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Mitochondrial dysfunction and defects in lipid homeostasis as therapeutic targets in neurodegeneration with brain iron accumulation

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ABSTRACT

The *PLA2G6* gene encodes a group VIA calcium independent phospholipase A2 (*iPLA2β*), which hydrolyses glycerophospholipids to release fatty acids and lysophospholipids. Mutations in *PLA2G6* are associated with a number of neurodegenerative disorders including neurodegeneration with brain iron accumulation (NBIA), infantile neuroaxonal dystrophy (INAD), and dystonia parkinsonism, collectively known as *PLA2G6*-associated neurodegeneration (PLAN). Recently Kinghorn *et al.* demonstrated in *Drosophila* and *PLA2G6* mutant fibroblasts that loss of normal *PLA2G6* activity is associated with mitochondrial dysfunction and mitochondrial lipid peroxidation. Furthermore, they were able to show the beneficial effects of deuterated polyunsaturated fatty acids (D-PUFAs), which reduce lipid peroxidation. D-PUFAs were able to rescue the locomotor deficits of flies lacking the fly ortholog of *PLA2G6* (*iPLA2-VIA*), as well as the mitochondrial abnormalities in *PLA2G6* mutant fibroblasts. This work demonstrated that the *iPLA2-VIA* knockout fly is a useful organism to dissect the mechanisms of pathogenesis of PLAN, and that further investigation is required to determine the therapeutic potential of D-PUFAs in patients with *PLA2G6* mutations. The fruit fly has also been used to study some of the other genetic causes of NBIA, and here we also describe what is known about the mechanisms of pathogenesis of these NBIA variants. Mitochondrial dysfunction, defects in lipid metabolism, as well as defective Coenzyme A (CoA) biosynthesis, have all been implicated in some genetic forms of NBIA, including *PANK2, CoASY, C12orf19* and *FA2H*.

Introduction

The PLA2G6 *gene and its functions*

Mutations in the *PLA2G6* gene are associated with a number of neurological disorders, including neurodegeneration with brain iron accumulation (NBIA) and infantile neuroaxonal dystrophy (INAD).[1,2,3] The *PLA2G6* gene encodes an 85-to 88-kDa group VIA calcium-independent phospholipase A2 (*iPLA2β*), which is a member of the PLA₂ superfamily.[4] This enzyme is responsible for the selective hydrolysis of the sn-2 ester bond of glycerophospholipids to release free polyunsaturated fatty acids (PUFAs), usually arachidonic acid, and lysophospholipids.[5] *PLA2G6* is expressed throughout the mammalian brain and is usually considered to reside in the cytosol.[6] However

it is present in rabbit cardiac inner mitochondrial membranes [7] and localization and translocation to mitochondria have been demonstrated in rat insulinoma cells.[8] It also exhibits variable subcellular localization to the nucleus, Golgi and endoplasmic reticulum (ER) in mammalian cells and is found at dendritic and axon terminals in primate brains.[9,6] The *iPLA2β* isoforms contain 8 N-terminal ankyrin repeats, a serine lipase consensus sequence (GXSXG), a caspase-3 cleavage site, a putative ATP-binding domain, a bipartite nuclear localization signal sequence, and a C-terminal calmodulin-binding domain,[10] in addition to its ability to form a signaling complex with CamKIIβ.[11] *iPLA2β* plays an important role in a number of cellular functions, including the maintenance, repair and remodeling of phospholipid

membranes via a cycle of deacylation and reacylation in concert with other enzymes including lysophospholipid acyltransferases. Specifically, it acts to deacylate phospholipids to release fatty acids from peroxidized phospholipids, with the replacement with new fatty acids by acyltransferases.[12,13,14,15,16] It is thus an important mediator of membrane remodeling and hence membrane homeostasis. The enzyme is also important in a number of other cellular processes such as signal transduction, cell proliferation and apoptosis.[10,17,18]

The clinical syndromes associated with PLA2G6 mutations

As mentioned above, the *PLA2G6* gene, at the PARK14 locus, has been implicated in a number of neurodegenerative disorders such as NBIA, INAD and Karak syndrome, as well as early adult onset dystonia-parkinsonism.[1,19] Furthermore, *PLA2G6* mutations have more recently been associated with early onset Parkinson disease, in addition to other neurological conditions such as bipolar disorder[20] and autism.[21] Collectively these syndromes are referred to as *PLA2G6*-associated neurodegeneration (PLAN).

PLA2G6 mutations have been identified in up to 92% of cases of INAD.[2,3,22] INAD is characterized by infantile onset of psychomotor regression with a rapidly progressive course. Infants develop symptoms such as hypotonia, weakness and areflexia with progression to dementia, ataxia, optic atrophy and spasticity. Death usually occurs before puberty.[23,3] More rarely, neuroaxonal dystrophy caused by *PLA2G6* mutations can present later and patients can survive into adulthood with slower disease progression. Such cases are referred to as atypical neuroaxonal dystrophy (ANAD).[3,24] The neuroradiological hallmark of INAD is the presence of cerebellar atrophy and cerebellar signal hyperintensity on T2-weighted magnetic resonance (MR) imaging, although a case of INAD caused by a *PLA2G6* mutation with isolated cerebellar atrophy has been described.[25] Iron accumulation within the globus pallidus on T2-weighted MR imaging is found in a significant proportion of patients.[3,26,22]

NBIA encompasses a genetically and phenotypically heterogeneous group of disorders with high basal ganglia iron deposition. It is most commonly associated with mutations in the *PANK2* gene, when it is referred to as NBIA type I, as well as mutations in fatty

acid hydroxylase 2 (*FA2H*), coenzyme A synthase (*COASY*), C9orf12, ferritin light chain and caeruloplasmin genes among others.[27] *PLA2G6* mutations are found in approximately 20% of patients with NBIA and are associated with onset earlier in childhood than *PLA2G6* mutation-negative cases. Neurological deficits include ataxia, dystonia, dysarthria and neurobehavioral disturbances. The majority of cases have cerebellar atrophy, in addition to brain iron accumulation in the globus pallidus on neuroimaging.[3]

Paisan-Ruiz *et al.* first described homozygous *PLA2G6* mutations in adult and childhood onset dystonia-parkinsonism without brain iron deposition on imaging.[28] Moreover a new homozygous *PLA2G6* mutation has recently been identified in an Italian family causing adult-onset dystonia-parkinsonism.[19] A number of reports have also implicated *PLA2G6* mutations in early onset Parkinsonism, lacking additional features such as supranuclear gaze palsy, dystonia and dementia.[29,30,31]

The neuropathological hallmarks of PLAN

Axonal swellings known as spheroids and tubulovesicular structures are often, but not invariably, seen in the nervous system of patients with PLAN.[1,2,3] They are distributed widely throughout the peripheral and central nervous systems and are thought to represent membrane-rich inclusions including mitochondria, lysosomal compartments, vacuoles and endoplasmic reticulum, as well as ubiquitin and other proteins.[32,33] In addition, progressive white matter disease consistent with demyelination has also been described.[2]

Given the interesting link between *PLA2G6* mutations and parkinsonian disorders, it is not surprising that the neuropathology observed in patients with *PLA2G6* mutations is similar to that seen in more common forms of neurodegeneration, such as Parkinson and Alzheimer diseases. Indeed widespread Lewy body pathology and tau accumulation was observed in the post-mortem brains of patients with childhood and adult-onset dystonia-parkinsonism.[34] Tau and Lewy body pathologies were also visualized in a patient with INAD and compound heterozygous *PLA2G6* mutations, in addition to numerous axonal spheroids and brain iron deposition.[24] Moreover, Gregory *et al.* described a patient with *PLA2G6*-positive ANAD with pathological features consistent with

classic Lewy bodies, dystrophic neurites, as well as neurofibrillary tangles.[3]

The mechanisms of pathogenesis of PLAN

The exact pathological mechanisms linking mutations in *PLA2G6* with neurodegeneration is not known. However, insights from clinical data, cell and mouse models suggest that it is loss of the normal *iPLA2β* function that leads to abnormalities in lipid homeostasis, resulting in the abnormal accumulation of cellular and mitochondrial lipid membranes.[35] Support for a loss of function of *iPLA2β* enzymatic activity in causing PLAN came from a study on the recombinant mutant and wild type *iPLA2β* proteins. This demonstrated that *PLA2G6* mutations associated with INAD and NBIA were associated with a low level of phospholipase activity, which was not seen with mutations associated with the less severe dystonia-parkinsonism phenotype. Engel *et al.* explained that the lack of a change in catalytic activity in mutations linked to dystonia-parkinsonism may be due to a mechanism not detected by the *in vitro* assays used, such as modulation of *iPLA2β* activity due to calmodulin binding.[36] Additional support for a loss of function hypothesis came from a Chinese study, which identified novel *PLA2G6* mutations that occurred in the heterozygous state, and which were associated with a decrease in phospholipid-hydrolyzing functions.[37] Furthermore, Gregory and colleagues noted a genotype-phenotype correlation in patients with PLAN. Patients with *PLA2G6* mutations and INAD, that would be predicted to lead to an absence of protein production, were associated with more severe clinical phenotypes, than those with compound heterozygous missense mutations and NBIA, characterized by a later age of onset. In the latter cases the *PLA2G6* mutations would be predicted to lead to protein with some residual enzyme activity.[3]

The lack of normal *iPLAβ2* activity has been hypothesized to cause neurodegeneration through the lack of the normal repair and remodeling of phospholipid membranes. In particular, the inner mitochondrial membrane contains a high proportion of cardiolipin, which is particularly susceptible to attack by ROS due to the high content of fatty acids.[38] This in turn is predicted to lead to a downstream pathogenic cascade of events involving release of cytochrome c and apoptosis.[39] In support of this, work in a rodent cell line demonstrated that expression of *iPLA2β* protects mitochondria, prevents loss of mitochondrial membrane potential and attenuates cytochrome c release in response to stress-induced apoptosis.[8]

A number of mouse models of INAD have been developed, including complete genetic ablation of *Pla2g6* and expression of *Pla2g6* pathogenic point mutations.[33,35,40] Such models have demonstrated an *in vivo* disturbance in phospholipid metabolism,[41] abnormal astrocyte calcium signaling,[42] as well as marked cerebellar atrophy.[43] In addition, mice lacking *Pla2g6* showed conserved neuropathology with widespread degeneration of axons and synapses, with spheroid formation and accumulation of ubiquitin.[33,44] A detailed study of the pathology at the ultrastructural level in *Pla2g6* knockout mice revealed significant mitochondrial and presynaptic membrane degeneration.[35]

New insights into PLAN using novel models

In order to further study how mutations in *PLA2G6* lead to neurodegeneration and mitochondrial membrane degeneration, Kinghorn *et al.* used a *Drosophila* model.[45] Flies lacking the *PLA2G6* ortholog, *iPLA2-VIA*, displayed locomotor deficits, reduced lifespan and organismal hypersensitivity to oxidative stress. Examination of the brains of these flies revealed widespread vacuolation representing neurodegeneration in the fly. Ultrastructural examination also demonstrated similar changes to those seen in *Pla2g6* knockout mice,[35] with widespread mitochondrial membrane degeneration and loss of the normal cristae structure (Fig. 1).

In order to confirm that mitochondrial dysfunction plays a role in the pathogenesis of PLAN, Kinghorn *et al.* studied the mitochondrial respiratory chain activity of the *iPLA2-VIA* knockout flies. They demonstrated that at a very young age (2 d old), when no ultrastructural mitochondrial abnormalities were seen using electron microscopy, that there were significant respiratory chain abnormalities in mitochondria from *iPLA2-VIA* deficient flies compared with age-matched controls. They also observed reduced mitochondrial membrane potential in flies lacking *iPLA2-VIA* activity. In accordance with these mitochondrial abnormalities, they showed that *iPLA2-VIA* knockout flies are sensitive to oxidative stressors such as hydrogen
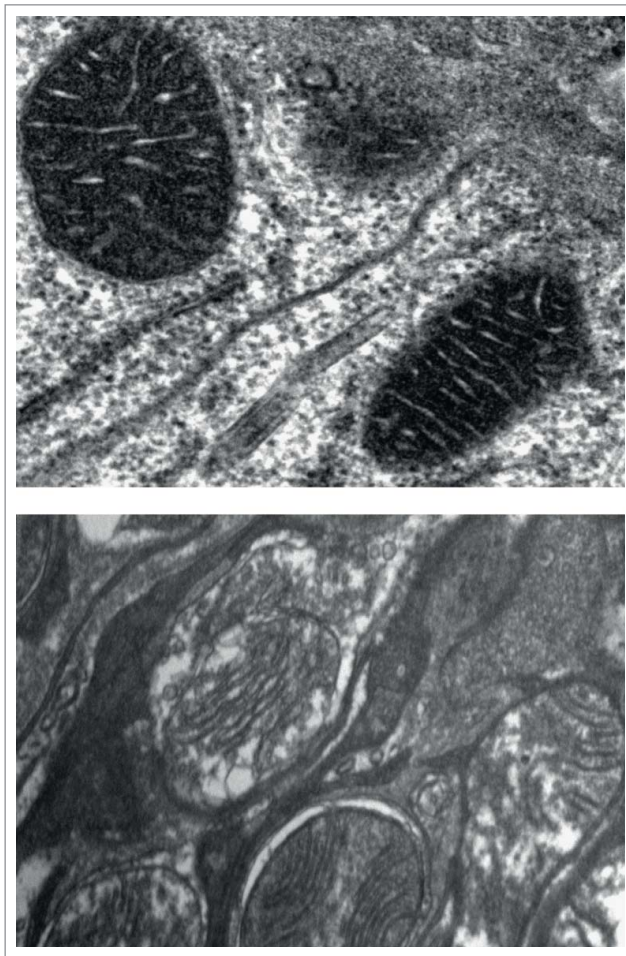
Figure 1. The brains of aged flies lacking the *iPLA2-VIA* gene show degenerate mitochondria with abnormal cristae at the ultrastructural level (lower panel), compared with age-matched control (top panel).

peroxide (H_2O_2) and paraquat, and had significantly reduced ATP levels when compared to control flies.

The role of mitochondrial lipid peroxidation in PLAN

As described above, oxidation of cardiolipin within the inner mitochondrial membrane is predicted to be an important toxic event in PLAN. Kinghorn *et al.* therefore used the fly model of PLAN to address whether indeed toxic oxidized cardiolipin is accumulating within the mitochondrial membranes. To their surprise they could not detect oxidized cardiolipin or significant changes in cardiolipin composition in the heads of *iPLA2-VIA* knockout flies. They did however show that loss of *iPLA2-VIA* activity leads to significant elevation in lipid peroxidation within the fly brain. To further confirm their findings they demonstrated increased lipid peroxidation levels in

fibroblasts taken from 2 patients with pathogenic *PLA2G6* mutations. Furthermore they showed that this lipid peroxidation was largely derived from the mitochondria within the cell, further supporting a role for mitochondrial lipid peroxidation in PLAN. These results are also supported by previous work in *Pla2g6* knockout mice showing an age-dependent increase in 4-HNE immunostaining in the spinal cord, suggesting increased lipid peroxidation.[35]

Although cardiolipin composition was not changed in the *iPLA2-VIA* knockout flies, caution should be taken when interpreting these results, as it may be related to the nature of cardiolipin in *Drosophila*. Palmitic acid is the main fatty acid side chain in *Drosophila* cardiolipin and is more resistant to oxidation than linoleic acid, which is the predominant side chain in mammalian cardiolipin. The possibility of redundancy must also be considered, as there are a number of enzymes involved in cardiolipin remodeling in the fruit fly, such as *tafazzin*.[46] Indeed Beck *et al.* demonstrated changes in cardiolipin composition in *pla2g6* knockout mice, but did not comment on oxidized cardiolipin.[35] Further study is therefore warranted to establish precisely whether cardiolipin is the main oxidized phospholipid in PLAN in humans.

The potential therapeutic benefits of reducing lipid peroxidation in PLAN

Finally, given the presence of abnormally oxidized mitochondrial membrane lipids in the fibroblast and fly models of PLAN, Kinghorn *et al.* assessed the potential beneficial effect of PUFAs. Natural PUFAs such as linoleic acid undergo autoxidation by reactive oxidative moieties (ROS), resulting in the production of toxic reactive carbonyl species, which lead to DNA damage and inflammation among other effects. However, deuterated PUFAs (D-PUFAs), such as deuterated linoleic acid, arrest this autoxidation and are therefore protective against oxidative stress. Accordingly D-PUFAs protected yeast from lipid peroxidation induced toxicity,[47,48] and *in vivo* they successfully reduced nigrostriatal degeneration in a Parkinson disease mouse model.[49] In keeping with the ability to reduce lipid peroxidation, deuterated linoleic acid was able to partially rescue the locomotor abnormalities of aged *iPLA2-VIA* knockout flies. Moreover, reduced lipid peroxidation levels in response to treatment with deuterated linoleic acid, was confirmed in 2 different

sets of *PLA2G6* mutants fibroblasts. This reduction in oxidized lipids was also associated with reversal of the abnormal mitochondrial membrane potential, a marker of mitochondrial health.

PANK2, CoASY and the role of CoA biosynthesis in NBIA

There is mounting evidence that aberrant oxidation of phospholipid membranes and subsequent mitochondrial dysfunction may play a role in a number of neurodegenerative diseases, particularly in NBIA. For example, in pathonate kinase-associated neurodegeneration (PKAN), mutations in the *PANK2* gene also lead to oxidative damage and mitochondrial dysfunction.[50,51] *PANK2* is an enzyme localized to mitochondria [52] and is involved in the
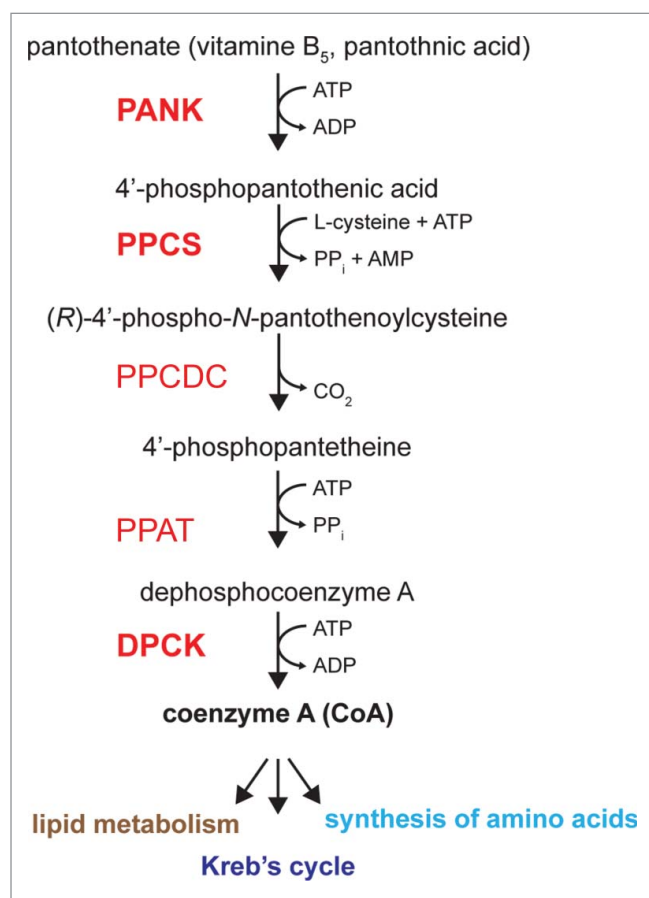

Figure 2. De novo synthesis of CoA is a highly conserved pathway that consists of 5 enzymatic steps: pathothenic acid phosphorylation, cysteine conjugation, decarboxylation, conjugation to an adenosyl group and phosphorylation. In mammals the first step is catalyzed by PANK2 and is the rate-limiting step, while the last 2 steps are catalyzed by CoASY and involve 2 enzyme activities: PPAT (4′-phosphopantetheine adenylyltransferase) and DPCK (dephospho-CoA kinase).

biosynthesis of coenzyme A (CoA),[53] a crucial cofactor in all organisms, which is involved in diverse cellular processes including the citric acid cycle, fatty acid, cholesterol and sphingolipid biosynthesis. The CoA biosynthetic pathway is conserved across taxa from prokaryotes to eukaryotes and utilizes ATP, panthonate (vitamin B_5) and cysteine (Fig. 2). Loss of normal *PANK2* activity leads to accumulation of the substrates N-pantothenyl cysteine and free cysteine. Cysteine in its free state can oxidise lipids in the presence of iron and produce ROS, leading to widespread oxidative damage.[54] Indeed human fibroblasts harboring *PANK2* mutations have increased levels of carbonylated proteins and elevated expression of antioxidant enzymes.[50] Moreover a mouse model of *PANK2* deficiency demonstrated reduced mitochondrial membrane potential, swollen mitochondria at the ultrastructural level, as well as defective mitochondrial respiration.[51] Mitochondrial dysfunction and elevated protein oxidation were also observed in a *Drosophila* model of PKAN, in addition to decreased levels of CoA and reduced longevity.[55] Interestingly, supplementation with pantethine (a dimeric form of pantothenic acid) was able to rescue the neurotoxic phenotypes in this *Drosophila* model of PKAN, including the reduced CoA, mitochondrial abnormalities and neurodegeneration.[56] It was also shown in *Drosophila* and human cell models of PKAN that defects in CoA biosynthesis cause abnormalities in histone and tubulin acetylation, leading to an impaired response to DNA damage and locomotor abnormalities.[57]

Furthermore, peroxidation of mitochondrial membranes, due to a lack of normal membrane homeostasis in PLAN, and possibly in PKAN, are predicted to perturb mitochondrial membrane structure, and alter ion permeability and surface charge.[58] This may in turn interfere with membrane-fusion-reliant processes such as mitophagy. Autophagy also decreases with age,[59] and so the autophagic clearance of damaged mitochondria would also be predicted to decline with age, leading to cellular build-up of abnormal mitochondria. These defective organelles would then accumulate, along with degenerated axon-terminal membranes and other damaged organelles, as spheroids within the nervous system, representing a common neuropathological hallmark of NBIA.

CoASY encodes CoA synthase, which catalyzes the final 2 steps of CoA biosynthesis, by coupling phosphopantetheine with ATP to generate dephosphoCoA, followed by its subsequent phosphorylation to generate CoA [60] (Fig. 2). In humans there are 3 tissue-specific isoforms of *CoASY*: CoASY *α*, which is ubiquitously expressed, the form predominantly expressed in the brain, COASY *β*,[61] and CoASY gamma.[62] The first 2 variants are localized in the mitochondrial matrix [63] or on the outer mitochondrial membrane [64] in contrast to *PANK2*, which is found in the intramembrane space.[65] Both homozygous and compound heterozygous CoASY mutations have been found in a small number of patients with NBIA, so-called *CoASY*-protein associated neurodegeneration (CoPAN).[62] *In vitro* studies have shown that the missense mutation p.Arg499Cys in *CoASY* results in reduced dephospho-CoA kinase (DPCK) activity, and an 80% reduction in CoA production.[62] Yeast deletion strains expressing the Arg499 mutant DPCK displayed reduced growth and the requirement for elevated levels of pantothenate, the substrate for the CoA biosynthetic pathway.[62] Moreover, in keeping with the brain iron accumulation, Arber et al. highlighted a possible role of iron metabolism in CoPAN with the identification, using a prediction tool, of an iron response element in the CoASY gene.[27]

By virtue of the fact that *CoASY* operates in the same pathway as *PANK2* (Fig. 2), it is likely that loss of its normal function causes NBIA through mechanisms shared with PKAN. Certainly the presence of 2 mutations in the same biosynthetic pathway supports the concept that abnormalities in CoA synthesis may play a critical role in the pathogenesis of NBIA. Indeed one study in *Drosophila* dissected the entire CoA biosynthesis route, including the study of *PANK2* (*dPANK/fmbl*) and *CoASY* (*dPPAT-DPCK*) mutants, and demonstrated that this pathway is important in maintaining DNA and cellular integrity. Both *dPANK/fmbl* and *CoASY* mutants displayed locomotor abnormalities, increased sensitivity to oxidative stress, altered lipid homeostasis, in addition to impaired DNA integrity. This work therefore demonstrated how defective CoA synthesis during development leads to CNS abnormalities.[55] Furthermore, in keeping with the importance of CoA in many cellular metabolic processes, one study on the serum of PKAN patients demonstrated reduced lipid and cholesterol biosynthesis, impaired bile acid metabolism, as well as a reduction in the levels of certain sphingomyelin species, critical components of myelin.[66]

C19orf12 and mitochondrial membrane protein associated neurodegeneration

Mutations in C19orf12 have recently been identified in patients with NBIA,[67,68] a variant referred to as mitochondrial membrane protein associated neurodegeneration (MPAN). C19orf12 mutations have also been implicated in a number of diseases, including Behr syndrome,[69] pallido-pyramidal syndrome,[70] and hereditary spastic paraplegia type 43 (SPG43).[71] Common clinical features in patients with MPAN include cognitive decline progressing to dementia, neuropsychiatric abnormalities and motor neuronopathy.[72] Two post-mortem neuropathological studies demonstrated iron-containing deposits, axonal spheroids, Lewy bodies, and hyperphosphorylated tau-positive inclusions, suggesting links to more common neurodegenerative diseases.[67,72] There was also loss of myelin in the pyramidal tract of the spinal cord and the optic nerve.[67] The C19orf12 gene encodes a 17-kDa protein of unknown function bound to the mitochondrial membrane,[67,73] as well as in the ER and Mitochondrial Associated Membrane.[73] It is expressed predominantly in the brain, adipocytes and blood cells, and analysis of transcriptomic profiles in MPAN, and C19orf12 co-regulated genes, has highlighted the role of mitochondrial dysfunction and CoA metabolism.[67] Molecular modeling predicts that the C19orf12 protein has transmembrane helices with glycine-zipper motifs and a soluble domain, with mutations hypothesized to structurally destabilize the transmembrane motif and the soluble domain.[73] Moreover C19orf12 mutations may result in mislocalisation of the mutant protein within the mitochondrial matrix.[73] Furthermore, work in fibroblasts from a C19orf12-positive patient demonstrated high mitochondrial calcium concentration and increased apoptosis in response to H_2O_2, while mutant C19orf12 protein re-localized to the cytosol following exposure to oxidative stress in cell lines.[73] C19orf12 has also been predicted to play a role in autophagy, and in support of this it was shown that overexpression of wild-type C19orf12 resulted in conversion of the autophagy

marker LC3 and reduction of p62, which usually accumulates when autophagy is blocked. C19orf12 mutants on the other hand failed to promote autophagy induction, with no change in basal levels of autophagy in response to oxidative stress.[73] Given the possible autophagy defects in MPAN, as with PKAN and PLAN, reduced mitophagy may lead to the accumulation of damaged mitochondria within spheroids. More studies are therefore required to further define the mitochondrial defects, as well as the role of autophagy in NBIA.

Finally, a *Drosophila* model of MPAN also exists with knockdown of the 2 *Drosophila* orthologues of C19orf12 resulting in reduced survival, age-dependent locomotor phenotypes, as well neurodegeneration.[74] This model is therefore well placed to begin unraveling the underlying biochemical and physiological abnormalities, including lipid metabolism and mitochondrial function.

FA2H and lipid dyhomeostasis

The fatty acid hydroxylase (FA2H) gene, previously implicated in hereditary spastic paraplegia[75] and a progressive familial leukodystrophy,[76] has more recently been identified as a rare cause of NBIA, known as FA2H-associated neurodegeneration (FAHN).[77] Affected individuals display a step-wise deterioration with an onset typically later and progression slower than in NAD, as well as atypical features including confluent white matter lesions and brainstem atrophy.[77] *FA2H* encodes fatty acid 2-hydroxylase, involved in the formation of 2-hydroxy galactolipids, which is important in lipid metabolism and required for normal myelin production.[78] Unlike in the other forms of NBIA discussed here, this enzyme is not known to be localized to mitochondria, rather this membrane-bound 43-kDa protein is found in the ER.[79] The neuropathology linking mutations in *FA2H* to NBIA remain unclear, but a number of possible pathogenic mechanisms have been hypothesized. One speculation is that abnormal myelin integrity caused by defects in FA2H function leads to abnormal iron homeostasis as a result of the disruption of the normal association between myelin and ferritin.[27,80] FA2H is also involved in the regulation of cell cycle and apoptosis [81,82] and is predicted to have widespread effects through its modulation of

ceramide generation, decreasing 2-hydroxyceramide production.[83] This in turn may lead to abnormalities in the composition of the ceramide pool with possible downstream effects on lipid turnover. Interestingly *PANK2* and *PLA2G6* are also predicted to affect ceramide signaling, via an inhibitory effect on acyl-CoA production, and promotion of sphingomyelinase activity respectively.[84,85] Indeed, ceramide metabolism is hypothesized to play a role in neurodegeneration and more specifically in Lewy body disease pathophysiology.[84,86] Further research in these forms of NBIA will be required to determine to what extent, if any, changes in ceramide metabolism contribute to the neuropathogenesis. Furthermore, the observed changes in sphingolipids, downstream of altered CoA biosynthetic pathways, in PKAN,[66] and likely in CoPAN, may be responsible for defective myelination within the brain. Indeed demyelination is a shared neuropathological feature among the different forms of NBIA.

Summary and discussion

PLAN is associated with mitochondrial dysfunction and elevated mitochondrial lipid peroxidation

In summary, using a novel fly model of PLAN Kinghorn *et al.* demonstrated that loss of normal *PLA2G6* activity is sufficient for mitochondrial dysfunction and neurodegeneration.[45] In particular they provided evidence that knockout of *iPLA2-VIA* in the fly leads to elevated levels of lipid peroxidation, likely as a result of the failure of *iPLA2β* to repair oxidative damage to membrane phospholipids. This resulted in a reduction in mitochondrial membrane potential, respiratory chain dysfunction and reduced ATP levels. Furthermore, *iPLA2-VIA* knockout flies showed frank degeneration of mitochondrial membranes. However, what is interesting is that there were clear mitochondrial respiratory chain abnormalities in very young day 2 *iPLA2-VIA* flies, when no mitochondrial abnormalities were seen at the ultrastructural level, confirming that mitochondrial dysfunction precedes membrane degeneration.[45] It is most likely that the tubulovesicular structures seen in INAD, at least in part, represent degenerated mitochondrial membranes and other abnormal cellular membranes. Indeed aged mice lacking *Pla2g6* have spheroids containing
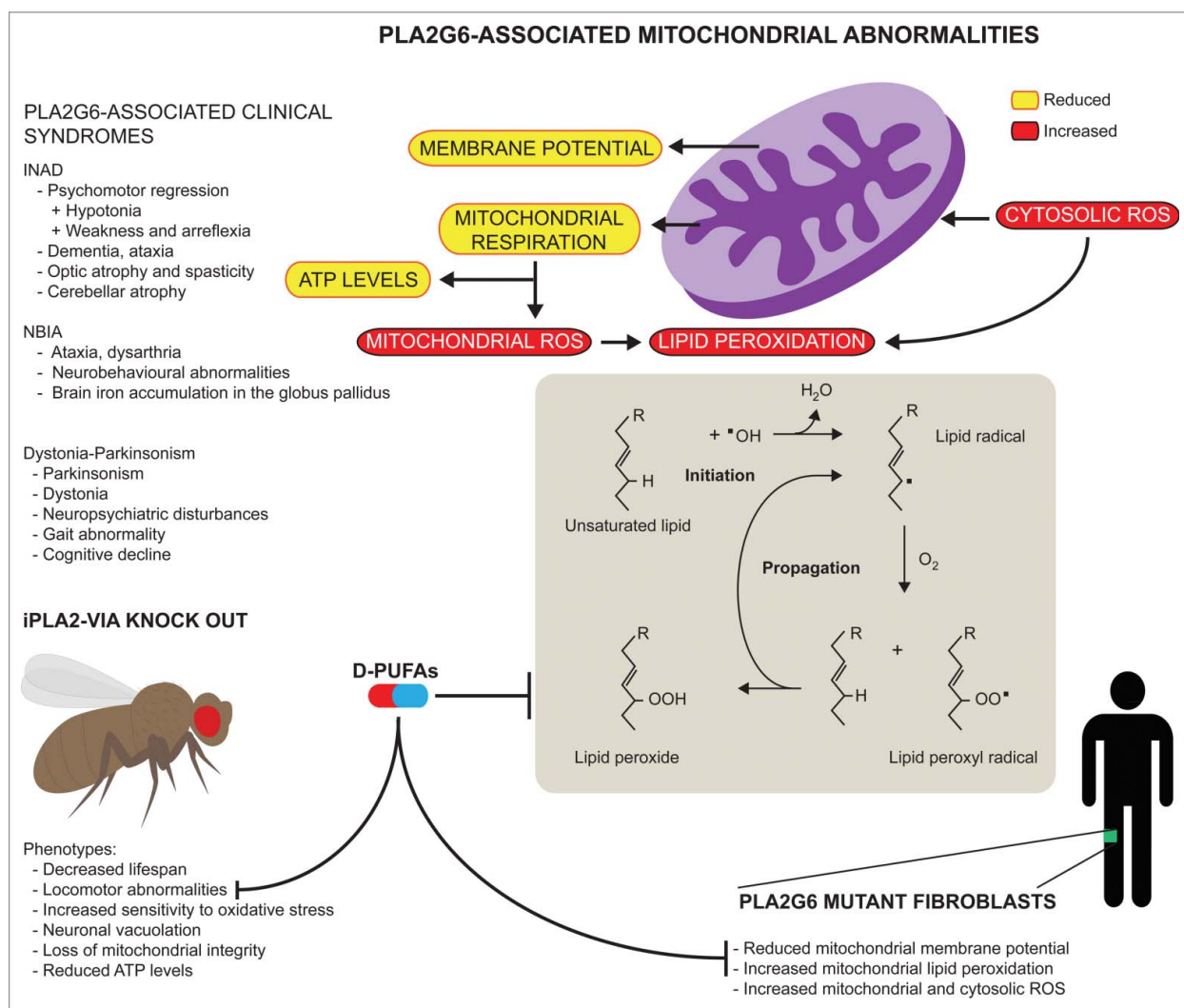
Figure 3. Schematic showing the clinical phenotypes of PLAN, as well as the role of lipid peroxidation and mitochondrial dysfunction in PLAN.

periodic-shift-positive granules consisting of degenerate inner mitochondrial membranes, as well as partial membrane loss at axon terminals.[35] This is consistent with oxidative damage to these membranes and degeneration in response to insufficient *Pla2g6* activity. It will also be interesting to see whether these membrane accumulations exist in PLAN, at least to some extent, due to an additional downstream failure of clearance of damaged mitochondria by autophagy. Furthermore, spheroids are not present in all cases of PLAN,[1,3] suggesting therefore that mitochondrial dysfunction is sufficient to cause neurotoxicity and disease in the absence of mitochondrial membrane degeneration.

Currently there are no effective disease-modifying treatments for PLAN and management is supportive, aimed at palliation and symptom control. Kinghorn

et al. highlighted the potential therapeutic benefits of lipid peroxidation-lowering strategies, such as D-PUFAs, in both *iPLA2-VIA* knockout flies and mutant *PLA2G6* fibroblasts [45] (Fig. 3).

Lipid dyshomeostasis and mitochondrial dysfunction as therapeutic targets in NBIA

Interlinking mechanisms in NBIA are emerging with many of the enzymes discussed here being linked to CoA biosynthesis, lipid metabolism and mitochondrial dysfunction. It is now becoming clearer how mutations in CoA biosynthetic enzyme genes (PANK2 and CoASY), associated with altered CoA availability in the cell, cause not only effects such reduced DNA intergrity,[55] but also altered glycerol/sphingophospholipid metabolism with predicted

defects in myelination and membrane remodeling.[87] *PLA2G6* and *FA2H* are predicted to act downstream of CoA production, with specific abnormalities in membrane remodeling, especially of inner mitochondrial membranes, and myelin production respectively.[45,78] Mitochondrial dysfunction is likely a common feature of most forms of NBIA, consistent with the mitochondrial localization of many of these NBIA-associated enzymes. A number of processes are predicted to lead to abnormal mitochondrial function, including abnormal membrane remodeling, increased mitochondrial lipid peroxidation and defective mitophagy.[45] Moreover, mitochondria play an important role in cellular iron homeostasis, and therefore mitochondrial dysfunction is likely to play a role in the iron dysregulation and iron deposition seen in neurodegenerative diseases such as NBIA and PD.[88]

To date there are no therapies available that modify disease progression in PLAN, PKAN or other forms of NBIA. Kinghorn *et al* demonstrated the first *in vivo* rescue of PLAN in a *Drosophila* model, as well as in *PLA2G6* mutant fibroblasts, suggesting a potential therapeutic role of PUFAs.[45] Therefore future work is required to further test the beneficial properties of D-PUFAs, and other compounds predicted to reduce lipid peroxidation, in order to develop disease-modifying agents for patients with PLAN and NBIA. Deuterated linoleic acid, the D-PUFA shown to rescue cellular and fly models of PLAN, is an antioxidant that reduces the autoxidation of natural PUFAs and the subsequent production of reactive carbonyl compounds,[47,48] protecting mitochondria against oxidative stress.[89] More conventional antioxidants have not been tested in PLAN, and despite the partial success of antioxidant moieties such as vitamins C, E and Coenzyme Q in experimental cellular and animal models of PD, human clinical studies have not have not shown conclusive benefits.[90] It may be that for antioxidants to be effective, they need to be targeted to the mitochondria, the site where most of the reactive oxygen species are produced.[90,91] Indeed a number of mitochondria-targeted antioxidants have been developed through the conjugation of the antioxidant moiety to a lipophilic triphenylphosphonium cation, including MitoQ,[92] but clinical benefits have not yet been proven.[90] New and improved methods of delivering antioxidants to mitochondria are therefore required and may involve the use of novel biologically active nanomaterials.[90] Moreover, combination strategies using more than one antioxidant may be required. It is also likely that effective antioxidants will need to be administered to patients before or early in the course of the disease, prior to the occurrence of any overt neuronal loss. Effective therapeutic strategies will therefore rely on the prompt and early diagnosis of these neurodegenerative syndromes.

As mentioned above pantethine was shown to rescue the neurodegenerative phenotypes in a *Drosophila* model of PKAN[56], and is a substrate for the CoA synthesis pathway, in which both PANK2 and CoASY act. It will therefore be interesting to see whether pantethine also rescues CoASY *Drosophila* mutants and other available models of CoPAN. Indeed, pantethine, aided by the fact that it is a naturally occurring vitamin that is already available for human consumption, could be directly tested in patients wtih NBIA caused by mutations in the CoA biosynthetic pathway genes (PANK2 and CoASY).

Only through a detailed understanding of the pathogenic mechanisms involved in the different forms of NBIA, will the overlapping and complex pathways in lipid and CoA metabolism, as well the role of mitochondrial dysfunction, become fully understood. In addition there are other genetic variants of NBIA that have not been considered here, which are caused by mutations in genes directly related to iron metabolism (neuroferritinopathy and aceruloplasminaemia) and lysosomal-autophagy defects (*β*-propellar-associated neurodegeneration (BPAN) and ATP13A2-associated NBIA).[27,87] Indeed, it is very likely that these too will also share common mechanisms of pathogenesis with those discussed here.

Conclusions

Faithful *Drosophila* models of NBIA now exist and can be used to further our knowledge of the *in vivo* mechanisms of pathogenesis associated with these diseases. In particular, a more detailed study of mitochondrial pathology and lipid homeostatic pathways in the genetic variants of NBIA discussed here will lead to further identification of new therapeutic targets. Specifically, the *Drosophila* models (including those of PLAN, PKAN, CoPAN and MPAN)[45,55,56,74] can now be used as platforms of drug discovery, including the identification of genetic modifiers and new therapeutic targets.

Finally, the study of NBIA, especially of PLAN and PKAN, given the similarities to the more common neurodegenerative disorders such as Parkinson and Alzheimer diseases, may provide important insights and therapeutic targets for these common forms of neurodegeneration.

Disclosure of potential conflicts of interest

Acknowledgments

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