CHAPTER 1

Introduction and aim of the thesis

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INTRODUCTION

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ABSTRACT

Coenzyme A (CoA) is an essential cofactor required for cellular metabolism. CoA is synthesized de novo from pantothenate (vitamin B5) by the action of five enzymes; pantothenate kinase (PANK), 4’-phosphopantothenoylcysteine synthetase (PPCS), (R)-4’-phospho-N-pantothenoylcysteine decarboxylase (PPCDC), 4’-phosphopantetheine adenylyltransferase (PPAT) and dephospho-CoA kinase (DPCK). These enzymes are well conserved among various species and the CoA biosynthesis pathway is biochemically well characterized. However, little is known about the physiological implications of an impaired CoA biosynthesis route in higher eukaryotes. More interestingly, in humans mutations in one gene coding for an enzyme of de novo CoA synthesis “PANK” have been genetically linked to the neurodegenerative disease “Pantothenate Kinase-Associated Neurodegeneration” or PKAN (OMIM 234200). The aim of this introductory chapter is to: 1) summarize knowledge about the role of CoA in cellular metabolism and highlight knowledge about CoA de novo biosynthesis, 2) summarize known physiological consequences of impaired CoA biosynthesis with a focus on the potential role of impaired CoA biosynthesis in PKAN pathogenesis, 3) discuss a potential niche for Drosophila as a model organism for PKAN-related research. The latter is of interest because an established animal model for PKAN is required to understand the physiological implication of impaired CoA biosynthesis; to understand PKAN etiology and pathogenesis and to screen for potential compounds which can ameliorate PKAN symptoms.
INTRODUCTION

1. Coenzyme A: a key metabolic cofactor

In 1945, Fritz Lipmann discovered a cofactor (a non-protein, enzyme helper molecule) that bound acetyl groups and this cofactor was named as Coenzyme A (CoA), in which “A” stood for “activation of acetate”. Later in 1953, Fritz Lipmann won the Nobel Prize for this remarkable discovery [1-3]. Over the past 60 years, biochemical studies reveal that CoA is a complex and highly polar molecule which is not only involved in acetyl carrier function but also is able to transfer various other acyl groups (like butyl, propyl etc.) in various metabolic reactions. As a metabolic cofactor, CoA is an indispensable compound in all living organisms ranging from prokaryotes to higher eukaryotes [4]. Over 5% of all reactions in intermediary metabolism, including the Krebs cycle and fatty acid metabolism, utilize CoA as an obligatory cofactor. In this introductory chapter, the following subjects will be highlighted: the key roles of CoA in cellular metabolism; the cellular and tissue distribution of CoA; the various mechanisms to maintain the dynamic levels of CoA intracellularly and the consequences of impaired CoA metabolism. Finally, the recently discovered link between impaired CoA metabolism and neurodegeneration will be discussed. This thesis work was inspired by the discovery of this unexpected association.

Various key roles of CoA in cellular metabolism: As a major acyl carrier, different acyl moieties can be attached to the terminal sulphahydryl group of CoA (denoted by X in Figure 1), and these compounds (thioesters) participate in the major anabolic and catabolic pathways critical to cell growth and function [5, 6]. Based on the chain length, these thioesters are classified into short-chain (2-3 carbons), medium-chain (4-12 carbons), long-chain (12-22 carbons) and very-long-chain (18-26 or more carbons) thioesters. Short-chain CoA thioesters, such as acetyl-CoA or succinyl-CoA, are the most abundant components.
of the CoA pool and are important intermediates in various metabolic reactions such as the Krebs cycle, synthesis of cholesterol & other steroids, synthesis of ketone bodies and lipogenesis [7]. Medium-chain thioesters are active components formed during the β-oxidation of medium chain fatty acids in mitochondria [8, 9]. Long chain CoA is an essential cofactor in long-chain fatty acid metabolism [10]. CoA along with mitochondrial carnitine-transferases transport all the intermediates of fatty acid-oxidation to and from the peroxisomes and mitochondria [11]. Apart from their role in fatty acid metabolism (lipid synthesis and β-oxidation) and energy production (Krebs cycle), CoA esters also regulate the activity of various enzymes such as for the enzyme “acetyl CoA carboxylase” [12, 13]. Long chain CoA thioesters also bind to certain hormone receptors and have signaling properties. CoA acyl-thioesters are also the substrates for the acyltransferases and desaturases involved in membrane phospholipid formation, triglyceride synthesis, and protein acylation [14-17]. Thus, all CoA thioester have high biological activity in various cellular reactions like energy metabolism [18] and regulating gene functions [19].

**Cellular and tissue distribution of CoA:** Since CoA thioesters have a high biological activity, eukaryotic cells contain a sequestered pool of CoA in various cellular compartments. The major pool of CoA in eukaryotic cells is located inside the mitochondria, peroxisomes and cytosol [20]. In animal tissues, the cytosolic concentration of CoA is low (0.02-0.14mM) and in these tissues CoA is used for lipid synthesis, protein modifications and membrane trafficking [20]. Mitochondrial concentration of CoA is significantly higher (2.2-5mM) and used for the Krebs cycle and the β-oxidation of fatty acids [20]. As compared to cytosolic CoA, peroxisomes also have a relatively high concentration of CoA (0.5-0.7mM) and in these organelles CoA is utilized for β-oxidation of long chain fatty acids [20, 21]. In animals, at the level of whole tissues the concentration of CoA differs over a wide range and depends on the metabolic status of the tissue [22]. Hepatic CoA levels are the highest and range between 136-434 nmol/g, whereas the heart contains approximately 100nmol/g tissue [22]. All other tissues are speculated to contain lower level of CoA than liver and heart; however, this suggestion requires further experimental verification. The tissue levels of CoA are also very dynamic and fluctuate with the metabolic state of the animal such as starvation or feeding conditions, diet, disease or treatment with several drugs [23, 24].

**Maintenance of the total cellular pool of CoA:** As the intracellular CoA homeostasis is very dynamic [24] it requires to be actively controlled through a balance between the de novo biosynthesis, degradation and recycling of CoA from various intracellular pools. The total cellular CoA pool consists of free CoA, acyl-CoA and CoA bound to proteins and to other -SH groups. It is speculated in several articles [4, 24, 25] that this intracellular pool of CoA is constantly recycling between the free and bound forms of CoA; however, this hypothesis still requires experimental validation. In bacteria, C.elegans, yeast, A.thaliana and mouse, a new class of enzymes called the “nudix family hydrolases” have been identified which can degrade CoA or acyl thioesters of CoA into 4’-phosphopantetheine, thus these enzymes are able to decrease the total CoA pool [4, 26-29]. In humans, CoA degradation is
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not well characterized, however, based on comparative genomics there are some orthologous human genes that may code for nudix family hydrolases (like human NUDT7, human NUDT8) [29]. Similarly, in *Drosophila* based on comparative genomics, the CG11095 gene is proposed to have a Nudix hydrolase domain (Flybase.org & [29]). Further characterization of these enzymes is required to elucidate a possible cellular CoA degradation pathway. Finally, *de novo* biosynthesis of CoA from pantothenic acid also contributes to maintenance of the CoA pool [4] Exact cues which can trigger the *de novo* synthesis of CoA are still unknown, however, it is speculated that this CoA *de novo* synthesis is essential in tissue in which a rapid turnover of CoA is required (e.g. liver, muscles, brain) to meet the high metabolic demand for proper functioning of these tissues. Also during development of the organism, there might be a high turnover of CoA required for the synthesis of new cellular components like cell membranes [30–32]. The next paragraphs summarize the current knowledge concerning *de novo* CoA biosynthesis.

2. *De novo* CoA biosynthesis

In most higher eukaryotes, including mammals, *de novo* CoA biosynthesis occurs mostly in the cytoplasm starting with the dietary precursor pantothenate (or pantothenic acid or Vitamin B5) (Figure 2A). In contrast, many bacteria, plant and yeast species are capable of synthesizing pantothenate *de novo* from β-alanine. Since pantothenate is so abundant in nearly all diets and it is also synthesized by the microflora of the gut [23], its deficiency is rarely reported in mammals. After being absorbed from the intestines, pantothenate like other water soluble vitamins is carried into various tissues by a mammalian pantothenate transporter which belongs to a family of the sodium coupled glucose transporters (Na+-dependent multivitamin transporter-SMVT) [33]. Once pantothenate reaches the cytoplasm, it is step wise converted into CoA by the subsequent action of five enzymes. The biosynthetic conversion pathway of pantothenate into CoA is discussed below.

Enzymes involved in *de novo* CoA biosynthesis: Intracellularly, pantothenic acid is enzymatically converted into CoA by the subsequent action of five enzymes: pantothenate kinase (PANK), 4’-phosphopantothenoylcysteine synthetase (*PPCS*), (R)-4’-phospho-N-pantothenoylcysteine decarboxylase (*PPCDC*), 4’-phosphopantetheine adenylyltransferase (*PPAT*) and dephospho-CoA kinase (*DPCK*) (Figure 2B). In the past decade, by comparative genomics most of the genes encoding for these five enzymes were identified and shown to be conserved in bacteria, plants and mammals with only a few exceptions (Figure 2C and reviewed in [4]). In bacteria, a single gene "*CoaA*" encodes for the prokaryotic PANK whereas there are four pantothenate kinase genes (*PANK1-4*) in humans and mice [4, 34, 35]. These four mammalian isoforms were shown to have different cellular localizations. *PANK1* transcripts are highly localized in liver, *PANK2* transcripts are more abundant in neuronal tissue whereas *PANK3* transcripts are highly expressed mostly in almost all tissues [36, 37]. The presence of multiple isoforms of PANK encoded by four genes in humans and
Figure 2: CoA de novo biosynthesis

A) Universal pathway representing the de novo CoA biosynthesis. Higher eukaryotes depend on dietary intake of pantothenic acid whereas prokaryotes and plants can synthesize pantothenic acid de novo. Pantothenic acid is converted into CoA by the subsequent action of 5 enzymes (PANK, PPCS, PPCDC, PPAT, and DPCK). Free CoA is converted into acyl CoA and then utilized in various cellular metabolic reactions including energy metabolism (TCA cycle), fatty acid biosynthesis, fatty acid catabolism (β-oxidation of fatty acid) and synthesis of various amino acids. B) Pantothenate is phosphorylated to 4'-phosphopantothenic acid by pantothenate kinase (PANK). Next a cysteine is added by 4'-phosphopantothenoylcysteine synthetase (PPCS) to form (R)-4'-phospho-N-pantothenoylcysteine which is decarboxylated by (R)-4'-phospho-N-pantothenoylcysteine decarboxylase (PPCDC) and produces 4'-phosphopantetheine. This 4'-phosphopantetheine receives an adenylyl group transferred from ATP mediated by 4'-phosphopantetheine adenylyltransferase (PPAT) and releases dephospho-CoA, which is then phosphorylated by dephospho-CoA kinase (DPCK) to finally the yield CoA. E.C. numbers of the enzymes are indicated in the boxes. C) The name of various genes encoding CoA synthesis enzymes and their differences
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in various species are shown in the table. ? indicates that at the time of start of the thesis the Drosophila genes coding for the CoA biosynthesis enzymes were unknown except for dPANK/fbl.

mice and their different cellular localizations clearly suggests a complexity associated with differential regulation of expression and differential activities of the PANK isoforms. Structurally, all 4 mammalian PANK isoforms have a common catalytic core with different amino-terminal (N-terminal) extensions [38, 39]. Interestingly, mammalian PANK4 also has a long carboxyl-terminal (C-terminal) extension of unknown function [40-42]. The specific role of each mammalian PANK isoforms (PANK1-4) remains to be elucidated; however, many recent studies have started to investigate the individual roles of the isoforms in cellular metabolism. Mammalian PANK1 mRNA expression and protein activity have been shown to regulate the intracellular CoA concentration upon benzofibrate administration via peroxisome proliferator-activated receptor regulation [43]. Using a Pank1(-/-) mouse it was shown that PanK1 plays an important role in maintaining the normal intracellular levels of CoA and PANK1 is required for the normal response to the increased demand for CoA during fasting [44]. During fasting, intermediary metabolism switches towards gluconeogenesis and β-oxidation of fatty acids [45, 46]. In order to achieve this response, elevated levels of CoA are required and PANK1 was shown to be crucial for increasing the intracellular levels of hepatic CoA [44]. Mouse PANK2 (-/-) was generated in an efforts towards understanding the pathology of PKAN however, this mouse model did not showed neurodegeneration except retinal degeneration and azoospermia [47]. Very little is known about the specific function of the PANK3 and PANK4 isoforms, although their cDNAs were identified and their specific RNA expression pattern was studied. Recently, it was suggested that PANK4 could modulate glucose metabolism via its interactions with pyruvate kinase muscle 2 (a regulator of glucose metabolism) [40-42]. Unlike human and mice, in Drosophila there is only one gene (CG5725, dPANK/fbl) which encodes 5 splice variants of PANK. These five splice variants differ in their 5’ exons (Figure 3). The longest isoform has the longest ORF and is referred as dPANK/fbl E and the shortest isoform is referred to as dPANK/fbl D.

In bacteria, the second and third enzyme, PPCS and PPCDC, exists as single bifunctional protein, encoded by one gene, however in human and mice these genes are expressed separately. Conversely, the final two enzymes, PPAT and DPCK, are expressed separately in bacteria but form a bi-functional gene/protein in human and mice [48]. Also the structural
analysis revealed significant differences between the eukaryotic and prokaryotic enzymes [49-52]. Bacterial PANK and PPAT differ significantly from the eukaryotic counterparts based on sequence similarity. This finding along with availability of the prokaryotic PANK and PPAT crystal structures make them an attractive target for the design of selective inhibitors of bacterial PANK and PPAT which could be used as antimicrobial targets [49, 53-55].

**Intracellular localization of de novo CoA synthesis enzymes:** Recent work has shed some light on the localization of CoA enzymes in higher eukaryotes and has shown that PANK2 in humans is localized in mitochondria, whereas, the other PANKs (1, 3 and 4) are cytoplasmic [4]. *Drosophila* PANK is encoded by a single gene but it has 5 splice variants. The longest one is *dPANK/fbl E* and is predicted to be targeted to mitochondria by the online programme MitoProt II (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html), whereas the other isoforms possess no predicted mitochondria targeting sequence in the 5'region. Recently this prediction was experimentally proven because it was shown that *fbl L* (synonym of *dPANK/fbl E*) is localized in mitochondria whereas the shorter isoforms (synonym for *dPANK/fbl A-D*) are not mitochondrial localized [56]. The mammalian PPAT-DPCK is also localized in mitochondria, either within the matrix or on the cytoplasmic membrane side [57, 58]. Interestingly both (PANK2 and PPAT-DPCK) which are mitochondrially localized are also proposed to be the rate limiting enzymes for the CoA biosynthesis pathway. The remaining two enzymes PPCS and PPCDC are proposed to be largely cytoplasmic based on comparative 3D modeling [59, 60] however, these predictions still need experimental validation.

**Regulation of the de novo CoA biosynthesis:** CoA is required for proper maintenance of the cellular metabolic status. So far, only one pathway to synthesize *de novo* CoA has been identified and this synthesis pathway is tightly controlled. In response to the metabolic status, *de novo* CoA biosynthesis is regulated by a feedback mechanism either at the level of PANK or at the level of PPAT enzymes by the concentration of free CoA or acyl thioesters of CoA [36, 38, 39, 58, 61-63]. PANK catalyzes the first step of the *de novo* CoA biosynthesis pathway and comprises the primary rate limiting step [36]. Acyl thioesters of CoA regulate PANK activity by a negative feedback regulation. It is currently not clear whether the four different PANKs in humans & mice play a similar role in the feedback regulation. The human PANK2 is a mitochondrial localized protein and is very sensitive to acyl CoA levels (especially long chain CoA, acetyl CoA and malonyl CoA, Figure 2A) whereas PANK2 is weakly sensitive to free CoA [61, 64]. It is speculated that PANK2 might be a master regulator of *de novo* CoA biosynthesis in humans. The feedback regulation of cytoplasmic human PANK (1, 3 and 4) is also highly likely to be regulated by the changing levels of CoA. The intercommunication between the cytoplasmic human PANKs (PANK1, 3 and 4) and mitochondrial human PANK (PANK2) is currently unknown. Palmitoylcarnitine, which is required for the mitochondrial transport of CoA, was also shown to be a potent activator of PANK2 [61]. In contrast to hPANK2, mouse PANK1 is activated by free CoA and weakly inhibited by acetyl-CoA instead of mouse PANK2 [36, 38, 39] indicating the existence of
a different manner of feedback regulation in mice. Another proposed rate limiting step in *de novo* CoA biosynthesis is at the level of PPAT-DPCK enzymes [36, 58, 62, 63, 65, 66]. This was demonstrated by the following observations. In an attempt to boost cellular CoA levels in bacteria, a mutant form of *PANK* (which is not sensitive to inhibition by CoA) was over expressed [66]. Interestingly, the levels of intracellular 4’-phosphopantethine were more elevated than CoA [66]. Thus it was indirectly concluded that PPAT-DPCK is, in addition to PANK, involved in a rate limiting step (Figure 2B). In mice after pantothenate, the second most abundant cellular metabolite of the CoA pathway is 4’-phosphopantethine which suggests that also in mammals both PPAT and PANK constitute the rate limiting step; because overexpression of mPANK1β results in reduction of the cellular pool of pan-tothenate and an increase in the CoA pool and more interestingly, 4’-phosphopantetheine was increased by 3-fold [36]. Thus comparable to bacteria, it is proposed that PPAT might be a second control point in CoA homeostasis in mice [36].

As discussed, so far the *de novo* CoA biosynthesis is crucial for maintaining the cellular CoA homeostasis and therefore it is reasonable to assume that there are various adverse physiological implications for an organism under circumstances of impaired *de novo* CoA biosynthesis. In the next section, implications of impaired CoA biosynthesis in various species will be discussed.

### 3. Impaired *de novo* CoA biosynthesis

Many human pathological conditions such as alcoholism, starvation, diabetes and certain tumors show alteration in CoA metabolism [23, 25]. Tissue levels of CoA generally change in response to chronic metabolic diseases most likely as an effort to utilize alternate fuel sources. However, it is very difficult to conclude whether the characteristics of these metabolic syndromes are due to CoA deficiency (a direct consequence of *de novo* CoA biosynthesis impairment) or due to accumulation and redistribution of toxic metabolites of the CoA pathway [25].

Another way to investigate the consequences of impaired CoA *de novo* biosynthesis is via a direct targeting of the biosynthesis enzymes. Moreover, in the current post genomic era, upcoming studies have started to investigate the specific role of these enzymes by studying specific phenotypes of CoA biosynthesis mutants. Consequently, mutational analysis of various CoA biosynthesis enzymes in bacteria and plants has broadened our knowledge about CoA metabolism. Bacterial *PANK* (E.coli) mutations causes a temperature-sensitive growth phenotype [67]. Recently, in *Arabidopsis thaliana*, the role of PANK [68], PPCDC [69] and PPAT [70] mutants has been thoroughly investigated. Null mutations in these enzymes led to embryonic lethality indicating that these enzymes are essential for life. Hypomorphic mutations in these enzymes led to a decrease in the total CoA pool and severe impairment in plant growth and seed production [68-70]. Similarly null mutations in the *Drosophila PANK* or *dPANK/fbl*) are lethal whereas hypomorphic mutants are viable, but
do show cytokinesis defects and a developmental delay [32]. Mutations in the *C. elegans* PANK (*pnk-1*) lead to nematodal larval developmental, growth and reproduction defects (www.wormbase.org). Major findings in the CoA metabolism research area occurred when a human neurodegenerative hereditary disease (PKAN) was genetically linked to the *PANK*2 gene. This discovery highlights an unexpected role of a well known metabolic enzyme in maintaining neuronal function. In the next section, the current knowledge concerning the PKAN disorder is summarized and possible explanations how *PANK*2 mutations might result in PKAN are being discussed.

4. Mutations in Human *PANK*2 are associated with neurodegeneration: an interesting link between a *de novo* CoA biosynthesis enzyme and neurodegeneration

The recently discovered genetic link between mutations in the human *PANK*2 (first rate limiting enzyme in CoA biosynthesis) gene and neurodegeneration has renewed the interest in investigating *de novo* CoA biosynthesis pathway. Here, we briefly introduce Pantothenate-Kinase Associated Neurodegeneration (PKAN), its genetic cause, its pathogenesis (which currently remains far from understood) and its current treatment alternatives.

**Pantothenate Kinase-Associated Neurodegeneration (PKAN):** PKAN is a neurodegenerative disorder with brain iron accumulation in specific areas of the brain [71]. It is also known as neurodegeneration with brain iron accumulation type 1 (NBIA1), Neuroaxonal dystrophy-juvenile-onset and formerly referred to as Hallervorden-Spatz syndrome. NBIA generally consists of rare, inherited, neurological movement disorders which are characterized by progressive degeneration of the nervous system and iron accumulation in the brain. Heterogeneous NBIA disorders are classified into two main groups, the first one consists of diseases associated with mutations in known genes; ferritin [72], ceruloplasmin [73], PLA2G6 [74] or PANK2 [37], while the other represents all cases of idiopathic origin [71, 75] (Figure 4). Among NBIA disorders, a *PANK*2 mutation is the most common cause of NBIA and accounts for approximately 50% of all the known cases. The precise incidence of PKAN is currently unknown however it is estimated to affect 1 to 3 per million people worldwide [75].

<table>
<thead>
<tr>
<th>Disease with known gene mutations</th>
<th>Idiopathic origin</th>
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<tbody>
<tr>
<td>PKAN (PANK2 mutation)</td>
<td>Causative reason unknown</td>
</tr>
<tr>
<td>INAD (PLA2G6 mutation)</td>
<td></td>
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<tr>
<td>Atypical NAD (PLA2G6 mutation)</td>
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<td>Neuroferritinopathy (FTL mutation)</td>
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<td>Aceruloplasminaemia (CP mutation)</td>
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Figure 4: Two main categories of NBIA disorder

Based on the causative genes the NBIA disorder is classified into two main categories, one for which the causative genes are known and other with unknown idiopathic origin.
Clinically, PKAN is characterized by progressive dystonia (repetitive uncontrollable muscle contractions), tremors and general weakness [76-80]. Based on the onset of the symptoms PKAN is classified into two main categories, classical and atypical PKAN. Classical PKAN symptoms are progressive and typically begin during childhood (before the age of ten years), often resulting in an early death. The symptoms include dystonia, dysphagia, tremor, dementia and retinitis pigmentosa. Another quarter of individuals experience atypical PKAN, an uncharacteristic form of PKAN that develops in children older than 10 years and follows a slower, more gradual pace of deterioration compared to the early onset form of PKAN. Typical and atypical PKAN individuals have intellectual defects, significant speech deficits accompanied by behavioral and psychiatric disturbances. Iron deposition in the basal ganglia and the globus pallidus give a characteristic MRI signal known as the “eye-of-the tiger sign” in both forms of PKAN [81].

Genetic cause of PKAN: PKAN is an autosomal recessive disorder caused by mutations in the PANK2 gene located at the chromosomal locus: 20p13-p12.3 (http://ghr.nlm.nih.gov/gene/PANK2). PANK2 encodes the protein Pantothenate kinase 2 which is a rate limiting enzyme in de novo CoA biosynthesis pathway (Figure 2). PANK2 is an essential regulatory enzyme in coenzyme A (CoA) biosynthesis, and is catalyzing the phosphorylation of pantothenate (vitamin B5). About hundred mutations in the PANK2 gene have been identified in people with pantothenate kinase-associated neurodegeneration (ref PANK2 International Mutation Database, [75]). The most frequent mutations are 1231G>A and 1253C>T and account for about one third of the disease alleles [75]. Although there are limited genotype-phenotype correlation studies, some general conclusion can be inferred, like individuals homozygous for null alleles usually have the classic form of PKAN [50, 78]. It is predicted by bioinformatics studies that patients with the typical, early-onset form of PKAN have PANK2 mutations causing severe disruption of the PANK protein structure inducing instability of the enzyme [78]. Patients affected by the atypical, later-onset form usually have mutations that only change single amino acids in the PANK enzyme and this enzymes may posses a residual function [50].

Treatment of PKAN: Currently there are no major breakthroughs in the treatment of PKAN, with most treatments being symptomatic and aimed towards easing or temporary relieving of PKAN symptoms. Supplementation of certain vitamins like pantothenate, Coenzyme Q10 and antioxidants have been speculated to have beneficial affect, however, proper clinical validation is lacking [82]. Future work is focused on the development of iron chelating agents that may reach the central nervous system by crossing the blood brain barrier (BBB). There is one ongoing clinical trial (In Italy by Dr. N Nardocci) with iron chelating agents (deferripirone which can cross the BBB), and results are awaited. Atypical PKAN patients might have partial PANK enzyme function and thus providing a high dose of pantothenic acid (substrate of enzyme) is reasoned to be beneficial, however this hypothesis requires experimental validation. Deep brain stimulation (DBS) is also an option for relieving some of the symptoms (such as dystonia) of PKAN patients. DBS is a surgical
treatment in which a medical device called a brain pacemaker is implanted in the brain, which sends electrical impulses to specific parts of the brain [83-85]. In an individual case study, DBS has been shown as a mode of symptomatic treatment of early onset PKAN [86]. Unfortunately, all the above treatment options are symptomatic and until now there is no therapy to delay or cure the PKAN neurodegeneration. In order to develop a possible treatment it is highly essential to gain more insight into the pathogenesis of the disease.

**PKAN pathogenesis:** Although the genetic cause of PKAN is known, it is currently unclear how mutations in the *PANK2* gene lead to the PKAN disorder. Various hypotheses have been postulated regarding the pathogenesis and progression of the disease. *PANK2* gene mutations lead to abnormal PANK2 enzyme function that could impair the *de novo* CoA biosynthesis. As CoA is involved in basic cellular functions like energy metabolism, fatty acid synthesis and phospholipid remodeling, impairment of CoA biosynthesis might lead to PKAN disease. However, as *PANK2* is ubiquitously expressed [64] the tissue specific clinical features of PKAN are rather unexpected. In *de novo* CoA biosynthesis pathway, cysteine is incorporated as a metabolite (see Figure 2B) and it is speculated that PANK2 dysfunction could lead to accumulation of this metabolite resulting in cell toxicity and high oxidative stress. Therefore, defective CoA metabolism and increased oxidative stress can be two main explanations for PKAN pathogenesis and will be discussed further below.

**Figure 5. Major hypotheses of PKAN pathogenesis.**
A) There are two main hypotheses which might be interrelated at the level of mitochondrial dysfunction. There can be high ROS generation due to cysteine or iron accumulation leading to mitochondrial hypofunction. This in turn leads to increased sensitivity to ROS and more oxidative stress. Alternatively PANK2 mutations lead to impaired CoA biosynthesis in the mitochondrial compartment thus leading to abnormal mitochondrial metabolism. B) Cysteine can drive the Fenton reaction leading to generation of free radicals and an increase in oxidative stress.

**High oxidative damage and PKAN pathogenesis:** An attractive model is proposed in which high oxidative damage could play an important role in PKAN pathogenesis [87]. High oxidative stress is associated with the development of a wide range of diseases including Alzheimer's disease [88, 89], Parkinson's disease [90], and neurodegeneration in motor neuron diseases [91]. However in many of these cases, it is unclear if high levels of oxidants
trigger the disease, or if they are produced as a secondary consequence of the disease [92, 93]. Similarly, high oxidative damage in PKAN patients could also play an important role in PKAN pathogenesis. High oxidative stress in PKAN could be either due to an increased generation of reactive oxygen species (ROS) or due to a decreased resistance to ROS or both.

There are various possibilities of high ROS generation in PKAN patients. Firstly, since cysteine incorporation in the de novo CoA pathway occurs downstream of PANK, impaired PANK could lead to accumulation of cysteine intracellularly and thereby drive the Fenton reaction (Figure 5B). The Fenton reaction generates free radicals leading to high oxidative damage. This hypothesis requires experimental validation and until now there is only one study showing high levels of cysteine in one patient suffering from Neurodegeneration with Brain Iron Accumulation, however it is unclear whether or not this patient carries a mutation in the PANK2 gene [94]. Secondly, a hallmark of PKAN is iron accumulation in the brain and iron is recognized as a potent pro-oxidant and a catalyst for the generation of ROS in biological systems [95]. The exact reason of iron accumulation in specific areas of the brain of PKAN patients is currently unknown. As cysteine-containing compounds have been implicated in iron chelation [95, 96] and PKAN patients might have high cysteine levels (as discussed above) this could result in high iron accumulation in PKAN patients. Another cause of iron accumulation could be mitochondrial dysfunction in PKAN patients. The release of iron from iron-binding proteins results in the formation of ROS possibly by driving the Fenton's reaction [97]. As mitochondria are the main consumers of iron where it is utilized for heme synthesis and for Fe-S cluster biogenesis [98-102], it is highly likely that impaired PANK2 functioning might hamper mitochondrial function and in turn this could impair Fe import into mitochondria and/or activity of enzymes involved in Fe-S cluster biogenesis. Impaired import of Fe could subsequently lead to increase Fe accumulation. In a recent study, hPANK2 silencing caused a strong induction of ferroportin mRNA (although only in a non neuronal cell line, hepatoma HepG2) thereby resulting in iron dysregulation [103]. Ultimately, Pank2-mediated alteration of ferroportin expression might alter iron transfer to the brain and this may lead to iron accumulation in PKAN patients and generation of high ROS. Future experiments should be directed to investigate which of the proposed mechanisms is at the basis of iron accumulation and whether this is linked to a high generation of ROS.

In addition to an increased ROS production, high oxidative stress could also be explained by decreased resistance and/or an increased sensitivity to ROS. Mitochondrial dysfunction is frequently associated with decreased resistance to ROS [104-106] and it is possible that PKAN patients do suffer from mitochondrial dysfunction (because human PANK2 is a mitochondrial enzyme). It is also highly likely that both the mechanisms, high ROS generation and low resistance to ROS, contribute to PKAN pathogenesis.

**Impaired de novo CoA biosynthesis and PKAN pathogenesis:** Another proposed hypoth-
esis is that PANK2 mutations are likely to result in impaired \textit{de novo} CoA biosynthesis. Among the total cellular pool of CoA, only a small proportion of CoA is synthesized \textit{de novo} thus it remains to be investigated how much these small changes in the total CoA pool contribute to PKAN symptoms. It is likely that although the change in total CoA is rather low, CoA levels in the crucial mitochondrial compartment are severely affected in PKAN patients, which may lead to $\beta$-oxidation defects and subsequently to the disease pathology. In support of this hypothesis, tissues with a higher energy demand will suffer more and this may explain why brain and muscle degeneration are prominent features in PKAN patients [75]. CoA plays an important role in several important pathways, including phospholipid biosynthesis and, therefore, membranogenesis. The \textit{Drosophila} pantothenate kinase mutant (dPANK/fumble) shows abnormal cell divisions which might be due to defective synthesis and redistribution of membranous structures, possibly due to CoA deficiency [32]. Similarly, \textit{hPANK2} mutations might affect tissues with high membrane synthetic demands such as rod cells in the retina and erythrocytes. Retina rod cells continually generate membranous discs, which may exceed the capacity for membranogenesis in PKAN. Erythrocytes frequently undergo trauma, and defective membrane repair has been implicated in acanthocyte formation [107, 108]. This observation in part could explain the changes in blood cell morphology (acanthocytosis) and the lipid abnormalities (hypoprebetalipoproteinemia), both are often found in PKAN patients [109]. To summarize, there are various speculations and many uncertainties regarding the role of \textit{PANK2} and CoA biosynthesis in PKAN disease pathogenesis.

\textbf{Model systems to investigate PKAN pathogenesis}: The discovery of the causative gene for PKAN, enabled the possibility to generate animal models for this disease. Model organisms are extremely useful to understand the molecular mechanisms underlying diseases and this in turn is required for the development of therapies. Especially for rare diseases, when patient material is scarce, animal models are of high value.

To investigate how PANK2 deficiency induces the symptoms of PKAN, a murine \textit{PANK2} knock-out model was generated [47]. Although \textit{PANK2} mutant mice suffer from azoospermia and retinal degeneration, \textit{PANK2} knock-out mice failed to develop typical neurological impairment and locomotor abnormalities associated with PKAN in humans [47]. Similar to humans, the mouse genome contains 4 PANKs, therefore it might be possible that a different isoform of PANK (and not PANK2) is required for normal neuronal functioning or that PANK1, 3 or 4 and not PANK2 are mitochondrial localized in mice. It is also possible that one of the other mouse PANK isoforms compensates for the loss of mouse PANK2. All together, these results demonstrate that \textit{PANK2} knockout mice are not a suitable genetic model to understand the pathophysiology of PKAN. Similar to \textit{PANK2 (-/-)} mice, \textit{PANK1 (-/-)} mice also did not show any signs of neurodegeneration [44, 47]. In another study mice deprived from pantothenic acid developed neurological symptoms [110] suggesting that maintenance of normal levels of CoA are required for proper neuronal functioning in mice. Also studies have been done in cultured cells to understand why specific mutations cause
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A more severe phenotype compared to others. Hereto mutant proteins were expressed in cells and pantothenate kinase activity of various mutated proteins was measured. However, this approach has led to confusing results since some of the mutant proteins (associated with a fast progressing form of PKAN in patients) are having either normal or even higher PANK activity than the normal wild-type PANK2 [111, 112]. It is speculated that either the presence of endogenous PANKs might interfere with these experiments resulting in inconclusive results or the impaired catalytic activity of PANK is not the sole underlying cause of PKAN and studies using cultured cells so far do not show clear answers how impaired function of PANK2 induces PKAN characteristics.

In summary, the pathophysiology of PKAN remains unclear and demands further investigations. As an alternative for studies in mice, Drosophila can also be used to understand mechanisms of health and disease [113]. In the final paragraph of this introductory chapter the possibilities of using Drosophila as model organism for PKAN related research will be discussed.

Drosophila as a model for investigating implications of impaired de novo CoA biosynthesis: Drosophila melanogaster (fruit fly) is one of the most studied organisms in biological research, particularly in genetics and developmental biology. Moreover, recently Drosophila is shown to be useful for the genetic characterization of various metabolic pathways [114-118] and more than 50% of all fly proteins have mammalian analogues [119, 120]. Together this makes the fruit fly a powerful candidate to investigate the implications of impaired de novo CoA biosynthesis pathway.

In 2001, just before PANK2 was identified as the causative gene for PKAN, a Drosophila mutant was characterized carrying a mutation in the Drosophila PANK gene: “dPANK/fumble” [32]. This fumble mutant was identified by others in a screen for male fertility and the male sterile phenotype was described in detail [121]. The neurodegenerative phenotype of this mutant remained largely unexplored. In the laboratory of prof. Sibon where this PhD thesis was performed a genetic screen was conducted to identify neurodegenerative mutants. One of the identified mutants carried a mutation in the gene coding for the second enzyme (PPCS; see Figure 2B) of the CoA biosynthetic pathway. The neurodegenerative phenotype of this identified CoA mutant and the availability of one other CoA mutant, the dPANK/fbl mutant, opened ways to investigate how defects in CoA biosynthesis are linked to neurodegeneration in a multicellular organism. These preliminary results also suggested that the Drosophila CoA mutants, especially the dPANK/fbl mutants may serve as a model for PKAN-related research.

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The aim of this research project was to investigate the physiological implication of impaired CoA biosynthesis using Drosophila as a model organism and to investigate whether the
**Drosophila** CoA mutants are a suitable model for PKAN-related research (Chapter 1). In case this could be established, the *Drosophila* model for PKAN enables research directed to understand the pathogenesis, which is currently largely unknown. In addition an established *Drosophila* PKAN model allows to screen for potential compounds, which can ameliorate the PKAN symptoms. When protective compounds can be identified, these can serve as starting points for a possible therapy to treat PKAN.

**Chapter 2: De novo CoA biosynthesis is required to maintain DNA integrity during development of the *Drosophila* nervous system**

In this chapter CoA mutants were either identified (dPPCS) during a forward genetic screen or obtained (dPANK/fbl) or specifically generated (dPPAT-DPCK) and the entire *Drosophila* de novo CoA biosynthesis pathway was annotated. Genes coding for this pathway appeared to be highly conserved. Subsequently a comprehensive analysis of the mutant phenotypes was performed. It is demonstrated that CoA de novo biosynthesis is required for maintaining DNA, cellular and neuronal integrity. Moreover, it was established that *Drosophila* PANK/fbl mutants can be used as a model for pantothenate kinase-associated neurodegeneration (PKAN).

**Chapter 3: Pantethine rescues a *Drosophila* model for pantothenate kinase-associated neurodegeneration**

In this chapter *dPANK/fbl* mutants were used to demonstrate that impaired function of pantothenate kinase induces a decrease in CoA levels, mitochondrial dysfunction, increased protein oxidation, a neurodegenerative phenotype and a reduced lifespan. Furthermore, compounds were tested for their ability to rescue these pertinent phenotypes of the *Drosophila* PKAN model. In this screen pantethine was identified. Pantethine feeding restores CoA levels, improves mitochondrial function, rescues brain degeneration, enhances locomotor abilities, and increases lifespan. Evidence is presented for the presence of a de novo CoA biosynthesis pathway in which pantethine is used as a precursor compound, a pathway independent from pantothenate kinase. Importantly, this pathway appears also to be effective in mammalian cells with impaired function of pantothenate kinase. These data suggest that pantethine may serve as a starting point to develop a possible treatment for PKAN.

**Chapter 4: Effects of various doses of pantethine in healthy mice**

After establishing that pantethine is protective in a *Drosophila* model for PKAN, we wished to investigate the potential of pantethine in mice. In this chapter, our objective was to investigate a possible toxicity (short-term) of pantethine in wild-type mice. Pantethine supplementation was given via the drinking water to wild-type mice. The following parameters were investigated in control mice and in mice treated with various concentrations of pantethine: daily weight gain, daily activity and total lifespan. After 3 weeks the mice were sacrificed and post mortem analysis was done on various organs to find out the affects of pantethine supplementation. The major finding of this pilot study is that pantethine is quite tolerable and 15 mg pantethine /ml did not induce any side effects.
Chapter 5 *Drosophila* phosphopantothenoylcysteine synthetase is required for tissue morphogenesis during oogenesis

In this chapter another feature of the phenotype of *Drosophila* CoA mutants was investigated and the female and male fertility and fecundity was described in detail. In general, our analysis demonstrates that the dPPCS, dPPAT-DPCK and dPANK/fbl genes are required for proper morphogenesis. Specifically it was demonstrated that a mutation in dPPCS disrupts the organization of the somatic and germ line cells, affects F-actin organization and results in abnormal PtdIns(4,5)P2 localization. Improper cell organization coincides with aberrant localization of the membrane molecules Gurken (Grk) and Notch, whose activities are required for specification of the follicle cells that pattern the eggshell. Mutations in dPPCS also induce alterations in scutellar patterning and cause wing vein abnormalities. Interestingly, mutations in dPANK and dPPAT-DPCK result in similar but not entirely identical patterning defects.

Chapter 6: Summarizing discussion

The presented work will be summarized in this chapter. *Drosophila* can be used as a model organism to gain insight into the PKAN pathogenesis (Chapter 2) and pantethine is a protective compound that rescues abnormalities of this PKAN *Drosophila* model (Chapter 3). In chapter 4, we also show that pantethine is well tolerated by the wild type mice when administered orally. In this chapter, we will discuss the possibilities of future studies concerning pantethine (in mice and humans) as a potential treatment for PKAN. Interestingly, with pantethine, CoA levels were restored; we could rescue all the neurodegeneration-associated phenotypes but not the male and female fertility as described in chapter 5. These data suggest that PANK has an additional function, other than synthesizing CoA. We will discuss other possible functions of PANK, which might be required for fertility and fecundity.

REFERENCES


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CHAPTER 1


