High-quality life extension by the enzyme peptide methionine sulfoxide reductase

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Cumulative oxidative damages to cell constituents are considered to contribute to aging and age-related diseases. The enzyme peptide methionine sulfoxide reductase A (MSRA) catalyzes the repair of oxidized methionine in proteins by reducing methionine sulfoxide back to methionine. However, whether MSRA plays a role in the aging process is poorly understood. Here we report that overexpression of the *msrA* gene predominantly in the nervous system markedly extends the lifespan of the fruit fly *Drosophila*. The MSRA transgenic animals are more resistant to paraquat-induced oxidative stress, and the onset of senescence-induced decline in the general activity level and reproductive capacity is delayed markedly. The results suggest that oxidative damage is an important determinant of lifespan, and MSRA may be important in increasing the lifespan in other organisms including humans.

Aging is a multifaceted process that governs the lifespan of an organism and is influenced by both genetic and environmental factors. Cumulative oxidative damages to cellular macromolecules such as nucleic acids, lipids, and proteins by reactive oxygen species (ROS) produced during normal metabolism are postulated to accelerate the aging process and shorten lifespan (1, 2). Several lines of evidence support this oxidative damage theory of aging, and oxidative modifications of proteins may be particularly important. One study estimated that up to 50% of proteins may be oxidized in an 80-year-old human (3). In the fruit fly *Drosophila*, the oxidized protein content also increases with age (4), and those animals with longer lifespans have greater resistance to ROS (5–8). Overexpression of superoxide dismutase and catalase, two enzymatic components in the cellular antioxidant system, together increases the lifespan of *Drosophila* (9–12), whereas genetic disruption of the superoxide dismutase/catalase antioxidant system in *Drosophila* shortens its lifespan (13, 14). Other interventions to extend lifespan such as caloric reduction and manipulations of genes involved in cell metabolism and growth-factor signaling cascades may work indirectly by decreasing the ROS production or increasing the level of antioxidants (2, 10).

Methionine (met) constitutes $\approx 2\%$ of amino acid residues in proteins (15), and is oxidized readily to met sulfoxide [met(O)] by physiological oxidants (16–18). The reduction of met(O) back to met is catalyzed by the enzyme peptide met(O) reductase A (MSRA; ref. 19). The presence of oxidized met residues in a variety of proteins affects biological activity (17), and MSRA has been postulated to act as a modulator of cellular excitability (20, 21). Evidence is emerging also that met/MSRA may constitute an important cellular antioxidant system. *Escherichia coli* and yeast strains lacking the *msrA* gene are more sensitive to oxidative stress (22, 23), and overexpression of the gene in yeast and human T lymphocyte cells protects them from oxidative stress (24). MSRA expression and activity decrease with age in rats (25), and the enzyme activity is also down-regulated in the brains of Alzheimer's disease patients (26). The content of oxidized met in calmodulin, which is important in cellular Ca^{2+}

homeostasis, has been shown to increase significantly with age in rats (27). A mitochondrial genotyping study of Japanese centenarians also suggests that met residues may play a role in longevity (28). A recent study by Moskovitz *et al.* (29) indicates that mice with reduced MSRA activity levels have shorter lifespans. Thus we hypothesized that the overexpression of MSRA to facilitate repair of oxidized met residues in proteins may limit the adverse age-related effects of ROS and extend lifespan.

Materials and Methods

Transgenic *Drosophila* Lines. To prepare the P-element insertion, we constructed the fusion gene *EGFP-msrA*, where enhanced green fluorescent protein (EGFP) was fused in-frame to the N terminus of bovine MSRA (bMSRA; ref. 30). The *bmsrA* gene was selected in part because the gene product had been studied extensively (24, 31–33). The enzymatic activity of this purified fusion enzyme, assayed *in vitro* as described (34), is indistinguishable from that of bMSRA (data not shown). An *Xba*I DNA fragment containing the fusion gene was subcloned into the *Xba*I site of the pUAST vector downstream from the UAS repeats. P element-mediated germline transformants were generated in *white* (*w*) mutant backgrounds (Oregon R) according to the method of Rubin and Spradling (35). Briefly, pUAST-EGFP-MSRA DNA was coinjected into syncytial blastoderm-stage embryos with pTURBO DNA (1 mg/ml) at a ratio of 8:1. Surviving flies were backcrossed to *w* mutant flies and transformants selected by the presence of an orange eye phenotype (*white*⁺) in the progeny. Single pair matings of heterozygous transformants were used to generate the homozygous stocks of pUAST-EGFP-MSRA transformed flies [*w*; $P(w^{+mC} = \text{UAS-AUG-EGFP-MSRA})^B, w$; $P(w^{+mC} = \text{UAS-AUG-EGFP-MSRA})^C, w$; $P(w^{+mC} = \text{UAS-AUG-EGFP-MSRA})^D, w$; $P(w^{+mC} = \text{UAS-AUG-EGFP-MSRA})^E$]. These homozygous UAS-responder lines are referred to as *MSRA*^B, *MSRA*^C, *MSRA*^D and *MSRA*^E.

The *GAL4* activator lines used in this study were *elav*^{C155} [*w*; $P(w^{+mW.hs} = \text{GawB})\text{elav}^{C155}$], *GMR* [*w*^{*}; $P[w^*]w^*$; $P(w + mC = \text{GAL4-ninaE.GMR})^{I2}$] and *Ubi* [*w*; $P(w^{+mW.hs} = \text{GawB})\text{Ubi}$]. The *Ubi-GAL4* activator line is a newly identified *hs-GAL4* P-element insertion on the second chromosome generated by mobilizing the

Abbreviations: ROS, reactive oxygen species; met, methionine; met(O), met sulfoxide; MSRA, met(O) reductase A; EGFP, enhanced green fluorescent protein; bMSRA, bovine MSRA; RT, reverse transcription.

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P(GAL4-Hsp70.PB) construct by using Delta2-3 transposase (36). The *GMR* line and the *P(GAL4-Hsp70.PB)* line were obtained from the *Drosophila* stock center (Bloomington, IN).

RT-PCR. RNA was prepared by homogenizing 50 animals in 500 μ l of RNazol (Tel-Test, Friendswood, TX). Reverse transcription (RT) followed by PCR was performed by using the Superscript One-Step RT-PCR kit (Life Technologies, Gaithersburg, MD). The sense and antisense oligonucleotides used were 5'-ATGGTGAGCAAGGGCGAGGAG-3' and 5'-CTTTTATATACCCAGGGGACAAGACACTCCGGTGCCCCC-3', which are complementary to the 5' and 3' termini of EGFP and bMSRA, respectively. Primers (0.5 μ g) were added to each sample. cDNA synthesis and predenaturation were carried out for one cycle each for 30 min at 52°C and 2 min at 94°C, respectively. PCR amplification was performed for 35 cycles for (i) denaturing step, 94°C for 15 s; (ii) annealing step, 58°C for 30 s; and (iii) extension step, 70°C for 90 s. The final extension was carried out for one cycle at 70°C for 10 min.

Lifespan Determination. Lifespan experiments typically were performed starting with 40–70 animals in each group with each vial containing standard cornmeal agar and 10 male and 10 female animals. The animals were transferred to fresh medium every 3–4 days and maintained at 25°C with a 12/12-h light/dark cycle. Embryonic, larval, and pupal animals were kept at room temperature before the start of a lifespan trial. Statistical significance was calculated by using the resampling-with-replacement method (37) implemented in IGOR PRO (WaveMetrics, Lake Oswego, OR). The data points were resampled >10,000 times to estimate the significance. Comparisons of lifespans were made only within the same run.

Paraquat Resistance and Food Intake. Twenty adult male flies were maintained in each vial, which contained a filter paper saturated with a 100- μ l solution of 10 mM paraquat, 1 mg/ml Brilliant Blue dye, and 1% sucrose at 25°C with a 12/12-h light/dark cycle. The number of animals alive after 24 h with paraquat was recorded to determine paraquat resistance. After removing the head, the animals were homogenized in distilled water. The dye concentration was quantified by using a spectrophotometer (model 340, Turner, Palo Alto, CA) at 630 nm to estimate the percentage of the dye intake by 20 animals over the amount of dye provided initially in each vial.

Met Oxidation Induced by Paraquat. A synthetic peptide with the sequence KIFMK was incubated with paraquat (10 μ M) for 10 min. Subsequently the amount of peptide with met(O) was assayed by matrix-assisted laser desorption ionization mass spectroscopy, yielding $\approx$ 75% met(O) and $\approx$ 25% met. The control peptide without paraquat treatment had no detectable met(O).

Body Weight Determination. In each group, 50 adult animals at the age of 10 days were anesthetized with ether, and their total weight was measured to estimate the mean body weight.

Physical Activity Assay. A reactive climbing assay (38) was used to assess the overall physical activity level of the animals. Adult male animals were transferred to empty glass vials containing 30 animals each. After a 2-min rest period, the vials were vibrated on a vortex mixer (S8233, Scientific Industries, Bohemia, NY) at the highest speed for 10 s. After vortexing, the animals were given a 10-s rest, and the number of animals climbing on the walls of the vial was recorded.

Results

We used the *GAL4-UAS* system (39) to overexpress bMSRA in the fruit fly *Drosophila melanogaster*. *Drosophila* is a good model

system to study aging because of its relatively short lifespan. We generated four homozygous transgenic lines of fruit flies, *MSRA^B*, *MSRA^C*, *MSRA^D*, and *MSRA^E*, representing four independent insertions of the gene into the genome by using the standard P-element insertion technique. Because previous studies suggested that protection from oxidative stress in the nervous system may be particularly important in extending the *Drosophila* lifespan (11), we overexpressed bMSRA by crossing the above *UAS-MSRA* responder lines with the homozygous *GAL4* activator line *elav^{C155}*, which promotes expression of MSRA predominantly in the nervous system (40). The expression of bMSRA in the total body homogenate of the resulting animals was verified by using RT-PCR (Fig. 1A). Tissue-specific expression of MSRA was confirmed by detecting the fluorescent signal of EGFP fused to the N terminus of bMSRA (Fig. 1B and C).

We examined the lifespan of the MSRA transgenic animals, and representative survivorship results are shown in Fig. 1D. Male and female animals were kept in separate vials, and their lifespans were recorded. The survival distributions of the two control heterozygous lines, which do not overexpress MSRA, were similar. The median lifespans of the female animals in these control groups in the trial shown were $\approx$ 58 days (55 and 61 days for *UAS-MSRA^C/+* and *elav-GAL4/+*, respectively). In the male animals, the median lifespans were $\approx$ 45 days (42 and 48 days for *UAS-MSRA^C/+* and *elav-GAL4/+*, respectively). Overexpression of MSRA predominantly in the nervous system of progeny derived from crossing the *MSRA^C* line with the *elav-GAL4* line dramatically increased the median lifespans to $\approx$ 95 days in female animals and $\approx$ 80 days in male animals, corresponding to a fractional increase of $\approx$ 70%. By using the resampling-with-replacement method (37), we confirmed that the lifespan increases were statistically significant ($P < 0.0001$). Other *Drosophila* studies testing the oxidative damage theory of aging typically reported 30–40% increases in lifespan (9–12). Near doubling of the median lifespan presented here is comparable to that found in *Drosophila* carrying a disruption of the *Indy* gene, which is homologous to a mammalian sodium dicarboxylate co-transporter gene (41).

The survival distributions were approximated by the Gompertz function (Fig. 1D, Lower), which has been used as a good descriptor of human and *Drosophila* mortality by multiple causes (4, 42). The death rate when 50% of the animals were alive/dead as indicated by the slope of the survival distribution was altered only slightly in the transgenic animals overexpressing MSRA when compared with those of the control groups (Fig. 1D, Upper). Thus the extended lifespan in these transgenic animals was achieved largely by shifting the survival distribution to the right, suggesting that the enzyme overexpression delays the onset of the same set of the mortality factors that normally determine the lifespan in the control groups.

The median lifespan of the transgenic animals overexpressing MSRA in the nervous system also was markedly greater than those of the two homozygous parental control lines, *elav-GAL4* and *MSRA^C* (Fig. 1E). The results shown were obtained by using male and female animals housed together. This may account for median lifespan values smaller than those obtained when male and female animals were segregated (Fig. 1D). The fractional increases in the median lifespan observed with the homozygous parental control groups were somewhat greater than those obtained with the heterozygous control groups (Fig. 1D). The median lifespan of the MSRA transgenic animals also was greater than that of the Oregon R wild-type animals ($\approx$ 31 days for male and $\approx$ 41 days for female). Comparison between progeny of a cross between *elav-GAL4* and *UAS-EGFP* with these parental lines indicated that the observed lifespan extension is not caused by EGFP overexpression (data not shown). To exclude the possibility that the lifespan extension induced by MSRA overexpression is caused by the chromosomal position effect, we repeated the same lifespan determination protocol

Fig. 1. Pan-neuronal MSRA overexpression and lifespan extension. (A) RT-PCR analysis. Lane 0, molecular weight markers; lane 1, *elav-GAL4/Y* adult male (parental control); lane 2, *UAS-MSRA^C/UAS-MSRA^C* (*MSRA^C*) adult male (parental control); lane 3, *elav-GAL4/Y;UAS-MSRA^C/+* adult male; lane 4, *elav-GAL4/+; UAS-MSRA^C/+* adult female; lane 5, *elav-GAL4; UAS-MSRA^C/+* male and female larvae. The expected product size is 1.4 kb. EGFP-MSRA signals are present in lanes 3, 4, and 5. The adult animals used were 5–8 days old. Expression of EGFP-MSRA in the nervous systems was achieved by crossing the *MSRA^C* line and the *elav-GAL4¹⁵⁵*-*GAL4* activator line. (B) Fluorescent micrographs of larval brain/ventral ganglion complexes. EGFP fluorescence was clearly observed in the *elav-GAL4; UAS-MSRA^C/+* larval nervous system (Right), but only weak signals were present in the parental control larval *MSRA^C* nervous system (Left), indicating that the non-*GAL4*-activated EGFP-MSRA expression was negligible. A third instar larva was dissected to expose the nervous system and observed by using an Olympus BX51 fluorescence microscope with a GFP filter. (Scale bars, 200 μ m.) E, eye-antennal disk; B, brain hemisphere; V, ventral ganglia. (C) Fluorescent micrographs of dissected adult brains. EGFP fluorescence was very faint in the parental control *MSRA^C* nervous system at the age of $\approx$ 8 days (Left) but clearly visible in the *elav-GAL4; UAS-MSRA^C/+* adult nervous system at the age of $\approx$ 8 days (Center) and also at 70 days (Right). Adult brains were dissected out in PBS, mounted in 50% glycerol/PBS, and viewed with an Olympus BX51 microscope with a GFP filter. Images were digitized with a Spot RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI) with monochrome settings and then pseudocolored. (Scale bars, 50 μ m.) O, optic lobe; B, central brain. (D) Survivorship curves of progeny animals resulting from crosses between *MSRA^C* and wild-type Oregon R

with two other independent lines of transgenic animals, *MSRA^B* and *MSRA^D*, using the *elav-GAL4* line. As found with *MSRA^C*, both *elav-GAL4/MSRA^B* and *elav-GAL4/MSRA^D* showed increased lifespans compared with their respective parental lines (mean fractional increase = $73 \pm 27\%$). *MSRA^B* and *MSRA^D* median lifespans (male/female), 31/49 and 20/19 days).

The specificity of MSRA-induced life extension is confirmed further by crossing *MSRA^C* with two other homozygous *GAL4* activator lines, *Ubi* and *GMR*. *Ubi* drives MSRA expression globally in the body, especially in muscle (Fig. 2A). *GMR* promotes more restricted gene expression primarily in the eye (ref. 43; Fig. 2B), and restricted expression of MSRA by *GMR* should have a smaller effect on the lifespan. The lifespan of the animals overexpressing MSRA in the whole body driven by *Ubi* was prolonged markedly as found with overexpression of MSRA preferentially in the nervous system (Fig. 2C). The median lifespan was increased by 44% in the male animals and 37% in the female animals when compared with those of the parental *Ubi-GAL4* animals, and 83 and 80% when compared with those of the parental *MSRA^C* animals (Fig. 2C; $P < 0.0001$). Similar results were obtained by crossing the *Ubi-GAL4* activator lines with *MSRA^B* and *MSRA^D*. In contrast, a noticeably smaller increase in the median lifespan was observed when MSRA overexpression was confined largely to the eye (Fig. 2D). In the female animals, the median lifespan was greater by 35 and 8% when compared with those of the parental control lines, *MSRA^C* and *GMR-GAL4*. The male lifespan increases were 45 and 15%, respectively. Similar results were obtained when *GMR-GAL4* was used to drive expression in *MSRA^B* and *MSRA^D*. The smaller lifespan extension by very restricted expression of MSRA argues against the possibility that crossing *UAS-MSRA* responder animals with any *GAL4* activator line increases the progeny lifespan.

Many animals with long lifespans often display increased resistance to common stresses such as starvation, heat exposure, and oxidative stress (44, 45). We tested the resistance of the animals overexpressing MSRA predominantly in the nervous system against oxidative stress. The herbicide paraquat has been used to induce oxidative stress in *Drosophila*, and we confirmed that paraquat

(*UAS-MSRA^C/+*), *elav-GAL4* and Oregon R (*elav-GAL4/+*), and *elav-GAL4* and *MSRA^C* (*elav-GAL4; UAS-MSRA^C/+*) shown in two different representations. *UAS-MSRA^C/+* (∇) and *elav-GAL4/+* ($\circ$) represent the control groups, and MSRA is overexpressed in *elav-GAL4; UAS-MSRA^C/+* ($\bullet$). The estimated median lifespan values of the female animals in these groups were 55, 61, and 95 days, respectively. The median values for the male animals were 42, 48, and 77 days. The smooth lines represent best fits of the data using the two-parameter Gompertz survivor distribution $S(t) = \exp\{(R_0/\alpha)[1 - \exp(-\alpha t)]\}$ where t is adult age in days, and R_0 and α are the Gompertz parameters. The values of R_0 and α for *UAS-MSRA^C/+*, *elav-GAL4/Y*, and *elav-GAL4/Y; UAS-MSRA^C/+* animals were $1.40 \times 10^{-3}/7.42 \times 10^{-2}$, $4.00 \times 10^{-3}/5.64 \times 10^{-3}$, and $5.13 \times 10^{-5}/9.29 \times 10^{-2}$ for males and $4.25 \times 10^{-4}/7.95 \times 10^{-2}$, $1.52 \times 10^{-3}/6.03 \times 10^{-2}$, and $1.65 \times 10^{-5}/8.65 \times 10^{-2}$ for females. Similar results were obtained in another trial and also when *MSRA^D/MSRA^D*, *elav-GAL4/+*, and *elav-GAL4; UAS-MSRA^D/+* were compared. Each group initially contained 60 animals, and each vial contained 20 male or female animals. (E) Survivorship curves of the parental control animals, *elav-GAL4* and *MSRA^C* and *elav-GAL4; UAS-MSRA^C/+* progeny that overexpresses MSRA in the nervous systems. The parental control lines *elav-GAL4/elav-GAL4* (or *elav-GAL4/Y*, $\square$) and *MSRA^C/MSRA^C* ($\circ$) shown were tested as homozygotes in Canton S and Oregon R genetic backgrounds, respectively. The estimated median lifespan values of *elav-GAL4* and *MSRA^C* were 40 and 35 days for male animals and 46 and 39 days for female animals. The median lifespans for the male and female *elav-GAL4;UAS-MSRA^C/+* ($\bullet$) animals were 69 and 75 days. The smooth lines represent best fits of the data using the Gompertz distribution. The values of R_0 and α for *MSRA^C* and *elav-GAL4/Y* and *elav-GAL4; UAS-MSRA^C/+* animals were $5.46 \times 10^{-3}/5.89 \times 10^{-2}$, $1.28 \times 10^{-3}/9.77 \times 10^{-3}$ and $9.55 \times 10^{-5}/9.56 \times 10^{-2}$ for males, and $4.01 \times 10^{-3}/6.15 \times 10^{-2}$, $1.15 \times 10^{-3}/8.19 \times 10^{-3}$, and $1.20 \times 10^{-5}/1.18 \times 10^{-1}$ for females, respectively. Each curve was based on 70 animals raised in vials, each containing 10 male and 10 female animals.

Fig. 2. Survivorship and MSRA expression driven by *Ubi* and *GMR*. (A and B) Fluorescent micrographs of the *Ubi-GAL4/+; UAS-MSRA^C/+* and *GMR-GAL4/+; UAS-MSRA^C/+* larvae. GFP fluorescence was detected diffusely in the whole body, especially in muscle fibers of *Ubi-GAL4/+; UAS-MSRA^C/+* (A) and primarily confined to the eye area of the eye-antennal discs of *GMR-GAL4/+; UAS-MSRA^C/+* (B). (Scale bar, 100 μ m.) O, optic lobe; B, brain hemisphere; V, ventral ganglia; E, eye-antennal disk; M, muscle fiber. Ectopic expression of EGFP-MSRA was achieved by crossing *MSRA^C* with the homozygous *Ubi-GAL4* or *GMR-GAL4* activator line. (C) Survivorship curves of *Ubi-GAL4/+; UAS-MSRA^C/+* (●) compared with the parental lines *Ubi-GAL4/+* (□) and *UAS-MSRA^C/+* (○). The smooth lines represent best fits of the data using the Gompertz survivor function. The estimated lifespan values for the male and female animals in the *Ubi-GAL4/+; UAS-MSRA^C/+* were 64 and 70 days. The values of R_0 and α for *MSRA^C/Ubi-GAL4* and *Ubi-GAL4/+; UAS-MSRA^C/+* animals were $5.46 \times 10^{-3}/5.89 \times 10^{-2}$, $1.10 \times 10^{-3}/8.73 \times 10^{-2}$, and $2.08 \times 10^{-5}/1.29 \times 10^{-2}$ for males and $4.01 \times 10^{-3}/6.15 \times 10^{-2}$, $3.28 \times 10^{-4}/1.05 \times 10^{-1}$, and $1.57 \times 10^{-4}/8.37 \times 10^{-3}$ for females. (D) Survivorship curves of *GMR-GAL4/+; MSRA^C/+* (●) compared with the parental lines *GMR-GAL4/+* (□) and *UAS-MSRA^C/+* (○). The smooth lines represent best fits of the data using the Gompertz survivor function. The estimated lifespan values for the male and female animals in the *GMR-GAL4/+; MSRA^C/+* were 64 and 70 days. The values of R_0 and α for *MSRA^C/MSRA^C*, *GMR-GAL4/GMR-GAL4*, and *GMR-GAL4/+; MSRA^C/+* animals were $5.46 \times 10^{-3}/5.89 \times 10^{-2}$, $3.78 \times 10^{-4}/1.23 \times 10^{-2}$, and $4.60 \times 10^{-4}/9.53 \times 10^{-2}$ for males and $4.01 \times 10^{-3}/6.15 \times 10^{-2}$, $1.65 \times 10^{-3}/6.77 \times 10^{-2}$, and $6.50 \times 10^{-4}/8.32 \times 10^{-2}$ for females. The control data in C and D are the same.

oxidized met to met(O) in a short synthetic peptide. If the MSRA-induced lifespan extension is achieved by conferring greater protection against oxidative stress, it should render the animals more resistant to paraquat. At the age of 30 days, dietary paraquat (10 mM) typically killed 60–70% of the male parental control animals after 24 h. In contrast, paraquat killed only 10% of the male animals that overexpressed MSRA in the nervous system (Fig. 3A). This finding suggests that the lifespan extension is caused by the antioxidant action of MSRA. Furthermore, the food intake was similar in all the groups examined (Fig. 3B), discounting the possibility that the greater resistance to paraquat is caused simply by reduced food intake.

Some genetically modified organisms with extended lifespans such as *daf-2* mutant *Caenorhabditis elegans* (46) and *mth* mutant

Fig. 3. Increased paraquat resistance by MSRA overexpression. (A) Paraquat resistance of 30-day-old male animals. In each trial, the experimental and control groups each consisted of 60 animals, and the number of animals alive after feeding for 24 h with paraquat was recorded. Each data point represents an average of 3–6 trials with medium containing the dye Brilliant Blue. Similar results were obtained when the dye was omitted. (B) Paraquat-containing food intake measurements on 30-day-old male animals. The average food intake of *elav-GAL4/Y; UAS-MSRA^C/+* progeny was similar to that of *elav-GAL4/Y* and slightly greater than that of *MSRA^C* animals. Intake of paraquat-sucrose solution was measured by using the dye Brilliant Blue as described for A.

Drosophila (45) show enhanced resistance to starvation. We found that overexpression of MSRA offered no significant protection against starvation (data not shown). It is worth noting that both *mth* *Drosophila* and *daf-2* *C. elegans* display increased body weights (44,

Fig. 4. MSRA overexpression delays senescence-induced declines in physical activity and reproductive levels. (A) Changes in the general activity level with age. The “active fraction” is defined as the fraction of animals on the wall after vortexing the vial. Each data point represents an average of 3–6 determinations, each of which involved 20–40 male flies. Separate groups of animals were tested at different ages. At a given age, the animals overexpressing MSRA predominantly in the nervous system (*elav-GAL4/Y; UAS-MSRA^C/+* progeny, ●) were more active than the parental control groups *elav-GAL4/Y* (□) and *MSRA^C/+* (○). (B) Changes in pupa production with age. The same groups of adult animals were transferred to fresh vials at fixed intervals as in the lifespan experiments, and the total number of the pupal progeny in each vial was counted. The results from the control groups *elav-GAL4/Y* (□) and *MSRA^C/+* (○) and the pan-neuronal MSRA-overexpression group *elav-GAL4/Y; UAS-MSRA^C/+* (●) are shown. The ordinate represents the number of pupae produced per day per animal in a vial. Each data point represents results from four vials. In all the groups, >95% of the pupae developed into adults. (C) Copulation probabilities in male animals of different genotypes. Twenty adult males (OR, *elav-GAL4/Y*, *MSRA^C*, *elav-GAL4/Y; UAS-MSRA^C/+*) of 4–5 days of age were placed with 20 virgin wild-type CS females (6–7 days old) in a 1% agar-plated Petri dish (10-cm diameter). The number of animal pairs copulating was counted every 3 min and normalized to obtain the probability values. Each data point represents an average of 2–4 determinations.

45) caused by fat accumulation (44), potentially contributing to the reported greater protection from starvation. We found that the average body weight of the MSRA-overexpression male animals was indistinguishable from that of either parental line at the age of 10 days (≈ 0.6 – 0.7 mg per animal).

Aging in many species is associated with a decline in physical activity and also in reproductive vitality. We observed that the transgenic flies overexpressing MSRA preferentially in the nervous system or generally in the whole body were noticeably more active than the control animals of the same age. We quantified this observation with a reactive climbing assay (38). The results shown in Fig. 4A demonstrate that in the animals in which MSRA was overexpressed predominantly in the nervous system, there was delayed onset of the senescence-induced decrease in the physical activity level. The observation that the MSRA transgenic animals were more physically active also suggests that the extended lifespan is not a simple consequence of reduced physical activity as found in some *Drosophila* mutants (47). Considering that the food intake in these animals is similar to that of the control animals (Fig. 3B), the MSRA transgenic animals may be more efficient metabolically.

We also found that overexpression of MSRA in the nervous system delays the onset of the decline in the overall reproductive vigor. We counted the number of pupae produced per animal in a fixed amount of time. The number of pupae produced per animal per day remained relatively stable up to ≈ 20 – 30 days of age but rapidly declined thereafter in the two parental strains (Fig. 4B). In the MSRA pan-neuronal overexpression group, the pupal production was greater and remained stable for longer. For example, at 50 days only a small number of pupae were produced in the control groups, whereas in the MSRA-overexpression group the pupal production was still at $\approx 75\%$ of the peak value observed in much younger animals. Because pupa production reflects multiple factors in reproduction, such as mating frequency, egg laying, and egg hatching, the prolonged pupa production period suggests that all the relevant steps in reproduction are preserved by overexpression of MSRA. We further examined the probability of copulation in the MSRA-overexpression group and the parental control strains. The peak copulation probability was greater and occurred faster in the MSRA-overexpression animals (Fig. 4C). Thus the results suggest that the greater copulation probability in the MSRA-overexpression group may contribute to the greater pupa production. These findings together suggest that the lifespan extension by MSRA overexpression does not occur at the expense of reproduction.

Although the adult lifespan is extended by overexpression of MSRA, the developmental time course up to the adult emergence was not altered in any of the MSRA-overexpression groups and remained ≈ 10 days long at room temperature. Other readily observable behavioral traits of the MSRA-overexpression animals such as feeding, flight, and geotaxis were indistinguishable from those of the control animals in early ages.

Discussion

The results presented here strongly suggest that overexpression of MSRA, which catalyzes the reduction of met(O) to met in proteins, is important in delaying death in *Drosophila*. The underlying molecular mechanism remains to be investigated further. Met oxidation in various proteins results in loss of biological activity (17). Thus one possibility is that met oxidation in some proteins is particularly crucial in determining the lifespan of an organism, and MSRA may act to protect these key proteins. For instance, met oxidation in calmodulin inhibits its ability to activate the plasma

membrane Ca^{2+} ATPase, and met oxidation in calmodulin progressively increases with age in the rat brain (27, 48). This observation may explain the age-related changes in intracellular Ca^{2+} homeostasis in neurons (49, 50). Alternatively, the reversible oxidation-reduction cycle of met involving MSRA may scavenge ROS to spare other cellular components from oxidative damage (51). It is possible also that overexpression of MSRA has an indirect effect, possibly involving alterations in gene expression patterns (52). Numerous redox-dependent cell signaling pathways and genes have been described (53).

Studies have postulated that met/MSRA forms a vital cellular antioxidant system (22–24). The oxidative damage theory of aging predicts that overexpression of MSRA extends the lifespan, and deletion of the gene shortens it. Our study confirms the first prediction. The second prediction is born out by the results by Moskovitz *et al.* (29), who showed that mice with a reduced MSRA activity had an $\approx 40\%$ shorter lifespan. These mice also displayed other noticeable behavioral abnormalities, suggesting that MSRA may be important in a variety of physiological functions.

Our results show that overexpression of MSRA predominantly in the nervous system extends lifespan, and restricted overexpression primarily in the eye had a much smaller impact on the lifespan. These results may indicate that *Drosophila* neurons have a high ROS production rate and/or that the endogenous MSRA activity is low, implying that the endogenous MSRA activity may be a limiting factor in lifespan determination of *Drosophila*. Because the *elav^{UAS}*-*GAL4*-mediated ectopic expression is unlikely to be absolutely specific for the nervous system, we cannot exclude the contribution from nonneuronal MSRA. Parkes *et al.* (11) reported that overexpression of superoxide dismutase/catalase in *Drosophila* motor neurons extends the mean lifespan by up to 40%. This study, however, noted that overexpression of the same enzymes using the *elav^{UAS}*-*GAL4* activator line did not extend the lifespan. The greater lifespan extension observed with MSRA overexpression in our study suggests that the antioxidant mechanism involving met and MSRA may be more robust than the superoxide dismutase/catalase system in lifespan determination.

In summary, we have shown that overexpression of MSRA predominantly in the *Drosophila* nervous system markedly extends lifespan. Unlike the lifespan extension induced by some experimental manipulations, the longer lifespan by MSRA overexpression is associated with many desirable features. The MSRA transgenic flies retain normal food intake and bodyweight, but they are more physically active. The MSRA transgenic animals also maintain high physical and reproductive activities much later into their lives than control animals. Some *Drosophila* mutants show extended lifespans, but their body weights are greater (45), potentially from fat accumulation. Many other mutations that extend the lifespans of model organisms often lower their physical activity and reproductive capacity. Mutant female *insulin-like receptor* flies with abnormal endocrine function live longer, but they are hypomorphic and sterile (54). Therefore, it may be argued that overexpression of MSRA in *Drosophila* extends its lifespan while maintaining quality of life. It will be of great interest to see whether overexpression of MSRA extends lifespan in mammals including humans.

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