Physiological Implications of impaired de novo Coenzyme A Biosynthesis in Drosophila melanogaster
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CHAPTER 6.

General discussion and summary
Coenzyme A: The metabolic key to many fundamental processes in eukaryotic systems

in preparation for a review
General discussion and summary

ABSTRACT
Coenzyme A constitutes an essential cofactor whose biosynthesis route is conserved amongst species. De novo CoA is synthesized from pantothenate by the subsequent action of five enzymes; PANK, PPCS, PPCDC, PPAT and DPCK. Although CoA biosynthesis is conserved, significant dissimilarities exist between the eukaryotic and bacterial enzymes, which makes these enzymes potential targets for antimicrobial drugs. Therefore, CoA production is well described at the biochemical level. However, little is known about the physiological implications of disruptions in the de novo CoA synthesis route in higher eukaryotes. Results presented in this thesis implicate a major role for CoA during morphogenesis, cell division, fertility, neuronal integrity, development and DNA integrity. In humans mutations in PANK2 result in the neurodegenerative disease: pantothenate kinase-associated neurodegeneration or PKAN. Here we describe the various aspects of the physiological implications of defects in the de novo CoA biosynthesis route in metazoans with a focus on Drosophila and we discuss which CoA dependent pathways might be responsible for the complex phenotypic characteristics of PKAN.

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**De novo CoA synthesis is essential and phylogenetically conserved**

As the major acyl carrier in all living organisms coenzyme A (CoA) and its derivatives constitute essential cofactors for approximately 5% of all reactions of intermediary metabolism. CoA is required for the tricarboxylic acid cycle (Kreb’s cycle), fatty acid metabolism and the synthesis of some amino acids. Biosynthesis of CoA is conserved amongst species and occurs from vitamin B₅ (pantothenate), which is modified by the subsequent action of five enzymes: pantothenate kinase (PANK), 4’-phosphopantothenoylcysteine synthetase (PPCS), (R)-4’-phospho-N-pantothenoylcysteine decarboxylase (PPCDC), 4’-phosphopantetheine adenyllyltransferase (PPAT), dephospho-CoA kinase (DPCK) (Figs. 1A-B). The complete CoA biosynthesis pathways in bacteria, archaea, plants, humans and *Drosophila* have been identified (CHAPTER 4 and ref. 3-6). Many bacteria, plants and yeast are capable of de novo synthesis of pantothenate from β-alanine, but higher eukaryotes depend on their dietary intake. In humans pantothenate and other water soluble vitamins such as biotin and lipoate are transported across the cell membrane by a Na⁺-dependent multivitamin transporter (SMVT). As a consequence of the dependency on dietary intake, vitamin B₅ deficiency in mammals causes many systemic defects including impaired motor responses, depressed heme synthesis, altered growth and maturation of the small intestine during neonatal and prenatal periods and increased prenatal mortality. Although many redundant biochemical routes exist that generate CoA or acyl esters of CoA, CoA is mainly synthesized de novo from pantothenate. Mammalian PANK can also utilize pantetheine and N-pantothenoylcysteine to produce CoA, but the contribution of these substrates for CoA biosynthesis is only marginal.

Despite CoA biosynthesis is functionally conserved, differences exist between the prokaryotic and the animal enzymes. In bacteria the second and the third enzymes in the pathway, PPCS and PPCDC, are fused into one protein/gene, but in animals these enzymes are expressed separately. Conversely, the final two enzymes in the pathway, PPAT and DPCK, are expressed separately in bacteria, but form a bifunctional protein/gene in animals. Similarly, structural analysis revealed significant differences between the eukaryotic and prokaryotic enzymes, and since CoA constitutes an indispensable cofactor these analyses may allow the rational design of antimicrobial drugs. Discussing the biochemical aspects of CoA biosynthesis are beyond the scope of this overview and these aspects of CoA synthesis have been described previously in full detail.

**CoA biosynthesis in vertebrates and *Drosophila***

The human genome contains four different autosomal loci encoding homologous pantothenate kinases, *PANK1-4*, whose expression are tissue specific. Expression of *PANK1* is highest in the liver, heart and kidneys. *PANK2* is most abundant in the retina and infant basal ganglia. The *PANK3* gene is expressed ubiquitously in the liver, while *PANK4* expression rises in muscle tissue. *Drosophila* has only one *PANK* gene (*dPANK/fumble*), which is closely related to human *PANK2*. The human and *Drosophila* genomes both contain single copies of *PPCS*, *PPCDC*, *PPAT-DPCK* and *DPCK* (CHAPTER 4 and ref. 4). Although the activity of the tentative fly homologs has not been demonstrated, comparative genomics and 3D remodeling indicate that these genes constitute functional homologs of their human counterparts (CHAPTER 4).

The main stores of CoA in eukaryotic cells are the peroxisomes and the mitochondria where CoA is utilized for fatty acid β-oxidation and as a cofactor in the Kreb’s cycle, respectively. As the first and the final two enzymes in the CoA synthesis pathway, PANK2...
and PPAT-DPCK, are targeted to the mitochondria, it was proposed that a complete \textit{de novo} CoA synthesis route is present in/at the mitochondria\textsuperscript{16,25-30}. Feedback regulation of \textit{de novo} CoA synthesis also occurs at the level of these two enzymes\textsuperscript{16,18,25,27,28,31}. PANK2 activity is stimulated by palmitoylcarnitine and carnitine, and inhibited by CoA and its acyl esters (palmitoyl-CoA, malonyl-CoA, acetyl-CoA), while the activity of PPAT-DPCK is stimulated by phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The remaining two enzymes, PPCS and PPCDC, may form large cytoplasmic enzyme complexes as determined by comparative 3D modeling\textsuperscript{32,33}.

Flies have 1 \textit{P ANK} gene, while humans possess 4 genes (\textit{PANK1-4}). In humans only PANK2 is targeted to the mitochondria and it could be that PANK1,3,4 are targeted elsewhere in the cell\textsuperscript{14}. In analogy, the \textit{Drosophila dP ANK} locus encodes multiple isoforms, whose predicted and immunohistochemically determined cellular localization differs and includes a mitochondrial targeted isoform (ref. 20 and unpublished data). Thus although CoA biosynthesis occurs at/in the mitochondria, production of this cofactor is likely not restricted to this organelle, but nonetheless mitochondrial CoA synthesis may represent a crucial production site\textsuperscript{28,34}.

\begin{figure}[h]
\centering
\caption{Pantothenate and lipid metabolism.}
\begin{enumerate}
\item \textbf{(A)} Higher eukaryotes depend on dietary pantothenate to produce CoA, which is proposed to take place mainly at the mitochondria\textsuperscript{16,25-28}. Pantothenate is phosphorylated to 4'-phosphopantothenate by pantothenate kinase (PANK). Next a cysteine is added by the 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 yield CoA\textsuperscript{2-5}. E.C. numbers are indicated.
\item \textbf{(B)} Schematic diagram of the \textit{de novo} CoA biosynthesis in animals. The \textit{Drosophila} genome encodes a single copy of \textit{PANK}, \textit{PPCS}, \textit{PPCDC}, a bifunctional \textit{PPAT-DPCK} and a \textit{DPCK}. 3D modelling revealed that hPPCS might form large enzymatic complexes with hPPCDC\textsuperscript{32,33}. Mammalian PANK can also utilize pantetheine and N-pantothenoylcysteine to produce 4'-phosphopantetheine and (R)-4'-phospho-N-pantothenoylcysteine, respectively\textsuperscript{13}. CoA and its acyl esters are utilized by many enzymes involved in the cellular metabolism including the Kreb’s cycle, fatty acid metabolism and the synthesis of some amino acids. Feed back regulation of CoA biosynthesis occurs at the level of PANK\textsuperscript{16,18,27,28,31} and PPAT-DPCK\textsuperscript{25}. (PE); phophatidylethanolamine, (PC); phosphatidylcholine.
\item \textbf{(C)} Simplified diagram of the biochemical routes towards the production of the major phospholipids reconstructed from the Kyoto Encyclopedia of Genes and Genomes database (www.genome.ad.jp). Although phospholipids can be synthesized from many alternative routes, the key step during \textit{de novo} phospholipid biosynthesis represents the formation of phosphatidic acid (PA). Formation of PA requires acyl-CoA (enzymatic steps depicted in dashed lines) and mutations in the \textit{de novo} CoA biosynthesis are predicted to reduce synthesis of this precursor. Enzymes are depicted in white spheres (Enzyme Commission (E.C.) numbers are indicated), while the lipids are depicted in black spheres.
\item \textbf{(D)} Schematic view of the major lipid synthesis routes that depend on CoA. Lipids are essentially synthesized from pantothenate and sugars, which are used to produce CoA and glycerol respectively. Sugars are converted to glycerol and pyruvate during glycolysis. In the mitochondrium pyruvate can be converted into acetyl-CoA and esters and subsequently enters the tricarboxylic acid cycle (Kreb’s cycle) used to synthesize CoA, CoA is acetylated and acetyl-CoA can be transported to the ER where it is used for the formation of O-acetylated gangliosides (spingoglycolipids). Acetyl-CoA is essential for the activation/transport of fatty acids to the peroxisomes and mitochondria, and subsequently for the \textit{β}-oxidation of these fatty acids in these compartments. Glycerol and acetyl-CoA are required for the production of CDP-diacyl-glycerol, which is the precursor for the synthesis of phospholipids. Glycerol can also be generated from the triglycerides (triacylglycerol). Although, triglycerides/triacylglycerol constitute the major form of the dietary fat and lipid storage, \textit{de novo} triglycerides can also be synthesized from CDP-diacyl-glycerol or fatty acids. The drawing is based on: the KEGG database and ref. 14,104.
\end{enumerate}
\end{figure}
Pantothenate kinase-associated neurodegeneration

Many pathological conditions such as alcoholism, starvation, diabetes, and certain tumors are associated with altered CoA metabolism\(^4,35\). Moreover, genetic analysis linked two neurological disorders to mutations in the \(PANK2\) gene: hypoprebetalipoproteinemia, acanthocytosis, retinitis pigmentosa, and pallidal degeneration (OMIM 234200; HARP)\(^36,37\) and pantothenate kinase-associated neurodegeneration (PKAN)\(^2,15,38\). Both syndromes are classified as neurodegeneration with brain iron accumulation (NBIA). NBIA comprises a heterogeneous group of disorders that are accompanied with iron accumulation in the brain. Essentially two groups exist, the first group comprises all cases with known mutations in \(ferritin\)\(^39\), \(ceruloplasmin\)\(^40\), \(PLA2G6\)\(^41\) or \(PANK2\)\(^2\), while the second group represents all cases of idiopathic origin. \(PANK2\) deficiency is the most common cause of NBIA and accounts for approximately 50% of all known cases.
PKAN patients display iron depositions in the basal ganglia and/or globus pallidus that give a characteristic MRI signal known as the eye-of-the-tiger sign\textsuperscript{42,43}. However, iron accumulation is not entirely pathognomonic for PKAN\textsuperscript{44,45}. PKAN is further characterized by motor symptoms such as dystonia or parkinsonism, mental retardation and retinitis pigmentosa\textsuperscript{2,46,47}. Acanthocytosis and abnormal plasma lipoproteins have also been associated with PKAN\textsuperscript{36,48}. Although the clinical and histological features of PKAN have been described in detail and the affected gene (\textit{PANK2}) has been cloned\textsuperscript{2}, the complex pathology of PKAN is far from understood and no therapies are available to delay the neurodegeneration. Moreover, several patients that show the clinical symptoms of PKAN do not carry a mutation in their \textit{PANK2} gene\textsuperscript{49,50}. This implies that there are other causative genes, but these have not been identified to date\textsuperscript{51}.

The origin of iron accumulation in the brains of PKAN patients is unclear, however, it was speculated that the chelation of iron by cysteine-containing moieties found in the substrates for PANK2 (\textit{N}-pantothenoylcysteine and pantetheine) and cysteine itself result in iron deposition\textsuperscript{2,52}. This hypothesis is supported by the findings that the brains of a few PKAN patients (although only clinically diagnosed) contained high cysteine levels\textsuperscript{53} and flies that carried a mutation in \textit{dPANK} were sensitive to high levels of cysteine in their food (CHAPTER 4). Alternatively, the PANK2 protein localizes to mitochondria and mutations in \textit{PANK2} may alter specifically neuronal mitochondrial CoA biosynthesis in PKAN, and it was hypothesized that disrupted mitochondrial lipid synthesis due to reduced CoA synthesis may also affect normal iron metabolism\textsuperscript{27}. The mitochondria are the main consumers of iron where it is used for heme synthesis and for Fe-S cluster biogenesis\textsuperscript{54,55}. Fe-S cluster formation in the mitochondria requires enzymes from the Kreb’s cycle and the respiratory chain and it is possible that mutations in \textit{PANK2} (but also in \textit{PLA2G6}, which encodes a phospholipase required for phospholipid homeostasis) affect membrane integrity thereby also influencing the import of Fe and/or the activity of enzymes involved in Fe-S cluster formation\textsuperscript{27,52}. Finally, altered heme synthesis may also contribute to the iron accumulation observed in PKAN patients\textsuperscript{56,57}. The first step of heme synthesis occurs in the mitochondria, where \textit{δ}-aminolevulinic acid (ALA) is formed from a condensation reaction of glycine and succinyl-CoA catalyzed by \textit{δ}-aminolevulinic acid synthase (ALAS)\textsuperscript{58}. The rate limiting step in heme biosynthesis in non-erythroid cells is the formation of ALA\textsuperscript{54}. Because the formation of ALA relies on succinyl-CoA, which is produced in the Kreb’s cycle, it is possible that reduced CoA synthesis in the mitochondria alters the production of succinyl-CoA thereby effectively slowing down heme precursor synthesis, which in turn results in an accumulation of iron. Recently, it was found in a chemically induced PANK knock-out mouse, that succinate, furamate and malate all were reduced in liver samples, indicating that the Kreb’s cycle was disrupted and thus could provide the basis for altered heme synthesis\textsuperscript{34}. Future experiments should be directed to investigate which of these proposed mechanisms (or other mechanism) is the basis for iron accumulation in the brains of PKAN patients.

Several hypotheses have been postulated that could explain PKAN pathogenesis. In addition to high iron, also high cysteine levels have been reported in the brains of a few patients that were clinically diagnosed with PKAN\textsuperscript{53}. High levels of cysteine might be explained, since cysteine incorporation occurs in a step downstream of PANK during \textit{de novo} CoA synthesis mutations in \textit{PANK2} may lead to the accumulation of free cysteine (\textbf{Figs. 1A-B}). It has been demonstrated that in the presence of iron, free cysteine rapidly autooxidizes (Fenton reaction), which leads to the production of hazardous free radicals that can induce oxidative damage\textsuperscript{59}. Patients with PANK often display Lewy bodies composed of abnormal \textit{α}-synuclein and/or
neurofibrillary tangles due to hyperphosphorylated tau aggregation, which may result from prolonged exposure to oxidative damage. Although these latter two histological markers are likely secondary to the main pathological process (ref. 64 and references herein), Fe/cysteine induced oxidative damage was proposed to underlie PKAN pathogenesis. Additionally, PANK2 deficiency likely results in impaired CoA synthesis. This may especially affect lipid homeostasis and could explain the changes in blood cell morphology (acanthocytosis) and the lipid abnormalities (hypoprebetalipoproteinemi) found in patients with PKAN. Fe/cysteine induced lipid peroxidation in cells already deprived of normal lipid metabolism could also enhance disease severity and/or progression. Finally, PKAN has been classified as a Parkinson related disease (Parkinsonism) associated with aberrant mitochondrial PANK2 function. The PANK2 protein is targeted to the mitochondria and disrupted PANK2 activity can cause mitochondrial dysfunction, which may lead to decreased resistance to reactive oxygen species (ROS), a feature implicated in Parkinson’s disease and Alzheimer’s disease pathogenesis, and as such cause neurological damage. Although increased oxidative damage due to mitochondrial dysfunction has been implicated in various neurodegenerative diseases (reviewed in ref. 68), studies performed with a Drosophila model of PKAN, generated in our laboratory (CHAPTER 4), indicate that increased oxidative damage is not the primary cause for neurodegeneration as will be discussed in the following paragraphs.

Animal models of PKAN
To investigate how PANK2 deficiency could elicited a neurodegenerative disorder, a PanK2 knock-out mouse was created. PanK2 mutant mice suffer from retinal degradation, but these mice do not display signs of neurodegeneration or impaired locomotor activity. Despite PanK2 mutant mice failed to develop a neurological phenotype, wild-type mice fed with a pantothenate deficient diet developed symptoms of neurological impairment. This suggest that a general impairment in CoA biosynthesis, rather than a mutation in PANK2, underlies PKAN. Although wild-type mice deprived of pantothenate might be useful for future studies, thus far no clear answers have been obtained about PKAN disease pathogenesis from PanK2 deficient mice.

On the contrary, Drosophila mutants that carry mutations in their dPANK, dPPCS or dPPAT-DPCK locus, exhibit progressive loss of locomotor activity, show abnormal muscle contraction, display reduced lifespan, become paralytic when exposed to heat and display features of retina/brain degeneration (CHAPTER 4, Table 1). These observations are in line with several studies in Drosophila that established a connection between neuronal dysfunction and neurodegeneration. Mutants that exhibit neurodegeneration frequently display a combination of observable phenotypes such as reduced lifespan, progressive loss of locomotor activity or become paralytic when exposed to heat (ref. 70 and references herein). The findings inferred from our analyses of the Drosophila CoA mutants demonstrate that mutations in CoA biosynthesis enzymes in general cause neuronal dysfunction and thus provide genetic evidence that supports the findings in mice that were fed with a pantothenate deficient diet. Therefore, other enzymes in the de novo CoA biosynthesis, PPCS, PPCDC, PPAT-DPCK and DPCK are candidate genes, that might be mutated in NBIA cases of idiopathic origin. Although some PKAN patients that did not carry a mutation in the PANK2 locus were screened for mutations in other CoA biosynthesis enzymes, no patient with such a mutation has been identified.
Neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease have been linked to mitochondrial dysfunction (reviewed in ref. 68). NBIA which includes PKAN has also been classified as a disease related to mitochondrial dysfunction and mitochondrial dysfunction associated with mutations in PANK2 has been reported in humans and mice. Mitochondrial dysfunction in flies is frequently associated with decreased resistance to ROS. Consistent with a role for CoA in maintaining mitochondrial integrity, young dPANK, dPPCS and dPPAT-DPCK mutant flies are decreased resistant to exogenously applied ROS (CHAPTER 4). Although mitochondrial (dys)function has not been investigated in CoA mutant flies, mitochondrial CoA synthesis likely represent a crucial production site and therefore it is possible that decreased resistance to ROS in CoA mutant flies is due to mitochondrial dysfunction. dPPCS mutants were

<table>
<thead>
<tr>
<th>Physiologic Abnormalities</th>
<th>dPANK/jumble</th>
<th>dPPAT-DPCK</th>
<th>dPPCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delayed larval development</td>
<td>++ (5)</td>
<td>+/- (5)</td>
<td>+ (5)</td>
</tr>
<tr>
<td>Reduced larval viability</td>
<td>++ (*)</td>
<td>- (* )</td>
<td>- (* )</td>
</tr>
<tr>
<td>Reduced pupal viability</td>
<td>+++ (1)</td>
<td>+++ (*)</td>
<td>+++ (3)</td>
</tr>
<tr>
<td>KO lethal</td>
<td>+++ (3)</td>
<td>+ (3)</td>
<td>- (3)</td>
</tr>
<tr>
<td>Reduced larval motility</td>
<td>+++ (3)</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>Reduced flight performance</td>
<td>+++ (3)</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>Abnormal muscle contraction</td>
<td>+++ (3)</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>Impaired geotaxis</td>
<td>+++ (3)</td>
<td>++ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>Progressive locomotor dysfunction</td>
<td>ND</td>
<td>++ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>Paralytic upon heat-shock</td>
<td>ND</td>
<td>+ (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>Decreased lifespan</td>
<td>+++ (3)</td>
<td>++ (3)</td>
<td>+ (3)</td>
</tr>
</tbody>
</table>

**Cellular/ Nuclear Abnormalities**

| Aberrant mitosis                                  | ++ (1,3)     | + (3)      | + (3) |
| Enhanced apoptosis                                | +++ (3)      | + (3)      | + (3,4)|
| Enhanced DNA damage                              | + (3)        | - (3)      | + (3,4)|
| Cytokinesis defects                               | ++ (1)       | + (* )     | ++ (5) |
| Abnormal chromatin                                | ++ (5)       | - (5)      | ++ (*,4)|
| Aberrant F-actin dynamics                         | ++ (1,4)     | ++ (4)     | +++ (4)|
| Reduced Akt/PKB phosphorylation                  | ++ (4,5)     | ++ (4,5)   | + (4,5)|
| G2/M checkpoint defects                           | ND           | ND         | - (*) |
| Impaired DSB repair during meiosis                | ND           | ND         | - (5) |

**Other**

| Sensitivity to IR                                 | ++ (3)       | +++ (3)    | ++ (2,3)|
| Sensitivity to HU                                  | ND           | ND         | ++ (2,*)|
| Sensitivity to MMS                                 | ND           | ND         | ++ (*)  |
| Sensitivity to cysteine                            | + (3)        | - (3)      | + (3)  |
| Reduced triglycerides                              | ++ (3)       | ++ (3)     | + (3)  |
| Abnormal neutral lipids                            | + (4)        | + (4)      | + (4)  |
| Reduced phospholipids                              | ND           | ND         | + (3)  |
| Abnormal PtdIns(4,5)P2                              | ND           | ND         | + (4)  |
| Reduced pericerebral fat body                      | ND           | ND         | +++ (3) |
| Neurodegeneration                                  | + (*)        | + (*)      | + (3)  |
| Retinal degeneration                               | ND           | ND         | + (3)  |
| Sensitive to oxidative stress                      | +++ (3)      | +++ (3)    | + (3)  |

**Fertility and patterning defects**

| Male fertility defects                             | ++ (1)       | ++ (*)     | + (5,*)|
| Female fertility defects                           | +++ (1,4)    | ++ (3,4)   | +++ (2,4)|
| Poorly developed ovaries                          | +++ (4)      | +++ (4)    | +++ (4) |
| Egg shell patterning defects                       | +++ (4)      | ++ (4)     | +++ (4) |
| Veneration patterning defects                      | ++ (4)       | ++ (4)     | + (4)  |
| Bristle patterning defects                         | + (4)        | ++ (4)     | + (4)  |
| Aberrant Notch, Grk, Egfr signaling               | ND           | ND         | ++ (4,*)|

(ND) not determined, (-) not present/affected, (+) present/affected, (+++) present/clearly affected, (++++) present/extremely affected, (1) Afshar et al., 2001, (2) CHAPTER 2, (3) CHAPTER 4, (4) CHAPTER 5, (5) CHAPTER 6, (*) unpublished.
only slightly sensitive to exogenously applied ROS compared to \textit{dPANK} and \textit{dPPAT-DPCK} mutants. Regulation of CoA biosynthesis occurs at the level of \textit{PANK} and \textit{PPAT-DPCK}, but not \textit{PPCS} \textsuperscript{16,18,25,27,28,31} and this difference could explain why \textit{dPPCS} mutants are less sensitive for exogenously applied ROS. In case \textit{dPPCS} is mutated \textit{dPANK} and/or \textit{dPPAT-DPCK} activity might be upregulated to compensate for reduced \textit{(R)-4’-phospho-N-pantothenoylcysteine} synthesis (\textbf{Fig. 1B}). Alternatively, phosphorylation of pantetheine and pantothenoylcysteine by \textit{PANK} \textsuperscript{13} could bypass \textit{PPCS}, but not \textit{PPAT-DPCK}, and these routes might be stimulated when \textit{dPPCS} is mutated.

An attractive model to explain PKAN disease pathogenesis involves enhanced oxidative damage as a result of impaired Fe/cysteine metabolism, which could drive the Fenton reaction \textsuperscript{59}, leading to the production of ROS. Because cysteine incorporation occurs upstream of \textit{dPANK} and \textit{dPPCS}, \textit{dPPAT-DPCK} mutants would be insensitive for cysteine intoxication. In agreement with this hypothesis survival of \textit{dPANK} and \textit{dPPCS} mutants, but not \textit{dPPAT-DPCK}, decreased when larvae were exposed to high cysteine concentrations (CHAPTER 4). Combined overexpression of three ROS scavengers (a CuZn-superoxide dismutase (SOD), a catalase (CAT) and a thioredoxin reductase (TRX), which are all present on one chromosome \textsuperscript{71}) could rescue the sensitivity to cysteine of the \textit{dPANK} and \textit{dPPCS} mutants, indicating that the observed cysteine induced lethality was likely due to elevated oxidative damage. Therefore a Fenton reaction might generate ROS in the \textit{dPANK} and \textit{dPPCS} mutants, but not in \textit{dPPAT-DPCK} mutants. Importantly, since \textit{dPPAT-DPCK} mutant flies are neurologically impaired, but do not display sensitivity to cysteine, these data imply that altered Fe/cysteine metabolism is not the primary cause for neuronal dysfunction in CoA mutant flies. This is further supported by findings that overexpression of ROS scavengers in young \textit{dPPCS} and \textit{dPPAT-DPCK} mutant flies could not rescue their inability to climb against gravity (CHAPTER 4). Nevertheless, it is likely that oxidative damage contributes to the progressive (secondary disease pathogenesis) loss of locomotor activity in aged flies, and mitochondrial dysfunction in the \textit{dPANK} and \textit{dPPAT-DPCK} mutants could also explain their more severe phenotype compared with the \textit{dPPCS} mutants, especially lifespan (\textbf{Table 1}) \textsuperscript{72,73}. Consistent with these findings in \textit{Drosophila}, the α-synuclein and tau pathology, which might be due to prolonged exposure to oxidative stress, in PKAN patients are likely secondary to the main pathological process (ref. 64 and references herein). Therefore we propose that, although mutations in the CoA biosynthesis enzymes result in decreased resistance to oxidative stress (possibly due to mitochondrial dysfunction) and increased production of ROS (possibly due to the Fenton reaction), the onset of neuronal dysfunction in flies and possibly PKAN patients originates from downstream effects, induced by impaired CoA biosynthesis, other than oxidative damage.

\textbf{PKAN and lipid metabolism}

CoA and its acyl esters constitute essential cofactors required for many metabolic reactions and especially lipid metabolism is known to make extensive use of these cofactors (\textbf{Fig. 1C}) \textsuperscript{14,34}. Acyl-CoA is required to synthesize phosphatidic acid (PA), which is a main precursor for many lipids, and CoA is utilized as a cofactor in the mitochondria and peroxisomes to support the β-oxidation of fatty acids (\textbf{Fig. 1D}). A major component of the very low density lipoproteins (VLDL) are the triglycerides, which play an important role in metabolism as energy sources and transporters of dietary fat. Abnormal plasma lipoproteins and acanthocytosis, features of aberrant lipid homeostasis, have been associated with PKAN \textsuperscript{36,48}. Because NBIA can also result
from mutations in $PLA2G6$\textsuperscript{41}, which encodes a calcium-independent group IV phospholipase A\textsubscript{2} that is important in phospholipid remodeling, arachidonic acid release, leukotriene and prostaglandin synthesis, and apoptosis\textsuperscript{34}, it was proposed that mutations in $PANK2$ or $PLA2G6$ cause changes in lipid homeostasis and as such cause neurodegeneration\textsuperscript{52} and retinal degeneration\textsuperscript{2}. Recently, it was demonstrated that PANK2 can bind to palmitoylcarnitine and it was hypothesized that PANK2 senses the levels of palmitoylcarnitine in mitochondria and up-regulates CoA biosynthesis in response to an increased mitochondrial demand for this cofactor to support β-oxidation of fatty acids\textsuperscript{28,34}. Secondary to changes in lipid homeostasis as the primary mechanism of PKAN pathogenesis, Fe/cysteine induced lipid peroxidation\textsuperscript{66} may enhance disease severity and/or progression\textsuperscript{25,76}.

Consistent with a general impairment in lipid homeostasis, ablation of $de$ $novo$ CoA biosynthesis in mice using the competitive inhibitor of PANK, hopantenate (HoPan), and profiling the changes in intracellular metabolism and gene expression in hepatocytes revealed that when CoA synthesis is reduced, the intracellular metabolism is shifted towards maintaining the pool of non-esterified CoA, which is critical for mitochondrial functions (gluconeogenesis, β-oxidation of fatty acids, Kreb’s cycle and urea cycle)\textsuperscript{34}. In addition, mice developed hypoglycemia and insulin levels were reduced, while glucagons and corticosterone were elevated, demonstrating an impairment in lipid/hormone homeostasis. Similarly, array analysis revealed changes in the expression of genes involved in lipid metabolism, but also revealed large changes in organic acid/carboxylic acid and protein metabolism, confirming the essential and central role of CoA in intermediary metabolism. This chemically induced knock-out mouse of PANK clearly demonstrated the essential role of CoA to maintain physiological integrity, but unfortunately the authors did not investigate whether HoPan treatment elicited a movement disorder in mice.

Like in humans and mice, mutations in the $Drosophila$ CoA enzymes also impaired lipid metabolism. Mutant females synthesized less neutral lipids (triacylglycerol (TAG) and sterol esters) during oogenesis, while adult flies contained reduced amounts of stored fatty acids in the form of triglycerides, had reduced amounts of phospholipids and the pericerebral fat bodies of $dPPCS$ mutant flies were decreased in size (CHAPTER 4-5). These findings in mice and flies support the hypothesis that changes in lipid metabolism may be the primary defect that underlies PKAN disease pathogenesis. In $Drosophila$ 4-hydroxy-2-nonenal (HNE), a marker for oxidative damage to lipids, primarily accumulates in the pericerebral fat body in an age dependent manner\textsuperscript{77}. Although levels of HNE or of malondialdehyde (MDA), the most abundant product derived from unstable lipid peroxides, were not measured in CoA mutant flies, depletion of the lipid stores and reduced production of lipids may slow the renewal of damaged membranes and lipid peroxides, which may be especially harmful\textsuperscript{75,76}, thereby affecting the integrity of tissues including the central nervous system (CNS) and thus enhance the neurological phenotype of CoA mutant flies. Future experiments should be aimed at identifying to what extent oxidative damage to lipids contributes to disease progression (decreased lifespan and progressive loss of locomotor function).

To date little is known about a possible link between lipid metabolism and neuronal dysfunction in flies. Currently only three genes have been characterized that when mutated cause neurodegeneration and affect lipid synthesis: $bubblegum$ ($bmg$), $löchrig$ ($loe$) and $swiss-cheese$ ($sws$)\textsuperscript{78-81}. Despite the fact that mutations in these three genes affect lipid homeostasis, no common mechanism (specific lipid biosynthesis route) that would explain the neurodegenerative phenotype of mutant flies can be identified: $bmg$ is required for β-oxidation of fatty acids, $loe$
is involved in cholesterol homeostasis, while sws is required for the synthesis of PC, TAG and sterol esters. Likewise, mutations in CoA synthesis enzymes affect several branches of lipid biosynthesis and it remains to be determined whether the neurodegenerative phenotype is the result of changes in the synthesis of a particular lipid. More importantly, although several studies demonstrated that impaired CoA biosynthesis is associated with changes in lipid homeostasis, experimental data that unambiguously places impaired lipid homeostasis at the basis of PKAN disease pathogenesis is still lacking.

CoA in response to nutritional changes

Besides biochemical feedback regulation, tissue levels of CoA and acyl-CoAs are also under transcriptional and nutritional control. The human PANK2 gene encodes two experimentally confirmed miRNAs\(^{82,83}\), which were proposed to act synergistically with their “host” (PANK2) to control cellular levels of acetyl-CoA and lipid levels\(^{84}\). In mice the peroxisome proliferator-activated receptor alpha (PPAR\(\alpha\)) transcription factor, which is a key regulator of lipid metabolism, regulates CoA concentrations via regulation of PanK1 gene expression\(^{19}\). PPAR\(\alpha\) also regulates gene expression of the acyl-CoA thioesterases, which hydrolyse acyl-CoA to the free fatty acid and CoA, to control intracellular concentrations of CoA (reviewed in ref. 85). In mice, liver PanK activity decreases following fasting and increases following feeding, increases during diabetes, and increases after treatment with hypolipidemic agents (reviewed in ref. 12, 14 and references herein). Because CoA is critical for intermediary metabolism, changes in the levels of acyl-CoA and CoA could have devastating consequences and therefore intracellular concentrations are mostly likely strictly maintained, which was clearly demonstrated in HoPan treated mice\(^{34}\). Interestingly, HoPan treated mice also had abnormal levels of insulin, glucagons and corticosterone, indicating that hormone signaling was disrupted. Moreover, PanK2 deficient mice suffered from growth retardation\(^{69}\). These observations suggest that reduced CoA synthesis mimics “intracellular” conditions of poor nutrition.

The insulin and insulin-like growth factor signaling routes are well characterized and conserved pathways that involve many factors that control protein translation, proliferation, apoptosis, lifespan, motility and growth\(^{86-97}\). Regulation of growth, body size and reproduction of Drosophila is largely controlled by transcriptional responses to amino acids, sugars and insulin/insulin-like growth factor signaling\(^{98-103}\). Array analysis revealed that many of the Drosophila genes involved in the conversion of sugars into fatty acids, which also depends on CoA and its acyl esters, are regulated by dietary sugars\(^{104}\). Although little is known about the regulation of CoA in Drosophila, these studies suggest that, like in mammalian systems, levels of CoA are strictly maintained and respond to nutritional changes. If true this also implies that when de novo CoA synthesis is disrupted in flies, processes downstream of insulin/insulin-like signaling would be affected. In fact many phenotypic characteristics of the CoA mutants point to a defect in insulin receptor (InR) signaling (Table 1). InR activation results in the conversion of PtdIns(4,5)P\(_2\) to PtdIns(3,4,5)P\(_3\) mediated by phosphatidylinositol 3-kinase (PI3K), which in turn results in the activation of several effector kinases (Fig. 2A). Drosophila CoA mutants produced approximately 50% less phospholipids (PE, phosphatidylserine (PS), PC) and no changes in the ratio between the individual phospholipids were detected compared to wild-type ratios (CHAPTER 4). Therefore it is likely that the synthesis of phosphatidylinositol (PtdIns), the precursor for all phosphoinositides\(^{105}\), would be affected as well (Fig. 1C), thereby effectively modifying insulin/insulin-like growth factor signaling. Consistent with this
hypothesis, membrane levels and localization of a PLCδ-PH-GFP fusion protein, which is able to bind to PtdIns(4,5)P₂, was disrupted during oogenesis in dPPCS mutants, demonstrating that PtdIns(4,5)P₂ homeostasis is changed in CoA mutant flies (CHAPTER 5).

A crucial mediator of insulin/insulin-like growth factor signaling is protein kinase B (Akt/PKB), which becomes phosphorylated upon stimulation and is an important regulator of actin dynamics, lipid storage, growth, proliferation, glycogen synthesis, and protein translation. Moreover, Akt/PKB is a regulator of cell survival and plays a role in the induction of apoptosis. Whereas hyperphosphorylation of Akt/PKB results in survival, decreased phosphorylation induces apoptosis. The brains of CoA mutant larvae displayed enhanced apoptosis, which correlated with decreased phosphorylation of Akt/PKB, suggesting that conversion of PtdIns(4,5)P₃ into PtdIns(3,4,5)P₅ was reduced (Fig. 2B). Neuronal survival during growth, development and maturation of the CNS depends on Akt/PKB signaling (reviewed in ref. 110,111) and aberrant PtdIns(4,5)P₂-Akt/PKB signaling due to defects in de novo CoA biosynthesis thus may affect neuronal survival during development of the CNS. Moreover, impaired development and maturation of the CNS could explain neuronal dysfunction in young flies and therefore may also explain the onset of the early locomotor symptoms in patients with classic PANK during childhood.

Several animal studies demonstrated that impaired insulin/insulin-like growth factor signaling inflicts a neurodegenerative phenotype similar to that found in patients with Alzheimer’s disease (reviewed in ref. 111). More specifically, since Alzheimer’s disease is associated with impaired insulin/insulin-like growth factor signaling within the brain it was proposed that Alzheimer’s disease represent a brain-specific form of diabetes (Type 3 diabetes). Alzheimer’s disease and PKAN are both associated with progressive neurodegeneration that shares similarities and it would be of interest to explore whether altered CoA synthesis causes a diabetes-like phenotype in the brains of PKAN patients.

Drosophila CoA mutants also displayed growth delay, had fecundity defects, had poorly developed ovaries, synthesized less lipids, displayed aberrant actin dynamics and had reduced lifespan (CHAPTER 4-5, Table 1). All these phenotypic defects may also result from downstream events caused by aberrant PtdIns(4,5)P₂-Akt/PKB signaling and will be discussed in more detail below (see Fig. 2A). Although depletion of CoA mimics intracellular conditions of poor nutrition and likely result in reduced production of many compounds including PtdIns(4,5)P₃, we cannot exclude the possibility that abnormal hormonal levels including insulin, which has been reported in HoPan treated mice, also disrupt PtdIns(4,5)P₂ signaling in CoA mutant flies by modifying InR activity directly.

PtdIns(4,5)P₂-Akt/PKB signaling

In cells, a highly dynamic pool of seven different inositol phospholipids exists whose spatial and temporal activity are strictly regulated (reviewed in ref. 113). PtdIns is the main precursor for all the different phosphoinositides and is produced from the condensation of CDP-diacylglycerol (CDP-DAG) and myo-inositol by phosphatidylinositol synthetase, which takes place mainly at the membranes of the ER and the plasma membrane. The PtdIns represent approximately 5-15% of the total pool of phospholipids. Because acyl-CoA is an essential precursor for the synthesis of CDP-DAG and thus the PtdIns (Fig. 1C), CoA biosynthesis likely interferes with PtdIns homeostasis. CDP-DAG and PA are also essential precursors for the synthesis of all major phospholipids, and reduced production of these precursors due to mutations in the CoA
biosynthesis enzymes could explain why dPPCS mutant flies produced less PC, PE and PS (CHAPTER 4).

PtdIns signaling is essential for all processes that require actin-cytoskeletal remodeling (reviewed in ref. 113,116,117). Remodeling of the actin cytoskeleton occurs during various processes of Drosophila oogenesis and include cyst budding, cyst encapsulation, the establishment of planar cell polarity, cytoplasmic dumping and cell migration (reviewed in ref. 118). Although actin dynamics during oogenesis are well studied and many components have been identified, the contribution of lipids during these processes is poorly understood. In CoA mutant female flies, egg chamber production during oogenesis is severely hampered and as a result females suffer from fertility and fecundity defects. Interestingly, disrupted egg chamber development coincided with altered F-actin remodeling in all CoA mutants and also abnormal membrane localization and levels of PtdIns(4,5)P₂ were found in dPPCS mutant females (CHAPTER 5). PtdIns(4,5)P₂ is one of the most abundant phosphoinositides and is mainly localized at the plasma membrane\(^\text{113}\). It has been demonstrated that PtdIns(4,5)P₂ can directly influence actin polymerization by promoting the dissociation of capping proteins and can also act in cooperation with small GTPases such as Cdc42 (and via GEFs) in controlling ARP2/3-mediated nucleation of actin networks\(^\text{113}\). A proper balance between PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ at distinct locations within the plasma membrane is required for actin remodeling\(^\text{106}\) and PtdIns(4,5)P₂ is essential to mediate cohesion between the plasma membrane and the underlying cytoskeleton\(^\text{119}\). Thus it is likely that reduced membrane levels of PtdIns(4,5)P₂ underlie the F-actin remodeling

Figure 2. Mutations in CoA synthesis affect PtdIns/growth factor signaling.
(A) In response to nutrients/insulin/insulin-like growth factor signaling PtdIns(4,5)P₂ is converted to PtdIns(3,4,5)P₃ mediated by PI3K to support various aspects of cell growth, division, motility and survival. The downstream effector kinases are shown; PDK1, S6K, RSK, Akt/PKB. PtdIns(3,4,5)P₃ is converted back to PtdIns(4,5)P₂ by the action of 3-phosphatidylinositide phosphatase PTEN to support survival in conditions of poor nutrition. DAG and InsP₃ produced from PtdIns(4,5)P₂ by phospholipase C (PLC) are known to modulate protein kinase C and calcium signaling. Various essential cellular processes that are regulated by PtdIns/growth factor signaling are indicated. Because many aspects of the PtdIns/growth factor signaling are disrupted in CoA mutant flies (light grey font), we propose that this signaling route is key in understanding the early developmental defects found in CoA mutant flies. References are indicated.
(B) Protein extracts from wild-type and CoA mutant third instar larval brains were analyzed for Akt/PKB Ser505 phosphorylation (Akt/PKB-P) by Western blotting. dPANK\(^\text{1/1}\), dPPAT-DPC1\(^\text{4/4}\) and dPPCS\(^\text{2/2}\) larval brains contain reduced amounts of Akt/PKB-P. Overexpression of P[dPPCS] in dPPCS\(^\text{2/2}\) (P[dPPCS]/ +;dPPCS\(^\text{1/1}\)) restored levels of Akt/PKB-P in this mutant. Blots were incubated with antibodies against β-actin as a loading control and levels of phosphorylated Akt/PKB were determined from 3 Westerns with ImageJ using β-actin as an internal control.
defects in the CoA mutants. Moreover, the ovaries from all CoA mutants contained reduced levels of phosphorylated Akt/PKB implying that, like in the larval brains, the conversion of PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃ is disrupted. Because F-actin remodeling also relies on Akt/PKB activity⁹⁵,¹²⁰,¹²¹, these findings strongly argue that impaired F-actin dynamics during oogenesis in the CoA mutants are due to, like in many other systems, aberrant PtdIns(4,5)P₂-Akt/PKB signaling possibly as a result of reduced PtdIns synthesis.

During Drosophila oogenesis, not only F-actin remodeling processes depend on PtdIns(4,5)₂-Akt/PKB signaling, but several studies in the Drosophila female reproductive system also implicated a link between defective neutral lipid droplet (TAG and sterol esters) synthesis and changes in PtdIns(4,5)₂-Akt/PKB signaling. For example, Akt/PKB signaling (via LSD2) is essential for the regulation of neutral lipid synthesis and lipid droplet formation during Drosophila oogenesis¹⁰⁷,¹²². Furthermore, a mutation in the DGAT/midway gene, which converts diacylglycerol (DAG) into TAG, also affects neutral lipid biosynthesis and causes aberrant F-actin remodeling possibly by modifying PKC signaling via DAG¹²³. In CoA mutant females both processes, F-actin remodeling and also neutral lipid droplet synthesis (CHAPTER 5), were disrupted, implying that a well maintained balance between PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ at the plasma membranes during oogenesis is the key for normal F-actin remodeling and neutral lipid droplet biosynthesis. To date aberrant PtdIns(4,5)P₂-Akt/PKB signaling in the Drosophila CoA mutants is still poorly investigated, but nonetheless analyses of the defects during oogenesis in the dPPCS mutant implicate a major role for CoA metabolism during morphogenesis possibly by modulating F-actin dynamics. Since morphogenesis during oogenesis is well characterized and relatively easy to study, the female reproductive system may be a favorable model to dissect the exact pathways that require CoA during morphogenesis.

**CoA and development**

Systematic genetic footprinting studies in *E. coli* demonstrated the importance of the CoA biosynthesis enzymes for viability¹²⁴. Moreover, bacteria deficient for CoA synthesis enzymes, could be complemented with genes coding for human CoA synthesis enzymes, confirming the functional conservation of the CoA biosynthesis pathway⁴. Knock-out alleles of *dPANK*, *dPPCS* and *dPPAT-DPCK* are also not viable (CHAPTER 4 and ref. 20). However, *dPPCS* null embryos deposited by heterozygous mothers hatched normally (73%, n=840), but did not reach the pupa stadium and died as first instar larvae, indicating that *dPPCS* (or CoA) has a maternal origin. In situ hybridization studies and developmental gene expression analysis revealed that *dPANK*, *dPPCS*, *dPPAT-DPCK* and the *dDPCK* mRNAs are indeed maternally supplied (ref. 125, http://genome.med.yale.edu/lifecyle, www.fruitfly.org). Furthermore, *dPANK* and *dPPCS* expression peaks upon entry into larval development, which may prelude larval CoA demand for growth and energy. Thus when larval growth commences, CoA biosynthesis is upregulated. CoA mutant larvae displayed delayed pupation entry and decreased viability, which likely reflect a reduction in the production of CoA during larval development. Delayed development of the CoA mutants was not accompanied by a visible reduction in body size of the larvae or the adult flies.

The *dPPCS* gene (GH03502 mRNA) was identified in a high throughput screen as a gene whose expression is controlled by the *glial cell missing* (*gcm*) transcription factor¹²⁶. During embryonic development, GH03502 was downregulated in the macrophage precursors in a *gcm*¹* gcm² background, while ectopic expression of *gcm* induced GH03502 transcription throughout the CNS. These results suggest that CoA synthesis is essential for the development
of the CNS and the immune system. However, none of the CoA enzymes displayed a specific \textit{in situ} expression pattern in embryos (ref. 125, www.fruitfly.org). Similarly, we were unable to confirm the \textit{in situ} dPPCS mRNA expression pattern during embryogenesis (unpublished). Nonetheless, CoA biosynthesis is required for tissue morphogenesis during development and \textit{dPANK}, \textit{dPPCS} and \textit{dPPAT-DPCK} mutant flies developed ectopic wing veins, had ectopic bristles (macrochaetae, sensory cells) on the scutellum and eggs produced by mutant females displayed chorion patterning defects (CHAPTER 5). How CoA regulates patterning remains elusive, but may involve aberrant receptor signaling due to changes in cytoskeletal remodeling as a result of altered PtdIns signaling.

Patterning defects were only investigated in \textit{dPPCS} mutant females and analyses revealed that Gurken (Grk, TGF\(\alpha\)-like ligand for Egfr) and Notch were abnormally localized during various stages of oogenesis (CHAPTER 5). Because both molecules are required for specification of the follicle cells that pattern the chorion\(^{127-130}\), it is likely that these signaling routes are affected in \textit{dPPCS} mutants. Moreover, flies homozygous for a mutation in epidermal growth factor receptor (Egfr) and \textit{dPPCS} were not viable, further suggesting that a mutation in \textit{dPPCS} can modulate Egfr signaling (unpublished). Aberrant follicle cell patterning in \textit{dPPCS} mutant females coincided with severe defects in F-actin dynamics and abnormal membrane levels of PtdIns(4,5)\(P_2\) during oogenesis (CHAPTER 5). Because PtdIns(4,5)\(P_2\) signaling is essential for F-actin remodeling, changes in PtdIns(4,5)\(P_2\) homeostasis due to impaired CoA biosynthesis may lead to cytoskeletal defects. In turn, impaired PtdIns(4,5)\(P_2\) homeostasis

\textbf{Figure 3.} \textit{dPPCS} mutant males suffer from cytokinesis defects.

2-h-old wild-type and mutant spermatids during the onion stage were investigated for cytokinetic and mitochondrial defects by phase contrast microscopy. 
\textbf{(A)} In wild-type testis a cyst of 16 interconnected primary spermatocytes undergo two synchronized meiotic divisions accompanied with incomplete cytokinesis and results in the production of 64 early spermatids that remain connected by ring canals. During division mitochondria are assembled at the central spindle and are equally distributed over both spermatids where they form one big mitochondria or nebenkern. This nebenkern (phase dark) remains associated with the nucleus (phase light) and its size and spherical structure resemble that of the nucleus during the onion stage. Changes in size and structure of the nebenkern accompanied with the presence of 2 or 4 normal sized nuclei are believed to result from defective cytokinesis, but not from improper karyokinesis\(^{165}\).

\textbf{(B)} \textit{dPPCS} mutant testis contain big nebenkern structures with 2 (boxed arrowheads) or 4 (arrowheads) nuclei and \textbf{(C)} frequently display abnormally persistent mitochondrial bridges (arrow).

\textbf{(D)} Approximately 17\% of \textit{dPPCS}\(^{1/1}\) male flies contain testis with >10\% abnormal spermatids, while this percentage is 82\% in \textit{dPPCS}\(^{1/3}\) males. The number of flies analysed is depicted at the top of each histogram.
and F-actin remodeling defects would affect the overall structural integrity of all membranous structures in cells and the organization of cells within a tissue. Concomitantly, membrane associated receptor signaling processes required for cell migration and cell specification will be abrogated.

In summary, the CoA enzymes are not required for cell specification/signaling \textit{an sich}, but affect morphogenesis indirectly possibly by modifying PtdIns levels. Like morphogenesis within the ovaries\textsuperscript{118,131,132}, wing vein and bristle patterning also requires Notch signaling and actin remodeling\textsuperscript{133-141}. Therefore, we hypothesize that patterning defects in the CoA mutants are the result of aberrant actin dynamics due to altered PtdIns signaling, which in turn cause dysregulation of signaling routes that specify the vein cells, the sensory cells and the follicle cells (CHAPTER 5).

\textbf{CoA during cell division}

During embryonic cell division and cellularization of the embryonic syncytium, an ubiquitously expressed FLAG-tagged dPANK protein localized to the metaphase furrow and the cellularization furrow, respectively\textsuperscript{20}. At these sites active membrane addition takes place and it was proposed that localized CoA biosynthesis might be required at sites of high lipid demand. During cell division PE relocalizes from the inner to the outer leaflet of the plasma membrane at the cleavage furrow (reviewed in ref. 142). Because immobilization of PE at the outer leaflet results in defective cytokinesis and \textit{dPANK} mutants exhibited abnormal mitosis, aberrant aster separation, disrupted contractile ring formation and cytokinetic failure, it was proposed that altered CoA biosynthesis specifically interfered with phospholipid biosynthesis during mitosis\textsuperscript{20}. However, \textit{dPANK} physically interacts with the actin depolymerising factor Twinstar (TSR) (cofilin/ADF homolog)\textsuperscript{143} and mutations in this gene affect chromosome segregation, cytokinesis and aster separation, suggesting that changes in actin dynamics also resulted in cytokinetic failure\textsuperscript{144} in \textit{dPANK} mutants. Interestingly, \textit{tsr} is also required for planar cell polarity patterning in various \textit{Drosophila} epithelia including the eye and the wing\textsuperscript{145}, demonstrating that \textit{trs} is also crucial during morphogenesis and provides further support for the hypothesis that the patterning defects in the \textit{dPANK} mutants might be due to defects in cytoskeletal remodeling. Although it is unlikely that \textit{dPANK} modulates TSR activity, recruitment of \textit{dPANK} by TSR might allow localized synthesis of lipids at sites of active actin remodeling/membrane synthesis. It has been demonstrated that mutations in \textit{Drosophila vibrator}, a PtdIns transfer protein, result in aberrant cytokinesis due to abnormal contractile ring formation\textsuperscript{146}. Similarly, the \textit{Drosophila} phosphatidylinositol 4-Kinase (\textit{four wheel drive}) and PtdIns(4,5)P\textsubscript{2}, which are important for membrane trafficking, are essential to ensure proper cytokinesis\textsuperscript{147-149}. This again points to changes in actin dynamics and PtdIns homeostasis as a key defect in the CoA mutants. Local changes in lipid synthesis due to disrupted CoA synthesis might also disrupt PtdIns signaling thereby altering localized actin remodeling processes (hence contractile ring formation) and as such lead to cytokinetic failure.

\textit{dPANK} was originally found in a genetic screen to identify mutations that affect male fertility\textsuperscript{150} and aberrant cytokinesis during meiotic divisions in the male testis resulted in abnormal spermatid production\textsuperscript{20}. Like the \textit{dPANK} mutants, mutations in \textit{dPPCS} also affect male fertility due to abnormalities in cytokinesis (\textbf{Fig. 3}) and during spermiogenesis the \textit{dPPCS} mutants did not form mature sperm tails (strings) (unpublished). Furthermore, a high incidence of anaphase defects such as lagging chromosomes or chromatids and fragmented chromosomes,
which can give rise to polyploid cells, were found within the brains of CoA mutant larvae during development (CHAPTER 4 and ref. 20).

Increased aberrant mitoses in the larval brain coincided with an increase in apoptotic cells, suggesting that disrupted mitosis (in combination with decreased phosphorylation of Akt/ PKB) results in the induction of apoptosis. Larval brains from dPANK and dPPCS mutants, but not dPPAT-DPCK mutants, also frequently exhibited abnormal chromatin condensation during mitosis (Fig. 4). Surprisingly, the larval brains from dPANK and dPPCS mutants, but not the dPPAT-DPCK mutant, also displayed increased staining of phosphorylated Histone 2AvD (γ-H2AvD) (CHAPTER 4), which is indicative for the presence of DNA double strand breaks (DSBs)\(^{151,152}\).

During stage 1-6 of oogenesis the somatically derived follicular epithelium that surrounds the germ line cells (nurse cells and oocyte) is mitotically active\(^{153,154}\). The mitotically active follicle cells in dPPCS mutant ovaries also displayed enhanced γ-H2AvD staining and were undergoing apoptosis (CHAPTER 4). After the mitotic-to-endocyte switch within the follicular epithelium, which occurs between stage 6-7, nurse cell chromatin dispersion occurs. Initially, the nurse cell chromosomes show a characteristic 5-lobed appearance and by stage 6 they are completely dispersed. In dPPCS mutant nurse cells chromatin (de)condensation was not completed at stage 7 and the chromatin was also frequently apoptotic, but the mitotically inactive nurse cells did not display increased γ-H2AvD staining (CHAPTER 5). Furthermore, the nuclei of the nurse cells were frequently heterogeneous in size, which may indicate that the switch from mitosis to endoreplication was misregulated. Thus the CoA biosynthesis enzymes are required to maintain chromatin integrity in mitotically active (follicle cells, brain cells) and inactive cells (nurse cells), but accumulation of DNA damage is restricted to mitotically active cells.

**Figure 4. Larval brains from dP4NK and dPPCS mutants display abnormal chromatin organization during mitosis.**

Third instar larval brains were labelled with an antibody directed against pH3Ser10 to visualize mitotic chromatin.

(A-D) Mitotic chromatin condensations that were frequently found in dPANK\(^{1/1}\) and dPPCS\(^{0/3}\) brains, but not in dPPAT-DPCK\(^{43/43}\). To our knowledge the presence of these profound chromatin structures has not been reported and currently we do not understand how these structures arise.

Phosphorylation of histone H3Ser10 initiates during prophase and is rapidly lost during progression through telophase\(^{166}\). The observed structures might represent prophase H3Ser10 phosphorylation. However, such chromatin structures are never that profound. To date, we speculate that these structures arise prior to the assembly of chromosomes at the metaphase plate and are therefore referred to as abnormal metaphase condensations.

(E) Quantification of abnormal metaphases as observed in A-D. The presence of metaphase defects is higher in dPANK\(^{1/1}\) and dPPCS\(^{0/3}\) brains, but not in dPPAT-DPCK\(^{43/43}\) compared with wild-type larval brains. Untreated; wild-type (n=21), dPANK\(^{1/1}\) (n=17), dPPCS\(^{0/3}\) (n=34), dPPAT-DPCK\(^{43/43}\) (n=14). (**p < 0.001 as determined by t-test)**
No genetic interaction could be detected when dPPCS was crossed with Drosophila cell cycle checkpoint mutants of grp/dChk1 or mnk/loki/dChk2 (unpublished). Furthermore, because the G2/M checkpoint in dPPCS mutant wing-disc cells was intact and no defects in the repair of DSBs generated during meiotic recombination were observed in mutant ovaries (Fig. 5), enhanced γ-H2AvD labeling in mitotically active cells does not result from global defects in cell cycle regulation and processing of DSBs. How mutations in the CoA biosynthesis enzymes affect DNA integrity is difficult to understand, but may involve aberrant growth factor/PtdIns signaling (see below).

CoA and DNA damage responses
Originally the dPPCS mutant was identified as a potent modifier of DNA damage responses during larval development. When larvae were fed with solutions containing methyl methanesulfonate (MMS) or hydroxyurea (HU), or when mutant larvae were exposed to ionising radiation (IR) the survival of homozygous individuals was severely decreased (CHAPTER 2, 4). Mutations in dPANK and dPPAT-DPCK also renders larvae hypersensitive to IR and caused pupal death, demonstrating that impaired de novo CoA biosynthesis in general causes hypersensitivity to DNA stress (CHAPTER 4, Table 1). Moreover, IR enhanced aberrant mitosis in CoA mutant third instar larval brains and IR also caused abnormal mitosis in wild-type larval brains. Interestingly, exposure to IR lead to impaired locomotor activity in young wild-type flies, indicating that IR can induce neuronal dysfunction in wild-type flies. Likewise, locomotor function of young dPPCS mutant flies, which is already impaired in control flies, was further reduced after exposure to IR, demonstrating that IR enhances locomotor dysfunction in dPPCS mutants. These findings implicate a novel role for de novo CoA synthesis in maintaining DNA and neuronal integrity (measured as locomotor activity). In the dPPCS mutant, no defects in G2/M cell cycle regulation nor in the repair of DSBs generated during meiosis were found, indicating that CoA (and lipid) shortage does not induce gross abnormalities in these DNA damage response pathways. Overexpression of ROS scavengers in the dPPCS mutant could also not suppress the sensitivity to IR (CHAPTER 4) and therefore it is unlikely that oxidative damage in the CoA mutants contributes to IR sensitization. Thus the question remains how impaired de novo CoA biosynthesis affects DNA integrity and results in IR sensitization.

In a recent study it was shown that Drosophila larvae carrying mutations in growth factor signaling genes (cdk4, chico, dmyc) or when wild-type larvae were grown under poor nutrient conditions are sensitive to IR155. Because defects in CoA biosynthesis are associated with impaired growth and growth factor signaling in both mice and Drosophila (Fig. 6 and ref. 34,69), it may be that reduced growth factor/PtdIns signaling (intracellular conditions of poor nutrition) causes IR sensitization. How impaired growth factor signaling and starvation exactly sensitzes larvae to IR remains to be established. One model posits that mutations in growth factor signaling and also conditions of starvation specifically modify the insulin/insulin-like growth factor signaling routes, which are essential for compensatory proliferation to restore normal tissue integrity after damage155. This model is supported by the observation that tumors deficient of PTEN, which converts PtdIns(3,4,5)P3 back into PtdIns(4,5)P2 (Fig. 2), are more resistant to radiation (ref. 155 and references herein). Although compensatory proliferation was not investigated in CoA mutant larvae and loss of regenerative capacity due to conditions of intracellular starvation in these mutants (reduced PtdIns(4,5)P2 synthesis and loss of Akt/PKB signaling) may result in IR sensitization, other mechanisms likely contribute as well.
Nuclear synthesis of CoA may also be essential for proper maintenance of chromatin structure by modulating PtdIns levels within the nucleus (reviewed in ref. 116). Preliminary analyses show that the dPANK and dPPCS proteins are also targeted to the nucleus/nuclear membrane, indicating that a CoA biosynthesis route may exist inside the nucleus (unpublished).

Figure 5. dPPCS1 does not affect DSB repair and G2/M cell cycle regulation.

(A-C) Meiotic recombination and DSBs repair was investigated in wild-type and mutant germaria. Ovaries were labelled with antibodies against γ-H2AvD to detect DSBs and Orb to visualize the oocytes, respectively. DAPI was used to label the DNA. DSBs are generated during meiotic recombination in the oocyte and pro-oocyte and these DSBs can be visualized with antibodies against γ-H2AvD (reviewed in ref. 167). In wild-type ovaries γ-H2AvD staining is observed in region 2a of the germarium and as meiosis proceeds DSBs are repaired, γ-H2AvD staining disappears and in stage 1 follicles the signal is absent. In DSB repair deficient mutants γ-H2AvD remains detectable in late stage (stage 7-8) oocytes, indicative for delayed repair of DSBs during meiosis168-171. Asterisks mark the oocytes in newly produced egg chambers.

(A) During wild-type cyst development the oocyte and the pro-oocyte undergo meiotic recombination in region 2a-b of the germarium (arrowheads). DSBs are generated during recombination and are fully repaired when egg chambers bud from the germarium (asterisks).

(B) In dPPCS1/1 germaria DSBs were generated during meiotic recombination and were normally repaired. Increased γ-H2AvD staining was found in the follicular epithelium (arrow) of dPPCS1/1 females compared to wild-types (see supplementary Figs. S4A-B).

(C) In P[dPPCS] transgenic flies no γ-H2AvD staining was detected in the follicular epithelium.

(D) G2/M cell cycle checkpoint entry and exit analysis in third instar larval imaginal discs. Imaginal discs from wild-type and homozygous dPPCS1 larvae were labelled with antibodies against pH3Ser10 to visualize mitotic cells 1 h after 20 Gy (checkpoint entry) and 5 h after 20 Gy (checkpoint exit). As a control Mei-41/dATR (CHAPTER 2) mutant imaginal discs were used. In wild-type and dPPCS1/1 mutant imaginal discs the amount of mitotic cells is severely reduced 1 h after 20 Gy and is back to normal levels 5 h after IR, which reflects entry into and exit from the G2/M checkpoint respectively. Mei-41/dATR mutant cells fail to activate the G2/M cell cycle checkpoint 1 h after IR and remain mitotically active. untreated; wild-type (n=12), mei-41 (n=12), dPPCS1 (n=4). 20 Gy: 1 h; wild-type (n=16), mei-41 (n=11), dPPCS1 (n=4). 20 Gy: 5 h; wild-type (n=14), mei-41 (n=9), dPPCS1 (n=3).
It has been demonstrated that PtdIns also act as nuclear second messengers to control cell division, the response to DNA damage, proliferation and chromatin condensation. Remodeling of the chromatin by PtdIns has been reported. Moreover, recent studies established a link between actin-depend chromatin remodeling and PtdIns signaling. In this view mutations in the CoA biosynthesis route may disrupt actin-dependent chromatin remodeling and/or PtdIns-dependent DNA damage signaling responses through modifying PtdIns levels. In turn disrupted chromatin organization might cause higher levels of sustained DNA damage, result in aberrant mitosis and sensitize cells to IR.

Figure 6. Mutations in CoA biosynthesis enzymes delay larval development.
Larval development in wild-type and CoA mutants. Heterozygous wild-type and CoA mutant flies were crossed for 48 h and pupation entry of siblings was measured at 22 °C on a daily basis (d 8-14) to determine developmental progression. Heterozygous and homozygous individuals were identified by the presence or absence of the Thl marker. Upon pupation entry (d 8-10) the ratio heterozygous to homozygous siblings is higher for CoA mutants compared with wild-type siblings and gradually drops below 2:1 (dashed line indicates expected ratio assuming Mendelian inheritance takes place), indicating that mutations in the CoA biosynthesis route delays larval development. Larval development was measured over at least 3 independent experiments.

Concluding remarks and perspectives
Although CoA has been extensively investigated over the past 60 years, surprisingly little is known about the physiological implications of altered de novo CoA synthesis in higher eukaryotes. Only recently, research in this area attracted the attention after PKAN was linked to defects in the PANK2 gene. Now, with the availability of mouse and Drosophila (CHAPTER 4-5) models to study the effects of altered CoA biosynthesis in animal systems, it is possible to unravel the mechanisms of PKAN disease pathogenesis (Fig. 7). Moreover, these model systems can be easily employed as platforms for screening potential therapeutic compounds. Unfortunately no clear answers about the disease pathogenesis of PKAN were obtained from the mouse PKAN model. However, studies performed in Drosophila strongly suggest that oxidative damage due to impaired Fe/cysteine metabolism does not contribute to the onset of neuronal dysfunction in young flies and possibly PKAN. Moreover, the phenotypic overlap of mutations in the Drosophila enzymes involved in CoA biosynthesis, suggest that PPCS, PPCDC, PPAT-DPCK and DPCK are candidate genes, that might be mutated in NBIA cases of idiopathic origin.

Studies in Drosophila and mice indicate that mitochondria may be involved in primary disease pathogenesis, but this is likely due to defects in the Kreb’s cycle and the β-oxidation of fatty acids, and not due to enhanced oxidative damage as a result of mitochondrial dysfunction. Defects in the Kreb’s cycle could result in accumulation of iron, while defects in fatty acid
metabolism would severely affect lipid metabolism in general. Nonetheless, increased sensitivity to ROS due to mitochondrial dysfunction and enhanced production of ROS as a result from the Fenton reaction likely contributes to disease progression, especially upon aging. Thus the primary defect that underlies PKAN most likely involves impaired lipid homeostasis, which is supported by the finding that mutations in \textit{PLA2G6} also cause NBIA\textsuperscript{41}. Therefore the preferred strategy for curing PKAN should also be aimed at restoring lipid metabolism.

\begin{center}
\textbf{Figure 7. Model to explain the physiological consequence of impaired de novo CoA biosynthesis.}
\end{center}

Depletion of \textit{de novo} CoA synthesis affects cysteine and lipid metabolism, and causes mitochondrial dysfunction (CHAPTER 4,5 and ref. 2,27,28,34,37,48,53,65). Impaired succinyl-CoA synthesis due to defects in the Kreb’s cycle\textsuperscript{34} may result in depressed heme synthesis and lead to iron accumulation\textsuperscript{54,58}. In turn, Fe/cysteine could drive the Fenton reaction\textsuperscript{59} and cause oxidative damage (CHAPTER 4 and ref. 2). Oxidative damage can be enhanced due to reduced resistance to ROS as a consequence of mitochondrial dysfunction (CHAPTER 4 and ref. 72,73). Although oxidative damage likely contributes to the formation of Lewy bodies composed of abnormal α-synuclein and/or neurofibrillary tangles due to hyperphosphorylated tau aggregation (inclusion bodies) found in PKAN patients, enhanced oxidative damage does not contribute to the onset of neuronal dysfunction in flies (CHAPTER 4) and possibly PKAN patients (ref. 64 and references herein). Impaired β-oxidation of fatty acids in the mitochondria alters lipid metabolism\textsuperscript{28,34}, which is further disrupted due to reduced synthesis of phospholipids from glycerol and acyl-CoA (CHAPTER 4). Impaired lipid metabolism could explain the observed blood cell abnormalities (acanthocythosis) and the reduced levels of plasma lipoproteins in some PKAN patients\textsuperscript{36,48}. Defects in growth factor/PtdIns signaling due to altered (phospho)lipid and hormone synthesis (CHAPTER 4-6 and ref. 34) induces a development delay (CHAPTER 4,6 and ref. 69) and affects neuronal survival during development of the CNS (CHAPTER 6 and ref. 108-110). Since the phototransduction cascade relies on lipids and PtdIns signaling\textsuperscript{172-174}, PKAN associated retinopathy could be due to defects in membrane lipid turnover\textsuperscript{2}. Altered PtdIns homeostasis may also disrupt cytoskeletal remodeling, nuclear integrity and affect DNA damage signaling\textsuperscript{116}, which in turn result in sustained levels of DNA damage, abnormal chromatin condensation and aberrant mitosis (CHAPTER 4-6). Impaired membrane integrity and aberrant actin remodeling possibly due to changes in PtdIns levels may also lead to defects in tissue morphogenesis (CHAPTER 5). The nuclear abnormalities associated with mutations in the CoA biosynthesis enzymes together with altered PtdIns/growth factor signaling likely result in apoptosis and cause sensitization to DNA damaging agents (CHAPTER 2, 4, 6 and ref. 155). Finally, results presented in this thesis suggest that in CoA mutant flies, impaired DNA integrity during CNS development represent a key defect that could
We hypothesize that as a consequence of altered lipid metabolism, growth factor/PtdIns signaling is impaired in CoA mutant flies, which could explain the observed changes in DNA integrity and hypersensitivity to IR during larval development. In wild-type flies induction of aberrant mitosis by IR correlated with a decrease in locomotor activity in young flies, two features that where enhanced in dPPCS mutants, indicating that neuronal dysfunction due to changes in CoA metabolism is linked to impaired DNA integrity during development of the CNS. Moreover, impaired DNA/neuronal integrity during development could at least partly explain neuronal dysfunction in young flies and similar effects may also explain the early symptoms of PKAN during childhood. Several neurodegenerative disorders have been linked to impaired DNA damage control including ataxia-telangiectasia and Cockayne syndrome (reviewed in ref. 161) and a challenging question would be to analyze if defects in CoA biosynthesis cause impaired DNA/neuronal integrity due to altered PtdIns/growth factor signaling in mammalian model systems and whether such defects actually contribute to PKAN disease pathogenesis.

An interesting question that should be addressed is that many disease associated mutations in PANK2 do not affect PANK2 processing, mitochondrial localization, activity, or stability. This suggests that PKAN is not only due to a loss of PANK2 activity. Many of the phenotypes associated with mutations in the Drosophila CoA enzymes indicate a loss of cellular structural integrity, possibly due to defects in membrane lipid homeostasis and PtdIns-dependent actin remodeling. In Drosophila, a FLAG-tagged dPANK protein localized at sites of membrane growth and it was proposed that localized synthesis of CoA is essential to maintain localized membrane integrity. Because dPANK can bind to the actin depolymerizing factor TSR, recruitment of dPANK (and possibly other CoA synthesis enzymes) by components of the cytoskeleton may facilitate localized synthesis of CoA to support lipid production at cellular sites of high lipid demand. These lipids are then used for membrane growth or converted into lipid derived second messengers to drive local cytoskeletal remodeling processes. If dPANK is recruited to specific cellular sites to facilitate localized CoA synthesis and modulates cellular structural integrity by providing a “regional gradient” of CoA required for the production of membrane lipids, one could speculate that domains within the dPANK protein required for protein-protein interactions to sequester dPANK to such cellular sites are essential. Structural comparison of the human PANK proteins and the Staphylococcus aureus PANK protein revealed that the human PANKs possesses two additional domains that are not conserved between animals and bacteria. Interestingly, approximately 30% of all known PKAN associated disease mutations (amino acid substitutions) are in these domains. Although their functional roles remain elusive, these domains in human PANKs may be required for feedback regulation or, additionally, could serve to facilitate protein-protein interactions. Loss of protein-protein interactions due to mutations within these domains in PANK2 could explain why many disease associated mutations in PANK2 do not impair the enzymatic properties of the protein nor its mitochondrial localization, but still can cause a neurodegenerative phenotype. Mutations that renders PANK2 catalytically inert can also cause neurodegeneration, therefore this hypothesis implies that both localized CoA synthesis and PANK2 protein-protein interactions are essential. Alternatively, it is possible that PANK2 protein-protein interactions and PANK2 activity are linked and that the binding of proteins to PANK2 depends on the activity of PANK2 itself. To address these questions the Drosophila CoA mutants provide a good model for future research.
General discussion and summary

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General discussion and summary


