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The role of impaired de novo Coenzyme A biosynthesis in pantothenate kinase-associated neurodegeneration

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CHAPTER 6

Summarizing discussion

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SUMMARIZING DISCUSSION

Pantothenate kinase-associated neurodegeneration (PKAN, OMIM 234200) is an autosomal recessive, progressive neurodegenerative disorder. PKAN is caused by mutations in pantothenate kinase 2 (*PANK2*), the gene that encodes an enzyme (*PANK2*) which is a rate limiting enzyme in *de novo* Coenzyme A (CoA) biosynthesis. Interestingly, in humans there are four highly homologous *PANK* genes (*PANK1-4*) however; only mutations in *PANK2* but not in the other *PANK* genes, are associated with PKAN. Mutations in *PANK1*, 3, 4 have also never been reported to be associated with any other diseases or pathologies, suggesting that, these genes are essential for life, or their functions may be redundant. Since *PANK* is the first and the rate limiting enzyme in *de novo* CoA biosynthesis, the suggestion was postulated that impairment of *de novo* CoA biosynthesis might underlie the pathogenesis of PKAN [1]. Interestingly, among the four *PANK*s, only *PANK2* is targeted to the mitochondria and these organelles have the highest concentration of total cellular pool of CoA. Therefore, it was also proposed that a mitochondrial deficiency of CoA biosynthesis results in the PKAN disorder. In order to investigate these hypotheses and to understand how a deficiency in *PANK2* might give rise to the neuropathology of PKAN, a mouse *PANK2* knock-out model was generated [2]. Unfortunately, the mouse model was unable to recapitulate the neurodegenerative symptoms of PKAN. Therefore, currently, the PKAN pathology remains complex and far from understood and due to the lack of a proper PKAN model, the speculated role of impaired *de novo* CoA remained uninvestigated. In addition, the rarity of PKAN patients (1:3,000,000) complicates investigating PKAN pathology, because patient material is extremely sparse. *Drosophila* is a well established model organism to understand mechanisms in health and disease and also based on the above, it would be helpful for PKAN-related research to have a *Drosophila* PKAN model. Here, we explored the possibilities of *Drosophila* as a model for PKAN and we investigated the implicated role of impaired *de novo* CoA biosynthesis in the pathogenesis of this devastating disease. Specifically we were interested in determining; 1) whether the *PANK* deficiency in *Drosophila* indeed leads to a CoA deficiency. 2) Whether CoA deficiency in an organism (*Drosophila*) can lead to a neurodegenerative disorder. If successful, our aim was to use the power of the *Drosophila* model to screen various compounds, which can ameliorate the PKAN disease characteristics.

Impaired *de novo* CoA biosynthesis in *Drosophila* results in PKAN like disorder

De novo CoA biosynthesis occurs in a conserved metabolic route where VitB5 is converted into CoA by the action of five enzymes: *PANK*, *PPCS*, *PPCDC*, *PPAT* and *DPCK*. We demonstrated that *Drosophila* mutants for *dPANK/fbl*, *dPPCS*, *dPPAT-DPCK* (referred to as CoA mutants) can be used to study the consequences of impaired *de novo* CoA biosynthesis in higher eukaryotes (Chapter 2). The *Drosophila* CoA mutants are neurologically impaired, are highly sensitive to ROS or irradiation and have a reduced life span (Chapter 2). These findings strengthened the hypothesis that defective CoA biosynthesis induces a

neurodegenerative phenotype and might be one of the underlying causes of PKAN pathogenesis. We also showed that *dPANK/fbl* mutants have severe deficits in the total cellular pool of CoA (Chapter 3). Clearly, impaired function of dPANK results in a decreased CoA pool and thereby, possibly inducing a neurodegenerative phenotype and a reduced lifespan in *dPANK* mutants. In support of this, recently a mice study demonstrated that deprivation of Vit B5 (substrate of PANK and precursor for CoA biosynthesis) results in movement disorders possibly due to impairment of CoA biosynthesis [3]. Similarly, chemically blocking the PANK enzyme function by a competitive inhibitor of Vit B5 (HoPan) also results in decreased levels of CoA and a reduced life span of the treated mice, although neurodegeneration was not reported [4]. These combined findings clearly implicate the role of impaired *de novo* CoA biosynthesis in life span determination and neurodegeneration.

Our study also demonstrated that *dPANK* mutants have severe mitochondrial defects (Chapter 3). Contrary to human and mice, *Drosophila* has only one *PANK* gene (dPANK). However, this *dPANK/fbl* gene has five splice isoforms and interestingly one of them (dPANK-E) is predicted to be targeted to mitochondria [5]. Remarkably, only mitochondrial targeted dPANK-E is the most potent isoform in rescuing the phenotype of *dPANK/fbl* mutants [5]. This finding indirectly indicates that mitochondrial defects might be the major contributors to the PKAN-like characteristics in *Drosophila*. hPANK2 and hPPAT-DPCK are the enzymes that catalyze the first and last step of CoA biosynthesis respectively and both of these enzymes are mitochondrial localized [1, 6, 7]. Recently, hPANK2 is shown to be a sensor of palmitoylcarnitine and thereby proposed to regulate the mitochondrial *de novo* CoA biosynthesis. Similar to mutations in *dPANK/fbl*, it is possible that defects in *hPANK2* also lead to severe mitochondrial perturbations. This is consistent with the observation in mice that chemical inhibition of PANK by HoPan lead to a decrease in the hepatic CoA pool and severe mitochondrial pathology (swollen mitochondria [4]). Mitochondrial defects are shown to affect tissues with a high energy demand like the brain [8-10] and this might explain why a defect in PANK2 results in a neurodegeneration of specific areas of the brain. There are several patients with clinical symptoms of PKAN (idiopathic PKAN) that do not carry mutations in PANK2 gene. This implies that there are more causative genes (yet to be investigated) and *hPPAT-DPCK* might be one of them. Although, a group of 72 NBIA patients was screened for gene mutations in other enzymes of CoA biosynthesis however, none of the genes coding for the other CoA enzymes were affected [11]. This finding however, does not exclude the possibility that in the future *hPPAT-DPCK* mutations might be identified in these idiopathic NBIA patients. It is also possible that mutations in *hPPAT-DPCK* are embryonic lethal and thus can never be identified.

In summary, impaired function of the PANK enzyme either due to mutations in the *PANK* gene (Chapter 2 and 3.) or due to chemical inhibition of PANK [4] lead to a decrease in *de novo* CoA biosynthesis. Furthermore, impairment of *de novo* CoA biosynthesis results in PKAN like characteristics in *Drosophila* (this thesis).

Pantethine restores CoA levels and rescues *Drosophila* dPANK/fbl mutants

Pantethine can rescue CoA deficiency and thereby rescues the PKAN-like characteristics in *Drosophila* (Chapter 3). Although the finding that pantethine can be converted into CoA is not novel [12-14], our finding that pantethine can be physiologically converted into CoA in the absence of PANK is quite remarkable (Chapter 3). This finding elucidates a novel pathway of pantethine conversion from pantethine to CoA independent from pantothenate kinase. This is very relevant for PKAN patients, who suffer from impaired function of PANK2.

Pantethine is known since 1950s as a naturally occurring compound that constitutes part of the CoA molecule [13]. It was initially identified as *L. bulgaricus* growth factor (LBF) [15, 16] and later biochemically shown to be a substrate for *de novo* CoA biosynthesis ([13] and figure 1).

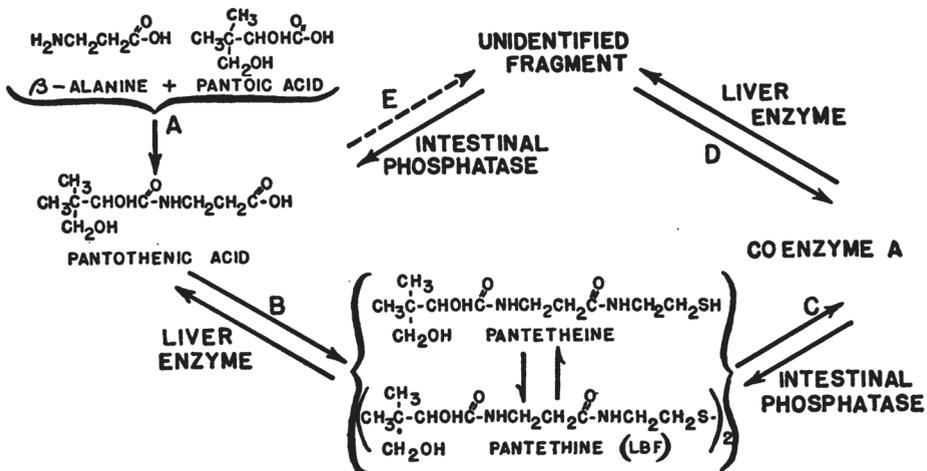


Figure 1: Figure adapted from J Bacteriology 1951 March; 61(3): 283-291.

One of the first few reports in which Pantethine was shown to be converted into pantetheine (monomeric form of pantethine). Later, Pantetheine was suggested to be converted into either CoA by the intestinal enzymes or Pantothenic acid by the liver enzymes.

In a biochemical conversion of pantethine into CoA, pantetheine must be phosphorylated into 4'-phosphopantetheine that is then suggested to be converted into CoA by the action of PPAT-DPCK [17, 18]. Using a crude cellular extract for an enzyme study, it was shown that a fraction from this crude mix has a potency to phosphorylate pantothenic acid and pantethine [19-21]. This enzyme fraction was termed as pantothenate kinase and since then it was assumed that pantothenate kinase is also the kinase, which phosphorylate pantetheine. Recently in a biochemical assay, pantetheine was shown to be readily phosphorylated by PANK3 [22]. Therefore, it might be possible that the earlier suggestions of

conversion of pantethine to 4'-phosphopantetheine by PANK is physiologically feasible. Although, presence of another kinase was never excluded that has a potency to phosphorylate pantetheine (a so called potential pantetheine kinase). In our experiments, pantetheine can also be converted into CoA however in absence, or at least in the presence of severely reduced levels of PANK. Our findings are in contrast to the earlier assumption that PANK is the only kinase capable of phosphorylating pantetheine and hints towards the presence of another potential, yet unidentified, pantetheine kinase.

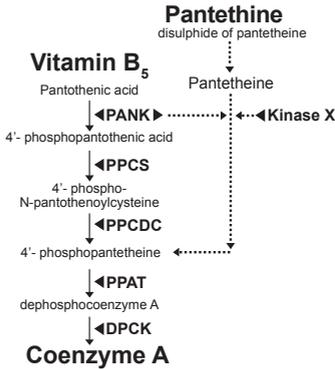


Figure 2: Possible pathways for *de novo* CoenzymeA biosynthesis.

In canonical de novo CoA biosynthesis pathway, vitamin B5 (pantothenic acid) is converted into CoA by the consecutive action of five enzymes: PANK, PPCS, PPCDC, PPAT and DPCK. Pantethine may be converted to pantetheine; pantetheine may be phosphorylated by a yet-unknown pantetheine kinase, other than pantothenate kinase (indicated by kinase X) Phosphorylated pantetheine (4'-phosphopantetheine) may enter the canonical de novo CoA biosynthesis pathway downstream of PPCDC and upstream of PPAT.

Based on the results of Chapter 3 and the above we hypothesize that at least in *Drosophila* there might be a so far unknown kinase which can catalyze the conversion of pantetheine into 4'-phosphopantetheine (Figure 2, kinase X). Identification of such a kinase is of prime importance because it strengthens the presence of a novel pathway of pantetheine conversion into CoA and it is of direct relevance to design strategies to treat PKAN.

We show that pantethine is a promising compound and can ameliorate the PKAN-like-characteristics in *Drosophila*. As a step further, studies must be undertaken to test its efficacy in mammalian models and then finally in PKAN patients. Pantethine is not a FDA approved drug and therefore it needs extensive further pharmacological tests. In our experiments, we have shown that pantethine at a high dose can be toxic (Chapter 3) and thus, toxicological studies for pantethine must be performed. We tested various doses in mice (Chapter 4) and demonstrated that around 4000 mg/body wt/day is well tolerated by mice and this study may serve as a base for future testing the potential of pantethine in mice. Pantethine has already been used in several human clinical trials as a drug for dyslipidaemia and patients can tolerate a dose of pantethine up to 15 mg/body wt/day. These observations surely suggest that testing pantethine in PKAN patients is possible within the near future. Because PKAN is a brain disease, it is highly relevant to investigate whether pantethine is able to cross the blood brain barrier (BBB). In a study aiming at treating cerebral malaria, thiol-pantethine appeared to be effective, suggesting that pantethine might cross the blood brain barrier (BBB) [23]. However, thorough investigations regarding pantethine's potency

to cross the BBB must be performed. In summary, pantethine may serve as a starting point to develop a possible treatment for PKAN however, there are still several key issues to be resolved.

Moonlighting role of PANK

Pantethine rescues CoA levels in the *Drosophila* model for PKAN (Chapter 3). Interestingly, restoration of CoA rescues all the neurodegeneration-associated phenotypes however, the male and female fertility (Chapter 5) were not rescued (unpublished observations). There can be several explanations for these observations. It is likely that pantethine, when supplemented through the food, is unable to reach the reproductive organs. Therefore, pantethine is unable to restore the local CoA deficits in the reproductive organs and fertility defects in male and females cannot be rescued. It is also possible that CoA levels needs to be tightly regulated in the reproductive organs and rescue by pantethine does not result in exact restoration of the endogenous intracellular levels of CoA. Intriguingly, another reason could be that dPANK/fbl might have an additional function apart from being a CoA biosynthesis enzyme. Recently, several proteins, including some metabolic enzymes, were reported to have more than one function in various cellular processes [24]. Such proteins are referred to as moonlight proteins and comprise an interesting subset of multifunctional proteins, in which two unrelated functions (moonlighting functions) are found in a single polypeptide chain [25]. It is an intriguing hypothesis that dPANK/fbl may be a moonlight protein that possesses an additional novel function apart from being a metabolic enzyme. In support of this hypothesis, in a yeast-two-hybrid experimental setup, dPANK/fbl was shown to physically interact with Twinstar (Tsr) [26]. Tsr is a human cofilin-like actin depolymerizing factor involved in the physiological turnover of actin and thereby influencing actin dynamics [27]. The physiological protein-protein interaction between dPANK/fbl and Tsr needs to be confirmed with additional essays and its biological relevance needs to be investigated as well. However, in case an interaction between dPANK/fbl and Tsr does exist, this points further to dPANK/fbl as a moonlighting protein. It is possible that in *dPANK/fbl* mutants, the interaction between dPANK/fbl and Tsr is disturbed and this explains the actin polymerization defects that we have reported in the *dPANK/fbl* mutants (Chapter 5). This is further supported by the fact that the actin defects in dPANK/fbl are not quite similar as the actin defects observed in the other CoA mutants and by the observation that pantethine treatment does not rescue the male and female fertility. Although, it will be a key experiment to measure CoA levels in the mutant testis and oocytes of the pantethine-treated mutants. This interesting hypothesis can be further investigated by determining the tsr-dPANK/fbl interaction sites, mutating these sites and thereby block their interaction and studying the induced phenotype. Further insight regarding possible moonlighting functions of dPANK might be obtained from a micro array study in which the transcriptome was compared between the following conditions. 1) Control Schneider S2 cells ; 2) Control Schneider S2 cells treated with pantethine; 3) dPANK/fbl depleted Schneider S2 cells; 4) *dPANK/fbl* depleted Schneider S2 cells treated with pantethine (Siudeja & Seinen

et. al manuscript in prep.). This analysis provides a unique opportunity to identify genes that do respond to the absence of dPANK/fbl protein in the background of normal CoA levels (compare 1 and 4). In other words, this analysis might unravel potential moonlight function of dPANK/fbl.

Interestingly, genetic mapping of the *PANK2* mutations in PKAN patients revealed that many (approximately 30%) disease-associated mutations in *PANK2* do not affect the mitochondrial localization of *PANK2* or the activity or stability of *PANK2* [28, 29]. This finding indicates the existence of a non-enzymatic function of *PANK2*. Alterations of this non-enzymatic function thereby can contribute to the pathogenesis of PKAN. Now with a *Drosophila* model for PKAN, we can address questions such as whether dPANK has a moonlighting function and whether disruption of this function can contribute to PKAN pathogenesis.

Concluding remarks and perspectives

Although patients suffering from a PKAN-like syndrome were already described in 1922, research in PKAN received momentum only in this decade after the discovery of the causative gene, *PANK2* [30]. PKAN research resulted in establishment of different models such as cell models, various mouse models and a *Drosophila* model [5, 31, 32]. These models are essential to understand the complex pathogenesis of PKAN and shed novel light on the cellular function of *PANK* protein and the consequences when it is impaired [2, 3, 5, 31-33]. Moreover, these models can also be used to screen for novel compounds, which might alleviate symptoms caused by the loss of *PANK*. Studies in mice and *Drosophila* suggest that a major part of the PKAN pathogenesis is due to reduced levels of CoA. Pantethine is a potential compound, which can rescue CoA levels and alleviates most of the disease related symptoms in the *Drosophila* model. Future studies must be directed towards investigating the potential of pantethine to treat PKAN. Identification of the so far elusive pantetheine kinase (Kinase X, Figure 2) that can generate 4'phosphopantetheine from pantetheine will further enhance our understanding regarding the non-canonical *de novo* CoA biosynthesis route. This might also be useful in designing more effective/potential treatment strategies regarding the usage of pantethine in PKAN treatment. Our study also surmises a possible moonlight function of *PANK*, which might unravel a completely new function of *PANK* and its role in PKAN pathogenesis.

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