Supporting Information

Rana et al. 10.1073/pnas.0912105107

SI Text

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

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

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

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

Measurement of CoA Levels by HPLC. For fly sample preparation, homozygous *dPANK/fbl* flies (6 days old), 60 female and 40 males per experiment, were collected and weighed. Flies were then snap-frozen in Liquid N_2, and 200 μL of solvent buffer (5% sulfosalicylic acid containing 50 μM DDT) was added. After thorough grinding, the samples were sonicated three times for 10 s on ice. Samples were centrifuged and supernatant was collected for HPLC analysis of CoA. Before analysis, 2 μL of Ammonia (25%) was added to 98 μL of the sample solutions. For *Drosophila* Schneider's S2 cell sample preparation, cells were pelleted, and 200 μL of solvent buffer (5% sulfosalicylic acid containing 50 μM DDT) was added. Samples were sonicated and centrifuged, and the supernatant was collected for HPLC analysis of CoA. Before analysis, 2 μL of ammonia (25%) was added to 98 μL of the sample solutions.

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

***Drosophila* Schneider's S2 Cell Culture and RNAi.** For generation of the dsRNA the following primers were used:

Gene	Primer
dPANK/Fbl	fwd-CGTGATACGCACCTACAGATG
	rev-GCCATTGGACCAGAACTCCAT
dPPCS	fwd-GGCACAACAAGCTCCAGAAT
	rev-CTTGCGTGTCTGCAGCACAT
dPPAT	I) fwd-GCGAGCCATCGAGAAGTACG
	rev-CCGAGTCATCCAGGAAGATTGT
dPPAT	II) fwd-GCCCACGTGATCGACTGCGAT
	rev-CCACTTCGCTCAACTTGTTGC

As a control, nonrelevant (human gene; hMAZ) dsRNA was used. dsRNA was produced and purified with MEGAscript RNAi Kit (Ambion) according to the manufacturer's instructions. Down-regulation of dPANK/Fbl protein was investigated by immunoblotting using dPANK/Fbl specific antibodies for every individual experiment (Fig. S2).

Mammalian Cell Culture and siRNA Knockdown of hPANK2. PANK2 knockdown in HEK293 cells was performed under conditions of regulated levels of pantothenic acid (vitB5) using custom made vitB5 free DMEM (ThermoScientific) supplemented with 0.4 mg/L vitB5 (Sigma), 10% dialyzed serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Two different human PANK2-specific small interfering RNAs (siRNAs) were used: siRNA1 (Dharmacon; D-003797-04) and siRNA2 (Ambion; AM51321). Nonsilencing control siRNA was purchased from Dharmacon (VOSMC 000005). HEK293 cells were transfected with 100 nM siRNA using siPORT Amine transfecting agent (Ambion) according to the manufacturer's

protocol. Knockdown efficiency was assayed by immunoblotting 48 h after transfection.

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

Gene	Primer
dPPCS	fwd- ACTTCACCGGCCAGCAGTTC
	rev- AATCGTCGGCGCTCCATCTC
dPPAT	fwd-GCGAGCCATCGAGAAGTACG
	rev-CCGAGTCATCCAGGAAGATTGT
rp49	fwd-GCACCAAGCACTTCATCC
	rev CGATCTCGCCGCAGTAAA

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4. Demoz A, Garras A, Asiedu DK, Netteland B, Berge RK (1995) Rapid method for the separation and detection of tissue short-chain coenzyme A esters by reversed-phase high-performance liquid chromatography. *J Chromatogr B Biomed Appl* 667:148–152.

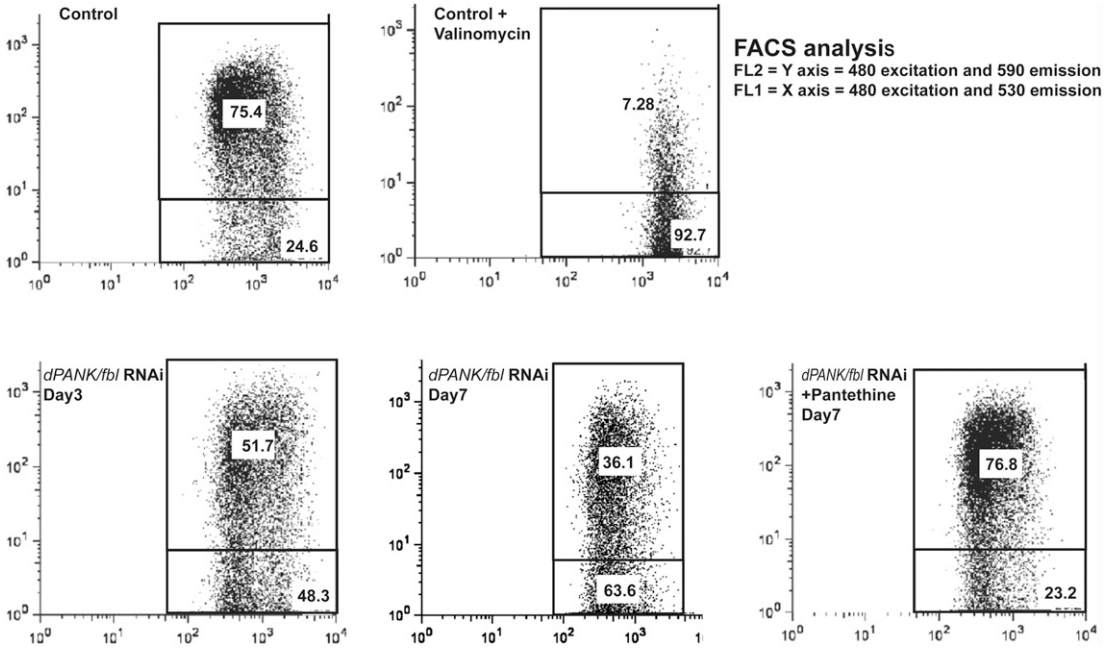

Fig. S1. Analysis of functional mitochondria in dPANK/Fbl depleted cells by FACS analysis. FACS analysis in combination with a JC-1 assay was used to measure changes in the mitochondria transmembrane potential, and this enabled the quantification of active mitochondria (detailed description of JC-1 assay described above in *SI Text*). Dot plots are shown for the following conditions: control cells; control cells treated with Valinomycin; dPANK/Fbl depleted cells (untreated and treated with pantethine). *Upper boxed areas* represent cells with a red and green fluorescence emission of 590 nm and 530 nm above a specific threshold, representing cells with active mitochondria. *Lower boxed areas* represent cells with a red fluorescence emission of 590 nm below a specific threshold (and with a green fluorescence emission of 530 nm above a specific threshold) representing cells with a disturbed mitochondrial membrane potential. Percentages of cells are indicated in boxed areas.

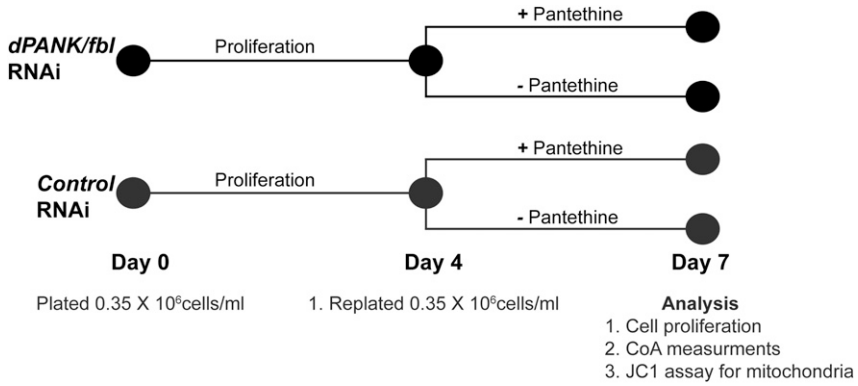

Fig. S2. Schematic representation of RNAi experiments. At day 0, cells were plated in equal densities and were treated with *dPANK/fbl* dsRNA or with control dsRNA. After 4 days, cells were replated in equal densities and left further untreated or were treated with pantethine. On day 7, various assays were performed. Down-regulation induced by the RNAi treatment for all assays was measured with Western blot analysis using dPANK/Fbl antibodies.

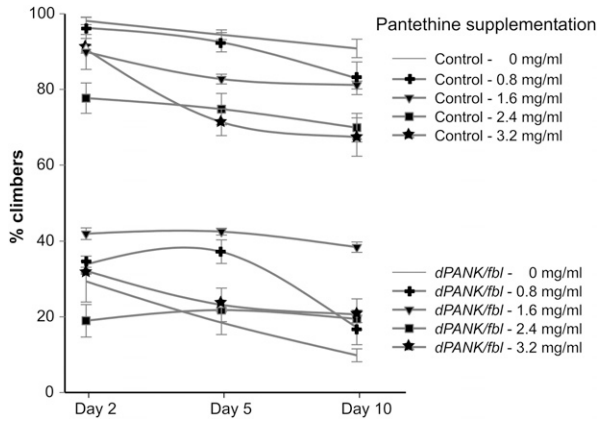

Fig. S3. A 1.6-mg quantity of pantethine per milliliter of food is the optimal concentration to rescue climbing ability. Various concentrations (0.8 mg/mL, 1.6 mg/mL, 2.4 mg/mL, and 3.2 mg/mL) of pantethine were added to the food of wild-type flies and *dPANK/fbl* mutants. Pantethine was added immediately after eclosion, and the food was refreshed every day. On days 2, 5, and 10, climbing activity was measured. Adding 1.6 mg of pantethine per milliliter of food induced a significant rescue of climbing activity. Moreover 1.6 mg pantethine showed only a mildly reduction of climbing activity in wild-type flies compared with 2.4 mg pantethine. Based on these results, 1.6 mg pantethine per milliliter of food was used for the experiments described in this article.

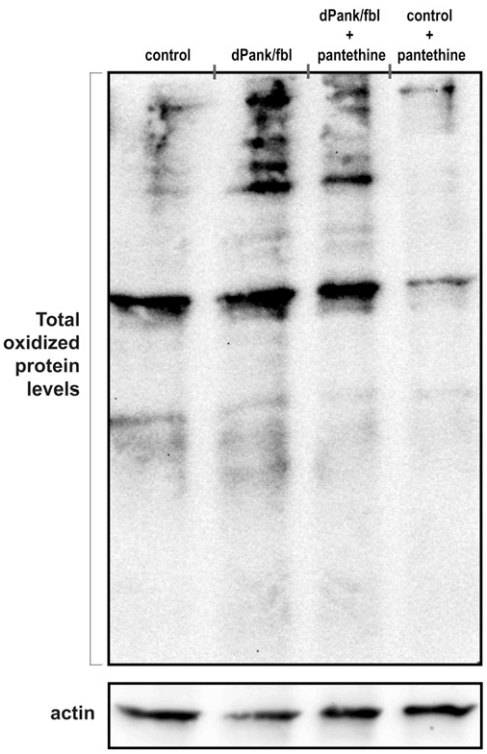

Fig. S4. *dPANK/fbl* flies have increased levels of oxidized proteins that are rescued upon pantethine feeding. Oxyblot analysis revealed that *dPANK/fbl* mutants have higher levels of total oxidized proteins as compared with wild type. Daily feeding of pantethine for 6 days immediately after eclosion results in reduction of oxidative damage to the proteins in *dPANK/fbl* mutants. Actin is used as loading control.

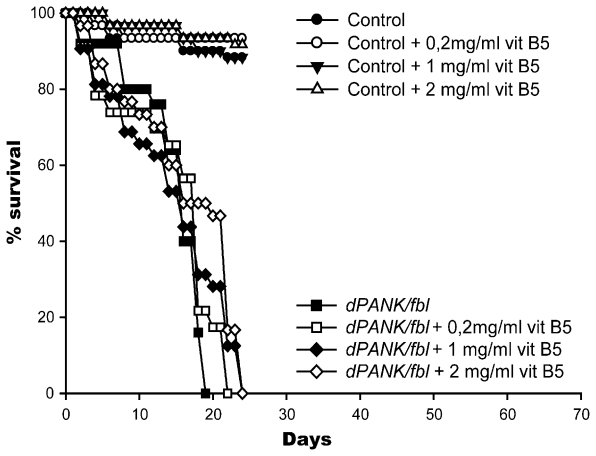

Fig. S5. Vitamin B5 does not increase the lifespan of *dPANK/fbl* mutants. To investigate the effect of vitamin B5, various concentrations (0.2 mg/mL, 1 mg/mL, and 2 mg/mL) of vitamin B5 were added to the food and tested for their potential to increase lifespan. At 22 days after feeding the various concentrations of vitamin B5, all *dPANK/fbl* mutants had died, and no significant increase in lifespan was observed.

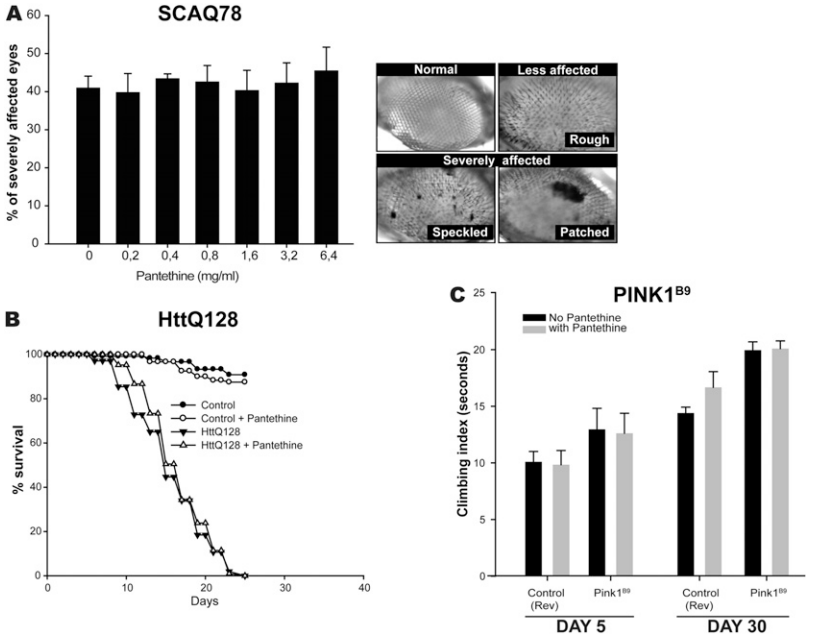

Fig. S6. *Drosophila* models for the neurodegenerative diseases: Spinocerebellar Ataxia-type 3 (SCA-3), Huntington, and Parkinson are not rescued by pantethine. (*A*) Flies expressing a truncated form of Ataxin 3 protein containing an expanded repeat of 78 glutamines in eyes show a rough-eye-phenotype (1) and are referred to as SCA3Q78 flies. This transgenic *Drosophila* strain is a model for SCA3 and has been used to investigate modifiers of toxicity induced by polyglutamine in SCA3 related neurodegeneration. Eye abnormalities are classified as "rough" or as "severely affected" as previously described (2). Protective compounds will reduce the percentage of severely affected eyes. Increasing concentrations of pantethine did not result in a significant decrease of the percentage of severely affected eyes (>250 eyes were scored for each condition). (*B*) Flies expressing a truncated form of Huntingtin containing an expanded repeat of 128 glutamines show a neurodegenerative phenotype including a reduced lifespan of 24 days and are referred to as HttQ128 flies (3). Addition of (1.6 mg/mL) pantethine to the food did not increase the lifespan of HttQ128 flies. For each condition, more than 100 flies were used. (*C*) Mutations in human PINK1 are linked to parkinsonism. The *Drosophila PINK1* gene is an ortholog of the human PINK1 gene, and *Drosophila PINK1*^{B9} mutants show a progressive impairment to climb as they age (4). Addition of (1.6 mg/mL) pantethine to the food did not improve the climbing ability of *PINK1*^{B9} mutants. For each time point, >100 flies were used. As a control, *PINK1*^{B9} revertants were used that overexpress the wild-type *Drosophila PINK1* gene in the *PINK1*^{B9} mutant background. Climbing index is defined as the average climbing time required to climb 15 cm by 50% of the flies (4).

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4. Park J, et al. (2006) Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature* 441:1157–1161.

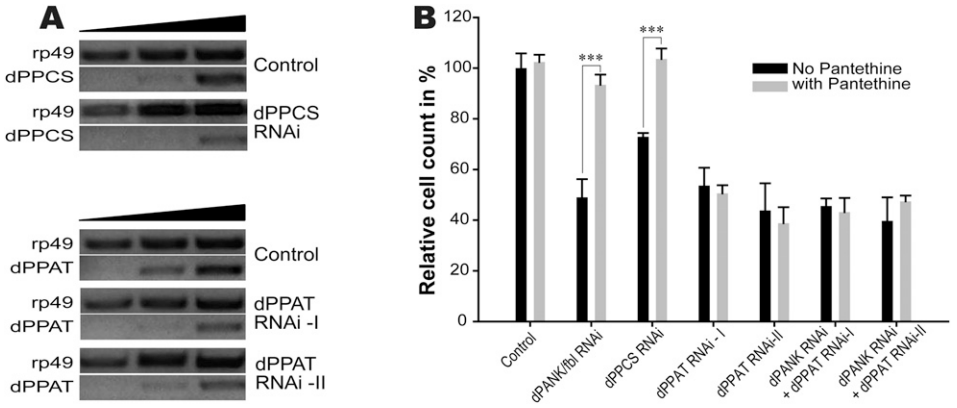

Fig. S7. Pantethine rescues cell count of dPPCS-depleted cells but not of dPPAT-depleted cells. RNAi was used to down-regulate dPPCS, dPPAT, or dPANK/Fbl and dPPAT simultaneously in *Drosophila* S2 cells. To down-regulate dPPAT, two independent nonoverlapping RNAi constructs were used (detailed description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

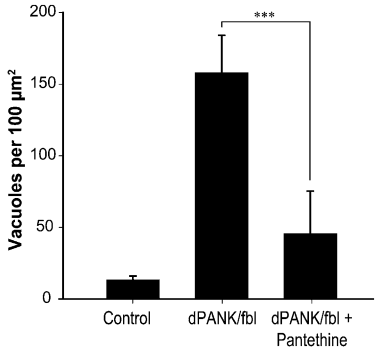

Fig. S8. Amount of brain vacuoles in *dPANK/fbl* mutant flies is decreased after pantethine treatment. Number of vacuoles in the brain region (indicated in Fig. 4*D)* was measure by National Institutes of Health Image J software (http://rsb.info.nih.gov/ij/index.html). The method of quantification is outlined in the Image J documentation ("Particle Analysis"). The total amount of vacuoles was calculated per 100 μm^2 from comparable regions (indicated in the boxed areas in Fig. 4*D*). For every condition, four brains were examined of 12-day-old flies. After eclosion, flies were kept on standard food or on standard food supplemented with 1.6 mg/mL pantethine. ***$P < 0.001$ (Student's *t* test). Error bars indicate SEM.