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Review

OXPHOS mutations and neurodegeneration

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Mitochondrial oxidative phosphorylation (OXPHOS) sustains organelle function and plays a central role in cellular energy metabolism. The OXPHOS system consists of 5 multisubunit complexes (CI–CV) that are built up of 92 different structural proteins encoded by the nuclear (nDNA) and mitochondrial DNA (mtDNA). Biogenesis of a functional OXPHOS system further requires the assistance of nDNA-encoded OXPHOS assembly factors, of which 35 are currently identified. In humans, mutations in both structural and assembly genes and in genes involved in mtDNA maintenance, replication, transcription, and translation induce 'primary' OXPHOS disorders that are associated with neurodegenerative diseases including Leigh syndrome (LS), which is probably the most classical OXPHOS disease during early childhood. Here, we present the current insights regarding function, biogenesis, regulation, and supramolecular architecture of the OXPHOS system, as well as its genetic origin. Next, we provide an inventory of OXPHOS structural and assembly genes which, when mutated, induce human neurodegenerative disorders. Finally, we discuss the consequences of mutations in OXPHOS structural and assembly genes at the single cell level and how this information has advanced our understanding of the role of OXPHOS dysfunction in neurodegeneration.

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Introduction

Nearly every activity of the cell is powered by the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). In order to maintain ATP homeostasis and, therefore, cell integrity and function, ATP must be continuously replenished. The energy required for this process comes from the stepwise oxidation of fuel molecules originating from three different carbon sources, i.e., monosaccharides, mainly glucose (GLC) but also fructose (FRC) and galactose (GAL), fatty acids (FAs) and amino acids. Following food uptake, these fuel molecules enter the body from the intestine, where they are produced upon the enzymatic breakdown of carbohydrates, triacylglycerols (TAGs) and proteins. Their distribution throughout the body occurs via the circulatory system and cells take up the required nutrients for energy production, biosynthesis and replenishment of intracellular glycogen stores (liver cells and skeletal muscle cells) and TAGs (fat cells). Liver cells convert excess GLC to TAGs, which they package in very low density lipoprotein (VLDL) particles for transport to the fat cells. In between feeding, the blood GLC level is maintained by the liver mobilizing its glycogen stores and producing GLC from lactate (LAC), glycerol and glucogenic amino acids. At the same time, fat cells mobilize their TAG stores to release FAs and glycerol. GLC is the only fuel molecule for red blood cells and, normally, brain cells and to limit its consumption, liver cells and skeletal muscle cells at rest primarily use FAs for the production of ATP. Mature red blood cells and skeletal muscle cells at work convert GLC to LAC, which they release in the circulation. This LAC is taken up mainly by the liver, which uses ATP derived from FAs to reconvert it to GLC.

For GLC and glycerol, the stepwise oxidation process starts in the cytosol, where a series of enzymes catalyse their partial oxidation to pyruvate (PYR; Figure 1). During this process, the major part of the chemical bond energy of the fuel molecule is transferred in the form of electrons to the electron carrier nicotinamide adenine dinucleotide (NAD^+) thus reducing to reduced nicotinamide adenine dinucleotide (NADH), whereas a smaller part is transferred in the form of a phosphoryl group to ADP. The latter process, referred to as substrate-level phosphorylation, uses a phosphorylated reactive intermediate as a donor. In the case of GLC, cytosolic oxidation yields two molecules each of PYR, ATP and NADH. Other contributions to the cytosolic PYR pool come from LAC and certain amino acids.

PYR oxidation involves the combined action of a series of enzymes located within the mitochondrial matrix. First, PYR is oxidatively decarboxylated by pyruvate dehydrogenase (PDH), yielding one molecule each of CO_2, NADH and Acetyl coenzyme A (AcCoA). Next, AcCoA is oxidized by the enzymes of the tricarboxylic acid (TCA), producing two molecules of CO_2, three molecules of NADH, one molecule of the reduced form of the electron carrier flavin adenine dinucleotide ($FADH_2$) and one molecule of GTP, by substrate-level phosphorylation.

The oxidation of FAs takes place entirely in the mitochondrial matrix by a process referred to as β oxidation. Also, this

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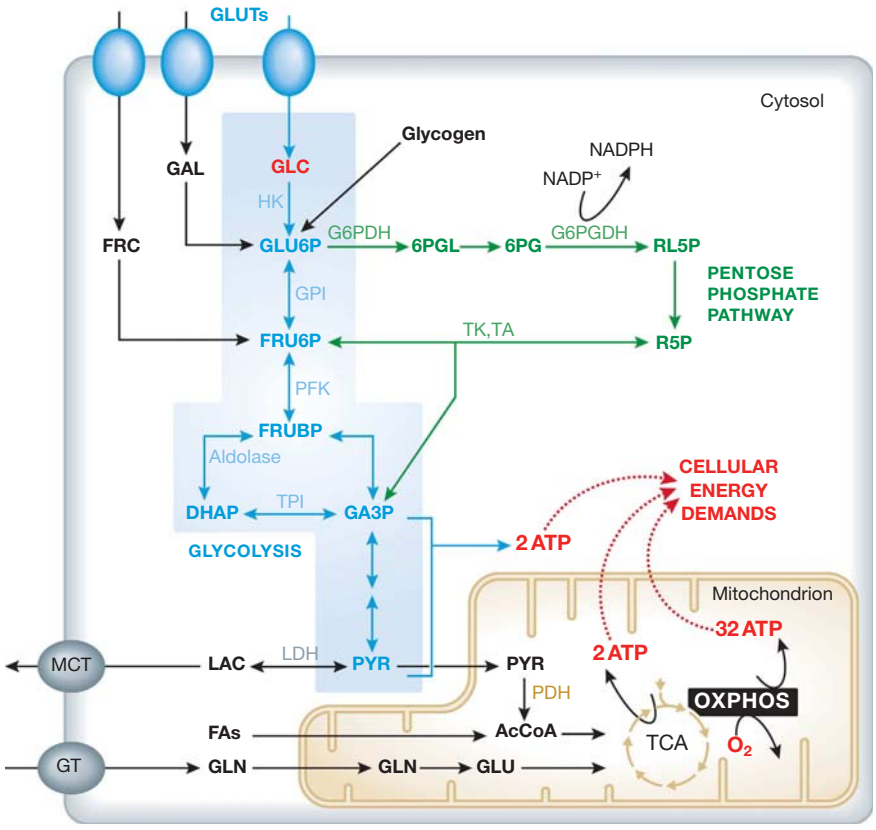

Figure 1 Energy metabolism in a typical mammalian cell. To meet cellular energy demands, ATP is generated by the glycolysis pathway (blue), the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation (OXPHOS) system. The main energy substrate glucose (GLC) enters the cell via GLC transporters (GLUTs) and is converted into pyruvate (PYR). Alternatively, surplus GLC can be stored as glycogen for later use or enter the pentose phosphate pathway (green). PYR can have two different fates: either it is converted into lactate (LAC) that leaves the cell, or it enters the mitochondrion (yellow) to form Acetyl coenzyme A (AcCoA). The latter is processed by the TCA cycle to yield NADH and $FADH_2$, which are substrates of the OXPHOS system. In addition to GLC also fructose (FRC), galactose (GAL), fatty acids (FAs) and glutamine (GLN) can enter the ATP producing system (see text for details). 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconolactone; DHAP, dihydroxyacetone phosphate; FRU6P, fructose 6-phosphate; FRUBP, fructose 1,6-bisphosphate; GA3P, glyceraldehyde 3-phosphate; GLU, glutamate; G6PDH, glucose 6-phosphate dehydrogenase; G6PGDH, 6-phosphogluconate dehydrogenase; GT, glutamine transporter; GPI, phosphoglycose isomerase; HK, hexokinase; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; PDH, pyruvate dehydrogenase; PFK, phospofructokinase; RL5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; TA, transaldolase; TK, transketolase; TPI, triosephosphate isomerase.

process occurs in a stepwise manner yielding one molecule each of AcCoA, NADH and $FADH_2$ per step. The end product is either AcCoA (even-numbered FAs) or propionyl-CoA (odd-numbered FAs). The latter molecule can be converted into succinyl-CoA, which is an intermediate of the TCA cycle. Also, the oxidation of amino acids occurs entirely in the mitochondrial matrix. To this end, amino acids are first deaminated and then, depending on the type of amino acid, processed to PYR, AcCoA or an intermediate of the TCA cycle (α-ketoglutarate, succinyl-CoA, fumarate or oxaloacetate) (Lunt and Vander Heiden, 2011).

Intermediates can be withdrawn from the above oxidation processes, e.g., for the synthesis of neurotransmitters and amino acids (Dienel, 2012). Furthermore, GLC can be metabolized through the pentose phosphate pathway (PPP; Figure 1), yielding reduced nicotinamide adenine dinucleotide phosphate (NADPH) for anabolic reactions and pentoses for the synthesis of nucleotides and aromatic amino acids.

For the oxidation processes to continue, reoxidation of the reduced electron carriers (NADH and $FADH_2$) is a prerequisite. This reoxidation can take place in the cytosol by the enzyme lactate dehydrogenase (LDH) and in the mitochondrial matrix by the combined action of the enzymes and electron carriers of the electron transport chain (ETC; Smeitink *et al*, 2001). During the LDH reaction, the NADH electrons are transferred to PYR, yielding LAC, whereas during the ETC reaction, the NADH and $FADH_2$ electrons are transferred to molecular oxygen (O_2), yielding H_2O. The mitochondrial inner membrane (MIM) is impermeable to NADH, and under normal conditions of oxygen supply the electrons of cytosolic NADH are transferred across this membrane by shuttle systems such as the malate-aspartate shuttle and the glycerol-phosphate shuttle, yielding cytosolic NAD^+ for continuation of glycolysis and mitochondrial NADH (malate-aspartate shuttle) or $FADH_2$ (glycerol-phosphate shuttle) for reoxidation by the ETC.

Together, the enzymes of the ETC convert the oxidation energy temporarily stored in NADH and $FADH_2$ into an electrochemical proton gradient across the MIM that is used by a proton-transporting enzyme (F_1F_o-ATP synthase) to produce ATP. This process is referred to as oxidative phosphorylation (OXPHOS). Here, it is important to realize that many other MIM transporters are driven by the electrochemical proton gradient and it is for that reason that a proper

electrochemical proton gradient is essential for the maintenance of mitochondrial integrity and many other aspects of mitochondrial function (apoptosis, innate immunity, redox control, calcium homeostasis and several biosynthetic processes) (Kwong *et al*, 2007; Wang and Youle, 2009; Koopman *et al*, 2010, 2012; Arnoult *et al*, 2011; Mammucari *et al*, 2011). In addition, some energy of the electrochemical proton gradient is used for thermogenesis.

The balance between cytosolic and mitochondrial ATP production depends on the type of cell and its physiological demands and environmental conditions (supply of fuel molecules and O_2). Some cells depend completely on cytosolic ATP production and produce LAC to reoxidize NADH (mature red blood cells), others depend largely on the complete oxidation of GLU (brain cells) or FAs (liver cells) and use O_2 as the final electron acceptor, again others oxidize mainly FAs at rest and GLU at a sudden burst of activity (skeletal muscle cells). In the latter case, LAC is produced because of a hampered supply of O_2. In terms of ATP production, the maximum yield per molecule of GLU is 2 ATP in the case of oxidation to LAC and $\sim$30 ATP in the case of full oxidation to CO_2 and H_2O (Rich, 2003). Under pathological conditions, the mechanism of ATP production can change dramatically. For instance, most cancer cells oxidize GLU to LAC to produce ATP, even in the presence of O_2 (Warburg effect) (Cairns *et al*, 2011). Other pathological conditions are caused by inborn errors of enzymes that convert energy from fuel molecules to NADH, $FADH_2$ and ATP by substrate phosphorylation or from NADH and $FADH_2$ to ATP by OXPHOS. Moreover, such errors can develop in time, e.g., as a consequence of insufficient control of reactive oxygen species (ROS) levels.

Neurons are high consumers of ATP and because they have no glycogen stores they depend entirely on the uninterrupted supply of GLU through the extracellular fluid. For the same reasons, neurons preferentially oxidize GLC to CO_2 and H_2O providing the highest yield of ATP per GLU. Therefore, maintenance of mitochondrial integrity and function is of highest priority to these cells. Mitochondria are motile organelles that exhibit fusion and fission and display a dynamic internal structure (Benard and Rossignol, 2008). The balance between these processes determines net mitochondrial (ultra)structure and distribution, which is linked to mitochondrial (dys)function and metabolism during healthy and pathological conditions including neurodegeneration (Knott *et al*, 2008; Lizana *et al*, 2008; Willems *et al*, 2009; Dieteren *et al*, 2011; Campbell *et al*, 2012; Court and Coleman, 2012; Kageyama *et al*, 2012). In humans, a (progressive) decrease in mitochondrial function in general, and of the OXPHOS system in particular, has been linked to neurodegeneration during normal ageing and many other conditions including inborn errors of energy metabolism, amyotrophic lateral sclerosis (ALS), Parkinson disease (PD), Alzheimer disease (AD), Huntington disease (HD), certain forms of (brain) cancer, diabetes, epilepsy, obesity, cognitive impairment, psychosis and anxiety (Chandra and Singh, 2011; Martin, 2011; Anglin *et al*, 2012; Costa and Scorrano, 2012; Finsterer and Mahjoub, 2012; Nunnari and Suomalainen, 2012; Schapira, 2012).

OXPHOS inhibition is also evoked by off-target (drug) effects, likely differentially affecting healthy individuals and patients with mitochondrial dysfunction (Wallace, 2008; Dimauro and Rustin, 2009; Cohen, 2010; Finsterer and Segall, 2010; Morán *et al*, 2012). For example, mice with fatal encephalomyopathy due to mitochondrial dysfunction were 2.5- to 3-fold more sensitive to the volatile anaesthetics isoflurane and halothane than wild-type (wt) mice (Quintana *et al*, 2012). Moreover, environmental toxins including rotenone and persistent organic pollutants (POPs) like the insecticide dichlorodiphenyltrichloroethane (DDT), the herbicide and industrial waste product 2,3,7,8-tetrachlorodibenzodioxin (TCCD) and the phenolic flame retardant tetrabromobisphenol A (TBBPA) directly or indirectly inhibit OXPHOS function (Lee *et al*, 2010; Schapira, 2010). During recent years, substantial progress has been made in understanding the role of mitochondrial dysfunction in neurodegeneration. We recently argued that understanding the cellular (patho)physiology of monogenic mitochondrial disorders, particularly those associated with (relatively rare) OXPHOS mutations, will not only enhance our understanding of mitochondrial (dys)function but is also therapeutically relevant for the many diseases in which OXPHOS function is disturbed (Koopman *et al*, 2012). Below we first provide a theoretical background regarding the OXPHOS system. This is followed by an inventory of OXPHOS genes that are, when mutated, associated with neurodegeneration in humans. Finally, we present the insights obtained from studying the consequences of mutations in OXPHOS structural and assembly genes in living cells.

The mitochondrial OXPHOS system

The OXPHOS system (Figure 2) consists of five MIM-embedded multisubunit complexes: complex I (CI or NADH:ubiquinone oxidoreductase; EC 1.6.5.3), complex II (CII or succinate:ubiquinone oxidoreductase; EC 1.3.5.1), complex III (CIII or ubiquinol:cytochrome c oxidoreductase; EC 1.10.2.2), complex IV (CIV or cytochrome-*c* oxidase; EC 1.9.3.1) and complex V (CV or F_oF_1-ATP-synthase; EC 3.6.1.34). These complexes are divided into two functional parts: (i) the four complexes (CI–CIV) of the ETC and (ii) CV that generates ATP (Distelmaier *et al*, 2009; Smeitink *et al*, 2001; Koopman *et al*, 2012). Genetically, 92 different genes encoding structural OXPHOS subunits have been identified (Figure 2). CII is exclusively derived from the nuclear DNA (nDNA), whereas the other OXPHOS complexes contain subunits that are encoded by nDNA and the mitochondrial DNA (mtDNA). In addition to the structural OXPHOS subunit genes, the mtDNA also contains genetic information for the 2 mitochondrial ribosomal RNAs (mt-rRNAs) and the 22 mitochondrial transfer RNAs (mt-tRNAs). All proteins involved in mtDNA repair, replication, transcription, translation and maintenance of the mitochondrial deoxynucleoside triphosphate (dNTP) pool, as well as mt-tRNA synthetases and mitochondrial ribosomal proteins, are nDNA encoded (Peralta *et al*, 2012). Biogenesis of a functional OXPHOS system further requires a large set ($>$75) of nDNA-encoded proteins (Supplementary Table 1).

CI is the largest OXPHOS enzyme proposed to consist of 45 different subunits. Recent evidence suggests a number of 44 subunits since the NDUFA4 protein hitherto classified as a CI constituent appears to be a component of CIV (Balsa *et al*, 2012). Seven CI subunits (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) are encoded by the mtDNA and the remainder by

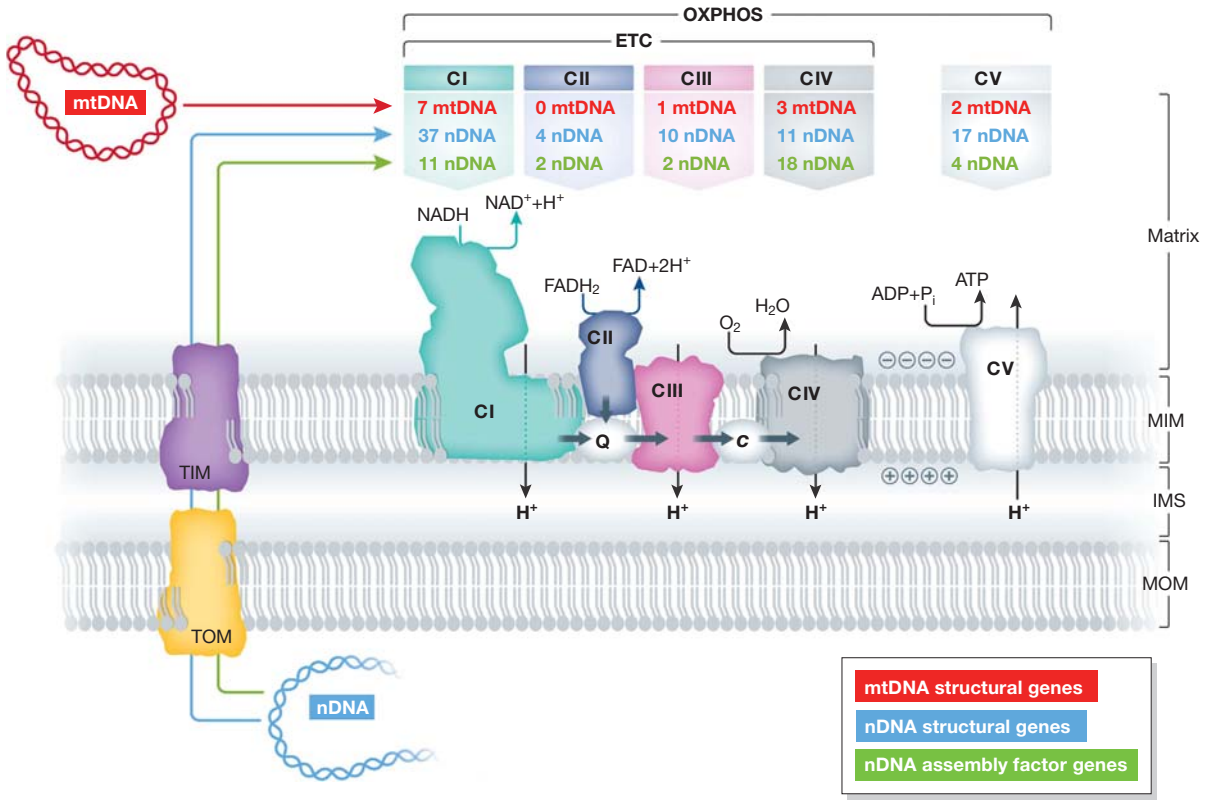

Figure 2 Genetic origin and functional interaction of the mitochondrial oxidative phosphorylation (OXPHOS) complexes. The mitochondrial OXPHOS system consists of five multisubunit complexes (CI–CV) that reside in the mitochondrial inner membrane (MIM). The MIM encloses the mitochondrial matrix and is surrounded by the mitochondrial outer membrane (MOM). An inter-membrane space (IMS) is located between the MIM and MOM. The subunits of CI, CIII, CIV and CV are encoded by the mitochondrial (mtDNA; red) and nuclear DNA (nDNA; blue), whereas CII exclusively consists of nDNA-encoded subunits (table at the top). OXPHOS biogenesis is mediated by nDNA-encoded assembly factors (green). The nDNA-encoded proteins are imported into the mitochondrial matrix via the TOM (translocator of the inner membrane) and TIM (translocator of the inner membrane) systems. At CI and CII, NADH and $FADH_2$ are oxidized, respectively, and the released electrons are transported to CIII via Coenzyme Q_{10} (CoQ_{10}; 'Q'). From thereon, electrons are transported to CIV via cytochrome-*c* (cyt-*c*; '*c*') and donated to oxygen (O_2). Together, CI–CIV constitute the electron transport chain (ETC). The energy derived from the electron transport is used to expel protons (H^+) from the mitochondrial matrix across the MIM. This establishes an electrochemical proton-motive force, associated with an inside-negative mitochondrial membrane potential ($\Delta\psi$) and increased matrix pH. The controlled backflow of H^+ is used by CV to drive the production of ATP (see text for details).

the nDNA (Figure 2; Supplementary Table 1). CI oxidizes NADH to NAD^+ and donates the released electrons to the electron carrier coenzyme Q_{10} (CoQ_{10}, a.k.a. ubiquinone). To perform its enzymatic reactions, CI only requires a set of 14 evolutionary conserved 'core subunits', consisting of the 7 mtDNA-encoded ND subunits and 7 nDNA-encoded subunits (NDUFV1, NDUFV2, NDUFS1, NDUFS3, NDUFS7, NDUFS8; Koopman *et al*, 2010; Hirst, 2011). The remaining subunits are denoted as 'accessory' or 'supernumerary'. Although the role of accessory subunits in CI biogenesis, stability and function still is incompletely understood, recent evidence in the aerobic yeast *Yarrowia lipolytica* suggests that they are important for CI stability (Angerer *et al*, 2011). Biogenesis of holo-CI is assisted by at least 11 assembly factors (NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, C8orf38, C20orf7, ACAD9, FOXRED1, ECSIT, NUBPL and OXA1L). Details about the CI assembly mechanism are provided elsewhere (e.g., Vogel *et al*, 2007; Dieteren *et al*, 2008, 2011; Koopman *et al*, 2010; Mckenzie and Ryan, 2010; Perales-Clemente *et al*, 2010; Moreno-Lastres *et al*, 2012; Nouws *et al*, 2012). In mammals, fungi and bacteria CI displays an L-shaped form consisting of a hydrophilic (matrix-protruding) and a lipophilic (MIM-embedded) arm (Clason *et al*, 2010). During recent years, significant progress has been made in understanding the link between electron and H^+ transport in CI (Sazanov and Hinchliffe, 2006; Efremov *et al*, 2010; Hunte *et al*, 2010; Efremov and Sazanov, 2011a, b). In the proposed coupling mechanism, electrons extracted from NADH are transported by a chain of iron-sulphur (Fe-S) clusters (Xu and Møller, 2011) to CoQ_{10} (Hinchliffe and Sazanov, 2005; Hayashi and Stuchebrukhov, 2010). This transport is linked to H^+ translocation due to long-range conformational changes within the complex (Onishi, 2010; Efremov and Sazanov, 2011a, b).

CII constitutes part of both the OXPHOS system and TCA cycle, oxidizes $FADH_2$ to flavin adenine dinucleotide (FAD) and also transfers the released electrons to CoQ_{10} (Figure 2). CII is a heterotetrameric complex consisting of four nDNA-encoded subunits (SDHA, SDHB, SDHC and SDHD) and its assembly is assisted by two assembly factors (SDHAF1 and SDHAF2; Supplementary Table 1). Details about CII biogenesis are provided elsewhere (Rutter *et al*, 2010). Structurally, the SDHC and SDHC subunits are embedded in the MIM, whereas SDHA and SDHB protrude in the mitochondrial matrix (Brière *et al*, 2005). SDH-encoding genes are tumour suppressors, and their mutation predisposes carriers to carotid body paragangliomas and adrenal gland pheochromocytomas (Raimundo *et al*, 2011). In addition to

CI and CII, also other enzymes can potentially donate electrons to CoQ_{10}. These include: (i) the MIM-associated electron-transferring flavoprotein (ETF)-ubiquinone oxidoreductase, which transfers electrons generated during the flavin-linked oxidation step in the catabolism of FAs, (ii) s,n-glycerophosphate dehydrogenase and (iii) dihydroorotate dehydrogenase, present only in certain types of mitochondria (see Koopman *et al*, 2010 and the references therein). Electrons from CoQ_{10} are received by CIII and transported further to CIV by the electron carrier cytochrome-*c* (cyt-*c*). Similarly to CoQ_{10}, cyt-*c* can receive electrons from an alternative source (especially in the liver) during oxidation of sulphur-containing amino acids by sulphite oxidase. However, this reaction usually occurs at a very low rate relative to other ETC inputs (see Koopman *et al*, 2010 and the references therein).

CIII contains 11 subunits, one of which is encoded by the mtDNA (CYB). Its assembly is described elsewhere (Smith *et al*, 2012) and requires the action of two identified assembly factors (BCS1L and UQCC; Supplementary Table 1). At CIV, electrons are donated to molecular oxygen (O_2) to form water. About 95% of the O_2 we breathe is consumed by CIV (Ferguson-Miller *et al*, 2012). CIV consists of 14 subunits, 3 of which are mtDNA-encoded (CO1, CO2, and CO3), and its biogenesis is assisted by at least 18 assembly factors (Supplementary Table 1), as discussed in detail elsewhere (Mick *et al*, 2011). At three sites in the ETC (CI, CIII and CIV), the energy released by the electron transport is used to drive the trans-MIM efflux of protons (H^+) from the mitochondrial matrix. As a consequence, a trans-MIM proton motive force (PMF or Δp_m) is established, which consists of an (inside negative) electric charge ($\Delta\psi$) and (inside more alkaline) pH (ΔpH) difference across the MIM (Mailloux and Harper, 2012; Figure 2).

At CV, the energy released by the controlled backflow of H^+ is coupled to the formation of ATP from ADP and inorganic phosphate (P_i). Experimental evidence in eukaryotes revealed that each ATP produced requires the CV-mediated backflow of 2.7 protons (Watt *et al*, 2010). CV is built up of 19 subunits, 2 of which are encoded by the mtDNA (ATP6 and ATP8), and its assembly requires 4 nDNA-encoded proteins (Supplementary Table 1). CV is a molecular machine composed of two mechanical rotary motors (F_o and F_1), which interconvert the chemical energy of ATP hydrolysis and H^+ electrochemical potential via a mechanical rotational mechanism (e.g., Okuno *et al*, 2011; Watanabe *et al*, 2011; Jonckheere *et al*, 2012a). This means that CV can either dissipate Δp_m to generate ATP, or use ATP to fuel the trans-MIM efflux of H^+. The latter condition sustains Δp_m and is known as the 'reverse-mode' of CV (Chinopoulos and Adam-Vizi, 2010). In addition to ATP generation, the $\Delta\psi$ and/or ΔpH gradient is also required for mitochondrial fusion, the import of mitochondrial preproteins and the exchange of metabolite and ions with the cytosol (Figure 3), as reviewed previously (Garlid and Paucek, 2003; Kaasik *et al*, 2007; O'Rourke, 2007; Klingenberg, 2008; Palmieri, 2008; Koopman *et al*, 2010; Becker *et al*, 2012).

Supramolecular architecture of the OXPHOS system

In bovine heart mitochondria, the unit stoichiometry of the OXPHOS system equalled 1/1.3/3/6.7/0.5 for CI/CII/CIII/CIV/CV and 2–5 units of the adenine nucleotide translocase (ANT; Lenaz and Genova, 2007), which mediates the trans-MIM exchange of ADP and ATP (Figure 3). Analysis of various rat tissues (Benard *et al*, 2006) revealed different molecular CII/CoQ_{10}/CIII/cyt-*c*/CIV ratios in heart (1:24:3:12:8), kidney (1:73:3:18:7), muscle (1:58:3:11:7), brain (1:58:3:35:8) and liver (1:135:3:9:7). This suggests that the amount of CoQ_{10} and cyt-*c* display tissue-dependent differences, whereas CII, CIII and CIV do not. Statistical analysis predicted that different tissues display different sensitivities to a pathological OXPHOS defect, with brain being more sensitive than liver and kidney tissue but less sensitive than skeletal muscle and heart tissue (Benard *et al*, 2006). Experimental evidence suggests that CI assembly/stability depends on its interaction with other OXPHOS complexes (Schägger *et al*, 2004). In addition, CIII is required to maintain CI (Acín-Pérez *et al*, 2004) and deficiency of CIV reduces CI function (Suthammarak *et al*, 2009). Moreover, in human patient cells the presence of a truncated CIV subunit destabilized not only CIV but also other ETC complexes, leading to their rapid clearance by mitochondrial quality control systems (Hornig-Do *et al*, 2012). These observations, supported by other experimental evidence (reviewed in Boekema and Braun, 2007; Wittig and Schägger, 2009; Dudkina *et al*, 2010; and Winge, 2012), are compatible with a model in which individual OXPHOS complexes are not randomly distributed but organized in supercomplexes (or 'respirasomes'). The finding that CIII and CIV are not essential for the assembly/stability of CI in fungi (Maas *et al*, 2009) suggests that respirasome formation and/or stability might be species and/or tissue dependent. Although it was previously suggested that CIII interacts with CII (Chen *et al*, 2008), the current view is that respirasomes consist of CI, CIII and CIV (Boekema and Braun, 2007; Wittig and Schägger, 2009; Dudkina *et al*, 2010; Althoff *et al*, 2011; Winge, 2012). In order of decreasing abundance, respirasome composition in bovine heart is predicted to be I-III_2-IV_1, I-III_2, I-III_2-IV_2 and I-III_2-IV_{3-4} (Schägger and Pfeiffer, 2001; Winge, 2012). *In silico* evidence highlighted the involvement of lipids in the gluing together of the OXPHOS complexes at the interfaces (Dudkina *et al*, 2011). Based on biochemical evidence, respiratory strings of CI, CIII and CIV have been proposed meaning that respirasomes might not be the highest level of organization of the OXPHOS system (Wittig and Schägger, 2009; Dudkina *et al*, 2010). Also CV forms higher oligomeric structures from dimeric building blocks, thought to be involved in maintaining cristae structure (Wittig and Schägger, 2009; Dudkina *et al*, 2010; Davies *et al*, 2011). Although no live-cell data are available yet, evidence demonstrating that the activity of supercomplexes as true respirasomes has been presented (Acín-Pérez *et al*, 2008). This study revealed that: (i) respirasome formation requires the presence of all of its constituting complexes, (ii) there is a time-lag between assembly of the individual OXPHOS complexes and respirasome formation. These findings support a model in which individual holoenzymes are first preassembled and subsequently combined into respirasomes. This view was recently challenged by a study demonstrating that respirasome biogenesis is mediated by a CI assembly intermediate, which acts as a scaffold for the combined incorporation of CIII and CIV subunits into the respirasome (Moreno-Lastres *et al*, 2012). The study of the Enriquez laboratory (Acín-Pérez *et al*, 2008) further

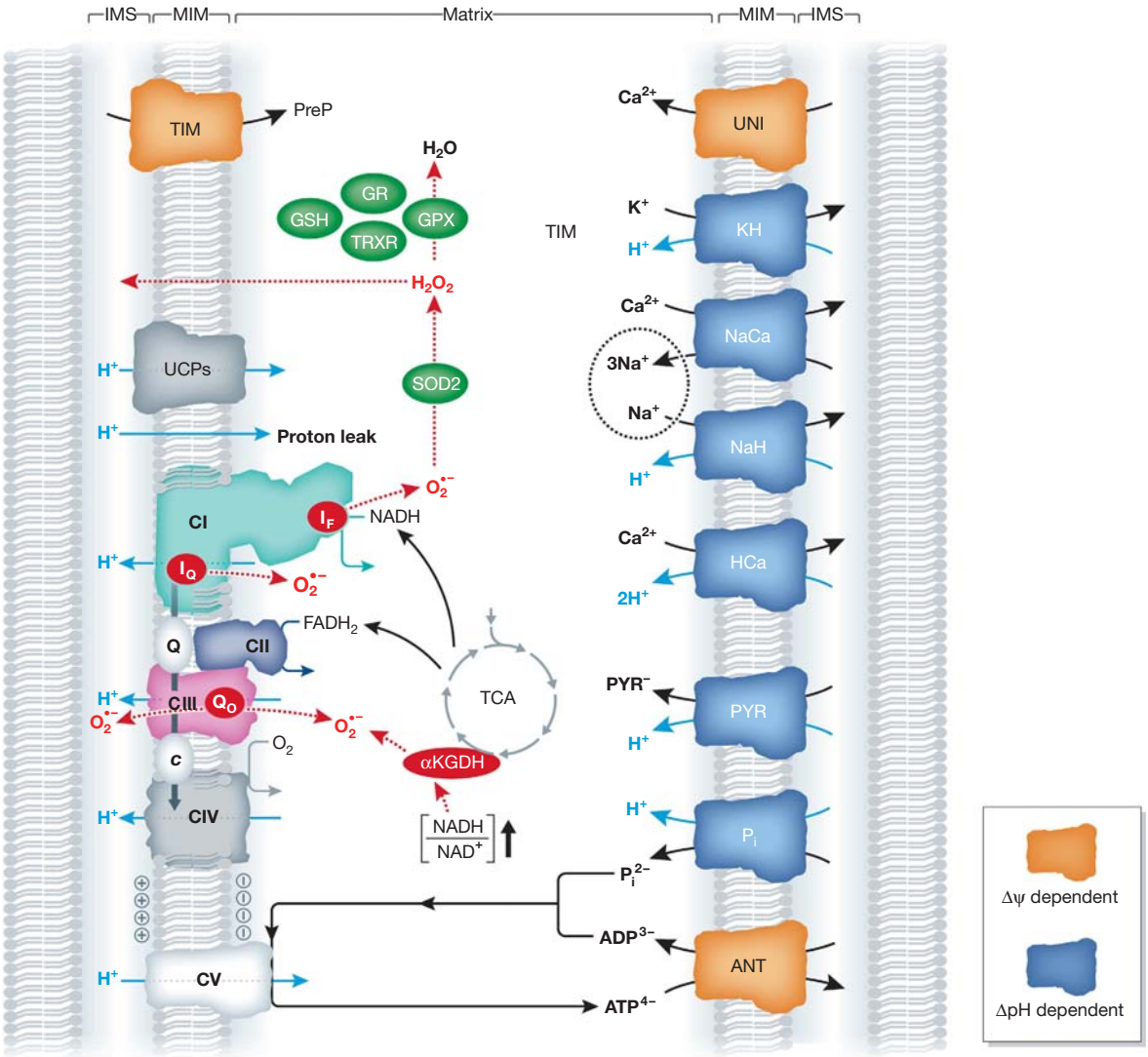

Figure 3 Integration of the OXPHOS system and mitochondrial metabolism. The five OXPHOS complexes, depicted on the lower left of the figure (see also Figure 2), maintain the inside-negative mitochondrial membrane potential (Δψ) and generate reactive oxygen species (ROS; red) in the form of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). ROS can also be generated by the TCA cycle enzyme α-ketoglutarate dehydrogenase (αKGDH), under conditions of elevated NADH/NAD$^+$ ratio. ROS are removed by several antioxidant systems (green). In addition to fuelling ATP generation by CV, a sufficiently negative Δψ is also crucial for import of nDNA-encoded mitochondrial preproteins (PreP) via the TIM system. Moreover, metabolite and ion exchange across the mitochondrial inner membrane (MIM; right part of the figure) is driven by Δψ (orange) or its associated pH gradient (ΔpH; blue) (see text for details). ANT, adenine nucleotide translocase; GR, glutathione reductase; GPX, glutathione peroxidase; GSH, glutathione; HCa, proton/calcium transporter; I_F, flavin site in CI; I_Q, CoQ_{10}-binding site in CI; KH, potassium/proton transporter; NaCa, sodium/calcium transporter; NaH, sodium/proton transporter; Pi, inorganic phosphate/proton transporter; PYR, pyruvate/proton transporter; Q_o, CoQ_{10}-binding site in CIII; SOD2, superoxide dismutase 2; TRXR, thioredoxin reductase; TIM, translocator of the inner membrane; UCP, uncoupling protein; UNI, mitochondrial calcium uniporter.

revealed that: (iii) isolated respirasomes mediate electron transfer from NADH to O_2 and (iv) respirasomes can contain CoQ_{10} and cyt-*c*. The latter suggests that also cyt-*c* is associated with respiratory supercomplexes, compatible with the observation that fibroblasts from cyt-*c* knockout mice lacked fully assembled CI and CIV and displayed lower levels of CIII (Vempati *et al*, 2009), and evidence that cyt-*c* and CoQ_{10} are functionally compartmentalized (Benard *et al*, 2008). The first protein factor (HIGD2A) required for respirasome assembly and stability in mammals was recently identified (Chen *et al*, 2012; Strogolova *et al*, 2012; Vukotic *et al*, 2012). HIGD2A is a homologue of Rcf1 (respiratory supercomplex factor 1), which mediates CIII/CIV supercomplex formation in yeast (Shoubridge, 2012). Respirasomes display a reduced stability in Barth syndrome patients, which carry a mutation in the tafazzin (*TAZ*) gene encoding a putative phospholipid acyltransferase involved in cardiolipin (CL) remodelling (McKenzie *et al*, 2006). This suggests that respirasomes are stabilized by CL (Wittig and Schägger, 2009), which is a mitochondria-specific lipid dimer consisting of two phosphatidyl groups bridged by a glycerol. CL is highly unsaturated and therefore has a high susceptibility to peroxidative attack. CL is tightly bound to CI and its oxidation reduces CI activity (Paradies *et al*, 2002). Furthermore, CL interacts with a variety of mitochondrial proteins including the other OXPHOS complexes, cyt-*c* and

the ANT (see Koopman *et al*, 2010 and the references therein). CL improves OXPHOS efficiency, stimulates mitochondrial filamentation, affects cristae morphology and provides a mitochondria-specific activation platform for caspase-8 downstream of apoptotic Fas signalling (Gonzalvez *et al*, 2008; Claypool and Koehler, 2012). The presence of CL is also critical for the degree of oligomerization in CV assemblies by promoting the ribbon like assembly of CV dimers and thereby the lateral organization and morphology of the cristae membrane (Acehan *et al*, 2011). In addition to respirasomes, structural evidence suggests the existence of 'ATPasomes' in the MIM (Bénit *et al*, 2010; Saks *et al*, 2010). These ATPasomes contain CV, the inorganic phosphate/proton transporter, and the ANT in a stoichiometry of 1:1:1. Their existence supports a mechanism through which mitochondrial ADP and P_i import, ATP synthesis and ATP export (Figure 3) occur in a highly localized manner. It remains to be established whether respirasomes can structurally combine with ATPasomes *in vivo* and if they are fixed structures or can be dynamically formed and disassembled on demand (Wittig and Schägger, 2009).

Regulation of OXPHOS function

At the cellular level, OXPHOS function can be controlled in many ways including expression regulation, post-translational modifications, metabolite-binding, second messenger systems, substrate availability and by uncoupling the ETC from CV. It is to be expected that these control mechanisms are disturbed and/or mediate adaptive responses during neurodegeneration. OXPHOS expression is controlled by the action of transcriptional activators (e.g., NRF-1 (nuclear respiratory factor 1), NRF-2, ERRa, CREB and YY1) and members of the peroxisome proliferator-activated receptor (PPAR) gamma coactivator (PGC-1) family (i.e., PGC1-α, PGC1-β and PGC-1-related coactivator; Scarpulla, 2012). Expression of these transcriptional (co)activators is regulated by other cues like temperature, nutrient availability and metabolic status (Handschin and Spiegelman, 2006; Scarpulla, 2008). With respect to OXPHOS structural proteins, several (regulatory) modifications have been described including: (i) phosphorylation (CI, CII, CIII, CIV and CV; Pagliarini and Dixon, 2006; Carlucci *et al*, 2008; Yadava *et al*, 2008; Kane and Van Eyk, 2009; Koopman *et al*, 2010; Hebert-Chatelain *et al*, 2012; Helling *et al*, 2012; Papa *et al*, 2012), (ii) acetylation (CI, CII and CV; Guan and Xiong, 2011), (iii) glycosylation (CI, CII and CV; Burnham-Marusich and Berninsone, 2012), (iv) cleavage by caspases (CI, CII and CV; Ricci *et al*, 2004; Martinvalet *et al*, 2008; Zhu *et al*, 2012) and (v) modification by ROS and/or reactive nitrogen species (RNS) mediated mechanisms (CI, CII, CIII, CIV and CV; Murray *et al*, 2003; Choksi *et al*, 2004; Galkin and Moncada, 2007; Chen *et al*, 2008; Hurd *et al*, 2008; Taylor and Moncada, 2009; Chinta and Andersen, 2011; Danielson *et al*, 2011; Wang *et al*, 2011). Recent evidence suggests that phosphorylation and acetylation of mitochondrial ribosomal proteins and translation factors allow for regulation of mitochondrial protein synthesis (Koc and Koc, 2012). In addition to phosphorylation, it was argued that CIV activity is (co)regulated by various biomolecules (e.g., ATP/ADP ratio, FAs and CL), as well as several of its nDNA-encoded subunits (e.g., subunit 5A and subunit 6A isoforms; Arnold, 2012). OXPHOS activity is also controlled by diverse second messenger systems (for detailed information, see Boneh, 2006 and Pagliarini and Dixon, 2006) including cyclic AMP (cAMP), Ca^{2+}, ceramide and ROS (also see below). In adipose tissue, the coupling between electron transport (ETC action) and ATP production (CV action) is reduced ('uncoupled') by uncoupling protein 1 (UCP1). UCPs mediate the trans-MIM backflow of H^+ and thereby bypass CV, resulting in heat generation (Divakaruni and Brand, 2011). Interestingly, analysis of live cells with a fluorescent molecular thermometer sensor revealed that the local temperature near mitochondria is higher than the temperature of the rest of the space in the cytosol (Okabe *et al*, 2012). Moreover, this temperature increased when cells were treated with the chemical uncoupling molecule 4-(trifluoromethoxy)phenylhydrazone (FCCP). It appears that members of the UCP family (UCP2, UCP4 and UCP5; Figure 3) are also expressed within the central nervous system (CNS; Andrews *et al*, 2005). Interestingly, these UCPs do not act as constitutive uncouplers, but are activated by ROS and free FAs. In this sense, neuronal UCPs can regulate mitochondrial biogenesis, Ca^{2+} flux, ROS production and local temperature, thereby directly affecting neurotransmission, synaptic plasticity and neurodegenerative processes (Andrews *et al*, 2005). ROS-induced UCP2 activation has also been implied in minimizing ROS emission from the ETC, thus providing a negative feedback loop for mitochondrial ROS production (Mailloux and Harper, 2012). Metabolic control analysis (MCA) of several tissues including brain suggests that the control over the flux through the OXPHOS system is shared by essentially all components of this system (Pathak and Davey, 2008; Korzeniewski, 2011). This means that in order to significantly increase this flux (for instance during cell activation), and at the same time maintain relatively constant concentrations of intermediate metabolites (e.g., ADP, ATP, P_i and NADH), direct activation of multiple components of the OXPHOS system is required (the 'multistep parallel activation' mechanism). Similarly, theoretical analysis of mathematical models of OXPHOS in heart, skeletal muscle and liver suggests that mitochondrial Ca^{2+} uptake during cell activation stimulates OXPHOS function at several sites (Korzeniewski, 2011). Although analysis of cardiac submitochondrial particles revealed that Ca^{2+} inhibits CI and thereby reduces electron transport activity (Matsuzaki and Szweda, 2007), the current consensus is that mitochondrial Ca^{2+} uptake from the cytosol directly or indirectly modulates the activity of mitochondrial transporters and enzymes including the glutamate/aspartate exchanger, PDH, the TCA cycle enzymes isocytrate dehydrogenase (ICD) and α-ketoglutarate dehydrogenase (αKGDH), CIII and CV (Willems *et al*, 2008; Gellerich *et al*, 2010; Glancy and Balaban, 2012). This suggests that Ca^{2+} might be a mediator of multistep parallel activation of the OXPHOS system during cell activation. Interestingly, experimental results in plants suggest that the activity of individual complexes within supercomplexes can be regulated (Ramírez-Aguilar *et al*, 2011). This process involved (reversible) dissociation of these complexes from the supercomplex depending on the O_2 tension and the pH of the mitochondrial matrix.

Role of the OXPHOS system in CNS energy metabolism

The central nervous system (CNS) consists of two types of cells: neurons and glial cells (Kandel *et al*, 1995). Neurons can be classified based upon the number of processes that originate from the cell body as unipolar (e.g., an invertebrate neuron), bipolar (e.g., a bipolar retinal cell), pseudo-unipolar (e.g., a dorsal root ganglion) or multipolar (e.g., a spinal cord motor neuron, a hippocampal pyramidal cell or a cerebellar Purkinje cell). The principal types of glial cells in the nervous system are oligodendrocytes and astrocytes (CNS) and Schwann cells (peripheral nervous system; Kandel *et al*, 1995). Brain functioning requires a large amount of energy, which is highly dependent on the external supply of energy substrates delivered by the circulation and OXPHOS action, as reflected by the tight coupling between GLC and O_2 delivery from the vasculature (Bélanger *et al*, 2011). Most evidence supports GLC as the major fuel for normal, metabolically active, brain (Dienel, 2012). In the latter condition, the majority of brain energy is consumed by synaptic transmission (Harris *et al*, 2012). GLC enters the brain from the circulation mediated by GLUT1 in the microvascular endothelial cells of the blood–brain barrier (BBB) and glial cells and GLUT3 in neurons (Simpson *et al*, 2007). Astrocytes represent the most abundant cell type in the brain and are also present in the spinal cord. Glial cells play an important role in many cellular processes including glutamate, ion and water homeostasis and ROS detoxification (Volterra and Meldolesi, 2005; Bélanger and Magistretti, 2009; Bélanger *et al*, 2011). Metabolically, evidence was provided that both neurons and astrocytes rely on OXPHOS for ATP generation whereas astrocytes also possess energy stores in the form of glycogen (Hertz *et al*, 2007; Bélanger *et al*, 2011). It appears that astrocytes and neurons are metabolically linked by LAC shuttling (for a critical review, see Dienel, 2012). Recent evidence (Choi *et al*, 2012) suggests that soluble adenylyl cyclase (sAC) in astrocytes becomes activated in response to bicarbonate (HCO_3^-), which enters via the electrogenic $NaHCO_3$ cotransporter (NBC). As a consequence, astrocyte cAMP levels increase, leading to the breakdown of glycogen, stimulation of glycolysis, and release of LAC. The latter is subsequently taken up by neurons for use as an energy substrate (Choi *et al*, 2012).

With respect to human ageing, magnetic resonance imaging (MRI) and positron emission tomography (PET) examinations revealed that, during normal ageing, cerebral blood flow (CBF) and to a lesser extent the cerebral rate of O_2 consumption ($CMRO_2$) decreased with age in extended regions of the brain, with sparing of primary sensory-motor neurons and occipital cortices (Aanerud *et al*, 2012). This study further revealed significant increases of O_2 extraction fraction (OEF) in frontal and parietal cortices, excluding primary motor and somatosensory regions, and in the temporal cortex. It was concluded that the increased OEF, which can compromise O_2 delivery to neurons, possibly perturbs energy turnover. This suggests a possible mechanism of progression from healthy to unhealthy brain ageing, as the regions most affected by age were the areas most vulnerable to neurodegeneration. Analysis of mouse and rat brain slices suggests that gamma oscillations (i.e., neuronal network oscillations in the 30–100 Hz range that occur in the electroencephalogram) in the cerebral cortex are associated with high energy demand (Kann, 2011). The latter might explain why higher cognitive functions including sensual perception and working memory become disturbed during neurodegeneration (Kann, 2011).

ROS generation by the OXPHOS system

OXPHOS action is inherently coupled to the production of ROS. Under normal conditions, mitochondrial and cytosolic ROS levels are controlled by mitochondrial and cytosolic antioxidant systems and exert a signalling function. However, in case the antioxidant systems fail to keep these ROS levels within safe limits, lipids, proteins and DNA molecules are at risk of being damaged. The latter process may occur over the years leading to a gradual decline in mitochondrial and cellular integrity and function. ROS are chemical entities that are formed upon incomplete reduction of O_2 (Forkink *et al*, 2010), and their generation by the mitochondrial ETC has been proposed to play a role in neurodegeneration (e.g., Abramov *et al*, 2010; Chinta and Andersen, 2011; Correia *et al*, 2012; Court and Coleman, 2012; Hedskog *et al*, 2012; Leuner *et al*, 2012). RNS-like nitric oxide (NO) can interact with ROS and also have been implicated in neurodegeneration, but this is discussed elsewhere (Calabrese *et al*, 2009; Brown, 2010; Nakamura *et al*, 2010; Doherty, 2011; Cambron *et al*, 2012; Ramalingam and Kim, 2012). In case of ROS, redox dysregulation and/or ROS-induced stress has been linked to various neurological presentations including AD (Hedskog *et al*, 2012; Leuner *et al*, 2012; Von Bernhardi and Eugenín, 2012), PD (Chinta and Andersen, 2008; Fato *et al*, 2008; Del Hoyo *et al*, 2010), Friedreich's ataxia (FRX; Calabrese *et al*, 2005), Down syndrome (Pagano and Castello, 2012), ALS (Martin, 2011) and psychiatric conditions like schizophrenia and bipolar disorder (Clay *et al*, 2011; Manji *et al*, 2012). The association between ageing in the CNS, OXPHOS malfunction, elevated mtDNA mutation load and increased ROS-induced damage has led to the 'vicious cycle' theory (e.g., Bandy and Davison, 1990 and Balaban *et al*, 2005). The latter states that there is a feedback mechanism connecting these events in ageing and age-associated neurodegeneration. However, it is not always trivial to unequivocally determine whether ROS molecules play a damaging and/or signalling role (see below) during neurodegeneration. In this sense, it needs to be kept in mind that most evidence supporting the vicious cycle model is obtained using pharmacological inhibition of mitochondrial enzymes, which not necessary reflects the physiological situation. Moreover, recent *in vivo* evidence also contradicts the vicious cycle model (e.g., Fukui and Moraes, 2008 and Frenzel *et al*, 2010). Within mammalian cells, ROS can originate from many sources (Brown and Borutaite, 2012) including: (i) mitochondria (CI, CII, CIII, glycerol 3-phosphate dehydrogenase, the ETF:Q oxidoreductase of FA β-oxidation, αKGDH, PYR and 2-oxoglutarate dehydrogenase, $p66^{shc}$), (ii) the endoplasmic reticulum (ER) (cytochrome *P*-450 and *b5*, diamine oxidase, Ero1), (iii) peroxisomes (FA oxidation, D-amino-acid oxidase, L-2-hydroxy acid oxidase and urate oxidase), (iv) the cytosol (NO synthases, lipoxygenases and PGH synthase), (v) the plasmamembrane (NADHP oxidases, lipoxygenase) and, (vi) the extracellular space (xanthine oxidase) (Boveris *et al*, 1972; Kukreja *et al*, 1986; Roy *et al*, 1994; O'Donnel and Azzi, 1996; McNally *et al*, 2003; Giorgio *et al*, 2005; Adam-Vizi and Chinopoulos, 2006; Gross *et al*, 2006; Starkov,

2008; Brand, 2010; Touyz *et al*, 2011; Quinlan *et al*, 2012). Although the relevance of each ROS source is cell-type dependent, the above suggests that mitochondria are not necessarily the main source of ROS in mammalian cells (Brown and Borutaite, 2012). However ROS generation by CI and CIII is considered relevant (Figure 3), both under physiological and pathological conditions (Adam-Vizi and Chinopoulos, 2006; Koopman *et al*, 2010; Pryde and Hirst, 2011; Treberg *et al*, 2011). In addition, recent evidence in rat skeletal muscle mitochondria suggests that CII can generate ROS at high rates under conditions that CI and CIII are inhibited and succinate concentration is low (Quinlan *et al*, 2012). Also αKGDH (Figure 3) is able to generate ROS in the mitochondrion when the $NADH/NAD^+$ ratio is increased (Tretter and Adam-Vizi, 2005). The ROS family consists of a large collection of molecules, but biologically most of them are derived from superoxide ($O_2^{\cdot -}$) and/or hydrogen peroxide (H_2O_2). The amount of cellular ROS generated, as well as its primary source, varies with the type of ROS, the type of cell, the organism from which the cells were derived, metabolic state, the (patho)physiological condition and the presence of ROS-detoxifying (or consuming) systems (Adam-Vizi and Chinopoulos, 2006; Brown and Borutaite, 2012). In case of the OXPHOS system, $O_2^{\cdot -}$ appears to be generated (in descending order of maximal capacity) by the CoQ_{10}-binding sites in CI (site I_Q) and CIII (site Q_o) and the flavin in CI (site I_F; Muller *et al*, 2004; Dröse and Brandt, 2008; Brand, 2010; Quinlan *et al*, 2011). These three sites all release $O_2^{\cdot -}$ into the mitochondrial matrix (Figure 3), whereas site Q_o also produces $O_2^{\cdot -}$ into the space between the MIM and MOM, the mitochondrial intermembrane space (IMS). How these individual sites contribute to ROS generation in the absence of ETC inhibitors is still unclear, but it is expected that this considerably varies with cell/tissue type, available substrates, energy demand and O_2 tension (Brand, 2010).

Classically, ROS are considered as damaging entities because they can react with and thereby damage or modify many biomolecules including proteins, lipids and (mt)DNA (e.g., Ahmad *et al*, 2005; Dröge and Schipper, 2007; Murphy, 2009; Kourtis and Tavernarakis, 2011). In this context, cells have available an elaborate ROS detoxifying apparatus (Figure 3) consisting of enzymatic (e.g., MnSOD (manganese superoxide dismutase)/SOD2, GPX, GR, TRXR (thioredoxin reductase)) and non-enzymatic (e.g., GSH, vitamin C, vitamin E, carotenoids, and flavonoids) systems (e.g., Koopman *et al*, 2010; Aon *et al*, 2012; and Miriyala *et al*, 2012). Importantly, ROS are not only damaging to biomolecules but also act as (redox) signalling entities, possibly specifically affecting mitochondrial function (e.g., Thannickal and Fanburg, 2000; Dröge and Schipper, 2007; Koopman *et al*, 2010; Lukosz *et al*, 2010; Murphy *et al*, 2011; Distelmaier *et al*, 2012; Handy and Loscalzo, 2012; Murphy, 2012; and Perjés *et al*, 2012). In this context, evidence was provided that CIV biogenesis involves several ROS and/or redox-regulated steps (Bourens *et al*, 2012) and ROS signalling pathways are implicated in cell proliferation, survival, differentiation and metabolism (mediated by ASK1 (apoptosis signal-regulated kinase 1), PI3K (phosphoinositide-3-kinase), PTP (protein tyrosine phosphatase) and Shc (Src homology 2 domain-containing)), antioxidant and anti-inflammatory responses (TRX (thioredoxin), Ref1 (redox-factor 1) and Nrf2 (NF-E2-related factor 2)), iron homeostasis (IRP) and DNA-damage responses (ATM (ataxia-telangiectasia mutated); Ray *et al*, 2012). Moreover, an increase in ROS levels, the spatiotemporal magnitude of which by itself depends on the balance between ROS production and detoxification, often serves to activate adaptive programs that counterbalance ROS stress (Collins *et al*, 2012). ROS further (co)control the removal of dysfunctional mitochondria by mitophagy (Gomes and Scorrano, 2012; Lee *et al*, 2012; Novak, 2012; Rugarli and Langer, 2012), thereby limiting the detrimental cellular consequences of mitochondrial dysfunction and increased ROS production.

Both in healthy cells and cells from patients with an OXPHOS disorder, changes in cellular ROS levels have also been linked to mitochondrial metabolic state and net morphology (Koopman *et al*, 2007; Benard and Rossignol, 2008; Distelmaier *et al*, 2012). Mitochondrial shape is governed by the balance between mitochondrial fusion, fission and motility. These dynamics are mediated by dedicated mitochondrial fusion (e.g., mitofusins or Mfns), fission (e.g., dynamin-related protein 1 or Drp1) and motor proteins (e.g., Milton), which are controlled by cell signalling mechanisms (Lovas and Wang, 2012; Wilson *et al*, 2012). According to a recent conceptual model presented by Westermann (2012), mitochondrial shape and OXPHOS activity are closely linked. This model states that mitochondria exist in three states in which their net morphology appears: (i) 'fragmented' and OXPHOS activity is low, (ii) 'normal' and OXPHOS activity is normal and (iii) 'hyperfused' and OXPHOS activity is high. We recently provided evidence (using primary human skin fibroblasts, Chinese hamster ovary cells and immortalized mouse embryonic fibroblasts (MEFs)) suggesting that the transition between these morphological states is controlled by Mfns in an ROS-dependent manner (Distelmaier *et al*, 2012). This implies that cell-governed changes in ROS level (for instance by altering the balance between their production and detoxification) may allow regulation of mitochondrial morphology and function. Moreover, it was observed that inhibition of GSH synthesis by L-buthionine-(S,R)-sulphoximine (BSO; 12.5 μM, 72 h) shifts the cytosolic and mitochondrial thiol redox environment towards a fully oxidized state in human skin fibroblasts (Verkaart *et al*, 2007b) and that this shift is paralleled by mitochondrial shortening (Distelmaier *et al*, 2012). In contrast, another study reported that BSO treatment induces mitochondrial hyperfusion (Shutt *et al*, 2012). However in the latter experiments a different cell type (HeLa) and BSO treatment regime (100 μM, 24 h) were used, suggesting that changes in thiol redox state affect mitochondrial morphology in a time-, concentration- and cell type-dependent manner. The effect of BSO-induced GSH depletion on the mitochondrial thiol redox environment and mitochondrial shortening was counter-balanced by overexpression of BOLA1, a glutaredoxin 5 (GLRX5)-interacting protein (Willems *et al*, 2012). Another member of the BOLA family (BOLA3) was suggested to be involved in Fe-S cluster assembly and also bind GLRX5 (Cameron *et al*, 2011b).

OXPHOS mutations and neurodegeneration

Analysis of the mitochondrial proteome in 19 different mouse tissues revealed that mitochondrial functioning requires

between 1100 and 1400 genes and mitochondria from different organs share ~75% of their proteins (Mootha *et al*, 2003; Calvo and Mootha, 2010). In humans, ~1000 genes have currently been identified (Human MitoCarta; www.broadinstitute.org). In principle, a mutation in any of these genes can lead to mitochondrial dysfunction and induce a 'primary mitochondrial disorder'. When mitochondrial dysfunction occurs for another reason, this gives rise to a 'secondary mitochondrial disorder' (Koopman *et al*, 2012).

Importantly, human nDNA-encoded mutations are generally inherited in an autosomal recessive manner (Smeitink *et al*, 2001). From this perspective, cells from patients with nDNA-encoded mutations are well suited for microscopy imaging analysis since they all contain the genetic defect. In case of mtDNA mutations, the situation is much more complex since each cell contains many mitochondria and each mitochondrion contains many mtDNA molecules. MtDNA is generally inherited exclusively from the mother (maternal inheritance) and mtDNA mutations display 'heteroplasmy' (Davis and Sue, 2011; Schapira, 2012; Schiff *et al*, 2012). The latter means that normal and mutated mitochondrial genomes coexist in the same cell. The percentage of mutated versus normal mtDNAs needs to exceed a certain threshold to induce pathology (the 'threshold effect'). This threshold is tissue specific leading to them being differentially sensitive to OXPHOS dysfunction (e.g., Rossignol *et al*, 1999). The latter might be explained by the fact that OXPHOS expression greatly differs between tissues. For instance, mitochondrial (protein) abundance was highest in mouse heart tissue and equalled 40–50% of this value in kidney, brain stem, spinal cord and skeletal muscle (Pagliarini *et al*, 2008). Even lower values were observed for large intestine, cerebellum, cerebrum, small intestine, stomach and liver (25–40%), and testis, adipose, thymus, placenta, fetal tissue, lung, spleen and eye (<25%). To complicate matters even further, by examining mouse chimeras with a mixture of normal and ETC-deficient neurons ('mosaic ETC deficiency') in cerebral cortex (Dufour *et al*, 2008), it was found that the presence of a low proportion of ETC-deficient neurons sufficed to induce symptoms whereas premature death occurred only at higher proportions. Interestingly, neurons with normal ETC function ameliorated disease progression and ETC-deficient neurons adversely affected normal adjacent neurons leading to trans-neuronal degeneration (Dufour *et al*, 2008).

In the strict sense of the word 'neurodegeneration' is defined as 'any pathological condition primarily affecting neurons' or 'a disease process in which neurons are selectively and gradually destroyed, leading to a progressive loss of nervous system structure and function' (Przedborski *et al*, 2003; Deuschl and Elble, 2009). This implies that neoplasm, oedema, haemorrhage and trauma of the nervous system are not considered to be neurodegenerative disorders. Diseases of the nervous system that implicate not neurons *per se* but rather their attributes, such as the myelin sheath as seen in multiple sclerosis, are not neurodegenerative disorders either, nor are pathologies in which neurons die as the result of a known cause such as hypoxia, poison or infections' (Przedborski *et al*, 2003). Neurodegenerative disorders manifest with a heterogeneous clinical and pathological picture, affecting specific regions of the nervous system. They may present acutely and rapidly progressive or symptoms may be subtle and slowly progressive. The clinical course is generally unfavourable and therapeutic options are mostly not available. During recent years, several studies focused on the role of mitochondrial dysfunction in neurodegenerative disorders (e.g., Finsterer, 2006; DiMauro and Schon, 2008; McFarland *et al*, 2010; Schon and Przedborski, 2011). These highlighted a plethora of clinical symptoms and phenotypes. Several of them could be defined as distinct syndromes: AD, ALS, FRX, HD, cardioencephalomyopathy, Charcot-Marie Tooth disease (CMT), familial bilateral striatal necrosis (FBSN), growth retardation, amino aciduria, cholestasis, iron overload, lactic acidosis and early death (GRACILE), hereditary spastic paraparesis (HSP), Kearns-Sayre syndrome (KSS), Leber hereditary optic neuropathy (LHON), Leigh syndrome (LS), mtDNA depletion syndrome (MDS), mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy associated with ragged-red fibres (MERRF), maternally inherited diabetes and deafness (MIDD), mitochondrial neurogastrointestinal encephalopathy (MNGIE), multiple systemic lipomatosis (MSL), neuropathy, ataxia and retinitis pigmentosa (NARP), optic atrophy (OA), PD, sensory ataxic neuropathy, dysarthria, and ophthalmoparesis (SANDO) and spinocerebellar ataxias (SCAs).

Mutations affecting OXPHOS function and biogenesis in human neurodegeneration

By combining information from the literature and various databases, we here present a list of nDNA- and mtDNA-encoded OXPHOS structural/biogenesis genes which, when mutated, are associated with neurodegeneration in humans (Supplementary Table 1; Figure 4). This analysis highlighted genes encoding: (i) structural OXPHOS subunits, (ii) OXPHOS assembly factors, (iv) Fe-S biogenesis enzymes, (v) enzymes involved in the synthesis of CoQ_{10} and cyt-*c*, (vi) mt-rRNAs, (vii) mt-tRNAs, (vii) mtDNA repair enzymes, (viii) mtDNA replication, transcription and translation factors, (ix) enzymes involved in the maintenance of the mitochondrial dNTP pool, (x) mitochondrial ribosomal proteins, (xi) mt-tRNA synthetases and (xii) nucleoid-associated proteins. The latter constitute mitochondrial nucleoprotein complexes consisting of mtDNA and its associated proteins involved in mtDNA organization and protection (e.g., Spelbrink, 2010; Brown *et al*, 2011; Cameron *et al*, 2011b; and He *et al*, 2012).

Clinical aspects

From a clinical perspective, OXPHOS dysfunction presents with a wide range of neurological symptoms, including developmental regression, failure to thrive, seizures, spasticity, dystonia (movement disorder with abnormal tonicity of muscle, characterized by prolonged, repetitive involuntary muscle contractions), ataxia (loss of coordination and balance with instability of gait) and nystagmus (abnormal/oscillating eye movement). Many of these symptoms cannot be categorized into defined clinical syndromes. Especially in severe OXPHOS deficiencies, disease onset may be already neonatal, presenting with severe encephalopathy (global brain dysfunction). Apart from these 'non-syndromic' entities, there are several classical OXPHOS diseases, which are associated with neurodegeneration. We used the data in Supplementary Table 1 to compile a summary of neurological disorders associated with mutations in structural OXPHOS

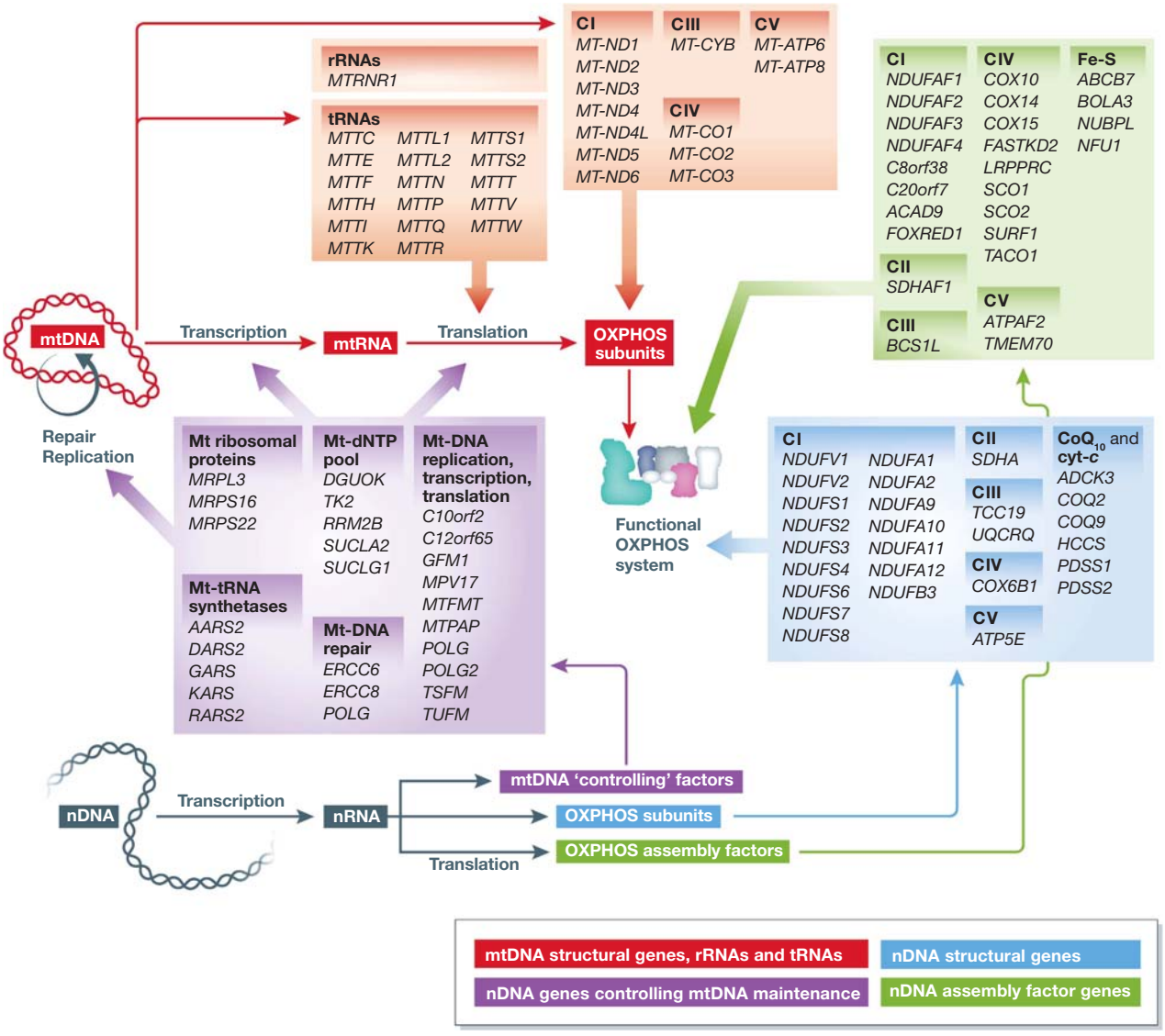

Figure 4 Biogenesis and neurodegeneration-associated mutations of the OXPHOS system. The mitochondrial DNA (mtDNA; red) encodes ribosomal RNAs (rRNAs), transfer RNAs (tRNAs) and OXPHOS subunits. Mitochondrial ribosomal proteins, tRNA synthetases, mtDNA repair proteins, dNTP (deoxynucleoside triphosphate) pool-maintaining proteins and proteins mediating mtDNA replication, transcription and translation are all encoded by the nuclear DNA (nDNA; purple). Also, OXPHOS assembly factors (green) and the remainder of the OXPHOS subunits (blue) are nDNA encoded. Mutated genes associated with neurodegeneration are indicated (italic). Gene names are given according to the HGNC (HUGO Gene Nomenclature Committee) standard (see text for details). cyt-*c*, cytochrome-c biogenesis; Fe-S, iron-sulphur cluster biogenesis; CoQ_{10}, CoQ_{10} biogenesis.

subunit and assembly factor genes (Table I). At first sight, mutations in nDNA-encoded genes are associated with different disorders than mutations in mtDNA-encoded genes. However, a certain disorder can be caused by mutations in different OXPHOS structural or assembly factor genes. Among the listed diseases, Leigh Syndrome (LS) is probably the most typical OXPHOS disease during early childhood. The main cause of LS is an isolated CI deficiency, caused by defects in its structural subunits (either mtDNA or nDNA encoded) or assembly factors. However, also mutations in other OXPHOS complexes (or in the respective assembly factors), as well as disturbances in CoQ_{10} metabolism or dysregulation in mitochondrial RNA/DNA maintenance may cause LS. This makes this syndrome one of the most frequent clinical entities. LS was first described by the British neuropathologist Denis Archibald Leigh (1916–1998) and is characterized by symmetrical necrotic lesions in the basal ganglia, especially in the putamen, or in variable areas within the brain stem (Leigh, 1951). However, lesions can also appear within other CNS regions such as the cerebellum, thalamus and even the spinal cord (e.g., Rossi *et al*, 2003; Friedman *et al*, 2010; Lebre *et al*, 2011). So far, the exact mechanism of neurodegeneration in LS is still unclear. There are no conclusive research studies, which could explain the exquisite vulnerability of circumscribed brain regions in LS patients (Wirtz and Schuelke, 2011). Classically, children with LS have a normal prenatal development and normal birth parameters. However, for respiratory chain defects in general, intrauterine growth retardation, leading to a low birth weight was reported (Yanicostas *et al*, 2011). In LS patients, the illness often takes a severe course within the first months of life, leading to developmental regression and failure to

Table I Mutated structural OXPHOS subunit and assembly factor genes associated with neurodegeneration in humans

Disorder/phenotype	Clinical features	Structural genes					Assembly factors				
		CI	CII	CIII	CIV	CV	CI	CII	CIII	CIV	CV
Bjornstad syndrome	Neurosensory deafness, twisted hair shafts (pili torti), mental retardation								N		
Generalized brain atrophy	Progressive loss of brain tissue with regression of memory and cognitive skills, altered consciousness, aphasia, failure to thrive	N		N			N				N
Dystonia	Involuntary muscle contractions, repetitive movements, abnormal postures	M									
Encephalopathy	Global brain dysfunction with altered mental state, possibly associated with respiratory abnormalities, seizures, failure to thrive	N		M	M	M	N		N	N	N
GRACILE syndrome	Severe intrauterine growth retardation, neonatal lactic acidosis, liver hemosiderosis, Fanconi-type amino aciduria								N		
Leigh (-like) syndrome	Characteristic symmetrical brain lesions, muscular hypotonia or spasticity, seizures, respiratory problems, abnormal eye movements, failure to thrive	B	N	N	M	M	N		N	N	
Leukoencephalopathy	Gradual decline in cognitive and motor functions, alteration of the sleep-wake cycle, rigidity, feeding difficulties	N			N			N			
LHON	Painless, central vision loss; may be associated with cardiac conduction defects, altered reflexes, ataxia, sensory neuropathy	M		M	M	M					
MELAS (-like) syndrome	Myoclonic epilepsy, migrainous headache, non-ischaemic strokes, muscle weakness, atrophy of the optic nerve, sensorineural deafness, ataxia	M									
NARP syndrome	Neuropathy, ataxia, retinitis pigmentosa, learning disabilities, developmental delay					M					
Parkinsonism/MELAS	Akinetic rigid motor performance, epileptic seizures, non-ischaemic strokes			M							
Sensorineural deafness	Progressive hearing loss or impairment, including total deafness	M			M						
Modifier of PD	Early-onset of PD with tremor, rigidity, slowness of movement, postural instability	M									
Susceptibility to AD	Genetic risk factor for developing AD with progressive dementia	M									
Susceptibility to PD	Genetic risk factor for developing PD (symptoms see above)	M									

AD, Alzheimer disease; B, both nDNA and mtDNA encoded; CI, complex I; CII, complex II; CIII, complex III, CIV, complex IV; CV, complex V; LHON, Leber hereditary optic neuropathy; M, mtDNA encoded; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; N, nDNA encoded; NARP, neuropathy, ataxia and retinitis pigmentosa; PD, Parkinson disease.

thrive (Distelmaier *et al*, 2009). As a consequence, most children die within the first years of life. The disease progression may suddenly accelerate, especially under the influence of intercurrent deleterious factors such as infection. Therapeutic options are generally unsatisfactory and palliative care is still a mainstay in the treatment of affected children.

In addition to LS, LHON represents an important OXPHOS-associated neurodegenerative disease. Although LHON is already known for about 150 years, it was first linked to mutations in mtDNA genes in 1988 (Wallace *et al*, 1988; Newman, 2005). As summarized in Table I, LHON may be caused by defects in CI, CIII, CIV or CV. The disease typically presents with painless visual loss. Funduscopic abnormalities include hyperaemia (increased blood flow) of the optic nerve head with obscuration of the disk margins, dilation and tortuosity of posterior pole vasculature (Newman, 2005). The pathogenesis of LHON is still not fully resolved. It was suggested that the unmyelinated, prelaminar portion of the optic nerve requires a high degree of ETC activity, which might explain the specific vulnerability of this tissue (Qi *et al*, 2003).

Another relevant clinical phenotype (Table I) is MELAS. In addition to mutations in mtDNA-encoded CI genes, this syndrome is mostly caused by a 3243A-G mutation in the *MTTL1* gene, leading to disturbed mitochondrial transcription. This frequently causes a biochemical CI and CIV deficiency (Koga *et al*, 2010). As a clinical syndrome, MELAS was first defined in the 1980s and is characterized by a combination of key symptoms, which are highlighted by the acronym for the disorder (Pavlakis *et al*, 1984). Apart from these symptoms, MELAS represents a true multisystemic disorder, potentially involving every organ, including gastrointestinal tract, heart, lungs, kidneys and skin (Sproule and Kaufmann, 2008). Almost 70% of patients present with initial symptoms between 2 and 20 years (Pavlakis *et al*, 1984). The pathogenesis of MELAS includes degenerative changes in small arteries and arterioles in the brain, accompanied by accumulation of mitochondria in vascular endothelial cells and smooth muscle cells. These abnormalities are thought to be involved in the genesis of non-ischaemic strokes in these patients. However, it was also suggested that the stroke-like episodes may reflect neuronal hyperexcitability with increases energy demand, causing an imbalance between energy requirements and inadequate ATP supply (Iizuka and Sakai, 2010; Koga *et al*, 2010).

Taken together, mutations in structural OXPHOS subunit and assembly factor genes are often associated with rare early-onset diseases displaying a devastating clinical course and some of them may present as defined clinical syndromes. Although, research is expanding our knowledge about molecular genetics and biochemistry of these diseases, numerous questions remain unsolved. Especially, the heterogeneous presentation of OXPHOS defects and the specific affection of certain tissues (e.g., the optic nerve in LHON patients, basal ganglia lesions in LS patients) in a subset of patients remain enigmatic.

Unfortunately, cell and organ material from patients is (extremely) scarce and information on brain mitochondrial function heavily relies on MRI/PET imaging and analysis of

post-mortem samples. Although certainly valuable, these techniques do not provide insight into the pathophysiology of OXPHOS gene mutations at the level of single living cells. Such information is important since mitochondrial and cellular functioning are intimately linked and generally associated with submaximal metabolic rates. Moreover, the cytosolic environment allows mitochondria to communicate with the rest of the cell and other organelles (Koopman *et al*, 2012). In the next sections, we discuss how quantitative fluorescence microscopy techniques are applied to study the pathophysiology of OXPHOS mutations at the level of single living cells.

Quantitative live-cell microscopy

Ideally, relevant (i.e., neuronal) patient-derived cell lines should be used to study the cellular (patho)physiology of mtDNA and nDNA-encoded OXPHOS mutations during neurodegeneration. Unfortunately, these cell lines are generally unavailable and often patient-derived primary skin fibroblasts are used for genetic, diagnostic and live-cell analysis of OXPHOS disorders. Given their flat morphology, (patient) fibroblasts are ideally suited for microscopy analysis. Alternatively, primary cells and/or immortalized cell lines can be derived from appropriate mouse models or healthy cells can be treated with OXPHOS inhibitors to induce mitochondrial dysfunction. After selecting an appropriate cell model and culturing conditions, protein-based and/or chemical fluorescent reporter molecules can be introduced into the cell using transfection techniques or dedicated incubation protocols (Figure 5A). Moreover, cellular/mitochondrial autofluorescence can also be measured to monitor mitochondrial function (e.g., NAD(P)H; Verkaart *et al*, 2007b; Rodrigues *et al*, 2011). When accompanied by the proper control experiments, fluorescence microscopy/spectroscopy allows a relatively non-invasive quantification of various physiological readouts at the (sub)cellular level including ROS levels, Ca^{2+} dynamics, $\Delta\psi$, NADH levels, thiol redox status, ATP levels, GLC levels, pH and mitochondrial dynamics and protein localization, mobility and concentration (e.g., Verkaart *et al*, 2007a; Benard *et al*, 2008; Dieteren *et al*, 2008, 2011; Koopman *et al*, 2008, 2012; Abramov *et al*, 2010; Dickinson *et al*, 2010; Digman and Gratton, 2011; Liemburg-Apers *et al*, 2011; Palmer *et al*, 2011; Distelmaier *et al*, 2012). Electron microscopy of fixed cells has been widely used to analyse the internal structure of the mitochondrion with the required high spatial resolution. Recently, a three-dimensional (3D) super-resolution microscopy technique (structured-illumination microscopy or SIM) was applied to visualize the temporal 3D structure of the mitochondrial matrix in living HeLa cells (Shao *et al*, 2011). This approach is important since it allows analysis of matrix volume, structure and topology that all appear to be linked to mitochondrial metabolic (dys)function (Hackenbrock *et al*, 1971; Rossignol *et al*, 2004; Mannella, 2008; Lizana *et al*, 2008; Dieteren *et al*, 2011). For multiparameter ('high-content') microscopy analysis of live cells, different reporter molecules that are spectrally compatible can be simultaneously introduced into the same cell. In case of overlapping emission spectra using multicoloured cells, spectral imaging during acquisition combined with linear unmixing of the image data can be applied (Zimmermann, 2005). The fluorescence signal(s) of the reporter molecule(s) can be quantified in space and time using live-cell fluorescence microscopy/spectroscopy and (quantitative) image analysis (Figure 5B–D; Koopman *et al*, 2008). Computer-controlled automated microscopy can be used to image cells cultured on multiwell plates, allowing investigation of multiple conditions in a large number of cells during a relatively short time period ('high-throughput'; Conrad and Gerlich, 2010). The latter strategy requires extensively validated protocols for cell staining, image acquisition/processing/quantification and classification (e.g., Ljosa and Carpenter, 2009; Jain *et al*, 2010; Horvath *et al*, 2011; Shariff *et al*, 2011).

The live-cell consequences of mutations in OXPHOS structural and assembly genes

Live-cell fluorescence microscopy analysis has been applied to study the consequences of both mtDNA- and nDNA-encoded mutations in OXPHOS structural and assembly genes. Below we provide some typical examples that illustrate this strategy and present a framework summarizing the cellular data. In general, the effect of mtDNA mutations is analysed using 'cytoplasmic hybrids' (cybrids). These cells are generated by fusing non-nucleated (patient-derived) cells (cytoplasts) with mtDNA-depleted cells (ρ0 cells). The resulting cybrid cell line receives the mtDNA from the (patient) cytoplast and the nDNA from the ρ0 cell. Because the cybrid cells are derived from a common ρ0 nuclear background they have equivalent nuclear genes and biochemical and/or molecular differences are expected to reflect differences between their mtDNA content (King and Attardi, 1988, 1989; Swerdlow, 2012). In case of CI deficiency during LHON, mtDNA mutations (G3460A, G11778A and T14484C) were associated with reduced mitochondrial O_2 consumption, $\Delta\psi$ depolarization, increased mitochondrial ROS production and reduced mitochondrial ATP production (see Pellegrini *et al*, 2012 and the references therein). Recently, an alternative strategy was presented to study the live-cell effects of mtDNA-encoded OXPHOS mutations. In this approach, neurons are used that are differentiated from mouse embryonic stem-cell cybrids containing mtDNA polymorphic variants or mutations (Kirby *et al*, 2009; Abramov *et al*, 2010; Trevelyan *et al*, 2010). These studies revealed that in neurons displaying a low residual CI activity ($<10\%$), $\Delta\psi$ was hyperpolarized (i.e., more negative and likely maintained by CV reverse-mode action), ROS levels were increased and GSH was depleted. In case of CIV deficiency, a 40% residual activity was associated with a normal $\Delta\psi$, increased ROS levels and normal GSH levels. This suggests that CI-deficient neurons display oxidative stress, whereas CIV-deficient neurons do not. The latter was supported by the observation that CI deficiency, but not CIV deficiency, increased neuronal death that was attenuated by ROS scavengers (Abramov *et al*, 2010). Analysis of Ca^{2+} signals in the same cell lines revealed that pathogenic mtDNA mutations did not affect the Ca^{2+} transient in response to single glutamatergic stimuli. However, in response to repeated stimuli, Ca^{2+} transients decayed more slowly in the mtDNA mutant cell lines (Trevelyan *et al*, 2010), suggesting insufficient fuelling of Ca^{2+} pumps on the ER with mitochondrial ATP (Willems *et al*, 2008). Although neuronal differentiation was observed, this parameter was impaired in cybrids displaying a large

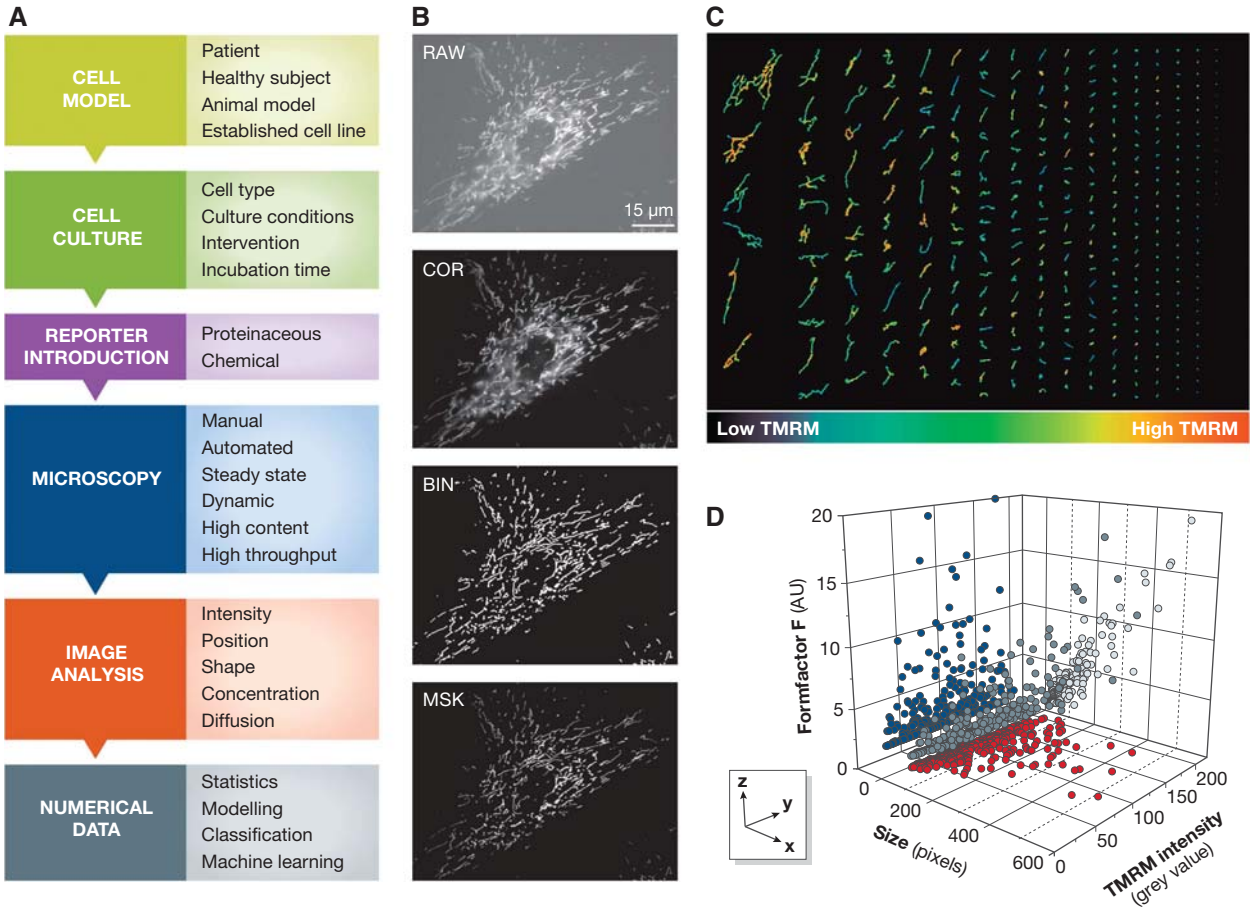

Figure 5 Quantitative analysis of mitochondrial (dys)function at the live-cell level. (**A**) Flow scheme illustrating how live-cell microscopy techniques can be applied to study OXPHOS dysfunction. Topics/decisions associated with the corresponding box are indicated at the right. (**B**) Image processing strategy allowing quantification of mitochondrial structure and function in a primary human skin fibroblast (#5120) from a healthy individual. Living cells were stained with the $\Delta\psi$-sensitive fluorescent cation tetramethylrhodamine (TMRM) and visualized using epifluorescence microscopy. The obtained image (RAW) was corrected for background fluorescence (COR) and binarized to highlight mitochondrial structures (BIN; white objects). By masking the COR image with the BIN image information about mitochondrial structure, number and position (BIN image) were combined with TMRM intensity information from the COR image. This allows simultaneous quantification of these parameters from the MSK image. In this example, the number of mitochondrial objects equals 341, the average size of a mitochondrion equals 69 ± 7 (s.e.m.) pixels, the average formfactor F (a combined measure of mitochondrial length and degree of filamentation) equals 2.7 ± 0.2 (s.e.m.) arbitrary units, and the average mitochondrial TMRM fluorescence intensity equals 100 ± 0.2 (s.e.m.) grey values. (**C**) Mitochondrial objects sorted (column-wise from top to bottom and from top left to lower right) based upon their size. The colour coding indicates the TMRM intensity, suggesting that $\Delta\psi$ is heterogeneous between individual mitochondrial objects. (**D**) Relationship between mitochondrial size (x axis), TMRM intensity (y axis) and formfactor (z axis) allowing multivariate analysis and multiparameter classification. Dark-grey spheres represent the original data points (each representing a mitochondrial object in the MSK figure), blue dots represent a projection of the data on the yz plane, red dots represent an projection of the data on the xy plane and light-grey dots represent a projection of the data on the xz plane). The latter reveals a linear correlation ($R = 0.97$, $P < 0.001$) between mitochondrial size and mitochondrial form factor F.

biochemical deficiency. Synaptic activity was detected in neurons with non-pathogenic mtDNA mutations or neurons with a mild defect of respiratory activity. However, mtDNA mutations that resulted in severe biochemical deficiency induced a marked reduction in post-synaptic events (Kirby *et al*, 2009). Taken together, these results suggest that neurons with a severe CI deficiency display oxidative stress, increased cell death, aberrant cytosolic Ca^{2+} handling due to limited mitochondrial ATP supply, impaired differentiation and a reduction in post-synaptic events. In contrast, other experimental evidence suggests that cytosolic Ca^{2+} clearance in cultured cerebellar granule cells during treatment with high K^+ artificial cerebrospinal fluid (ACSF), is largely fuelled by glycolytic ATP and mediated by the plasma membrane Ca^{2+}-ATPase (PMCA; Ivannikov *et al*, 2010). The latter study reported similar results for Purkinje cells in acutely prepared slices during electrical stimulation and further revealed that ER Ca^{2+} pumps are fuelled by both glycolytic and mitochondrial ATP. Mutations in the nDNA-encoded CIV assembly factor SURF1 are associated with LS in humans (Table I). Interestingly, analysis of a recombinant mouse model lacking this assembly factor ($SURF1^{-/-}$ mice), revealed that spontaneous neurodegeneration was absent, lifespan was markedly prolonged and animals were fully protected from kainic acid-induced Ca^{2+}-dependent neurotoxicity (Dell'agnello *et al*, 2007). These results might be due to the fact that although CIV biochemical and assembly defects were present in $SURF1^{-/-}$ mice, they were milder than in humans. Analysis of primary neuronal cultures from $SURF1^{-/-}$ mice revealed that glutamate-induced cytosolic Ca^{2+} signals were of lower amplitude than in neurons from $SURF1^{+/+}$ mice. The fact that $\Delta\psi$ was similar between

SURF1$^{-/-}$ and SURF1$^{+/+}$ neurons suggests that the above effects are independent of mitochondrial bioenergetics (Dell'agnello *et al*, 2007).

Integrating our own experimental results (largely obtained with primary skin fibroblasts from LS patients with isolated CI deficiency) with those in the literature revealed that primary monogenic mitochondrial disorders (i.e., those caused by a mutation in one of the nDNA-encoded proteins that make up the mitochondrial proteome) have only a limited number of (identified) consequences at the cellular level (see Koopman *et al*, 2012 and the references therein). Likely, this also holds true for defects in mtDNA/nDNA-encoded OXPHOS subunits and nDNA-encoded OXPHOS assembly factors since they represent a subset of the mitochondrial proteome. Due to the mutation, a mitochondrial protein defect is induced that is associated with its altered expression and/or activity. Subsequently, the protein defect will trigger (a combination of) 'primary cellular consequences' including: $\Delta\psi$ aberrations, altered mitochondrial shape/movement/positioning, increased ROS levels, and/or substrate accumulation. The magnitude of these changes, as well as the triggering of 'secondary cellular consequences' (e.g., altered ATP production, glycolysis upregulation, changes in redox state, mitophagy, ionic imbalance and mitochondrial biogenesis), depends on the nature of the mutation, the cell type, culture conditions and metabolic state (see Koopman *et al*, 2012 and the references therein). Importantly, both primary and secondary consequences might constitute part of an adaptive (signalling) mechanism attempting to counterbalance the consequences of the mutation. For example, the loss of a CIV assembly factor (SURF1) in fibroblasts from LS patients was associated with upregulation of CI, CIII and CV due to a post-transcriptional compensatory mechanism (Kovářová *et al*, 2012). Similarly, fibroblasts from patients with a mutated CV assembly factor (TMEM70), associated with reduced CV protein levels and ATP production, displayed $\Delta\psi$ hyperpolarization, increased ROS levels and compensatory upregulation of CIII and CIV (Havlíčková Karbanová *et al*, 2012). A parallel study with fibroblasts from a patient with a novel TMEM70 gene deletion revealed that reduced CI and CV activity was paralleled by mitochondrial fragmentation and aberrations in cristae structure (Jonckheere *et al*, 2012b). Analysis of primary fibroblasts from LS patients with isolated CI deficiency revealed increased ROS levels (Koopman *et al*, 2007; Verkaart *et al*, 2007a) but no detectable downstream effects on lipid peroxidation or thiol redox status (Verkaart *et al*, 2007b). Further experiments demonstrated that greatly reduced CI activity was associated with greatly increased ROS levels and mitochondrial fragmentation, whereas moderately reduced CI activity was paralleled by a minor increase in ROS levels and no effect on mitochondrial morphology (Koopman *et al*, 2005, 2007). The consequences of OXPHOS dysfunction with respect to ROS generation are also cell-type dependent. This is illustrated by our recent analysis of different fibroblast types derived from the NDUFS4$^{-/-}$ KO mouse (Kruse *et al*, 2008), which is the first animal model of isolated CI deficiency and LS (Roestenberg *et al*, 2012). In agreement with our primary patient fibroblast data, it was observed that primary mouse muscle and skin fibroblasts displayed increased ROS levels (WJHK, unpublished observation). In contrast, immortalized MEFs did not display this increase (Valsecchi *et al*, 2012). When the latter cells were placed in a medium containing GAL instead of GLU (to stimulate OXPHOS-mediated ATP generation; Rossignol *et al*, 2004), ROS levels were increased in NDUFS4$^{-/-}$ MEFs relative to MEFs from a wt animal (Valsecchi *et al*, unpublished observation) This suggests that immortalized cells and/or (high) GLU culture conditions (Marroquin *et al*, 2007) might not be ideal to study the (patho)physiology of OXPHOS (dys)function. Also cell differentiation can affect cellular bioenergetics and responses to oxidative stress (e.g., Schneider *et al*, 2011). The latter study revealed that differentiation of SH-SY5Y neuroblastoma cells to a neuronal phenotype induced $\Delta\psi$ hyperpolarization, increased stimulation of mitochondrial respiration by uncoupling (linked to increased CIV expression), and higher resistance to exogenous ROS application (linked to increased MnSOD expression). These results agree with the observation that real neurons rely on OXPHOS for ATP generation (Bélanger *et al*, 2011) and suggests that substantial changes in mitochondrial metabolism and antioxidant defences occur upon differentiation of neuroblastoma cells to a neuron-like phenotype (Schneider *et al*, 2011). Taken together, these results demonstrate that aberrations at the cellular level induced by OXPHOS dysfunction likely constitute a (cell-type and culture-condition dependent) convolution of primary and secondary (adaptive) effects, which requires careful interpretation. On the other hand, experimental analysis of the adaptive program will deliver valuable information about its molecular mechanism (e.g., Benard *et al*, 2012). In this sense, exogenous stimulation of this adaptive program might constitute a potential intervention strategy (e.g., Stranahan and Mattson, 2012).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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