Chapter 4

Design and validation of a model to identify enhancers and suppressors of phenotypes induced by impaired function of pantothenate kinase

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Pantothenate kinase-associated neurodegeneration (PKAN) is a neurodegenerative disease with a multifaceted phenotype caused by impairment of pantothenate kinase (PANK), an enzyme catalyzing the first step of Coenzyme A biosynthesis. Due to its multifaceted pathogenesis, the underlying molecular processes of PKAN are poorly understood. Genetic modifiers that delay or speed up the progression of the neurodegenerative symptoms are also completely unknown. The identification of PKAN disease modifiers will be of help to understand mechanisms behind the symptoms and to find strategies for possible treatments. In order to identify modifiers a straightforward screenable phenotype and large numbers of affected flies are a prerequisite. One limitation of the existing PKAN models is their low survival rates and therefore the available models are not suitable for large-scale studies. In this study, we design and validate a Drosophila wing model for PKAN with three modifiable phenotypes. Also, we demonstrate that modulation of the hydrogen sulfide (H$_2$S) pathway, upregulation of which has previously been shown to positively affect neurodegeneration, is able to modify one of the phenotypes of the PKAN model. We present and validate a convenient PKAN model to study molecular processes of the disease in an isolated organ and to perform large-scale chemical and genetic screens in the future. Moreover, our research proposes a novel modifier of PKAN, the H$_2$S biosynthesis pathway.
INTRODUCTION

Coenzyme A (CoA) is an essential metabolic cofactor, required for multiple metabolic reactions in the citric acid cycle, fatty acid metabolism, amino acid metabolism and many more. In many organisms, CoA is synthesized de novo in five steps, each of which requires the action(s) of a specific enzyme. Vitamin B5 is first converted into 4’-phosphopantothenate. This reaction is catalyzed by pantothenate kinase (PANK) and is rate-limiting. Then 4’-phosphopantothenate condenses with cysteine in the presence of 4’-phosphopantothenate synthetase (PPCS). In the third step, the product of the previous reaction, 4’-phosphopantothenoylcysteine, is decarboxylated by 4’-phosphopantothenoylcysteine decarboxylase (PPCDC). The last two steps of CoA biosynthesis are ATP-dependent. In the forth reaction, an adenylyl group is attached to 4’-phosphopantetheine by 4’-phosphopantetheinyltransferase (PPAT). Finally, CoA is produced by phosphorylation of dephospho-CoA, which is formed in the previous reaction, by dephospho-CoA kinase (DPCK) 3, 4. In Drosophila melanogaster and humans, the last two enzymes of the pathway, PPAT and DPCK, form one bifunctional protein CoA synthase (COASY) 3, 4 (Figure 1A).

In addition to a central role in metabolism, recently, it was demonstrated that CoA levels also influence acetylation of histones and CoA binds and activates CAMKII, indicating that CoA is also involved in signal transduction and epigenetics 5-7. Because of its central role in metabolism and its broad influence on other cellular processes, consequences of decreased levels of CoA are multiple and diverse. Decrease in CoA levels is associated with abnormal morphogenesis 8, sterility 9, impaired cell cycle progression 9, 10, increased DNA damage 10, abnormal actin organization 11 and neurodegeneration 4, 12 among others. The link between transduction and epigenetics 5-7. Because of its central role in metabolism and its broad influence on other processes, CoA depletion further and to identify enhancers and suppressors of the phenotypes. Together our results show proof of principle that the developed wing model can be used for high throughput studies to understand consequences of pantothenate kinase deletion further and to identify enhancers and suppressors of the phenotypes.

MATERIALS AND METHODS

Drosophila maintenance and lines

Drosophila stocks and crosses were maintained at either 25°C or 29°C on commercially available Nutri-Fly Bloomington food (containing Brewer’s Yeast, Sucrose, Agar Type II, Glucose, Yeast Extract, MgSO_4 x 6H_2O, Peptone, CaCl_2) (Genesee Scientific). The original stocks were either obtained from the Bloomington Stock Centre (Indiana University, USA), or the VDRC (Vienna Drosophila RNAi Collection, Vienna, Austria). The stocks used in this study were: w1118; engrailed-Gal4 (en-Gal4, Bloomington 30564), UAS-CFP (w[1118], P[w]<mC::UAS-CFP>rib1344, Bloomington 4775), UAS-dPANK/fbl RNAi (VDRC 101437), UAS-γ-lyase RNAi (VDRC 104495), UAS-CSE RNAi (VDRC 103779). The following lines were obtained or generated by recombination in our lab: en-Gal4-UAS-CFP (en-Gal4-UAS-CFP; gift from H. Richardson), en-Gal4-UAS-dPANK/fbl-RNAi/CyO (en-Gal4-UAS-fbl-RNAi/CyO, N. Grzeschik, this study), en-Gal4-UAS-γ-lyase RNAi/CyO (en-Gal4-UAS-γ-lyase; N. Grzeschik, this study), UAS-CSE (Chapter 2 of this thesis).

For further details, see Supplementary Materials and Methods.
RESULTS

Depletion of dPANK/Fbl in the posterior part of the wing induces a wing phenotype

Reported phenotypes induced by impaired function of PANK are diverse, poorly understood and genetic enhancers or suppressors are unknown. Our aim was to design and validate a new in vivo model that could be used for both systematical characterization of the molecular processes and identification of potential suppressors and enhancers of PANK-depleted phenotypes. Drosophila eyes and wings are powerful target organs for these types of screens. Overexpression of disease causing polyQ stretches in Drosophila eyes induces a rough eye phenotype that coincides with dark neurodegenerative patches in a certain percentage of the eyes. This phenotype has been extensively used to identify enhancers (more eyes with neurodegenerative patches) or suppressors (fewer eyes with neurodegenerative patches). We first investigated whether downregulation of Drosophila pantothenate kinase (further referred to as dPANK/Fbl) by RNAi induced a phenotype in the eye. Using the UAS-GAL4 system that can drive expression of dPANK/Fbl RNAi constructs in eye tissue did not result in a phenotype visible at the exterior of the adult eyes (data not shown). Next, we tested whether a phenotype could be evoked in wing tissue as wing morphology and size can be modulated by suppressors or enhancers and this organ is non-vital for fly development and survival.

Initially, to confirm that dPANK/Fbl protein was expressed in the wing imaginal disc and to investigate whether overexpression of dPANK/Fbl RNAi under control of the engrailed-Gal4 driver led to decreased protein expression solely in the posterior compartment of the larval imaginal disc, immunohistochemistry of the larval imaginal discs using a dPANK/Fbl antibody was performed. In our experiment, cells that express the RNAi construct could readily be identified because of co-expression of fluorescent protein in the posterior compartment of the imaginal disc by overexpression of one copy of dPANK/Fbl RNAi driven by the engrailed-Gal4 driver (marked in green) in the posterior compartment of the larval imaginal disc (upper scheme) and the adult wing (bottom scheme). Downregulation of dPANK/Fbl in the posterior compartment of the wing resulted in viable offspring with a strong phenotype in the adult wing.

Characterization of the Drosophila wing model

Further characterization of the dPANK/Fbl depletion wing phenotype revealed three characteristic sub-phenotypes that can be distinguished in the wing: a smaller size (Figure 2A-D, 3A, 3B), the presence of dark patches or blisters (Figure 2D, 3D) and a held-out position of wings (Figure 2M, 2N, 3C). In control flies neither blisters nor held-out wings were observed. A prerequisite of a suitable model is that the observed
Smaller wing size and wing blistering phenotypes induced by overexpression of one copy of either (A-F. phenotypes RNAi at 25°C, three different wing positions were observed: normal, partially held-out and fully held-out (fbl) dPANK/fbl (further this position is referred to as ‘normal’) (Figure 2P), whereas in the adult flies expressing Adult control fly wings always (over 500 flies were scored) partially overlapped with each other at rest. At 29°C, the wing area was relatively smaller compared to control wings and the difference in size between dPANK/fbl depleted wings and control was stronger when raised at 29°C (Figure 2A-D, 3A, 3B). Together these data demonstrate that it is possible to modulate the phenotypes of the model, which can be used in modifier screens in future studies.

To validate the model further and to exclude the possibility that the wing phenotypes originate from an off-target effect of the dPANK/fbl RNAi expression, we expressed an RNAi, targeting another enzyme of the de novo biosynthesis pathway of CoA, Phosphopantothenoylcysteine Decarboxylase (PPCDC), which is downstream of dPANK/Fbl. Downregulation of this enzyme should result in a similar phenotype. Indeed, when PPCDC was downregulated by expressing an RNAi construct under control of the en-Gal4 driver, comparable wing phenotypes were observed (Figure 2E, 2F, 2O). Moreover, combined overexpression of PPCDC RNAi and dPANK/fbl RNAi produced a more pronounced phenotype than the ones with dPANK/fbl or PPCDC RNAi expression alone (Figure 2C, 2D, 2F, 2I, 2J). Together these results confirm that the phenotypes develop specifically due to impairment of the CoA biosynthesis pathway and not due to off-target effects.

Addition of pantethine suppresses the small wing phenotype induced by impaired CoA homeostasis

Previously, we demonstrated that adding pantetheine to the food of dPANK/fbl impaired adult flies rescued various phenotypes. In this study, we tested whether addition of pantetheine to the food of the larvae also partially rescued the adult wing phenotype. Supplementation of 2mM pantetheine partially rescued the small wing phenotype in en-Gal4-dPANK/fbl RNAi flies at 25°C and 29°C (Figure 3A, 3B). Likewise, the smaller wing area of en-Gal4-PPCDC RNAi expressing flies was also partially restored upon pantetheine supplementation at 29°C (Figure 4B) but not at 25°C (Figure 4A). The blister phenotype and the held-out phenotype were not rescued by addition of pantetheine to the food (Figure 3C, 3D, 4C, 4D). Although it is currently unclear why specifically the wing-size phenotype is rescued by supplementation of pantetheine and not the blisters and the held-out phenotype, these results do confirm the suitability of the model to screen for modifiers of the phenotype. This data also indicate that at least the wing-size phenotype is associated with impaired CoA de novo biosynthesis because this phenotype was partially rescued by the addition of pantetheine.

Figure 2. Depletion of dPANK/Fbl in the posterior part of the wing causes a smaller wing size, blistering and held-out wing phenotypes

A-F. Smaller wing size and wing blistering phenotypes induced by overexpression of one copy of either (C, D) dPANK/fbl RNAi or (E, F) PPCDC RNAi under control of the engrailed-Gal4 driver in comparison with (A, B) control wings at (A, C, E, 29°C and (B, D, F) 25°C. The blister areas are indicated with arrows. G-L. Additive effect on the wing size and blistering phenotypes induced by overexpression of either (C, H) dPANK/fbl RNAi under control of the engrailed-Gal4 driver combined with another copy of dPANK/fbl RNAi, J-L, two copies of dPANK/fbl RNAi under control of the engrailed-Gal4 driver at (C, I, K) 29°C and (H, J, L) 29°C. The blister areas are indicated with arrows. M-O. Fully penetrant held-out wing position phenotype developing upon overexpression of one copy of either dPANK/ fbl RNAi or PPCDC RNAi under control of the engrailed-Gal4 driver. Control flies show normal position of the wings at 29°C. P-R. Representative example of respectively as (P) normal, (Q) partially held-out and (R) fully held-out wing position phenotype at 29°C.

Adult control fly wings (over 500 flies were scored) partially overlapped with each other at rest (further this position is referred to as ‘normal’) (Figure 2P), whereas in the adult flies expressing dPANK/fbl RNAi at 25°C, three different wing positions were observed: normal, partially held-out and fully held-out (visualized in Figure 2P-R, see also Figure 3C). This changed towards a more severe phenotype when raised at 29°C, where the held-out position of the wings became fully penetrant (over 250 flies were scored in 5 independent experiments).

Wings of wild type flies are normally transparent as were those of dPANK/fbl RNAi expressing flies raised at 25°C. The blistering phenotype developed only at 29°C with around 40% of wings of the dPANK/fbl RNAi expressing flies exhibiting darker patches of various sizes (Figure 2A-D, 3D). These darker patches or blisters are caused by separation of dorsal and ventral surfaces of the adult wing with hemolymph and cell debris collecting between the layers. In addition to increased temperatures, flies bearing two dPANK/fbl RNAi overexpressing alleles also exhibited stronger phenotypes of all the three reported characteristics than the ones with one RNAi overexpressing allele only, representative images are shown (Figure 2C, 2D, 2G, 2H, 2I, 2L). To test whether addition of pantetheine to the food rescues the small wing phenotype, we expressed the RNAi, targeting another enzyme of the de novo biosynthesis pathway of CoA, Phosphopantothenoylcysteine Decarboxylase (PPCDC), which is downstream of dPANK/Fbl. Downregulation of this enzyme should result in a similar phenotype. Indeed, when PPCDC was downregulated by expressing an RNAi construct under control of the en-Gal4 driver, comparable wing phenotypes were observed (Figure 2E, 2F, 2O). Moreover, combined overexpression of PPCDC RNAi and dPANK/fbl RNAi produced a more pronounced phenotype than the ones with dPANK/fbl or PPCDC RNAi expression alone (Figure 2C, 2D, 2F, 2I, 2J). Together these results confirm that the phenotypes develop specifically due to impairment of the CoA biosynthesis pathway and not due to off-target effects.

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Overexpression of cystathionine γ-lyase suppresses the held-out phenotype of en-Gal4-dPANK/fbl RNAi expressing flies, whereas downregulation of cystathionine γ-lyase enhances the phenotype

We wished to test whether we could provoke a rescue in the developed wing model using potential suppressors other than CoA or CoA precursors. Recently we demonstrated that overexpression of cystathionine γ-lyase (CSE) suppressed neurodegeneration in a Drosophila model of SCA3\(^{20-22, 35, 36}\). CSE is one of the main hydrogen sulfide-producing enzymes\(^{33, 34}\), and both administration of H\(_2\)S donors and induction of its biosynthesis have been shown protective in various experimental models of disease\(^{20-22, 35, 36}\).

We demonstrated that suppression of SCA3-induced neurodegeneration by overexpression of CSE is a suppressor and decreased expression of CSE is an enhancer of the held-out wing phenotype. It also indicates that suppressors not directly related to the CoA de novo biosynthesis pathway can be identified.

To perform this experiment, flies bearing Drosophila ortholog of CSE, which were previously generated in the lab, were used. The flies were crossed with en-Gal4-dPANK/fbl RNAi flies. The offspring overexpressing both dPANK/fbl RNAi and CSE under control of the engrailed-Gal4 driver was then analyzed.

Overexpression of CSE significantly decreased the fraction of fully held-out wings, whereas downregulation of CSE expression by RNAi caused the opposite effect implying an association between modulation of hydrogen sulfide biosynthesis pathway and dPANK/fbl impaired wing phenotype (Figure 5A). The smaller wing size and blistering phenotype were not affected by CSE overexpression (Figure S8, S5). These data demonstrate that increased expression of CSE is a suppressor and decreased expression of CSE is an enhancer of the held-out wing phenotype.
DISCUSSION

Our results demonstrate that in our wing model, dPANK/fbl is downregulated in the posterior compartment of the imaginal wing discs, which results in viable offspring with a visible phenotype in the adult wings. The severity of this phenotype can be modulated via a genetic or chemical way in a positive and in a negative way. The wing phenotype shows three characteristics: a smaller size, a held-out position of the wings and the presence of blisters. The decrease in size could be due to impaired cell proliferation, impairment of cell growth or increased apoptosis. Increased apoptosis and decreased cell proliferation are consistent with reported phenotypes of pantothenate kinase impaired cells and organisms. The presence of blisters is a novel phenotype, which has neither been reported before in relation to impaired pantothenate kinase nor in relation to impaired CoA metabolism. Based on existing literature, this phenotype is closely associated with cell polarity and/or cell adhesion. It would be of interest to investigate further, using available markers, whether indeed tissue polarity is affected in the posterior parts of the dPANK/fbl depleted wings. It is currently unknown what the underlying mechanism of the held-out wings is. Interestingly, this phenotype particularly is suppressed by overexpression of CSE and enhanced by downregulation of CSE. H₂S protects against oxidative stress and CSE is an H₂S producing enzyme, overexpression of which has been shown to increase protein persulfidation, a posttranslational protein modification activated in the presence of hydrogen sulfide. Thus, it may be possible that the held-out wing phenotype is caused by oxidative stress. This is consistent with previous results, demonstrating that oxidative stress is increased as a result of dPANK/fbl downregulation. Addition of pantethine rescues the smaller wing size, however the held-out and blister phenotypes remain unaffected. It may be possible that the held-out and blister phenotypes arise during the pupal stage. A phenotype being formed during this developmental period, in which no food is consumed, may be more difficult to rescue via compounds added to the food. Rescue of such phenotypes is maybe more successful via genetic manipulations. Indeed, overexpression of CSE rescues the held-out wing phenotype. CSE overexpression does not rescue the blisters, suggesting that the blisters are caused by another downstream consequence of dPANK/fbl depletion than the held-out wing position and, therefore, can not be modulated by CSE overexpression.

In summary, the presented wing model is suitable to identify enhancers or suppressors (genetically or in the form of compounds) of all the three reported phenotypes induced by impaired CoA homeostasis.

Our data together with further investigation of the mechanisms behind the reported phenotypes will help to solve the numerous questions surrounding CoA homeostasis in health and disease.
REFERENCES


**SUPPLEMENTARY MATERIALS AND METHODS**

**Drosophila crosses**

All UAS/UAS-RNAi constructs were expressed using the UAS-GAL4 binary system for targeted gene expression. For the immunohistochemical stainings (Figure 1C), UAS-dPANK/fbl RNAi was crossed with engrailed-Gal4-UAS-CFP; so F1 wing imaginal discs would show expression of CFP in addition to dPANK/fbl knockdown in the posterior compartment. For all genetic interaction experiments and subsequent scoring of adult wings the recombinant engrailed-Gal4-UAS-dPANK/fbl-RNAi/CyO was crossed with other UAS/UAS-RNAi constructs and non-CyO adults were scored. For the phenotypic analysis at different temperatures and rescue with Pantethine the recombinants engrailed-Gal4-UAS-dPANK/fbl-RNAi/CyO and engrailed-Gal4-UAS-dPPCDC-RNAi/CyO were crossed with w1118 and non-CyO adults scored.

**Supplementation of pantethine**

For pantethine rescue experiments, pantethine (Sigma) was added to the freshly made fly food to the final concentration of 2mM, which, based on previous experiments, proved to be the optimal concentration (data not shown). Adult flies (5 females and 3 males per vial, genotypes see above) were put into the vials with or without supplemented pantethine at 25°C or 29°C, allowed to lay eggs for 5 days and discarded afterwards. Non-CyO progeny from this cross were transferred to fresh vials and scored 3 days after eclosion (to allow for proper wing inflation and clearing of cell debris in the meantime).

**Scoring of “held-out” wings**

On day 3 after eclosion, the wing position of non-CyO adults was analyzed by eye in living flies using a Leica dissection microscope. The wing position was considered “normal” if the wings were partially overlapping above the abdomen (Figure 2P), “partially held-out” if the wings were further apart from each other (Figure 2Q) or “fully held-out” if both wings were completely separate from each other and not touching above the abdomen (Figure 2R). Males and females were scored separately. To visualize the results, Adobe Photoshop and Illustrator (Adobe Systems Incorporated, San Jose, California, USA) and GraphPad Prism software (GraphPad Software, San Diego, CA, USA) were used.

**Scoring of wing size and wing blisters**

After the scoring of held-out wings, the flies were transferred to 70% ethanol and stored for 2-3 days. Afterwards the wings were removed with forceps, mounted on slides in 80% glycerol and imaged on an Olympus BX-51 microscope with Olympus software at 2x magnification. The images were analyzed using Adobe Photoshop software. To score the wing size, the area of the whole wing was measured in pixels and recalculated into mm² using Microsoft Excel software. For the count of wing blisters, imaged wings were scored as “normal”, if no brown patches were observed (Figure 2B), indicating that hemolymph and cell debris were removed from the wing and no blisters had formed. They were scored as “blistered”, if brown patches occurred, indicating that hemolymph and cell debris were not removed and still trapped between the two wing layers, producing a wing blister (Figure 2D). Male and female wings were scored separately and each wing was scored as an individual entity. To visualize the results, Adobe Photoshop and Illustrator (Adobe Systems Incorporated, San Jose, California, USA) and GraphPad Prism software (GraphPad Software, San Diego, CA, USA) were used.

**Immunohistochemical analysis of third instar wing imaginal discs**

For immunohistochemical analysis of the larval wing discs, third instar larvae (day 5) were collected in cold phosphate-buffered saline PBS and their wing discs dissected. The discs were fixed in 4% formaldehyde (from methanol-free 16% Formaldehyde Solution, Thermo Scientific) for 30 min at RT and the fixed tissue washed in PBS-T (PBS + 0.1% Triton-X-100) for 1 hr at RT. Afterwards the discs were incubated in rabbit anti-Fbl antibody, used at a 1:500 dilution, overnight at 4°C. After a wash in PBS-T for 1 hr at RT, the samples were incubated in secondary antibody (rabbit-Alexa-Fluor-594 (Molecular Probes, Invitrogen), used at a 1:500 dilution) and the DNA marker DAPI (0.2 μg/ml) for 2 hrs at RT. After one final wash in PBS-T for 1 hr at RT the samples were mounted in 80% glycerol, imaged with a Zeiss LSM780 NLO confocal microscope and data analysis performed with Zeiss Zen software. For data assembly Adobe Photoshop and Illustrator (Adobe Systems Incorporated, San Jose, California, USA) were used.

**Statistical analysis**

Each experiment was performed at least three times, the data are presented as a mean ± standard error of the mean (SEM) unless indicated otherwise. The unpaired Student’s t-test was used for comparisons between the two wing layers, producing a wing blister (Figure 2D). Male and female wings were scored separately and each wing was scored as an individual entity. To visualize the results, Adobe Photoshop and Illustrator (Adobe Systems Incorporated, San Jose, California, USA) and GraphPad Prism software (GraphPad Software, San Diego, CA, USA) were used.

**SUPPLEMENTARY REFERENCES**