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RESEARCH ARTICLE

Increase of *Drosophila melanogaster* lifespan due to *D-GADD45* overexpression in the nervous system

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Abstract The GADD45 protein family plays an important role in stress signaling and participates in the integration of cellular response to environmental and physiological factors. GADD45 proteins are involved in cell cycle control, DNA repair, apoptosis, cell survival and aging, and inflammatory response by complicated protein–protein interactions. In *Drosophila melanogaster* a single *D-GADD45* ortholog (*GG1086*) has been described. Our data show that overexpression of the *D-GADD45* gene in the nervous system leads to a significantly increase of *Drosophila* lifespan without a decrease in fecundity and locomotor activity. The lifespan extension effect is more pronounced in males than in females, which agrees with the sex-dependent expression of this gene. The longevity of *D. melanogaster* with *D-GADD45* overexpression is apparently due to more efficient recognition and repair of DNA damage, as the DNA comet assay showed that the spontaneous DNA damage in the larva neuroblasts is reduced with statistical significance.

Keywords Aging · Longevity · Stress response · Stress signaling · GADD45 · Nervous system

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Introduction

The ability of the cell and organism to respond adequately to stress is important requirement for a long lifespan (Rattan 2005; Le Bourg 2009). Long-living individuals of various species are usually characterized by increased resistance to environmental and physiological damaging agents, which is determined by higher efficiency of the processes of recognition, signaling, repair and elimination of damaged cell macromolecules (Gilad et al. 1987; Sohal et al. 1990; Lithgow et al. 1995; Murakami and Johnson 1996; Lin et al. 1998; Guarente and Kenyon 2000; Fabrizio et al. 2001; Landis et al. 2003; Morrow et al. 2004; Brown-Borg 2006; Masse et al. 2008; Ungvari et al. 2008; Labinskyy et al. 2009; Perez et al. 2009; Amrit et al. 2010; Slack et al. 2010). Cellular signaling mechanisms are involved in diverse processes, including cellular metabolism, growth, proliferation, differentiation and survival of cells. In particular, they play an important role in the cellular response to stress and in the determination of the organism lifespan (Niedernhofer and Robbins 2008).

In mammals, stress signaling in response to environmental and physiological factors involves the GADD45 (Growth Arrest and DNA Damage) protein family. The GADD45 proteins play an important role in the cell cycle control, DNA repair, apoptosis, cell survival and proliferation by complicated protein–protein interactions with PCNA, p21, Cdc2/CyclinB1, p38 and JNK (Liebermann and Hoffman 2008).

In *Drosophila* a single *D-GADD45* ortholog (*GG11086*) has been identified (Peretz et al. 2007).

Since the GADD45 protein family has an important function in the system of cellular interactions of the stress response, it may be involved in the control of aging processes. We assumed that overexpression of the *D-GADD45* gene in the nervous system of *Drosophila melanogaster* would result in lifespan increase.

This work is aimed at studying the effects of the *D-GADD45* overexpression on the *D. melanogaster* lifespan, and estimating the age-dependent dynamics of physiological life quality characteristics (fecundity, locomotor activity) and cytogenetic parameters (DNA damage level, frequency of apoptosis).

Materials and methods

D. melanogaster strains

The following laboratory strains of *D. melanogaster* were used: *Canton-S* as a wild type strain; *UAS-D-GADD45,* containing an additional copy of the *D-GADD45* gene controlled by the *UAS* promoter (a kind gift from Dr. Uri Abdu, Ben-Gurion University, Israel); *GAL4-1407* (genotype: *w**; *P{GawB}1407*), carrying the constitutively expressed *GAL4* driver in the nervous system (kindly provided by the Bloomington Stock Center, USA); *ELAV* (genotype: *y w*; *P{ELAV-GeneSwitch}*), carrying the mifepristone-inducible *GAL4* driver in the nervous system (kindly granted by Dr. Haig Keshishian, Yale University, USA).

Activation of overexpression of the *D-GADD45* gene

For constitutive overexpression of the *D-GADD45* gene in *Drosophila* nervous system, *UAS-D-GADD45* females were crossed with *GAL4-1407* males. Parental strain individuals were used as control.

For conditional overexpression of the *D-GADD45* gene in the *Drosophila* nervous system, *UAS-D-GADD45* females were crossed with *ELAV* males, and the offspring were kept on a medium with mifepristone (conditional inducer of GAL4 expression). It is known that mifepristone on its own does not affect the *D. melanogaster* lifespan (Ford et al. 2007), therefore the changes in the studied parameters of experimental animals were caused by *D-GADD45* overexpression and/or genetic background. Flies obtained by crossing *UAS-D-GADD45* females to *ELAV* males and kept on a medium without mifepristone as well as flies of the parental *UAS-D-GADD45* and *ELAV* genotypes were used as control when studying the effects of conditional *D-GADD45* overexpression.

Drosophila maintenance

Flies were kept under standard conditions at 25°C and 12*:*12 h light–dark regime on an agar/semolina/sugar/yeast medium in all experiments (Ashburner 1989). 25 pairs of parents with synchronized 24 h egg laying were used to obtain the experimental flies. The flies were extracted from vials immediately after imago eclosion.

Mifepristone treatment

For mifepristone treatment, stock solution of RU486 (Mifepristone, Sigma) with 25 mg/ml concentration in 100% ethanol was used. It was mixed with yeast paste (60.4% water, 39% active yeast, 0.5% acetic acid, 0.1% ethanol) in proportion of 0.1 ml initial RU486 solution per 100 ml yeast paste. 0.3 ml of prepared yeast paste was added to the surface of the medium (Poirier et al. 2008). For experiments without mifepristone, the medium surface was covered with yeast paste that was prepared in the same way but with an equivalent amount of ethyl alcohol added instead of RU486 solution.

Quantitative real-time RT-PCR

Fifty 5–7-day-old adult flies' heads per experiment were used to determine the level of the expression of the *D-GADD45* gene in the nervous system. Gene expression was evaluated in males and females separately.

The heads were cut off from the bodies, placed in the TRIzol Reagent solution (Invitrogen) and homogenized using the SilentCrusher S homogenizer (Heidolph). RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. cDNA synthesis using the prepared RNA was done using the Oligo(dT) primer (Invitrogen) and SuperScript III First-Stand Synthesis System reverse transcriptase

(Invitrogen) according to the manufacturer's protocol. In order to avoid RNA samples contamination by DNA, one of the PCRs was performed using *β-Tubulin* primers (forward primer: 5′-GCAACTCCACTGCCATCC-3′; reverse primer: 5′-CCTGCTCCTCCTCGAACT-3′) without reverse transcription.

Real-time PCR was carried out in 30 μl volume in 200-μl PCR tubes according to the manufacturer's protocol using a reaction mixture containing SYBR Green PCR Master Mix dye (Applied Biosystems) and the following primers (Syntol, Russia): *D-GADD45* (forward primer: 5′-GCAAACGCACAACCAAAC-3′; reverse primer: 5′-GGCCATCAGGCAGAAGAG-3′), *β-Tubulin* (forward primer: 5′-GCAACTCCACTGCC ATCC-3′; reverse primer: 5′-CCTGCTCCTCCTCGA-ACT-3′). The reaction was carried out in the ANK-32 amplifier (Institute of Analytical Instrumentation, Russia) using a the following program: (1) denaturing at 95°C for 10 m, (2) denaturing at 95°C for 15 s, (3) annealing at 60°C for 30 s, (4) elongation at 60°C for 30 s, (5) stages 2–4 were repeated 50 times. *D-GADD45* expression was normalized to the *β-Tubulin* housekeeping gene. Amplification of *D-GADD45* and *β-Tubulin* was carried out in individual tubes. 4–8 measurements were performed per each experiment.

The relative level of *D-GADD45* expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak, Schmittgen, 2001) using cycle threshold (Ct) values obtained using the ANK32 1.1software (Institute of Analytical Instrumentation, Russia). ΔΔCt was calculated as ΔΔCt = ΔCt(*D-GADD45* overexpression)—ΔCt(without*D-GADD45* overexpression), and each value of ΔCt = Ct(*D-GADD45*)—Ct(*β-Tubulin*).

A two-way ANOVA was used to compare differences between expression levels of *GADD45* in the reference and the overexpression groups according to the model $Y = \mu + E + S + E \times S + e$, where Y is the observed value, μ designates the overall mean, E represents the effect of overexpression ('yes' or 'no'), S is the effect of the sex, $E \times S$ is the effect due to the interaction between expression and sex, and e is the error variance.

Lifespan assay

To estimate the longevity 150–250 flies were collected (about 30 adult flies per 120 ml vial) for each experimental variant. Males and non-virgin females were kept separately. Flies were transferred to a fresh medium twice a week. Dead flies were calculated daily.

The data were used to plot survival curves and to calculate the mean, median, minimum and maximum lifespan and the age of 90% mortality. It is known that the lifespan distribution does not obey a normal law (Gavrilov and Gavrilova 1991), therefore parametric statistical tests do not apply. In order to estimate the statistical significance between experimental and control groups, nonparametric methods were used: the Kolmogorov–Smirnov test (for the comparison of survival functions between sample groups) (Fleming et al. 1980), Gehan-Breslow-Wilcoxon (Breslow 1970) and Mantel-Cox tests (Mantel 1966) (for the comparison of median lifespan values). The significance of differences in maximum lifespan was evaluated using the Wang-Allison test. According Wang-Allison test each animal in each experimental variant was categorized into one of two groups: either lifespan above the 90th percentile or lifespan below the 90th percentile. The two by two contingency table was used to record data. The ordinary χ^2-test was used for independence test of two groups (Wang et al. 2004).

In addition, the α and R_0 parameters of the Gompertz equation ($\mu(x) = R_0 e^{\alpha x}$) and the mortality rate doubling time ($MRDT = ln(2)/\alpha$) were calculated, and the trajectories of logarithmic mortality rate were plotted. The significance of differences in age-dependent mortality rate and initial mortality rate (parameters α and R_0 in the Gompertz equation) was evaluated using the maximum likelihood method (Pletcher 1999). Statistical analysis was carried out using *Statistica* version 6.1, StatSoft, Inc. and *WinModest* version 1.0.2 (Pletcher 1999) software.

Fecundity assay

50 flies per experiment were collected. They were transferred to vials with wild-type *Canton-S* males (five females and five males per vial). Flies were kept under the same conditions and transferred to a fresh medium twice a week. Dead flies were counted daily. Every month, old *Canton-S* males were replaced with young males. Daily egg production was calculated once a week. In addition, the numbers of pupae developed from the eggs by the 10–14th day after egg laying were counted. The significance of differences between distributions of frequencies (a number of

eggs or pupae per female) in sample groups at different ages was evaluated using the χ^2-test.

Locomotor activity assay

To estimate the locomotor activity parameters, 90 flies were collected (30 flies per vial) for each experiment. Flies were kept under identical conditions and transferred to a fresh medium every week. Males and females were studied separately. Measurements of locomotor activity were carried out using the *Drosophila* Population Monitor (TriKinetics) hardware-software complex. For the evaluation of spontaneous activity, measurements were performed for 3 min. For the negative geotaxis test, flies were shaken to the bottom of the vial and measurements of climbing were carried out for 20 s. The test was repeated 3 times for each vial. The significance of differences between distributions of frequencies in sample groups at different ages was evaluated using the χ^2-test. Data were analyzed with three-way ANOVA according to the model $Y = \mu + E + S + A + E \times S + E \times A + S \times A + E \times S \times A + e$, where Y is the observed value, μ designates the overall mean, E represents the effect of *GADD45* overexpression ('yes' or 'no'), S is the effect of the sex, A is the effect of the age, $E \times S$, $E \times A$, and $S \times A$ are the effects due to the interactions between factors, and e is the error variance.

Comet assay

The DNA damage level was evaluated using comet assay (single cell gel electrophoresis) as described previously (Mukhopadhyay et al. 2004) with some modifications. Brain ganglia of third-instar *Drosophila* larvae were isolated and mechanically suspended in the Poel's salt solution (15 mM NaCl, 6.4 mM NaH_2PO_4, 42 mM KCl, 7.9 mM $CaCl_2$, 1.8 mM $KHCO_3$, 20.8 mM $MgSO_4$; pH 6.95). Neuroblasts were embedded in 0.75% agarose on slides. To prepare a slide 4 ganglia were used. After that slides were placed in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100; pH 10) for 1 h at 4°C. Then slides were incubated in an alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) for 10 min followed by electrophoresis at 15 V/300 mA for 10 min at 4°C. Slides were washed 3 times in 20 mM Tris, pH 7.5 and 3 times in distilled water. After washing slides were fixed in ethanol for 10 min.

The dried slides were stained with acridine orange. Comet images were captured using the MIKMED var. 11 luminescent microscope (LOMO, Russia) and a video system based on an Olympus C-7070 digital camera. For DNA comet assay, the CometScore software (TriTek Corp., USA) was used. The DNA damage level was characterized by the median tail moment for 300–400 cells (eight slides at 30–50 cells per slide) per experimental variant. The tail moment magnitude was estimated according to P. Olive (Olive et al. 1990). The differences between sample groups were estimated using the Student's *t*-test.

DNA diffusion assay

The frequency of apoptosis was evaluated using DNA diffusion assay as described previously (Singh 2000) with some modifications. Neuroblasts (suspensions were prepared as described for the comet assay) were embedded in 0.75% low melting agarose on slides. Then slides were placed in a lysing solution (1.25 M NaCl, 1 mM EDTA, 5 mM Tris, 0.3 M NaOH; pH 13) for 10 min. After lysis slides were incubated with neutralizing and DNA precipitating solution (50% Ethanol, 20 mM Tris-HCl; pH 7.4) for 40 min (2 times for 20 min with fresh solution). Slides were dried at 50°C and stained with acridine orange (2 μg/ml). The frequency of apoptosis was determined in 4,000 cells (eight slides at 500 cells per slide) per experiment. The differences between sample groups were estimated using the φ-Fisher test.

Results

Level of the *D-GADD45* gene expression

To confirm the occurrence of constitutive *D-GADD45* overexpression, expression levels of the *D-GADD45* gene in the nervous tissue were compared in males and females with the *UAS-D-GADD45/GAL4-1407* genotype (flies with expected *D-GADD45* overexpression in the nervous system) and with the *Canton-S/GAL4* genotype. We used the F1 progeny flies of crosses between *Canton-S* wild type females and *GAL-4-1407* males to avoid the heterosis effect. Analysis of the relative *D-GADD45* expression values revealed 10-fold overexpression in *UAS-D-GADD45/GAL4-1407* males and 3-fold overexpression in

females. ANOVA revealed a significant effect of the *D-GADD45* overexpression ($P < 0.001$) and sex ($P < 0.001$) as well as their interaction ($P < 0.001$).

To detect the conditional *D-GADD45* overexpression effect, F1 progeny flies of crosses between *UAS-D-GADD45* females and *ELAV* males were kept on a medium with mifepristone (inductor of the *ELAV* driver). Comparison of the relative *D-GADD45* expression values in the nervous tissue of flies with *UAS-D-GADD45/ELAV* genotype not treated with mifepristone exhibited a 3-fold overexpression in males and 2-fold overexpression in females. The ANOVA showed a significant effect of the *D-GADD45* overexpression ($P < 0.001$) and sex ($P < 0.001$) as well as their interaction ($P < 0.001$).

Effects of the *D-GADD45* overexpression on the *D. melanogaster* lifespan

The influence of constitutive and conditional *D-GADD45* overexpression in the nervous system on *D. melanogaster* males' and females' life span was studied.

In the first experiment we estimated the effect of the constitutive *D-GADD45* overexpression. Survival curves showed a lifespan increase in both males and females with constitutive *D-GADD45* overexpression as compared to flies with the parental *UAS-D-GADD45* and *GAL4-1407* genotypes ($P < 0.001$, Kolmogorov–Smirnov test) (Fig. 1). The median life span assay confirmed this result. The median lifespan of males with constitutive *D-GADD45* overexpression in the nervous system was extended by 77 and 73% compared to the median lifespan of males of parental *UAS-D-GADD45* and *GAL4-1407* strains, respectively ($P < 0.001$, Gehan-Breslow-Wilcoxon test), and in females, by 22 and 46%, respectively ($P < 0.001$) (Table 1). In addition an increase of the maximum life span was detected. The age of 90% mortality of males with constitutive *D-GADD45* overexpression was increased by 25 and 59% compared to *UAS-D-GADD45* and *GAL4-1407* males ($P < 0.001$, Wang-Allison test), and in females by 11 and 50%, respectively ($P < 0.001$) (Table 1).

The assay of Gompertz equation parameters demonstrated that constitutive overexpression of *D-GADD45* gene leads to delayed and slower aging of *D. melanogaster* individuals. On this account in males with *D-GADD45* overexpression the R_0 Gompertz equation parameter was 41 and 6 times lower than in males of the parental *UAS-D-GADD45* and *GAL4-1407* strains, and in females—2.3 and 1.3 times lower, respectively (Table 1). The α Gompertz equation parameter characterizing the age-depended mortality rate was decreased by 11–37% in flies with *D-GADD45* overexpression compared to that in flies of the *GAL4-1407* genotype (Table 1). The trajectories of logarithmic mortality rate for males and females with constitutive *D-GADD45* overexpression illustrate these results. Ranges are shifted to the right compared to the individuals of the parental *UAS-D-GADD45* and *GAL4-1407* strains ($P < 0.001$, maximum likelihood method) (Fig. 2).

Thus the data obtained indicate that constitutive *D-GADD45* overexpression in the nervous system increases lifespan and delays aging in *D. melanogaster* males and females. This effect is more considerable in males than in females.

In order to avoid the influence of heterosis and differences in genetic background and possible harmful effect of *D-GADD45* overexpression induction at the embryo and larvae stages, in the second experiment we studied the effect of conditional *D-GADD45* overexpression in the nervous system on the *Drosophila* lifespan.

Survival curves demonstrate a lifespan increase in males with the *UAS-D-GADD45/ELAV* genotype treated with mifepristone (conditional *D-GADD45* overexpression in the nervous system) compared to those kept without mifepristone and males of parental strains *UAS-D-GADD45* and *ELAV* ($P < 0.001$, Kolmogorov–Smirnov test) (Fig. 3). Specifically, the median lifespan of males with mifepristone-inducible *D-GADD45* overexpression in the nervous system was higher by 40–42% compared to males with the same genotype not treated with mifepristone ($P < 0.001$, Gehan-Breslow-Wilkinson test); in females, by 3–6% ($P < 0.05$). The median lifespan of males with conditional *D-GADD45* overexpression in the nervous system was increased by 50–70 and 62–84%, compared to the median lifespan of parental *UAS-D-GADD45* and *ELAV* strain males, respectively ($P < 0.001$); in females, by 27–29 and 100–102%, respectively ($P < 0.001$) (Table 2). Furthermore, the increased maximum lifespan value (the age of 90% mortality) by 5–54% ($P < 0.001$, Wang-Allison test) was obtained in flies with conditional *D-GADD45* overexpression in the nervous system.

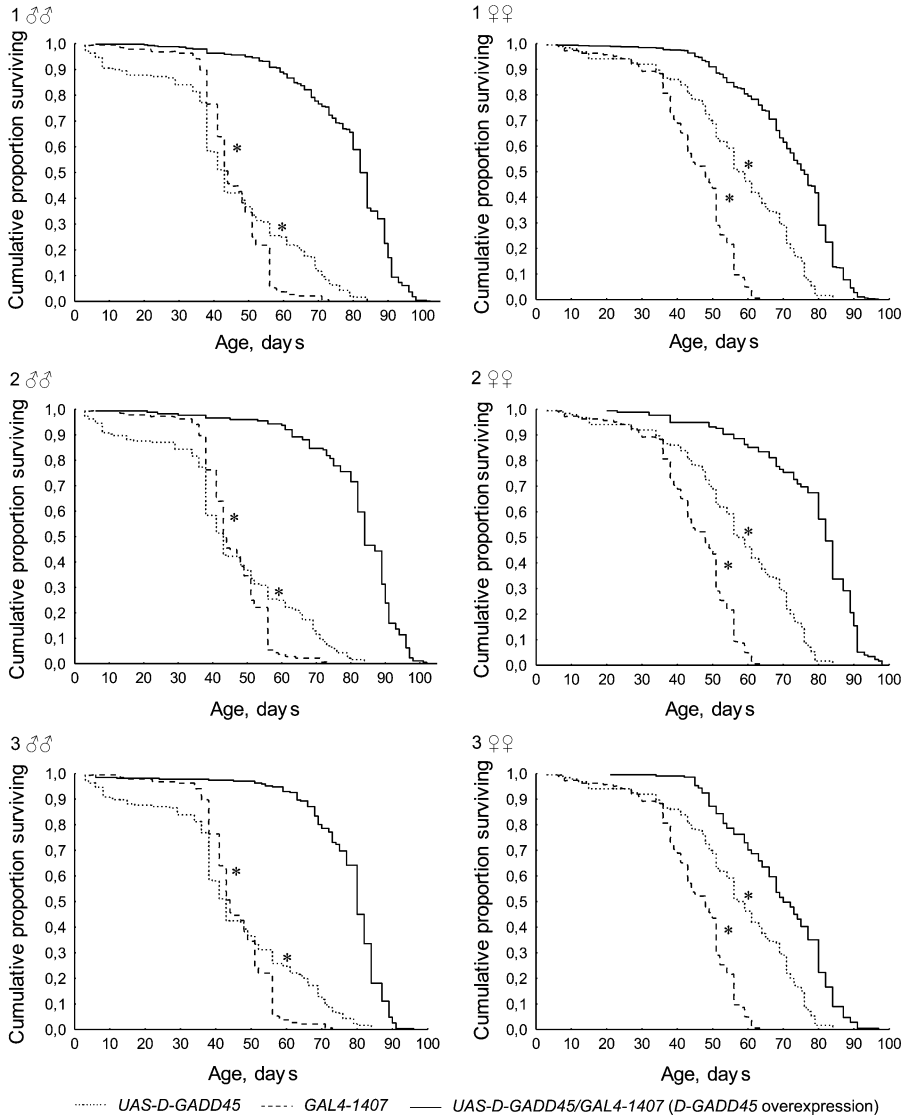

Fig. 1 Survival curves for *Drosophila melanogaster* males (♂♂) and females (♀♀) with the parental *UAS-D-GADD45* and *GAL4-1407* genotypes and constitutive *D-GADD45* overexpression; 1, 2, 3 replications; $*P < 0.001$ (Kolmogorov–Smirnov test)

In males with conditional *D-GADD45* overexpression in the nervous system the delayed aging was revealed compared to males with the same genotype not treated with mifepristone. It is illustrated by the 5–11-fold decrease of the R_0 Gompertz equation parameter in males, and the 2.5-fold decrease in females. Similar effect was shown in comparison with the parental *ELAV* genotype (Table 2). The age-depended rate of mortality declined in flies with conditional *D-GADD45* overexpression compared to flies of the parental *UAS-D-GADD45* strain because the α Gompertz equation parameter was 1.5–2.2 times lower (Table 2). These results are illustrated by the trajectories of logarithmic mortality rate (Fig. 4).

Thus the data obtained indicate that conditional *D-GADD45* overexpression in the nervous system increases the *D. melanogaster* lifespan, and this effect is not affected by the genetic background or heterosis effect.

Effect of the *D-GADD45* overexpression on the *D. melanogaster* fecundity

Some mutations which promote longevity in laboratory experiments are accompanied by decreased reproductive activity and fecundity which is demonstrated by lower life quality (Friedman and Johnson

Table 1 Effects of constitutive *D-GADD45* overexpression in the nervous system on the *D. melanogaster* lifespan

Variant	M	$\bar{X} \pm \Delta m$	90 (%)	Min	Max	α	R_0	MRDT	N
♂ *UAS-D-GADD45* *	43*	45.0 ± 1.2	71*	3	84	0.05	0.0041	13.7	258
♀ *UAS-D-GADD45* *	57*	56.6 ± 1.2	76*	3	84	0.07	0.0007	9.9	211
♂ *GAL4-1407* *	44*	45.6 ± 0.8	56*	4	73	0.10	0.0006	6.9	136
♀ *GAL4-1407* *	48*	45.0 ± 1.0	56*	6	64	0.11	0.0004	6.3	138
♂ *UAS-D-GADD45/GAL4-1407* (*D-GADD45* overexpression) (1)	76	74.2 ± 1.4	89	34	98	0.09	0.0001	7.7	186
♂ *UAS-D-GADD45/GAL4-1407* (*D-GADD45* overexpression) (2)	84	81.8 ± 1.1	96	6	102	0.11	0.0000	6.3	176
♂ *UAS-D-GADD45/GAL4-1407* (*D-GADD45* overexpression) (3)	82	77.8 ± 1.2	91	20	98	0.10	0.0000	6.9	175
♀ *UAS-D-GADD45/GAL4-1407* (*D-GADD45* overexpression) (1)	70	67.0 ± 1.1	84	15	98	0.08	0.0003	8.7	173
♀ *UAS-D-GADD45/GAL4-1407* (*D-GADD45* overexpression) (2)	80	77.0 ± 0.8	89	6	96	0.13	0.0000	5.3	271
♀ *UAS-D-GADD45/GAL4-1407* (*D-GADD45* overexpression) (3)	70	69.1 ± 1.0	84	21	97	0.09	0.0001	7.7	211

M median lifespan; $\bar{X} \pm \Delta m$ mean lifespan and standard error of mean; 90 (%) age of 90% mortality; *Min* minimum lifespan; *Max* maximum lifespan; α and R_0 Gompertz equation parameters; *MRTD* mortality rate time of doubling; *N* number of flies in a sample; ♂ males; ♀ females; *1, 2, 3* replications; $*P < 0.001$; $**P < 0.05$ (*first column* Kolmogorov–Smirnov test, *second column* Gehan-Breslow-Wilcoxon test; *fourth column* Wang-Allison test)

1988; Guarente and Kenyon 2000; Tatar et al. 2001; Giannakou et al. 2004; Kenyon 2005; Slack et al. 2010). Therefore, we studied the fecundity of flies with constitutive and conditional *D-GADD45* overexpression in the nervous system.

In females with constitutive *D-GADD45* overexpression in the nervous system fecundity was higher over the entire course of lifetime, compared to the parental strains females (Fig. 5). The average number of eggs laid daily by a female increased 2- and 2.3-fold in comparison to *UAS-D-GADD45* and *GAL4-1407* females, respectively ($P < 0.001$, χ^2-test), and the number of pupae per female was 3.7 and 4.4 times higher, respectively ($P < 0.001$).

In females with conditional *D-GADD45*overexpression in the nervous system the average number of eggs laid daily by a female increased 1.7 times compared to the *ELAV* strain ($P < 0.001$), while the difference from parental *UAS-D-GADD45* strain and *UAS-D-GADD45/ELAV* females not treated with mifepristone was insignificant. The number of pupae per female was 1.5 and 2.9 times higher compared to the *UAS-D-GADD45* ($P < 0.05$) and *ELAV* females ($P < 0.001$), and did not significantly differ from that for *UAS-D-GADD45/ELAV* females not treated with mifepristone (Fig. 6).

Thereby the data obtained indicate that *D-GADD45* overexpression in the nervous system did not reduce the *Drosophila* fecundity. Furthermore, the fecundity of flies with constitutive *D-GADD45* overexpression in the nervous system was higher than in females of the parental genotypes.

Effect of the *D-GADD45* overexpression on the *D. melanogaster* locomotor activity

The age-dependent dynamics of the locomotor activity are also a characteristic of life quality. We showed that the locomotor activity of males and females with constitutive *D-GADD45* overexpression in the nervous system was increased in comparison with males and females of the parental *UAS-D-GADD45* and *GAL4-1407* strains throughout the whole lifetime, both in terms of spontaneous locomotor activity ($P < 0.001$, χ^2-test) and according to the negative geotaxis test ($P < 0.001$, χ^2-test) (Figs. 7, 8). The ANOVA demonstrates a significant effect of *D-GADD45* overexpression ($P < 0.001$) and age ($P < 0.05$) but neither sex ($P > 0.05$), nor their interaction ($P > 0.05$).

The negative geotaxis test showed an increase in locomotor activity in males (on day 4–29) and in females (on day 12–36) with the conditional

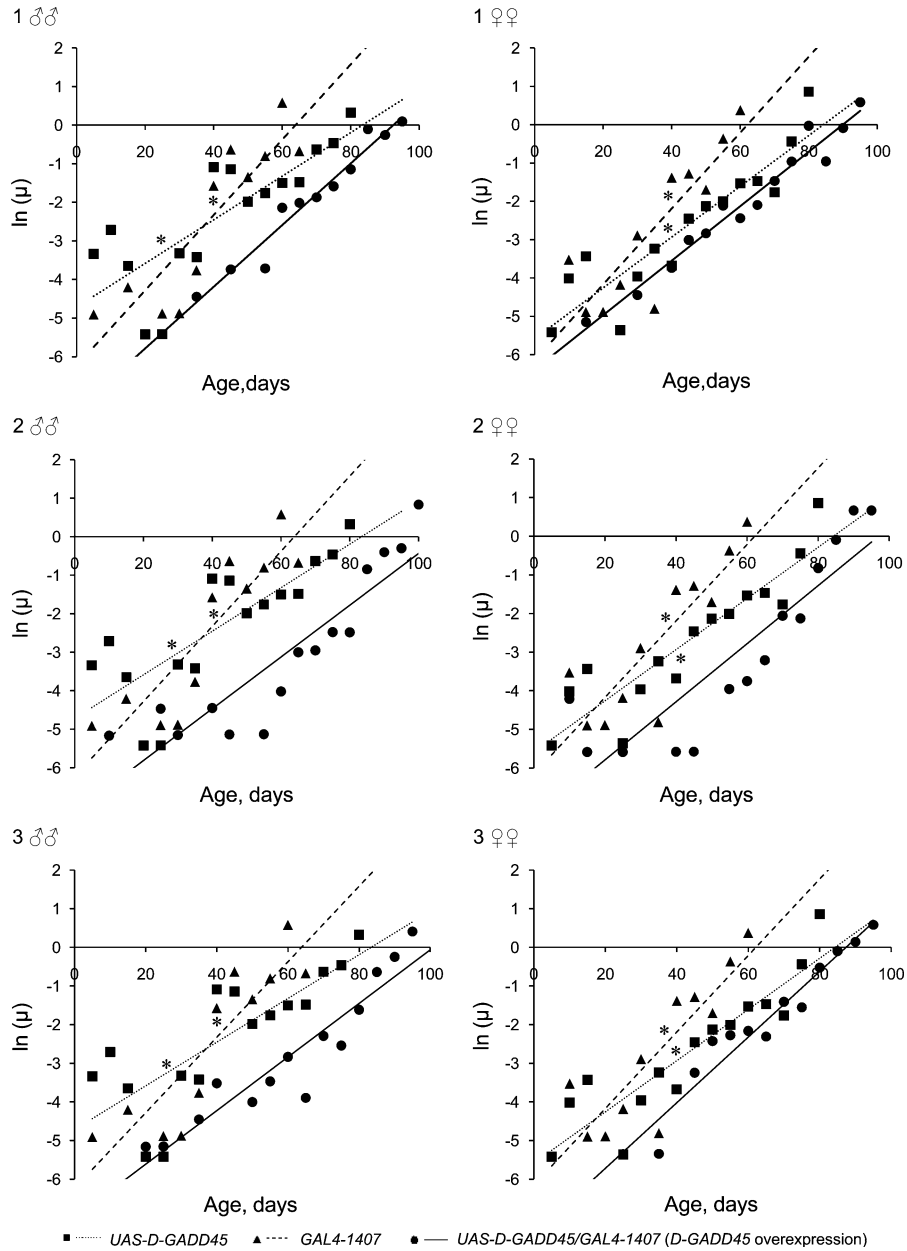

Fig. 2 Trajectories of logarithmic mortality rate for *D. melanogaster* males (♂♂) and females (♀♀) with the parental *UAS-D-GADD45* and *GAL4-1407* genotypes and constitutive *D-GADD45* overexpression; *1, 2, 3* replications; $*P < 0.001$ (maximum likelihood method)

D-GADD45 overexpression in the nervous system and those not treated with mifepristone ($P < 0.001$) (Fig. 9). However, a factorial ANOVA failed to reveal a significant effect of *D-GADD45* overexpression or sex and their interaction ($P > 0.05$). Significant differences between spontaneous locomotor activity values are absent (Fig. 10).

The locomotor activity of males and females with conditional *D-GADD45* overexpression in the nervous system is increased and differed with statistical significance from the locomotor activity of the *UAS-D-GADD45* and *ELAV* flies ($P < 0.001$). But differences in spontaneous activity level were absent between males with conditional *D-GADD45* overexpression and males of the *UAS-D-GADD45* parental strain. The ANOVA failed to show any significant effects of *D-GADD45* overexpression or sex, or interaction between them ($P > 0.05$).

Thus, constitutive and conditional *D-GADD45* overexpression in the nervous system did not impair

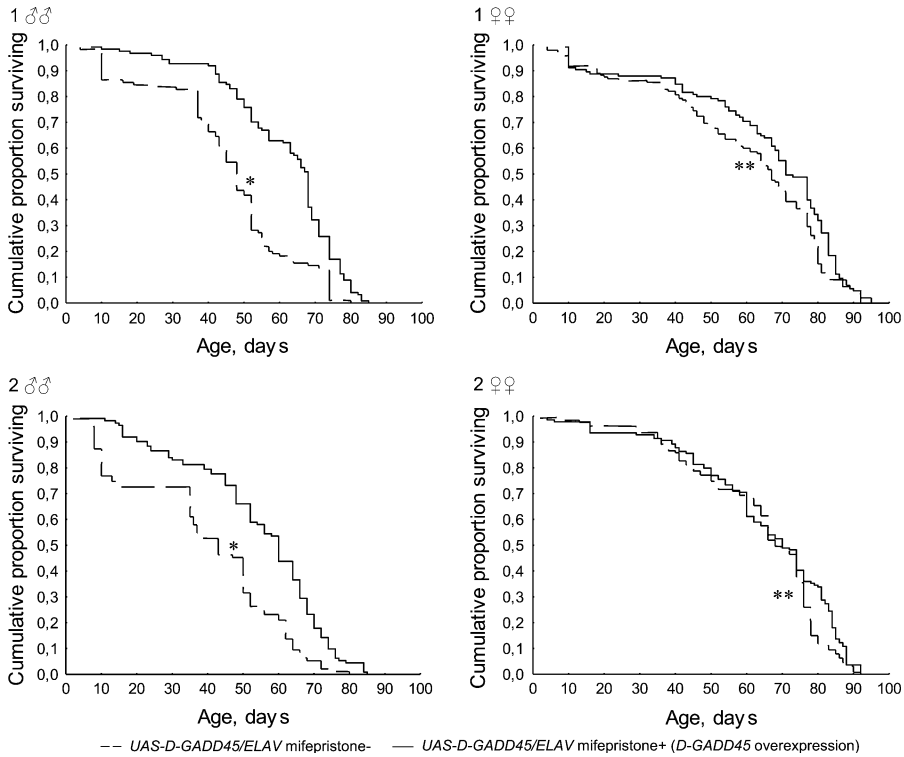

Fig. 3 Survival curves for *D. melanogaster* males (♂♂) and females (♀♀) with the *UAS-D-GADD45/ELAV* genotype not treated with mifepristone and treated with mifepristone (conditional *D-GADD45* overexpression); *1, 2* replications; $*P < 0.001$; $**P < 0.05$ (Kolmogorov–Smirnov test)

Table 2 Effects of conditional *D-GADD45* overexpression in the nervous system on the *D. melanogaster* lifespan

Variant	M	$\bar{X} \pm \Delta m$	90 (%)	Min	Max	MRDT	α	R_0	N
♂ *UAS-D-GADD45* *	40*	39.1 ± 0.7	52*	10	54	5.2	0.13	0.0004	162
♀ *UAS-D-GADD45* *	55*	56.1 ± 0.9	69*	4	85	7.7	0.09	0.0004	173
♂ *ELAV* *	37*	32.5 ± 1.2	57*	1	70	15.4	0.05	0.0092	221
♀ *ELAV* *	35*	33.8 ± 1.1	57*	4	63	12.8	0.05	0.0067	223
♂ *UAS-D-GADD45/ELAV* mifepristone- (1) *	48*	45.4 ± 1.8	74**	4	80	13.1	0.05	0.0032	110
♂ *UAS-D-GADD45/ELAV* mifepristone- (2) *	43*	39.5 ± 2.1	64**	2	80	16.1	0.04	0.0064	105
♀ *UAS-D-GADD45/ELAV* mifepristone- (1) **	67**	59.9 ± 2.0	83	4	95	13.6	0.05	0.0015	145
♀ *UAS-D-GADD45/ELAV* mifepristone- (2) **	68	63.7 ± 1.7	83	2	92	9.9	0.07	0.0005	127
♂ *UAS-D-GADD45/ELAV* mifepristone + (*D-GADD45* overexpression) (1)	68	61.3 ± 1.5	78	7	85	8.3	0.08	0.0003	124
♂ *UAS-D-GADD45/ELAV* mifepristone + (*D-GADD45* overexpression) (2)	60	54.4 ± 1.8	74	4	85	11.0	0.06	0.0012	112
♀ *UAS-D-GADD45/ELAV* mifepristone + (*D-GADD45* overexpression) (1)	71	65.0 ± 2.1	86	9	92	11.4	0.06	0.0006	125
♀ *UAS-D-GADD45/ELAV* mifepristone + (*D-GADD45* overexpression) (2)	70	65.4 ± 1.8	88	4	85	10.8	0.06	0.0006	139

M median lifespan; $\bar{X} \pm \Delta m$ mean lifespan and standard error of mean; 90 (%) age of 90% mortality; *Min* minimum lifespan; *Max* maximum lifespan; α and R_0 Gompertz equation parameters; *MRTD* mortality rate time of doubling; *N* number of flies in a sample; ♂ males; ♀ females; *1, 2* replications; $*P < 0.001$; $**P < 0.05$ (*first column* Kolmogorov–Smirnov test, *second column* Gehan-Breslow-Wilcoxon test; *fourth column* Wang-Allison test)

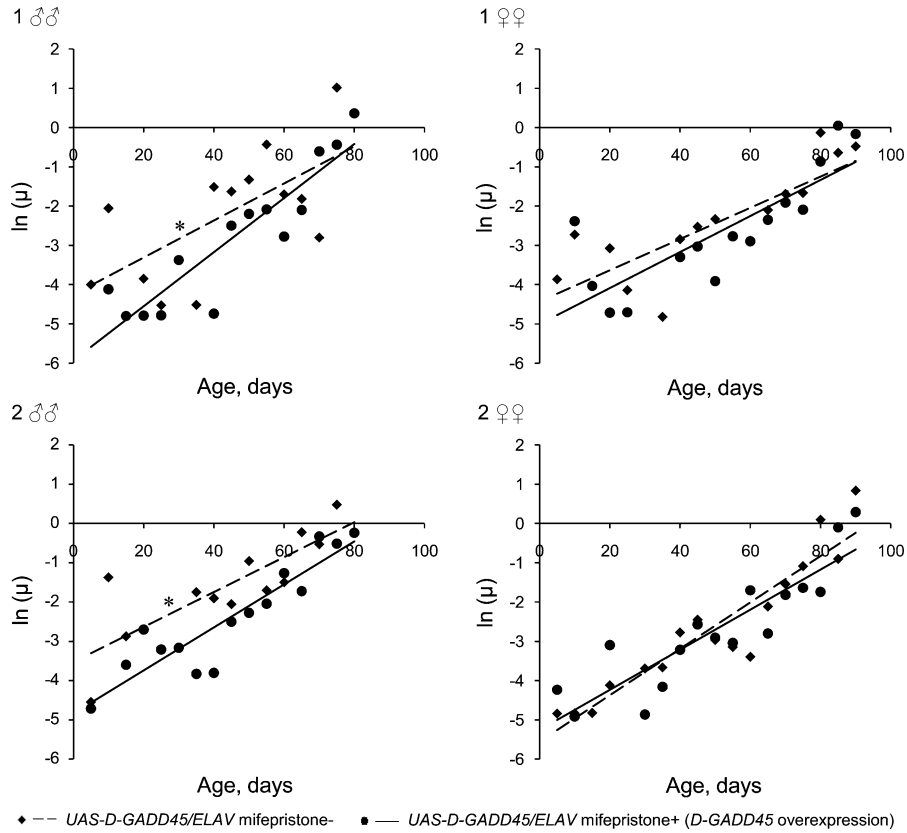

Fig. 4 Trajectories of logarithmic mortality rate for *D. melanogaster* males (♂♂) and females (♀♀) with the *UAS-D-GADD45/ELAV* genotype not treated with mifepristone and treated with mifepristone (conditional *D-GADD45* overexpression); *1, 2* replications; $*P < 0.05$ (maximum likelihood method)

the locomotor activity of *Drosophila* males and females.

Effect of the *D-GADD45* overexpression on the DNA damage level and apoptosis frequency in *D. melanogaster*

To understand the possible mechanisms of the positive effect of the *D-GADD45* gene on the *Drosophila* lifespan, the spontaneous level of the single-strand DNA breaks (using single cell gel electrophoresis) and frequency of apoptosis (DNA diffusion assay) were estimated, since the *GADD45* gene in the mammalian cells is involved in the stress signaling, DNA repair and apoptosis.

In the neuroblasts of *D. melanogaster* larvae with constitutive and conditional *D-GADD45* overexpression in the nervous system, the DNA damage level is reduced by 21–27% with statistical significance ($P < 0.001$, Student's *t*-test) compared to the larvae without *D-GADD45* overexpression (larvae with the *Canton-S/GAL4-1407* genotype and larvae with the *UAS-D-GADD45/ELAV* genotype not treated with mifepristone) (Fig. 11). However, the apoptosis frequency in larva neuroblasts does not differ significantly (data not shown).

Discussion

The dysfunction of numerous genes results in decreased lifespan, but this can be caused not only by the acceleration of healthy aging, but also by specific aging-independent pathologies. An alternative approach would be to perform screening for genes the knock-out or knock-down of which increases the lifespan. Longevity genes can also be identified by estimating the lifespan of organisms with overexpressed candidate genes. A wide range of evolutionarily conserved mechanisms responsible for the processes of aging and the lifespan were studied using the knock-out approach. For example, it was shown that

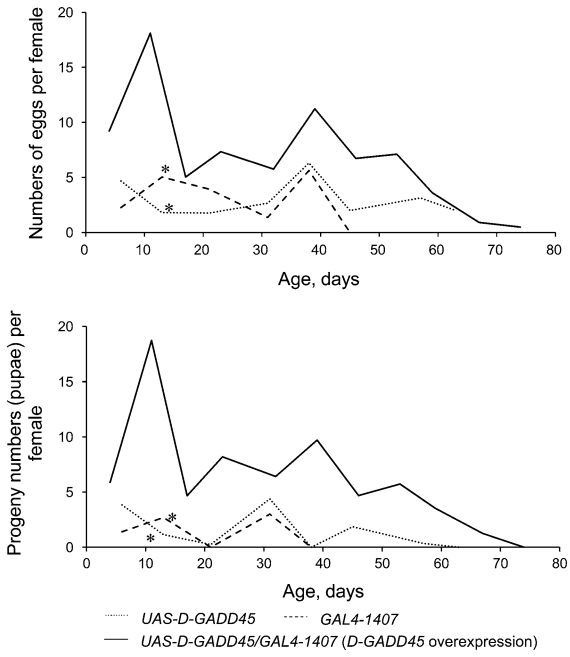

Fig. 5 Fecundity of *D. melanogaster* females with the parental *UAS-D-GADD45* and *GAL4-1407* genotypes and constitutive *D-GADD45* overexpression; $*P < 0.001$ (χ^2-test)

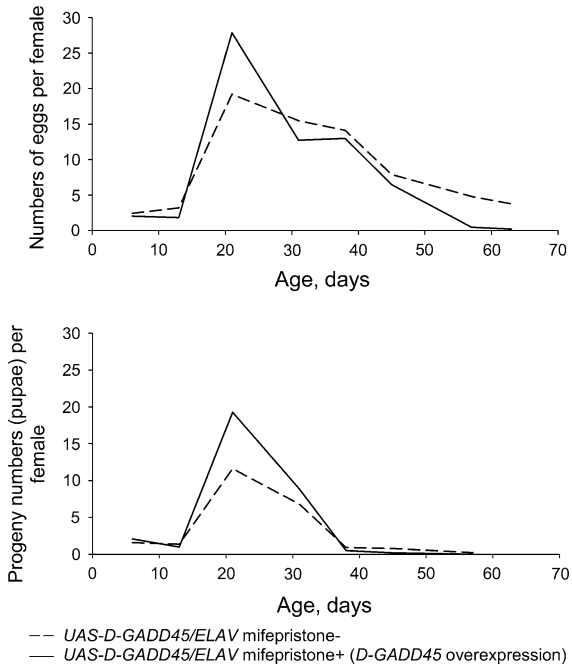

Fig. 6 Fecundity of *D. melanogaster* females with the *UAS-D-GADD45/ELAV* genotype not treated with mifepristone and treated with mifepristone (conditional *D-GADD45* overexpression)

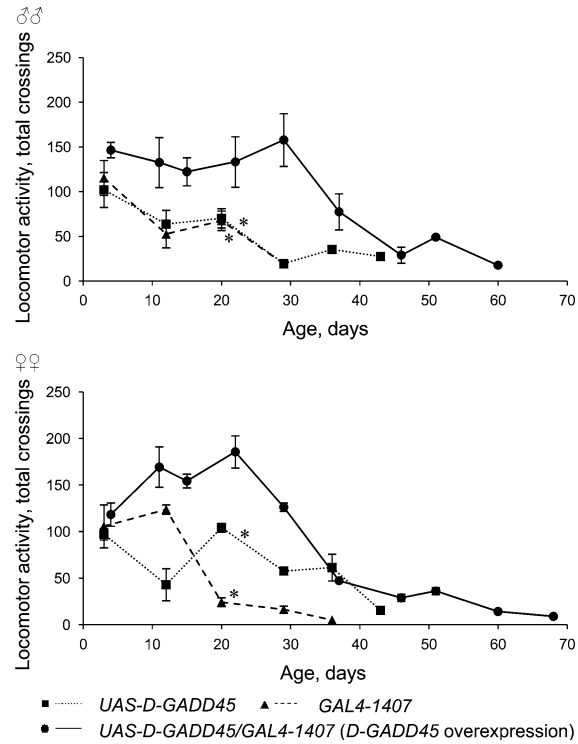

Fig. 7 Spontaneous locomotor activity of the *D. melanogaster* males (♂♂) and females (♀♀) with the parental *UAS-D-GADD45* and *GAL4-1407* genotypes and constitutive *D-GADD45* overexpression; $*P < 0.001$ (χ^2-test)

mutations in the genes of insulin/IGF-1 and TOR signaling pathways may extend the lifespan in living organism of various species (Friedman and Johnson 1988; Larsen et al. 1995; Lithgow et al. 1995; Guarente and Kenyon 2000; Barsyte et al. 2001; Clancy et al. 2001; Lee et al. 2001; Tatar et al. 2001; Meissner et al. 2004; Halaschek-Wiener et al. 2005; Kenyon 2005; Sun 2006; Selman et al. 2008; Henis-Korenblit et al. 2010). However, mutations in genes encoding FOXO transcription factor family reduce the lifespan significantly (Larsen et al. 1995; Lee et al. 2001; Giannakou et al. 2008; Li et al. 2008).

Lifespan extension caused by mutations in certain genes is often accompanied by a decline in other parameters, for example, delayed development and decline of metabolism, or decreased reproductive capacity. This trade off is a consequence of interference with regulations of various functions at cell and organism level. In the absence of such negative effects one would expect random hypomorphic alleles of these genes, leading to increased lifespan,

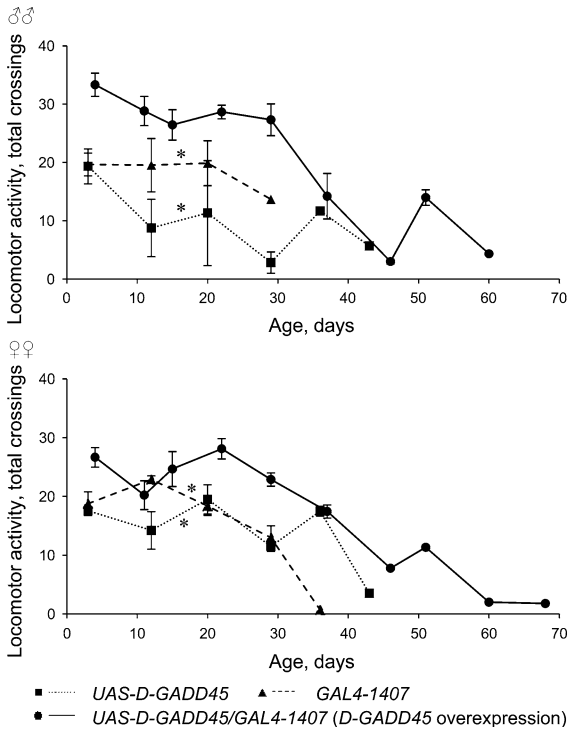

Fig. 8 Results of the negative geotaxis test for the *D. melanogaster* males (♂♂) and females (♀♀) with the parental *UAS-D-GADD45* and *GAL4-1407* genotypes and constitutive *D-GADD45* overexpression; $^*P < 0.001$ (χ^2-test)

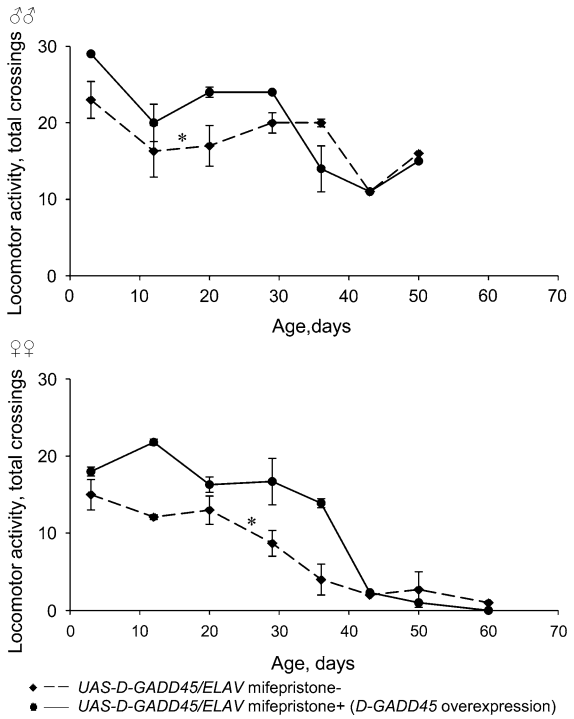

Fig. 9 Results of the negative geotaxis test for the *D. melanogaster* males (♂♂) and females (♀♀) with the *UAS-D-GADD45/ELAV* genotype not treated with mifepristone and treated with mifepristone (conditional *D-GADD45* overexpression); $^*P < 0.001$ (χ^2-test)

would be stabilized in the population, or even displace the normal variant of gene which is not observed under natural conditions.

Therefore, studies of the lifespan of animals with overexpressed candidate genes appear more promising. Using this approach, it was found that *dFOXO* overexpression in the fat body results in an increase of *D. melanogaster* lifespan (Giannakou et al. 2004). Overexpression of the *Hsp22* gene in motor neurons increases the lifespan of *D. melanogaster* along with the increase in locomotor activity and oxidative and thermal stress resistance (Morrow et al. 2004). A significant increase in the *Drosophila* lifespan and oxidative stress resistance was also observed as a result of overexpression of the methionine sulfoxide-reductase gene *msrA* (Ruan et al. 2002).

In the present work, we have studied the effect of overexpression of the stress signaling gene *D-GADD45* on the lifespan of *D. melanogaster*. The GADD45 proteins are most well-studied in mammals. In mammal cells, three types of GADD45 family proteins are found: GADD45α (GADD45), GADD45β (MyD118) and GADD45γ (CR6). They are evolutionarily conservative cytoplasm-localized small protein molecules (118 kDa) with similar structure (the amino acid sequence similarity is 55–57%) and having acidic properties (pH 4.0–4.2) (Liebermann and Hoffman 2008). The *GADD45* genes are targets for the p53 and FOXO transcription factors, and their expression is induced in response to various stress factors (Carrier et al. 1999; Furukawa-Hibi et al. 2002). Proteins of the GADD45 family provide an adequate reaction of the cell to the influence of damaging agents by interactions with other proteins. Specific binding of GADD45 proteins with the Cdc2/CyclinB1 complex inhibits its activity and thus is involved in cellular cycle control under genotoxic stress (Wang et al. 1999; Zhan et al. 1999; Liebermann and Hoffman 2008). GADD45 proteins can interact with the DNA repair and replication protein PCNA. In particular, binding of GADD45α or GADD45β to PCNA promotes excision repair of DNA breaks (Hall et al. 1995; Vairapandi et al. 2000; Liebermann and Hoffman 2008).

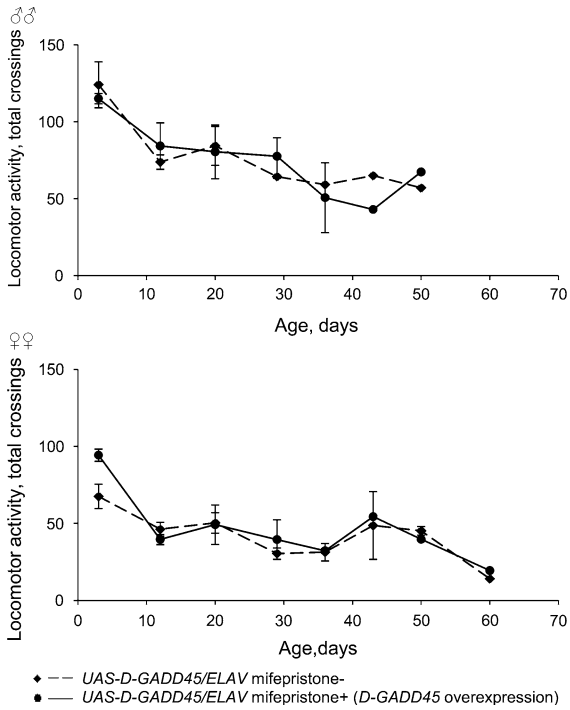

Fig. 10 Spontaneous locomotor activity of the *D. melanogaster* males (♂♂) and females (♀♀) with the *UAS-D-GADD45/ELAV* genotype not treated with mifepristone and treated with mifepristone (conditional *D-GADD45* overexpression)

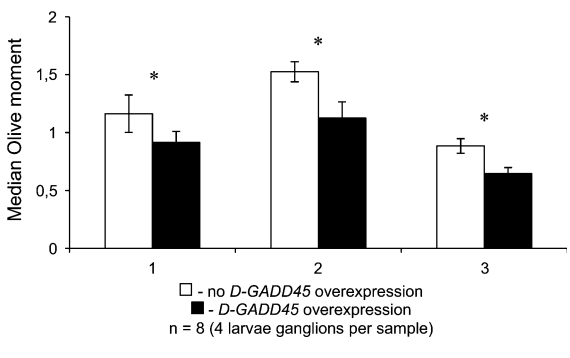

Fig. 11 DNA damage level in the neuroblasts of *D. melanogaster* larvae with constitutive (1, 2) and conditional (3) *D-GADD45* overexpression; $*P < 0.001$ (Student's *t*-test)

Additionally, by being involved in p38/JNK signaling, GADD45 proteins regulate apoptosis in various tissues of an organism, particularly, in response to inflammatory and genotoxic influences (Kojima et al. 1999; Yoo et al. 2003; Gupta et al. 2006; Liebermann and Hoffman 2008; Papa et al. 2008). The involvement of GADD45 proteins in the response to inflammatory stress and infection has been established. After an infection of *D. melanogaster* with Gramm-positive and Gramm-negative bacteria, an increase of the *D-GADD45* expression occurs (Peretz et al. 2007). Furthermore, the GADD45α protein is known to play a role in the stimulation of epigenetic gene activation by DNA demethylation (Liebermann and Hoffman 2008). Therefore, proteins of the GADD45 family play a role in the response to stress and inflammation, in cell cycle control, DNA repair and apoptosis. It is well known that these processes define the stress resistance and affect the rate of aging in living organisms.

G. Peretz et al. have shown that *D-GADD45* overexpression in *D. melanogaster* has diverse phenotypic manifestations depending on the target tissue. Ubiquitous overexpression of this gene in *Drosophila* individuals is lethal (Peretz et al. 2007). Therefore, we investigated the constitutive *D-GADD45* overexpression in the nervous system instead of ubiquitous overexpression. Our data indicate that constitutive *D-GADD45* overexpression in the nervous system of *D. melanogaster* leads to median lifespan extension in comparison with flies of parental strains (by up to 77%). Furthermore, not only the median, but also the maximum lifespan was increased, which is an evidence of slowing down aging. Nevertheless lifespan increasing could be partly due to heterosis or/and genetic background. To avoid these uncontrolled effects we also studied the lifespan of the F1 offspring of crosses between *UAS-D-GADD45* females and *ELAV* males, carrying the mifepristone-inducible *GAL4* driver in the nervous system. The use of a conditional (mifepristone-inducible) driver allowed us to control not just the tissue, but also the stage of transgene expression induction, and to exclude the effect of dissimilar genetic background on the lifespan. Finally, it is known that mifepristone does not have a significant effect on the lifespan of *D. melanogaster* (Ford et al. 2007). We found that the median lifespan of individuals with conditional overexpression of the *D-GADD45* gene in the nervous system was higher in comparison with animals with the same genotype kept on a medium without (by 42%). Thus, it has been demonstrated that the lifespan extension effect is caused by *D-GADD45* overexpression irrespective of the effects of heterosis and genetic background. Moreover, our data suggest that in order to increase the lifespan of *D. melanogaster*, it is sufficient to overexpress

D-GADD45 in the nervous system at the imago stage only and not during the whole lifetime.

It has to be noted that the extension of lifespan resulting from *D-GADD45* overexpression in the nervous system is more pronounced in males than in females. Expression enhancement of this gene in flies with the overexpressing genotype compared to animals without overexpression genotype has also been stronger in males (3–10-fold) than in females (only 2–3-fold). Therefore, the higher increase in the *D-GADD45* gene expression in the nervous system correlates with the stronger increase of lifespan.

We overexpressed the *D-GADD45* gene in the nervous system, since this system plays a key role in the regulation of the function of an organism. Signals coming from the nervous system to other tissues and changes in the expression of certain stress response genes in the nervous tissue can affect the stress resistance and lifespan of the organism as a whole. The IGF-I and growth hormone produced by neurosecretory cells in the brain can regulate the lifespan in mammals (Kappeler et al. 2008; Holzenberger 2009). On the other hand, the lifespan of *Caenorhabditis elegans* with the mutation of *age-1* gene can be completely restored to wild genotype level upon expression of AGE-1 in just the neurons, whereas expression in other tissues results in no such effect (Morley and Morimoto 2004). Aging of the nervous system is a dramatic limitation for the lifespan of an organism. The majority of cells of the nervous system are postmitotic in many animal species. Therefore, the recovery of the nervous tissue from damage is substantially slower than in other tissue types. Furthermore, the nervous system is characterized by high metabolic activity, as a result of which, a lot of damage is accumulated in neurons during aging (Cortopassi et al. 1992; Hamilton et al. 2001). Aging of the brain causes progression of neurological disorders which accelerate and aggravate the aging process (Lee et al. 2000). Therefore, the condition and functioning of the nervous system affects resistance to the effects of various environmental factors, aging processes and the lifespan of an organism.

We proposed that *D-GADD45* overexpression causes an extension of *D. melanogaster* lifespan due to more effective functioning of stress response mechanisms which include DNA repair or apoptosis. First, overexpression of the *D-GADD45* gene in *Drosophila* nervous system could result in more efficient recognition and elimination of spontaneous DNA damage caused by physiological processes and environmental factors. Second, as a result of increased sensitivity to apoptosis induction, selection of neuroblasts that are most resistant to DNA damage and perform better at its repair could occur. Our data have shown that in the neuroblasts of *D. melanogaster* larvae with *D-GADD45* overexpression, a decrease of DNA damage level is observed in two independent repeats of the experiment, which confirm the first assumption. At the same time, the frequency of apoptosis in the neuroblasts of *D. melanogaster* larvae with and without *D-GADD45* overexpression does not differ with statistical significance.

As a rule, the increase of the organism lifespan resulting from decreased expression of certain genes is accompanied by decreased fecundity, metabolism deterioration and delayed development, or impair of locomotor activity (Friedman and Johnson 1988; Guarente and Kenyon 2000; Tatar et al. 2001; Giannakou et al. 2004; Kenyon 2005; Slack et al. 2010). However, the data on fecundity and locomotor activity of *D. melanogaster* individuals with *D-GADD45* overexpression in the nervous system exhibit that their increased lifespan is not accompanied by decreases in the studied parameters, and, conversely, in some cases has positive effects: for example, fecundity increases as compared to parental strain females, as well as the increase of spontaneous locomotor activity in comparison with flies without *D-GADD45* overexpression.

Thus, our data show that *D-GADD45* overexpression in the nervous system of *D. melanogaster* significantly extends lifespan without reducing fecundity and locomotor activity, which is probably caused by more efficient recognition and repair of spontaneous DNA damage generated by physiological processes and environmental factors.

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