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Review

Mitochondrial complex assembly in epilepsy of primary mitochondrial disease origin

Gavin P. McStay

School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, Merseyside L3 3AF, United Kingdom

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ABSTRACT

Primary mitochondrial diseases are caused by mutations in genes required for expression, function or assembly of the mitochondrial oxidative phosphorylation system. The pathology of primary mitochondrial diseases is varied and a subset of these are associated with epilepsy and seizures. Mutations are found in each of the 5 complexes of the oxidative phosphorylation system in both structural subunits and assembly factors along with mitochondrially encoded components of the protein synthesis machinery. This review will highlight the mutations identified in clinical case studies that are associated with epilepsy and seizures and include the studies using cell systems and other model organisms where molecular characterisation of oxidative phosphorylation is more extensive. The molecular causes of epilepsy have not been well characterised in the relevant cells. This review identifies gaps in knowledge and suggestions for future studies to advance the understanding of the molecular pathogenesis of epilepsy that is associated with primary mitochondrial disease.

1. Introduction

Epilepsy is a neurological condition resulting in uncontrolled seizures comprised of loss of consciousness, awareness, or movement control and with effects on senses, emotions, and behaviours. The seizures are due to a variety of causes including genetics, brain injury or developmental disorders with unclear mechanisms. Epileptic seizures can be treated with medications, in addition to surgical or neurostimulatory interventions. However, the wide variety of causes makes epilepsy difficult to diagnose and treat successfully [1].

At the cellular level, epilepsy is caused by a synchronised overstimulation of neuronal activity. This hyperactivation of neurons can be caused by increasing activity of excitatory neurons, or decreased activity of inhibitory neurons. Neurons can obtain epileptogenic properties through a variety of dysfunctions in ion channel function, issues with neurotransmitter release, re-uptake or receptors, glial cell dysfunction or mitochondrial dysfunction [2]. This review will focus on the mitochondrial role in epilepsy.

Neurons are the main communicating cell in the brain and rely on intercellular communication by neurotransmitters but also require electrical signalling to propagate signalling along the length of the cell. These requirements for cellular function have high demands for energy meaning neurons have an enormous dependence on mitochondrial function. Loss of mitochondrial function is associated with very many neurological conditions ranging from neurodegenerative, neuromuscular and neurodevelopmental disorders amongst others. In addition to the role of mitochondria in energy provision, mitochondria play essential roles in cell death, calcium signalling, reactive oxygen species (ROS) generation and metabolism – all of utmost importance for neuronal function [3].

To support the neuronal network in the brain, glial cells, such as astrocytes function to maintain the neuronal environment to enable efficient neurotransmission. Astrocytes have an abundant mitochondrial network, which is comparable to the mass found in neurons. Astrocytes are heavily involved in glutamate metabolism to generate glutamate for release from neurons and recover the vast majority released into the synaptic cleft to prevent excessive accumulation. Mitochondria are crucial for these aspects of glutamate homeostasis. Mitochondria are also critical for calcium ion and ATP homeostasis in astrocytes that can have an indirect impact on neurotransmission if there are defects in homeostasis [4].

2. Mitochondrial role in neuronal cells

Mitochondrial functions have expanded recently from their original role in ATP synthesis to fuel cellular activities. The role in energy production has typically been seen as the cause of disease. Neuronal cells require a high supply of energy to enable high activity for

E-mail address: g.p.mcstay@ljmu.ac.uk.

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electrochemical signalling and neurotransmission. However, the more recently characterised functions could also influence cellular and tissue activity, such as, cell death, immune responses, development, and intracellular signalling, and evidence supports the role in some of these processes in epileptogenesis. These functions could impact in all aspects of neuronal cell and tissue development and maintenance to cause seizures because of inappropriate neuronal differentiation, connections, function or longevity [3].

3. Mitochondrial oxidative phosphorylation complex assembly in neuronal cells

Mitochondrial function depends on the generation of an electrochemical gradient, known as the proton motive force (PMF), across the inner mitochondrial membrane (IMM). This is mainly driven by the oxidation of carbon-based substrates that result in the capture of electrons by carriers such as NADH/NAD^+ (nicotinamide adenine dinucleotide) and $FADH_2$/FAD (flavin adenine dinucleotide). These directly supply electrons to the electron transport chain (ETC) and passed through a series of electron carriers within or associated with the IMM ultimately being captured by molecular oxygen to form water. Alongside the passage of electrons there is a coupled translocation of protons from the matrix to the inter-membrane space (IMS). This PMF provides a strong gradient of charge and pH across the IMM which drives ATP synthesis as well as other functions such as import of proteins and small metabolites through specific transporters and carriers. The translocation of protons from the matrix to the IMS occurs through three of the four ETC components, Complex I (NADH:ubiquinone oxidoreductase), Complex III (ubiquinone:cytochrome *c* oxidoreductase) and Complex IV (cytochrome *c* oxidase). These complexes are made up of multiple subunits that are derived from the nuclear genome and the separate genome found within the mitochondria.

Complexes of the oxidative phosphorylation (OXPHOS) system follow a highly regulated process of assembly to ensure subunit and cofactor availability, correct subunit folding, and post-translational modification. These control mechanisms are mediated by a series of assembly factors that guide and monitor the assembly of each complex. These assembly factors are not associated with the final assembled complex but associate transiently with a specific sub-assembly [5]. Most of the OXPHOS complexes are made up of structural and functional modules that assemble independently before final association to form the fully assembled enzyme complex. Some of these sub-assembly modules have been detected in mitochondria as latent species awaiting other modules for final incorporation.

Defects in assembly and activity of these complexes results in mitochondrial dysfunction and is often associated with inherited mitochondrial diseases. These primary mitochondrial diseases follow two types of inheritance patterns, a typical Mendelian inheritance pattern where parental alleles will determine genotype and phenotype. These diseases can result from mutations in the structural subunits of complexes, but also from mutations in complex-specific assembly factors. In addition to these, mutations found on mitochondrial DNA can cause mitochondrial disease. Inheritance in this manner is mediated entirely through the maternal line as only mitochondrial DNA is passed from mother to child. To complicate matters further, mitochondrial DNA exists in multiple copies in a cell, as well as with different genotypes, a scenario known as heteroplasmy. The inheritance pattern, copy number and heteroplasmy have made it extremely difficult to diagnose and treat these diseases [6]. Not all genes found in mitochondrial DNA harbour mutations that result in epileptic symptoms. Both mitochondrial DNA-encoded ATP synthase genes, MT-ATP6 and MT-ATP8 are causative for epileptic conditions. Most Complex I genes have been associated with epilepsy. The single mitochondrially encoded Complex III gene MT-CYTB is associated with epilepsy. Several tRNA genes harbour mutations associated with epilepsy. However, the 3 genes of Complex IV, MT-COI, MT-COII or MTCOIII, nor the ribosomal RNA genes, MT-RNR1 or MT-RNR2 have been associated with epilepsy (Fig. 1). There are also many nuclear encoded genes, including both structural subunits and assembly factors associated with epilepsy.

In addition to the ETC complexes, a final complex in the OXPHOS process mediates the conversion of PMF gradient into chemical energy in the form of ATP. F_1F_O-ATP synthase (Complex V), also located in the IMM translocates protons from the IMS to the matrix, down the proton concentration gradient, capturing the potential energy through a mechanical process resulting in a chemical process to synthesise ATP. ATP synthase also is made up of nuclear and mitochondrial encoded subunits, meaning a requirement for assembly factors which can also lead to mitochondrial diseases similar to those associated with the ETC [5].

Defective assembly or activity of OXPHOS complexes leads to primary mitochondrial diseases. These affect approximately 1/5000 people. Severity of disease is difficult to predict; symptoms are hard to use to diagnose disease and the treatments available only impact to extend life and decrease suffering without curing the disease. Mitochondrial diseases can be sub-classified based on pathologies, such as Leigh Syndrome and Myoclonic Epilepsy with Ragged-Red Fibres (MERRF), as examples where epilepsy is associated. However, each of these diseases can be caused by different mutations in one of several genes, on either the nuclear or mitochondrial genome, leading to differences in presentation and pathology.

Clinical analysis of patients and families has led to the identification of many genes associated with epilepsy. This involves determination of the genetic nature of the disease by identifying the responsible gene and inheritance pattern. Histological techniques are used to determine which tissues display defects. These are usually done through biopsies of muscle which can be obtained and analysed routinely. Biochemical analysis is performed on muscle or skin biopsies, along with blood samples to determine defects in individual OXPHOS components. Each complex can be biochemically assayed to determine where dysfunction lies measured as a significant decrease in activity. However, there is no universal standard of deficiency as biochemical tests of each OXPHOS complex can result in differences from laboratory to laboratory. Therefore internal laboratory controls and normalisation are required, such as through the measurement of a mitochondrial marker, such as citrate synthase [7]. For example, deficiencies in normalised Complex I activity ranged from 24 % to 38 % on peripheral blood leukocytes in a study of 67 children in China [8]. This analysis does not necessarily reflect the actual loss of activity of a particular complex in another tissue, such as the muscle, or a tissue that cannot be readily accessed, such as the brain. Continuing to use Complex I as an example, activity lower than 20 % or 30 % of control, depending on tissue or cell type is considered a major deficiency, while <20 % to 40 % is considered a mild or possible deficiency [8]. Despite this, a well-established pipeline of diagnosis of the genetic nature of the disease is followed. However, the molecular pathology causing the disease has been difficult to determine. A variety of mutations have been identified that cause the mitochondrial basis of epilepsy, but characterisation of each of these genetic changes requires analysis using expensive and labour-intensive molecular approaches. In the context of epilepsy, the physiological location and nature of the condition make it extremely difficult to analyse human brain tissue or generate human-relevant models of the disease in neuronal cell systems. This review will focus on the genetic causes of epilepsy in humans with particular focus on case studies in conjunction with other molecular-based analysis using model systems [6].

The main primary mitochondrial diseases where epilepsy is associated are LHON (Leber's hereditary optic neuropathy, Leigh Syndrome, MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes), MERRF and NARP (neuropathy, ataxia, and retinitis pigmentosa). LHON is estimated to affect between 1 in 30,000 to 1 in 50,000 people in the UK and is primarily associated with loss of central vision in both eyes. LHON Plus is a more severe form of LHON that has symptoms occurring outside of the eye, including movement disorders, tremors, cardiac defect, muscle weakness, poor coordination and

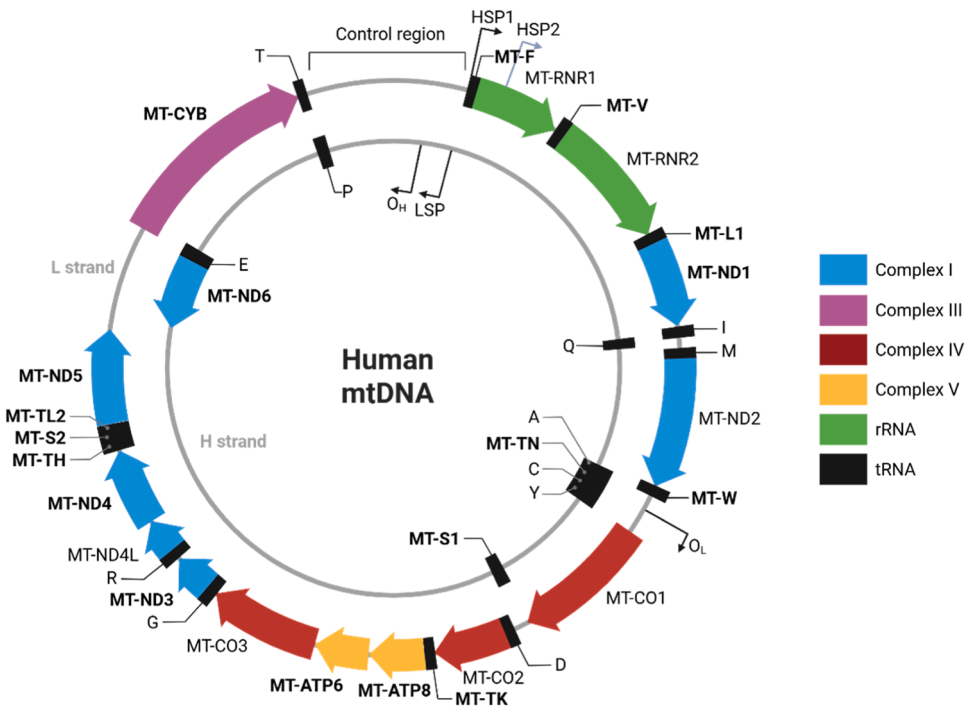

Fig. 1. Mitochondrial DNA encoded genes associated with diseases where epilepsy and seizures are associated. Genes where mutations are associated with epilepsy or seizures are highlighted in bold. Transfer RNAs with mutations associated with epilepsy or seizures are labelled as MT-T to highlight these from those not associated with the condition. Figure created in BioRender.

seizures. Leigh Syndrome has a prevalence of approximately 1 in 40,000 births and is a progressive neurodegenerative disorder of children initiating by the age of 2 years. Symptoms include developmental delay, ataxia, dystonia and ophthalmological disorders [9]. MERRF, is a disease characterised by myoclonic epilepsy and histological observation of muscle fibres, that primarily affects children. The estimated prevalence of the disease in <1 in 100,000 individuals. MELAS, has a strong association with epilepsy. It has a frequency of 1 in 6000 individuals and is associated with stroke-like episodes, muscle weakness and other symptoms with clinical heterogeneity [10]. NARP is a condition associated with seizures, in addition to neuropathy, ataxia, retinitis pigmentosa, muscle weakness, dementia, developmental delay and sensory neuropathy. Other isolated cases of epilepsy have been attributed to mutations in mitochondrial OXPHOS assembly, but symptoms do not fully align to LHON or MERRF and therefore have not been diagnosed as such.

This review will provide a systematic overview of the molecular basis of epilepsy associated with mitochondrial diseases using case studies, genome analysis, molecular characterisation and model systems. This will highlight the current understanding in the field with the aim of identifying gaps in knowledge for future research.

4. Complex I assembly defects resulting in epilepsy

Complex I (NADH:ubiquinone oxidoreductase) is the largest complex of the OXPHOS system with an estimated 47 structural subunits present in the final assembled human enzyme. The multi-subunit enzyme is composed of 3 main functional modules: The N module (NADH dehydrogenase) accepts electrons from NADH to pass on to a series of Fe-S clusters and flavin mononucleotide (FMN) co-factors, that then pass onto the Q module, responsible for electron transfer to oxidised Coenzyme Q10 (ubiquinone/ubiquinol). The P module is coupled to the N and Q modules to enable proton translocation from the matrix to the IMS to fuel the mitochondrial electrochemical potential.

Mutations that cause seizures and epilepsy are found in all 3 modules of Complex I and are found in both mitochondrial and nuclear genes as well as in assembly factors (Table 1).

5. Mitochondrially encoded complex I genes with mutations associated with epilepsy

MT-ND1 is located in the P module and is involved with the association with the Q module. The first report of mutation in MT-ND1 associated with seizures was in 2009 of a 10-month-old presenting with mitochondrial encephalomyopathy including tonic seizes with absence along with episodes of respiratory and metabolic distress. Complex I activity was found to be reduced in muscle and fibroblasts. The mutation was found to be homoplasmic for 3688G>*A* resulting in an Alanine to Threonine mutation at position 218. This mutation resulted in specific reduction of Complex I subunits and fully assembled complex [11]. Several years later, two unrelated children from China with seizures that started after approximately 6 months were found to have the 3955G>*A* variant. Both children did not have a family history of this mutation leading to the suspicion of a *de novo* mutation. The mutation was found to have a heteroplasmy frequency greater than 70 % in the tissues tested. The mutation results in an Alanine to Threonine alteration at the highly conserved amino acid position of 217, the preceding amino acid from the other case reported initially in 2009. Analysis of the atomic structure of Complex I predicts this mutation to interfere with neighbouring subunits and results in destabilisation. MT-ND1 protein was expressed at approximately 50 % of wild-type in cybrid cells and family-derived fibroblasts. Other Complex I subunits displayed decreased expression by western blotting, which was also seen with native gel electrophoresis of the fully assembled Complex I and supercomplexes. Other OXPHOS complexes were not affected in the mutant cells. The defects in Complex I protein expression resulted in decreased respiration of cells that was specifically due to Complex I. Cybrid cells with this mutation also displayed enhanced amounts of reactive oxygen species and decreased mitochondrial membrane potential [12].

MT-ND3 is a structural subunit of the P module encoded by mitochondrial DNA. The first description of a mutation in the *MT-ND3* gene associated with epilepsy was described in 2001 in a 42-year-old man. Symptoms emerged at 24 years of age including migraines, ataxia and decreasing cognitive function. Complex I activity was substantially

Table 1
Mutations in Complex I genes associated with epilepsy. Gene names in bold are associated with epilepsy, others have not been associated with epilepsy. N, P, and Q indicate which module the subunit or assembly factor is involved in.

Complex I structural subunit		Complex I assembly factors
Nuclear	**Mitochondrial**	**NDUFAF2 (N/Q)**
NDUFA1 (P)	**MT-ND1 (P)**	**NDUFAF3 (Q/P)**
NDUFS1 (N)	**MT-ND3 (P)**	**NDUFAF4 (Q/P)**
NDUFS4 (N)	**MT-ND5 (P)**	**NDUFAF6 (Q/P)**
NDUFV1 (N)		**FOXRED1 (P)**
NDUFS8 (Q)		
– N module -		– N module ——
NDUFV2		NUBPL
NDUFA2		
NDUFV3		
NDUFA12		
NDUFS6		
— Q module –		– Q/P module ————
NDUFA7		TIMMDC1
NDUFAB1		
NDUFA6		
NDUFA9		
NDUFA5		
NDUFS2		
NDUFS3		
NDUFS7		
– P module –	– P module –	– P module ——
NDUFA3	MT-ND4L	TMEM186
NDUFA8	**MT-ND6**	COA1
MDUFA13	MT-ND2	ACAD9
NDUFC1	**MT-ND4**	ECSIT
NDUFC2		NDUFAF1
NDUFA10		TMEM70
NDUFS5		ATP5SL
NDUFA11		
NDUFB5		
NDUFB10		
NDUFB11		
NDUFB6		
NDUFB1		
NDUFB4		
NDUFB7		
NDUFB3		
NDUFB8		
NDUFB9		
NDUFB2		

decreased compared to the activity of other complexes in skeletal muscle mitochondria [13]. Following on from this, twelve published cases of the *MT-ND3* mutation 10191T>*C* associated with seizures were summarised in 2012. The mutation 10191T>*C* resulted in a Serine to Proline change at conserved position 45. The patients displayed varying ages of onset and heteroplasmy. Mutation in this subunit was found in a rare adult-onset case of Leigh Syndrome. A 24-year-old female with a history of epilepsy for 6 years was diagnosed with MELAS-LS overlap syndrome. This report also highlighted the exceptional variety of symptoms of this mutation in a comparison of 28 cases, where 15 were associated with seizures [14].

Mutations in *MT-ND4* have been predicted to be causative of temporal lobe epilepsy in genome analysis studies and in Han Chinese populations. The 11994C>*T* mutation was identified as significantly associated with patients in a study of mesial temporal lobe epilepsies and hippocampal sclerosis. This mutation results in a Threonine to Isoleucine change at amino acid 412, but this was not characterised further [15]. A 11087T>*C* mutation that would cause a Phenylalanine to Leucine change was predicted to deleteriously impact MT-ND4 through computational analysis [16]. However, further clinical or biochemical characterisation of this mutation was not performed. Other variations in *MT-ND4* sequence were identified in the population but the predicted impacts on protein structure did not suggest a deleterious effect would be observed.

MT-ND5 is a structural subunit of the P module encoded by mitochondrial DNA and three published clinical cases of disease-causing mutation exist. A patient with MERFF was found to have a 13042G>*A* transition resulting in a substitution of the Alanine to Threonine at position 236. The patient was healthy until at the age of 17 years a seizure occurred. Following on from this, strokes and neurological dysfunction occurred, and he was assessed at the age of 25 years. The mutation was found to be 90 % heteroplasmic in muscle and 50 % in blood and Complex I activity in the muscle was 7-fold lower than in control samples [17]. In another case, a patient displaying 3-way overlap syndrome of MELAS, LHON and Leigh Syndrome was found to have the mutation 13042G>*A* which resulted in a Methionine to Leucine substitution at position 237. This patient presented with seizures at the age of 7, but without typical loss of Complex I function or histological markers of the pathology [18]. A third case of an 11-year-old child analysed for mitochondrial encephalomyopathy was described in 2009. The child presented with occasional seizures and deteriorated generally after the age of 7 years, along with ophthalmoplegia, facial weakness and swallowing difficulties. The mutation 13094T>*C* caused a Valine to Alanine change at position 253 with heteroplasmy of 30–50 %. This patient maintained close to normal activity of Complex I in muscle and fibroblasts but displayed low expression of fully assembled Complex I [11]. Genome analysis also identified a 13231A>*C* mutation is significantly associated with mesial temporal lobe epilepsy-hippocampal sclerosis patients. This results in a Lysine to Glutamine change at amino acid position 299, but molecular analysis is still needed [15]. The milder phenotypes associated with MT-ND5 may be due to the late stage at which this subunit associates.

MT-ND6 association with epilepsy is from a Belgian family harbouring the 14487T>*C* mutation. This mutation results in a Methionine to Valine change at position 63. This mutation was rated as a pathogenic mutation based on protein structure and conservation. The severity of the condition was closely linked to the level of heteroplasmy, although not all family members displayed epilepsy, but displayed encephalomyopathy and other symptoms associated with LHON [19]. Biochemical analysis of a separate case using cybrid cells demonstrated a loss in Complex I activity that correlated with heteroplasmic state. The cybrid cells contained less fully assembled Complex I and increased levels of lower molecular weight complexes indicating incomplete assembly [20].

6. Nuclear encoded genes of complex I with mutations associated with epilepsy

NDUFA1 is a structural subunit found in the P module of Complex I that is encoded by the nuclear genome. One patient presenting with epilepsy at age 9, along with delayed walking and language skills was identified with a mutation in the NDUFA1 subunit. A transition at 251G>*C* resulted in an Arginine to Serine substitution at this highly conserved position 37 in a domain involved in interactions with other subunits. This mutation is associated with decreased Complex I activity as measured by blue native PAGE, as well as a substantial decrease in abundance of the fully assembled Complex I. Interestingly, there was an increase in the activity of other OXPHOS complexes, perhaps as a compensatory mechanism for Complex I deficiency [21].

NDUFS1 is a structural subunit in the N module of Complex I, encoded by the nuclear genome. It is the largest subunits of Complex I and contains one of the Fe-S cluster. Mutations in *NDUFS1* have been identified in Leigh Syndrome patients, a disease typically presenting with seizures. However, several patients have been described with NDUFS1 mutations, without report of seizures [22–24]. This perhaps indicates mutations in *NDUFS1* are not associated with epilepsy or these

have not been described due to severity of the pathology associated with this form of Leigh Syndrome.

NDUFS4 is a structural subunit in the N module of Complex I encoded by the nuclear genome. This small subunit has a phosphorylation site for cyclic AMP-dependent protein kinase. Mutations in this subunit cause Leigh Syndrome-like symptoms, but no patients have been reported with epilepsy. However, mouse models of Leigh Syndrome, with a whole body or tissue-specific knockout of the *NDUFS4* gene display epileptic seizures. The whole-body knockout mice died at day 50 due to a set of complex phenotypes [25]. The brain-specific knockout driven from the Nestin locus, resembles the whole-body knockout indicating the phenotype is due to the neuronal pathology. Some mice displayed tonic-clonic seizures while a larger proportion developed seizures due to handling, which increased as the disease progressed. The brains of total body and brain-specific knockout displayed very low Complex I activity [25,26]. Using more specific neuronal knockout of NDUFS4 determined that GABAergic neurons lacking NDUFS4 led to a phenotype where severe epileptic seizures occurred [27]. The impact on Complex I assembly in the brain has not been reported but is expected to be absent due to the central nature of this subunit on the Complex I structure as seen in other mitochondrial diseases.

NDUFV1 is a structural subunit found in the N module of Complex I that is encoded by the nuclear genome. NDUFV1 harbours the flavin cofactor and is also a site of ROS production. Various mutations in several families have been identified that result in epilepsy. Compound heterozygosity of *NDUFV1* was identified to be the cause of disease for 2 brothers. A 1268C>*T* transition resulted in a Threonine at position 423 to Methionine in the maternal allele, while a 175C>*T* transition resulted in a termination codon instead of Arginine 59, which caused nonsense-mediated degradation and almost complete absence of the paternal allele. The young brothers suffered from severe encephalopathy and died before the age of 2 years old [28]. In another patient, compound heterozygosity was again identified as the cause of neurologic abnormalities. A transition at 640G>*C* resulted in a Glutamine to Lysine substitution at position 214. The second mutation interfered with mRNA splicing at intron 8 and resulted in an unstable mRNA [29]. To determine the deficiency associated with each of these amino acid substitutions mutations were introduced into a yeast model suitable for Complex I analysis. Introduction of the T423M mutation result in complete absence of Complex I expression, explaining the severe pathogenic phenotype seen in the young brothers. The E214K mutation caused a decreased association of FMN with the subunit, lower Complex I activity, accompanied with increased ROS. Comparison with other pathogenic mutations in *NDUFV1* in this model revealed that mutations can result in complete absence of Complex I, decrease in FMN association, and impaired activity [30].

NDUFS8 is part of the Q module and houses an Fe-S cluster. *NDUFS8* was identified as the first nuclear-encoded subunit of Complex I to be associated with Leigh Syndrome. A 5-week-old patient feeding poorly, with apnoea and cyanosis was found to have compound heterozygous mutations in NDUFS8 at position 79, Proline to Leucine, and at position 102, Arginine to Histidine, caused by 236C>*T* and 305G>*A* mutations respectively. Complex I activity was found to be severely decreased compared to control sample in muscle, skin fibroblasts, heart, liver and brain [31]. A similar set of compound heterozygous mutations were identified, where a 254C>*T* transition alongside a 413G>*A* transition resulted in mutation of Proline at position 85 to Leucine and Arginine 138 to Histidine [32]. More recently, three separate pathogenic mutations were identified in 2 patients, 2 compound mutations in 1 patient, 229C>*T* and 476C.A, causing Arginine to Tryptophan mutation at position 77, and Alanine to Aspartate at position 159. A homozygous mutation was found in the other patient, 187G>*C*, causing Glutamate to Glutamine change at position 63. These resulted in specific defects in Complex I activity and decreased abundance of Complex I-containing supercomplexes [33]. NDUFS8 may have a regulatory role in Complex I assembly as patients with *NDUFS8* mutations express decreased protein levels of other Complex I subunits [32].

7. Complex I assembly factors

NDUFAF2 is an assembly factor involved in bringing together the N and Q modules of Complex I. It stabilises an 830 kDa complex containing NDUFS1, NDUFV1 in the absence of NUDFS4 and NDUFA12 [34]. A female patient with a *NDUFAF2* mutation was identified with decreased complex I NADH:ubiquinone reductase activity in both muscle and fibroblast mitochondria. The mutation in the patient, ATG -> TTG, resulted in a substitution of Leucine at the initiation codon. This resulted in undetectable amounts of NDUFAF2 in fibroblasts. The patient died at 2 years of age [35]. A second patient that died at 2 years old had a homozygous deletion in the *NDUFAF2* gene, which resulted in a severe Complex I activity deficiency in muscle [36]. Despite complete absence of this assembly factor Complex I activity was still detected indicating NDUFAF2 is not essential for assembly. However, the deficiency is so severe to cause death at a young age. Patients with other types of pathologies due to *NDUFAF2* mutations also display undetectable levels of the protein, but moderate effects on Complex I activity, indicating the tolerance to defects in this protein, but these generally result in death at a young age [37–41]. Molecular investigation in knockout cell lines demonstrated that absence of *NDUFAF2* resulted in increased ROS production, loss of mitochondrial DNA, while maintaining Complex I abundance and activity. These observations potentially explain the severe clinical results associated with patients harbouring *NDUFAF2* mutations [42].

NDUFAF3 is an assembly factor involved in bringing the Q and P modules together. A patient with a compound heterozygous mutation at 2T>*C* and 365G>*C* resulting in Methionine to Threonine substitution at position 1 and Arginine to Proline at 122 respectively. This resulted in Complex I activity at one-third-of control activity. The patient died before the age of 6 months [43]. A 10-year-old patient presenting with neurodevelopmental disorders and exercise intolerance along with epileptiform discharges carried a compound heterozygous mutation. The maternal allele contained a 302G>*A* transition resulting in a Serine to Asparagine substitution at position 101. The paternal allele harboured a mutation resulting in disruption of a donor splice site. Fibroblast mitochondria from the patient displayed reduced Complex I and supercomplexes [44]. Other mutations in *NDUFAF3* cause mitochondrial diseases without evidence of seizures [45,46]. NDUFAF3 exists in a tight complex with NDUFAF4 which is also associated with mitochondrial diseases where epilepsy is observed [47].

NDUFAF4 functions to assemble Q and P modules during Complex I biogenesis. A homozygous 7G>*C* transition resulting in an Alanine to Proline substitution at position 3 was identified, resulting in death at 21 months. This mutation resulted in severe loss of Complex I activity and abundance [48]. A second patient was report with a 478G>*T* mutation resulting in a premature termination codon at position 160 in place of Glutamate. This resulted in decreased stability of NDUFAF4 along with other Complex I structural subunits [49].

FOXRED1 is an assembly factor involved in the assembly of the P module. A patient identified from a cohort of Complex I-deficient individuals was found to be compound heterozygous harbouring 694C>*T* and 1289A>*G* mutations causing exon skipping and Asparagine to Serine substitution at position 430. This resulted in 20 % of control Complex I activity in patient fibroblasts that could be restored with wild-type *FOXRED1* expression [37]. A second patient, presenting with

epilepsy accompanied by psychomotor retardation, was identified with a homozygous mutation, 1308G>*A* resulting in a Valine to Methionine substitution at position 421. This resulted in decrease abundance of Complex I and accumulation of Complex I sub-assemblies [50]. A third patient, with severe complications and who survived until the age of 3 months, had a compound heterozygous mutation, with a 4-nucleotide duplication at position 612, resulting in a frameshift and 874G>*A* resulting in a Glycine to Arginine substitution at position 292. This resulted in decreased Complex I activity and abundance compared to the reference ranges [51]. A fourth patient, at the age of 2 months the symptoms of mitochondrial disease began to display until the age of 15 years at the time of the report. He displayed refractory epilepsy and compound heterozygosity. A previously reported mutation, 920G>*A* resulting in a Glycine to Glutamate substitution at position 307, alongside a 733+1G>*A* splicing variant. This resulted in Complex I-specific deficiency resulting in loss of activity [52].

NDUFAF6 is associated with the assembly of the Q module. Mutations in NDUFAF6 were initially identified in children of first-cousin parents, presenting with focal right-hand seizures, weakness, ataxia and rigidity. A homozygous missense mutation 296A>*G* resulting in Glutamine to Arginine mutation in a conserved amino acid at position 99, also potentially affecting splicing at end of exon 2. This resulted in Complex I-only defect in skeletal muscle, liver and fibroblasts, while other complexes were unaffected [53]. A second patient was identified with a *NDUFAF6* mutation, resulting in an Alanine to Proline mutation at position 178, caused by a 532G>*C* mutation. The patient was heterozygous for this mutation, but the defect caused by the other allele could not be identified. A single mutated allele is not predicted to result in pathogenicity, highlighting the requirement for homozygous mutation of *NDUFAF6*. This mutation did not result in seizures, but neurological defects in gait and verbal fluency [54]. The second mutation was later identified separate patients in an intron, resulting in an alternative splice site [55]. Further studies identified 3 more patients with *NDUFAF6* mutations resulting in Isoleucine to Threonine mutation at position 124 in one allele and a frameshift mutation in the other. Again, neurological defects were observed, but not seizures [56]. These results indicate that the severity of neurological pathology depends on the mutation. Deficiency of Complex I expression and supercomplex assembly was confirmed in 2 patients displaying mild neurological defects [57]. Recently, deep mutational scanning was used to determine that NDUFAF6 is responsible for the incorporation of NDUFS8 into a 125 kDa assembly intermediate as part of Q module biogenesis [58].

As Complex I is the largest OXPHOS complex and the most complicated it would be expected that many mutations are associated with epilepsy, and this is the case. Approximately one-quarter of subunits are associated with epilepsy, however the remainder are not, indicating they have not been detected due to a very low frequency. This could be due to tolerance of mutations in neuronal cells or increased severity preventing successful embryogenesis and foetal development. Genetic models of Complex I have provided insights into the cellular and molecular nature of epilepsy through cell-specific knockout of specific Complex I genes.

8. Mutations in complex II structural subunits and assembly factors associated with epilepsy

Succinate dehydrogenase, or Complex II, is responsible for the oxidation of $FADH_2$, and transfer of electrons into the ETC derived from the citric acid cycle and β oxidation of fatty acids. Electrons are carried

Table 2
Mutations in Complexes II, III, IV and V associated with epilepsy. Gene name in bold are associated with epilepsy, others have not been associated with epilepsy. The module each gene is associated with is in parentheses.

	Complex II	Complex III		Complex IV		Complex V	
Structural subunits	**Nuclear** **SDHA** ——— SDHB SDHC SDHD	**Nuclear** – CYT B – UQCRQ UQCRB UQCR1 UQCR2 – CYT C1 – CYC1 UQCRH – RIP ——- UQCRFS1 – BC1 —— UQCR10 UQCR11	**Mitochondrial** **CYB (CYT B)**	**Nuclear** **COX6B1** **COX8A** **COXFA4** – MT-CO1 – COX4–1 Cox5a Cox7c – MT-CO3 – Cox5b Cox7a Cox6a – ??? – Cox6c Cox6b	**Mitochondrial** MT-CO1 MT-CO2 MT-CO3	**Nuclear** **ATP5F1A (F1)** **ATP5F1D (F1)** **ATP5PO(Fo)** – F1 —— ATP5F1B ATP5F1C ATP5IF1 ATP5F1E – FO —— ATP5PB ATP5PD ATP5PF ATP5MF ATP5MC1 ATP5MC2 ATP5C3 – Dimer – ATP5MK ATP5MG ATP5ME	**Mitochondrial** **MT-ATP6 (Fo)** **MT-ATP8 (Fo)**
Assembly factors	**SDHAF1 (B)** ———— SDHAF2 (A) SDHAF4 (A) SDHAF3 (B)	**BCS1L (RIP)** – CYT B —— UQCC1 UQCC2 UQCC3 – CYT C1 — HCCS – RIP — LYRM7		**LRPPRC (1)** **SURF1 (1)** **SCO2 (2)** **COX10 (1)** **COX11 (1)** **COX15 (1)** – MT-CO1 ———- Cmc1 Coa1 Coa3 Cox14 – MT-CO2– Coa6 Sco1 Cox20 Cox18		**ATPAF2** **TMEM70** – F1 – ATPAF1 – F0 – ATP23	

by co-factors such as FAD, Fe-S cluster, and haem to the mobile electron carrier co-enzyme Q10 to Complex III. SDH is a tetrameric enzyme, and all subunits are encoded by the nuclear genome. Mutations in one structural subunit and an assembly factor have been identified that are associated with epilepsy (Table 2).

SDHA is a peripheral membrane protein that harbours the FAD co-factor required for electron transfer and is part of the catalytic core. A 9-year-old girl with epilepsy as part of a wider range of mitochondrial disease associated pathologies was found to be compound heterozygous for *SDHA*. The paternal allele results in a nonsense mutation at Tryptophan at position 119. The maternal allele resulted in a missense mutation at position 38, resulting in an Alanine to Valine mutation. Protein analysis revealed a decrease in expression of this subunit, but further characterisation was not performed. Muscle biopsies demonstrated a 23 % level of activity compared to control [59]. In a second case, a whole-exome study identified a mutation in an 11-month boy, where epileptic spasms began at 6 months. The patient carried the compound heterozygous mutations 1A>*G* and 409G>*C*, resulting in a methionine mutation at the start codon and an Aspartate to Histidine mutation at position 137, which was deemed to be a pathogenic mutation by *in silico* prediction [60].

SDHAF1 is an assembly factor for SDHB and assists in the incorporation of an Fe-S cluster into the catalytic subunit. A 16-year-old patient with infantile leukoencephalopathy since age 20 months, presenting with single epileptic seizures, harboured a homozygous nonsense mutation at position 22 of the *SDHAF1* gene, which resulted in premature termination of the polypeptide. Fibroblasts from the patient had 16 % of control SDH activity, while other complex activities were unaffected [61]. SDHAF1 associates with SDHB to recruit the Fe-S donor complex [62]. Interestingly, other *SDHAF1* pathogenic mutations are known which result in infantile leukoencephalopathy, without seizures, but presenting with neurological dysfunction [63].

As Complex II is the smallest and least complex member of the OXPHOS system, it is not surprising few mutations are associated with epilepsy. However, these rare cases indicate that Complex II mutation can still be the cause of disease and should not be overlooked.

9. Mutations in complex III structural subunits and assembly factors associated with epilepsy

Complex III, or ubiquinone:cytochrome *c* oxidoreductase, transfers electron from co-enzyme Q to cytochrome *c* along with the translocation of protons. It is an 11 subunit multi-subunit protein complex in humans and has several assembly factors required for correct biogenesis. It contains two haem prosthetic groups in the form of haem *b* and c_1. Mutations in the mitochondrial encoded subunit *MT-CYTB* and an assembly factor are associated with mitochondrial disease displaying epileptic characteristics (Table 2).

MT-CYTB is the only mitochondrially encoded subunit of Complex III. It harbours haem prosthetic groups within the whole complex. The first mutation in *MT-CYTB* associated with epilepsy was a 4-base pair deletion in a boy aged 15 years where epileptic seizures increased in severity over time. The deletion is found early in the coding sequence resulting in a frame-shift mutation at amino acid 13. MT-CYTB is 380 amino acids in length, so this deletion will essentially result in the absence of functional protein. Skeletal muscle was found to harbour 95 % mutant *MT-CYTB* while other tissues contained about 60 % [64]. Several years later a single nucleotide mutation was identified in *MT-CYTB* in a 15-year-old girl (14864T>*C*). This resulted in a change from Cysteine to Arginine at the highly conserved amino acid position 40. Seizures were observed at the age of 9 years following physical exertion. Complex III activity in muscle was reduced to just below 70 %, while other complexes displayed altered activity from 68 % to 112 %. The mutation was found to be present at 39 % in muscle and ranged from 32 % to 57 % in other tested tissues. The mutation was predicted to have a damaging impact through computational analysis as it is in the ubiquinone binding site [65]. More recently a 12-month-old boy in Bangladesh was found to have the 14766C>*T* mutation. The resulting Threonine to Isoleucine change at amino acid position 7 was predicted to be "likely pathogenic" through computational methods [66].

BCS1L is an IMM localised protein required for correct assembly of the bc1 subunit of Complex III. The proposed function is to promote the addition of the Rieske Fe-S protein during assembly. In one case, mutations in the *BCS1L* gene were identified in two separate families with children displaying epilepsy. These two patients had decreased Complex III activity compared to control fibroblasts, lymphoblasts and muscle. The two patients were compound heterozygous for *BCS1L*, and each patient carrying different sets of mutations. The mutations in one patient were Arginine to Cysteine at the conserved position 73, and Phenylalanine to Isoleucine at also conserved position 368. The R73C mutation is part of the mitochondrial localisation sequence, while the F368I mutation is in the AAA ATPase domain, proposed to be responsible for activity of this protein. The second set of mutations were located at position 183 and 184 and both mutated Arginine to Cysteine. Patient samples contained unusual complexes and sub-assemblies not found in the control [67]. Several years later, in a second study, a 7-month-old girl presented with mitochondrial disease associated symptoms, including epilepsy. This child was compound heterozygous, carrying the R183C mutation alongside a 3-nucleotide insertion mutation at position 1061 resulting in the addition of a Tyrosine residue at position 354. This patient was also diagnosed with Bjornstad syndrome, which is characterised by sensorineural hearing loss and twisted, brittle hair [68].

These mutations in Complex III reveal that defects in both assembly and function can lead to epilepsy of mitochondrial origin in two specific genes. As Complex III has 11 subunits and several assembly factors, the limited number of mutations associated with epilepsy indicates a lower tolerance to changes in Complex III activity.

10. Complex IV structure and assembly

Cytochrome *c* oxidase, Complex IV, is the terminal electron acceptor of the ETC. Electrons passed from cytochrome *c* are carried through a series of haem and copper co-factors to reduce molecular oxygen to water. Complex IV is made up of 13 subunits in humans, 3 from the mitochondrial genome and the remaining 10 from the nuclear genome. The 3 mitochondrial subunits are the catalytic site of Complex IV and subunits MT-CO1 and MT-CO2 carry the haem and copper groups. Electrons are received from cytochrome c by CuA in MT-CO2 and then passed to haem A in MT-COI, followed by transfer to the MT-COI binuclear centre of haem a_3 and CuB where oxygen is reduced of water. Along with electron transport Complex IV is also a proton pump.

Assembly of Complex IV is a modular biogenesis pathway where MT-CO1, MT-CO2, and MT-CO3 form separate sub-assemblies before joining together with nuclear-encoded peripheral subunits to form the fully assembled enzyme complex. For the sub-assemblies to form, assembly factors regulate the process based on subunit and co-factor abundance along with maturation of subunits through post-translational mechanisms and membrane incorporation. Translation of mitochondrially encoded Complex IV subunits is coupled to the presence of certain assembly factors that ensure subsequent steps can be executed fully [69].

Mutations in both structural subunits and assembly factors have been assigned as causative in conditions where epilepsy is seen (Table 2). In addition, Complex IV activity has been used as a marker for the seizure-onset zone. In young patients with focal cortical dysplasia type II Complex IV activity was found to be lower than surrounding tissue. This potentially points to Complex IV activity being a general feature of epilepsy, whether it is of mitochondrial origin or not [70].

11. Mutations in complex IV structural subunit genes associated with epilepsy

COX6B1 is a ubiquitously expressed peripheral nuclear-encoded subunit. It is located on the IMS face of Complex IV and proposed to stabilise Complex IV dimers and contribute to the cytochrome *c* binding site [71]. COX6B1 is incorporated into Complex IV at a late stage as part of the MT-COIII module. Recent reports suggest COX6B1 is required for assembly of early intermediates of the MT-COII module [72]. A recent clinical report of a mutation in *COX6B1* identified a 7-year-old boy presenting with status epilepticus, resulting in an amino acid mutation at position 31 resulting in a substitution of a Tryptophan for Arginine [73]. While COX6B1 is the ubiquitously expressed isoform of this subunit, the other isoform COX6B2 is testes-specific and there are no reports of any pathological mutations to date.

COX8A is the smallest subunit of Complex IV and associates with MT-COII during assembly and in the final structure. A patient has been characterised who harboured a mutation in this subunit. The girl presented with symptoms from 6 months, and by the age of 8 years started to present with focal and generalised epileptic tonic-clonic seizures and eventually died at the age of 12 due to complications. The mutation in *COX8A* caused a severe loss of Complex IV activity caused by a decreased abundance of mRNA and protein, as well as unexpected mRNA splicing. The mutation caused a frameshift altering the sequence and causing a truncation resulting in a low-expressed and nonfunctional protein. Fully assembled Complex IV was decreased in expression, while other OXPHOS complexes remained at control amounts. The alternative human COX8 isoform COX8C was not expressed in the fibroblasts of the patient indicating lack of a potential compensatory response in these cells [74].

COXFA4 is a recently assigned subunit to Complex IV, required for full functional activity and potentially required for assembly. A family harbouring a splice site mutation had several members displaying Leigh Syndrome-like symptoms, including seizures in one of them. The mutation resulted in aberrant splicing after exon 1 and complete absence of the protein in muscle and fibroblasts. The absence of COXFA4 in Complex IV in these patient samples results in decreased activity, but activity is still present, explaining the milder severity of these cases, where some individuals were still living beyond 30 years of age. However, the family displayed a variety of symptoms, and not all associated with seizures [75].

12. Mutations in complex IV assembly factor genes associated with epilepsy

Leucine-rich PPR motif containing protein, LRPPRC, functions to regulate gene expression of mitochondrially encoded RNA transcripts. *LRPPRC* was first identified as the causative gene for a variation of Leigh Syndrome in Quebec and caused a deficiency of COX in the brain and liver. The impact on the brain resulted in a severe form of Leigh Syndrome with its associated seizure-like symptoms. The mutation was not identified but a decrease of 50 % in brain and liver was observed, but not in other tissues [76]. Whole genome sequencing enabled the identification of a recessive mutation associated with this form of Leigh Syndrome. A conserved residue, Alanine at position 354, was found to be mutated to a Valine, by a Cytosine to Thymine transition at position 119. This mutation was found to be heterozygous in almost all the parents in the study [77]. This mutant form of LRPPRC was found to be associated with defective synthesis and stability of mitochondrially encoded proteins, especially the three COX subunits, MT-COI, MT-COII and MT-COIII, in patient fibroblasts. This was found to be due to decreased stability of the mRNAs of these protein subunits. The *LRPPRC* mutation caused a decrease in expression of the protein and binding partner SLIRP (Steroid receptor RNA activator-stem Loop Interacting RNA-binding protein) [78]. In a later case, a boy from a non-Quebec family was found to harbour *LRPPRC* mutations had episodes of seizures and ultimately died at 6 weeks of age. The mutation was compound heterozygous resulting in a frameshift mutation and a splicing mutation. Fibroblasts derived from the patient had decreased expression of LRPPRC, resulting in decreased activity of COX along with Complex I, III and IV [79]. Recent structural studies have determined that LRPPRC functions to deliver mRNAs to the mitochondrial ribosome [80] and as a holdase to maintain mRNA folding [81].

SURF1 encodes an assembly factor for Complex IV that is commonly mutated and the cause of Leigh Syndrome. SURF1 functions to mature MT-COI in the assembly intermediate MITRAC (mitochondrial translation regulation assembly intermediate of cytochrome c oxidase) and possibly with a role in haem insertion [82,83]. MITRAC is a multi-protein complex assembly intermediate containing MT-COI, structural subunits COX4–1, COX6C and assembly factors MITRAC7 and MITRAC12. A series of Leigh Syndrome patients were determined to have <10 % Complex IV activity, due to frameshift, splice-site and nonsense mutations in this gene resulting in a nonfunctional and truncated protein [84]. Further review of many reports of SURF1 mutations around this time determined that many mutations were deletions or insertions (12 of 30), followed by missense or nonsense mutations (10 of 30) and then splice mutants (8 of 30) with an equal likelihood of being homozygous or compound heterozygous and all mutations being different [85]. A missense mutation was identified in a family, at position 227 causing a Tryptophan to Arginine mutation resulting in a mild form of Leigh Syndrome [86]. Two identified human mutations in *SURF1* were tested in the *S. cerevisiae* orthologue Shy1p to understand their impact on Complex IV. Glycine at position 137 and Tyrosine at position 344 were mutated to the corresponding residue in the yeast protein. These mutations resulted in decrease expression of fully assembled Complex IV. These mutants appeared to be unstable compared to wild-type Shy1p and associate with altered assembly intermediates that did not contain the translational activator Mss51p. These mutations impacted control of MT-COI biogenesis resulting in Complex IV deficiency [87]. At this point >40 mutations in SURF1 have been identified, but this has not helped to understand the function of this protein or use the genotype to predict the severity or pathology of disease [88].

SCO2 is a protein involved in the incorporation of copper ions into the MT-COII subunit of Complex IV. SCO2 works with its close homologue SCO1 to capture copper ions and then deliver to MT-COII at the appropriate stage in Complex IV biogenesis [89]. Mutations in *SCO2* are the cause of cytochrome *c* oxidase deficiency accompanied with seizures. The most common mutation is a substitution of Glutamate to Lysine at position 140. This is adjacent to the copper ion binding site, which is required for SCO2 function. This resulted in a mild decrease in Complex IV subunit expression. A separate mutation at position 171 was identified where Arginine was substituted by Tryptophan in a patient that was compound heterozygous [90].

Complex IV relies on the presence of a haem A prosthetic for function. Maturation of the haem group is carried out by two assembly factors COX10 and COX15. Haem requires chemical modification by a farnesyl group – this reaction is catalysed by COX10. A form of Leigh Syndrome is associated with *COX10* mutation, which does not have seizures as a symptom. The patient died at the age of 4 months. Biochemical analysis determined <20 % Complex IV activity in muscle biopsies, while other OXPHOS activities remained close to control levels. Complex IV was expressed at 15 % of control amounts, and assembly sub-complexes were not detected. The patient harboured two missense mutations at amino acid position 336, where Aspartate was replaced by Valine and Glycine [91]. This position is evolutionarily conserved pointing to the importance of this residue. This residue is in a matrix-exposed loop between two trans-membrane helices. This loop is implicated in the stabilisation of the substrate haem B before modification [92].

COX11 is a chaperone required for the incorporation of copper ions into the MT-COI subunit of Complex IV. Two patients displaying

epileptic episodes have been shown to have mutations in *COX11*. One patient, that died at the age of 9 months, displayed seizure-like episodes at 6 weeks. Complex IV activity was slightly decreased in this patient, with some other OXPHOS complexes displaying small decreases in activity the muscle and liver. The patient was found to have an Alanine to Proline mutation at position 244, which is a conserved residue and predicted to be damaging by several *in silico* prediction tools. This resulted in mild defects in Complex IV expression and assembly without effect on other OXPHOS complexes [93]. A second patient, in the same study, displayed epileptiform episodes at 7 months of age. This patient had developmental delays and had occasional focal seizures by the time she was aged 3. This patient harboured a Valine to Glycine mutation at amino acid position 12, located in the mitochondrial targeting sequence, because of a deletion and insertion of position 35 and 36 in the coding sequence, resulting in a premature termination codon at amino acid position 21. Further biochemical analysis of this mutation could not be performed due to a lack of sample, but mRNA expression of the *COX1* transcript was not impaired. *In silico* prediction tools determined that there would be a low likelihood of mitochondrial targeting. However, a premature termination codon soon after the mitochondrial targeting sequence would predict a nonfunctional truncated COX11 polypeptide lacking any functional regions of the protein [93]. A patient with Leigh Syndrome-like symptoms was also identified with a mutation in *COX11*. This patient did not exhibit epileptic episodes but harboured a compound heterozygous mutation resulting in one mutation at amino acid residue 247, changing Proline to Threonine, and one premature termination at amino acid position 21 positions from the carboxy terminus with an altered sequence. A mild reduction of <50 % of control levels was seen for COX11 in muscle and minimal effect on Complex IV activity. Complementation assays in yeast demonstrated the truncated variant was a null allele for COX11 function, while the mutation corresponding to P247T behaved like wild-type [94].

COX15 is a haem synthase responsible for the methylation of haem O to form haem A, which is found in the MT-CO1 subunit of Complex IV. Several patients with epileptic characteristics have been identified with mutations in *COX15*. The first patient identified with a *COX15* compound heterozygous mutation was a 6-day old girl who died at the age of 24 days who presented with seizures. The patient has severely decreased Complex IV activity, <30 % of control in both heart and fibroblast samples. Mitochondria contained extremely reduced amounts of assembled Complex IV, with some sub-complexes apparent, without any impact on assembly of other OXPHOS complexes. The patient expressed a mutant form of COX15, with a mutation at position 217, causing an Arginine to Tryptophan mutation, and a splice site mutation in the other allele resulting in a transcript missing exon 4, a causing premature termination in the polypeptide. This resulted in a dysfunctional enzyme as haem A levels were 10 time less than in control, while haem O accumulated [95]. A patient presenting Leigh Syndrome-like symptoms was found to be homozygous for the R217W mutation but did not display epileptic episodes [96].

Complex IV is unique in that there are no mutations found in mitochondrially encoded genes that are associated with epilepsy. The mutations are found in genes that are peripheral subunits or assembly factors. This suggests mutations in the mitochondrially coded genes are not tolerated, whereas mutations in peripheral subunits and assembly factors still allow for a sufficient level of Complex IV assembly and activity to support cellular survival, but not enough to support correct neuronal function.

13. Complex V structure and assembly

F_1F_O-ATP synthase, Complex V, is the final component of the OXPHOS system, which translocates protons to generate ATP. Complex V is made up of 3 modules, the stalk/stator, rotor and ring which function mechanically to convert electrochemical energy into chemical energy captured in the high-energy bond in ATP. The F_O complex acts as a proton channel in the IMM. Complex V also follows a modular biogenesis pathway, where each module assembles independently before final association to form the fully assembled enzyme complex and mutations in genes in all modules are associated with epilepsy (Table 2). Neurons are highly dependent on ATP to maintain energy reserves for all vital activities. If there is a defect in ATP synthase that inhibits activity, then neurons will be dysfunctional and this will lead to different pathologies. In the case of epilepsy, this could lead to defects of ion pumps and ion channel function, as well as disrupted calcium homeostasis, leading to altered release of neurotransmitters. Certain neurons do not survive low ATP conditions, especially where ROS levels are high, causing problems in neuronal connectivity and regulation of neuronal signalling, leading to hyperexcitability seen in epilepsy [97]. As the main function of ATP synthase is to synthesis ATP, any defects in the function or assembly of this enzyme, will cause severe issues in high energy demanding tissues, such as the brain.

14. Mutations associated with complex V mitochondrially encoded genes

MT-ATP6 is encoded on the mitochondrial genome and is present as part of the F_O complex as a single subunit embedded in the IMM. There are several pathogenic mutations that result in epilepsy, and this gene is the most well characterised gene causing epilepsy and seizures. The first described mutation was in a family with various forms of epilepsy, as part of the NARP syndrome, with a mutation in *MT-ATP6* at nucleotide position 8993 changing a Thymine to a Guanine, resulting in a Leucine to Arginine substitution at the evolutionarily conserved position 156 in the ATP synthase **a** subunit. The mutation when modelled in *S. cerevisiae* resulted in a decrease in growth dependent on mitochondrial function because of a 90 % decrease in ATP synthase function. ATP synthase existed as the fully assembled complex, but at a decreased level that corresponded with activity. The mutation interrupts proton release from the channel c-ring. Studies in human cell lines and patient samples have been less conclusive, with suggestions of structural defects, complex stability, association of regulatory proteins, perhaps due to the impact of heteroplasmy and also the variety of models used [98–101].

A second mutation at nucleotide position 8993, has also been identified, resulting in a Thymine to Cytosine mutation, and a substitution of Leucine to Proline. A comparative study of these different mutations at position 8993 in cybrids revealed that ATP synthase could exist in different sub-complexes, expressed at different relative amounts and even with very low expression [102]. A girl displayed epileptic seizures from 22 months, with family members also displaying varying neurological conditions. The severity of the pathology was associated with an increasing proportion of the T9176G mutation. ATP synthase was found to be assembled correctly, however displayed approximately 20 % of activity or less in fibroblasts whilst possessing and increased mitochondrial membrane potential [103]. Further investigation of this mutation also revealed it as associated with NARP. The variation in disease, severity and onset has also been attributed to other underlying genetic factors, which could relate to mitochondrial haplotypes leading to defective assembly of the OXPHOS system [104].

A mutation at position 9185, causing a Thymine to Cytosine transition, and a Proline to Leucine substitution at the evolutionarily conserved position 220 was originally described as causing late-onset Leigh Syndrome, but has also been shown to affect newborns as well [105]. This mutation results in approximately 50 % decrease in ATP synthase activity in patient lymphoblasts [106].

A deletion at the intersection of a bicistronic gene was also identified in a patient displaying seizures. *ATP6* is transcribed with the *MT-COIII* gene in a single bicistronic mRNA named RNA14 that is cleaved resulting in the two single transcripts. The girl displayed seizures 3 days after birth. The 2-nucleotide microdeletion causes a loss of the termination codon of ATP6, resulting in decreased abundance of RNA14, increased deadenylation and degradation [107,108] as well as loss of

protein expression [109]. The presence of the microdeletion in *MT-ATP6* in mitochondria results in activation of mitochondrial stress response when proteotoxic stress is engaged by heat or a translational or degradation defect when either *AFGL32* or *OXA1L* are absent, pointing to impacts on co-translational quality control of protein degradation or protein insertion into the IMM [110,109].

Truncation mutations have also been described in the *MT-ATP6* gene, resulting in shorter than full-length ATP6 polypeptide. A 32-year-old female patient was diagnosed with myoclonic epilepsy, along with her mother who exhibited focal-onset epilepsy. The truncation mutant converted Glycine at position 86 to a termination codon. Cybrid fibroblasts derived from this patient contained altered ATP synthase complexes indicating a change in assembly or stability, while ATP6 was expressed at lower levels it was still incorporated into the fully assembled complex. The same mutation in a different patient did not present with epilepsy but also displayed altered ATP synthase complexes [111]. These differences could be explained by heteroplasmy of the mutation.

A mutation in *MT-ATP6* was found to cause MLASA (mitochondrial myopathy, lactic acidosis, and sideroblastic anaemia) with seizures in a 6-year-old boy. The mutation was a Guanine to Adenine mutation at position 8969 resulting in a Serine to Asparagine substitution at the highly conserved position 148. The patient-derived fibroblasts displayed decreased respiration overall and were insensitive to the ATP synthase inhibitor oligomycin [112].

A *Drosophila melanogaster* model of *MT-ATP6* mutation results in a seizure-like phenotype late in the shortened lifespan of these flies. The mutation results in a Glycine to Glutamate substitution at position 116. This resulted in decreased ATP and decreased expression of ATP synthase dimers and surprisingly Complex I supercomplexes. Metabolic analysis of these *MT-ATP6* mutant flies revealed complex remodelling of glycolysis, ketogenesis and Kreb's cycle to compensate for decreased OXPHOS activity [113]. These observations revealed that a ketogenic diet can delay the seizure-like activity of the *MT-ATP6* mutant flies, through modulation of the Kreb's cycle. However, standard anti-epileptic treatments did not improve epileptic episodes, perhaps undermining this model [114].

The other mitochondrial encoded gene in ATP synthase is *MT-ATP8* is also associated with epilepsy with 3 separate examples. The first case in 2006 was an 8-year-old boy who displayed seizures that were managed with anti-epileptic treatment. However, this valproate treatment caused a deterioration in his condition, and when this was stopped there was a clinical improvement. The causative mutation was found to be a mutation in *MT-ATP8*, at nucleotide 8383 resulting in a Cytosine to Thymine transition, and a consequent Proline to Serine substitution [115]. In 2010, a 10-year-old boy who had congenital deafness and delayed psychomotor development, developed seizures from the age of 2, a progressively worsened until he died at the age of 10-years-old. The causative mutation, Adenine to Guanine at position 8411, resulted in a Methionine to Valine change at amino acid position 16 which is in a conserved region. This was a *de novo* mutation that was found to have a close to 100 % mutant heteroplasmic load [116]. Four years later, a genome analysis of patients with mesial temporal lobe epilepsies-hippocampal sclerosis was performed. A mutation in *MT-ATP8* was identified as the most significant in patients. The Adenine to Threonine mutation at position 8502 resulted in an Asparagine to Isoleucine mutation at amino acid position 46. No further characterisation of this mutation was performed to determine impacts on mitochondrial function of ATP synthase activity of assembly [15].

15. Mutations in complex V nuclear encoded structural genes associated with epilepsy

ATP5F1A encodes the α subunit which is found in the F_1 complex in 3 copies along with 3 copies of the β subunit to form a hexameric ring. A 13-day-old-patient was found to have a mutation in *ATP5F1A* causing an amino acid change at position 207, resulting in an Arginine to Histidine substitution, interfering with the association between the α and β subunits. This mutation caused a decrease in ATP5F1A protein expression and altered assemblies of ATP synthase. Seizures occurred after gavage feeding of breast milk and resolved by 18 months of age [117].

ATP5F1D encodes the δ subunit which is part of the stator stalk that prevents rotation of the F_1 complex as the central stalk and F_o complex rotate to couple proton translation with ATP synthesis. A 4-year-old boy with a tonic-clonic seizure, amongst other issues, was found to have a mutation in *ATP5F1D*, at nucleotide position 317, causing a Thymine to Guanine mutation, and Valine to Glycine substitution at amino acid position 106. Patient fibroblasts displayed <20 % control-ATP synthase activity. ATP5F1D protein expression was unaffected, however other ATP synthase subunits were expressed less than control. Expression of fully assembled ATP synthase complex was decreased while other OXPHOS complexes remained unaffected in patient fibroblasts and muscle [118].

ATP5F1E codes for the ε subunit which is the least conserved subunit and may not be an essential component of F_1-F_o ATP synthase. Decreased expression of ATP5F1E causes decreases in ATP synthase expression and activity and hinders assembly [119]. Mutations in the F_1 subunit, *ATP5F1E*, have been identified in patients displaying seizures. The homozygous mutation at nucleotide position 35 where an Adenine to Guanine change caused a Tyrosine to Cysteine mutation at amino acid position 12. Patient fibroblasts and blood cells were found to display reduced levels of the ATP5F1E protein as well as decreased oxygen consumption [120].

ATP5PO is a subunit of the peripheral stalk stator complex, connecting the F_1 catalytic head to the F_o rotor and confers sensitivity to oligomycin, a well-characterised inhibitor of ATP synthase. A patient with a truncation and splice-site mutation in *ATP5PO* has been identified. The mutation at nucleotide position 34, changing Cytosine to Thymine caused termination at amino acid 12. The splice-site mutation at position 329 to 320 caused loss of exons 4 and 5, resulting in a loss of function mutant. This resulted in decreased fully assembled ATP synthase and accumulation of a sub-complex [120].

16. Mutations in complex V assembly factors genes associated with epilepsy

Mutation in the F_1 complex assembly factor *ATPAF2* (formerly ATP12) was one of the first assembly factors to be associated with causing seizures. ATPAF2 is an assembly factor for the F_1 complex. This patient displayed decreased ATP synthase assembly and activity in skeletal muscle, liver and fibroblasts. The mutation resulted in a Tryptophan to Arginine mutation at amino acid position 94. This patient was born with developmental problems and dysmorphic features and died by at 14 months [121].

TMEM70 is an assembly factor for ATP synthase. A mutation in this protein was found in a patient displaying status epilepticus at 1 month of age. Patient fibroblast mitochondrial activity was 50 % control level, had severely decreased expression of TMEM70 and ATP synthase subunits. Patient fibroblast mitochondria were deficient in fully assembled ATP synthase, but the F_1 complex was assembled and active. The mutation in this resulted in incorrect splicing and absence of a mature TMEM70 transcript [122].

As it can be seen, mutations in several ATP synthase structural subunits genes and assembly factors lead to mitochondrial disease with epilepsy, encoded by both the nuclear and mitochondrial genome. However, mutations in the remaining subunits of ATP synthase have not been reported, indicating either mutations cannot be tolerated in these subunits or there is a very low occurrence of these in the general population. Further screening and study of this enzyme will uncover any unidentified mutations.

17. Mutations impacting on translation of mitochondrial genes

Mitochondrial DNA encodes the 20 tRNAs required to fulfil translation of the mitochondrially encoded protein-coding genes. Mitochondrial tRNAs need to be folded correctly, post-transcriptionally modified, charged by the corresponding aminoacyl-tRNA synthetase, and associate with the ribosome at the appropriate time. Many mitochondrial diseases and those associated with epilepsy are caused by mutations in mitochondrial tRNAs [123]. Mitochondrial tRNA mutations lead to MERFF and Leigh Syndrome. Mutations in tRNA for Lysine, Leucine, Histidine, Serine and Phenylalanine cause MERFF, which mutations in Valine, Lysine, Tryptophan and Leucine are associated with Leigh Syndrome. These mutations result in alterations to transfer RNA structure and modifications which lead to defects in tRNA charging with amino acids and association with the ribosome. This results in synthesis of polypeptides that may contain substituted amino acids or altered polypeptide synthesis kinetics that stalls OXPHOS complex assembly and results in degradation of subunits. This has been seen with the A3243G mutation in MT-TL(UUR) and the A8344G mutation in MT-TK [124–126]. More recently two mutations were found in mitochondrial tRNA for asparagine (MT-TN) at positions 5688 and 5691, resulting in Thymine to Cytosine and Guanine to Adenine. These changes are predicted to cause structural alterations of the typical tRNA structure, which would lead to inefficiency of amino acid charging, ribosome association and impact of incorporation into polypeptides. Mature MT-TN expression was decreased in these mutants, decreasing respiratory activity in patient fibroblasts, and decreased activity of Complex I, III, and IV, with mutation specific loss of expression of different OXPHOS complex subunits. These MT-TN mutations resulted in increased mitophagy, as well as increased mitochondrial biogenesis [127]. Similar observations have been made for the transfer RNAs for lysine, leucine, histidine, serine, phenylalanine, valine and tryptophan. Multiple patients have been identified with a variety of mutations in each transfer RNA gene. However, the prediction of pathogenicity based on mutation is very difficult due to pleiotropic effects and heteroplasmy in different tissues [128].

Mutations in the aminoacyl-tRNA synthetases that charge mitochondrial tRNA have also been reported to cause several mitochondrial diseases, including those associated with epilepsy. A patient with a mutation in the phenylalanine-tRNA synthetase, FARS, displayed a deficiency of Complex IV activity in muscle and myoblasts, but not fibroblasts, and no other OXPHOS complexes. Myoblasts had decreased expression of Complex IV subunits and phenylalanine tRNA, but not general mitochondrial protein synthesis. This FARS2 mutant had decreased tRNA charging function and decreased ATP binding [129]. The consequence of mutations in either transfer RNA or aminoacyl-tRNA synthetases causing disease, whilst increasing mitophagy and mitochondrial biogenesis, suggests a similarly broad impact on mitochondrial function.

Mitochondrial DNA also encodes rRNA, in the form of *MT-RNR1* and *MT-RNR2*, mutations in these genes have been detected, however, none are associated with epilepsy, and are generally not thought to cause disease.

Defects in mitochondria translation would cause widespread alterations in mitochondria leading to dysfunction. As many mutations in the translation machinery are associated with epilepsy this indicates there is a tolerance to these mutations, indicating translation can proceed at a required level to maintain certain functions, but which can lead to dysfunctions that result in epilepsy.

18. Conclusion

The various forms of epilepsy that are associated with mitochondrial diseases vary greatly in presentation and severity in the patients reported up to date. This is due to the intense variability of mitochondrial functions and properties found in the human population and in patients. Mitochondrial background, heteroplasmy, exposure to stressors and other underlying genetic factors will all play a role in the presentation of the final disease. Most studies report on clinical cases to diagnose the deficiency and determine the genetic cause. This approach has resulted in the identification of dozens of mutations in both mitochondrial DNA and nuclear DNA that are associated with epilepsy. However, because of the variation in these cases, there is likely to be a vast number of missed epilepsy cases. In severe cases, patients may not survive long enough to exhibit epileptic episodes.

While diagnosis of these diseases is becoming more comprehensive, enabling a causative genetic alteration to be assigned, the opportunities for therapy are extremely limited. Conventional anti-epileptic therapies are used for most patients, to manage the condition, however some remain resistant to these, while some will exacerbate the pathology, and ultimately these will not successfully cure the patients. Newly developed therapies of mitochondrial replacement therapy could be used in the future in cases where inheritance of mutated mitochondrial DNA is suspected. This experimental therapy is currently undergoing clinical trials in limited locations with report success [130]. For nuclear inherited mutations, pre-implantation screening can be used to identify non-pathogenic zygotes or embryos in families with a medical history. For unexpected cases therapies will still need to be developed to alleviate suffering and shortened life spans.

Several models are being used to characterise the cellular and molecular pathways invoked by the mutations found in the patients. Many clinical investigations rely on easily obtained samples, such as fibroblasts, blood and muscle which can be used to identify a mutation and characterise the impact of that mutation in the tissue. However, in the case of epilepsy, there have been no attempts to determine the impact of the mutation in the brain and how these impacts on neuronal signalling would lead to epilepsy. This means better neuronal models need to be developed so neuronal function can be investigated on the background of the mutation. There are examples of rodent and fly models used to study epileptic episodes in model organisms, but these are extremely limited. The extensive use of stem cells that can be differentiated into appropriate cell types and grown into a structure resembling that found *in vivo* will lead to further understanding of what is happening in the brains of these patients with the consequential development of therapies. Expertise from many areas of biomedical research will be needed to develop interdisciplinary collaborations for better diagnosis, management of symptoms and ultimately generation of novel therapies.

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Declaration of competing interest

None.

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