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

Rana, Anil

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Pantethine rescues a *Drosophila* model for pantothenate kinase-associated neurodegeneration

Anil Rana\(^a\), Erwin Seinen\(^a\), Katarzyna Siudeja\(^a\), Remco Muntendam\(^b\), Balaji Srinivasan\(^a\), Johannes J. van der Want\(^a\), Susan Hayflick\(^d\), Dirk-Jan Reijngoud\(^e\), Oliver Kayser\(^b\), and Ody C. M. Sibon\(^a\).

\(^a\)Department of Cell Biology, Radiation and Stress Cell Biology, University Medical Center Groningen, The Netherlands; \(^b\)Department of Pharmaceutical Biology, University of Groningen, The Netherlands; \(^c\)Molecular Imaging and Electron Microscopy, University Medical Center Groningen, The Netherlands; \(^d\)Department of Molecular and Medical Genetics, Oregon Health and Science University, Portland USA; and \(^e\)Department of Laboratory Medicine, Center for Liver Digestive and Metabolic Diseases, University Medical Center Groningen, The Netherlands

Proc Natl Acad Sci U S A. 2010 Apr 13;107(15):6988-93
ABSTRACT

Pantothenate kinase–associated neurodegeneration (PKAN), a progressive neurodegenerative disorder, is associated with impairment of pantothenate kinase function. Pantothenate kinase is the first enzyme required for de novo synthesis of CoA, an essential metabolic cofactor. The pathophysiology of PKAN is not understood, and there is no cure to halt or reverse the symptoms of this devastating disease. Recently, we and others presented a PKAN Drosophila model, and we demonstrated that impaired function of pantothenate kinase induces a neurodegenerative phenotype and a reduced lifespan. We have explored this Drosophila model further and have demonstrated that impairment of pantothenate kinase is associated with decreased levels of CoA, mitochondrial dysfunction, and increased protein oxidation. Furthermore, we searched for compounds that can rescue pertinent phenotypes of the Drosophila PKAN model and identified pantethine. Pantethine feeding restores CoA levels, improves mitochondrial function, rescues brain degeneration, enhances locomotor abilities, and increases lifespan. We show evidence for the presence of a de novo CoA biosynthesis pathway in which pantethine is used as a precursor compound. Importantly, this pathway is effective in the presence of disrupted pantothenate kinase function. Our data suggest that pantethine may serve as a starting point to develop a possible treatment for PKAN.
INTRODUCTION

CoA is a ubiquitous and essential cofactor for various metabolic reactions, including the tricarboxylic acid cycle and fatty acid metabolism (1). The canonical pathway leading to de novo synthesis of CoA starting at vitamin B5 (also known as pantothenate or pantothenic acid, further referred to as VitB5) is well known. All genes encoding the CoA biosynthetic enzymes have been identified and are highly conserved between different species (1–6) (Fig. 1A).

The biosynthesis of CoA, especially the CoA biosynthetic enzyme pantothenate kinase (PANK; EC 2.7.1.33), received renewed interest after the discovery that the Hallervorden-Spatz syndrome, a hereditary disease mainly affecting children, is caused by a mutation in the human PANK2 gene, one of the four human pantothenate kinase genes (PANK1-4), rendering the enzyme inactive (7). Accordingly, this syndrome has been referred to pantothenate kinase–associated neurodegeneration (PKAN). This finding uncovered a completely unknown role of CoA biosynthesis in cellular functioning. Patients with the autosomal recessive disorder PKAN show progressive impairment of speech, locomotor, and cognitive function (8). The pathophysiology of PKAN is not understood, and there is no cure to revert or delay the neurodegeneration. It is not known whether there are decreased levels of CoA in the affected tissues and thus whether decreased levels of CoA coincide with impaired neurological and locomotor function. Although a Pank2 mouse knock-out has been generated, this murine model did not show any signs of neurodegeneration (9), leaving the question unanswered as to whether decreased levels of CoA are causative in PKAN.

Recently, we and others have demonstrated that mutations in Drosophila CoA biosynthesis enzymes, including the Drosophila homolog of PANK2 (further referred to as dPANK/fbl mutants), induce a neurodegenerative phenotype; and these flies can be used as a model for PKAN-related research (2, 4, 10). Drosophila is not only a powerful model to understand the mechanisms of various human neurodegenerative diseases (11), but Drosophila disease models are also of value to identify compounds that are able to rescue disease-associated characteristics (12).

In the present study, we used the Drosophila dPANK/fbl mutants and dPANK/Fbl down-regulated Drosophila cultured S2 cells to address the following questions: (i) Does depletion of dPANK/Fbl correlate with decreased levels of CoA? (ii) If dPANK/Fbl depletion does induce decreased levels of CoA, are there ways to restore CoA levels in this background? (iii) If we are able to restore CoA levels, does this lead to a rescue of the phenotypes induced by dPANK/Fbl depletion?

Our results show that dPANK/Fbl depletion results in a significant decrease of CoA. Furthermore, we tested several compounds for their potential to restore CoA levels in the
presence of impaired dPANK/Fbl function. One of the compounds tested was pantethine (the disulphide of pantetheine). Previously, it has been demonstrated that purified enzymatic extracts were able to convert both pantetheine and pantethine into 4’-phosphopantetheine (13–15), an intermediate in the canonical de novo CoA biosynthesis pathway (Fig. 1A). However, it has never been tested whether this alternative pathway is functional in a PANK-impaired background, although this knowledge is highly relevant in light of a possible PKAN therapy. Feeding pantetheine to dPANK/fbl mutant flies or adding pantetheine to dPANK/Fbl–down-regulated S2 cells restored CoA levels and rescued nearly all tested phenotypes, including the neurodegenerative phenotype. Our data further indicate that pantethine rescued mitochondrial abnormalities in hPANK2-depleted mammalian cells. Our results strongly suggest that pantethine can serve as a precursor compound to generate CoA even in the absence of a functional pantothenate kinase. Our findings may serve as a starting point to develop a possible treatment for PKAN.

RESULTS

**dPANK/fbl Mutant Flies Show Reduced Levels of CoA**

Pantothenate kinase is the enzyme required for the first step in the canonical biosynthetic route of CoA (Fig. 1A). In hypomorphic dPANK/fbl mutant flies the dPANK/Fbl protein
content was severely decreased (Fig. 1C). Although there is a dynamic turnover of CoA in numerous intracellular metabolic reactions, there is only one route known that leads to the \textit{de novo} synthesis of CoA (1). Therefore, we hypothesized that low levels of CoA caused the phenotype of \textit{dPANK/fbl} mutants. Indeed, HPLC analysis clearly revealed significantly lower levels of CoA in homozygous \textit{dPANK/fbl} mutants compared with wild type (Fig. 1B). To further test the effect of impaired function of \textit{dPANK/fbl} on CoA levels, \textit{dPANK/Fbl} protein levels were down-regulated in \textit{Drosophila} S2 cells by RNAi (experimental setup in Fig. S2). Four days after the addition of \textit{dPANK/fbl} dsRNA, \textit{dPANK/Fbl} protein levels were strongly decreased (Fig. 1D). Under these circumstances, CoA levels were also significantly decreased to 24% of levels of control cells (Fig. 1E), and cell counts were significantly lower as compared with control cells (Fig. 2A). This suggested that \textit{de novo} synthesis of CoA is required for maintenance of normal levels of CoA in \textit{Drosophila} cells and accordingly for normal cell growth in culture.

\textbf{Pantethine Addition Restores Normal Growth of \textit{dPANK/Fbl}-Depleted Cells}

Our data strongly suggested that reduced levels of CoA might be the primary cause for the decreased cell count of \textit{dPANK/Fbl}-depleted cells and for the mutant phenotype of \textit{dPANK/fbl} homozygous flies. Accordingly, restoring CoA levels in \textit{dPANK/Fbl}-depleted cells and in \textit{dPANK/fbl} mutants should lead to a rescue of the related phenotypes. We checked several compounds related to CoA biosynthesis (CoA, VitB5, and pantethine) for their ability to rescue growth of \textit{dPANK/Fbl}-depleted S2 cells and, when successful, for their ability to restore CoA levels. CoA was tested because adding CoA as a supplement may directly restore CoA levels. VitB5 was tested because adding large doses of VitB5 may compensate for decreased activity of \textit{dPANK/Fbl} enzyme in a \textit{dPANK/fbl} hypomorphic mutant background. Pantethine was tested because previously it has been reported that pantethine can be converted into the normally occurring CoA intermediate 4’-phosphopantetheine (13–15) (Fig. 1A). Our results showed that although high concentrations of all compounds were toxic, CoA and pantethine were effective in restoring cell counts of \textit{dPANK/Fbl}-depleted cells in a concentration-dependent manner, whereas VitB5 was ineffective (Fig. 2B–D). Because rescue with pantethine was most effective for \textit{dPANK/Fbl}-depleted cells and pantethine was less toxic compared with CoA, our further analyses were focused on the rescuing potential of pantethine. The optimal effective concentration of pantethine for cells was 100 μM (Fig. 2D).

\textbf{Pantethine Rescues Levels of CoA in \textit{dPANK/Fbl}-Depleted Cells and in \textit{dPANK/fbl} Mutant Flies}

First, we tested whether addition of pantethine to the cell culture medium of \textit{dPANK/Fbl} down-regulated cells could restore CoA levels. Indeed, a restoration of CoA was observed (Fig. 2E). Next, we addressed the question whether pantethine addition to the food also rescues \textit{dPANK/fbl} mutants. To identify the effective pantethine concentration in the fly
food, various doses of pantethine were tested (Fig. S3). The addition of pantethine at a concentration of 1.6 mg/mL induced a significant increase in climbing activity in dPANK/fbl mutants while inducing only a moderate side affect in wild types. This concentration was used in all further experiments unless indicated otherwise. Concomitantly, levels of CoA were restored upon feeding pantethine directly to dPANK/fbl mutants via the food (Fig. 2F).
These data suggested that there is a dPANK/Fbl-independent way to generate CoA from pantethine in both Drosophila S2 cells and in Drosophila dPANK/fbl mutant flies. Our data also indicated that pantethine provided via the food can still exert its CoA levels-restoring function.

Fig. 3. Impaired mitochondrial integrity and increased oxidative damage induced by disruption of dPANK/Fbl function is rescued by pantethine.

(A) Morphological analysis of wild-type (12 days old) and dPANK/fbl mutant (7 days and 12 days old) flight muscles (untreated and treated with pantethine) was performed by light microscopy. (B) Ultrastructural analysis of mitochondria in flight muscle of 12-day-old wild types and dPANK/fbl mutants (untreated and treated with pantethine). (Lower) Higher magnifications of the indicated areas in Upper. (C and D) JC-1 assay was used to quantify mitochondrial function in control cells and in dPANK/fbl RNAi treated cells (C) in the absence and presence of pantethine and in dPANK/fbl mutants (D) in the absence and presence of pantethine. Valinomycin was used as a positive control. (E) Oxyblots were used to measure levels of oxidative damage to proteins in dPANK/fbl mutants and the effect of supplementation of pantethine was investigated. ***P < 0.001 (Student’s t test). Error bars indicate SEM.
Mitochondrial Structure and Function Are Severely Affected in dPANK/fbl Mutants, and Pantethine Rescues These Phenotypes

Mitochondrial dysfunction is associated with a number of neurodegenerative diseases (16, 17). Several findings suggest that most likely PKAN is also a neurodegenerative disorder associated with impaired mitochondrial function; it has been shown that human PANK2 is localized in mitochondria (18), and that chemical inhibition of pantothenate kinase activity in primary hepatocytes induces abnormal mitochondrial morphology (19). In addition, it was shown that a specific splice isoform of Drosophila PANK/fbl was localized in mitochondria of Drosophila S2 cells (10). Together these data strongly suggest that human PANK2 and Drosophila dPANK/Fbl have a mitochondrial function and that impairment of pantothenate kinase might lead to mitochondrial abnormalities. To investigate this, mitochondrial morphology was examined. Flight muscles contain numerous densely packed mitochondria, and therefore this tissue was analyzed. Visual inspection of flight muscles with bright field microscopy revealed that the structure had a more “loose” appearance and contained more ruptures in dPANK/fbl mutants as compared with controls (Fig. 3A). Moreover, the muscular degeneration was progressive with age in dPANK/fbl mutants. Electron microscopic analysis was performed for a more detailed analysis, and this revealed that, in contrast to wild types, mitochondria of dPANK/fbl mutants were severely affected. The mutant mitochondria were swollen and showed damaged cristae and ruptured membranes (Fig. 3B). This analysis showed that low levels of CoA coincide with severely damaged mitochondrial structures in dPANK/fbl mutants. Pantethine feeding significantly reversed the morphological mitochondrial defects (Fig. 3A and B).

In addition to this analysis, the more quantitative JC-1 assay (Fig. S1 and SI Text) was used to measure the percentage of functional mitochondria. Under control conditions, 70.8% of S2 cells had functional mitochondria (Fig. 3C). As a positive control for this assay, valinomycin was added to the media, and the percentage of cells with functional mitochondria dropped to 18% (Fig. 3C). Under normal culturing conditions, in dPANK/Fbl down-regulated S2 cells, mitochondrial activity was less than 54% after 4 days of RNAi treatment and was less than 32% after 7 days of RNAi treatment compared with control cells (Fig. 3C). Mitochondrial function was rescued to the levels of control cells after adding pantethine to the medium of dPANK/Fbl-depleted cells (Fig. 3C). A similar assay was performed on isolated mitochondria from Drosophila flies. The results showed that dPANK/fbl mutants have 42% reduced mitochondrial function at day 6 as compared with control flies (Fig. 3D). Interestingly, feeding pantethine to dPANK/fbl mutants restored their mitochondrial function up to 84% of wild-type controls (Fig. 3D).

Pantethine Reduces Levels of Increased Oxidative Damage of Proteins in dPANK/fbl Mutants

Previously, we showed that Drosophila CoA mutants displayed an increased sensitivity to
Fig. 4. Pantethine is a protective compound in mutant flies and human cells.
(A) Larval crawling abilities were measured in wild types and in dPANK/fbl mutants untreated and treated with pantethine. ***P < 0.001 (Student’s t test). (B) Percentage of climbers was measured in aging wild types and in aging dPANK/fbl mutants untreated and treated with pantethine. (C) Cohorts of more than 120 flies (n = 3) of wild types and dPANK/fbl mutants were followed and survival curves were generated in the absence and presence of pantethine. All four curves were significantly different compared with each other (log-rank test, P < 0.001). (D) Brain morphology was investigated at the light-microscopic level in (12-day-old) wildtypes, dPANK/fbl mutants untreated and treated with pantethine. (Left) Low magnification. (Bars, 100 μm.) (Right) Higher magnifications of boxed areas. (Bars, 20 μm.) Vacuoles are marked by arrows. (E) Human HEK293 cells were treated with two independent siRNAs (siRNA 1 and siRNA 2) directed against human PANK2 mRNA. Western blot analysis with specific hPANK2 antibodies was used to investigate the effect of the RNAi treatment (48 h after
CHAPTER 3

oxidative stress (4). Here we investigated whether pantethine was able to reduce increased levels of oxidative stress in dPANK/fbl mutants by using Oxyblot assays (Fig. S4). Clearly, dPANK/fbl mutants showed increased levels of oxidative damage of proteins compared with wild type, and these levels were strongly reduced by addition of pantethine to the food (Fig. 3E).

Pantethine Improves Locomotor Abilities and Rescues Brain Degeneration in dPANK/fbl Mutants

So far, our results have demonstrated that pantethine restores CoA levels, improves mitochondrial function and reduces levels of oxidative damage in a Drosophila model for PKAN. Next, we investigated whether all these beneficial effects resulted in improvement of locomotor function in dPANK/fbl mutants. On average, wild-type larvae were able to crawl 50 cm in 9 min, whereas homozygous dPANK/fbl mutants reached only 20 cm (Fig. 4A). Addition of pantethine to the larval food significantly improved larval crawling abilities, and an average distance of 30 cm was reached (Fig. 4A). Although a strong improvement in larval crawling activity was observed, pantethine feeding was unable to completely rescue the mutant phenotypes. Our data were inconclusive as to whether incomplete rescue was because dPANK/Fbl has additional functions (other than the production of CoA) or whether pantethine has side effects that hampered complete recovery. The latter explanation was supported by our observation that, in wild types, crawling activity was also reduced after pantethine feeding (Fig. 4A).

Previously, we demonstrated that dPANK/fbl flies showed reduced ability to climb at a young age (4). Here we assayed whether this reduced ability to climb further deteriorates with age. Climbing tests of wild-type and homozygous dPANK/fbl flies at increasing age (2, 5, 10, 16, and 21 days), showed that mutants not only possessed impaired climbing abilities following eclosion but that they also experienced a steeper decline of the already-reduced climbing activity over time as compared with wild type (Fig. 4B). Pantethine feeding significantly prevented the rapid decline of climbing ability of dPANK/fbl mutant flies (Fig. 4B). Consistent with the data presented in Fig. 4A, pantethine feeding induced a decrease in climbing activity in wild-type flies. In addition to these behavioral assays we tested a possible protective function of pantethine on neurodegeneration more directly by analyzing dPANK/fbl mutant brain tissue. dPANK/fbl mutants show increased numbers of vacuoles in their brains (4), indicating brain degeneration (Fig. 4D). Pantethine also rescues this...
Pantethine rescues Drosophila model of PKAN

**Pantethine Increases Lifespan of dPANK/fbl Mutants**

Previously, we have demonstrated that dPANK/fbl mutants showed a severe reduction in lifespan (4). We investigated whether rescue of all of the above-mentioned phenotypes with pantethine also coincides with increased lifespan of dPANK/fbl mutants. The maximal and median lifespan of dPANK/fbl mutants were 23 and 15 days, respectively (Fig. 4C). Under these circumstances, wild-type flies showed maximal and median lifespans of 81 and 45 days, respectively (Fig. 4C). Pantethine feeding increased the maximal lifespan of dPANK/fbl mutants from 23 days to 41 days and the median lifespan from 15 to 22 days (Fig. 4C). Consistent with the data presented in Fig. 4 A and B, pantethine feeding induced a reduction in lifespan in wild-type flies. Regardless of these deleterious side effects of pantethine in wild types, pantethine feeding clearly rescued various relevant abnormalities of dPANK/fbl mutants.

**In Mammalian HEK293 Cells with Down-Regulated PANK2 Levels, Pantethine Also Improved Mitochondrial Function**

Finally, we addressed the question of whether pantethine was also capable of rescuing an abnormal phenotype induced by impaired function of endogenous PANK2 in human cells, and for this we used mitochondria integrity as a read-out. First we tested whether down-regulation of PANK2 in HEK293 cells also results in decreased mitochondrial activity [quantified by the mitochondrial JC-1 assay (SI Text)]. Indeed, depletion of human PANK2 using two independent siRNAs resulted in decreased levels of PANK2 and decreased mitochondrial activity (Fig. 4E). Addition of pantethine to the medium of PANK2-depleted cells resulted in a significant rescue of mitochondrial activity (Fig. 4E). These data indicate that, also in human cells, pantethine was capable of protecting (albeit partly) against consequences of impaired PANK2 enzyme function.

**DISCUSSION**

In the current study, we used a Drosophila model for PKAN to investigate the consequences of impaired pantothenate kinase function and to identify possible protective compounds against the mutant phenotypes. We demonstrate that in Drosophila dPANK/fbl mutants and in dPANK/Fbl down-regulated S2 cells, CoA levels are significantly decreased. Low levels of CoA coincide with impaired mitochondrial integrity, increased levels of oxidized proteins, increased loss of locomotor function, neurodegeneration, and decreased lifespan. Our data are consistent with published data demonstrating that numerous neurodegenerative disorders are tightly linked to mitochondrial dysfunction and increased levels of oxidative
stress (16, 17). All of the dPANK/fbl phenotypes, including neurodegeneration, were more or less rescued by addition of the compound pantethine to the food. Our data support that the mechanism underlying pantethine protection in dPANK/fbl flies is specific and not general, because three other neurodegenerative (Parkinson’s and two PolyQ) Drosophila models are not rescued by pantethine treatment (Fig. S6). Pantethine has been already proved to be an effective treatment for hyperlipoproteinemia and dyslipidemia in human patients, and a dose of up to 1,200 mg pantethine per day for 24 weeks is tolerated without any side effects (20, 21). Unfortunately it is currently unknown whether pantethine crosses the blood–brain barrier, although this knowledge is highly relevant to develop pantethine further as a possible treatment for PKAN.

For the first time, we show genetic evidence for the existence of a parallel pathway to the canonical de novo CoA biosynthesis starting from pantethine, which at least bypasses the first step of the pathway. We demonstrated that decreased levels of CoA were a clear consequence of impaired dPANK/Fbl function in Drosophila. Although this was an anticipated result, this consequence of impaired pantothenate kinase function has not been investigated in multicellular animals or in human patients. However, there are several reports that indirectly support our observations. Chemical inhibition of PanK1, PanK2, and PanK3 by HoPan in isolated murine hepatocytes resulted in a reduction of de novo CoA synthesis and reduction of total levels of CoA (19). In Arabidopsis thaliana, it was demonstrated that mutations in several genes coding CoA biosynthesis enzymes resulted in impaired CoA biosynthesis and decreased levels of CoA (22–24). Together, these and our data show that impaired function of CoA biosynthesis enzymes (including PANK) lead to a decreased rate of de novo CoA synthesis, and that normal de novo synthesis of CoA is required to maintain the physiological levels of CoA.

After establishing that CoA levels were indeed below detection in the Drosophila PKAN model, we demonstrated that pantethine is a very potent compound that can act as a starting substrate for generating CoA in a dPANK/fbl mutant background. How, exactly, pantethine can be converted into CoA is currently unclear. Classic biochemical studies using cell extracts showed that pantethine can be reduced into pantetheine (25, 26) and that this can be converted into 4’-phosphopantetheine (14). The latter is an intermediate of the canonical de novo biosynthesis pathway and thus here, upstream from PPAT, the CoA de novo synthesis pathways starting from vitB5 and from pantethine may converge (Fig. 4F). This is further supported by experiments showing that the decreased cell counts of dPPCS-depleted cells, but not of dPPAT-depleted cells, is rescued by pantethine (Fig. S7). Possible enzymes that catalyze the phosphorylation of pantetheine have never been genetically identified. However, it has been shown that specific purified enzyme preparations were able to phosphorylate both VitB5 and pantethine with similar kinetics (13). Based on these studies it was assumed, but never tested, that pantothenate kinase is the only enzyme present that can phosphorylate pantetheine. However, these earlier studies did not exclude the presence of additional kinases (other than pantothenate kinase) in the purified enzyme preparations.
with pantetheine kinase activities. It is also possible that pantetheine can be converted into CoA not via 4’-phosphopantetheine but via a completely alternative route. Regardless of the exact route, our data suggest that this pathway can most likely occur independently from pantothenate kinase based on the following. (i) In both dPANK/fbl mutants and in dPANK/fbl down-regulated cells, the levels of dPANK/fbl are severely reduced and it is unlikely that these reduced dPANK/fbl protein levels are responsible for the restoration of CoA levels after pantetheine addition. (ii) If some residual pantothenate kinase activity were present in dPANK/fbl mutants and in dPANK/Fbl down-regulated cells, addition of extra VitB5 should have been beneficial also. However, addition of extra vitB5 did not lead to rescue in dPANK/fbl (Fig. S5) mutants and in dPANK/Fbl down-regulated cells (Fig. 2B). Thus all of the above results suggest the presence of an alternative route for de novo synthesis of CoA independent from pantothenate kinase. Apart from whether residual activity of pantothenate kinase is required for pantetheine rescue, our findings are still relevant for PKAN-related research because most of the patients with PANK2 gene mutations still have some residual activity of pantothenate kinase (18).

Regardless of the mechanisms behind pantetheine toxicity in wild-type cells, the exact pathway of pantetheine conversion into CoA, and whether residual activity of pantothenate kinase is required for pantetheine rescue, our data at least suggest the existence of an alternative pathway that uses pantetheine as a primary compound to generate de novo CoA in the presence of impaired pantothenate kinase function. This knowledge allows the development of a possible future therapy for PKAN.

**MATERIAL AND METHODS**

*Drosophila Strains:* The *Drosophila* strain y’w1118 was used as a wild-type control (Bloomington Stock Centre). The hypomorphic dPANK/fbl1 mutant flies were used for all assays (2, 4).

**Pantethine Supplementation:** D-Pantethine (Sigma) was added at a concentration of 1.6 mg/mL in standard fly food; 100 μM of D-Pantethine was added to S2 cell and HEK293 medium, except where mentioned otherwise.

**Physiological Assays:** To study larval crawling, late third instar homozygous dPANK/fbl larvae were placed on 1% nonnutritive agar in a Petri dish. Total distance crawled by the larvae during 9 min was measured. To study the adult lifespan, newly eclosed flies (n > 100, 1 or 2 days old) were collected and raised on standard medium at 25 °C in a dry Petri dish with food (2.29 cm2; with or without pantetheine) at the center of the Petri dish. The number of dead flies was counted every 2 days. Each experiment was repeated three times. For a climbing assay, adult flies were used to investigate climbing performance as previously described (27). The experiment was repeated three times (n > 100).
CoA Measurements: CoA levels were measured from fly extracts (100 flies, 6 days old) or from *Drosophila* Schneider’s S2 cells using HPLC (sample preparation and HPLC analysis are described in SI Text).

Cell Culture and PANK Knockdown: *Drosophila* Schneider’s S2 Cells were cultured, and RNAi knockdown of dPANK/Fbl was performed as previously described (28) (SI Text). Mammalian cell culture and siRNA knockdown of hPANK2 are described in SI Text.

Electron and Light Microscopy: Flies were immersed in fixative solution (2.5% glutaraldehyde in 0.1 M cacodylate, pH7.8). Postfixation was performed in 2% OsO$_4$ for 2 h at 4 °C. Dehydration was carried out with graded ethanol series followed by a propylene wash and preembedding in (1:1) propylene:epon solution. Embedding of the flies was performed in EPON. For light microscopy, sections (1–2 μm) were cut using a Reichert Ultracut microtome and stained with Toluidine Blue. For ultrastructural analysis of mitochondria, thin sections (60 nm) were cut from the same samples and analyzed by electron microscopy.

Mitochondrial Assays: Mitochondria were isolated from 7-day-old flies as previously described (29). For measurement of mitochondrial membrane potential, J-aggregate-forming lipophilic cation (JC-1) was used to evaluate mitochondrial damage (30). The JC-1 assay (Sigma) was performed according to the manufacturer’s manual (SI Text and Fig. S1).

Protein Oxidation Detection: Protein lysates were prepared in RIPA buffer containing 2% β-mercaptoethanol. Total protein solutions were incubated with 2,4-dinitrophenylhydrazine (DNP) according to the OxyBlot protein oxidation detection Kit (Chemicon). The total amount of oxidized proteins was quantified for each sample by measuring chemiluminescence from the whole lane and oxidized protein levels were normalized using β-actin as a loading control (Fig. S4).

Antibodies: dPANK/Fbl (1:4,000) (4), hPANK2 (1:2,000; a gift from J. Gitschier, University of California–San Francisco), GAPDH (1:10,000; Fitzgerald Industries), β-actin, and γ-tubulin (Sigma) were used. HRP-conjugated antimouse or antirabbit antibodies were used (1:2,000; Amersham) as secondary antibodies.

ACKNOWLEDGEMENTS

We thank Floris Bosveld and Harm Kampinga for stimulating discussions. This work was supported by a VIDI grant (to O.S.), a Neurodegeneration with Brain Iron Accumulation Disorders Association grant (to O.S and S.H.), and a Topmaster grant from the Graduate School GUIDE (to A.R.).
REFERENCES


23. Rubio, S., et al., The coenzyme a biosynthetic enzyme phosphopantetheine adenylyltransferase plays a crucial role in plant growth, salt/osmotic


SUPPORTING INFORMATION - Chapter 3

SI Text:

**Evaluation of Functional Mitochondria Using a JC-1 Assay:** Mitochondrial function can be quantitatively assessed by measuring changes in the mitochondria transmembrane potential using JC-1 which is J-aggregate-forming lipophilic cationic fluorochrome (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (Sigma) assay (1). At high mitochondrial membrane potentials, JC-1 accumulates in the mitochondria and forms J-aggregates that show a red fluorescence emission at 590 nm. At lower mitochondrial potentials, less dye enters mitochondria, resulting in monomers that show green fluorescence emission at 530 nm. By using this assay, one can quantify highly active mitochondria (with both red and green fluorescence) and depolarized mitochondria (with green fluorescence only). In addition, by using this assay, it is possible to investigate the mitochondria integrity of suspension cells, of attached cells and to investigate integrity of isolated mitochondria from tissues. For these assays, valinomycin is added as a control, because this K+ ionophor depolarizes the mitochondrial membrane and induces a sharp decrease in red fluorescence emission at 590 nm, representing an increase in impaired mitochondria integrity.

Mitochondrial potential in Drosophila S2 cells was estimated using the flow cytometer analysis method for JC-1 probe (Molecular Probes protocol: MitoProbe JC-1 assay kit for Flow Cytometry (M34152). Briefly: 1 × 10^6 cells were suspended in 500 μL of growth medium and incubated with valinomycin or left untreated. Cells were then centrifuged at 400 rpm (5 min at 4 °C) and resuspended in JC-1 solution buffer (10 μg/mL). After incubation (15 min), cells were pelleted, resuspended in ice-cold PBS, and analyzed immediately using a flow cytometer. Monomers and J-aggregates of JC-1 were simultaneously excited using 488-nm laser, and emission was quantified in FL1 (530 nm) and FL2 (590 nm) channels. Mitochondria containing red JC-1 aggregates (mitochondria with a normal membrane potential; active mitochondria) from viable cells were detectable in FL2 channel, and green JC-1 monomers (mitochondria with a depolarized membrane; impaired mitochondria) were detectable in FL1 channel (Fig. S1). The results were plotted as the percentage of cell with active mitochondria (FL2) from total number of cells analyzed (20,000 cells per analysis) (Fig. 3C).

Analysis of mitochondria isolated from flies was performed according to the manufacturer’s protocol (Sigma; mitochondrial isolation Kit). In short: 100 μL of the JC-1 Staining Solution was added to 10 μL of isolated mitochondria resuspended in mitochondrial maintenance medium (Sigma; mitochondrial isolation kit) in a 96-well plate. Fluorescence was measured in a spectrofluorometer (FL600 Biotek) using the following settings: excitation wavelength, 490 nm; emission wavelength, 590 nm. Fluorescence produced (FLU) per well was recorded, and total FLU per milligram of proteins (FLU/mgP) was calculated. FLU/
mgP is an indication of the amount of J-aggregate formation and a measurement of active mitochondria. In control cells this was set to 100%. The amount of FLU/mgP was indicated for every condition as a percentage of the FLU/mgP in control cells (Fig. 3D).

Analysis of mitochondria in HEK293 cells was performed according to protocols for adherent cells (2, 3). In short, adherent cells were incubated with JC-1 solution (10 μg/mL) in growth medium for 15 min, washed twice with ice-cold PBS, and fluorescence measured in a spectrofluorometer. To normalize for the amount of cells, the ratio of FLU for active mitochondria (590 nm alone) to the total FLU from the well (sum of 530 nm and 590 nm) was calculated, and in control cells this was set to 100% (Fig. 4D).

**Measurement of CoA Levels by HPLC:** For fly sample preparation, homozygous *dPANK/fbl* flies (6 days old), 60 female and 40 males per experiment, were collected and weighed. Flies were then snapfrozen in Liquid N2, and 200 μL of solvent buffer (5% sulfosalicylic acid containing 50 μM DDT) was added. After thorough grinding, the samples were sonicated three times for 10 s on ice. Samples were centrifuged and supernatant was collected for HPLC analysis of CoA. Before analysis, 2 μL of Ammonia (25%) was added to 98 μL of the sample solutions. For Drosophila Schneider’s S2 cell sample preparation, cells were pelleted, and 200 μL of solvent buffer (5% sulfosalicylic acid containing 50 μM DDT) was added. Samples were sonicated and centrifuged, and the supernatant was collected for HPLC analysis of CoA. Before analysis, 2 μL of ammonia (25%) was added to 98 μL of the sample solutions.

CoA was measured according to a slightly modified, previously described method (4) using HPLC. A Nucleosil 120 C18 (4.6 × 150 mm, 3-μm) column was used, together with an Agilent Technologies Guard column C18 (4.6×12.5mm, 5 μm), with an injection volume of 30 μL per sample. Mobile phase A consisted of 100 mmol/L sodium dihydrogen phosphate and 75 mmol/L sodium acetate. The pH of the buffer was set at 4.6 with phosphoric acid. Mobile phase B consisted of 30% methanol and mobile phase A 70%. The temperature of the column was maintained at 35°C. The solvent gradient consisted of 10–40% B in 10 min and 40–90% in 8 min. The column was equilibrated with 10% B between each sample analysis. The flow rate was maintained at 1.2 mL/min. HPLC analysis was performed using a Shimadzu-VP system (Shimadzu).

**Drosophila Schneider’s S2 Cell Culture and RNAi:** For generation of the dsRNA the following primers were used: As a control, nonrelevant (human gene; hMAZ) dsRNA was used. dsRNA was produced and purified with MEGAscript RNAi Kit (Ambion) according to the manufacturer’s instructions. Down-regulation of dPANK/Fbl protein was investigated by immunoblotting using dPANK/Fbl specific antibodies for every individual experiment (Fig. S2).

**Mammalian Cell Culture and siRNA Knockdown of hPANK2:** PANK2 knockdown in HEK293 cells was performed under conditions of regulated levels of pantothenic acid.
Pantethine rescues Drosophila model of PKAN

Table 1: Gene Primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
</table>
| dPANK/Fbl | fwd-CGTGATACGCACCTACAGATG  
            | rev-GCCATTGGACCAGAACTCCAT |
| dPPCS   | fwd-GGCACAACAAGCTCCAGAAAT  
            | rev-CTTGGTGCTGACGCACCAT   |
| dPPAT-I  | fwd-GCGAGCCATCGAGAAGTGCGT  
            | rev-CCGAGTCATCCAGGAAGATGTTGT |
| dPPAT-II | fwd-GCCCACGTGATCGACTCGAT    
            | rev-CCACTTCGCTCAAeCTGTTG    |

(vitB5) using custom made vitB5 free DMEM ThermoScientific) supplemented with 0.4 mg/L vitB5 (Sigma), 10% dialyzed serum (Gibco), 100U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Two different human PANK2-specific small interfering RNAs (siRNAs) were used: siRNA1 (Dharmacon; D-003797-04) and siRNA2 (Ambion; AM51321). Nonsilencing control siRNA was purchased from Dharmacon (VOSMC 000005). HEK293 cells were transfected with 100 nM siRNA using siPORT Amine transfecting agent (Ambion) according to the manufacturer’s protocol. Knockdown efficiency was assayed by immunoblotting 48 h after transfection.

RT-PCR Analysis: Total RNA was extracted from the S2 cells using the Absolutely RNA kit (Stratagene). A 1-μg quantity of total RNA was used for cDNA transcription (Invitrogen). Semiquantitative PCR was performed using the following primers to amplify parts of rp49, dPPCS, and dPPAT (product was amplified for 21, 25, and 29 cycles):

Table 2: Gene Primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
</table>
| dPPCS | fwd- ACTTCACCAGGAGCGAGGTTC  
            | rev- AATCCTCGAGGCTCCCATTCCT |
| dPPAT | fwd-GCGAGCCATCGAGAAGTGCGT  
            | rev-CCGAGTCATCCAGGAAGATGTTGT |
| rp49  | fwd-GCAACCGACCTCATCC      
            | rev CGATCCTCAGCGAGGTAAA     |

SUPPLEMENTARY REFERENCES


Fig. S1. Analysis of functional mitochondria in dPANK/Fbl depleted cells by FACS analysis.

FACS analysis in combination with a JC-1 assay was used to measure changes in the mitochondria transmembrane potential, and this enabled the quantification of active mitochondria (detailed description of JC-1 assay described above in SI Text). Dot plots are shown for the following conditions: control cells; control cells treated with Valinomycin; dPANK/Fbl depleted cells (untreated and treated with pantethine). Upper boxed areas represent cells with a red and green fluorescence emission of 590 nm and 530 nm above a specific threshold, representing cells with active mitochondria. Lower boxed areas represent cells with a red fluorescence emission of 590 nm below a specific threshold (and with a green fluorescence emission of 530 nm above a specific threshold) representing cells with a disturbed mitochondrial membrane potential. Percentages of cells are indicated in boxed areas.

Fig. S2. Schematic representation of RNAi experiments.

At day 0, cells were plated in equal densities and were treated with dPANK/fbl dsRNA or with control dsRNA. After 4 days, cells were replated in equal densities and left further untreated or were treated with pantethine. On day 7, various assays were performed. Down-regulation induced by the RNAi treatment for all assays was measured with Western blot analysis using dPANK/Fbl antibodies.
Pantethine rescues Drosophila model of PKAN

Fig. S3. A 1.6-mg quantity of pantethine per milliliter of food is the optimal concentration to rescue climbing ability.

Various concentrations (0.8 mg/mL, 1.6 mg/mL, 2.4 mg/mL, and 3.2 mg/mL) of pantethine were added to the food of wild-type flies and dPANK/fbl mutants. Pantethine was added immediately after eclosion, and the food was refreshed every day. On days 2, 5, and 10, climbing activity was measured. Adding 1.6 mg of pantethine per milliliter of food induced a significant rescue of climbing activity. Moreover, 1.6 mg pantethine showed only a mildly reduction of climbing activity in wild-type flies compared with 2.4 mg pantethine. Based on these results, 1.6 mg pantethine per milliliter of food was used for the experiments described in this article.

Fig. S4. dPANK/fbl flies have increased levels of oxidized proteins that are rescued upon pantethine feeding.

Oxyblot analysis revealed that dPANK/fbl mutants have higher levels of total oxidized proteins as compared with wild type. Daily feeding of pantethine for 6 days immediately after eclosion results in reduction of oxidative damage to the proteins in dPANK/fbl mutants. Actin is used as loading control.

Fig. S5. Vitamin B5 does not increase the lifespan of dPANK/fbl mutants.

To investigate the effect of vitamin B5, various concentrations (0.2 mg/mL, 1 mg/mL, and 2 mg/mL) of vitamin B5 were added to the food and tested for their potential to increase lifespan. At 22 days after feeding the various concentrations of vitamin B5, all dPANK/fbl mutants had died, and no significant increase in lifespan was observed.
Fig. S6. Drosophila models for the neurodegenerative diseases: Spinocerebellar Ataxia-type 3 (SCA-3), Huntington, and Parkinson are not rescued by pantethine.

(A) Flies expressing a truncated form of Ataxin 3 protein containing an expanded repeat of 78 glutamines in eyes show a rough-eye-phenotype (1) and are referred to as SCA3Q78 flies. This transgenic Drosophila strain is a model for SCA3 and has been used to investigate modifiers of toxicity induced by polyglutamine in SCA3 related neurodegeneration. Eye abnormalities are classified as “rough” or as “severely affected” as previously described (2). Protective compounds will reduce the percentage of severely affected eyes. Increasing concentrations of pantethine did not result in a significant decrease of the percentage of severely affected eyes (>250 eyes were scored for each condition). (B) Flies expressing a truncated form of Huntingtin containing an expanded repeat of 128 glutamines show a neurodegenerative phenotype including a reduced lifespan of 24 days and are referred to as HttQ128 flies (3). Addition of (1.6 mg/mL) pantethine to the food did not increase the lifespan of HttQ128 flies. For each condition, more than 100 flies were used. (C) Mutations in human PINK1 are linked to parkinsonism. The Drosophila PINK1 gene is an ortholog of the human PINK1 gene, and Drosophila PINK1B9 mutants show a progressive impairment to climb as they age (4). Addition of (1.6 mg/mL) pantethine to the food did not improve the climbing ability of PINK1B9 mutants. For each time point, >100 flies were used. As a control, PINK1B9 revertants were used that overexpress the wild-type Drosophila PINK1 gene in the PINK1B9 mutant background. Climbing index is defined as the average climbing time required to climb 15 cm by 50% of the flies (4).

Fig. S7. Pantethine rescues cell count of dPPCS-depleted cells but not of dPPAT-depleted cells.
RNAi was used to down-regulate dPPCS, dPPAT, or dPANK/Fbl and dPPAT simultaneously in Drosophila S2 cells. To down-regulate dPPAT, two independent nonoverlapping RNAi constructs were used (detailed description of constructs is provided above in SI Text). Down-regulation induced by the RNAi treatment of dPPCS and dPPAT was investigated by RT-PCR. Down-regulation of dPANK/Fbl induced by RNAi treatment was controlled by using Western blotting (as in Fig. 1C). (A) PCR products revealed a significant down-regulation of dPPCS and dPPAT mRNA after RNAi treatment. (B) In dPANK/Fbl-depleted cells, dPPCS-depleted cells, dPPAT-depleted cells, and dPANK/Fbl-dPPAT-double-depleted cells, the cell count was decreased as compared with control cells. Pantethine addition to the cell culture medium significantly increased the cell count of dPANK/Fbl-depleted cells and of dPPCS-depleted cells but not the cell count of dPPAT-depleted cells and of dPANK/Fbl-dPPAT-double-depleted cells. These data strongly suggest that dPPAT is required for the pantethine rescue of dPANK/Fbl-depleted cells. ***P < 0.001 (Student’s t test). Error bars indicate SEM.

Fig. S8. Amount of brain vacuoles in dPANK/fbl mutant flies is decreased after pantethine treatment.
Number of vacuoles in the brain region (indicated in Fig. 4D) was measured by National Institutes of Health Image J software (http://rsb.info.nih.gov/ij/index.html). The method of quantification is outlined in the Image J documentation (“Particle Analysis”). The total amount of vacuoles was calculated per 100 μm² from comparable regions (indicated in the boxed areas in Fig. 4D). For every condition, four brains were examined of 12-day-old flies. After eclosion, flies were kept on standard food or on standard food supplemented with 1.6 mg/mL pantethine. ***P < 0.001 (Student’s t test). Error bars indicate SEM.