of control; ↓↓, 30 – 50% of control/mean control value; ↓, > 50% of control/mean control value; ↑↑, 160% of mean control value; ↑↑↑, > 200% of mean control value; N, normal; nd, not done; ^{sub}-assembly intermediates of indexed complex were present; ^D - dilated; ^H - hypertrophic.

* relative steady-state protein levels (Western blot) correlated with activities of respiratory chain complexes (spectrophotometry), (Fig. 3).

Table 2

Oxygen consumption by saponin-permeabilized skeletal muscle fibers with 8363G>A and 8344A>G mutations

<i>Mitochondrial respiration (nmol O₂ . min⁻¹ per mg of wet weight)</i>			
	8363G>A	8344A>G	Controls (n = 9)
pyruvate / malate	6.9	14.7	16 - 26
glutamate / malate	5.1	16.5	10 - 22
succinate	21.4	30.0	9 - 18
ascorbate / TMPD	66.0	57.0	43 - 83

<i>Value normalized to respiration after succinate addition</i>			
	8363G>A	8344A>G	Controls (n = 9)
pyruvate / malate	0.32	0.49	0.96 - 1.85
glutamate / malate	0.24	0.55	0.83 - 1.72
ascorbate / TMPD	3.08	1.90	3.50 - 5.98

Polarographic measurements were performed as multiple substrate-inhibitor titration in the presence of 1 mM ADP with subsequent additions of pyruvate (10 mM)/malate (5 mM) or glutamate (10 mM)/malate (5 mM); succinate (10 mM); ascorbate (2 mM)/TMPD (500 μM).