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Review article

PKAN pathogenesis and treatment

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ABSTRACT

Studies aimed at supporting different treatment approaches for pantothenate kinase-associated neurodegeneration (PKAN) have revealed the complexity of coenzyme A (CoA) metabolism and the limits of our current knowledge about disease pathogenesis. Here we offer a foundation for critically evaluating the myriad approaches, argue for the importance of unbiased disease models, and highlight some of the outstanding questions that are central to our understanding and treating PKAN.

Contents

1. Introduction

Pantothenate kinase-associated neurodegeneration (PKAN) is an inborn error of coenzyme A (CoA) metabolism that manifests with dystonia, parkinsonism, pigmentary retinopathy, acanthocytosis, basal ganglia iron accumulation, and early death [1]. The brain phenotype is exquisitely localized, with damage limited to the globus pallidus and, occasionally, substantia nigra [2]. Mutations in *PANK2*, encoding the mitochondrially-targeted pantothenate kinase 2, cause this autosomal recessive disorder [3]. Despite gains in knowledge during the two

Abbreviations: PKAN, pantothenate kinase-associated neurodegeneration; CoA, coenzyme A; PANK, pantothenate kinase; PPCS, phosphopantothenoylcysteine synthetase; PPCDC, phosphopantothenoylcysteine decarboxylase; COASY, CoA synthase; 3′,5′-ADP, 3′,5′-adenosine diphosphate; mtACP, mitochondrial acyl carrier protein; GP, globus pallidus; CoPAN, COASY protein-associated neurodegeneration; IMS, intermembranous space; IBM, inner boundary membrane; CM, crista membrane.

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decades since the disease gene discovery, important gaps remain. As the field moves towards rational therapeutics for this ultra-rare disorder, highlighting these gaps is important in order to critically assess the most promising approaches.

High interest in PKAN therapeutics is underscored by the publication of six reviews in the past three years describing the myriad approaches [4–9]. What has been missing, however, is an exploration of the limits of knowledge of PKAN pathophysiology and CoA biochemistry and acknowledgement of what is known versus assumed about the underlying disease biology. Here we highlight important questions to be addressed in advancing therapeutics.

The CoA synthesis pathway appears to be simple, straightforward, and well understood, yet fundamental questions remain unanswered (Fig. 1). PANK2 is one of four mammalian pantothenate kinase isoforms, yet the reason cells require this redundancy is still largely speculative. Different patterns of tissue expression and organellar localization are well-documented for PANK1α, PANK1β, PANK2, and PANK3, the four mammalian isozymes with pantothenate kinase activity [10–14]; however, we do not understand their respective cellular roles, their temporal and spatial expression differences, their specific kinase activities and substrate utilization in various tissues and cellular compartments, or their capacity to regulate other isoforms or compensate for their dysfunction. Moreover, we lack a clear understanding of the unique cellular role of PANK2 and why only certain tissues are selectively vulnerable to its loss.

Our limited understanding of the enzyme defect is compounded by the widespread assumption that PKAN results from a cellular deficiency of CoA. This has led to the corollary hypothesis that increasing or replenishing total CoA levels will improve outcomes. Acknowledging this assumption is important because it has served as the basis for development of putative disease models, including some of our own, [15–19] and for the advancement of four approaches to treating PKAN [16,19–21]. Though such a deficiency may eventually be proven, current data suggest a more complex basis for disease. While only a clinical interventional trial can demonstrate the true therapeutic potential of a compound, use of high-fidelity model systems of disease that make no assumptions about pathogenesis provide a complementary resource for therapeutics development.

One role of PANK2 related to CoA homeostasis is its influence on the activation of mitochondrial acyl carrier protein (mtACP). Normal PANK2 levels are required in order to maintain normal levels of activated mtACP [22–24]. A CoA-derived 4′-phosphopantetheine moiety binds to and activates mtACP supporting mitochondrial fatty acid synthesis, iron-sulfur cluster biogenesis, and electron transport [25,26]. Activation of carrier proteins is the only CoA-dependent reaction that consumes rather than recycles this molecule (Fig. 1). How then might this process be sensitive to a defect in PANK2? The simplest answer is that CoA levels are decreased; however, studies using a variety of disease models challenge this idea.

1.1. CoA metabolism and PKAN models

Fly models of PKAN have revealed critical insights into pathogenesis and provided early evidence for efficacy of targeted therapeutics [18,22,27]. While fly models of PKAN demonstrate a decrease in total CoA levels [18,27], no such difference has been found in a variety of mammalian models, including human PKAN primary cells, in human cell lines with knock-down of *PANK2* by RNA-interference, or in induced pluripotent stem cells derived from PKAN patient fibroblasts and differentiated into cortical neurons [22,28]. Normal CoA levels were also found in whole cell and mitochondrial fractions derived from *Pank2* KO mouse GP and from cultured patient fibroblasts (our unpublished results), despite the presence of a clear disease-relevant molecular phenotype in both model systems [29]. These studies did not address the possibility of decreased CoA levels occurring in only a subset of brain cell types (e.g., astrocytes) or at only specific stages of development. Indeed, a transient decrease in whole brain CoA levels was reported in the early postnatal period in an independently generated *Pank2* KO mouse [30], but this change is difficult to reconcile with the sustained and localized biochemical perturbations that are present in murine brain long past the early postnatal period and after brain CoA levels were documented to be normal [29,30].

These important observations lead to a central divergence in concepts of PKAN pathogenesis and thus in approaches to treatment. Is PKAN associated with a global deficiency in CoA, and can it be treated by a general increase in CoA synthesis effected by up-regulating an alternate pantothenate kinase isoform? Or, based on demonstration that a defect in pantothenate kinase 2 selectively disrupts the CoA-derived 4′-phosphopantetheinylation of carrier proteins in the context of normal levels of CoA, does treatment instead require targeted replenishment to recover this specific function [22,29]? It is tempting to think that activating a general increase in CoA will serve to also replenish

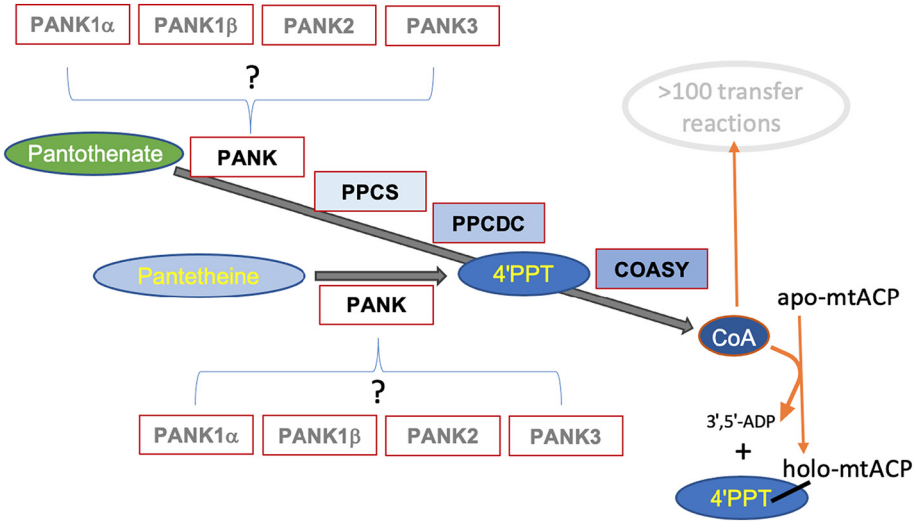

Fig. 1. CoA synthesis and consumption. Both pantothenate and pantetheine serve as substrates for pantothenate kinase (PANK). The relative contribution of each PANK isoform likely differs by cell type and developmental stage. 4'PPT is both a pathway intermediate and the activating moiety of mtACP. PPCS = phosphopantothenoylcysteine synthetase; PPCDC = phosphopantothenoylcysteine decarboxylase; 4'PPT = 4′-phosphopantetheine; COASY = CoA synthase; 3′,5'-ADP = 3′,5′-adenosine diphosphate; mtACP = mitochondrial acyl carrier protein.

the CoA needed for carrier protein activation, and while this may ultimately be shown to be true, there is no direct evidence demonstrating this. A general CoA-increase strategy views CoA synthesis as linear with a single substrate and regulatory step by pantothenate kinase. In fact, the synthesis of CoA can utilize at least two substrates [27] (Fig. 1) and requires a complex orchestration of events to produce this energy-costly molecule necessary for more than a hundred biochemical reactions [31].

The therapeutic approach that focuses on general CoA replenishment via upregulation of other PANK isoforms has been supported using data derived from CoA-depleted model systems [16,21]. While this approach may ultimately prove beneficial in human studies, published results have not addressed efficacy in correcting any disease-specific changes that are attributable to a defect exclusively in pantothenate kinase 2. Instead, the approach has relied on compound models developed to manifest a measurable decrease in cellular CoA levels, employing interventions that impede the activity of multiple pantothenate kinase isoforms [15–17]. The neuron-specific *Pank1 Pank2* double knock-out animal model, as well as those with increased CoA degradation from neuronal overexpression of a nudix hydrolase, show a global decrease in brain CoA and general neurologic defects that are relieved by increasing CoA levels. While neurons are reasonable starting targets for genetic manipulations, the specific cell types that are vulnerable to loss of pantothenate kinase 2 have not been identified. In these animals, there is no evidence of a disease process that selectively impairs globus pallidus function. Instead, thalamus and hippocampus were reported to show spectroscopic evidence of neuronal injury [32]. These findings suggest that widespread neuronal dysfunction is a likely result of the genetic knock-out of multiple pantothenate kinase genes and suggest a basis for the neurological features that extends beyond GP dysfunction alone. The double knock-out mice show a locomotor phenotype that is rescued by replenishing CoA levels. Not yet clear is why these animals, even when treated to increase CoA levels, show a markedly shortened lifespan. The non-specific features that manifest in these models are similar to those resulting from hopantenate, the metabolic toxin that impedes CoA synthesis.

In addition to the neuronal *Pank1 Pank2* double knock-out animal model, there is also a record of utilizing hopantenate to generate putative models of PKAN [19,33,34]. Hopantenate was initially thought to competitively inhibit pantothenate kinase [28] but was recently discovered to inhibit PPCS (Fig. 1) [35]. Hopantenate is phosphorylated by pantothenate kinase producing phosphorylated hopantenate, which inhibits PPCS and thereby blocks CoA synthesis [31]. Thus, hopantenate is a substrate for and not an inhibitor of pantothenate kinase and leads to impaired CoA synthesis through the activities of all pantothenate kinase isozymes. The depletion of CoA that occurs in mice fed hopantenate causes neurological disease and a markedly shortened lifespan. Although the new insights into its mechanism of action limit the utility of hopantenate in modelling PKAN, it still serves as an important tool for studying CoA synthesis and possible salvage pathways. The question remains of how to align data from these toxic and compound genetic models with those of PKAN, and whether increasing CoA synthesis by alternate isoform upregulation represents a rational therapeutic approach.

The framework typically used for comparing one disease model to another considers their fidelity in recapitulating key aspects of the human disorder. For PKAN, those features are: 1) dystonia/parkinsonism; 2) GP selective dysfunction with preservation of normal brain function outside of that region; 3) GP iron accumulation; and 4) pigmentary retinopathy [1]. A further comparator would be the etiologic basis used to create the model and whether a phenotype is directly attributable to mutations in the *PANK2* ortholog or whether additional manipulations (genetic, toxic, or dietary) are required to elicit a phenotype. Table 1 provides a summary of models and approaches to intervention.

High value has been placed on mouse models with clinical features that mimic the manifestations of human disease. As a corollary, animals showing only biochemical and molecular changes have garnered less favor as models, even when those changes are specifically attributable to the primary cause of disease. In fact, such models might arguably be more informative for investigating the critical early disease changes before cells and tissues sustain complex, manifold abnormalities that overwhelm normal neurological function.

Though the *Pank2* KO mouse manifests no neurological abnormalities other than retinopathy, four key observations establish its relevance to PKAN in humans, including genetic, biochemical, anatomic, and therapeutic datasets that have been corroborated in primary human cell lines from people with PKAN [29]. (1) Dysfunction of PANK2 alone underlies all defects in the KO mice and in PKAN. (2) The KO mouse brain metabolic defects of iron dyshomeostasis and dopamine dysmetabolism are also found in people with PKAN (3). In KO mouse brain, disease changes are limited to the globus pallidus-containing brain region, which is a central feature of the human disease. Finally, (4) correction of CoA dysmetabolism by 4′-phosphopantetheine also corrects secondary iron and dopamine metabolic defects. These features have not been reported in other putative mammalian models of PKAN. This last observation implicates a defect in CoA metabolism, though not necessarily a deficiency of CoA, as central to PKAN pathogenesis.

Among the most compelling observations in the *Pank2* KO mouse is brain iron accumulation. As in humans, this defect occurs only in the GP region and can be specifically attributed to a defect in PANK2. This is the sole model system with GP iron accumulation. Moreover, 4′-phosphopantetheine is the only molecule targeting the primary defect that corrects the secondary iron defect and other molecular sequelae found in brain [29].

Studies in cultured primary fibroblast cell lines derived from people with PKAN corroborated the murine findings by demonstrating CoA dysmetabolism, mitochondrial dysfunction, and correction of all defects by 4′-phosphopantetheine [29]. Iron dyshomeostasis and dopamine dysmetabolism are not observed in tissues outside of GP, consistent with their absence in cultured human cells [29]. Abnormalities found in the *Pank2* KO mice and in patient-derived cell lines are specifically attributable to a defect in pantothenate kinase 2. The recapitulation in the *Pank2* KO mouse of key elements of PKAN in humans establishes confidence in this model as a tool to interrogate pathogenesis and test potential treatments. A disadvantage of this model is that no neurological features have been observed.

Numerous genetic mouse models of human neurodegenerative disorders lack overt clinical features [39]; and, the lack of an overt neurological phenotype in the *Pank2* KO mice requires an explanation. If the documented biochemical perturbations found in these animals also underlie the neurological symptoms in the human disease but are insufficient to cause a similar phenotype in mice, then the value of the model holds. Alternatively, if the biochemical perturbations are not causative of the clinical features, the model offers little translational value. The secondary disruption of dopamine metabolism in this model is hard to ignore even if it is insufficient to cause overt dystonia-parkinsonism. Nevertheless, direct evidence of causation will be needed to settle this question. Until then, we view the body of evidence as supporting the utility of these animals in studying PKAN pathogenesis and testing treatments. The advantages and disadvantages of the existing PKAN mouse models indicate complementarity for their use in advancing potential therapeutics.

1.2. Are defects in mtACP central to PKAN?

Multiple lines of evidence indicate that a defect in mitochondrial acyl carrier protein (mtACP) is likely to be central to PKAN pathogenesis [22–24,29]. The pathways that require mtACP include electron transfer, mitochondrial fatty acid synthesis needed for lipoylation of target proteins, iron-sulfur cluster biogenesis, and mitochondrial ribosome biogenesis and protein translation [40–44]. Loss of pantothenate kinase 2 function leads to decreased phosphopantetheinyl-activated mtACP

Table 1
Comparison of putative animal models of PKAN and their fidelity to the human disease.

Human disease features		Animal Disease Models					
			CoA depletion				
	PKAN	*Pank2*$^{-/-}$ mouse	*Pank1*$^{-/-}$ n*Pank2*$^{-/-}$ mouse	n*Pank1*$^{-/-}$ n*Pank2*$^{-/-}$ mouse	n*Nudt7*$^{++}$ mouse	*fumble* *Drosophila*	**Other findings**
Dystonia parkinsonism	+	-*	-	-	-	-	
Selective GP impairment	+	+	N/A	N/A	N/A	N/A	
Increased GP iron	+	+	N/A	N/A	N/A	-	
Retinopathy	+	+	N/A	N/A	N/A	retinal defect	
	not measured	normal^	decreased	decreased	decreased	Whole body levels decreased	Brain CoA levels
	dystonia parkinson-ism	none*	forelimb deformity	locomotor defect	motor incoordi-nation	impaired climbing, flying	Neurologic features
	sperm defect	sperm defect	median survival 17d; glucose dyshomeo stasis	median survival 52d	none	sperm defect	Other
Therapeutic approach in disease model		4'PPT	PANK3 activation	PANK3 activation	none reported	4'PPT	
References	[1]	[29, 30, 36]	[17]	[32]	[15]	[18, 37, 38]	

* dopamine metabolism is perturbed, but clinical neurological features are absent; '+' means present; '-'means absent; 'n' before a gene name refers to neuronal-specific knock-out; N/A = not assessed; ^- see text for detailed discussion.

and to impairment of processes that depend on its function [22,29]. In addition, down-regulation of *PANK2* by RNA interference in the SH-SY5Y human neuroblastoma cell line leads to decreased levels of holo-mtACP [22]. These experiments along with extensive studies in *Drosophila* directly link a defect in pantothenate kinase 2 with decreased levels of activated mtACP. Complementary indirect evidence from mouse and human model systems further implicate a defect in mtACP [29]. These include decreased complex I and PDH activities as well as iron dyshomeostasis specifically in GP in *Pank2* KO mice. Decreased complex I activity was also seen in cultured primary cells derived from people with PKAN. Together, these data implicate the loss of pantothenate kinase 2 activity in causing decreased levels of holo-mtACP. What is not clear is the mechanism.

The simple answer is that less CoA is synthesized and, though low levels of CoA have not been detected, several studies can be interpreted to support this. Impeding CoA synthesis in *Drosophila* S2 cells via the toxin hopantenate was associated with reduced holo-mtACP levels, reduced protein lipoylation, and decreased pyruvate dehydrogenase activity, and these changes were recovered by adding CoA to the culture media, though these results are also fully consistent instead with a direct action of 4′-phosphopantetheine derived from the added CoA [22]. Similarly, in PKAN mammalian model systems the correction of biochemical changes by 4′-phosphopantetheine may reflect either a direct action or its use as a substrate for CoA synthesis (Fig. 1). From these studies, one can infer that impaired PANK2-dependent CoA metabolism, with or without decreased levels of CoA, can be causally linked to reduced activation of mtACP. Measuring levels of *holo*-mtACP in the GP of various mouse models at baseline and under treatment conditions may provide important insights.

How do we now reconcile our current knowledge in order to generate a cohesive hypothesis explaining the function of PANK2 and thereby the pathophysiology of PKAN? Perhaps PANK2 regulates or participates directly in the transfer of 4′-phosphopantetheine to mitochondrial carrier proteins by a mechanism that is independent of the concentration of CoA. Alternatively, perhaps PANK2 is essential for the synthesis of specific CoA molecules that are directly transferred, or 'tunneled', to activate mitochondrial carrier proteins, though it is difficult to imagine how these CoA molecules might be differentiated from others, especially given the shared CoA synthesis enzymes downstream of pantothenate kinase and their different compartmental locations. Many aspects of phosphopantetheinyl-activation of mtACP are not clear, including whether there is a mitochondrial phosphopantetheinyl transferase in mammalian cells like there is in yeast and whether 4′-phosphopantetheine can promote this transfer directly as a substrate or via a different action; currently there is no evidence for either of these [45,46]. In summary, the mechanism by which a defect in pantothenate kinase 2 causes decreased holo-mtACP remains enigmatic, with the most parsimonious interpretation of available data being that the CoA needed to activate mtACP is insufficient.

1.3. Selective vulnerability of globus pallidus in PKAN

While questions about the specific cellular function of PANK2 need further study, existing data provide new insight into the basis for the GP vulnerability in PKAN. In the *Pank2* KO mouse the localization of myriad biochemical changes to only GP suggests that cells in this region are selectively vulnerable perhaps because they are more heavily reliant on pathways that require mtACP. While numerous hypotheses abound, published data suggest that GP vulnerability is caused by regional differences in CoA metabolism itself [29]. Specifically, changes in CoA synthesis enzyme gene expression are limited to just this region despite the loss of PANK2 in all cells in KO animals.

Perhaps the GP requires PANK2-dependent CoA synthesis and is unable to compensate for its loss, whereas other brain regions either do not share this requirement or have developed compensatory mechanisms. The concept of PANK2-dependent CoA synthesis is supported by data derived from other disorders that also selectively damage GP and suggests its unique metabolic vulnerability [22,47]. Work is needed

to identify the functional basis for this vulnerability by investigating distinct cell types, metabolic regulation, substrate availability, and other factors that are unique to GP. Ultimately, differences in the primary pathway itself seem to account for GP tissue vulnerability in PKAN, even if the mechanism itself remains enigmatic.

We do not yet understand why some of the molecular perturbations that are otherwise specific to GP are also present in peripheral cells [29]. Cultured patient fibroblasts are exposed to an artificial milieu of growth and other serum factors, abundant glucose, relative hyperoxia, and different metabolic substrates [48,49]. In PKAN cultured cells, we observe a glycolytic shift with decreased oxidative phosphorylation, likely from a combination of decreased complex I activity and limited iron-sulfur cluster biogenesis needed for the activities of other OXPHOS complexes [29]. The cells behave as if they are experiencing hypoxia, but our studies of hypoxia-inducible factor 1 (Hif-1) activation have been inconclusive. In contrast, evidence supporting a role for hypoxia in PKAN pathogenesis has been found in neuropathologic studies. GP neurons in PKAN show pathologic changes that are similar to those found when these same neurons are exposed to hypoxia [50]. The cellular response to loss of PANK2 by primary fibroblasts in vitro and also by lymphocytes in circulation causes a glycolytic shift and mimics the cellular response observed in vivo by a mixed cell population in GP. CoA synthesis is perturbed in all, while CoA levels are normal in all. Thus, these cell types can serve as model systems to reveal their common vulnerabilities as well as their specific dependence on PANK2.

In disease models where perturbations can be specifically attributed to a defect in PANK2, 4′-phosphopantetheine corrects those changes. In addition to correcting CoA metabolic defects, secondary changes are normalized as well, including iron dyshomeostasis, dopamine dysmetabolism, and mitochondrial dysfunction [29]. The power of this observation is in linking these secondary changes to a primary defect in CoA metabolism. 4′-phosphopantetheine recovers the primary metabolic process to a sufficient level to correct the secondary cellular processes that are linked to disease pathogenesis. 4′-phosphopantetheine is hypothesized to effect rescue by bypassing the PANK2 defect and normalizing activation of mitochondrial carrier proteins.

In the same way that pantothenate is a vitamin in healthy people, 4′-phosphopantetheine may be able to serve as the essential vitamin form in PKAN. At baseline, people with PKAN are unable to carry out PANK2-dependent CoA synthesis, thus creating a unique nutritional need. The biophysical properties of 4′-phosphopantetheine and its ability to recover PANK2-dependent CoA metabolism implicate it as an essential nutrient in PKAN.

1.4. Other genetic diseases linked to CoA biosynthesis enzymes

Normal CoA levels were also found in fibroblasts from people with CoPAN, a related neurodegenerative disorder with iron accumulation caused by mutations in *COASY* [51]. Only those cells from people with mutations in *PPCS* showed a detectable decrease in CoA, among the three human disorders arising from inborn errors in CoA metabolism. Interestingly, mutations in *PPCS* cause cardiomyopathy, suggesting a fundamentally different disease mechanism [52]. The overlap in phenotypes between PKAN and CoPAN and the observation that cellular CoA levels are normal in both suggests that they share a common pathophysiology. Since the neuropathology of CoPAN involves more brain structures than in PKAN, these data also suggest that two mechanisms may be at work and that GP iron accumulation in CoPAN arises from a mechanism similar to that of PKAN [22,29], whereas the perturbations found in other brain regions may be caused by a different mechanism, possibly reflecting PANK2-dependent and -independent CoA synthesis, respectively. COASY protein has been found in association with the outer mitochondrial membrane, in matrix, and in cytosol [51,53–55]. Perhaps mitochondrial COASY activity is needed for PANK2-dependent CoA synthesis necessary for carrier protein activation and essential for normal GP function. In contrast, cytosolic COASY activity may be needed to replenish recycled CoA from a much smaller pool given the low turnover rate for CoA [56]. Cytosolic COASY activity may be essential for normal function of cells in all brain regions. This hypothesis would provide a common mechanism for GP vulnerability in both PKAN and CoPAN while also explaining the wider neuropathology of CoPAN.

1.5. Where is CoA synthesized?

We know that the CoA concentration in cytosol is a small fraction of that in mitochondria [57,58]. This implies that CoA does not move freely between these compartments. CoA is synthesized in cells and cannot passively diffuse across membranes, and although a putative CoA transporter has been reported, data showing active transport in vivo is lacking [59]. Interestingly, people with mutations in the gene encoding the putative CoA transporter had only mildly decreased levels of CoA in cultured fibroblasts [60]. Though the question of active transport remains unanswered, separate synthesis processes in cytosol and mitochondria may explain the genesis of these distinct pools. While separate de novo synthesis processes would require a full complement of enzymes to be co-located, a phenomenon that is not known to occur in any sub-cellular compartment in brain cells, the transit of 4′-phosphopantetheine across membranes and the presence of COASY activity in both mitochondria and cytosol suggests that on-demand CoA synthesis from this precursor may be possible in both compartments [27].

Subsets of CoA synthesis enzymes localize to specific organelles (Fig. 2). Co-localization may enable these enzymes to physically interact or form biomolecular condensates in order to facilitate transfer of intermediates [61–63]. Those enzymes found in or associated with mitochondria include PANK2, PPCS, and COASY [10–12,29,64]. COASY has been found in matrix and on the outer mitochondrial membrane facing the cytosol [53,64]; PANK1β, PANK3, and PPCDC all localize to cytosol [14,29]. We have shown that PPCS localizes to nuclei and mitochondria in mouse globus pallidus, substantia nigra, and cerebellum [29]; conflicting datasets leave unanswered the question of whether PPCS also localizes to cytosol [52,63], but we find no evidence for that in brain. Tissue or cell type differences may lead to localization of these proteins to different compartments. Importantly in mouse brain, PPCS was found only in mitochondrial and nuclear fractions [29]. Nuclei harbor PANK2 and PANK1α, which suggests a possible role for these proteins in transcriptional regulation or for regulation by their substrates or products.

PANK2 has been reported to localize to the intermembranous space (IMS) in mitochondria, possibly tethered to the inner membrane [65,66]. The IMS can be effectively divided into two compartments: one defined by the inner boundary membrane (IBM) and the outer membrane forming the bulk IMS, and the other bounded on all sides by the crista membrane (CM) and forming the crista lumen. Though the IBM and CM are continuous, the compartments are functionally distinct while also communicating intimately [67–70]. Cristae are highly dynamic membrane compartments that traverse the mitochondrial interior and are essential for organellar function. All of the respiratory chain complexes localize to cristae and are embedded in the CM [68]. Their co-location and specific siting within the CM are essential for the generation of the electrochemical gradient across the mitochondrial inner membrane that drives ATP synthesis [71]. Proteins needed for iron-sulfur biogenesis, including ISD11 and NFS1, also localize to the CM suggesting that at least some steps in this process are active specifically in the crista lumen [69]. Most proteins show lateral diffusion within membranes, and movement of proteins between the IBM and CM seems to be driven by metabolic changes that influence the structure of cristae and their membrane contents [72]. The special milieu of cristae becomes relevant to PKAN with a careful re-examination of its likely sub-cellular location.

Early studies of murine PANK2 localized the protein to the IMS [66], and further high resolution electron micrography using immuno-gold

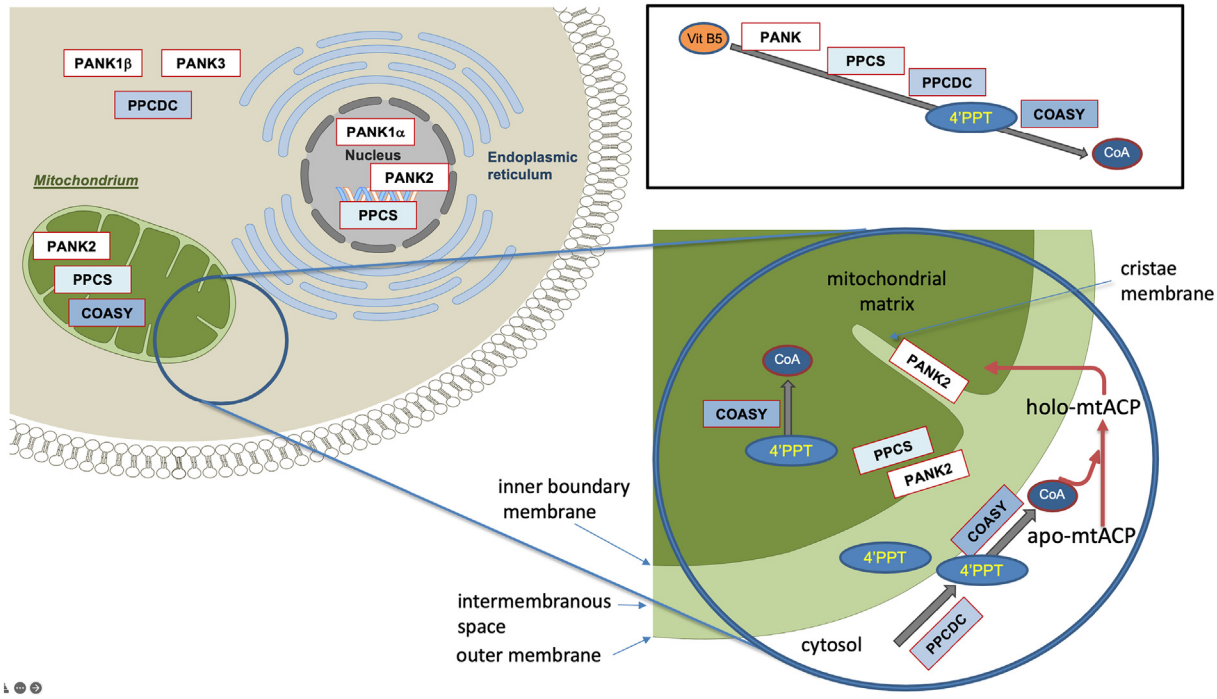

Fig. 2. The CoA synthesis pathway and subcellular localization of its various enzymes. In brain, no compartment contains all enzymes necessary for the synthesis of CoA from pantothenate. Abbreviations are listed in Fig. 1.

labelling of PANK2 refines the localization to cristae rather than the IMS (Fig. 3). In addition to the images shown below from Leydig cells, a similar pattern of decoration was observed in rod photoreceptor cell mitochondria, which is a tissue damaged by loss of PANK2 [66]. Though we cannot exclude localization also to the IBM and bulk IMS space, clear signal across and throughout each mitochondrion supports localization to cristae. Earlier predictions that PANK2 has two trans-membrane domains raised the possibility that it is anchored to the inner mitochondrial membrane with its catalytic domain in the IMS [66]. Perhaps instead the protein is in the CM with its active site in the crista lumen. This leaves open the possibility that PANK2 physically interacts with other cristae proteins or functions directly in local processes, including mtACP-dependent ones. Further studies using mammalian brain are needed.

Since no sub-cellular compartment in brain has been conclusively shown to contain all CoA synthesis enzymes, the transfer of intermediates across organellar membranes may be necessary in order to produce CoA. Transfer would be required to complete synthesis that had begun in a different compartment, an idea supported by findings of passive diffusion of 4′-phosphopantetheine through membranes [27,73].

The physico-chemical properties of 4′-phosphopantetheine lend further support to its potential to function as the essential vitamin

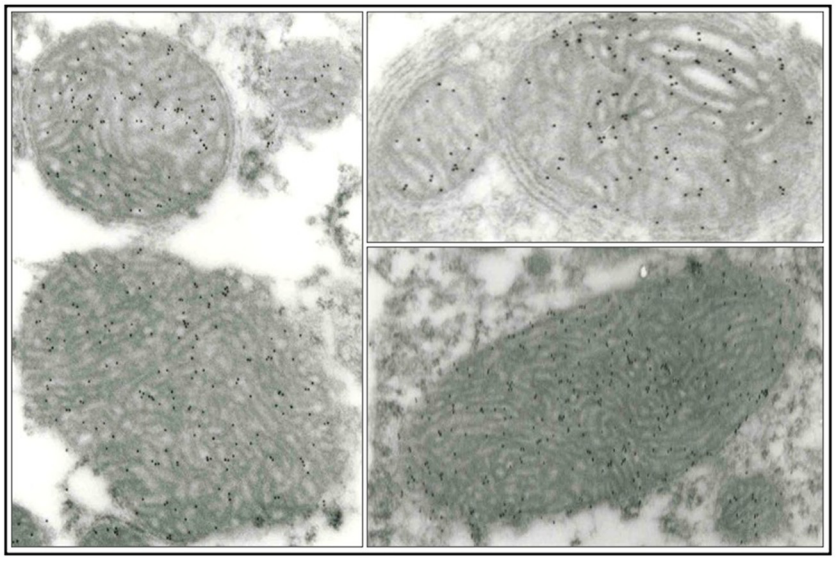

Fig. 3. Electron micrographs showing gold particles distributed throughout the mitochondria of Leydig cells in mouse testis (rabbit anti-Pank2 visualized with secondary goat anti-rabbit gold, 19,000×, image by Yien Ming Kuo).

form in PKAN. Despite predictions, 4′-phosphopantetheine is stable in mammalian serum and can cross cell membranes [27,29,73]. Moreover, endogenous 4′-phosphopantetheine is present in mammalian serum, which suggests that it can be transferred between cells and tissues and presumably serve as an alternate source for CoA synthesis [27]. Newly published data show that CoA intermediates, including 4′-phosphopantetheine, are transferred from mother to progeny and from gut microbiome to the host [74], further bolstering the importance of this intermediate in supporting a widening range of functions.

Additional evidence of 4′-phosphopantetheine transit across membranes, albeit indirect, shows that when fed orally to *Pank2* KO mice 4′-phosphopantetheine can normalize blood and brain biomarkers of disease. In order to do so, 4′-phosphopantetheine would have to cross multiple layers of membranes in gut, vasculature, and brain [29]. The most parsimonious interpretation of these data is that they demonstrate that 4′-phosphopantetheine is orally bioavailable, is not degraded by intestinal phosphatases, distributes widely in tissues including in circulating blood, and is able to cross plasma and nuclear membranes to effect gene expression changes. While it is possible that a metabolite of 4′-phosphopantetheine is instead effecting rescue, none of the candidate molecules, including pantothenate, pantetheine, or CoA, produced a similar effect when fed orally, making this alternative unlikely [29]. CoA can rescue PANK2 defective phenotypes only when supplied in media to cultured cells, a condition where it has been shown to be converted to 4′-phosphopantetheine [27]. Orally administered CoA is catabolized to products that include 4′-phosphopantetheine. Therefore, the question remains why oral CoA does not normalize features in the *Pank2* KO mice [29]. It may be that levels of 4′-phosphopantetheine arising from oral CoA are not sufficient to induce a rescue, although molar equivalent doses were administered. Experiments tracing labelled-4′-phosphopantetheine in vivo are required in order to answer these open questions.

In mammalian brain tissue, the data on compartmental localization of individual CoA synthesis enzymes consistently separates PPCS from PPCDC. Mitochondria and nuclei harbor the first and second pathway enzymes, but PPCDC is found only in cytosol. The product of PPCS and substrate for PPCDC, 4′-phosphopantothenoylcysteine, must reach cytosol, possibly through an as yet undiscovered transporter. A transporter of the CoA intermediate dephospho-CoA has been described in *Drosophila*, but this is the only intermediate with a known or suspected transporter in mitochondrial membrane [75]. Though classic predictions would argue against passive diffusion through membranes by 4′-phosphopantothenoylcysteine, similar predictions about 4′-phosphopantetheine were clearly contradictory to the experimental evidence [8,27]. Therefore, the transit of these intermediates may connect CoA synthesis across compartments, with pathway regulation being primarily via enzyme kinetics and secondarily via compartmentalization. Whereas co-locating the components of a pathway can increase reaction kinetics, segregating them into separate compartments can slow or inhibit reactions and may provide a secondary dimension of control of this critically important pathway.

Distinct subsets of pantothenate kinase isoforms are found in each compartment. This primary regulator of CoA synthesis has been shown in vitro to phosphorylate any of three substrates [76,77]. Pantothenate, pantothenoylcysteine, and pantetheine can each serve as a substrate for pantothenate kinase, potentially providing distinct entry points for synthesis [78–80]. Though only pantothenate has been detected in cells, compartmental concentration differences of these substrates and of 4′-phosphopantetheine might also influence biochemical flux through the CoA pathway.

Also relevant to PKAN and posing further questions about compartmentalized processes is the observation in mammalian cells that transfer of the 4′-phosphopantetheinyl moiety occurs only in cytosol; no mitochondrial process has been found despite this being the target organelle for localization of two proteins activated by this post-translational modification [45,46]. Based on current knowledge, only CoA can serve as the substrate for the phosphopantetheinyl transferase enzyme; 4′-phosphopantetheine is unable to be transferred directly. How then might the mitochondrial pantothenate kinase primarily influence a cytosolic process but only when it is being used to activate proteins destined for mitochondria?

To further challenge our understanding of the role of PANK2 in CoA metabolism, a recent publication suggests the possibility of an entirely novel role for this protein: a role in phosphoinositide-3-kinase (PI3K) signaling [81]. Studies revealed that PANK2 as well as PANK4 are substrates for the PI3K effector kinase AKT. It remains unclear how phosphorylation by the protein kinase AKT influences PANK2 and thereby possibly CoA biosynthesis. However, it was demonstrated that PANK4, which lacks pantothenate kinase activity, is a rate-limiting suppressor of CoA synthesis via its phosphatase activity targeting 4′-phosphopantetheine [81]. PANK4 is a substrate for and itself regulated by phosphorylation via AKT. This potentially strong influence of PANK4 on CoA levels, possibly via regulation of 4′-phosphopantetheine levels, in combination with the discovery that PANK2 and PANK4 are both targeted by AKT, adds a new dimension of complexity to CoA metabolism that needs further exploration.

These questions and unexplained observations demand a better understanding of the full complexities of CoA synthesis and sequestration in order to advance our thinking about PKAN pathogenesis. Tools are needed to directly measure 4′-phosphopantetheinyl activation in vivo, to differentially tag the products in situ of each pantothenate kinase and also of the phosphatase PANK4 [81,82], and to determine at high-resolution the sub-cellular location(s) of each of these proteins in cells of interest. Such resources will provide substantial insight into the complex interplay of CoA metabolic proteins.

With so many unanswered questions about CoA and PKAN biology, we risk becoming too protective of and invested in our ideas, even when they are in conflict with the data. At the same time, people with PKAN are waiting for scientific advances that will lead to treatments to improve their lives. For this reason, clinicians and scientists are motivated to translate their work, but this aim must be balanced against a responsibility to retain a clear view of what is known versus what is believed. Uncertainty should not hold back therapeutics development, but it should be openly acknowledged.

The future prospects for advancing PKAN research and treatment design are extremely promising. The field is growing in numbers of investigators and in diversity of disciplines. More funding agencies, both public and private, are recognizing the value of investing in science to understand the highly tractable single gene disorders, which includes nearly all metabolic disorders. Knowing the precise primary defect causing a disorder is immensely powerful for targeting treatments. More and more examples reveal how that knowledge enables the design of precision therapeutics, with increasing numbers of compounds advancing to clinical trials. Several interventional trials in PKAN are underway or have been completed, with plans for additional trials in the next few years. The promise for changing the outcome in PKAN has never been brighter.

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Data availability

Data will be made available on request.

Declaration of Competing Interest

SJH and OCMS are Directors of Stichting Lepelaar, and OCMS is a Director of the Spoonbill Foundation, not-for-profit foundations dedicated to the goal to discover, develop, and deliver therapeutics for PKAN and all NBIA disorders in partnership with the NBIA community.

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