Pantothenate kinase-associated neurodegeneration: insights from a *Drosophila* model

Zhihao Wu, Chenghua Li, Shan Lv and Bing Zhou*

State Key Laboratory of Biomembrane and Membrane Biotechnology, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

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Pantothenate-Kinase-Associated-Neurodegeneration (PKAN) is a devastating disease, resulting from mutations in pantothenate kinase 2 (*PANK2*), one of the four human pantothenate kinase genes (*PANK1-4*). Interestingly, PanK2 appears to be the only mitochondria-targeted human PanK. It is unknown whether the mitochondria-targeted PanK is associated with any unique function, nor whether PKAN is due solely to the loss of pantothenate kinase activity. *Drosophila PANK* [*fumble (fbl)*] encodes several isoforms of pantothenate kinase products, one of which localizes to mitochondria and the others cytosol. *fbl* flies exhibit many characteristic features reminiscent of PKAN patients. Various forms of *Drosophila fbl* and human *PANK2* were introduced into *fbl* flies to study their *in vivo* functions. Only mitochondria-targeted Fbl or human PanK2 was able to rescue *fbl* mutation, with the rescuing ability sensitive to the expression level of the transgene. Transgenic lines with low expression of normal Fbl or PanK2 displayed similar phenotypes as *PANK2* mutant transgenic flies. These PanK2 mutants all showed reduced and phenotype severity-correlated *in vitro* pantothenate kinase activities. Amazingly, cytosolic PanK3 and PanK4 could mostly, but not fully, rescue *fbl* defects except the male sterility. Therefore, *fbl* appears to be the orthologue of human *PANK2*, and PanK2 is functionally more potent than PanK3 and PanK4 *in vivo*. We suggest that mitochondria-located pantothenate kinase is required to achieve the maximal enzymatic activity to fulfill the most challenging task such as maintaining male fertility and optimal neuronal functions, and PKAN features are mainly due to the reduction of the total cellular pantothenate kinase activity in the most susceptible regions.

INTRODUCTION

Hallervorden-Spatz syndrome or more recently renamed as Neurodegeneration with Brain Iron Accumulation (NBIA), is a devastating neurological disease characterized by iron accumulation in the globus pallidus in the basal ganglia, culminating in a typical image called ‘eye of the tiger’ on T2-weighted magnetic resonance (OMIM 234200) (1,2). Typical cases of this parkinsonism-like syndrome present in early childhood with motor abnormalities, dementia, dysarthria, dysphagia and retina degeneration (3,4). Although heterogeneous by nature, the major group of NBIA is Pantothenate-Kinase-Associated-Neurodegeneration (PKAN), so called because it is caused by mutations in the human pantothenate kinase 2 (*PANK2*) gene (5,6), which presumably catalyses the first step of Coenzyme A (CoA) biosynthesis route (7).

There are altogether four potential pantothenate kinase genes (*PANK1-4*) in human. Intriguingly, although human PanK1, PanK3 and PanK4 all probably reside in cytosol (8,9), PanK2 is mainly targeted to mitochondria (10–12), although minor cytosolic isoforms might also exist based on the bioinformatic analysis of alternative splicing products in the public accessible database. This led to the suggestion that PKAN may be due to metabolic abnormality in mitochondria (13). Efforts to link pantothenate kinase activity of human mutations with the severity of PKAN patients have been taken, with mammalian cell culture as a testing paradigm. Surprisingly, some of the mutants expressed in these cultures appear to result in normal or even higher total pantothenate...
Figure 1. Different Drosophila PanK (Fbl) isoforms are associated with different subcellular localizations. (A) ORFs of Drosophila fbl gene. Five alternatively spliced forms of fbl, designated as fbl-RA, fbl-RB, fbl-RC, fbl-RD and fbl-RE in the Flybase (http://flybase.org/), are shown here. fbl-RC and fbl-RD are associated with the same and the shortest ORF, which is a part of the other isoforms. fbl-RA, fbl-RB and fbl-RE, renamed here as fblS1 (short form 1), fblS2 (short form 2) and fblL (long form), include additional different N terminals. (B) Subcellular localizations of different Drosophila PanK isoforms. (Ba, Bd and Bg) Transient transfected Drosophila S2 cells expressing Fbl isoforms (FblL, FblS1 and FblS2) fused with EGFP (Green). (Bb, Be and Bf) Mitochondria were marked with MitoTracker red CMXRos (Red). (Bc, Bf and Bi) Merged images for the Fbl-EGFP fusion proteins and the MitoTracker. FblL was predominantly localized to the mitochondria, although FblS1 and FblS2 were in the cytosol. Scales, 10 μm.

kinase activity than the normal wild-type PanK2 (10,14). It remains unclear whether the existence of endogenous PanKs in these cell lines could complicate the interpretation of the study as it is known that PanKs function as dimers (8,10,15,16). In essence, the etiology of PKAN remains controversial (10,14). A PanK2 knockout murine model was established to explore how the deficiency in PanK2 might give rise to the specific and consistent neuropathology in PKAN patients. As in human, mice pantothenate kinases also comprise four PanKs. Unfortunately, PanK2 knockout mice, although showing weight decrease, retinal degeneration and male infertility, exhibit no dystonia and little alteration in basal ganglia, both cardinal features of PKAN patients (17). Nevertheless, application of chemical inhibitor of pantothenate kinase (Hopantenate) or deprivation of dietary pantothetic acid leads to movement disorder and azoosperma (18,19). Taken together, it seems that the PanK2 knockout mice in general do not serve as an excellent genetic model for the PKAN study.

The fruit fly Drosophila melanogaster has been demonstrated to be a powerful model to investigate the mechanism of the human neurodegenerative diseases (20–22). In contrast to human and mouse, Drosophila has only one pantothenate kinase gene [fumble (fbl)]. fbl gene exhibits maternal effect, as evidenced by that flies carrying even null alleles of fbl (fbl0 and fbl*) can survive to pupae. fbl+, a hypomorphic allele, is due to a P-element insertion near the fbl locus. fbl+ flies manifest much decreased pantothenate kinase activity, male and female sterility, movement coordination defect and neurological degeneration (23–25), and normally die during the pupal stage or soon after eclosion. Interestingly, Fbl is also associated with several isoforms—one of them is predicted to be targeted to mitochondria (26), whereas the others are cytosolic.

Powerful genetic tools available together with the existence of only one PanK in Drosophila could simplify the study of underlying nature of PKAN. In this work, we introduced various forms of PANK genes into Drosophila fbl, a relatively ‘clean’ background, to study their functions at the organismal level. We found that only the mitochondrial isoform of Fbl or PanK2 can rescue the phenotypes of fbl flies, although other isoforms can not. When three mutant forms of PANK2 found in PKAN patients were expressed in fbl flies, we observed a shortened lifespan, male infertility, progressive locomotion defect, and degenerations in both central nervous system and retina. Surprisingly, transgenic lines with human PanK3 or PanK4 or a low expression level of mitochondria-targeted Fbl or human PanK2 displayed almost identical phenotypes as the transgenic flies of human PANK2 mutants. These results indicate that the level of decreased pantothenate kinase activity determines the severity of phenotypes in the fbl fly model, and suggest a similar scenario in PKAN patients. Our studies also provide a glimpse into the functional differentiation of various forms of PanKs in vivo.

RESULTS

Drosophila fbl gene encodes several isoforms of PanK with different subcellular localizations

Drosophila, unlike human and murine, has only one PANK gene (fbl, CG5725) with five splicing isoforms. These splice variants differ in their 5’ exons as evidenced in the expressed-sequence-tagged database of Berkeley Drosophila Genome Project (www.fruitfly.org/index.html) (Fig. 1A). The isoform with the longest Open Reading Frame (ORF) (referred here as fblL or FblL by protein) is predicted to be targeted to mitochondria by MitoProt II (v1.101) (26). But the other isoforms or shorter ones possess no predicted mitochondria targeting sequence.

To validate the bioinformatic prediction, we engineered fusion protein constructs of these isoforms (fblL, fblS1, fblS2) to the reporter Enhanced Green Fluorescence Protein (EGFP), called FblL-EGFP, FblS1-EGFP and FblS2-EGFP, respectively, and transfected them into Drosophila Schneider 2 (S2) cell. The resultant cell lines were then examined to see whether the EGFP fluorescence signals were colocalized
with the mitochondria as marked by MitoTracker red CMXRos. FblL, the longest isoform of Fbl, was observed essentially in the mitochondria, whereas the two shorter ones (FblS1 and FblS2) were found in the cytosol (Fig. 1B). Thus, different splice variants of Fbl are indeed associated with different subcellular localizations.

Fbl isoforms differ in their abilities of rescuing fbl flies

To analyze the functions of these differently targeted Fbl isoforms, three fbl splicing isoforms fblL, fblS1 and fblS2 were subcloned into the Drosophila expression vector pUAST, and made transgenic flies. Expression of these transgenes was made possible through the use of the GAL4/UAS bipartite expression system, in which the transgene is activated by the UAS binding factor GAL4. Gene expression was confirmed by RT-PCR (data not shown). For each isoform at least 2–3 transgenic lines were analyzed.

fbl1 mutants normally die during the pupal stage with a few escapers managing to eclose and survive several additional days (22,25). These rare survivals exhibit impaired locomotion ability, infertility and neurodegeneration in the brain and retina. We compared the different rescuing efficiencies of these diverse fbl isoforms by ubiquitously expressing them under the control of actin-GAL4. Interestingly, not all the isoforms showed effective rescue. FblL, the mitochondria-targeted isoform, virtually completely rescued the eclosion defect of fbl1 flies with the eclosion rate reaching 100% of the expected theoretical ratio (Fig. 2A). Lifespans of these flies in many cases also recovered to nearly the normal level.
However, the extent of lifespan rescue by FblL varied somewhat from transgenic line to line. For example, FblL-2 almost fully restored the decreased lifespan, but another line FblL-5 extended the lifespan to a median level of 40 days although the normal control was around 60 days (Fig. 2B). On the other hand, fbl1 flies expressing the short or the cytosol-targeted isoforms (FblS1 and FblS2) showed little improvement in eclosion (Fig. 2A) and lifespan (Fig. 2B). The locomotion defect in these flies also corresponded well with the eclosion and lifespan: those rescued by FblL displayed little locomotion defects whereas those by FblS1 and FblS2 showed no apparent improvement over fbl1 flies at all (Fig. 2C).

fbl1 flies are both male and female sterile. Consistent with the above observations, only the FblL form but not the FblS forms could restore fertility to fbl1 flies. Unexpectedly, the weaker rescuer FblL5 could only reverse the female’s fertility but not the male’s (Fig. 2D). It thus appears that rescuing male fertility, as well as full restoration of lifespan (not only eclosion), demands more FblL activity.

Given the hypomorphic nature of fbl1, we additionally repeated the eclosion rescue with these Fbl forms in flies carrying the null allele fbl3, and obtained essentially identical results. We conclude not all the Fbl isoforms are functional in vivo. The mitochondrial form (fblL) is the only functional isoform and can completely substitute the original fbl gene in all aspects examined. In contrast, the cytosolic ones (fblS1 and fblS2) seems insignificant.

Fbl is the orthologue of human PanK2
Among the four human PanKs, it appears that only PanK2 is mitochondrial (8–12). Nevertheless, expressed sequence tag (EST) analysis revealed that there might be other splicing variants of PanK2 that could miss a potential mitochondria targeting signal (11). We made a presumably mitochondria-targeted PanK2-EGFP fusion (PanK2m-EGFP) and a cytosol-targeted fusion (PanK2c-EGFP) construct, differing by the presence or absence of a mitochondria signal at the N-terminal, and transiently transfected them into Drosophila S2 cells. Consistent with previous results in COS-7 cell (6,11,12), PanK2m-EGFP fusion protein was essentially mitochondrial whereas PanK2c-EGFP cytosolic (Fig. 3B). The subcellular locations of these isoforms were later further confirmed with direct antibody staining of PanK2m and PanK2c expression (without the EGFP tag) in S2 cells (data not shown).

These features of human PanK2, reminiscent of that of Fbl, suggest that they might be functionally similar. To ascertain whether mitochondria- or cytosol-targeted human PanK2 can complement fbl defects, PANK2m and PANK2c splicing variants were introduced into Drosophila. Ubiquitous expression of PanK2m could rescue essentially every aspect of defect in fbl1 flies. On the contrary, expression of PanK2c had no effect (Fig. 3C–F). Again, we further tested the eclosion rescue of these in fbl1 flies and obtained similar results as in fbl1 flies. The sequence homology, subcellular localization similarity and functional equivalency between human PanK2 and Drosophila Fbl indicate that Fbl, the only PanK in fly, is the orthologue of human PanK2.

Modeling human PKAN mutations in Drosophila: behavior analysis
A number of mutations, associated with either early or late onset PKAN, were identified at the PANK2 gene of PKAN patients (5,6,27–29). We studied three relatively common or representative PKAN mutations found in patients: G411R, T418M and S241P (5,6,27,28). After these mutant forms of PanK2m (simplified here as G411R, T418M and S241P) were introduced into fbl1 flies, their rescuing efficiencies on eclosion rate, lifespan and locomotion ability were analyzed. PanK expression levels in these lines were largely comparable based on RT-PCR and western blot analysis (data not shown). Occasionally slightly higher, but never lower, expression of mutant protein was noted in one or two lines. In the eclosion analysis, T418M and S241P were able to dramatically increase the eclosion rate, to a level comparable to that of the normal human PanK2m flies, although G411R could not at all (Fig. 3D). G411R also failed to rescue fbl1 obviously in the other parameters including lifespan and locomotion ability (Fig. 3C–F).

In the lifespan analysis, T418M or S241P rescued significantly but not fully the lifespan of fbl1 flies. The median lifespan was extended to 30–35 days, although the normal control median lifespan was typically around 60 days (Fig. 3D). In addition, the survival curves of these mutants were shaped abnormally, giving a typical disease model pattern—the population smoothly and gradually diminished in size along the whole duration. This surviving pattern was also observed in some other Drosophila neurological disease models such as that of the tauopathy (30).

Similar results were also recorded in the locomotion assays, in that T418M and S241P could only partially suppress the impaired locomotion phenotype of fbl1 flies. Progressive loss of the movement ability in fbl1 mutants was difficult to quantitate as they were extremely short-lived (24). When T418M or S241P was introduced, 10 days after the eclosion, the climbing ability of the mutants lost quickly and progressively until they died (Fig. 3E).

We also investigated how various PanK2 mutants could rescue the fbl1 sterility. Normal mitochondria-targeted PanK2 could fully rescue both the male and female sterility of fbl1 flies, whereas G411R had no effect. Interestingly, although female fertility was restored by introducing T418M or S241P, the male flies remained sterile when carrying T418M or S241P.

In summary, the various phenotypes observed in the transgenic flies could mimic some key features of the disease symptoms revealed in the corresponding PKAN patients.

Modeling human PKAN mutations in Drosophila: pathological study
After the behavior assays, we then performed histological examinations of these mutants. A cardinal pathological feature of PKAN or NBIA is neurodegeneration. fbl1 flies also manifest neurodegeneration in the brain and retinal tissues. We thus examined brain and retina sections of these fbl1 flies carrying various PanK2 forms. A vacuolar pathology may reflect the relatively rapid tempo of
neurodegeneration in flies. Through H&E staining, vacuolization was displayed in all of the 12-day-old samples of fbl1 flies and G411R mutants, and also in 30-day-old T418M, S241P homozygotes. As expected, the vacuoles were not observed in the brains of 30-day-old normal control and PanK2m transfectants (Fig. 4A).

Because of the severity of the disease associated with PKN, fertility has not been well recorded or tested in the brains of 30-day-old normal control and PanK2m transfectants (Fig. 4A).

Figure 3. Modeling PKAN mutations in Drosophila. (A) A summary of human PanK2 variants used in this study. (B) Subcellular localizations of PanK2m and PanK2c. Ba and Bd are transiently transfected Drosophila S2 cells expressing PanK2 isoforms (PanK2m and PanK2c) fused with EGFP (Green). Bb and Be show cell mitochondria as marked by MitoTracker red CMXRos (Red). Bc and Bf are the merged images for the EGFP fusion proteins and MitoTracker. Scales, 10 μm. (C) Eclosion rescue of fbl flies by human PanK2 variants. Normal control stands for actin-GAL4/++; fbl/TM3. PanK2m-2 (PanK2m transgenic line 2) stands for actin-GAL4/UAS-PANK2m-2; fbl/TM3, and so on for the others. Background control (fbl) is actin-GAL4/++; fbl/TM3. Normal PanK2 greatly increased the eclosion rate, although PanK2c failed to benefit. T418M and S241P also could significantly improve the eclosion rate, whereas no meaningful difference was found between G411R lines and fbl. Data are presented as means ± SE (n ≥ 4). * and ** P < 0.05, *** and **** P < 0.005, two tailed Student’s t-test. * Compare with the normal control flies. (D) Lifespan rescue by human PanK2 variants. Presented here are typical lines of each genotype. For each genotype at least two individual lines were analyzed. Median survival: normal control (59 days), PanK2m-2 (56 days), PanK2c-1 (12 days), T418M-4 (35 days), S241P-4 (26 days), G411R-26 (12 days), and fbl (9 days). PanK2c and G411R did not increase the median lifespan (log-rank, P < 0.05). (E) Locomotion rescue by PanK2 variants. Normal PanK2m, mutant T418M and S241P were able to restore the locomotion ability of fbl significantly, but PanK2c or G411R could not. Although progressive loss of locomotion ability could be seen in all the tested groups, T418M and G411R exhibited much faster loss than the normal control and PanK2m flies. Data are presented as means ± SE (n ≥ 4). * P < 0.05, ** P < 0.005, two tailed Student’s t-test. * Compare with the normal control flies. (F) Fertility rescued by PanK2 variants. For normal control and PanK2, both males and females were fertile; for T418M and S241P, only females were fertile but males were sterile; G411R, fbl and PanK2c were sterile in both sexes. ○ and x denotes fertile and infertile, respectively.
Figure 4. Histological analysis of fbl rescue by human PanK2 variants. (A) (Aa-Af) Horizontal paraffin head sections of fbl1 (12-day-old), human PanK2 variants (12-day-old G411R, 30-day-old T418M, S241P and normal PanK2m), and normal control flies stained with H&E. Green arrows signify the neurodegenerative phenotypes. Ten brains of each genotype were analyzed, and the statistic result was presented (Ag). Severe neurodegeneration could be observed in fbl and G411R at early stages. Obvious vacuoles showed up in T418M and S241P at 30 days. Data are presented as means ± SD (n = 10). * and ** p < 0.005, two tailed Student’s t-test. * Compare with 12-day-old normal controls, # compare with 30-day-old normal controls. ND: not determined. (Ah–Am) Retinal
human PKAN patients, in particular classical PKAN patients. Mice PanK2 knockout results in male infertility and fewer offspring for the females (17). In fly, both female and male fbl1 flies are sterile. Histology analysis of fbl1 flies revealed great malformations in reproductive tissues. The female ovary was dramatically reduced in size (data not shown) whereas no mature sperms could be seen in the male testis (Fig. 4B) (23,25). In fbl1 flies, Nebenkern spermatids carry large nebenkern and micronuclei at onion stage. Hemizygous have degenerative spermatocytes. Testes are short and nearly devoid of germinal content (31). Approximately 10% of the testis cysts in fbl1 male contain spermatids with mitochondrial aggregates of abnormal size and shape, and multinucleate nuclei of different sizes (25). Testes from flies with various PanK2 forms were dissected and examined under the phase contrast microscope with DAPI staining. The sterile males with mutant PanK2 all displayed developmental deformity in the testes (Fig. 4B). By MitoTracker red CMXRos staining, we observed positive signals of mitochondria aggregated near the end of these testes (Fig. 4C), where no nuclei (DAPI-positive) signals were detected. These results indicate that although some PanK2 mutants can restore substantially (though not completely) other functions to fbl1 flies, they do little in improving the spermatogenesis.

**Levels of pantothenate kinase activity determine the rescuing abilities and the phenotypes**

When analyzing the rescuing capability of Drosophila PanK isoform FblL, we found some transgenic lines (stronger lines) performed better than the others (weaker lines). The stronger line L-2 could restore the median lifespan of fbl1 close to that of the normal control (61 versus 63 days). In comparison, the median survival time of weaker lines (L-5 and L-6) was shorter (40 days for L-5 and 37 days for L-6) (Fig. 5B). Interestingly, L-5 and L-6 also manifested progressive loss of locomotion ability (Fig. 5C) and male sterility (Fig. 5D), very similar to what we observed in PanK2 mutant flies. To examine whether the different rescuing abilities associated with these transgenic lines are due to various expression levels of FblL, we analyzed the expressed mRNA levels in these flies. Indeed, the weaker lines expressed much lower levels of FblL than the stronger lines (Fig. 5A). When we combined the weaker lines to generate a recombinant fly strain with more copies of the transgene (L-5, L-6) so that FblL level is increased, the resultant flies could live with a median survival of 51 days (Fig. 5B). Accordingly, the locomotion was also dramatically improved and fertility restored (Fig. 5C and D). Similar observation was also made in human PanK2 flies: expression in stronger rescuer PanK2m lines is higher than in weaker lines. Although the increased expression of FblL could enhance the rescuing ability, this phenomenon was not observed for human PanK2 mutant lines: adding extra copies of mutant human PanK2 did not significantly further improve the mutant phenotypes such as movement disorder or male sterility (data not shown), indicating that under this scenario the mutant phenotype arose from non-optimal specific activity of the mutant enzyme instead of lacking sufficient corresponding protein product.

Biochemical function analysis of the three human PanK2 mutants was also performed to examine how these mutations could affect the pantothenate kinase enzymatic activity. In the background of Drosophila fbl1, which carries undetectable level of fblL transcript (Fig. 5A, last lane), the human PanK2 mutants showed significantly lower kinase activity than that of the human wild-type form: G411R possesses no activity; S241P demonstrated ~40% and T418M ~70% activities as compared with the wild-type (Fig. 5E). We also tested the relative activity of two FblL lines—L-2 and L-5: L-2 displayed normal activity, whereas L-5, the weaker line, only ~40% kinase activity.

The analysis suggests that the level of pantothenate kinase activity, as determined by the amount of enzyme and its specific activity, underlies the phenotype: the lower activity, the more severe phenotype.

**Other human PanKs can significantly rescue most fbl defects except the male sterility**

PanK2 is probably the only mitochondrial PanK isoform in human. On the basis of a set of in vitro experiments, it has been suggested that PanK2 could only function in mitochondria whereas other PanKs function in cytosol (8–12,32). It is not clear whether PanK2 is associated with any other unique functions that are important for PKAN etiology and that the other PanKs may lack. To explore whether mitochondrial PanK2 activity is necessary for the fbl rescue, we engineered transgenic lines carrying PANK3 or PANK4 (12,32), two cytoplasmic pantothenate kinases, that are (besides PanK2) relatively more abundant in the brain and testis tissues (PANK1 is mostly found in the liver but not much in the brain, and in vitro PanK1 displays comparable specific activity to PanK3) (6,33). Interestingly, both PanK3 and PanK4 dramatically increased the eclosion rate of fbl1 flies (Fig. 6A), and all the transformants lived much longer than the fbl1 control, with the median survival increased from 10 days (fbl1) to 40 days (PanK4) and 50 days (PanK3), respectively (Fig. 6B). In the locomotion test, PanK3 and PanK4 also normalized the climbing defect of fbl1 flies when young, although after 30 days of aging their climbing ability scores fell to 50% of the normal degeneration appeared in fbl1 (12-day-old fbl1) and human mutants (12-day-old G411R, 30-day-old T418M and S241P), but not in 30-day-old normal control or PanK2m. Scales, 50 μm. (B) Phase contrast imaging (Ba, Bd, Bg, Bj, Bm and Bp) and DAPI staining (Bb, Be, Bk, Bn and Bq) of the fly testes. fbl1 (fbl1) and human mutants (S241P, T418M and G411R) were male sterile and displayed abnormality during their spermatogenesis, whereas PanK2m appeared normally. Green arrows denote the approximate positions where the late differentiated spermatids are located. (Bc, BF, BI, Bl, Bo and Br) Zooming-in pictures of DAPI staining, showing the concomitant nuclei of spermatids in the normal control and PanK2m testes, and the lack of pre-mature spermatids in the fbl1 and human mutants. Scales, 100 μm. (C) Mitochondria in the testes. Mitochondria (Red, MitoTracker) are localized tightly with the nuclei (Blue, DAPI staining) in the normal control and PanK2m (Ca–Cc, Cp–Cr). The green arrows point to the merged signals. In testes of fbl1 and human mutants, intense and massive mitochondria indicated degeneration and fused spermatids. No late differentiated spermatids were found in fbl1 and human mutants’ testes. Yellow arrows designate the abnormal mitochondrial signals. Scales, 50 μm.
control (Fig. 6C), indicating weaker rescue ability than PanK2m. Intriguingly, despite rescue was seen in most of these aspects, including the fertility of female, the male sterility was not restored at all (Fig. 6D).

In somatic tissues the central nervous system is most susceptible to PanK reduction

PanK is a housekeeping protein and essential for cellular growth. \textit{fbl}^1 flies display severe defects in neuronal functions, fertility, and possibly other less severe defects, suggesting some tissues are more susceptible to the loss of PanK. Tissuespecific expression of \textit{Drosophila} PanK was utilized to find which somatic tissue is vulnerable to the loss of PanK function. Interestingly, panneural expression of mitochondrial isoform (FblL), driven by \textit{elav-GAL4}, greatly complemented \textit{fbl}^1 flies for their eclosion, lifespan and movement coordination, but not the fertility, increasing the lifespan medium from 10 days to near 40 days (compared with normal about 60 days). This suggests that in somatic tissues nervous system is most

Figure 5. Levels of pantothenate kinase activities determine rescuing abilities. (A) RT-PCR analysis from the normal control, \textit{fbl}^1 and \textit{fblL} transgenic male flies. Relative mRNA levels of \textit{fblL} were determined by semi-quantitative PCR, using the ribosomal \textit{rp49} mRNA as the internal control. Compared with the normal, \textit{fblL} mRNA was decreased in L-5 (58.5 ± 3.65%) and L-6 (60.9 ± 6.11%), although L-2 had a much higher expression level (170.5 ± 3.47%) and \textit{fbl}^1 almost had no mRNA in adults (5.25 ± 0.22%). (B–D) The expression levels of \textit{Drosophila} \textit{fblL} correlated to their rescuing abilities. (B) Low expression \textit{fblL} lines showed similar longevity phenotypes as PanK mutants: significantly long-lived than \textit{fbl}, but shorter than the normal. L-5, L-6 is a recombination line of L-5 and L-6 (i.e. contains both the L-5 and L-6 transgene insertions) displayed longer median lifespan (48 days). Log-rank, \(P < 0.05\) (C) Low expression of FblL also caused the faster progressive loss of locomotion ability, like the PanK2 mutant, although increased expression level would greatly ameliorate that. Data are presented as means ± SE (\(n \geq 4\)). * \(P < 0.05\), ** \(P < 0.005\), two tailed Student’s \(t\)-test. * Compare with the normal control. (D) Fertility is also determined by the expression level. Male fertility appears to demand higher PanK activity. (E) Pantothenate kinase activities of mitochondria-targeted PanK2 mutants are correlated with their rescuing abilities. The kinase activities were normalized based on the total cellular protein amounts. Compared with the normal (100%), G411R exhibited no activity at all, although T418M and S241P showed decreased activities. L-5 had much lower activity than L-2. Data are presented as means ± SE (\(n \geq 3\)). * \(P < 0.05\), ** \(P < 0.005\), two tailed Student’s \(t\)-test. * Compare with the normal control.
susceptible to the loss of PanK and other somatic tissues need much less PanK to be functional. In fbl null (fbl<sup>−</sup>) background, however, this rescue was not observed for the panneural expression of FblL, consistent with the idea that all cells need PanK but neuronal tissues are more sensitive to PanK reduction. We further explored possible differential effects by expressing FblL in two types of neurons with different neurotransmitters: dopaminergic neurons (driven by ddc-GAL4) (34) and cholinergic neurons (driven by cha-GAL4) (35) in the central nervous system. Expression of FblL in dopaminergic neurons resulted in drastically better improvement in lifespan than that in cholinergic neurons, although the latter are the major neuron type in the fly brain (Fig. 7B). In contrast, in the movement coordination assay, better improvement was recorded when FblL was expressed in cholinergic neurons than dopaminergic neurons (Fig. 7C). To address the concern that these variations might have arisen from expression differences as a result of use of different drivers, we crossed ddc-GAL4 and cha-GAL4 separately to UAS-GFP flies. Intensity of GFP signals from both types of flies turned out to be comparable at individual cell levels, although there are many more cholinergic neurons than dopaminergic neurons (Fig. 7D). Therefore, although both types of neurons are involved in causing the lifespan and movement defects in fbl, differential effects are indicated: the dopaminergic neurons are more relevant than the cholinergic neurons in the presentation of the shortened lifespan of fbl, but cholinergic neurons appear slightly more involved in the movement defect.

Figure 6. PanK3 and PanK4 can substantially rescue fbl defects. (A) Eclosion rescue of fbl by PanK3 and PanK4. PANK3 and PANK4 can greatly improve the eclosion rate of fbl<sup>1</sup>. PanK3-1 stands for actin-GAL4/UAST-PANK3-1; fbl<sup>1</sup>/fbl<sup>1</sup>, others likewise. Negative control (fbl<sup>−</sup>) is actin-GAL4/þ; fbl<sup>−</sup>/fbl<sup>−</sup> in A–D. Data are presented as means ± SE (n ≥ 4). * and ** P < 0.05, *** and **** P < 0.005, two tailed Student’s t-test. * Compare with the full rescue rate; # compare with fbl. (B) Lifespan of fbl rescued by PanK3 and PanK4. Much longer lifespan was observed when PANK3 or PANK4 transgene was introduced to fbl<sup>1</sup> (log-rank, P < 0.05). (C) Locomotion rescue by PanK3 and PanK4. Although significantly improved over fbl, rescued lines still exhibited faster progressive loss of locomotion ability. Data are presented as means ± SE (n ≥ 4). * P < 0.05, ** P < 0.005, two tailed Student’s t-test. * Compare with the normal. (D) Fertility of fbl rescued by PanK3 and PanK4. PanK3 and PanK4 could only rescue the fertility of fbl females, but not males. ○ and × denotes fertile and infertile, respectively.
In this work, we analyzed the functions of the cytosol- and mitochondria-targeted isoforms of Fbl and human PanK2, and used the Drosophila fbl mutant to create fly models to study human PKAN mutations. Fbl and human PanK2 appear to function only in mitochondria, but not in cytosol, whereas both human PanK3 and human PanK4 work in cytosol. Cytosol-targeted PanK3 and PanK4 can mostly complement fbl defects except male sterility, similar to the weaker lines of FblL or PanK2 or to PanK2 mutant transgenic flies, suggesting PanK3 or PanK4 is functionally less potent in vivo than mitochondria-targeted PanK2 or Drosophila Fbl. In consistent to this, in vitro experiments have shown that murine PanK2 has the highest specific activity, much higher than that of PanK3 and PanK1α (18). In experiments not presented in this work, we also tried targeting PanK3 to mitochondria by adding an N-terminal mitochondrial signal. However, this engineered PanK3 failed to function well in the fbl complementation test, indicating PanK3 can only function properly in the cytosol, but not well in mitochondria. It is thus apparent that various PanKs have to be located in appropriate subcellular environments to be fully functional, presumably due to their different regulatory properties as proposed before based on in vitro biochemical experiments (36).

In our study, severity of the phenotypes is strictly correlated with PanK activities: higher expression and potency are associated with better rescue. For mitochondria-targeted
Drosophila Fbl or human PanK2, high expression levels are critical for full restoration of the mutant phenotypes. However, for PanK2 mutants, additional increase of the protein level can increase in vivo total enzymatic activity but will not help much in vivo, indicating potency is another issue. It thus seems there are two limiting factors: one is the expression level of the enzyme, and the second is the catalytic potency of the molecule. In an in vivo system when the amount of protein reaches a threshold level the specific activity of the PanK becomes critical. Nevertheless, it is still intriguing that some mutant forms of PanK2 displayed significant pantothenate kinase activity (40–70% for S241P and T418M) and still manifested some mutant phenotypes in the flies. These partial activities most likely reflect specific activity as the protein expression level is comparable to that of the normal one. On the basis of current data, we speculate that neither the amount of PanK protein nor the associated specific activity in a normal fly is in far excess. In consistent with this, it has been reported that even heterozygous fbl flies could show subtle pathological abnormality in the spermatogenesis (31). In this context, it is conceivable that 50% drop of specific activity could be a serious event for the fly.

Previous in vitro biochemical data suggest T418M is associated with no loss of PanK activity, although S241P results in a little decrease (10,14). In that assay, different mutant PanK2 was transfected to HEK 293T cells and the pantothenate kinase activity of each mutant was determined by deducting the original endogenous activity from the total cellular kinase activity of the transfectant. Our in vivo and in vitro data, however, indicate they are only partially active. Although the in vitro activities of these PanK2 mutants correspond well with the in vivo phenotypes in our hands, some caution has to be taken when interpreting this in vitro data because biochemical assay condition is never the same as the in vivo environment. In particular, PanK2 is targeted and functions in mitochondria.

PANK3 encodes a single cytosolic form of PanK (8), which has been extensively studied. Although PANK4 encodes a product with sequence similarity to other PanKs, it lacks an evolutionarily conserved and proposed catalytic Glu/Asp residue, based on structural and biochemical analysis of Staphylococcus aureus PanK (CoaA) (37), and possesses a long carboxyl-terminal extension of unknown functions. It has even been reported that PanK4 has no pantothenate kinase activity in vitro (18). Nevertheless, this residue may not be essential to all PanKs as alteration of it in Pseudomonas aeruginosa PanK failed to diminish the associated PanK activity (37). Our observation that either of PanK3 and PanK4 can significantly complement fbl mutation indicates in vivo they are both functional pantothenate kinase. The discrepancy of in vivo and in vitro results reinforces the notion that in vitro activity needs to be interpreted cautiously, and suggests the possible existence of some special and currently unknown catalytic/regulatory features about PanK4.

Among all the phenotypes examined, restoration of male fertility seems to be one of the most demanding. Although recovery of eclosion rate is the first sign of rescue, more activity is needed to improve other features (movement, life-span, neurodegeneration and fertility). Male fertility is restored only when all other features are close to that of the normal flies. Why male fertility is the most challenging obstacle for the rescue? It is possible that spermatogenesis may require the highest PanK activity—the spermatogenesis of Drosophila involves successive mitosis and meiosis divisions and further differentiation of stem cell to produce the late elongating spermatids and finally the mature sperms. When PanK expression level is low (such as in some mitochondrial PANK2 transgenic lines) or the specific activity of PanK (potency) does not reach a threshold such as in the case of PanK2 mutants, the spermatogenesis process might be impaired. Because during the differentiation and maturation of sperms most cytoplasm and organelles are gradually discarded and only nucleus and mitochondria remain (38), it is also interesting to consider whether mitochondrial PanK has further advantage over cytosolic PanK in surviving this process. However, we do not have evidence to support this later speculation unambiguously.

PanK2 mutant mice did not present the cardinal PKAN neuronal phenotypes. It is likely that the corresponding brain tissues in mice are less vulnerable to the reduction of PanK activity. As lack of pantothenate via dietary control or the chemical inhibitor still reproduced movement disorder in mice (18,19), it has also been proposed that in mice the susceptible brain regions might be associated with higher expression of the other, cytosolic PanKs, which could complement most of the PanK2 functions (33). Indeed, in the fly model cytosolic PanK3 or PanK4 can restore most, though not full, neuronal functions to fbl—the fly orthologue of PANK2. It is thus possible that one or the combination of both contributes to the neuronal invulnerability of mice to the lack of PanK2 activity.

MATERIALS AND METHODS

Drosophila stocks and genetics

Flies were raised at 25°C on standard cornmeal food. Stocks from Bloomington Stock Center (Indiana University, USA) include act-GAL4/Cyo, elav-GAL4, ddc-GAL4, cha-GAL4 and fbl1 fbl2 was a gift from Dr S. Wasserman’s lab (University of California, USA). Transgenic flies were generated in w1118 background for each UAS construct by P-element mediated transformation. Tissue-specific GAL4 lines and transgenic flies were crossed with fbl1 or fbl2 to generate flies that carry the fbl mutant background.

DNA constructs

For the transfection of Drosophila S2 cells, FbIL-EGFP, FbIS1-EGFP, FbIS2-EGFP, PanK2m-EGFP and PanK2c-EGFP fusion were all constructed in the pPac5c-PL backbone and fused with a C-terminus EGFP. Though the translational start point of human PANK2 remains controversial (5,12), mature product is relatively certain. PANK2m was made by replacing the original CTG at the N-terminal of the mature PanK2 product (5) with ATG and PANK2c with CAG (so that an ATG shortly downstream will be used), and placed in the pUAST vector, resulting the expression of the mature PanK2 with or without a mitochondrial signal peptide, as reported before (11). The position of PKAN-associated point
mutations is numbered relative to the CTG starting site as proposed in (5), corresponding to 110 in the amino acid position if translated with an upstream ATG (10,14). Amino acid changes mimicking PKAN mutations were aided by PCR-mediated DNA site mutagenesis and confirmed by sequencing.

**Drosophila S2 cells culturing and transfection**

*Drosophila* S2 cells were propagated at 25°C in Schneider’s Medium (Invitrogen, USA) supplemented with 10% heat-inactivated FBS (Gibco, USA) and penicillin/streptomycin. For *Drosophila* S2 transfection, cells were cultured until 1 x 10^6 cells per 35 mm dish, and then transfected using Cellfectin (Invitrogen) according to the procedure recommended by the manufacturer. A DNA/liposome ratio of 1 μg/5 μl was applied per 35 mm dish. Mito Tracker red CMXRoS (Molecular Probes, USA) was used as the mitochondrial marker.

**RT-PCR and western blot analysis**

Total RNA was extracted from transgenic flies using TRIzol® Reagent (Invitrogen). cDNA was transcribed from 1 μg total RNA with Superscript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Semi-quantitative RT-PCR was performed using primers to amplify parts of rp49 (39) and fblL. Intensity of gel bands was quantitated using ImageJ (http://rsb.info.nih.gov/ij/, National Institutes of Health, USA) densitometry. Primers used for RT-PCR are listed below: 5'-GCACCAAGCATT CATCC-3' (rp49 forward); 5'-CGATCTCGCGGCAGTAAA -3' (rp49 reverse); 5'- CACAGCAGGTGCAACAGCT-3' (fblL forward); 5'- GTAAGTGCGATACGATGT-3' (fblL reverse).

For western blotting, a polyclonal mouse anti-human PanK2 antibody (a gift from Dr Yien Kuo, University of California, San Francisco, USA) against a 14-mer synthetic peptide (AEGTRRDRLGSYSG) corresponding to amino acids 68–82 (GenBank accession no. DAA00004) located downstream of the mitochondria-targeting sequence was used. Fly samples were homogenized in buffer containing 1% Trixon-100 plus 10% proteinase inhibitor cocktail (Sigma, USA), centrifuged, separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore, UK). Rabbit polyclonal anti-β-actin (sc-1616-R, Santa Cruz Biotechnology, USA) was used as the control antibody. Secondary antibody was HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG (Zhongshan Goldenbridge Biotechnology, Co., Ltd., China). Signals were developed using ECL detection kit (Vigorous Biotechnology, China). Protein bands were quantitated using ImageJ densitometry (National Institutes of Health, USA).

**Behavior assays**

For behavior analyses, all GAL4 lines and transgenic lines were prior outcrossed with w^{118} for six generations for isoge- nization. For survival assay, 25 flies were placed in a food vial. Each vial was kept on its side at 25°C and 70% humidity under a 12 h-light-dark cycle. Food vials were changed every 2–3 days, and flies were simultaneously counted. At least 200 flies were prepared for each genotype, and the experiments were carried out no less than three times. For locomotion assay, 20 male flies were placed in a plastic vial and gently tapped to the bottom. The number of flies at the top of the vial was counted after 18 s of climbing under red light (Kodak, GBX-2, Safelight Filter). The experiment was repeated more than three times, and the data shown represent results from a cohort of flies tested serially for 30–50 days. For fertility testing, a mutant male was crossed with three virginal wild-type (w^{118}) females, or conversely, a virginal mutant female with three young wild-type males. After several days, the progenies were counted. For each genotype, at least five individuals were tested.

**Histological study**

Fly brains were fixed in PBS+4% formaldehyde overnight at room temperature, dehydrated, embedded in paraffin and cut (4 μm thickness) as described (40). Sections were stained with Mayer’s H&E (Zhongshan Goldenbridge Biotechnology) and examined under a Nikon ECLIPES 80i microscope attached to a Nikon DXM1200F digital camera. Continuous sections were analyzed and the numbers of vacuoles in each brain were recorded, and at least 10 brains were counted for each genotype. For examining retinal degeneration, heads from properly aged flies were fixed in 4% formaldehyde and embedded in Epon. One micrometer horizontal sections were stained with 1% toluidine blue and checked under the microscope. At least six heads were counted for each genotype. Tests were dissected in PBS solution and fixed in PBS+4% formaldehyde at room temperature. After fixing for 60 min, tests were washed three times in PBT (PBS+0.1% Triton X-100) and subsequently stained in 0.1 ng/μl DAPI (Sigma) for 30 min. Then they were transferred to the mounting media (PBS+70% glycerol) and mounted on glass slides before microscopic examination.

**Fly lysates and pantothene kinase activity assay**

Flies were ground and lysed in the lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 10 mM NaF and 5 μg/ml of leupep- tin). The lysate was centrifugated at 5000 x g for 5 min at 4°C to remove the cell debris. The supernatant was mixed with equal volume of saturated ammonium sulfate. After incubation on ice for 1 h, the protein precipitate was collected by centrifugation at 3000 x g for 30 min. The precipitated protein was resuspended in the lysis buffer without leupeptin and dialyzed against the lysis buffer overnight at 4°C.

Pantothene kinase assay was performed with D-[1-14C] pantothene (90 μM; specific activity 40–60 mCi/mmol; American Radiolabeled Chemicals, Inc., USA), in the presence of ATP (2.5 mM, pH 7.0), MgCl₂ (10 mM), Tris–HCl (0.1 M, pH 7.5) and the fly extract containing PanK2, in a total volume of 40 μl (41,42). The mixture was incubated at 37°C for 10 min and the reaction was terminated by adding 4 μl of 10% (v/v) acetic acid. Forty microliter of the mixture was then deposited onto a Whatman DE81 ion-exchange filter disk followed by three changes of washes of 1% acetic acid in 95% ethanol to remove unreacted pantothene. 4'-Phosphopantetheine was quantified by...
counting each dried disk in scintillation fluid by Microplate Scintillation & Luminescence Counter TopCount. NXT™ (Packard, USA). Counts from the fbl background were subtracted from the data. Measured enzymatic activities were linear with respect to protein concentrations and their final kinase activities were normalized to the total protein levels of the fly lysates.

Statistical analysis

$P$-value were calculated using the Student’s t-test (* or #, $P < 0.05$; ** or ##, $P < 0.005$). Survival curves were analyzed by the method of Kaplan and Meier using SYSTAT 12.

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REFERENCES


