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Review

Modelling biochemical features of mitochondrial neuropathology ☆

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ABSTRACT

Background: The neuropathology of mitochondrial disease is well characterised. However, pathophysiological mechanisms at the level of biochemistry and cell biology are less clear. Progress in this area has been hampered by the limited accessibility of neurologically relevant material for analysis.
Scope of review: Here we discuss the recent development of a variety of model systems that have greatly extended our capacity to understand the biochemical features associated with mitochondrial neuropathology. These include animal and cell based models, with mutations in both nuclear and mitochondrial DNA encoded genes, which aim to recapitulate the neuropathology and cellular biochemistry of mitochondrial diseases.
Major conclusions: Analysis of neurological tissue and cells from these models suggests that although there is no unifying mode of pathogenesis, dysfunction of the oxidative phosphorylation (OXPHOS) system is often central. This can be associated with altered reactive oxygen species (ROS) generation, disruption of the mitochondrial membrane potential ($\Delta\Psi_m$) and inadequate ATP synthesis. Thus, other cellular processes such as calcium (Ca^{2+}) homeostasis, cellular signaling and mitochondrial morphology could be altered, ultimately compromising viability of neuronal cells.
General significance: Mechanisms of neuronal dysfunction in mitochondrial disease are only just beginning to be characterised, are system dependent and complex, and not merely driven by energy deficiency. The diversity of pathogenic mechanisms emphasises the need for characterisation in a wide range of models, as different therapeutic strategies are likely to be needed for different diseases. This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

1. Introduction

Mitochondrial disease is highly variable in its presentation, potentially affecting any organ, in isolation or combination, at any age, with any severity [1]. Similarly, the genetic basis of the disease is complex. Mutations have been characterised in over 100 genes, encoded either by the nuclear genome (nDNA) and inherited in a Mendelian manner, or by the mitochondrial genome (mtDNA) and inherited through the maternal lineage only [1,2]. Mutations inherited through the mtDNA are further complicated in that each cell typically contains thousands of copies of the mtDNA genome [3]. Accordingly, mutations of this type are often inherited in a heteroplasmic manner, where the cell contains a mixture of wild-type and mutant mtDNA. Disease may only manifest in such cases when the mutant load exceeds a certain threshold.

Mitochondrial disease, although diverse in presentation, typically affects the more metabolically active tissues and organs, most notably the brain [1,2,4]. Common neurological symptoms of mitochondrial disease include developmental delay or regression, optic atrophy, hearing loss, ataxia, seizures and stroke [5]. That the disease frequently manifests as a neurological disorder of the central nervous system (CNS) is perhaps not so surprising considering that the human brain comprises only 2% of our body weight and yet accounts for 20% of oxygen consumption [6]. This underscores the high dependence of the CNS on energy supply from the mitochondrial oxidative phosphorylation (OXPHOS) system (Fig. 1), the chain of mitochondrial protein complexes that creates an electrochemical gradient of protons, ultimately driving ATP synthesis [7]. Just how and where the brain spends this very large energy budget is still contentious, but is central to understanding the specificity of mitochondrial diseases. It is likely though that most energy is consumed in the dual process of firing action potentials and repackaging neurotransmitters [8]. Neurons however are not the only cell type in the brain with an appetite for energy. Astrocytes, for example, are the most common cell type in the brain, and are intricately linked to neurons for proper function [9]. They are also reported to be highly metabolically active, not merely suppliers of glutamate and lactate to neurons, but potentially also receiving some of these same products back from neurons to drive OXPHOS, and propagating signals of their own [9–13].

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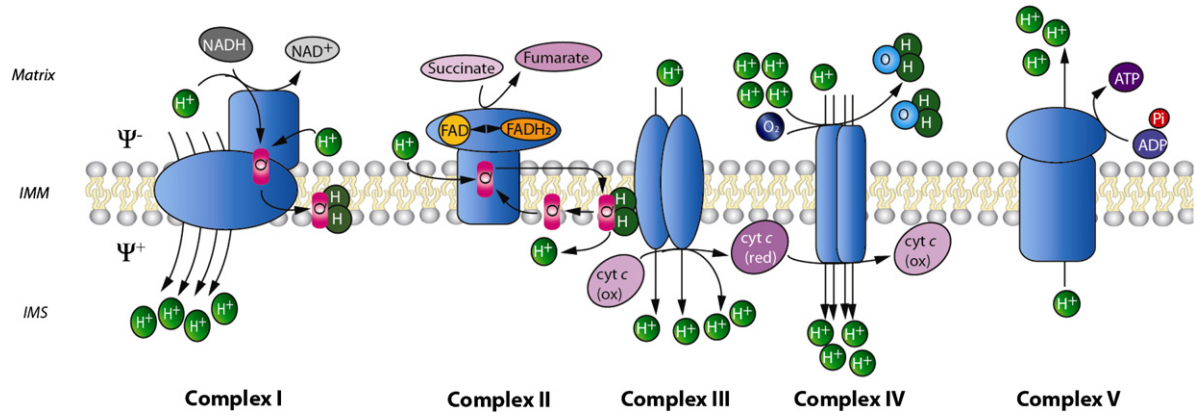

Fig. 1. The OXPHOS system. The mitochondrial OXPHOS system (blue) pumps H^+ protons (green) across the IMM and into the IMS at complexes I, III and IV to maintain the $\Delta\Psi_m$. Electrons enter OXPHOS by oxidation of NADH at CI and succinate at CII; they are transferred by the mobile electron carrier Q to CIII and then via cyt *c* to CIV, where they are used to reduce molecular oxygen to form water. CV utilises the electrochemical gradient, and couples proton flow through the complex with ATP synthesis from ADP and inorganic phosphate. Abbreviations: cyt *c*, cytochrome *c*; IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; ox, oxidised; Pi, inorganic phosphate; Q, ubiquinone; QH_2, ubiquinol; red, reduced; Ψ, charge.

Although the clinical manifestations of mitochondrial disease have generally been well characterised, the difficulty in obtaining relevant fresh neurological tissue has limited our understanding of the biochemical features at a cellular level. To this end, a number of animal and cellular model systems of primary mitochondrial dysfunction that mimic neurological mitochondrial disorders have recently been generated (Fig. 2). Additionally, a number of model systems have been generated that mimic disorders where mitochondrial dysfunction may be secondary but appears to be central to neurological pathogenesis (Fig. 2).

Such models have provided the opportunity to further our understanding of the neuropathology of disease involving mitochondrial dysfunction, through biochemical characterisation of mitochondrial function and structure. Perhaps the most fruitful of these models to date has been the mouse. Mouse models have provided the opportunity to characterise neurological biochemistry *in vivo* in the mouse brain (Fig. 2, Table. 1 and extended in Supplementary Table. 1), as well as *in vitro* (Fig. 2, Table. 2), utilising primary cell cultures of neurologically relevant cell types such as neurons and glial cells.

These mouse models have faithfully recapitulated aspects of common mitochondrial disorders with neurological involvement. For example, the *Ndufs4* (nuclear encoded) knockout (KO) mouse models exhibit key features of Leigh syndrome (LS), such as bilateral lesions in the brain stem, ataxia, increased serum lactate, blindness and breathing abnormalities [14–17]. More recently, the mtDNA-encoded *mt-Nd4* and *mt-Nd6* transgenic mouse models were shown to develop optic atrophy, similar to patients with Leber's hereditary optic neuropathy (LHON) [18,19]. It is of note that the generation of any model system with mtDNA-encoded mutations, not least in mouse, has been a great challenge to the field. Restraining such advances are the lack of genetic tools available to manipulate mtDNA, along with the further complication that the mode of inheritance is not Mendelian in nature and mtDNA can exist in either a heteroplasmic or homoplasmic state as discussed above.

Until recently, mouse models have been restricted to nuclear gene defects due to technical difficulties in introducing mitochondrial DNA (mtDNA) mutations into germ cells. Here, cybrid technology — the fusion of an enucleated patient cell with a host cell whose mtDNA has been chemically ablated — has provided the opportunity to model biochemical dysfunction in neuronal-like cells with a broad range of pathogenic mtDNA mutations [20,21] (Fig. 2, Table. 2).

Although yet to be fully exploited, further transforming our capacity to study a wide variety of mitochondrial disease-causing mutations is induced pluripotent stem (iPS) cell technology (Fig. 2, Table. 2) [22]. The technique is characterised by the cellular transfection of four key transcription factors that, when expressed simultaneously in mouse or human cells, can confer pluripotency. These pluripotent cells can subsequently be induced to differentiate into the cell type of choice, such as the neurologically relevant neurons and astrocytes.

In addition to studies of classical or primary mitochondrial disorders, there are also a number of models that mimic disorders where secondary mitochondrial dysfunction appears to contribute to disease pathogenesis. These include: the *Sod1* (nuclear encoded) transgenic mice with large scale death of motor neurons, as seen in patients with amyotrophic lateral sclerosis (ALS) [23–26]; and a number of cellular and mouse models which seek to recapitulate Friedreich's Ataxia (FA), albeit with varying degrees of success [27–32].

Biochemical studies of mitochondrial disease in model systems have often focused on primary defects that directly affect the mitochondrial OXPHOS system (Fig. 1). The OXPHOS system plays a central role in maintaining a host of mitochondrial processes [7] by pumping protons across the inner mitochondrial membrane (IMM) and into the intermembrane space (IMS) to create an electrical and chemical gradient of protons, the mitochondrial membrane potential ($\Delta\Psi_m$) (Table. 2) [3]. The $\Delta\Psi_m$ drives a number of critical cellular functions, chiefly the synthesis of ATP by complex V (CV) [33]. Other critical processes dependent on the $\Delta\Psi_m$ include: modulating rates of superoxide production from CI and CIII [34]; regulating mitochondrial ultrastructure [35]; maintaining cellular calcium homeostasis [36]; and acting as a regulator of cell death pathways [37]. Regulation, however, does not always occur in a straightforward linear relationship. Cellular calcium, for example, is not just regulated by mitochondria, but itself regulates mitochondrial function [38]. This review focuses on the interplay between each of these mitochondrial processes, as modeled in both animal and cellular systems of mitochondrial disease, and how this may contribute to disease pathogenesis.

2. Dysfunction of OXPHOS

The OXPHOS system is comprised of five-multi subunit complexes (CI - V) embedded in the IMM, collectively encoded for by 12 mtDNA and 74 nDNA genes (Fig. 1) [7]. The system oxidises the reduced coenzymes NADH and $FADH_2$ at CI and II respectively. Liberated electrons are then passed along the OXPHOS complexes via ubiquinone (Q) to CIII, which shuttles the electrons to CIV via cytochrome *c* (cyt *c*), where finally the electrons are donated to molecular oxygen (O_2) to form water (H_2O). Electron transport is coupled to proton pumping at CI, III and IV, which take protons from the mitochondrial matrix, and deposit them in the mitochondrial intermembrane space to maintain the $\Delta\Psi_m$. The fifth OXPHOS complex, CV, utilises the $\Delta\Psi_m$ by mechanically coupling the

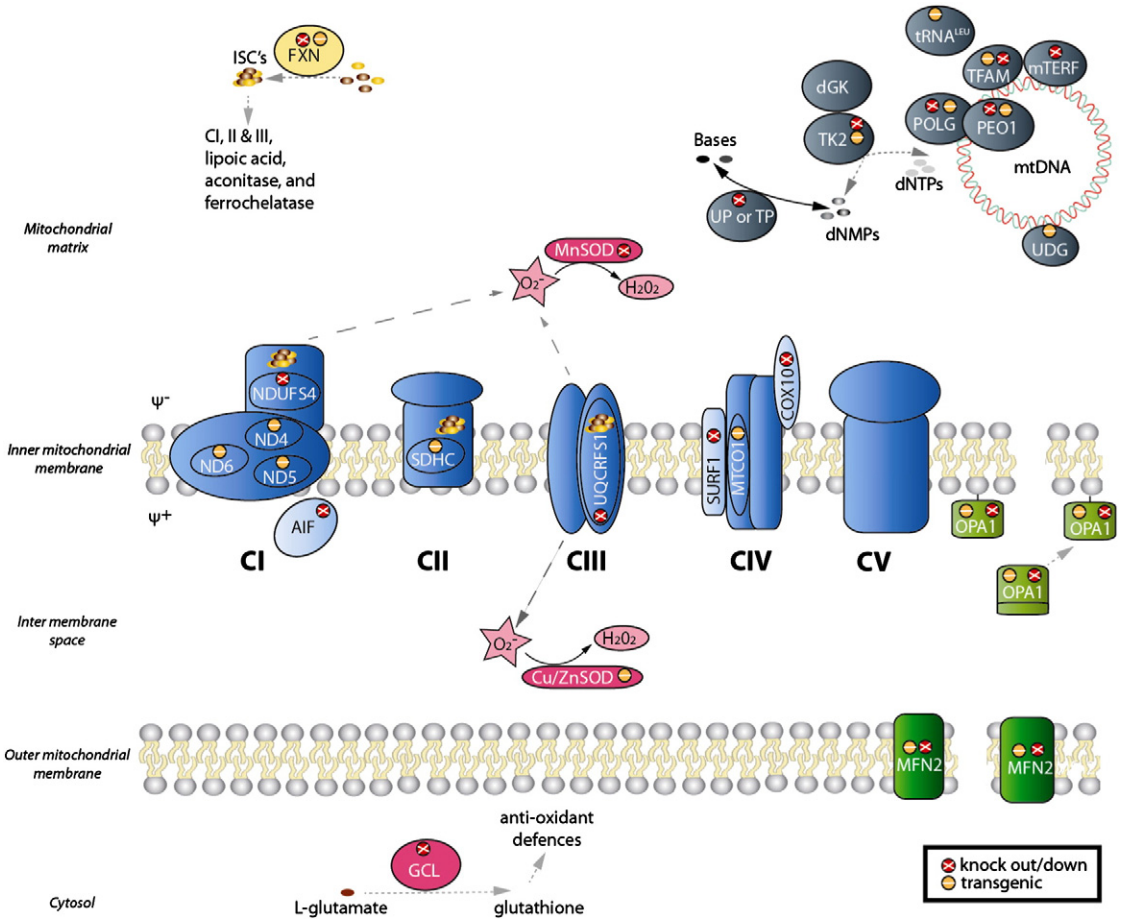

Fig. 2. Mouse and cellular models of mitochondrial disease with associated neuropathology. A number of mouse and cellular models of mitochondrial disease have been developed that demonstrate associated neuropathology. The mutations described in this review have been broadly classified as being located in: Dark blue, subunits of the oxidative phosphorylation system (NDUFS4, ND4, 5 and 6, SDHC, UQCRFS1 and MTCOI); light blue, OXPHOS assembly factors (AIF, SURF1 and COX10); grey, mtDNA replication and maintenance machinery and mtRNA processing machinery (TFAM, POLG, TK2, mTERF, UP/TP, UDG, PEO1 and $tRNA^{Leu}$); green, mitochondrial fusion components (MFN2 and OPA1); pink, reactive oxygen species defences (SOD1 and 2, GCL); and yellow, iron sulfur cluster biogenesis (FXN). A red circle with a white cross denotes knock out and knock down models, and an orange circle with a white dash indicates transgenic models. Abbreviations: AIF, apoptosis inducing factor; CI–V, complexes I–V; dGK, deoxyguanosine kinase; dNMPs, deoxynucleoside monophosphates; dNTPs, deoxynucleoside triphosphates; GCL, glutamate-cysteine ligase; ISCs iron sulfur clusters; mtDNA, mitochondrial DNA; TP, thymidine phosphorylase; TK2, thymidine kinase 2; UP, uridine phosphorylase.

flow of protons through the complex down the electrochemical gradient, driving ATP synthesis from ADP and inorganic phosphate [33].

Impairment of OXPHOS is the most common feature of mitochondrial disease, and disruption of OXPHOS would be expected to compromise the $\Delta\Psi_m$ and associated processes [33]. This is not a consistent relationship however, as discussed in detail later in this review. As such, here we take the opportunity to examine not only the integrity of the OXPHOS complexes in each of the models described, but also the relationship with associated biochemical features including bioenergetics, O_2 consumption, redox balance ($NADH/NAD^+$ pools) and mitochondrial metabolomics.

2.1. Isolated OXPHOS defects

2.1.1. Complex I models

A number of models have been described with mutations either directly or indirectly affecting the function and activity of the OXPHOS complexes. As would be expected, these models typically display severe defects in the OXPHOS pathway. For instance, there are several models described that directly affect complex I (CI), the largest OXPHOS complex, comprising 45 subunits (although recent data suggests that one subunit, NDUFA4, is actually part of CIV [39]) and estimated to provide ≈ 40% of the proton motive force [40,41]. Disease causing mutations have been previously identified in 19 of its 45 subunits [2], and at least 10 assembly factors [42]. Mutations in CI subunits and assembly factors have been associated with a large number of mitochondrial disorders featuring neurological impairment, including LS and LHON.

Among such models with isolated CI deficiency are the *Ndufs4* (nuclear encoded) knockout mice. These mice display neurological features and early death that mimic aspects of LS. Analyses of whole brain and isolated mitochondria from primary mesencephalic neurons reveal that CI is reduced in abundance and forms a ≈830 kDa crippled complex on blue-native (BN) PAGE with less than 25% residual activity, while activities of CII, III and IV activities are unaffected [14,15,43,44]. Oxygen (O_2) consumption linked to CI substrates is also reduced by 50% [43,44]. An elevated pool of NADH was also observed, indicating a redox imbalance that may also contribute to pathogenesis [45].

The Harlequin mice also have a mutation in a nDNA encoded gene, *Aifm1*, and develop cerebellar ataxia and blindness [46,47] with a severe CI deficiency (reduced expression and activity) [46–48] implying that AIF is specifically involved in CI assembly [49]. While these mice show an isolated CI defect, patients with *AIF* gene mutations have enzyme defects affecting multiple OXPHOS complexes [50,51], so it remains unclear whether the roles of AIF differ in humans and mice.

It is exciting to see that two mouse models have recently been described with mutations in the mtDNA encoded CI subunits ND4 and ND6 [18,19]. Lin et al. [18] reported a transgenic mouse model of

Table 1
Neurologically relevant biochemical features from mouse models of mitochondrial disease with associated neuropathology.

Gene(s)	Mutation	Biochemical & morphological features	Ref(s)
OXPHOS structural subunits			
Ndufs4	KO (whole animal and CNS)	Brain – ↓ CI act. ↓ CI linked ATP synthesis. Ox dam to prot. ↑ cleaved casp-8. Δ mito morph	[14–16,43–45,121]
mt-ND4	Tg human mt.G11778A	Optic nerve – Axonal degeneration. Δ mito dist	[19]
mt-Nd6	Tg (p.P25L)	Brain & optic nerve – CI, II & III norm exp.↑ CI linked, &↓ CII linked ROS prod. Ox damage to prot. Demyelination of neurons. Δ mito morph	[18]
Sdhc	Tg (p.V69E)	Brain & pups – CI & II norm act. ↑ CIII exp. ↑ ROS prod. Ox dam to prot. TUNEL + ve neurons	[56]
Uqcrfs1	KO (neuronal)	Brain – ↓ CIII act. CIV & CS norm act. CI, II, III & IV norm exp. Ox dam to prot & DNA. ↑ SOD2 exp & SOD1 norm. TUNEL + ve neurons	[60]
OXPHOS assembly factors			
Cox10	KO (neuronal specific)	Brain – ↓ CIV act. CII + III norm act. ↓ CIV exp. CI, II & III norm exp. Ox dam to DNA & lipid. ↑ SOD2 exp & ↓ SOD1. TUNEL + ve neurons	[60]
Surf1	KO	Brain – ↓ CIV act	[63]
Aifm1	KD	Brain & retina – ↓ CI act. ↓ CI exp. Ox dam to DNA & lipid. ↑ cleaved casp-3 & TUNEL + ve neurons. Δ mito morph	[47]
Aifm1	KO (early embryo stage)	Embryos – ↓ CI act. CII + III & IV norm act. ↓ CI exp. CIII norm exp	[48]
Aifm1	KO (midbrain & cerebellum)	Brain – Neuron cell cycle arrest	[122]
mtDNA, transcription, translation, maintenance & mitochondrial RNA processing			
Tfam	KO (neuronal)	Brain – ↓ CI & IV act. CII norm act. No ev ox dam. ↑ GPRX exp. SOD2 exp norm. ↓ mtDNA	[69]
Tfam	KO (DA neurons)	Brain – ↓ CIV act. TUNEL + ve neurons. Δ mito morph	[123,124]
Tfam	Tg (over expression human TFAM)	Brain – ↑ CI & IV act. CII & III norm act. ↓ ox dam to DNA & prot	[125]
Polga	Tg (p.D257A)	Brain & cochlear – ↑ cleaved casp-3 & TUNEL + ve neurons. Mutated mtDNA	[120,126,127]
Tk2	Tg (p.H126N)	Brain & spinal cord – ↓ CI & IV act. ↓ CI & IV exp. Gliosis. ↓ mtDNA	[67,118]
Tk2	KO	Brain – ↓ CI, III & IV exp. TUNEL + ve neurons	[74,119]
Upp1/Tymp	Double KO	Brain & spinal cord – ↓ CI & IV act. CS norm act. ↓ IV exp. ↓ mtDNA	[68,128]
UDG	Tg (hUNG1-p.Y147A, forebrain)	Brain – ↓ CI & CII linked O2 cons. TUNEL + ve neurons	[75]
Mterfd3	KO	Brain – CI & IV norm act stand diet. ↓ CI & V act on keto diet. CI & IV exp norm stand diet. ↓ CI & IV exp on keto diet. Norm mtDNA	[70]
Peo1	Tg (dup 353–365)	Brain – ↓ IV exp. ↓ mtDNA & mtDNA deletions	[71]
Anti-oxidant defence			
Sod2	KO	Brain & brain stem – Δ mito morph. Mutated mtDNA	[112,113,115]
Sod2	KO + MnTBAP	Brain – Gliosis & neuronal demyelination	[113]
Sod2	KO (post natal motor neuron)	Spinal cord – ↑ ROS production. No ev of ox dam. Δ mito morph	[114]
Sod1/Gclm	Tg hSOD1 (p.G93A) + KO GCLM	Spinal cord – ↓ CIV exp. Ox dam to prot. ↓ GSH exp & SOD1 norm. Loss of large motor neurons. Δ mito morph	[26]
Sod1/Gclm	Tg hSOD1 (p.G93A)	Spinal cord – ↓ CI, II & IV act. Δ mito morph	[23,24,26]
Mitochondrial fusion			
Opa1	KD	Brain & optic nerve – CI & IV norm exp in brain. ↓ CIV exp in optic nerve. Axonal demyelination. ↑ mtDNA	[85]
Opa1	KD (enu/+)	Retina – Loss of retinal ganglion cells. Δ mito morph	[102,129,130]
Opa1	Tg (p.Q285X)	Optic nerve – ↑ autophagosomes. Δ mito morph	[103]
Mfn2	KO (cerebellar primordia)	Brain – ↑ CIV act. Purkinje cell loss. Δ mito dist	[87]
Mfn2	KO (Purkinje cell)	Brain – ↓ CIV act. ↑CIII act. Purkinje cell loss	[87]
Mfn2	Tg (p.T105M, motor neuron)	Motor neurons – Δ mito morph & dist	[100]
Mfn2	Tg (p.R94Q, neuron)	Brain – Δ mito dis	[101]
Iron sulfur cluster biogenesis			
Fxn	KD (neuronal)	Brain – Purkinje cell loss. Δ mito morph	[28,79]
Fxn	Tg (h*FXN* with GAA repeat expansion) / KO mouse *Fxn*	Brain – Ox dam to prot & lipid. ↑ SOD1 & 2 exp. Axonal demyelination	[117]

Abbreviations: ↑, increased; ↓, decreased; + ve, positive; act, enzyme activity; CI–IV, complex I – IV; ev, evidence; exp, protein expression; GCL, glutamate-cysteine ligase; GSH, glutathione; KD, knock down; keto, ketogenic; KO, knock out; mtDNA, mitochondrial DNA; norm, normal; ox dam, oxidative damage; p., point mutation; prot, protein; stand, standard; Tg, transgenic; Δ mito dist, altered mitochondrial distribution; Δ mito morph, altered mitochondrial morphology.

LHON with a p.P25L mutation in the mtDNA encoded CI subunit ND6. This variant is equivalent to the human mt.14600G > A (p.P25L) mutation found at homoplasmy in a child presenting with LS and at a heteroplasmic mutant load of 50% in a maternal aunt with optic atrophy [52]. Measurements in forebrain lysates from the ND6 mice do not suggest any adaptive changes in OXPHOS expression, where expression of representative subunits from CI, CII and III were all stable [18]. Perhaps this reflects the nature of the missense mutation, which may have no impact on CI assembly but leads to disrupted activity. Unfortunately there are currently no data on CI activity in neuronal tissue. There is however evidence that CI linked O_2 consumption is reduced in neuronal synaptosomes to ≈ 75% of control, which implies CI activity is impaired in brain. Yu et al. [19] also recently reported an alternative mouse model of LHON, where a mitochondrial targeted adenovirus containing either mutant mt.11778G > A or wild-type *mt-ND4* (CI subunit) was injected into vitreous cavities of contralateral eyes. Injection of mutant *mt-ND4* specifically correlated with optic nerve atrophy and thinning of the retina, however there are currently no data on OXPHOS activities.

There is also a cybrid model of severe CI deficiency [53] with mutations in both the mitochondrially encoded *mt-Nd5* and *mt-Nd6* genes (CI subunits). These cybrids have been differentiated into neuronal and astrocytic like cells, but currently CI activity has only been measured in undifferentiated cybrids, CI activity is reduced to

Table 2
Biochemical features from cell culture models of mitochondrial disease with associated neuropathology.

Gene	Mutation	Model	Biochemical and morphological features	Ref(s)
OXPHOS structural subunits				
Ndufs4	KO	Prim mouse mesencephalic neurons	↓ CI act & impaired capacity to adapt from states 1 to 2 respiration. ↑ NADH. Un-stimulated ROS prod norm, ↑ ROS prod with + rotenone. Cleaved casp-3 norm. ↑ L-dopamine levels in + rotenone cells	[43,45]
mt-Nd5/6	Tg (mt.13887 C ins and G12273A)	Mouse cybrid derived neurons and astrocytes	↑ ROS in neurons & ↓ GSH in astrocytes & neurons. ↑ $\Delta\Psi_m$ in astrocytes and neurons resting, ↓ with oligomycin. Ca^{2+} response to single glut pulse is nom in astrocytes & neurons. Neurons repeatedly challenged fail to return to baseline Ca^{2+} concentration.	[53,91,96]
mt-Co1	Tg (mt.T6589C)	Mouse cybrid derived neurons and astrocytes	↑ ROS in neurons. $\Delta\Psi_m$ normal. Ca^{2+} response to a single glut pulse norm, cells repeatedly challenged fail to return to baseline Ca^{2+} concentration	[53,91,96]
OXPHOS assembly factors				
Aifm1	KD	Prim mouse granule & cortical neurons	No cell death in granule cells & cortical neurons under stand culture conditions but granule cells more sensitive to H_2O_2 & glut	[108]
Surf1	KO	Mouse prim neurons	$\Delta\Psi_m$ normal. Δ cytosolic Ca^{2+}. ↓ sensitivity to glut toxicity	[63]
mtDNA, maintenance & mitochondrial RNA processing				
Tk2	KO	Mixed prim mouse cerebellar neurons	↓ exp of CI & IV. CII, III & V exp norm. ↓ OCR. ↓ ATP. ECAR norm. Δ mito morph. ↓ mtDNA	[74]
$tRNA^{Leu}$	Tg (mt.A3243G)	Cybrid glioblastoma	↓ CIV activity. ↓ ATP. ↓ $\Delta\Psi_m$, pronounced by gluc deprived. ROS prod norm. ↑ cleaved casp-3	[72]
$tRNA^{Leu}$	Tg (mt.A3243G)	Human cybrid derived neurons	In 100% mutant - ↓ CI act & exp. Δ CI assembly. ↑ CII act, assembly norm. ↓ CIV act & assembly norm. ATP & SOD2 exp norm. ↑ LDH activity, gluc consumption, lactate and alanine pools. ↓ Pyruvate, GSH pools, & aconitase act	[73]
$tRNA^{Leu}$	Tg (mt.A3243G)	Human cybrid derived neurons	In 70% mutant - ↑ CI, II and IV act. Assembly norm for CII and IV and slightly disrupted in CI. Norm ATP amount, SOD2 exp & pools of pyruvate and alanine. LDH activity, glucose consumption and pool of lactate all ↑. ↓ GSH. ↓ aconitase act	[73]
Mitochondrial fusion				
Mfn2	KO	Mouse immortalised cerebellar neurons	Δ mito dist. Stunted dendritic arbor growth & impaired ability to different into Purkinje cells	[87,131,132]
Mfn2	KO	Mouse prim spinal cord neurons	Δ mito dist	[100]
Iron sulfur cluster biogenesis				
Fxn	GAA expansion (P1 600/800, P2 900/400)	Human iPS derived neurons	↓ $\Delta\Psi_m$. Neurons fail to generate action potentials (spontaneous or induced)	[32]

Abbreviations. ↑, increased; ↓, decreased; act, enzyme activity; CI–IV, complex I – IV; ECAR, extra cellular acidification rate; exp, protein expression; glut, glutamate; GSH, glutathione; KD, knock down; KO, knock out; mtDNA, mitochondrial DNA; $\Delta\Psi_m$, mitochondrial membrane potential; norm, normal; OCR, oxygen consumption rate; p., point mutation; p1 & 2, patient 1 & 2; prod, production; prim, primary; Tg, transgenic; Δ mito dist, altered mitochondrial distribution; Δ mito morph, altered mitochondrial morphology.

less than 10% of wild-type, while CII, III and IV activity are normal. These cells also had an impaired capacity to differentiate into neuronal cell types, suggesting that CI deficiency may not only compromise cellular function, but developmental processes.

2.1.2. Complex II models

Complex II is the smallest of the OXPHOS complexes comprising 4 subunits, with no proton pumping capacity [54]. Mutations, although rare, have been reported in all 4 structural genes and 2 assembly factors [2,42]. Autosomal recessive mutations in the nuclear encoded *SDHA* and *SDHAF1* genes cause neurodegenerative diseases and autosomal dominant mutations in the other 4 nuclear encoded genes are associated with paragangliomas and phaeochromocytomas [55]. The nuclear encoded *Sdhc* (CII subunit) dominant transgenic mouse, with a p.V69E substitution located within the functional ubiquinone-binding region of CII shows normal CII activity; CI activity was also normal [56]. However, in this model, expression of SDHC itself was up-regulated by about 50%, which suggests compensation.

2.1.3. Complex III models

Complex III consists of 11 structural subunits and couples the reduction of cytochrome *c* to the oxidation of CoQ to ultimately drive proton pumping [57]. Disease causing mutations have been identified in 4 structural subunits and 3 assembly factors [2,42,58,59]. A neuronal-specific KO mouse model of the CIII subunit nuclear encoded UQCRFS1 (formerly known as RISP) showed no overt behavioral change, but died suddenly at 5 months of age with smaller brains than controls [60]. In the piriform cortex, CIII activity was severely impaired from postnatal day 30 (≈ 50% of control), despite stable expression of the nuclear encoded CIII subunit UQCRC2. There was also no evidence for altered activity or expression of representative subunits for CI or IV. The *Uqcrfs1* KO mouse brain had levels of lactate and glutamate in the normal range, as measured by nuclear magnetic resonance spectroscopy (MRS), which indicated that glycolysis rates are unchanged.

2.1.4. Complex IV models

Complex IV comprises 13 subunits and is the last complex of the OXPHOS system. It receives electrons from cytochrome *c* and donates them to molecular O_2 to form water, coupling this to proton pumping [57]. Disease causing mutations have been characterised in 5 structural genes, and as many as 8 assembly factors [2,42]. These mutations cause a number of neurological diseases, including LS and sensorineural deafness [4,42,61]. KO mouse models have been developed for the nuclear encoded CIV assembly factors SURF1 [62,63] and COX10 [60]. Similar to other models of isolated OXPHOS deficiencies, *Surf1* KO mice, despite exhibiting severe CIV deficiency in brain by 3 months of age, failed to recapitulate aspects of LS as exhibited in patients, were resistant to Ca^{2+} induced cytotoxicity, and even had a prolonged lifespan [63]. In comparison, the *Cox10* KO mice had normal CIV activity at 20 days, which progressively declined over 4 months in the cortex until death, with progressive loss of CIV subunit expression [60]. CII + III and CS activity were not altered in the *Cox10* KO mouse. The brains at 4 months of age from the *Cox10* KO mice also had elevated levels of lactate, but glutamate levels within normal range, as detected by nuclear MRS, which may indicate increased rates of glycolysis, consistent with a block in the OXPHOS system. The mtDNA encoded MTCOI (CIV subunit) has also been mutated in undifferentiated cybrid

cells, where CIV activity is down to ≈40% of wild-type, although CI, II and III activity was in the normal range [53].

2.2. OXPHOS dysfunction caused by defects in mtDNA replication, maintenance or mtRNA processing

Dysfunction of the OXPHOS pathway is not limited to mutations in its subunits and associated assembly factors. Mutations in the mtDNA replication and maintenance machinery, as well as mitochondrial RNA (mtRNA) processing machinery, are also associated with OXPHOS defects. Such mutations are associated with a number of classical mitochondrial disorders including mitochondrial neuro-gastro-intestinal encephalomyopathy (MNGIE), progressive external ophthalmoplegia, Alpers syndrome and mitochondrial encephalomyopathy with lactic acidosis and stroke like episodes (MELAS) [4,64,65]. Mutations in mtDNA transcription and mtRNA translation components are most frequently associated with reduced expression and activity of CI and IV. This is consistent with human mtDNA encoding 13 protein subunits of the OXPHOS system, 7 of which are embedded in CI, a further 3 in CIV, 2 in CV, and only a single subunit in CIII [3,66].

Where examined, CI and IV activity and expression in mouse brain and relevant cell lines were almost universally impaired in all models affecting mtDNA and mtRNA machineries. Among these models with neurologically relevant features, or associated with neurological disorders in patients, are those with defects in nucleotide biogenesis. These include the nuclear encoded genes thymidine kinase II (*Tk2*) KO mouse [67], and the uridine phosphorylase (*Upp1*) and thymidine phosphorylase (*Tymp*) double KO mouse [68]. Likewise, similar defects were observed in models of defective mtDNA replication and maintenance, such as the nuclear encoded *Tfam* KO mouse [69], *Mterfd3* KO mouse [70], and *Peo1* Tg mouse [71]. Similarly, CI and IV activities were impaired in the mtRNA translational defective $tRNA^{Leu}$ (mitochondrially encoded) transgenic cybrids [72,73]. In these models, additional hallmarks of OXPHOS dysfunction were also observed, including reductions in O_2 consumption rates, and spare respiratory capacity in primary cerebellar neurons from the *Tymp* KO mouse [74]. As well, neuronal-like and glioblastoma-derived cells from mitochondrial $tRNA^{Leu}$ transgenic cybrids exhibited an increased rate of glucose consumption with increased lactate and alanine production and increased lactate dehydrogenase activity, consistent with a switch toward glycolytic energy supply [72,73]. Further, CI and II linked O_2 consumption were reduced in hippocampus from the mtDNA replication defective forebrain specific *UDG* (nuclear encoded) transgenic mouse, which removes thymine and uracil from mtDNA [75].

2.3. Friedreich's ataxia and OXPHOS dysfunction

FA is caused by a GAA repeat expansion in the first intron of the *FXN* gene (nuclear encoded). The mitochondrial protein frataxin (FXN) is thought to be involved in iron sulfur cluster (ISC) biogenesis [27–31,76]. In FA patients, biochemical features observed include evidence of oxidative damage, deposits of intracellular iron, and dysfunction of enzymes containing ISCs such as aconitase and OXPHOS complexes I-III [77,78]. However, although mouse and cell models of the disease also feature oxidative damage and evidence of reduced aconitase activity in brain, there are no conclusive reports of dysfunction in the OXPHOS pathway in these models [28,79].

2.4. Mutations in anti-oxidant defence and OXPHOS dysfunction

Mitochondria are significant sources of ROS, which although known to have signaling functions within cells, are rapidly dismutated and cleared [34]. A host of intracellular anti-oxidant defences achieve this, and alterations in these components are associated with damage to cellular structures including DNA, proteins, carbohydrates and lipids [80]. The disruption of the OXPHOS pathway observed in these models is likely associated with defective mitochondrial anti-oxidant defences, involving direct damage to complexes and mutations in mtDNA.

One such model is the nuclear encoded glutamate-cysteine ligase modifier (*Gclm*) KO mouse [26]. Glutamate-cysteine ligase (GCL) is the rate-limiting enzyme in the synthesis of glutathione, an essential component of many anti-oxidant defence pathways. In this mouse, CIV activity was found to be impaired in the spinal cord, despite there being no evidence of damage to proteins.

In contrast to the *Gclm* KO model, dominant mutations in the nuclear encoded *Sod1* (Cu/Zn SOD) transgenic mouse models of amyotrophic lateral sclerosis (ALS) likely represent a gain of function [81]. It has been suggested that mutant SOD1 binds to and blocks the voltage dependent anion channel (VDAC), preventing flux of metabolites such as ATP and ADP across the channel [25,82]. Thus ALS is suggested to be a channelopathy, which could disrupt the ATP/ADP balance, ultimately perturbing the OXPHOS system. Consistent with this, CI, CII and CIV activity were all impaired in spinal cord of *Sod1* transgenic mice, notably correlating with disease progression [24,26].

2.5. Mitochondrial fusion mutations and OXPHOS dysfunction

Mitochondria are highly dynamic organelles, their networks continually undergoing fission and fusion in cells [83]. The IMM is highly folded, increasing the ratio of surface area-to-mitochondrial matrix and thus the potential for OXPHOS activity and linked ATP synthesis [5]. Mutations in mitochondrial fusion are frequently associated with mitochondrial morphological defects. Disruption of mitochondrial fusion also prevents the mixing of mitochondrial contents, and appears to sensitise cells to mitophagy [84]. It is therefore reasonable to consider whether these mutations and mitochondrial structural defects affect OXPHOS function.

In an *Opa1* (nuclear encoded) transgenic mouse model, where OPA1 is involved in fusion of the IMM [85], CI and IV activity are unchanged in whole brain, as was CIV-linked O_2 consumption. However, CIV activity was reduced in the retina and optic nerve. Notably, the optic nerve is also prominently affected in patients with OPA1 mutations who often develop dominant optic atrophy [86].

Likewise, in a model of outer mitochondrial membrane fusion dysfunction, the *Mfn2* (nuclear encoded) Purkinje cell KO mouse [87], CIV activity in cerebellum was impaired and preceded Purkinje cell death. Disruption of mitochondrial fusion is suggested to affect the arrangement of cardiolipins in the IMM, which may be the cause of CIV deficiency in these models [88]. It was also observed that mtDNA nucleoid formation was disrupted in the *Mfn2* fusion deficient cells, which may contribute to CIV deficiency [87].

2.6. Mechanism of disease associated with OXPHOS dysfunction is unclear

OXPHOS dysfunction presumably forms the basis for the neuropathology in the models described with direct mutations in subunits and assembly factors of OXPHOS itself. Similarly, in models of defective mtDNA replication and maintenance, disruption of the OXPHOS pathway is well documented and almost certainly contributes to disease pathogenesis. What is less clear is how the defects observed in other mitochondrial disease models, such as those impacting on mitochondrial fusion and anti-oxidant defence, are correlated with disease.

Overall, it also remains to be elucidated how disruption of OXPHOS is directly correlated with disease. As the central function of the OXPHOS pathway is considered to be proton pumping, one might expect to observe an altered $\Delta\Psi_m$ and subsequent impairment of ATP synthesis as a consequence of these defects. However, as now discussed, this is a poorly conserved relationship.

3. Mitochondrial membrane potential and ATP synthesis

Maintenance of the $\Delta\Psi_m$ is essential not only for driving ATP synthesis via CV, but also for a number of other cellular processes including regulating cell death [37], importing proteins and substrates into the mitochondrial matrix [89], regulating ROS production [34] and modulating cellular Ca^{2+} dynamics [36,90]. Accordingly, the $\Delta\Psi_m$ is tightly regulated, and disturbances are rarely detected.

3.1. Dysfunction of the OXPHOS pathway correlates poorly with disruptions in $\Delta\Psi_m$ and ATP synthesis

In the severe CI deficient *mt-Nd5/6* transgenic neuronal and cybrid cell model systems, although CI activity was reduced to ≈10% of control, the $\Delta\Psi_m$ was found to be elevated in both neurons and astrocytes by as much as 40% under resting conditions [91]. However, the $\Delta\Psi_m$ collapsed with application of the CV inhibitor oligomycin, consistent with the theory that CV can operate in reverse at the expense of ATP hydrolysis to maintain the electrochemical gradient [92]. Therefore, OXPHOS defects in this model do appear to result in a reduced ability to maintain a $\Delta\Psi_m$. Nonetheless, the ATP synthesis capacity of cells was not reported and therefore the impact on CV function cannot be fully assessed.

In contrast, no perturbation of $\Delta\Psi_m$ was detected in either the CIV deficient *mt-Co1* (mtDNA encoded) transgenic neurons and astrocytes, or primary neurons from the *Surf1* KO mouse brain [63], despite the CIV activity being reduced to less than 50% in both models.

Similarly, in the isolated CI deficient *Ndufs4* KO mouse model, the rate of ATP synthesis linked to CI substrates in brain is reduced by ≈ 25% [15]. However, CI activity is reduced to less than 20% of wild-type in brain [15,44]. These observations emphasise that the impact on overall flux through OXPHOS is often milder than the extent of impairment of a single OXPHOS complex. Unfortunately there are currently no data available in neurologically relevant samples on the $\Delta\Psi_m$ to compare to CI activity and rates of ATP synthesis.

3.2. Evidence of disrupted $\Delta\Psi_m$ and altered ATP levels in mtDNA deficient models

As previously indicated, mutations in mtDNA replication, maintenance and mtRNA translational machinery are frequently associated with combined CI and IV deficiency. These combined OXPHOS deficiencies might have a greater impact on the $\Delta\Psi_m$ and associated ATP synthesis capacity of cells, as opposed to models of isolated OXPHOS complex dysfunction.

Indeed alterations in the $\Delta\Psi_m$ and associated ATP synthesis were observed in a number of models with defective mtDNA machinery. For instance, disruption of the $\Delta\Psi_m$ by ≈30% compared to control was observed in the $tRNA^{LEU}$ glioblastoma cells carrying an mt.3243A > G point mutation, which was further compromised following glucose deprivation [72]. This also correlated with reduced steady state levels of ATP in this model to ≈50% of wild-type [72]. However, a parallel study in neuronal-like cybrid cells could not confirm these findings [73]. Total ATP was also reduced in another mutant affecting the nucleotide pool, the *Tk2* KO mouse, where ATP in the primary mouse cerebellar neurons at *in vitro* day 12 was reduced again to ≈50% of wild-type [74].

3.3. Depleted ADP, not ATP in Sod1 transgenic model

In contrast to the reported depletion of ATP in the models above, the pool of ADP was slightly depleted (by ≈20%) in isolated mitochondria from the spinal cord of p.G93A *Sod1* transgenic mice, although no data were provided for ATP levels [25]. In addition to the previous observation that OXPHOS is perturbed in this model, the data further support the proposal that ALS is a channelopathy, where mutant SOD1 binds VDAC, thus preventing the exchange of small metabolites such as ADP [82].

3.4. Variable presentation likely reflects the critical nature of $\Delta\Psi_m$, and steady state levels of ATP

Maintenance of the $\Delta\Psi_m$ is essential for cellular viability, even at the expense of ATP synthesis, as observed in the *mt-Nd5/6* transgenic cybrid cells [91]. The critical nature of maintaining the $\Delta\Psi_m$ is further highlighted in a Rho^0 cell model that has undetectable levels of mtDNA, yet remarkably still exhibits a $\Delta\Psi_m$ of ≈50% compared to control Rho^+ cells [92,93]. It is therefore likely that even small alterations in the $\Delta\Psi_m$, such as in the $tRNA^{LEU}$ transgenic cybrid cells, are pathologically relevant [72].

The intracellular ADP to ATP ratio is also finely tuned and highly stable *in vivo*. This was demonstrated by measurements of the ADP to ATP ratio in bicarbonate buffer perfused wild-type rat heart tissue, which found no variation in this ratio from the resting level despite an increased work output of up to 4-fold [94]. Accordingly, evidence of altered ATP and ADP levels, such as observed in the $tRNA^{LEU}$ glioblastoma cells [72] and *Sod1* transgenic mice [25], suggests that mitochondrial function is compromised. Further evidence for this is the reduced rate of ATP synthesis observed in brains from the *Ndufs4* KO mice [15].

Depletion of cellular ATP is of particular relevance in a neurological setting, considering the high energy consumption of the brain, relative to its mass [10]. It is also important to consider that although neurons are highly oxidative, they may not be the only neurological cell type affected by such an imbalance [10]. Mathematical modeling of astrocytes for example, a glial cell type performing a number of critical neurological functions, suggests that they are also highly oxidative [10–12]. Therefore, both neurons and astrocytes could be hypersensitive to dysfunction of OXPHOS, even if the impacts on ATP synthesis and $\Delta\Psi_m$ are minor.

4. Calcium dynamics

Mitochondria have a large capacity to accumulate Ca^{2+}, a function that is intricately linked with the $\Delta\Psi_m$ as above, as well as being regulated by energy dependent mitochondrial pumps [36]. Mitochondrial Ca^{2+} regulates a number of physiological processes including: the rate and frequency of Ca^{2+} signaling in a spatiotemporal manner; stimulating key mitochondrial enzymes that drive OXPHOS; regulating mitochondrial sub-cellular localisation; activating the permeability transition pore; and playing a central role in initiating cell death pathways [36,95]. Given mitochondrial function and Ca^{2+} homeostasis are closely linked, it could be expected that dysfunction of this relationship would be frequently involved in disease pathogenesis. And yet, the relationship is poorly researched in a neurological context.

4.1. Altered calcium signalling in mutant models of OXPHOS

Comprehensive studies of Ca^{2+} dynamics have been performed though in the CIV deficient *mt-Co1* transgenic cybrids and the severe CI deficient *mt-Nd5/6* double transgenic neurons and astrocyte derived cybrids [91,96]. Here, astrocytes and neurons challenged with a single glutamatergic stimulus responded with Ca^{2+} transients indistinguishable from controls. However, when *mt-Nd5/6* transgenic (and to a lesser extent the *mt-Co1* transgenic) neuronal derived cybrids were repeatedly challenged, the mutant cells, unlike the wild-type cells, were unable to return to a resting baseline cytosolic Ca^{2+} concentration. This trend was strongly associated with cells that showed the greatest reduction in OXPHOS capacity. In the double transgenic, the $\Delta\Psi_m$, which is essential for maintaining Ca^{2+} homeostasis, was also severely disrupted [91].

Primary neurons from the CIV deficient *Surf1* KO mouse also exhibit Ca^{2+} dysfunction [63]. Primary neurons challenged with a single glutamatergic stimulus responded with a markedly reduced rise of

cytosolic and mitochondrial Ca^{2+}. Further, *Surf1* KO neurons were found to be resistant to kainic acid induced Ca^{2+}-mediated excitotoxicity. Perhaps this is mechanistically associated with the increased life span of such mice as mentioned earlier, noting that the $\Delta\Psi_m$ was not altered, as in the *mt-Nd5/6* double transgenic cybrid cells.

4.2. Calcium dysfunction and neuronal cell dysfunction

Disruption of cellular Ca^{2+} dynamics could compromise the specificity of cell signaling and increase the susceptibility of the cell to entering apoptotic or necrotic cell death pathways. This is of particular relevance in neuronal cell types, which rely heavily on Ca^{2+} transients as a mechanism for signal transduction [97]. Limited evidence points to disruption of Ca^{2+} homeostasis in isolated OXPHOS models of neuronal dysfunction, but further study in other models is required to determine if it is a common feature impacting the neuropathology of mitochondrial disease.

5. Mitochondrial morphological defects

Fusion, like mitochondrial Ca^{2+} homeostasis, is also dependent on the $\Delta\Psi_m$, and is an energy dependent process [35], thus correlating with disease more broadly than just in fusion deficient cells. Mitochondria are highly dynamic intracellular organelles constantly undergoing both fission and fusion. This dynamic state is considered to be essential for the cellular positioning of mitochondria near energy demanding sources, such as the neuronal synapse and endoplasmic reticulum [98], and to allow mixing of mitochondrial pools [84]. Disruption of fusion in particular is associated with mitochondrial diseases including Charcot–Marie–Tooth 2A (CMT2A) and OPA1 dominant optic atrophy [86,99].

5.1. Mitochondrial morphological defects associated with OXPHOS deficiency

In mouse models of isolated CI deficiency, mitochondrial morphological abnormalities were frequently observed. For instance, in nerve terminals from the *Ndufs4* KO mouse with a CI-linked ATP synthesis defect, there was accumulation of mitochondria with irregular cristae, preceding spongiform degeneration [44]. Similarly in the *Aifm1* KO mouse with a severe CI deficiency, there were degenerative mitochondria in neurons, which also preceded gliosis [46]. In the *mt-ND4* transgenic mice, mitochondria were observed to aggregate and fuse in the optic nerve, correlating with axonal degeneration [19]. Lastly, there was proliferation of abnormal mitochondria in the optic tract of the *mt-Nd6* transgenic mice that correlated with axonal demyelination [18].

Mitochondrial morphological defects were also observed in *Tk2* transgenic primary cerebellar neurons with swollen mitochondria and abnormal cristae [74], and fragmented mitochondria in the $tRNA^{LEU}$ transgenic neuronal derived cybrid cells [73].

5.2. Defective mitochondrial fusion machinery predictably correlates with morphological defects

Mitochondrial morphological defects and their impact on function have been well characterised in *Mfn2* mutant models. In the *Mfn2* cerebellar cell specific KO mouse there was a redistribution of mitochondria with swollen cristae from the axons to the soma of Purkinje cells [87], which is likely to disrupt signal transduction along axons. Similarly in the *Mfn2* transgenic motor neuron specific KO mouse, mitochondria clustered tightly in axons from motor neurons [100]. There was also an increased density of mitochondria in axons less than 3.5 μm in diameter in the neuron specific transgenic mouse [101].

Opa1 transgenic mouse models have similarly well characterised mitochondrial morphology. Features included morphological defects, reduced numbers of axons in optic nerve [102], and increased numbers of electron dense mitochondria in axons from optic nerve. This may represent an increase in the density of cristae to fulfil the energy requirements of the axon [103]. An increase in the number of autophagosomes in the retinal ganglion cell layer was also reported [103].

5.3. Anti-oxidant defence and mitochondrial morphological defects

Mitochondrial morphological defects were also detected in *Sod1* transgenic mice, which exhibited swollen dendritic mitochondria with disorganised cristae and broken outer membranes [23]. *Sod1* transgenic mice on a *Gclm* KO background showed mitochondrial damage and vacuolisation in motor neurons [26].

5.4. Contribution of mitochondrial morphological defects to disease

The pathogenesis of mitochondrial morphological defects is likely to be two-fold. Firstly, disruption of mitochondrial ultrastructure may disrupt the capacity and activity of the OXPHOS pathway, as well as sensitising the cells to mitophagy. Secondly, altered morphology may contribute to disrupted mitochondrial transport, thus modifying the spatial intracellular distribution of mitochondria and accordingly region-specific processes such as neuronal signalling along axons.

6. Oxidative damage

Dysfunction of the OXPHOS complexes, altered $\Delta\Psi_m$ and decreased ATP synthesis will all impact on energy dependent processes, and are all thought to be prime contributors to the neuropathology as discussed above. Oxidative damage though has also been proposed to be involved in cellular damage and dysfunction. ROS and reactive nitrogen species are chemically reactive molecules containing O_2 or nitrogen. The majority of ROS generated in a mammalian cell is believed to be generated in the mitochondria at CI and CIII [34]. Here, it is estimated that up to 1–2% of O_2 is only partially reduced to form superoxide (O_2^-), which can then be converted to form hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH^-) [104]. These molecules can react with and damage proteins, DNA, polysaccharides and lipids, and have been implicated in disease and ageing. There is a growing body of evidence that increased ROS production is a central tenet of mitochondrial neurodegenerative disorders, such as FA [105], LS [106] and LHON [107].

6.1. Evidence of excessive ROS production and cellular damage in OXPHOS mutants

It is widely accepted that if the electron transport chain (OXPHOS complexes CI to IV) becomes highly reduced, then excessive ROS may be produced [66]. This could be expected with mutations that allow the OXPHOS complexes to oxidise substrates but block efficient electron transfer, such as mutations in many OXPHOS subunits or dysfunctional ISC biogenesis.

Indeed evidence for this has been observed in several neurologically relevant models of isolated CI deficiency, including the *Ndufs4* KO mouse model [44,45], *mt-Nd5/6* double transgenic cybrids [91], ND6 transgenic mouse [18] and *Aifm1* KO mouse [108]. In these models, elevated production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) were frequently observed, correlating with damage to proteins and lipids in regions with pronounced neuropathology. Specifically, in dopaminergic (DA) neurons of the *Ndufs4* KO mouse, H_2O_2 production was normal at rest, but increased with the addition of the CI inhibitor rotenone more than controls [45]. There was also evidence of ROS damage to proteins in the olfactory bulb, an area prominently affected by the disease, as noted by an increase in cleaved caspase-8 and ultrastructural features of necrotic cell death [44]. In addition, *mt-Nd5/6* transgenic cybrid cells

show evidence of elevated O_2^- production [91]. This was accompanied by a severely depleted pool of the free radical scavenger glutathione in neurons and astrocytes [91]. Similarly the *mt-Nd6* transgenic mouse also showed evidence of elevated H_2O_2 production linked to CI substrates in isolated synaptosomal brain mitochondria, while ROS production for CII substrates was reduced [18]. Again, there was also evidence of ROS damage to proteins in whole brain extracts from this model. Lastly, in the Harlequin mouse (*Aifm1* KO), there was increased catalase activity in the cerebellar neurons, elevated pools of glutathione in brain and evidence of ROS damage to DNA and lipids, while SOD1 and 2 levels were unchanged [108]. Primary granule cells isolated from these mice were also observed to be more sensitive to H_2O_2 treatment and glutamate induced toxicity, perhaps consistent with granule cell death in these mice.

Similar to the isolated CI deficient models, there is also suggestion of excessive ROS production and ROS-mediated damage in isolated models of CII, III and IV. For example, in the transgenic *Sdhc* mouse O_2^- production in brain linked to CII substrates was elevated, with ROS damage to proteins [56]. Likewise, in the neuronal specific *Uqcrfs1* and *Cox10* KO mice, SOD2 expression was elevated and there was evidence of ROS damage to DNA, proteins and lipids in the piriform cortex, an area notably affected in the *Uqcrfs1* KO mouse, as suggested by prominent cell death [60].

6.2. Little evidence of ROS damage in mutant models of mtDNA replication, maintenance and mtRNA processing

As with the models of isolated OXPHOS complex defects, models of mtDNA replication, maintenance and mtRNA dysfunction are strongly associated with CI and IV deficiency. However, there has been no evidence for elevated ROS production or damage in such models. Rather, only slight alterations in the anti-oxidant defence machinery were observed in the *Tfam* KO mouse [69] and $tRNA^{Leu}$ transgenic cybrids [72,73]. This may reflect that the OXPHOS machinery is still functional in such models, albeit with reduced flux through the pathway, and accordingly may actually produce less ROS. This model is supported again by Rho^0 cells, which were observed to produce significantly less superoxide than control Rho^+ cells, consistent with the theory that reduced flux through OXPHOS may produce less ROS [93].

In the neuronal specific *Tfam* KO mouse, glutathione peroxidase expression, but not activity, was marginally up-regulated [69]. The function of the glutathione peroxidases in anti-oxidant defences is to reduce H_2O_2 and lipid hydroperoxides to water [109]. However, there was no evidence of ROS damage. Likewise, in the $tRNA^{Leu}$ transgenic neuronal and glioblastoma derived cybrid cells there was also no indication of elevated ROS production or altered ROS defences [72,73], although a smaller pool of GSH (reduced glutathione) was observed in the neuronal derived cybrids [73].

6.3. Oxidative stress associated with mutant models of anti-oxidant defences

There are a number of key mitochondrial and cytosolic enzymes that detoxify ROS and prevent ROS-mediated cellular damage, including the superoxide dismutases (SOD1 and 2) [104], peroxiredoxins (PRX1-6) [80], catalase [110] and the glutathione peroxidases (GPRX1-4) [109,111].

Mutations in SOD1 are perhaps the most relevant class of mutations in anti-oxidant defence machinery causing neurodegeneration and disease, such as that associated with ALS [25,81,82]. Nonetheless, mouse models of ALS with SOD1 mutations are surprisingly poorly characterised for ROS production and associated damage. The only relevant report is of *Sod1* transgenic mice on a glutamate-cysteine ligase KO background, which manifest oxidative damage to proteins in spinal cord extracts [24,26].

Sod2 (nuclear encoded) KO mouse also manifest neurologically relevant symptoms (spongiform encephalopathy) [112–114]. Here, there was clear evidence of ROS damage to DNA in brains from neonatal mice in *Sod2* KO mice [115], correlating with swollen mitochondria in neurons and cellular degeneration [112]. In an alternative *Sod2* KO mouse, the life span of the mice could be doubled (mean 8 days to 16 days) following treatment with the anti-oxidant MnTBAP [113]. In contrast, in the *Sod2* post-natal motor neuron specific KO mouse, O_2^- production is increased in spinal cord, but evidence of ROS damage is lacking [114].

6.4. ROS production, damage and defence in FA models is poorly characterised

Evidence suggests that patients with FA produce elevated levels of ROS, and may be more susceptible to ROS mediated cellular damage [77,78]. However, mechanistically, this relationship is poorly understood. It is possible that excessive ROS production is linked to OXPHOS dysfunction and a highly reduced electron transport chain [76]. Alternately, it may be linked to increased free iron, which can enhance production of more reactive species via Fenton chemistry [76]. Dysfunctional ISC biogenesis could also indirectly lead to dysfunction of anti-oxidant defence components such as glutathione production [116]. Biochemical analyses of ROS production and associated damage however, have been poorly characterised in neurologically relevant disease models. The only report of alterations to anti-oxidant defence is observed in the cerebrum, brain stem and cerebellum in mice with knock-in human *FXN* with GAA repeat expansions (90/190 GAA repeats) on a mouse *Fxn* KO background [117]. In these tissues, SOD1 and SOD2 expression are slightly elevated and there are slight increases in protein and lipid oxidation [117].

6.5. ROS dysfunction and the relationship with mitochondrial associated neuropathology

Analysis of neuronal models for mitochondrial disease suggests that oxidative damage is a biochemical feature in some cases, as elevated ROS, cellular damage and altered anti-oxidant defences are observed. Frequently, this damage appears to correlate with disease progression, as in granular cell neurons from the *Aifm1* KO mouse [108], the olfactory bulb in the *Ndufs4* KO mouse [44] and in the piriform cortex from *Uqcrfs1* KO mouse [60]. However, this may simply be correlative and there are equally as many models where ROS production does not seem to match disease progression, such as the *Sdhc* transgenic mouse [56], *Tfam* KO mouse [69] and Fxn/*FXN* KO/transgenic mouse [117].

7. Integrity of mtDNA

Compromised mtDNA is most commonly associated with defective mtDNA replication and maintenance machinery, but may also result from other types of mutations, such as those in the mitochondrial fusion machinery.

7.1. mtDNA mutations and mtDNA depletion are frequently associated with deficient and defective translation and transcription machinery

It is of little surprise that mutations in mtDNA replication, maintenance or nucleotide synthesis machinery frequently result in depletion of mtDNA. This has been observed in *Tk2* KO mice [67,118,119], *Tfam* transgenic mice [69], *UDG* transgenic mice [75], and *Peo1* transgenic mice [71]. As well as mtDNA depletion, alterations in nucleotide pools were also observed in the *Tk2* transgenic mouse (decreased dTTP), *Tymp/Upp1* double KO mouse (reduced pool of dCTP and elevated pool of dTTP) and *UDG* transgenic mice (elevated pool of dTTP). Lastly, a very high mutational frequency of mtDNA in

brain from the nuclear encoded *Polga* transgenic (mutator) mice was also detected [120].

7.2. Alteration of mtDNA in mitochondrial fusion-deficient model

In the *Opa1* transgenic mouse, OPA1 expression is reduced by ≈50% [85]. The optic nerve is notably affected in this model, an area found to have mild mtDNA proliferation, perhaps indicative of compensation. The mice also manifested premature age-related axonal and myelin degeneration.

7.3. mtDNA and mitochondrial disease

As previously discussed, mutations in mtDNA associated machinery typically correlated with CI and IV deficiency. The decreased mtDNA copy numbers are expected to cause reduced expression of mitochondrial encoded OXPHOS subunits. Accordingly, dysfunction of OXPHOS is likely the basis of disease in this group of mutations. mtDNA proliferation can also correlate with disease however, as was observed in the *Opa1* transgenic mouse [85], which may be representative of a compensatory stress response to OXPHOS dysfunction.

8. Common biochemical features of mitochondrial neuropathology

Neurological dysfunction associated with mitochondrial disease most typically manifests as impairment of OXPHOS. It was therefore traditionally considered that the $\Delta\Psi_m$ would be disrupted and consequently, ATP synthesis. The disease was thought to be an energy deficiency disorder, as evidenced by the 'metabolically' active tissues that were primarily affected such as the heart, muscle, and brain. As discussed above, although it is clear that altered $\Delta\Psi_m$ and ATP synthesis contribute to neuropathogenesis in a limited number of models, the biochemical mechanisms of disease are far more diverse and complex. For example, it is now critical to understand which pathways are sensitive to such disturbances in neurologically relevant tissues. Such an example is Ca^{2+} homeostasis, where dysfunction was so clearly defined in cybrid models of isolated CI and CIV deficiency, providing a window into neuron-specific dysfunction.

Altered mitochondrial structure and localisation were also commonly observed, as best characterised in the fusion deficient MFN2 and OPA1 disease models. Mitochondrial structure is critical for maintenance of OXPHOS and associated ATP synthesis. Structural alterations are likely to disrupt these processes, and are also known to prevent the mixing of mitochondrial contents, which sensitises the cell to mitophagy. The subcellular localisation of mitochondria is also of critical importance to cellular function, as regulated by Ca^{2+} gradients. Redistribution of mitochondria in neurons e.g., from the axons to soma in the *Mfn2* KO mouse may also give us clues about the tissue-specific nature of the disease.

ROS production is also a hallmark of mitochondrial disease, with excess production and altered defences observed in a wide range of models including isolated OXPHOS deficient models, mutations in ROS defence machinery, and to a lesser extent mtDNA maintenance and replication machinery. ROS damage to cellular structures such as DNA, proteins and lipid has been duly characterised in many of these models, and almost certainly contributes to pathogenesis. As before however, the understanding of ROS production and defence in mitochondrial disease is still at a very basic level. Altered ROS homeostasis for example is likely to also involve altered signalling pathways, which have not been discussed in any detail in this review.

OXPHOS dysfunction, altered $\Delta\Psi_m$ and associated ATP synthesis, disturbances in cellular processes such as Ca^{2+} homeostasis, ROS imbalances, and mitochondrial morphological defects are all hallmarks of neurological dysfunction. However, other pathogenic mechanisms are also certainly involved. Other emerging pathogenic mechanisms not discussed in any detail in this review include altered cell signalling and disturbances in metabolite pools.

Accordingly, the mechanisms of neuronal dysfunction in mitochondrial disease are only just beginning to be understood, are system dependent and complex, and not merely driven by energy deficiency. The diversity of pathogenic mechanisms emphasises the need to characterise these in a wide range of models, as different therapeutic strategies are likely to be needed for different diseases.

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