Rescue strategies in Drosophila models of neurodegenerative diseases
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Chapter 3

Extracellular 4’-phosphopantetheine is a source for intracellular Coenzyme A synthesis


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SUMMARY

The metabolic cofactor Coenzyme A (CoA) gained renewed attention because of its role in neurodegeneration, protein acetylation, autophagy and signal transduction. The longstanding dogma is that eukaryotic cells obtain CoA exclusively via the uptake of extracellular precursors, especially vitamin B5, which is intracellularly converted through five conserved enzymatic reactions into CoA. We demonstrate that cells and organisms possess an alternative mechanism to influence intracellular CoA levels with the use of exogenous CoA. CoA is hydrolyzed extracellularly by ecto-nucleotide-pyrophosphatases to 4’-phosphopantetheine, a biologically stable molecule, able to translocate through membranes via passive diffusion. Inside the cell, 4’-phosphopantetheine is enzymatically converted back to CoA by the bifunctional enzyme CoA synthase. Phenotypes induced by intracellular CoA deprivation are reversed when exogenous CoA is provided. Our findings answer long-standing questions in fundamental cell biology and have major implications for understanding CoA-related diseases and therapies.
INTRODUCTION

Coenzyme A (CoA) was identified more than 60 years ago and as a carrier of acyl groups, CoA is essential for over 100 metabolic reactions. It is estimated that CoA is an obligatory cofactor for 9% of known enzymatic reactions. CoA and acetyl-CoA influence protein acetylation levels in various model organisms. Protein acetylation is an essential posttranslational modification, catalyzed by acetyltransferases that use acetyl-CoA as the source. Acetyl-CoA levels also affect autophagy, and CoA promotes oocyte survival in Xenopus laevis by binding to and activating calcium/calmodulin-dependent protein kinase II (CaMKII). Taken together, intracellular concentrations of acetyl-CoA and CoA are critical to a broad range of cellular processes.

Current thinking about how cells and organisms obtain this indispensable molecule originates from experiments performed in the 1950's, which demonstrate how a specific sequential order of enzymatic activities result in the formation of CoA in vitro when Vitamin B5 was used as a substrate. These enzymes are, in order, pantothenate kinase (PANK), phosphopantothenoylcysteine synthetase (PPCS), phosphopantothenoylcysteine decarboxylase (PPCDC), phosphopantetheine adenylyltransferase (PRAT) and dephosphoCoA kinase (DPCK) (Figure 1A). Later, genes encoding these enzymes were identified in a wide range of organisms and references therein. In some organisms, including Drosophila melanogaster, mice and humans, PRAT and DPCK enzyme activities are combined into a single bifunctional protein, referred to as CoA synthase or COASY. In vitro experiments show that in addition to Vitamin B5, pantetheine can also be phosphorylated by pantothenate kinase activity, and the formed product, 4'-phosphopantetheine, can serve as a precursor for CoA. However, direct evidence that cells take up intact pantetheine and utilize it for CoA biosynthesis is still lacking.

In addition to renewed interest in the CoA molecule and its cellular roles, the biosynthetic route gained attention because of its connection with specific forms of neurodegeneration. Two enzymes in the CoA de novo biosynthetic route, PANK (first step) and COASY (combined last 2 steps) are associated with a neurodegenerative disease classified as NBIA (Neurodegeneration with Brain Iron Accumulation). Mutations in the gene encoding PANK2 (one of four human PANK genes) cause an NBIA disorder, called pantothenate kinase-associated neurodegeneration (PKAN). Patients experience progressive dystonia. When CoA was added to the medium of cultured cells rescued the cell count in a concentration-dependent manner (Figure 1C) and restored histone acetylation levels (Figure 1D-E), as previously demonstrated. Addition of CoA to the medium of cultured cells rescued the cell count in a concentration-dependent manner (Figure 1C) and restored histone acetylation phenotype (Figure 1F). Next, we questioned whether this rescue also applied to other cell types and systems of impaired CoA biosynthesis. Treating Drosophila S2 cells with the chemical PANK inhibitor Hopantenate (HoPan), also induced a decrease in cell count (Supplementary Results) and histone acetylation levels (Supplementary Figure 1B), as previously demonstrated. Addition of CoA to the medium of the cultured cells rescued the cell count in a concentration-dependent manner (Figure 1C) and restored the histone acetylation phenotype (Figure 1F). Next, we questioned whether this rescue also applied to other cell types and systems of impaired CoA biosynthesis. Treating Drosophila S2 cells with the chemical PANK inhibitor Hopantenate (HoPan), also induced a decrease in cell count (Supplementary Results) and histone acetylation levels (Supplementary Figure 1A) and histone acetylation levels (Supplementary Figure 1B). This HoPan-induced phenotype was also rescued by direct supplementation of CoA to the medium of the cells (Supplementary Figure 1A, 1D). Next, we studied the effects of HoPan in mammalian HEK293 cells to address the possibility that the beneficial effects of exogenous CoA were cell-specific. When HEK293 cells were treated with HoPan, they showed a phenotype similar to Drosophila S2 cells, with decreased cell count and impaired histone acetylation. When CoA was added to the culture medium both the decreased cell count and impaired histone acetylation, in addition to the histone acetylation phenotype (Figure 1F) were rescued. These in vitro results confirmed the potencies of exogenous CoA to rescue phenotypes induced by impaired PANK in diverse cellular systems.
CHAPTER 3

EXTRACELLULAR 4'-PHOSPHOPANTETHINE IS A SOURCE FOR COA SYNTHESIS

**Figure 1. CoA supplementation rescues PANK impaired phenotypes**

A. Canonical de novo CoA biosynthesis pathway. Vitamin B5 (pantethenate) is taken up and intracellularly converted to CoA by the enzymes PANK, PPRC, and COASY (PANK - Pantethenate Kinase, PPRC - Phosphopantetheine oxidoreductase, COASY - CoA synthase).

B. Relative Drosophila S2 cell count of control (100%) and dPANK/ffiRNA treated cells. "Mann" - western blot of dPANK/ffiRNA protein levels in control and dPANK/ffiRNA treated cells, tubulin as loading control. Data represent mean ± SD (n=3), two-tailed unpaired Student's t-test was used for statistical analysis (***P < 0.001).

C. Relative cell count of control (100%) and dPANK/ffiRNA treated cells in the presence of increasing concentrations of CoA. Data represent mean ± SD (n=3), two-tailed unpaired Student's t-test was used (***P < 0.001).

D. Immunofluorescence showing protein acetylation levels in control (D), and dPANK/ffiRNA treated cells without (E) and with CoA (F). Anti-acetylated lysine antibodies (green), Rhodamin-Phalloidin (red, F-actin) and DAPI (blue, DNA) were used. Scale bar indicates 20 mm.

**Figure 2. CoA rescues impaired PANK phenotypes of C. elegans and Drosophila**

A. Motility (bends per 30 sec) was determined in C. elegans ptk-1 mutants and wild-type with and without CoA treatment. Error bars indicate ± SD (n=10), analysed with two-tailed unpaired Student's t-test (***P < 0.001) and ptk-1 mutants with (n=50) and without CoA treatment compared to wild-type with (n=50) and without (n=50) CoA treatment. Survival curves were significant (P = 0.005), analysed with Log-rank (Mantel-Cox) test between untreated and CoA treated ptk-1 mutants.

B. Eclosion rate of adult control flies (set as 100%) and flies treated with increasing concentrations of HoPan, added to the food during development. Data indicates mean ± SD (n=25); two-tailed unpaired Student's t-test was used (***P < 0.001). A relative eclosion rate of adult control flies and flies treated with 2.5 mM HoPan, added to the food during development, in the presence of increasing concentrations of CoA. Data represent mean ± SD (n=3). A. Intracellular CoA levels measured with HPLC in Drosophila S2 control cells (000%) and cells treated with HoPan alone or with HoPan and CoA. E. Intracellular CoA levels measured with HPLC in HEK293 control cells (100%) and HoPan treated cells with and without CoA. Data in F represent mean ± SD (n=3), analysed with two-tailed unpaired Student's t-test (***P < 0.001, **P < 0.01).
To test the effect of CoA supplementation in vivo, we used homozygous Caenorhabditis elegans (C. elegans) pantothenate kinase (pntk) mutants, which showed decreased motility (Figure 2A, Supplementary Figure 2A) and a decreased lifespan (Figure 2B). Addition of CoA to the food of these mutants improved these phenotypes significantly (Figure 2A, 2B and Supplementary Figure 2A-E). Furthermore, when a Drosophila w1118 control fly line was treated with HoPan, larval lethality was induced and a decreased eclosion (emerging from the pupal case) rate was observed (Figure 2C). This HoPan-induced phenotype was fully rescued by the addition of CoA to the food of the larvae (Figure 2D).

These data demonstrated that supplementation of CoA reverted the phenotypes arising from impaired de novo CoA biosynthesis, an effect that was observed in diverse eukaryotic cell types and organisms.

External supply of CoA influences intracellular CoA

The observed rescue effect could occur in several ways. Either intracellular CoA levels could have been restored, or rescue was achieved independent of the restoration of CoA levels in the cells. If the latter was true, intracellular levels of CoA would not be restored by exogenous CoA. To investigate this, a sensitive HPLC method was developed consisting of pre-column thiol-specific derivatization of samples with ammonium 7-fluorobenzofuran-4-sulfonate (SBDF), followed by chromatographic separation by gradient elution on a C18 column and fluorescence detection (see Supplementary Materials and Methods). The HPLC-CoA analysis showed that intracellular CoA levels were significantly reduced in extracts of HoPan-treated S2 and HEK293 cells, addition of CoA to the culture medium restored the intracellular concentration of CoA (Figure 2E, 2F). These results suggested that extracellular CoA exerted its beneficial effects in CoA-depleted cells by increasing and thereby “normalizing” intracellular CoA concentrations.

In serum, CoA is degraded to stable 4′-phosphopantetheine

The mechanism behind this alternative CoA route was not known. The observations in Figure 1 and 2 indicated that either 1) CoA entered cells directly, although such a transport process has not been described; 2) CoA was converted to an intermediate product that entered the cell and was converted back to CoA in a PANK-independent manner. Previous research found that CoA is not stable in liver extracts and degrades to 50% at -20°C after a week 23, however, the stability of CoA in an extracellular environment such as in aqueous or in standard cell culture medium is unknown. Moreover, these early reports did not identify specific degraded or converted products. We measured the stability of CoA in PBS, serum-free medium, medium containing fetal calf serum and in fetal calf serum (FCS) during a 3hrs incubation. HPLC analysis revealed that CoA was relatively stable in PBS and serum free medium, with >95% of the initial concentration still present after 3hrs (Supplementary Figure 3, A4A). However, in the presence of fetal calf serum, CoA was rapidly degraded (Figure 3A, Supplementary Figure 4B). After 3hrs of incubation only 10% of the initial concentration was detectable (Supplementary Figure 3, 4B). Detailed stability analysis at different time points in PBS and fetal calf serum revealed that 90% of CoA was already degraded after 30 min in fetal calf serum (Figure 3A). Disappearance of CoA coincided with the appearance of one unknown thiol-containing product in the HPLC chromatogram, which migrated at 18.273 minutes.
and remained stable over the time course of 3hrs (Figure 3B, Supplementary Figure 4B). Since this extra peak had to be a thiol-containing molecule, we speculated that it could be a CoA degradation product, namely dephospho-CoA, 4’-phosphopantetheine (PPhSH), or pantetheine. In contrast to dephospho-CoA and pantetheine, 4’-phosphopantetheine is not commercially available and hereto, we chemically synthesized this compound (Supplementary Note) in order to complete our analysis. HPLC analysis and comparison with standards demonstrated that the thiol-containing degradation product of CoA was neither dephospho-CoA nor pantetheine (Supplementary Figure 4A-E), but it exactly matched the retention time of 4’-phosphopantetheine standard (Figure 3C, 3D, Supplementary Figure 4B, 4C). These results indicated that CoA was converted into 4’-phosphopantetheine in serum and was stable. This is in contrast to pantetheine which is not stable in serum (Supplementary Figure 5A).

We further investigated the conversion of CoA in mouse serum and in human serum. In sera from both species, including serum derived from PKAN patients (Supplementary Figure S8), we found that CoA was also converted to 4’-phosphopantetheine (Figure 3E, Supplementary Figure S8).

To investigate whether this conversion also occurred in vivo, Drosophila larvae were fed CoA, and L1 and L2 stage larval extracts were obtained after 2 days and 3 days of feeding, respectively. HPLC analysis showed that externally added CoA resulted in increased levels of 4’-phosphopantetheine in both L1 (20 fold) and L2 larva (>60 fold) (Figure 3F). To investigate whether this conversion also occurred in higher organisms, different concentrations of CoA were injected intravenously into adult mice, and plasma was collected after 30 min and 6 hrs. HPLC analysis in combination with mass spectrometry revealed the presence of low levels of endogenous 4’-phosphopantetheine in fresh mouse serum (Supplementary Figure 6A-C) and showed that the injected CoA was rapidly converted to 4’-phosphopantetheine after 30 min (Figure 3C). Moreover we demonstrated using mass spectrometry that elevated levels of 4’-phosphopantetheine were still present in the plasma 6 hrs after CoA injection (Supplementary Figure 6D).

These data indicated that CoA is converted into 4’-phosphopantetheine in vitro and in vivo. Furthermore, these results suggested that 4’-phosphopantetheine could be the principal molecule that was taken up by CoA-depleted cells, converted back into CoA intracellularly and this resulted in rescue of the CoA-depleted phenotypes.

Conversion of CoA into 4’-phosphopantetheine by ENPPs

Next we questioned which factors could be responsible for the conversion of CoA into 4’-phosphopantetheine in serum. To identify candidate enzymes, serum from various species (fetal calf, mouse and human) was pre-conditioned, and CoA conversion into 4’-phosphopantetheine was assessed. First, the effect of heat inactivation of the serum was studied. HPLC analysis showed that heating the serum at 50°C for 30 min completely abolished the conversion of CoA to 4’-phosphopantetheine (Figure 4A), indicating the involvement of enzymes or proteins in the process. Second, the conversion of CoA to 4’-phosphopantetheine requires the hydrolysis of a phosphoanhydride bond, which is typically catalyzed by (pyro)phosphatas and hydrolases. The majority of enzymes in the known family of (pyro)phosphatas and hydrolases depend on metal ions for their activity. To test these candidates, ethylenediaminetetraacetic acid (EDTA) was added to serum to chelate metal ions. Treatment of serum with EDTA completely

Figure 4. Conversion of CoA into stable 4’-phosphopantetheine (PPhSH) in serum is mediated by heat unstable and metal-activated enzymes

A. CoA was incubated in heat-inactivated fetal calf serum, mouse serum and human serum for 3 hrs and CoA stability was measured using HPLC analysis. B. CoA stability was determined in fetal calf serum, mouse serum and human serum pre-treated with EDTA (10mM) and CoA levels were measured after 3 hrs using HPLC analysis. C. CoA was incubated in fetal calf serum, mouse serum and human serum pre-treated with ATP or ADP (both 10mM), and CoA levels were measured after 3 hrs. D. CoA stability was determined in fetal calf serum, mouse serum and human serum pre-treated with sodium fluoride (NaF), levamisole, suramin or 4’,4’-diselidocyanatothio-benzene-2,2’-dithiolic acid (DIDS) (all 10mM) and CoA levels were measured after 3 hrs. Data in all the above represent mean ± S.D (n=3). Two-tailed unpaired Student’s t-test was used for statistical analysis to compare indicated subsets (* P ≤ 0.05, **P ≤ 0.01, *** P ≤ 0.001). In all the above experiments CoA was added to the indicated sera to a final concentration of 10uM, and percentages relative to CoA stability for 3 hrs in PBS (100%) are indicated (see Supplementary Materials and Methods for detailed protocol).
prevented the formation of 4'-phosphopantetheine (Figure 4B). This strongly suggested that metal ions were required for the CoA conversion. The most likely hydrolase or (pyro)phosphatase candidates, which possess the ability to convert CoA and which are metal-ion dependent for their activity, are nucid hydrolases, alkaline phosphatases and ecto-nucleotide pyrophosphatases (ENPPs)25-35. These candidate enzymes are also known for their ability to hydrolyze adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP)36-38. Therefore, we tested the conversion of CoA into 4'-phosphopantetheine in serum after addition of excess ATP and ADP. Both competitively blocked the conversion in all sera tested, further underscoring the involvement of one of these enzymes (Figure 4C). Alkaline phosphatase and ENPPs are excreted by cells and are present in serum25,30; however, an additional possible extracellular role for this class of hydrolases cannot be excluded.

Next, we used sodium fluoride (NaF), and levamisole to inhibit nucid hydrolases, and alkaline phosphatase respectively, and in addition we also used two different ENPP inhibitors, suramin and 4,4'-disothiocyanatostilbene-2,2' disulphonic acid (DIDS)33-35. Our data showed that only suramin and DIDS were able to efficiently abolish the degradation of CoA into 4'-phosphopantetheine in all the sera, unlike levamisole, and sodium fluoride (NaF) which showed only mild if no inhibition of CoA degradation into 4'-phosphopantetheine, respectively (Figure 4D). Sodium fluoride (NaF) did not influence CoA degradation in serum, which indicated that either nucid hydrolases were not present or did not degrade CoA in serum. These experiments implicated ENPPs as the most likely class of enzymes to hydrolyze CoA into 4'-phosphopantetheine in serum. Moreover, in all of the CoA serum stability experiments listed above, there was an inverse correlation between the levels of CoA and 4'-phosphopantetheine (Supplementary Figure 7A-C), which underscored that CoA degradation into 4'-phosphopantetheine was mediated by ENPPs.

**4'-phosphopantetheine rescues CoA-depleted phenotypes**

Our data so far predicted that PANK impairment not only induced decreased CoA levels but also decreased levels of 4'-phosphopantetheine. Furthermore, it predicted that addition of 4'-phosphopantetheine to CoA-depleted cells could rescue the induced phenotypes. HPRC analysis of Hopan treated Drosophila S2 cells indeed showed reduced levels of 4'-phosphopantetheine, and external supplementation with either CoA or 4'-phosphopantetheine significantly increased intracellular levels of 4'-phosphopantetheine (Figure 5A). Moreover, when 4'-phosphopantetheine was added to Drosophila S2 cells treated with Hopan (Figure 5B) or dPANK/Rβ RNAi (Figure 5C) the CoA-depleted phenotype was again rescued. 4'-Phosphopantetheine supplementation also rescued the histone acetylation defect in Drosophila S2 cells treated with dPANK/Rβ RNAi (Supplementary Figure 8A-C) or Hopan (Supplementary Figure 8D-F). Finally, we tested the rescue effect of 4'-phosphopantetheine in Hopan-treated mammalian HEK293 cells and found that it also rescued the Hopan-induced reduction in cell count (Supplementary Figure 8G), intracellular CoA levels (Supplementary Figure 8H-D) and histone acetylation levels (Supplementary Figure 8I). Next we investigated whether intact 4'-phosphopantetheine entered cells and whether it was subsequently converted into CoA. First we treated intact cultured Drosophila S2 cells with stable isotope-labelled 4'-phosphopantetheine under various conditions, and mass spectrometry analysis was used to measure the levels of stable isotope-labelled CoA and 4'-phosphopantetheine (Supplementary Figure 9A-D) within the harvested cell extracts.

When labelled 4'-phosphopantetheine is added to the cell culture medium, labelled CoA was detected in harvested cell extracts (Figure 5D). In the presence of Hopan, CoA levels were decreased and replenished in the form of labelled CoA when labelled 4'-phosphopantetheine was added. These data demonstrated that exogenously provided 4'-phosphopantetheine was able to enter cells and intracellularly converted into CoA under normal culturing conditions and under conditions of impaired CoA biosynthesis by Hopan. Next we investigated the characteristics of the passage of 4'-phosphopantetheine over the cell membrane. First,
within 30 min after the incubation of cells with labelled 4′-phosphopantetheine, the intracellular presence of labelled 4′-phosphopantetheine was detected in cells cultured at 25°C (normal culturing temperature of S2 cells) and 4°C. There was no significant difference in the intracellular levels of labelled 4′-phosphopantetheine between these two conditions (Figure 5E). Next we investigated whether under these conditions, the levels of intracellular 4′-phosphopantetheine increased to the same extent as the externally added increased concentrations of 4′-phosphopantetheine. Hereinafter an increasing concentration series (0–100–1000μM) of labelled 4′-phosphopantetheine was added to the cells. This appeared to be the case (Figure 5F). These results indicated that the capacity of cells to accumulate the externally provided 4′-phosphopantetheine was not influenced by temperature and was determined by extracellularly provided concentrations. Finally we investigated the membrane permeating efficiency of 4′-phosphopantetheine using a Parallel Artificial Membrane Permeability Assay (PAMPA assay). Based on this assay 4′-phosphopantetheine but not CoA showed membrane permeating properties (Supplementary Figure 9E–F). Altogether, these results pointed to a capacity of 4′-phosphopantetheine to permeate membranes via passive diffusion.

CoA rescues dPANK/fbl, dPPCDC but not dCOASY phenotypes

Our data showed that CoA from external sources could replenish intracellular CoA levels through its hydrolysis product 4′-phosphopantetheine and subsequent conversion back to CoA. The most likely candidate for the latter conversion is the last bifunctional enzyme, COASY, of the classic CoA biosynthetic pathway. This hypothesis (Supplementary Figure 10) predicts that CoA but not Vitamin B5 can rescue phenotypes caused by mutations in genes encoding enzymes upstream of 4′-phosphopantetheine in the CoA pathway. As a corollary, CoA would not be predicted to rescue CoASY mutant phenotypes.

We aimed to test this hypothesis. In the genome of Drosophila single orthologs were identified for all the enzymes involved in CoA biosynthesis12, including dPANK/fbl, dPPCDC and dCOASY. A set of Drosophila strains was obtained, carrying either mutations in genes encoding these enzymes or carrying a UAS–RNAi construct. Homozygous mutants or flies ubiquitously expressing the RNAi construct showed a significant reduction of CoA, but not 4′-phosphopantetheine (Supplementary Figure 12F).

(On the right) Figure 6. External supplementation of CoA rescues dPANK/fbl, dPPCDC but not dCOASY-impaired phenotypes

A. Lifespan analysis of dPANK/fbl mutants (n=260) or without (n=207) CoA treatment, compared with control flies (n=175) or without (n=179) CoA treatment. Survival curves were significant with Log-rank (Mantel-Cox) test between untreated and CoA (9mM) treated dPANK/fbl mutants (P<0.01). B. Number of pupae of dPANK/fbl mutants after treatment of increasing concentrations of CoA or Vitamin B5. C. Number of pupae of dPPCDC mutants untreated after treatment with CoA or Vitamin B5. D. Lifespan analysis of the dPPCDC RNAi line untreated (n=111), treated with CoA (n=102) or Vitamin B5 (n=102). Survival curves were significant with Log-rank (Mantel-Cox) test between untreated and CoA (18mM) treated dPPCDC RNAi flies (P<0.001). E. Ovarian size of control females, untreated or treated with CoA or Vitamin B5, imaged with light microscopy. Scale bar = 200μm. F. Amount of eclosed adults of dPPCDC RNAi females, crossed with control males raised on control food or supplemented with CoA or Vitamin B5. G. Amount of L1 and L2 dCOASY mutant larvae and controls untreated or treated with CoA or Vitamin B5. H. Proposed non-canonical CoA supply route starting with extracellular CoA. ENPPs are ecto-nucleotide pyrophosphatases. Data represents mean ± SD (n=3) in A, C, F. Solid thick bars without error bars indicate no pupae or eclosed flies observed.
It should be stressed that not all mutants with defects in CoA biosynthesis enzymes showed an identical phenotype, which can be explained by the type of fly lines (RNAi expressing lines, hypomorphic or null mutants) used. This has been reported previously not only for Drosophila but also for other organisms.13,17 Regardless of the severity and developmental stage in which the phenotypes manifested, the determination of the rescue potential of CoA in the available mutants was a valuable tool to test our hypothesis. A scheme of the hypothesis, Drosophila life span and the phenotypes of the used fly lines are presented in Supplementary Figure 10.

We first tested 2 available mutants for dPANK/Fbl, the hypomorphic dPANK/Fbl and the null mutant dPANK/Fbl. Homozygous dPANK/Fbl mutants showed reduced levels of dPANK/Fbl protein, and in homozygous dPANK/Fbl/Fbl mutants, levels of dPANK/Fbl protein were below detection (Supplementary Figure 1A). Correlating with this, homozygous dPANK/Fbl/Fbl mutants showed a reduced adult lifespan (Figure 6A, Supplementary Figure 1A).18,19 While homozygous dPANK/Fbl/Fbl mutants only developed until an early L2 larval stage and pupae were not observed (Figure 6B). Addition of CoA to the food of the homozygous dPANK/Fbl/Fbl mutants increased the life span from 20 to 40 days (Figure 6A, Supplementary Figure 1A), and CoA addition to the food of homozygous dPANK/Fbl/Fbl mutants extended development from the L2 stage to early pupal development (Figure 6B). These results supported our hypothesis. Remarkably, in dPANK/Fbl null mutants low CoA and 4'-phosphopantetheine levels were detected (Supplementary Figure 12C). This may come from a maternal supply or the flyfood as possible resources (Supplementary Figure 1B).

To compromise dPPCDC, the enzyme carrying out the third step of the CoA biosynthesis pathway, we used a UAS-RNAi line (dPPCDC RNAi) as well as a dPPCDC mutant and investigated rescue by CoA. Homozygous dPPCDC mutants showed lethality at early second instar larval stage L2 (Figure 6C). dPPCDC RNAi expressing flies showed a milder phenotype, where adult flies were viable but showed a reduced lifespan (Figure 6D). Females also showed sterility associated with small ovaries and no eggs were produced (Figure 6E, Supplementary Figure 1A-D). Addition of CoA to the food of homozygous dPPCDC mutants extended larval development to late pupal stage (Figure 6C). For the dPPCDC RNAi expressing flies, supplementation of CoA to the food increased the maximal lifespan from 10 days to 30 days (Figure 6D, Supplementary Figure 1E). In addition, sterility was rescued based on the observations of egg production and eclosion of viable offspring (Figure 6E, 6F, Supplementary Figure 1E-D). These results were also consistent with our hypothesis.

Finally, we tested a mutant line of the bifunctional enzyme dCOASY, downstream of 4'-phosphopantetheine. Homozygous dCOASY mutants developed until first instar larval stage and addition of CoA to the food did not result in a significant rescue (Figure 6G). As a negative control for all rescue experiments, vitamin B5 was added to the food, and this did not result in any significant rescue of the phenotypes. A summary of the rescue with CoA in all flylines is presented in Supplementary Figure 10.

To test our hypothesis further, COASY was downregulated with RNAi in mammalian HEK293 cells. Under these conditions levels of COASY protein, CoA and histone acetylation were significantly reduced (Supplementary Figure 1A-F). As in dCOASY mutants, levels of 4'-phosphopantetheine remained unaltered in COASY-compromised mammalian cells (Supplementary Figure 1G). Addition of CoA to the medium neither rescued the COASY RNAi-induced decrease in intracellular CoA levels (Supplementary Figure 1H) nor restored histone acetylation levels (Supplementary Figure 1H). These results were also in agreement with our hypothesis.

T.Taken together, these results demonstrated that impairment of the CoA biosynthetic pathway by genetic manipulation could give rise to highly complex pleiotropic effects affecting lifespan, development and fecundity. These phenotypes can be (partially) rescued by the addition of CoA to the food of the animals, which is then hydrolyzed to 4'-phosphopantetheine, which crosses the plasma membrane via passive diffusion before being converted back to CoA intracellularly, a step requiring COASY (Figure 6H).

DISCUSSION

In our study we addressed the basic question of whether cells and organisms possess alternative ways to obtain the essential molecule CoA in addition to the canonical pathway utilizing Vitamin B5. We demonstrate that cells and organisms are able to acquire exogenous CoA, which is converted into the stable molecule 4'-phosphopantetheine, which enters cells and is converted again into CoA. These newly characterized features of 4'-phosphopantetheine suggest that this molecule can serve as a transport form of CoA or stable reservoir for rapid access and conversion. The proposed mechanism hypothetically allows a net flow of CoA or 4'-phosphopantetheine between cells and between membrane-bound cellular compartments. Our data further suggest that not all cells or organelles within an organism need to harbor all CoA biosynthetic enzymes in order to obtain CoA and that the route to CoA does not necessarily need to follow the archetypal direction starting from the uptake of Vitamin B5. Moreover, these observations hold promise for therapeutic intervention for PKAN because 4'-phosphopantetheine is stable in serum and passes through cell membranes, thereby allowing for restoration of intracellular CoA levels in cells with defective CoA synthesis. The stability of 4'-phosphopantetheine is in strong contrast to characteristics of the dephosphorylated form, pantetheine, which is degraded rapidly in serum by pantetheinases into vitamin B5 and cysteamine.20,21 These results show that the phosphate group protects the molecule from degradation and allows 4'-phosphopantetheine to serve as an effective substrate for CoA biosynthesis from its ready reserve in the circulation.

One intriguing question is whether the proposed route shown here has a physiological function or whether it is artificially provoked by manipulating concentrations of extracellular CoA. Compared to CoA concentrations in cytoplasm (0.02-0.14nM) and mitochondria (2.2-5nM),22 the concentrations used in our study (nM range) are relatively low. Answers may come from previous studies demonstrating that bacteria are able to excrete, but not take up 4'-phosphopantetheine from their environment, suggesting that bacteria-derived 4'-phosphopantetheine may be present in the digestive system.23 The presence of endogenous 4'-phosphopantetheine in mouse serum and in dPANK/Fbl null mutants is consistent with a possible source of extracellular 4'-phosphopantetheine. The function of such a ‘ready’ pool of CoA-precursor may be for transport from one organ to another. In addition to being a source for intracellular CoA, extracellular CoA or 4'-phosphopantetheine may have signaling functions based on reports of an
CHAPTER 3

EXTRACELLULAR 4’-PHOSPHOPANTETHINE IS A SOURCE FOR COA SYNTHESIS

The ability of 4’-phosphopantetheine to translocate across membranes answers long-standing questions regarding the intracellular distribution of CoA and its biosynthetic enzymes. CoA is present in the cytoplasm and in organelles including mitochondria. All CoA biosynthetic enzymes are present in cytoplasm but only a subset has been found in mitochondria. It remains unclear how mitochondria obtain CoA, and the localization of COASY (but not the other CoA biosynthetic enzymes) to the mitochondrial matrix, is also unexplained. It has been postulated that CoA synthesized in the cytosol can be transported into the mitochondrial matrix by specific CoA transporters localized in the mitochondrial inner membrane. Indeed evidence for the presence of such CoA transporters has been presented. Based on our observations we hypothesize that 4’-phosphopantetheine is able to pass over the mitochondrial inner membrane into the matrix and be subsequently converted into CoA by matrix COASY. This may explain the localization of COASY in the mitochondrial matrix.

The presence of a 4’-phosphopantetheine uptake mechanism may have large public health implications. Pathogens and parasites acquire resistance to current treatments, and species-specific inhibitors of CoA biosynthetic enzymes are attractive candidates for a new class of antibiotics and anti-malarial drugs. Such inhibitors may be more effective anti-microbials when 4’-phosphopantetheine uptake is blocked as well. Alternatively, differences in the uptake capacity of 4’-phosphopantetheine by eukaryotic cells (this manuscript) and bacteria may be further explored as possible targets for antimicrobial strategies.

CoA is essential for coordinating key aspects of cell function. It is therefore not surprising that an extracellular pool exists to facilitate swift replenishment and that it relies on the formation of a stable intermediate. While these novel observations raise many new questions about CoA metabolism, they also suggest therapeutic approaches for a range of life-threatening human diseases.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS


COMPETING FINANCIAL INTEREST

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REFERENCES

**SUPPLEMENTARY MATERIALS AND METHODS**

**Drosophila S2 cell culture, RNA Interference, and CoA and 4’-phosphopantetheine treatment**

*Drosophila* Schneider’s S2 cells were maintained at 25°C in Schneider’s *Drosophila* medium (Invitrogen) supplemented with 10% fetal calf serum (Gibco) and antibiotics (pencillin/streptomycin, Invitrogen) under laboratory conditions. Synthesis of RNAi constructs and RNA interference (dsRNA) was carried out as described previously. Non-relevant (human gene, hMAZ) dsRNA was used as control. The cells were incubated for 4 days to induce an efficient knock-down. Cells were then subcultured, with or without CoA (Sigma-Aldrich, Cat. No. C4780, 95% – which is used for all the experiments wherever stated below) or 4’-phosphopantetheine (PPanSH) (Acies Bio, >92%) at different concentrations and were maintained for additional 3 days until analysis for rescue efficiency of the compounds was performed. The stock solutions of compounds were made in sterile water and stored in -20°C until use.

**HoPan treatment of *Drosophila* S2 Cell in combination with CoA or 4’-phosphopantetheine treatment**

*Drosophila* Schneider’s S2 cells were maintained at standard conditions as explained above. Cells in the exponential phase of growth were used for all the experiments. Different indicated concentrations of CoA or 4’-phosphopantetheine (deuterium labelled PPalnSH(D4) or unlabelled PPalnSH) were added to S2 cells either in the presence or absence of 0.5mM HoPan (Zhou Fang Pharm Chemical,; 99%) for 48hrs. Similarly, *Drosophila* S2 cells were treated with different concentrations of PPalnSH(D4) at either 25°C or 4°C and cells were then harvested at various time points to access transport of PPalnSH(D4). Stable isotope labelled PPalnSH containing 4 deuterium atoms was purchased as a sodium salt (from Syncom; synthesized as previously described, 99.7%). As a read out, cell count, intracellular total CoA and PPalnSH levels (both labelled and unlabelled levels wherever appropriate) and histone acetylation levels were analyzed as explained below.

**Drosophila S2 cell immunofluorescence staining**

For immunofluorescence *Drosophila* S2 cells were seeded on Poly-L-Lysine coated (Sigma-Aldrich) glass microscope slides and allowed to settle for 45min. Cells were fixed with 3.7% formaldehyde (Sigma Aldrich) for 20min, washed briefly with phosphate-buffered saline (PBS) + 0.1% Triton-X-100 (Sigma Aldrich) and permeabilized with PBS + 0.2% Triton-X-100 for 20min. The slides were incubated in primary antibody (rabbit anti-AcLys, Cell Signaling Cat No. 9441, 15000) to visualize histone acetylation levels in PBS + 0.1% Triton-X-100 overnight and after an additional washing step in PBS + 0.1% Triton-X-100 they were incubated in secondary goat anti-rabbit-Alexa488 antibody (Molecular Probes) for two hours at room temperature (RT). F-actin was detected with Rhodamin-Phalloidin (20U/ml) (Invitrogen) and DNA by staining with DAPI (0.2mg/ml) (Thermo Scientific). Finally the samples were mounted in 80% glycerol and analyzed using a Leica fluorescence microscope with Leica software. Adobe Photoshop and Illustrator (Adobe Systems Incorporated, San Jose, California, USA) were used for image assembly.

**HoPan treatment of mammalian HEK293 Cells in combination with CoA and 4’-phosphopantetheine treatment**

HEK293 cells were maintained in dMEM (Invitrogen) supplemented with 10% fetal calf serum (Gibco) and antibiotics (pencillin/streptomycin, Invitrogen). For HoPan treatment, cells were cultured in custom made dMEM without Vitamin B5 (Thermo Scientific) supplemented with dialyzed FCS (Thermo Scientific). CoA or PPalnSH was added to HEK293 cells for the final concentration of 25μM, either in the presence or absence of HoPan (0.5mM) for 4 days, followed by analysis for phenotype and rescue efficiency of CoA and PPalnSH.

**Knockdown of COASY by siRNA in mammalian HEK293 cells**

HEK293 cells were maintained as described above. HEK293 cells were transfected with 200nM COASY siRNA (GE Healthcare human COASY 80347 smartpool Cat no: M-006751-00-0010) or non-targeting siRNA (GE Healthcare Cat no D-001206-13-20) using Lipofectamine 2000 (Invitrogen). 4hrs after transfection CoA was added in a final concentration of 25μM. Cells were cultured for 3 days and then harvested for HPLC analysis of total CoA and PPalnSH levels and Western blot (histone acetylation) as described below.

**Western blot analysis and antibodies**

For Western blot analysis, cells were collected and washed with PBS, followed by centrifugation. The cells were lyzed and sonicated in 1X Laemml Sample Buffer and boiled for 5min with 5% β-mercaptoethanol (Sigma). Protein content was determined using DC protein assay (BioRad). Equal amounts of protein were loaded on a 10 or 12.5% SDS-PAGE gel, transferred onto PVDF membranes and blocked with 5% milk in PBS/0.1% Tween, followed by overnight incubation with primary antibodies. The primary antibodies used were: rabbit-anti dPANK/fbl, 1:4000 Eurogentec; mouse anti-tubulin (Sigma Aldrich Cat no: T5168, 15000), anti-acetyl-Histone (Active Motif Cat no: 39139, 12000), anti GAPDH (Fitzgerald Cat no 10R-C109a, 110000), rabbit anti COASY (Abcam Cat no: AB2912, 11001). Appropriate HRP-conjugated secondary antibodies (Amersham) were used and detection was performed using enhanced chemi-luminescence (Pierce cat nog: 32106) and Amersham hyperfilm (GE healthcare). Band intensities were quantified with Image-studio software. Full uncit gel images for all Westerns displayed in this paper are shown in Supplementary Figures 15 and 16.

**C. elegans media and strains**

Standard culturing conditions were used for *C. elegans* maintenance at 20°C. N2 strain was used as a wild-type control. VC927, the PANK deletion mutant, pnk-1(ok1435)/Hf72(bli – d(e937)) let-7(rf214) was obtained from the Caenorhabditis Genetics Center. To obtain synchronous cultures, worms were
bleached with hypochlorite, and allowed to hatch in M9 buffer (3g KH2PO4, 6g Na2HPO4, 5g NaCl, 1ml 1M MgSO4, H2O to 1 liter) overnight and cultured on standard Nematode Growth Medium (NGM) plates seeded with OP50 strain of Escherichia coli.

**C. elegans lifespan assay**

After synchronization, C. elegans L1 animals were grown on control NGM plates or NGM plates supplemented with 400μM CoA. The life span experiments were started by transferring 100 one-day old adults per condition on NGM plates, which contained 5-fluoro-2'-deoxy-uridine (FUDR) to inhibit growth of offspring. Once a day surviving animals were counted, the worms that disappeared or crawled out of the plate were excluded from the analysis.

**C. elegans motility assay**

After synchronization, L1 C. elegans were grown on control NGM plates or NGM plates containing various concentrations of CoA. One-day old adults were placed in a drop of M9 buffer and allowed to recover for 30sec. During the following 30sec, the number of body bends was counted. A movement was scored as a bend when both the anterior and posterior ends of the animal turned to the same side. At least 15 worms were scored per condition and each experiment was repeated twice. The sequential light microscopy images demonstrating movements of C. elegans in M9 buffer were acquired using Leica MZ16 FA microscope at 52x magnification within the time frame of 1sec and processed using ImageJ software (National Institutes of Health, Maryland, USA) and Adobe Photoshop (Adobe Systems Incorporated, San Jose, California, USA).

**Drosophila maintenance and crosses**

Drosophila melanogaster stocks/crosses were raised on standard cornmeal agar fly food (containing water, agar 17 g/L, yeast extract 26 g/L and nipagin 13 g/L) at 25°C. The stocks were either obtained from the Bloomington Stock Centre (Indiana University, USA), VDRC (Vienna Drosophila RNAi Collection, Vienna, Austria) or from the Exelixis Collection (Harvard Medical School) and rebalanced over eGFP-positive balancers to identify homozygous (eGFP negative) progeny. The stocks used were: w[1118], dPANK/fbl hypomorph 12,19; dPANK/fbl hypomorph 12,19; dPANK/fbl hypomorph 12,19; dPANK/fbl null (y[1] w[∗]; PBac{w[+mC]=WH}Ppcdc[f00839]/CyO, Bloomington 18377), UAS-dPPCDC RNAi line (VDRC 104495); dCOASY mutant (P{w[+mC]=Act5C-GAL4}25FO1/CyO, Bloomington 16341), UAS-RNAi constructs were ubiquitously expressed under the control of Actin-Gal4. The crosses were raised at 25°C. F1 RNAi-construct expressing females and control females were cultured shortly after eclosion and transferred to standard fly food or food containing Vitamin B5 or CoA (18mM). Flies were maintained for 2 days on this food at 25°C. After this period extra yeast and w1118 control males were added and the crosses were kept at 25°C for another 2 days. After this 4-day period ovaries were dissected and stained for further analysis. The vials (or plates) from the crosses (with eggs that were being laid during the 4 day period of CoA treatment) were kept for another 10 days and offspring numbers were counted after eclosion.

**Drosophila larval collection and larval count experiment**

One week old flies (in the ratio 10 females and 5 males) were kept on 5ml of standard fly food in a vial at 25°C with or without various concentrations of CoA or Vitamin B5 (Sigma, Cat. No. PS155). The flies were allowed to lay eggs for 2 days and parent flies were then discarded. The L1, L2 and L3 larvae were collected from the food with 20% sucrose at appropriate time (day 4, 6 and 8 respectively) for larval counting and stored in -80°C until analysis. The pupal count was performed between 10-12 days.

**Drosophila life span**

One-day old female adult flies of Drosophila homoyzous mutants or RNAi-constructs expressing lines, were collected with appropriate controls and were kept on standard fly food at 25°C with or without CoA or Vitamin B5 (Sigma) at necessary concentration (in 50μl added on top of the fly food and dried before flies were added). The flies were counted every 12-24hrs and flipped to new fly food vials with or without CoA or Vitamin B5.

**Drosophila ovary rescue experiment**

UAS-dPPCDC RNA constructs were ubiquitously expressed under the control of Actin-Gal4. The crosses were raised at 25°C. F1 RNAi-construct expressing females and control females were cultured shortly after eclosion and transferred to standard fly food or food containing Vitamin B5 or CoA (18mM). Flies were maintained for 2 days on this food at 25°C. After this period extra yeast and w1118 control males were added and the crosses were kept at 25°C for another 2 days. After this 4-period ovaries were dissected and stained for further analysis. The vials (or plates) from the crosses (with eggs that were being laid during the 4 day period of CoA treatment) were kept for another 10 days and offspring numbers were counted after eclosion.

**RNA isolation, quantitative Real-Time PCR, and primers:**

Drosophila larvae and samples of 1-day old adult flies or larvae were collected for homoyzous dPPCDC mutants, dPPCDC RNAi-construct expressing lines and for homoyzous dCOASY mutants, followed by brief washing with PBS. The samples were lysed in TRIzol (Invitrogen) for RNA extraction and reverse transcribed using M-MLV (Invitrogen) and oligo(dT) 12-18 (Invitrogen). SYBR green (Bio-Rad) and Bio-Rad Real-Time PCR with specific primers were used for gene expression level analysis. The expression levels were normalized for rp49 (house-keeping gene). The Primer sequences used were dPPCDC (TCACATTCTGATGCAATCC; TCCGCTGAACCCCGCTATAA), dCOASY (GCCGTGCACGCGCGATTATCG; CGCGTAAACGCTGCTTCC) and rp49 (GCACAAACACTTCTACCC; CGATCTCCCACGCTAAA) (Bioligo).
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Drosophila ovary dissection and staining
Drosophila ovaries were collected in cold PBS and fixed in 4% formaldehyde (from methanol-free 16% Formaldehyde Solution, Thermo Scientific) for 45min at RT. The fixed tissue was washed in PBS + 0.1% Triton-X-100 for 1hr at RT and afterwards permeabilized in PBS + 0.2% Triton-X-100 for 1hr. Finally the ovaries were stained with Rhodamin-Phalloidin (20U/ml) to detect F-actin and DAPI (0.2mg/ml) for DNA. Finally the samples were mounted in 80% glycerol and analyzed on a Zeiss-LSM780 NLO confocal microscope with Zeiss Zen software. Adobe Photoshop and Illustrator (Adobe Systems Incorporated, San Jose, California, USA) were used for image assembly.

PAMPA assay procedure
Parallel Artificial Membrane Permeability Assay (PAMPA) was performed and processed according to manufacturer’s instructions (BD-Designer Pre-coated PAMPA plates). Briefly, two superimposed wells were separated by an artificial lipid-oil-lipid membrane. The compound of interest (PPanSH, CoA, caffeine, amiloride) was added to the bottom well in phosphate-buffered saline, whereas the top well was filled with phosphate-buffered saline alone. After 5hrs of incubation at RT, concentrations of the different compounds were measured using UV-VIS absorption spectroscopy (BMC Labtech SPECTROStar Omega) along with calibration curves for all compounds. The permeability efficiency was further calculated according to manufacturer’s instructions (Supplementary figure 9E). For caffeine and amiloride, four replicates were performed, for PPanSH and CoA twelve replicates were performed. Caffeine and amiloride were obtained from Sigma.

Serum collection from PKAN patients
Serum was collected from the blood of PKAN patients and respective healthy family members (control) using standard protocols. Briefly, venous blood was collected in commercial lyophilized available red top Vacutainer tubes (Becton Dickinson) and allowed to remain at RT for 15-30min undisturbed for the blood clotting. The tubes were then centrifuged at 2,000 g for 10min at 4°C. The resulting supernatant serum was immediately transferred to 2ml cryovials and maintained at -80°C until CoA stability assessments were performed. Blood samples and clinical data were obtained under OHSU’s IRB-approved repository protocol #7232 following informed consent.

CoA and pantetheine serum stability measurements
CoA serum stability studies were conducted in commercially obtained serum and in serum collected from PKAN patient and healthy family members as controls. Human and Mice sera were purchased from Sigma and Fetal calf serum was purchased from Gibco. Additionally, dMEM medium with or without 10% fetal calf serum was used for evaluating CoA stability. Briefly, all sera and samples were incubated for 30min at 37°C with or without pre-conditioning compounds at final concentration 10mM (Adenosine 5’-triphosphate (ATP), Adenosine 5’-diphosphate (ADP), Ethylenediaminetetraacetic acid (EDTA), Levamisole, Suramin, 4,4’-Disothiocyanostilbene-2,2’-disulfonic acid disodium (DIDS) and sodium fluoride (NaF), all purchased from Sigma) followed by addition of CoA 20µM in the ratio of 11 and incubated at 37°C after brief vortex for indicated time intervals. For heat inactivation, all sera were incubated for 30min at 50°C after which CoA was added. Serum samples at different time points were collected, deproteinized and analyzed by HPLC as described below. For pantetheine serum stability, pantetheine (Sigma) was incubated in fetal calf serum, mice serum and human serum for 15min in 37°C and total levels of pantetheine and cysteamine was measured using HPLC.

Mice and CoA intravenous injection study
Adult male mice of C57BL/6J (19-21g) mixed genetic background were used for this study. Two mice (approximately 25-30g wt) were used for each condition. 0.1mg or 0.5mg CoA in 0.25ml saline solution was injected intravenously (i.v) into the tail vein. Saline solution (0.25ml) was injected to control groups. After 30min and 6hrs blood samples were collected and further processed to obtain plasma followed by sample preparation for HPLC or LC-MS analysis as indicated below. All animal studies were approved by the Ethics Committee of the Foundation IRCCS Neurological Institute C. Besta, in accordance with guidelines of the Italian Ministry of Health. Project no. BT4/2014. The use and care of animals followed the Italian Law D.L. 116/1992 and the EU directive 2010/63/EU.

HPLC sample preparation protocol for total CoA and 4’-phosphopantetheine measurement
Samples were briefly washed with ice-cold PBS solution. Samples were sonicated thoroughly in 100µl ice-cold PBS and centrifuged for 10-15min at 4°C to collect supernatant. Tris(2-carboxyethyl)phosphine hydrochloride (Sigma) (50mM; 10µl) was added to 50µl sample supernatant and were incubated at RT for 15min after vortex-mixing. Saturated ammonium sulfate solution or Millipore 3KD centrifugal filter units were used to remove proteins. The samples were centrifuged at 14,000 rpm for 5min at 4°C. The clear supernatant (50µl) or the filtrate was derivatized with 45µl of ammonium 7-flurobenzo-2-oxa-1,3-doazole-4-sulfonate (SBD-F, Sigma) (1mg/ml in borax buffer - 0.1W containing 1mM EDTA dioxidum, pH 9.5), and 5µl ammonia solution (25.5% v/v Merck Millipore) for 60min at 37°C. The derivatized samples were placed in a refrigerated autosampler (10°C) in the Shimadzu HPLC system, and injected for total CoA and PPanSH analysis using optimized chromatographic separation conditions combined with fluorescence detection (described below).

Chromatography separation condition
Chromatographic analysis was performed with a Shimadzu LC-10AC liquid chromatograph, SCL-10A system controller, SIL-10AC automatic sample injector and LC-10AT solvent delivery system. Shimadzu RF-20Axs fluorescence detector was used for derivatized sample extract analysis. The fluorescence detector was set at excitation and emission wavelengths of 385nm and 515nm, respectively. Signal output was collected digitally with Shimadzu Labsolution software and postrun analysis were performed. Chromatographic separation of the analytes was achieved with a Phenomenex Gemini C18 guard column (4 x 3mm) connected to a Phenomenex Gemini NX-C18 analytical column (4.6 x 150mm; 3µm particles) at 45°C. The two mobile phases consisted of A 100mM ammonium acetate buffer (pH 4.5) and B
acetonitrile. Flow rate was maintained at 0.8 ml/min with a slow gradient elution: 0% B till 7 min, 20% B at 20 min, 20% B at 22 min, 50% B at 23 min, maintained at 50% B till 27 min, 0% B at 28 min and 7-10 min for column re-equilibration.

Sample preparation for mass spectrometry and instrumental parameters

Samples were briefly washed with ice-cold PBS solution. Samples were then sonicated thoroughly in 100 μl ice-cold milliQ (MQ) water containing 50 mM Tris(2-carboxyethyl)phosphine hydrochloride. Subsequently 100 μl saturated ammonium sulfate was added to each sample and centrifuged for 20 min at 10°C, 16,100 rcf to collect supernatant. To 150 μl of supernatant, 15 μl of ammonium hydroxide (25%) was added and 20 μl was injected for LC-MS (liquid chromatography-mass spectrometry) analysis. For mouse plasma analysis, 50 μl of MQ water containing 50 mM Tris(2-carboxyethyl)phosphine hydrochloride was added to 50 μl of plasma and processed further as mentioned above. Appropriate dilution series of standard CoA, PPanSH and PPanSH(D4) was processed similarly before analysis. The LC separation of metabolites were obtained using Phenomenex Gemini NX-C18 analytical column (4.6 x 150 mm, 3 μm particles) at 45°C. The flow was maintained at 1 ml/min with optimized mobile phase gradient of MQ water (A), 200 mM ammonium acetate (NH₄Ac) in 95/5 MQ water/acetonitrile adjusted to pH 4.5 with acetic acid (B), and acetonitrile (C). The separated analytes were detected with positive mode mass spectrometry (Sciex API5500 Q-trap) under unit resolution. The targeted Q1/Q3 mass/charge ions of PPanSH, PPanSH(D4), CoA and CoA(D4) were 359.1/261.1, 363.1/265.1, 768/261.1, and 772/265.1 respectively. The absolute concentration was finally calculated using linear regression analysis of respective standard compounds, except CoA(D4) which was estimated indirectly using CoA standards.

Statistical analysis

All experimental results are presented as mean of at least 3 independent experiments ± SD, unless otherwise stated. Statistical significance was determined by a two-tailed unpaired Student’s t test between appropriate groups wherever applicable. For life span survival curve, more than 80 flies or C.elegans were used in each group and statistical significance was determined using Log-rank (Mantel-Cox) test (See figure legends for exact number or flies/C.elegans used in survival analysis). Statistical P values < 0.05 were considered significant (* P < 0.05, ** P < 0.01, *** P < 0.001). Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Supplementary Figure 1. CoA supplementation rescues HoPan induced phenotypes in Drosophila S2 cultured cells

A. Relative cell counts of control (100%) and HoPan treated cells in the presence of increasing concentrations of CoA. Data points represent mean ± SD (n=3). B-D. Protein acetylation levels were visualized in control (B) and HoPan treated cells without (C) and with + CoA. An antibody against acetylated lysine (green), Rhodamin-phalloidin (red), marking F-actin, and DAPI (blue, DNA) were used. Scale bars indicate 20 μm.

**Supplementary Figure 2. Phenotypes induced by impaired pantothenate kinase in C. elegans are rescued by external supplementation of CoA**

A. Quantification of motility in C. elegans pantothenate kinase (pkn-1) mutants with and without addition of different CoA concentrations (0, 5, 10, 20, 400, and 800µM) to the food. Error bars indicate ± SD (n=15, except for 0µM where n=45) and two-tailed unpaired Student’s t-test was used to assess statistical significance (* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001).

B. Lifespan analysis of C. elegans pkn-1 mutants untreated (n=96) and with CoA treatment (100µM; n=101 and 400µM; n=90). Survival curves were found to be significant with P value < 0.001, analyzed with Log-rank (Mantel-Cox) test, between untreated and CoA treated pkn-1 mutants.

C-E. Representative serial images demonstrating movements of C. elegans wild-type (C) and pkn-1 mutants without (D) and with (E) CoA treatment (400µM); still images are given; “merged” indicates superimposed images. Scale bar represents 200µm.

**Supplementary Figure 3. CoA degradation profile in medium and fetal calf serum**

Relative CoA levels determined by HPLC analysis in PBS, medium, medium containing fetal calf serum and in fetal calf serum (FCS) after 3 hrs incubation. Each bar represents mean data value (n=3) ± SD and two-tailed unpaired Student’s t-test was used to analyse significance (** ** P ≤ 0.001).
Supplementary Figure 4. Chromatogram profile of CoA and its thiol containing conversion product in fetal calf serum
A-E. HPLC chromatogram profile of CoA stability in PBS (A) and FCS (B) compared with standard 4’-phosphopantetheine (PPanSH, C), Pantetheine (D) and Diphospho-CoA (E). CoA is migrating at 17.64 min; PPanSH at 18.27 min; Pantetheine at 21.61 min and Diphospho-CoA at 18.85. CoA is stable in PBS and converted in serum into a thiol-containing compound exactly migrating as PPanSH standard at 18.27 min.

Supplementary Figure 5. Pantetheine stability, CoA conversion in patient’s serum and CoA and 4’-phosphopantetheine stability in human serum
A. Pantetheine is rapidly degraded in serum. Pantetheine was incubated for 15 min at 37°C in fetal calf serum, mice serum and human serum and levels of total pantetheine and cysteamine were measured using HPLC. B. CoA hydrolysis to PPanSH in serum derived from PKAN patients and their healthy family members as a control. CoA (20μM) was incubated for 3 hrs at 37°C in serum derived from PKAN patient and in serum derived from their healthy family members as a control. Levels of CoA and PPanSH were measured using HPLC analysis. In patient’s serum CoA was as efficiently hydrolysed into PPanSH as in serum derived from healthy family members. Error bars represent ± SD where applicable for mean values (n=3). Genders were indicated as female=F and male=M. C. CoA was added to human serum and concentrations of CoA and PPanSH in human serum over 6 hrs were determined by HPLC analysis. Data points indicate mean value ± SD (n=3).
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Supplementary Figure 6. HPLC chromatogram profile and confirmation by mass spectrometry of endogenous 4'-phosphopantetheine in mouse serum

A. HPLC chromatogram profile in untreated fresh mouse serum (solid line), shows a peak which co-migrates exactly with PPanSH as visible when the sample was spiked with standard PPanSH (dotted line). These results indicate the presence of endogenous PPanSH.

B-C. Confirmation of endogenous PPanSH by mass spectrometry in mouse plasma (C) compared to standard PPanSH (B). D. Mass spectrometry was used to confirm the presence of elevated levels of PPanSH in plasma, 6 hrs after CoA injection (0.5mg) in mice.

Supplementary Figure 7. CoA degradation correlates with the appearance of 4'-phosphopantetheine

A-C. 4'-phosphopantetheine measurements of the experiment shown in main Figure 4. Fetal calf serum (A), mouse serum (B) and human serum (C) were heat-inactivated or pre-treated with 10mM of EDTA, or ATP or ADP, or with the inhibitors Sodium fluoride (NaF) or Suramin and levels of PPanSH were determined as described for main Figure 4. Data represents mean value ± SD (n=3) and solid black bars without error bars indicate no PPanSH was detected, wherever applicable.
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\[(CD \times VD) + (CA \times VA)\]

**Supplementary Figure 8.** External supplementation of 4’-phosphopantetheine rescues CoA-deprived phenotypes

**A-F.** Immunofluorescence was used to visualize protein acetylation levels in control (**A, D**), PPaSH(D4) treated (**B**), and HoPan treated (**C, F**) S2 cells with and without PPaSH (**C, F**). An antibody against acetylated lysine (green), Rhodamin Phalloidin (red), marking F-actin, and DAPI (blue, DNA) were used. Addition of PPaSH rescues acetylation defects of iPAN/FR RNAi and HoPan treated S2 cells. Scale bars indicate 20 μm. **G.** Cell count of mammalian HEK293 control cells (100%) treated with HoPan with and without CoA or PPaSH added to the medium. Data indicate mean values ± SD (n=3) and two-tailed unpaired Students t-test was used for statistical analysis. **H.** Relative CoA levels were determined by HPLC of control (100%) and HoPan treated HEK293 cells with and without CoA or PPaSH added to the medium. Data indicate mean values ± SD (n=3) and two-tailed unpaired Students t-test was used for statistical analysis. **I.** Western blot analysis and quantification to determine histone acetylation levels of control HEK293 cells, cells treated with HoPan with and without CoA or PPaSH. Data represents mean values ± SD (n=3) and two-tailed unpaired Students t-test was used for statistical analysis.

In all the above data representation, statistical significance was indicated as applicable: *P < 0.05, **P < 0.01, ***P < 0.001.

**Supplementary Figure 9.** 4’-phosphopantetheine is taken up by Drosophila S2 cells and converted in CoA

**A-D.** Mass spectrometry was used to detect the levels of 4’phosphopantetheine labelled with stable isotope (deuterium) PPaSH(D4) and CoA labelled with stable isotope (deuterium) (CoA(D4)). S2 cells were left untreated (**A, B**) or treated with HoPan (**C, D**) and PPaSH(D4) was added to the medium of untreated and HoPan treated cells. Levels of PPaSH(D4) (**A, C**) and levels of CoA(D4) (**B, D**) were measured. Chemical structures of PPaSH(D4) and CoA(D4) are given. **E-F.** Schematic overview of a Parallel Artificial Membrane Permeability Assay (PAMPA assay). Experiments were performed and the permeability parameter was calculated according to the manufacturer’s instructions. Formulas are depicted in the figure and the parameters are explained below. **G.** Compounds that are below the assay threshold are predicted to be unable to pass membranes passively, whereas compounds above the threshold are able to pass membranes passively. In contrast to CoA, PPaSH shows a permeating property (although the permeability parameter of PPaSH is lower compared to the positive control). Caffeine and amiloride were used as a positive and negative control respectively. Data values represent mean ± SD with replicates for PPaSH and CoA (n=12) and caffeine and amiloride (n=4). Abbreviations are as follows: Ceq = Equilibrium Concentration, VA = Volume of acceptor well (0.2ml), CD = Concentration in donor well, PF = Permeability, VD = Volume of donor well (0.3ml), S = Membrane area (0.3cm²), CA = Concentration in acceptor well, t = Incubation time (1800 s).
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Supplementary Figure 10. External supplementation of CoA rescues dPANK/fbl- and dPPCDC- but not COASY-impaired phenotypes. From left to right; Overview of the well-known CoA biosynthesis route in which the enzymatic conversion steps 1, 2 and 3, upstream of PPanSH and the combined enzymatic step 4-5 downstream of PPanSH are indicated. Mutant lines and/or RNAi lines used for manipulating conversion steps upstream and downstream of PPanSH are indicated. The upper image represents time scale and images of normal Drosophila developmental and adult stages. Fly line and mutant-specific developmental arrest is indicated under control conditions (dotted line) and after CoA supplementation to the food (solid line).

Supplementary Figure 11. Quantitative real-time PCR data demonstrates reduced mRNA expression of dPPCDC and dCOASY in Drosophila RNAi lines and mutant flies. A. mRNA expression levels of dPPCDC normalized with house-keeping gene (rp49) expression levels in 1-day old adult dPPCDC RNAi Drosophila female flies and in age-matched control flies. B. mRNA expression levels of dPPCDC normalized with house-keeping gene (rp49) expression levels in L2 stage control larvae and in L2 stage dPPCDC mutant larvae. C. mRNA expression levels of dCOASY normalized with house-keeping gene (rp49) expression levels in L1 stage control larvae and in L1 stage dCOASY mutant larvae. All data presented above indicate the mean ± SD (n=3) and two-tailed unpaired Student’s t-test was used for statistical analysis (* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001).
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Supplementary Figure 12. External supplementation of CoA rescues dPANK/fbl- and dPPCDC- but not COASY-impaired phenotypes

A. Western blot analysis of dPANK/Fbl protein expression levels of control animals, homozygous hypomorphic (dPANK/fbl1) mutants and homozygous null (dPANK/fblnull) mutants. Tubulin as loading control. B. CoA and PPanSH levels measured by HPLC analysis in 1-day old homozygous (dPANK/fblnull) and control adult flies. CoA and PPanSH levels in mutant larvae are presented as percentages of CoA levels in control larvae.

C. CoA and PPanSH levels measured by HPLC in early L2 stage null homozygous (dPANK/fblnull) mutant and control larvae. CoA levels in mutant larvae are presented as percentages of CoA levels in control larvae. D. Relative CoA and PPanSH levels measured by HPLC in early L2 stage of control and homozygous dPPCDC mutant larvae. E. Relative CoA and PPanSH levels measured by HPLC of the L2 larval stage of control and homozygous dCOASY mutant larvae. F. Relative CoA and PPanSH levels measured by HPLC of 1-day old homozygous (dCOASYmutant) larvae compared to control.

All the data sets in B-F indicate mean ± SD (n=3) in the above representations and two-tailed unpaired Student’s t-test was used for statistical analysis (* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001).

Supplementary Figure 13. Life span survival of Drosophila dPANK mutant flies and endogenous 4'-phosphopantetheine levels in food sources

A. Lifespan analysis of hypomorphic (dPANK/fbl) homozygous female mutants untreated (n=207) and treated with various concentrations of CoA (6mM, n= 105; 9mM, n=115; and 12mM, n=88) added to the food. Survival curves were found to be significant with P value < 0.001, analyzed with Log-rank (Mantel-Cox) test, between untreated and all CoA treated dPANK/fbl mutants. B. In various animal food sources (yeast, E.coli and mouse liver) levels of CoA and PPanSH were measured and found to be present (n=2).
Supplementary Figure 14. External supplementation of CoA rescues phenotypes of dPPCDC RNAi lines and COASY is required for CoA rescue in mammalian cells

A-C. Ovaries of 4-day old control and dPPCDC RNAi expressing flies, stained with Rhodamin-Phalloidin (magenta; marking F-actin) and the nuclear marker DAPI (green) and imaged with confocal microscopy. A) In wild-type ovarioles strings of developing egg-chambers, from the gerarium up to stage 9 were visible. Mature eggs were also found (marked by asterisks), identifiable by the presence of yolk. B) In ovarioles of the dPPCDC RNAi expressing flies, egg-chambers developed normally until stage 7. From stage 8 on, fragmented and condensed DNA was visible, indicating apoptosis (marked by white arrowheads). No egg-chambers older than stage 8/9 or mature eggs were found in these ovarioles. C) CoA treatment of the dPPCDC RNAi expressing flies improved egg-production significantly and eggs developed to maturity (marked by asterisks). Scale bars =10µm; D) Increased fertility of dPPCDC RNAi expressing females. Untreated, Vitamin B5 treated and CoA treated (dPPCDC RNAi) females were mated with control males and put onto apple juice plates to allow egg laying for 4 days. For untreated and Vitamin B5 treated females, no or only very few eggs were observed on the plates (compare Figure 1H). The part of the Western blot used for the figure is outlined by a dashed line. 

Original blots used for Figure 1b

Supplementary Figure 15. Full uncut gel images for Western blots presented in Figures 1B and 1H and Supplementary Figure 8i. A-D) Full uncut gel images for the anti-acH3 and anti-Tubulin Western blots presented in Figure 1B. The part of the Western blot used for the figure is outlined by a dashed line. E-F) Full uncut gel images for the anti-act-H3 and anti-GAPDH Western blots presented in Figure 1H. The part of the Western blot used for the figure is outlined by a dashed line.
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Supplementary Figure 16. Full uncut gel images for Western blots presented in Supplementary Figures 12A and 14F
A-B. Full uncut gel images for the anti-dPANK/Fbl and anti-Tubulin Western blots presented in Supplementary Figure 12A. The part of the Western blot used for the figure is outlined by a dashed line. C-E. Full uncut gel images for the anti-COASY, anti-acH3 and anti-GAPDH Western blots presented in Supplementary Figure 14F. The part of the Western blot used for the figure is outlined by a dashed line.

SUPPLEMENTARY NOTE

Synthesis of 4'-phosphopantetheine (PPanSH)

A

B

C

D

E

Supplementary Note

Synthesis of 4'-phosphopantetheine (PPanSH)

a. Coupling Reaction

b. Phosphorylation
c. Deprotection

EDC / HOBl

Na / naphthalene

S-tritylpantetheine

Dibenzyl chlorophosphate

S-trityl-4'-dibenzylphosphopantetheine

4'-phosphopantetheine
4'-Phosphopantetheine (PPanSH) Synthesis Protocol

4'-Phosphopantetheine (PPanSH) was synthesized in a three-step procedure as described below (a/b/c) (Supplementary Note). In the first step, commercially available pantothenic acid was coupled with synthesized S-tritylcysteamine. The obtained S-tritylpantetheine was then phosphorylated with freshly prepared dibenzylchlorophosphate. Finally, removal of benzyl groups provided 4'-phosphopantetheine.

D-Pantothenic acid was prepared from its hemicalcium salt (Aldrich, > 99.0%) by reacting with oxalic acid in distilled water. The precipitated calcium oxalate was filtered off, while the protonated form of D-pantothenic acid was obtained by evaporation of water. S-tritylcysteamine was synthesized from cysteamine hydrochloride and triyl chloride. Dibenzychlorophosphate was prepared by reacting dibenzylphosphate with N-chlorosuccinimide in toluene as a solvent. All other chemicals were obtained from commercial sources and used without further purification; cysteamine hydrochloride (Aldrich, > 98.0%), triyl chloride (Aldrich, 97.0%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) (Aldrich, > 97.0%), 1-hydroxybenzotriazole hydrate (HOBt) (Aldrich, technical grade), N-chlorosuccinimide (Aldrich, 98.0%). Column chromatography was carried out using Silica gel 60 Å, 60-100 mesh (Aldrich). Cation exchange chromatography was performed on DOWEX 50WX2, hydrogen form, 100-200 mesh (Aldrich). 1 H and 13 C NMR were recorded at 25°C with Varian Unity Inova 300 MHz spectrometer (300 MHz/75 MHz). The chemical shifts (δ) relative to TMS as an internal standard where spectra recorded in CDCl3 or relative to residual solvent signal in D2O were reported in ppm units.

**4'-Phosphopantetheine (PPanSH) Synthesis Protocol**

**a) Coupling reaction – synthesis of S-tritylpantetheine**

In dried acetone (100ml) the following were prepared separately: (A) D-pantothenic acid (2.19g, 10.0mmol), (B) S-tritylcysteamine (3.9g, 10.0mmol) and (C) N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) (1.55g, 10.0mmol) together with 1-hydroxybenzotriazole hydrate (HOBt) (1.35g, 10.0mmol). After A, B and C were mixed together, triethylamine (10.4ml, 75mmol) was added. The mixture was stirred for 2 h while maintaining the temperature below –30°C. The reaction was quenched by addition of water (100ml) and then dichloromethane (200ml) was added. Phases were separated and the aqueous phase (together 500ml) was washed with dichloromethane (200ml) and diethyl ether (3x200ml), concentrated under vacuum and passed through the cation exchange column (DOWEX 50WX2, 200g). Fractions were analyzed by LCMS and those containing the product were pooled and concentrated under vacuum. 4'-phosphopantetheine was precipitated with addition of Ca(OH)2 as a calcium salt (332mg, 0.838mmol, 35%). The structure of the product was confirmed by comparison of NMR data with the literature1,3 and by HRMS. 1 H NMR (300 MHz, D2O) δ 0.86 (s, 3H), 1.08 (s, 3H), 2.54 (app d, J = 6.3 Hz, 2H), 3.01 (m, 2H), 3.38-3.49 (m, 4H), 3.92 (s, 3H), 6.20 (t, J = 5.7 Hz, 1H, NH), 7.14-7.29 (m, 10H), 7.36-7.45 (m, 5H).

**b) Phosphorylation – synthesis of S-trityl-4'-dibenzylphosphopantetheine**

Dibenzychlorophosphate was freshly prepared by allowing a reaction of dibenzylphosphate (2.14g, 8.24mmol) with N-chlorosuccinimide (1.29g, 9.60mmol) in toluene (40ml) at room temperature for 2 h. The mixture was filtered and the filtrate was evaporated under vacuum and added to a solution of S-tritylpantetheine (3.53g, 68%) was synthesized as pale-yellow crystals. 1 H NMR (300 MHz, CDCl3) δ 0.85 (s, 3H), 1.03 (s, 3H), 2.30 (app t, J = 6.2 Hz, 2H), 2.15 (td, J = 2.3, 6.6, 6.8 Hz, 2H), 3.01 (m, 2H), 3.38-3.49 (m, 4H), 3.92 (s, 3H), 6.20 (t, J = 5.7 Hz, 1H, NH), 7.14-7.29 (m, 10H), 7.36-7.45 (m, 5H).

**c) Deprotection – synthesis of 4'-phosphopantetheine**

Naphthalene (12.9g, 100.6mmol) dissolved in tetrahydrofuran (70ml) was added to sodium metal (Na) (2.21g, 96.1mmol) in tetrahydrofuran (50mL). After 2 h the solution was cooled to –35±5°C and 5- and 4'-dibenzylphosphopantetheine (1.85g, 2.37mmol) dissolved in tetrahydrofuran (70ml) was slowly added. The mixture was stirred for 2 h while maintaining the temperature below –30°C. The reaction was quenched by addition of water (100ml) and then dichloromethane (200ml) was added. Phases were separated and the aqueous phase (together 500ml) was washed with dichloromethane (200ml) and diethyl ether (3x200ml), concentrated under vacuum and passed through the cation exchange column (DOWEX 50WX2, 200g). Fractions were analyzed by LCMS and those containing the product were pooled and concentrated under vacuum. 4'-phosphopantetheine was precipitated with addition of Ca(OH)2 as a calcium salt (332mg, 0.838mmol, 35%). The structure of the product was confirmed by comparison of NMR data with the literature11 and by HRMS. 1 H NMR (300 MHz, D2O) δ 0.86 (s, 3H), 1.08 (s, 3H), 2.54 (app d, J = 6.3 Hz, 2H), 2.87 (app t, J = 6.3 Hz, 2H), 3.43 (dd, J = 10.3, 5.0 Hz, 1H), 3.54 (m, 4H), 3.76 (dd, J = 10.3, 6.5 Hz, 1H), 4.14 (s, 1H). The HRMS mass for C11 H22 N 2 O 7 SP [M-H] - was found to be 357.0880, which corresponds to the expected mass of 357.0885. The purity of the compound was determined to be >92%, using HPLC coupled with UV detection at 205nm.

**SUPPLEMENTARY REFERENCES**