The role of impaired de novo Coenzyme A biosynthesis in pantothenate kinase-associated neurodegeneration
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De novo CoA biosynthesis is required to maintain DNA integrity during development of the Drosophila nervous system

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CHAPTER 2

ABSTRACT

In a forward genetic screen in *Drosophila melanogaster*, aimed to identify genes required for normal locomotor function, we isolated dPPCS (the second enzyme of the Coenzyme A biosynthesis pathway). The entire *Drosophila* CoA synthesis route was dissected, annotated and additional CoA mutants were obtained (dPANK/fumble) or generated (dPPAT-DPCK). *Drosophila* CoA mutants suffer from neurodegeneration, altered lipid homeostasis, and the larval brains display increased apoptosis. Also, *de novo* CoA biosynthesis is required to maintain DNA integrity during the development of the central nervous system. In humans, mutations in the PANK2 gene, the first enzyme in the CoA synthesis route, are associated with Pantothenate Kinase-Associated Neurodegeneration (PKAN). Currently, the pathogenesis of this neurodegenerative disease is poorly understood. We provide the first comprehensive analysis of the physiological implications of mutations in the entire CoA biosynthesis route in an animal model system. Surprisingly, our findings reveal a major role of this conserved pathway in maintaining DNA and cellular integrity, explaining how impaired CoA synthesis during CNS development can elicit a neurodegenerative phenotype.
INTRODUCTION

Drosophila melanogaster has proven to be a powerful system to study the underlying mechanisms of various neurodegenerative diseases (1,2). In a forward genetic screen aimed to identify genes required to maintain neuronal integrity, we identified dPPCS, the second enzyme of the conserved Coenzyme A (CoA) biosynthesis route, as a novel gene required for normal locomotor behavior. CoA is produced from vitamin B5 which is subsequently converted by the action of five enzymes: PANK, PPCS, PPCDC, PPAT and DPCK (3–5). The consequences of impaired CoA synthesis in higher eukaryotes have not been investigated in detail. However, genetic analysis linked a neurological disorder to mutations in the human PANK2 gene: pantothenate kinase-associated neurodegeneration or PKAN (OMIM 234200) (6). The clinical symptoms and histological features of this Parkinson-like disorder (7,8) are well documented and although it has been suggested that increased oxidative stress is the underlying cause for neurodegeneration (9–11), it is still largely unknown how a deficiency in PANK2 affects neuronal survival and which cellular alterations are responsible for the onset and the progression of the disease. A PanK2 knockout mouse was generated, but although mutant mice exhibit retinal degeneration, no neurological abnormalities were observed (12). Like in humans, mice possess 4 PanK genes (PanK1-4) and it may be possible that in mice but not in humans other PanK genes compensate for loss of PanK2 function.

We took advantage of Drosophila to study the effect of mutations in the de novo CoA biosynthesis route, and the phenotypes of mutations in the first (dPANK/fbl) (13)), second (dPPCS) and final bifunctional enzyme (dPPAT-DPCK) were analyzed in detail. CoA biosynthesis is essential for all life forms and therefore mainly hypomorphic alleles were analyzed: dPANK/fbl1, dPPCS1 and dPPAT-DPCK43. We demonstrate that CoA mutants exhibit abnormal locomotor function which is progressive in time, have altered lipid homeostasis and show a reduced life span. Interestingly, although young mutant flies display decreased resistance to oxidative stress, oxidative damage is not the sole cause of locomotor dysfunction in young flies. Surprisingly, CoA mutant larvae show elevated levels of DNA damage during development and are hypersensitive to ionizing irradiation (IR). Exposure to IR leads to impaired locomotor activity in young wild-type flies and locomotor dysfunction is further enhanced in CoA mutants by IR, indicating that IR hypersensitivity, DNA damage and neuronal dysfunction are linked in CoA mutant flies.

RESULTS

Mutations in the phosphopantothenoylcysteine synthetase locus induce impaired locomotor function

In a forward genetic screen using P element insertion lines, aimed to identify genes required
for normal neuronal function, we identified a mutant showing uncoordinated behavior (see below and Fig. 1). In the mutant, the P element was mapped within the 5'-UTR of an uncharacterized gene CG5629 (Fig. 2A). This gene encodes two mRNAs that only differ in their 3'-UTR and that both encode a 313 amino acid protein. In silico analyses revealed that CG5629 encodes the structural ortholog of the human phosphopantothenoylcysteine synthetase (PPCS), which is the second enzyme in the biosynthesis of CoA (Supplementary Material, Figs S1–S2 and Table S1). The mutant will be referred to as dPPCS1. We cloned the 5'-UTR of the CG5629 mRNA from dPPCS1 mutants and found it to be truncated ~250 bp (Fig. 2A). It is possible that this truncation of the 5'-UTR affects mRNA processing, stability, transport, signaling or translation. Consistently, western blot analysis revealed that

![Figure 1](image-url)

**Figure 1. Mutations in genes coding for CoA biosynthesis enzymes cause progressive locomotor dysfunction, abnormal wing position and lifespan reduction.**

(A and B) Locomotor function in young (7-day-old) and aged (14-day-old) wild-type and mutant males. Only 5-day-old dPANK/fbl1/1 males were assayed. (A) The ability to initiate flight is reduced in young dPANK/fbl, dPPCS and dPPAT-DPCK mutant flies. Young dPANK/fbl1/1 flies also showed reduced flight performance, while the ability to climb against gravity was impaired in all CoA mutants compared to wild-type. Asterisks mark significant changes compared to wild-type flies. (B) Wild-type flies showed no age-related decrease in flight behavior, but the ability to climb against gravity was significantly changed. Aged dPPAT-DPCK mutants showed no differences in the initiation of flight compared to young flies, but flight performance and the ability to climb against gravity were affected compared to young adults. Aged dPPCS mutants showed a decrease in flight behavior, but no decrease in geotaxis was found compared to young flies. Double-rowed asterisks mark significant changes compared to young flies. (C) Wing phenotypes in wild-type (Ca and c) and mutant 14-day-old flies (5-day-old dPANK/fbl) (Cb and d). dPANK/fbl, dPPCS and dPPAT-DPCK mutant flies have held-out (Cb) and held-up (Cd) wings, quantified in (E), which are indicative of abnormal indirect flight muscle contraction. (D) Lifespan curves of wild-type and mutant flies. Median survival of: wild-type (50 days, n = 1045), dPANK/fbl1/1 (8 days, n = 240), dPPCS1/1 (42 days, n = 1230), dPPAT-DPCK43/43 (32 days, n = 1043), and P[dPPCS]+/--;dPPCS1/1 (61 days, n = 614). Lifespan curves of dPANK/fbl1/1, dPPCS1/1 and dPPAT-DPCK43/43 significantly differ from the wild-type (log-rank, p < 0.001).
Figure 2. The Drosophila CoA biosynthesis route.

(A) Physical map of the dPPCS locus. The region comprises cytological position 91F3–91F4 of chromosome 3R and harbors the CG5629 gene. CG5629 encodes two mRNAs and both transcripts have five exons that encrypt the tentative fly PPCS ortholog (Supplementary Material, Figs S1–S2). The P-lacW insertion allele dPPCS1 is indicated by the triangle. Transcripts are indicated by black boxes and their encoded proteins are depicted underneath (white boxes). dPPCS1 causes a ~250 bp truncation of the dPPCS mRNA (gray box). The genomic region missing in the imprecise excision allele, dPPCS33, is indicated (dashed line). (B) The de novo CoA synthesis route. Higher eukaryotes depend on dietary pantothenate to produce CoA (21–23, 43). 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 (3–6). E.C. numbers are indicated. (C) Diagram of the de novo CoA biosynthesis in animals. The Drosophila genome encodes a single copy of PANK, PPCS, PPCDC, a bifunctional PPAT-DPCK and a DPCK (Supplementary Material, Figs S1–S2). Mammalian PANK can also utilize pantetheine and N-pantothenoylcysteine to produce 4’-phosphopantetheine and (R)-4’-phospho-N-pantothenoylcysteine, respectively (34). CoA and its acyl esters are utilized by many enzymes involved in cellular metabolism including the Krebs cycle, fatty acid production and the synthesis of some amino acids. (D) Western blot analyses of wild-type, dPPCS1/1, dPPCS1/33 and dPANK/Fbl1/1 third instar larval brain extracts by using polyclonal antibodies raised against dPPCS (left panel) and dPANK/Fbl (right panel). dPPCS protein expression is reduced in dPPCS1/1 (~13% reduction (±4 SEM, n=4)) and further reduced in dPPCS1/33 (~28% reduction (±9 SEM, n = 4)) compared to wild-type. dPANK expression is severely reduced in dPANK/Fbl1/1 brains. Antibodies against β-actin or γ-tubulin were used as loading controls. (E) RT–PCR analysis of mRNA levels from wild-type and dPPAT-DPCK43/43 males. Relative mRNA levels of dPPAT-DPCK and the neighboring gene CG5505 were determined by semi-quantitative PCR (incrementing cycles are denoted), using the ribosomal rP49 mRNA as internal control. Compared to wild-type, the CG5505 mRNA is not affected in dPPAT-DPCK43/43 (5% reduction ± 11 SEM, n = 3), but the expression of the dPPAT-DPCK mRNA is 23% lower (±5 SEM, n = 3) compared to the wild-type.
levels of dPPCS protein are reduced in dPPCS^{1/1} mutants (Fig. 2D). Ubiquitous ectopic expression of a FLAG-tagged dPPCS cDNA (P[dPPCS]) in the dPPCS^{1/1} background (P[dPPCS]/++;dPPCS^{1/1}) rescued the impaired locomotor phenotype and the transgene essentially suppressed all the major phenotypes (decreased life span, decreased levels of triglycerides, decreased negative geotaxis, increased sensitivity to ionizing radiation (IR), impaired DNA integrity and increased apoptosis) associated with the dPPCS^{1} mutation (Figs 1D, 3A, 4C, 5A and D). A presumed null mutation (dPPCS^{33}) was created by imprecise P element excision (14) (Fig. 2A) and in contrast to dPPCS^{1/1} flies, dPPCS^{33/33} mutants die as first instar larvae. However, dPPCS^{1/33} mutants were viable, show locomotor impairment (Fig. 1A) and these transheterozygous mutants generally have a more severe phenotype especially in proliferating tissue as compared to dPPCS^{1/1} mutants (Fig. 6, Supplementary Material, Fig. S3). Together these results demonstrate that dPPCS^{1} constitutes a hypomorph and viable allele of the dPPCS gene, while dPPCS^{33} is most likely a null and lethal allele, and imply that dPPCS is essential in Drosophila. Intriguingly, mutations in dPPCS induce locomotor dysfunction, suggesting a link between CoA biosynthesis and neuronal integrity.

The de novo CoA synthesis pathway is conserved in Drosophila

De novo synthesis of CoA occurs in a conserved route in which vitamin B5 is subsequently modified by the action of five enzymes: PANK, PPCS, PPCDC, PPAT and DPCK (Fig. 2B) (3–5,15). Unlike in other model organisms, CoA biosynthesis in Drosophila is largely unknown. We performed in silico analyses and found five Drosophila loci coding for orthologs of PANK (dPANK/fbl), PPCS (dPPCS), PPCDC (dPPCDC), a bifunctional PPAT-DPCK (dPPAT-DPCK) and a single DPCK (dDPCK) (Fig. 2C, Supplementary Material, Figs S1–S2). Of these genes, only PANK, also known as fumble or fbl, has been studied previously and was described as a gene required for mitosis and male fertility (13).

To further analyze the Drosophila CoA enzymes, antibodies were raised against dPPCS and dPANK/Fbl. The expression levels of dPPCS and dPANK/Fbl (Fig. 2D) are affected in dPPCS and dPANK/fbl mutants, respectively. These results also indicate that the dPPCS and dPANK/fbl mutants under investigation are hypomorphs. Consistent with their essential role in cellular metabolism, dPPCS and dPANK/fbl proteins are ubiquitously expressed during development and in all wild-type tissues investigated (not shown).

Mutations in genes required for de novo CoA biosynthesis cause locomotor dysfunction and a reduced life span

Previously, it was reported that dPANK/fbl flies show movement abnormalities (13), but this was never further investigated. The availability of a dPANK/fbl mutant (13) and a dPPCS mutant allowed us to examine whether the movement abnormalities in these mutants are comparable and if so, enables us to investigate how impaired CoA biosynthesis
in general gives rise to behavioral abnormalities and whether this is due to neurodegeneration. In addition, we created an allelic series of the \textit{dPPAT-DPCK} gene by mobilizing the P\textsuperscript{SUPor-P}\textsubscript{KG04801} insertion, which is located \textasciitilde 50 bp upstream of \textit{dPPAT-DPCK} (www.flybase.org), and recovered a hypomorphic allele, \textit{dPPAT-DPCK}\textsuperscript{43} (see Materials and methods and Fig. 2E). First we tested in detail the CoA mutants for locomotor defects, an indicator of neuronal dysfunction (16). To assess locomotor behavior, we evaluated the ability to climb and fly in young (7-day-old) and aged (14-day-old) flies. As can be seen in Figure 1A, all the CoA mutants suffered from loss of locomotor function and locomotor dysfunction worsened in time (Fig. 1B). Progressive loss of locomotor activity could not be monitored in \textit{dPANK/fbl} mutants as they were extremely short-lived (see below). Consistent with their poor flight performance, \textit{dPANK/fbl}, \textit{dPPCS} and \textit{dPPAT-DPCK} mutant flies had held-up and held-out wings, indicative of abnormal indirect flight muscle contraction (Fig. 1C). In addition to adult locomotor defects, \textit{dPPCS} and \textit{dPPAT-DPCK} mutant adults become paralytic when exposed to heat and \textit{dPANK/fbl} and \textit{dPPAT-DPCK} mutant larvae displayed locomotor defects (Supplementary Material, Fig. S4A and B), features also associated with impaired function of the central nervous system (CNS).

Figure 3. \textit{dPPCS} mutant flies display retinal and neurodegeneration.

(A–E) Horizontal head sections of 30-day-old wild-type (A and C) and \textit{dPPCS}\textsubscript{1/33} (B and D) and 14-day-old \textit{dPANK/fbl1/1} mutants (E) stained with toluidine-blue. (B) In \textit{dPPCS}\textsubscript{1/33} brains vacuoles are present, suggesting a neurodegenerative phenotype (arrows). (D) \textit{dPPCS}\textsubscript{1/33} flies display degeneration of photoreceptor cells visible by loss of retinal structure and formation of vacuoles (arrows). (E) \textit{dPANK/fbl1/1} brains display vacuoles (arrows) and note that throughout the brain, a punctuated pattern of little 'vacuoles' can be found. Scale: 50 \textmu m.

Neurologically impaired flies often display reduced lifespan (16); therefore, we also investigated longevity in the CoA mutants. Median survival of wild-type flies was 50 days. This was reduced to 8 days for \textit{dPANK/fbl1/1} flies, 42 days for \textit{dPPCS}\textsuperscript{1/1} flies and 32 days for \textit{dPPAT-DPCK}\textsuperscript{33/43} flies (Fig. 1D). Finally, to conclusively link defective CoA metabolism to
locomotor dysfunction and neurodegeneration, we analyzed the brains from 30-day-old wild-type and dPPCS1/33 and 14-day-old dPANK/fbl1/1 flies (Fig. 3). Although the phenotype differs between the two CoA mutants, the brains from dPPCS1/33 and dPANK/fbl1/1 both displayed vacuolization, indicative for neurodegeneration (Fig. 3B and E). In addition, dPPCS1/33 flies suffered from retinal degradation as visible by the formation of vacuoles and loss of retinal structure (Fig. 3D).

In summary, although the severity of the phenotypes varies amongst the CoA mutants (Supplementary Material, Table S2), our genetic analysis suggests that disrupted CoA biosynthesis in general, underlies neuronal dysfunction.

**Lipid biosynthesis is disrupted in Drosophila CoA mutants**

CoA is an essential cofactor for the synthesis of many lipids (17) and mutations in lipid metabolism can cause neurodegeneration in flies (18–20). Therefore, it was investigated whether lipid homeostasis was disrupted in CoA mutant flies. Consistent with a role in lipid homeostasis, the stored fatty acids in the form of triglycerides were reduced with ~25% in CoA mutant flies compared to wild-type (Fig. 4A). Similarly, the synthesis of neutral lipids during oogenesis was reduced in the CoA mutants (not shown). Moreover, a 2-fold reduction in phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was found in head homogenates from dPPCS1/33 flies (Fig. 4B). This reduction in phospholipids likely reflects a global effect on phospholipid production, since the percentages of PS, PE and PC of the total pool of phospholipids were similar to the percentages found in wild-type heads (Fig. 4B). Finally, consistent with a defect in lipid

**Figure 4. CoA mutant flies display impaired lipid homeostasis.**

(A) The amount of triglycerides was determined in young dPANK/fbl (5-day-old) & aged dPPCS and dPPAT-DPCK mutants (14-day-old), and normalized to levels of wt flies of identical age. All CoA mutants have ~25% less TGA compared to wild-type flies. (B) Phospholipids/mg protein were determined in wild-type and dPPCS1/33 flies. Heads from 14-day-old dPPCS1/33 flies contain ~2-fold less PS, PC & PE compared to wild-type flies. (C and D) Head sections stained with toluidine-blue showing the pericerebral fat bodies (arrows) in 30-day-old wild-type (C) and dPPCS1/33 (D) flies. The pericerebral fat body is almost absent in dPPCS1/33 flies compared to wild-type flies (dashed lines). Scale; 100 µm.
synthesis, the pericerebral fat body was almost absent in 30-day-old dPPCS1/33 flies compared to wild-type flies (Fig. 4C and D). These data demonstrate that mutations in CoA synthesis affect lipid homeostasis and since lipid metabolism was impaired in all CoA mutants; this strongly suggests that this common feature, downstream of de novo CoA synthesis, contributes to the abnormal locomotor behavior in the fly mutants.

**CoA mutants are hypersensitive to ROS, but overexpression of SOD, CAT and TRX does not rescue locomotor dysfunction in young flies**

Neurodegenerative diseases such as neurodegeneration with iron accumulation, which includes PKAN, are classified as disorders associated with mitochondrial dysfunction (reviewed in 8), and mitochondrial dysfunction associated with PANK deficiency has been reported in humans (21,22) and mice (23). Because mitochondrial dysfunction can lead to decreased resistance to reactive oxygen species (ROS), increased oxidative stress may be one of the factors that induce neurodegeneration in case CoA synthesis is disrupted. Neurodegeneration in *Drosophila* is also frequently associated with increased oxidative stress (24,25). To investigate whether oxidative stress contributes to the CoA mutant phenotype, we analyzed the sensitivity of young (2-day-old) CoA mutant males to paraquat, dithiothreitol (DTT) and H$_2$O$_2$. In general, dPPCS mutants are less sensitive to the ROS-inducing agents when compared with the other CoA mutants (see also discussion), but all CoA mutants were hypersensitive to at least two of these ROS inducing agents (Fig. 5A). dPANK/fbl1/1 also displayed mild sensitivity when only H$_2$O was added to the medium. This phenotype is frequently found in mutants that also exhibit reduced lifespan and sensitivity to ROS and is referred to as ‘wet starvation conditions’ (26,27). This observation suggests that dPANK/fbl mutants show a reduced resistance to starvation. Overexpression of ROS scavengers [CuZn-superoxide dismutase (SOD), catalase (CAT), thioredoxin reductase (TRX)] (28) in the dPPCS1/1 flies (SOD–CAT–TRX;dPPCS1/1) suppressed the sensitivity to DTT, H$_2$O$_2$ and paraquat supporting that oxidative stress was indeed the main cause for the toxicity under these conditions and that CoA mutants are hypersensitive to ROS-mediated toxicity.

In the CoA synthesis route, cysteine incorporation occurs by PPCS in a step downstream of PANK2 and it was postulated that PKAN disease pathogenesis is due to enhanced oxidative damage as a result of impaired Fe/cysteine metabolism which could drive the Fenton reaction (29) leading to the production of ROS (6). (Fig. 2B and C). In theory, impaired function of dPANK/fbl or dPPCS, but not dPPAT-DPCK would lead to an accumulation of cysteine. If correct this will result in hypersensitivity to cysteine for dPANK/fbl and dPPCS mutants, but not for dPPAT-DPCK mutants. Consistent with this hypothesis, survival of dPANK/fbl and dPPCS mutants, but not dPPAT-DPCK mutants, decreased when larvae were fed with a 100 mm cysteine solution (Fig. 5B). This cysteine induced lethality was likely due to elevated oxidative damage, because survival of SOD–CAT–TRX;dPANK/fbl1/1 and SOD–CAT–TRX;dPPCS1/1 mutants was restored to wild-type levels in the presence of cysteine (Fig. 5B). These data suggest the presence of a Fenton reaction that generates ROS.
Figure 5. Oxidative damage is not the primary cause of neuronal dysfunction in CoA mutant flies. (A) Two-day-old CoA mutant males were exposed to H2O and to ROS inducing agents (H2O2, DTT and paraquat dissolved in H2O + 5% glucose) and their survival was analyzed (mean survival ± standard error is depicted, log-rank ***P < 0.001). dPANK/fbl1/1 mutants display sensitivity to H2O. dPANK/fbl1/1 and dPPAT-DPCK43/43 flies are extremely sensitive to H2O2, DTT and paraquat, while dPPCS1/1 flies show a decrease in resistance to H2O2 and DTT. Overexpression of ROS scavengers (SOD–CAT–TRX) (28) in dPPCS1/1 (SOD–CAT–TRX;dPPCS1/1) suppressed the dPPCS1/1 associated sensitivity to H2O2 and DTT, and increased the resistance to paraquat of this mutant. (B) Cysteine survival graph. The percentage of homozygous survivors was calculated under control conditions (H2O) and after feeding larvae a 100 mm cysteine solution. The percentage homozygous survivors under control conditions were set to 100%. dPANK1/1 and dPPCS1/1, but not dPPAT-DPCK43/43 larvae are sensitive to 100 mm cysteine. The sensitivity of the dPPCS1/1 and dPANK/fbl1/1 mutants could be suppressed by overexpression of ROS scavengers (SOD–CAT–TRX;dPPCS1/1 and SOD–CAT–TRX;dPANK/fbl1/1), suggesting that cysteine inflicts an oxidative stress response. (C) Ability to climb in the presence of ROS scavengers. Seven-day-old dPPCS1/1 and dPPAT-DPCK43/43 males have impaired negative geotaxis when compared with wild-type males. Flies overexpressing ROS scavengers (SOD–CAT–TRX) have normal climbing behavior when compared with wild-type flies. Overexpression of SOD–CAT–TRX in a dPPCS1/1 (SOD–CAT–TRX;dPPCS1/1) or in a dPPAT-DPCK43/43 (SOD–CAT–TRX;dPPAT-DPCK43/43) mutant background did not suppress the dPPCS1/1 and the dPPAT-DPCK43/43 associated inability to climb against gravity, while, as a positive control, overexpression of P[dPPCS] (P[dPPCS]/+;dPPCS1/1) rescued the locomotor defects associated with a mutation in dPPCS.

dPANK/fbl and dPPCS mutants.

To further characterize the role of decreased resistance to ROS, we tested whether the ROS scavengers could also rescue the locomotor defects of young (7-day-old) CoA mutants.
mutant flies. As can be seen in Figure 5C, geotaxis was still compromised in SOD–CAT–TRX;dPPCS1/1 and SOD–CAT–TRX;dPPAT-DPCK43/43 flies. Although not quantified with large numbers (due to low viability), climbing of SOD–CAT–TRX;dPANK/fbl1/1 flies was also not improved (not shown). Because overexpression of SOD–CAT–TRX fully restored hypersensitivity to oxidative stress, additional causes, other than oxidative damage per se, are involved in the onset of locomotor dysfunction and possibly neuronal dysfunction in young CoA mutant flies.

**Impaired CoA biosynthesis affects DNA integrity and cellular survival in the developing brain**

Because oxidative stress is most likely not causing locomotor dysfunction and possibly neuronal dysfunction in CoA mutant flies, other cellular abnormalities were explored. Previously, it was reported that larval brains of dPANK/fbl mutants show increased levels of abnormal mitotic chromosomes (13). Abnormal mitotic structures are indicative for the presence of higher levels of sustained damaged DNA (30). Moreover, several neurodegenerative diseases are associated with impaired DNA integrity (31). We investigated whether impaired CoA biosynthesis in general leads to spontaneous higher levels of DNA damage in proliferating cells and we analyzed mitotic chromosomes in larval brains of the CoA mutants (Fig. 6Aa–g). In CoA mutants, a high incidence of abnormal mitotic chromosomes was found, indicating that impaired CoA biosynthesis spontaneously affects DNA integrity. An exception to this is the fact that in dPPCS1/1 mutants, no aberrant mitoses were observed in the untreated brains. It is possible that in dPPCS1/1 mutants (and not in dPPCS1/33, dPANK/fbl1/1 and dPPAT-DPCK43/43), the levels of CoA are just sufficient to maintain chromosome integrity under normal growing conditions. Differences in phenotypic strength between dPPCS1/1 and dPPCS1/33 are also evident in other proliferating tissues [spermatogenesis (Supplementary Material, Fig. S3), developing brains (Fig. 6), oogenesis (not shown)]. Next we investigated whether the IR causes elevated levels of DNA damage in CoA mutants compared to wild-type. The frequency of IR-induced abnormal mitotic structures was higher in all three mutants compared to wild-type, indicating that CoA is required for a proper DNA damage response (Fig. 6Aa). After IR, levels of CoA may not be sufficient to allow repair and maintain genome integrity resulting in increased numbers of abnormal mitotic chromosomes.

Next, we analyzed the presence of phosphorylated Histone 2AvD (γ-H2AvD) (32), a marker for the presence of damaged DNA (33), to test whether aberrant mitoses indeed correlated with higher levels of sustained damaged DNA. The brains of dPANK and dPPCS mutant larvae (but not of dPPAT-DPCK43/43 larvae) contained more cells that stained positive for γ-H2AvD than wt brains (Fig. 6Ba–e and Da). In contrast to other described phenotypes (for which one copy of the dPPCS transgene is sufficient to rescue the phenotype), two copies of dPPCS transgene expression are required to rescue the increased levels of γ-H2AvD. It should be mentioned that the dPPCS transgene lacks the endogenous 5’/-3’-UTRs and the
Figure 6. CoA mutant larval brains display impaired DNA integrity during larval development. (Aa–g) Mitotic chromosomes from third instar wild-type and mutant larval brains, 5 h after recovery from 20 Gy IR or left untreated, were visualized with antibodies against pH3Ser10 and analyzed for mitotic abnormalities. (Aa) Untreated dPANK/fbl1/1, dPPCS1/1 and dPPAT-DPCK43/43 brains contained a high % of abnormal mitoses. The amount of abnormal mitoses increased in all mutants 5 h after recovery from 20 Gy. (Ab–d) Normal mitotic chromosomes: metaphase (Ab), anaphase bridge (Ac) and anaphase (Ad). (Ae–g) Abnormal mitotic chromosomes: fragmented chromosomes (Ae), lagging chromosome (Af), multiple centrosomes (Ag).

The amount of brains investigated was: (untreated); wild-type n = 21, dPANK1/1 n = 17, dPPCS1/1 n = 17, dPPCS1/33 n = 34, dPPAT-DPCK43/43 n = 14, P[dPPCS]/+;dPPCS1/1 n = 19 (20 Gy: 5 h recovery); wild-type n = 24, dPANK1/1 n = 15, dPPCS1/1 n = 21, dPPCS1/33 n = 35, dPPAT-DPCK43/43 n = 12, P[dPPCS]/+;dPPCS1/1 n = 16. (Ba–e) 3rd instar larval brains were labeled with an antibody against γ-H2AvD to detect DSBs. (Ba and b) Representative images of a wild-type and a mutant third instar larval optic lobe showing enhanced DNA damage. (Bc–e) High magnification of a field of three cells that contain γ-H2AvD foci (arrows). The DNA is visualized with DAPI. (Ca and b) Freshly dissected wild-type and mutant larval brains were stained with acridine orange and investigated by FM to detect apoptotic cells. (Ca and b) Representative images of a wild-type and a mutant third instar larval optic lobe showing enhanced apoptosis. Twenty larval brains were analyzed for wild-type and each mutant. (Da) Quantification of the total amount of γ-H2AvD positive cells in third instar larval brains. dPANK/fbl1/1, dPPCS1/1 and dPPCS1/33, but not dPPAT-DPCK43/43 third instar larval brains contained significantly more cells that stained positive for γ-H2AvD, indicating that these brains accumulate elevated levels of DSBs. Ectopic expression of one copy of the P[dPPCS] transgene reduced the amount of γ-H2AvD positive cells in dPPCS1/1 (P[dPPCS]/+;dPPCS1/1), and two copies (P[dPPCS];dPPCS1/1) reduced the amount of γ-H2AvD positive cells back to wild-type levels. The amount of brains investigated was: wild-type n = 25, dPANK1/1 n = 25, dPPAT-DPCK43/43 n = 12, dPPCS1/1 n = 17, dPPCS1/33 n = 23, P[dPPCS]/+;dPPCS1/1 n = 19, P[dPPCS];dPPCS1/1 n = 16. (Db) Quantification of apoptosis in third instar larval brains. Brains were scored as non-apoptotic (Ca) or as enhanced apoptotic (Cb). Compared with wild-type brains, the brains of the CoA mutant larvae display enhanced acridine orange staining, indicating that cells within these brains are apoptotic. The amount of brains investigated was: wild-type n = 20, dPANK1/1 n = 20, dPPAT-DPCK43/43 n = 21, dPPCS1/33 n = 20, dPPCS1/1.
transgene has a FLAG-tag. It is possible that these aspects of the FLAG-dPPCS mRNA/protein interfere with normal dPPCS activity/function and optimal dPPCS activity may be required to prevent accumulation of γ-H2AvD.

We also investigated whether impaired DNA integrity coincided with increased cell death. Acridine orange uptake in freshly dissected brains revealed that all three CoA mutants displayed enhanced staining compared to wild-type brains, demonstrating that apoptosis was enhanced in mutant larval brains (Fig. 6Ca–b and Db). Increased γ-H2AvD and TUNEL staining was also found in dPPCS/1 follicle cells during early oogenesis, indicating that mutations in CoA synthesis also affects DNA integrity in other proliferating tissues (Supplementary Material, Fig. S5). Although differences exist between mutants (see also discussion), together our findings clearly demonstrate that mutations in CoA biosynthesis result in impaired DNA integrity in proliferating cells, which can be enhanced after exposure to IR.

Increased levels of DNA damage are indicative for defects in DNA damage responses and if CoA mutants exhibit aberrant DNA damage responses then the prediction is that CoA mutants are increased sensitive to exogenously induced DNA damage. To test this, CoA mutant larvae were subjected to 20 Gy and the survival rate of homozygous adults was determined. dPPCS, dPANK/fbl and dPPAT-DPCK mutants were hypersensitive to IR, demonstrating that altered CoA biosynthesis causes DNA damage hypersensitivity (Fig. 6Dc). Since CoA mutant flies are increased sensitive to oxidative stress, we tested whether there was a link between oxidative damage and sensitivity to IR in CoA mutants. However, SOD–CAT–TRX;dPPCS/1 were also sensitive to IR, while survival was restored in P[dPPCS]/+;dPPCS/1 after 20 Gy, suggesting that IR sensitivity is not due to enhanced ROS. Scale; 50 µm (Ba and b and Ca and b), 6 µm (Bc–e).

Finally, to establish a link between IR hypersensitivity and neuronal dysfunction, we analyzed the absolute climbing behavior of young (24-h-old) wild-type and dPPCS/1 flies under control conditions and after IR. In case DNA damage applied to the developing larval brains is linked to locomotor dysfunction in young flies, then IR should induce behavioral defects in a wild-type background. For this assay, the percent of untreated flies that was able to climb was divided by the percent of flies that was able to climb after exposure to 20 Gy. Indeed, this ratio for wild-type adults was 1.69 ± 0.13 SEM (n = 10), demonstrating that the ability to climb was decreased in young wild-type flies after exposure to IR. Interestingly, in dPPCS/1 this decreased ability to climb was enhanced compared to wild-type (ratio of untreated to irradiated was 3.23 ± 0.56 SEM, n = 10, P < 0.05) and was restored in P[dPPCS]/+;dPPCS/1 flies (1.97 ± 0.15 SEM, n = 10, P < 0.05). These results suggest an
intricate link between impaired DNA integrity and CoA deficiency-associated neuronal dysfunction in young flies.

DISCUSSION

Here we demonstrate that defective CoA biosynthesis induces a pleomorphic phenotype. Mutations in genes coding for enzymes of this conserved pathway lead to increased sensitivity to ROS, impaired DNA integrity in proliferating cells of the developing CNS, cause changes in lipid homeostasis, induce vacuoles in adult brains and result in abnormal locomotor function in adult flies. Moreover, CoA mutant flies are hypersensitive to IR and our data show that locomotor dysfunction can be enhanced by IR in CoA mutants and induced in wild-type. Combined, our findings suggest a link between IR hypersensitivity, neuronal dysfunction and impaired DNA integrity.

Although the CoA mutants share many phenotypic characteristics, the strength of the various phenotypes differ between mutants and some characteristics are even absent in specific CoA mutants. Several factors could contribute to these differences. De novo CoA synthesis is essential and therefore hypomorphic alleles of the CoA mutants (dPANK/fbl1, dPPAT-DPCK43, dPPCS) were used to investigate the consequences of impaired de novo CoA biosynthesis. Differences between the CoA mutants may, therefore, reflect differences in the expression levels of their respective affected enzymes. Phenotypic differences might also be related to the position (first, second etc.) the individual CoA enzymes occupy within the CoA synthesis pathway and thus could be directly linked to their cofactor/substrate utilization. Regulation of CoA biosynthesis occurs at the level of mitochondrial localized PANK and PPAT-DPCK, but not PPCS (17). In case dPPCS is mutated, dPANK/fbl and/or dPPAT-DPCK activity might be upregulated to compensate for reduced (R)-4’-phospho-N-pantothenoylcysteine synthesis (Fig. 2C). Alternatively, phosphorylation of pantetheine and pantetheinoylcysteine by dPANK/fbl could bypass dPPCS, but not dPPAT-DPCK (34), and these routes might be stimulated when dPPCS is mutated. Combined, these differences could explain the generally less severe phenotype of the dPPCS mutants (especially the sensitivity to exogenously applied ROS) as compared to the other CoA mutants. Furthermore, differences between the dPANK/fbl and dPPAT-DPCK mutants could be due to the presence of the conserved dDPCK gene that may partially compensate for loss of dPPAT-DPCK activity. Finally, dPPAT-DPCK mutants do not suffer from impaired cysteine metabolism and thus, in contrast with dPANK/fbl and dPPCS mutants, dPPAT-DPCK mutants likely also not form enhanced ROS via the Fenton reaction.

Consistent with the latter hypothesis and our current understanding of the CoA synthesis route, in which cysteine is incorporated in the second step of this pathway (Fig. 1B–C), we found that dPPCS and dPANK/fbl, but not PPAT-DPCK mutants are hypersensitive to cysteine. Increased levels of cysteine have been found in the brains of a few patients
with clinical symptoms of PKAN (35), suggesting that cysteine metabolism is disrupted in PKAN patients. PKAN patients also display iron accumulation in specific areas of the brain (9) and together with high levels of cysteine, this led to the hypothesis that impaired iron/cysteine metabolism may drive the Fenton reaction (29), leading to the production of ROS (6). We show that in flies overexpression of ROS scavengers can protect against cysteine induced lethality, indicating that this lethality is due to enhanced oxidative stress. These data, together with the increased sensitivity of CoA mutants to ROS, indicate that impaired CoA metabolism can lead to enhanced levels of oxidative stress. However, our data also suggest that in addition to oxidative stress and altered cysteine metabolism, there are other consequences of impaired de novo CoA synthesis that induce locomotor impairment in Drosophila. This is based on the observation that PPAT-DPCK mutants, which are not hypersensitive to cysteine, also develop locomotor abnormalities and is based on the observation that overexpression of ROS scavengers did not improve the locomotor defects in young flies. Although it should be noted that SOD, CAT and TRX may not be effective in scavenging ROS in every cellular compartment and it is also unknown whether the ROS scavengers are equally effective in all tissues such as in brain cells.

The Drosophila CoA mutants also display abnormalities in lipid homeostasis, which is consistent with the fact that CoA is required for lipid metabolism (17,23). Impaired lipid metabolism has also been implicated in PKAN disease pathogenesis (6,36,37). In addition to abnormal lipid and cysteine metabolism and enhanced levels of oxidative stress, we found that CoA mutant larvae were hypersensitive to IR and their brains displayed increased levels of impaired DNA integrity. Hypersensitivity to IR, enhanced levels of γ-H2AvD, abnormal mitosis and induction of locomotor dysfunction by IR indicate that locomotor dysfunction in young flies is at least partly due to impaired DNA integrity. We demonstrate that abnormal chromosome organization and increased levels of DNA damage are present in CoA mutant cells during mitosis, suggesting that CoA is required for proper cytokinesis. This is supported by the observation that cytokinesis is abrogated in the testis of dPANK/fbl mutants (13) and dPPCS mutants (Supplementary Material, Fig. S3). Because both lipid homeostasis and DNA integrity are affected in all the mutants, we favor the following explanations for locomotor impairment caused by impaired CoA biosynthesis. Changes in lipid homeostasis, especially phospholipids, will disrupt the integrity of membranes in the CoA mutants. Depletion of the lipid stores and reduced production of lipids may hamper the renewal of damaged membranes and lipid peroxides, thereby affecting the integrity of tissues including the CNS leading to neurodegeneration. Alternatively and not mutually exclusive, aberrant membrane lipid composition might disturb the dynamic interplay between cytoskeletal components, membrane structures and lipid signaling, leading to division errors during CNS development that in turn may result in impaired DNA integrity. Therefore, it is possible that abnormal lipid homeostasis directly leads to impaired neuronal function and this neuronal dysfunction is enhanced by increased DNA damage as a result of these lipid abnormalities. Several neurological disorders are associated with defects in DNA repair (31), but increased DNA damage has never been linked to altered CoA biosynthesis.
All together our data show that impaired DNA integrity is a novel cellular consequence when the \textit{de novo} CoA biosynthesis pathway is affected and, the impaired DNA integrity is tightly linked to impaired locomotor function.

\section*{MATERIAL AND METHODS}

\textit{Drosophila} stocks and genetics: Fly stocks were maintained at 22°C according to standard protocols. For wild-type preparations \textit{y^w118} was used. Stocks were obtained from the Bloomington Stock Centre (Indiana University, USA). ZnSod-Cat-Trx was a gift from R.S. Sohal (Southern Methodist University, USA). \textit{dPPCS}^{33} and \textit{dPPAT-DPCK}^{43} were generated by imprecise excision of \textit{P[lacW]dPPCS}^{1} and \textit{P[SUPor-P]KG0480} respectively (14). Imprecise excisions were detected by PCR, cloned and the affected regions were sequenced to determine the scar left behind by the P-element. \textit{dPANK/fbl} \textsuperscript{1} is a hypomorphic allele of \textit{dPANK/fbl} and previously described (13). The hypomorphic allele \textit{dPPCS}^{1} (\textit{P[lacW]dPPCS}^{1}) was created by transposon mutagenesis (38) and the insertion mapped using plasmid rescue analysis (39). The transposon landed inside the 5'-UTR of the \textit{CG5629} gene, causing a truncation of the mRNA. The \textit{dPPCS}^{33} allele was generated by imprecise excision of the P-element. When \textit{dPPCS}^{1} was placed over \textit{Df(3R)Dl-KX23}, covering at least 50 genes surrounding the \textit{dPPCS} locus, transheterozygous flies were viable, sterile and were approximately equally sensitive to IR as \textit{dPPCS}^{1/33} mutant flies (not shown). On the contrary transheterozygous \textit{Df(3R)Dl-KX23/dPPCS}^{33} are not viable, suggesting that \textit{dPPCS}^{33} is a null allele of \textit{dPPCS}. Likewise, \textit{dPPCS}^{1/33} is lethal in the first instar larval stadium and sequence analysis revealed that imprecise P-element excision removed the entire first exon (including the ATG initiation codon) of the \textit{CG5629} gene. In \textit{dPPAT-DPCK}^{43}, the transposon left behind a scar of \textasciitilde450 bp P-element specific DNA \textasciitilde50 bp upstream of the \textit{dPPAT-DPCK} gene (\textit{CG10575} at 64E7). Homozygous \textit{dPPAT-DPCK}^{43} is sensitive to IR and females are impaired fertile. When \textit{dPPAT-DPCK}^{43} was placed over \textit{Df(3L)ZN47} (64C;65C), transheterozygous flies were sensitive to IR and displayed fertility defects (not shown). These phenotypes were not caused by a contribution of the neighboring gene (\textit{CG5505}) since the mRNA expression levels (as determined by RT–PCR) of \textit{CG5505} did not show significant changes in \textit{dPPAT-DPCK}^{43} mutant flies compared to wild-type expression levels (\(P < 0.4, n = 3\)), while the expression of \textit{CG10575} was reduced in \textit{dPPAT-DPCK}^{43/43} males (\(P < 0.03, n = 3\)) (Fig. 2E), demonstrating that the \textit{dPPAT-DPCK}^{43} allele is a hypomorphic allele of the \textit{dPPAT-DPCK} gene.

\textbf{Sensitivity screening:} Heterozygous flies were crossed (days 1–3) in vials and on day 5 larvae were exposed to 20 Gy of \textgamma-rays delivered by a Cs137 unit (IBL 637, CIS Biointernational) or left untreated. At days 14–21, the percentage of homozygous adults in the F1 generation was determined. In a similar screen larvae were fed a 0.5 ml 100 mm L-cysteine solution in H2O. At least three independent experiments were performed.
**Molecular techniques:** The dPPCS cDNA was cloned into pBUF, provided by J. Sekelsky (University of Carolina, USA), behind the ubiquitin promoter and in frame with a NH2-terminal FLAG. The promoter-FLAG-cDNA was cloned in front of a Bgh polyadenylation signal present in pCaSpeR4-Pme1-Bgh provided by L.G. Fradkin (LUMC, The Netherlands). Transgenic flies were created by Genetic Services Inc. (USA). The 5′-UTR was cloned from dPPCS1/mRNA using the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech). Affinity purified polyclonal antibodies were raised in rabbits against synthetic peptides (dPANK/Fbl YFEPKDITPDEQDREC and CDEPPEKAPTSKHSTR) and dPPCS (CDMMPTHKMQSGDGAP and CVQKHGEFISNAQQR) and affinity purified (Eurogentec, Belgium). RT–PCR analysis was performed with the following primers; (dPPAT-DPCK) 5′-AATTCATGCCCGTCTTACCC-3′ and 5′-CAGCAAGCCAAACATCT- TCC-3′, (CG5505) 5′-TCGTGGTAGTTGGGCACCTC-3′ and 5′-GCGAAGCGGCGAAT- GTCCAG-3′. mRNA levels were determined with ImageJ (http://rsb.info.nih.gov/ij/) using Rp49 (40) as an internal reference. The area × mean intensity of the reference was divided by the area × mean intensity of CG5505 or dPPAT-DPCK and normalized to wt ratios.

**Immunoblot analysis:** Dissections were performed in PBS. Samples were lysed in RIPA buffer, sonicated and the protein content determined using the DC protein assay kit (Bio-Rad). Extracts were separated on 12.5% SDS–PAGE gels, transferred to nitrocellulose membranes, blocked in PBS + 0.1% Tween20 (PBST) supplemented with 5% non-fat milk and the membranes were incubated with anti-dPANK (1:4000) or anti-dPPCS (1:1500) antibodies in blocking buffer. Blots were incubated with HRP-goat-anti-rabbit IgGs (1:2000, Amersham). Antibody complexes were visualized using the ECL kit (Amersham). The same blots were incubated with mouse anti-γ-tubulin (1:5000, Sigma) or mouse anti-β-actin (1:5000, Abcam). Protein levels were determined with ImageJ (http://rsb.info.nih.gov/ij/) using β-actin or γ-tubulin as references. The area × mean intensity of the reference was divided by the area × mean intensity of dPPCS or dPANK/Fbl and normalized to wt ratios.

**Lipid analysis:** Triglycerides were measured using the Stanbio LiquiColor assay kit as described in (41). Triglyceride levels were normalized to percentages of wild-type levels and represent the average of three independent experiments. To determine the amount of phospholipids, approximately 20 mg of fly heads from a mixture of 14-day-old female and male adults was homogenized and the protein content determined. Lipids were extracted, separated and quantified essentially as described in (20).

**Immunohistochemistry:** Larval brains were fixed in PBS + 4% formaldehyde and incubated with antibodies in PBS + 0.3% Triton X-100 + 5% BSA. Antibodies used included mouse anti-pH3Ser10 (1:100, Cell Signaling), rabbit anti-pH2AvDS137 (1:100, Rockland) and FITC-goat-anti-mouse IgG (1:200) and Cy3-goat-anti-rabbit IgG (1:200, Jackson ImmunoResearch). Brains were analyzed by fluorescent microscopy (FM) or confocal laser scanning microscopy (CLSM). CLSM images represent maximal projections of a z-stack.
(0.5–1 µm/scan) and were obtained with a Leica TCS SP2 DM RXE. Brains were dissected in serum-free Schneider’s Drosophila medium (S2) (Invitrogen), incubated for 4 m in S2 + 1.6 µM acridine orange, washed and directly investigated by FM (Leica CTR6000). pH3Ser10 labeled chromatin was analyzed using a 100× magnification with 1.5× optivar (Olympus IMT-2). Mitotic figures were scored per focal plane and the percentage aberrant mitosis was calculated from the SUM of 7–11 focal planes scored per brain. The total amount of pH2AvDS137 (γ-H2AvD) positive cells was counted in entire brains. Images were processed using Leica Software and Paint Shop Pro. Heads from 30-day-old flies were fixed in 4% formaldehyde and embedded in epon. One micrometer horizontal sections were stained with 1% toluidine-blue and 1% borax and investigated with an Olympus BX50 light microscope.

Physiological assays: Performance assays were carried out with male flies. Wing abnormalities were scored in 50–150 flies. Flight was assayed according to Benzer (42) and the performance was determined as described in (16). Geotaxis was assayed according to (16). The absolute climbing ability was determined by tapping 10–20 flies to the bottom of a food vial and counting the flies that were attached to the side after 30 s. Ten trails were performed for each cohort and the average climbing ability was calculated from >7 cohorts. The absolute ability to climb was measured 24 h AE to determine the effect of IR on adult performance. The percent of untreated flies that was able to climb was compared with the percent of flies that was able to climb after larval exposure to IR. ROS sensitivity was measured by placing 15–25 24-h-old male flies (100–200 were tested), after 6 h starvation, in vials containing filter paper soaked in H2O + 5% glucose and with 100 mm DTT, 5% H2O2 or 20 mm paraquat. Twice a day death flies were scored. Longevity was measured for both females and males and both sexes were present in equal proportions. Every 2–4 days, flies (10–20/vial) were transferred to fresh vials and the death flies were scored. Chemicals were obtained from Sigma.

Statistical analysis: P-values were calculated using the Student’s t-test (*P < 0.05, **P < 0.005, ***P < 0.001). Survival curves were analyzed by the method of Kaplan and Meier using SigmaStat 3.5.

ACKNOWLEDGEMENTS

We thank R.S. Sohal for providing the ZnSod-Cat-Trx fly line; J. Sekelsky for the pBUF plasmid; L.G. Fradkin for the pCaSpeR4-Pme1-Bgh plasmid; J. Hageman, E.B. van Lacum, M.A. Rujano, E. Seinen, P. Post and J.M. van der Wouden for technical advice and assistance; P. Rump and G. de Haan for critical reading of this manuscript and valuable comments.
REFERENCES


SUPPORTING INFORMATION - CHAPTER 2

METHODS

Bioinformatics analysis: Protein sequences were aligned with CLUSTAL W (1), manually edited and used to create a structural alignment with the program DEEP-VIEW and modeled by the SWISS-MODEL server (2). Between D251 and L252 of dPANK/Fbl 3 insertions and between S122 and V123 of dPPCS 2 insertions were allowed in the initial model making process and were religated prior to structure comparison and validation. Structures were not refined and represent crude models. Monomers were individually reconstructed during remodelling and analysed independently. Differences between monomers reflect slight differences in the template monomers used during modelling. DaliLite (3), PROCHECK (4) and WHATCHECK (5) were used for pair wise structure comparison and validation. Figures were prepared with ESPript (6) and YASARA (7).

Physiological assays: Larval crawling was analysed by placing third instar larvae in the centre of a petri dish containing non-nutritive agar (0.8%) and the path length that larvae crawled within 5 m was recorded (8,9). The trail traversed by each larva was drawn on the dish, scanned, and the tract length for at least 25 larvae was measured using UDruler (AVPSoft). Paralysis was assayed by analysing the absolute climbing ability of 7-d-old males, prior, directly after heat exposure (2 h 37 oC) and 15 m after recovery from heat exposure. The absolute climbing ability was determined by tapping 10-20 flies to the bottom of a standard food vial and counting the flies that were attached to the side after 30 s. 10 trails were performed for each cohort and the average climbing ability was calculated from at least 7 cohorts.

Immunostaining and apoptosis: Phase-contrast microscopy analysis of onion stage spermatids was performed as described (10). Dissection, fixation and immunolabelling of ovaries was performed as described in ref. 11. Rabbit anti-Histone H2AvD pS137 (1:100, Rockland) was used as primary antibody and Cy3-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch) was used as the secondary antibody. After immunolabelling ovaries were stained with 0.2 μg/ml DAPI and mounted in citifluor (Agar Scientific). Apoptosis was measured in 6 day old flies that were kept for 3 days in vials containing yeast paste. The TUNEL cell death assay was performed following the ApopTag Fluorescien in Situ Apoptosis Detection Kit (Chemicon). Ovaries were fixed in devitellizing buffer/heptane (11) (1:6 per volume) and pretreated with proteinase K (20 μg/ml in PBS + 0.1% tween-20 ) for 15 m at RT. Ovaries were analysed by CLSM. CLSM images represent maximal projec-
tions of a z-stack (0.5-1 μm/scan) and were obtained with a 63X/1.32 oil lens (Leica TCS SP2 DM RXE, Wetzlar).

**Statistical analysis:** P-values were calculated using the Student’s t-test (2-tailed & where appropriate with equal or unequal variance). P-values < 0.05 were considered significant.

**REFERENCES**


Figure S1. Drosophila Coenzyme A biosynthesis is conserved.

The Drosophila de novo CoA biosynthesis route was reconstructed by bioinformatics analysis. (A–E) Multiple sequence alignments of pantothenate kinase (PANK), 4'-phosphopantothenoylcysteine synthetase (PPCS), (R)-4'-phospho-N-pantothenoylcysteine decarboxylase (PPCDC), 4'-phosphopantetheine adenylyltransferase (PPAT) and dephospho-CoA kinase (DPCK). Higher eukaryotes have a bifunctional PPAT-DPCK, while bacteria have a bifunctional PPCS-PPCDC. The Drosophila genome encodes a single copy of PANK (CG5725), PPCS (CG5629), PPCDC (CG30290), a bifunctional PPAT-DPCK (CG10575) and a DPCK (CG1939). Humans have multiple copies of the CoA biosynthesis enzymes. Here the enzymes that displayed the strongest sequence homology with Drosophila are shown. The % identity and similarity between the human and the Drosophila enzymes is: PANK2 (46%, 61%), PPCS (39%, 59%), PPDC (48%, 66%), PPAT-DPCK (31%, 47%) and DPCK (38%, 60%). (A) Multiple sequence alignment of PANK; D. melanogaster (gi17864532), H. sapiens (gi24430171, PANK2), M. musculus (gi23943834), C. elegans (gi32565377), S. cerevisiae (gi6320740) and A. thaliana (gi30696417). (B) Multiple sequence alignment of PPCS; D. melanogaster (gi4972686), H. sapiens (gi13375919), M. musculus (gi18255582), C. elegans (gi17560194), S. cerevisiae (gi577131), A. thaliana (gi26451224) and E. coli (gi16131510, aa 177-430). (C) Multiple sequence alignment of PPCDC; D. melanogaster (gi24657262), H. sapiens (gi15680133), M. musculus (gi28849879), C. elegans (gi17560194), S. cerevisiae (gi6322762, aa 267-571), A. thaliana (gi13124313), ) and E. coli (gi16131510, aa 1-218) (D) Multiple sequence alignment of bifunctional PPAT-DPCK; D. melanogaster (gi10728128), H. sapiens (gi17981025), M. musculus (gi27229125) and C. elegans (gi25143409). (E) Multiple sequence alignment of monofunctional DPCK; D. melanogaster (gi24644728) and H. sapiens (gi19923601). For sequence alignments of S. cerevisiae and E. coli amino acids (aa) that correspond with their PPCS and PPCDC portions were used. Strictly conserved residues are shaded in black, while similar amino acids are boxed and in the bold letter type. Sequences were aligned with CLUSTAL W(1) and the figures were prepared with ESPript(6). GenBank identifiers (gi) are denoted between brackets.
Figure S2. Drosophila melanogaster dPANK/Fumble, CG5629 and CG30290 encode the structural homologs of human PANK, PPCS and PPCDC.

To explore functional conservation of the CoA biosynthesis route 3D models of dPANK, dPPCS and dPPCDC were created by modelling. Models were created with known x-ray structures of human PANK3 (2I7P), PPCS (1P9O) and PPCDC (1QZU), to explore functional conservation and were not refined for in depth structural analysis. Despite the % of residues within the most favoured region of the Ramachandran plot are below 90% (83%-88%) and the confidence factors (B-factor) are >50 (52-55), which likely reflect their sequence identities (39-48%), we conclude from this in silico approach that dPANK/Fbl, dPPCS and dPPCDC represent the struc-
tural/functional homologs of their human relatives. These are the only genes present in the Drosophila genome whose ORFs produce significant hits with a pantothenate kinase, a phosphopantothenoylcysteine synthetase and phosphopantothenoylcysteine decarboxylase and the analysis of bond lengths, angles, and Φ-Ψ properties from the models show that they resemble acceptable drafts of their human relatives (Table S1). (A) dPANK/Fbl and hPANK3 display sequence homology and topology. (B) Superimposition of the hPANK3 homodimer (yellow) and the dPANK/Fbl model (blue) (rmsd = 0.8 Å; 354 Ca atoms aligned per monomer). (C) dPPCS and hPPCS display sequence homology and topology. Conserved residues involved in phosphopantothenate and ATP binding, based on structural data obtained from the hPPCS, are highlighted in blue (and black from the adjacent monomer) and pink, respectively (12). (D) Superimposition of the hPPCS homodimer (yellow) and the dPPCS model (blue) (rmsd = 0.5 Å; 264 Ca atoms aligned per monomer). (E) The dPPCDC protein signature follows the universal signature of PPCDC (13). (F) dPPCDC and hPPCDC display sequence homology and topology. (G) Superimposition of the hPPCDC homotrimer (yellow) and the dPPCDC model (blue) (rmsd = 0.9 Å; 154 Ca atoms aligned per monomer). (A, C and F) Amino acids are marked according to their physico-chemical properties. Strictly conserved residues are highlighted in red boxes and similar residues with red letters. Amino acids considered similar are: HKR (polar positive), DE (polar negative), STNQ (polar neutral), AVLIM (non-polar aliphatic), FYW (non-polar aromatic), PG, C. Secondary structure conformations are denoted at the top and underneath the sequence alignment. (α) α-helix; (β) β-strand; (η) 310 helix; (TT) turn.

Table S1. dPANK, dPPCS and dPPCDC model statistics

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Monomers were individually reconstructed and analyzed independently. Representative statistics of 1 monomer are indicated. a WHATCHECK (Hooft et al., 1996), b PROCHECK (Laskowski et al., 1993), c YASARA (Krieger and Vriend, 2002)

Figure S3. dPPCS mutants display cytokinesis defects during spermatogenesis. Morphological analysis of wt and mutant spermatids.

1-d-old wt & dPPCS mutant testis were investigated for cytokinesis defects by phase contrast microscopy. (A) In wt testis a cyst of 16 interconnected primary spermatocytes undergo 2 synchronized meiotic divisions accompanied with incomplete cytokinesis & 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) & its size and spherical structure resemble that of the nucleus during the onion stage. (B-C) dPPCS mutant testis contain big nebenkern structures with 2 (boxed arrowheads) or 4 (arrow-
heads) nuclei, and (C) contain abnormally persistent mitochondrial bridges (arrows), indicative for cytokinesis defects (16). (D) Quantification of abnormal spermatids in wt and dPPCS mutant males. Approximately 17% of dPPCS1/1 male flies contain testis with >10% abnormal spermatids, while this percentage is 82% in dPPCS1/33 males. The number of flies analysed is shown at the top of each histogram.

Figure S4. Mutations in CoA biosynthesis impairs larval crawling and cause paralysis after heat exposure. 

(A) Quantification of locomotor activity of wt and mutant third instar larvae. Larval locomotor activity of dPANK/fbl1/1 larvae is severely impaired, while dPPCS1/1 show no decrease in activity and the activity of the dPPAT-DPCK43/43 larvae is slightly decreased. Impaired larval motility has been implicated in impaired CNS function(14). (B) Absolute ability to climb after heat exposure of 7-d-old wt and mutant males. dPPCS1/1 and dPPAT-DPCK43/43 mutants become paralyzed after heat exposure (2 h 37 °C) and recover to near normal activity 15' min after heat exposure as determined by analysing the absolute ability to climb. A paralytic phenotype after heat exposure is frequently associated with defects in the CNS(15). (*p < 0.05, **p < 0.005, ***p < 0.001 as determined by t-test).

Figure S5. dPPCS1/1 follicle cells display increased DNA damage and apoptosis. γ-H2AvDS staining of wt and mutant ovaries. DAPI was used to label the DNA.

(A) The follicular epithelium of dPPCS1/1 stage 3-7 follicles contained cells that stained positive for γ-H2AvD (arrows). (B) Quantification of stage 3-7 follicles that contained γ-H2AvD positive cells. Numbers depicted at the top of each histogram represent the amount of follicles investigated. (C) A TUNEL assay was performed to detect apoptotic cells. Follicle cells (FCs) of the follicular epithelium of dPPCS1/1 stage 3-7 follicles were frequently positive for TUNEL staining, indicating that these FCs were apoptotic. (D) Quantification of the amount of stage 3-7 TUNEL positive follicles. Numbers depicted at the top of each histogram represent the amount of follicles investigated. Scale bars; 20 μm
Table S2. Summary of phenotypes caused by mutations in the *Drosophila* CoA biosynthesis enzymes

<table>
<thead>
<tr>
<th></th>
<th>dPANK</th>
<th>dPPAT-DPCK</th>
<th>dPPCS</th>
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<tr>
<td><strong>Physiologic abnormalities</strong></td>
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<td>reduced flight performance</td>
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<tr>
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</table>

(ND) not determined, (-) not affected, (+) affected, (1) Afshar et al., 2001, (*) communicated elsewhere