Overexpression of Glutamate-Cysteine Ligase Extends Life Span in Drosophila melanogaster*

The hypothesis that overexpression of glutamate-cysteine ligase (GCL), which catalyzes the rate-limiting reaction in de novo glutathione biosynthesis, could extend life span was tested in the fruit fly, Drosophila melanogaster. The GAL4-UAS binary transgenic system was used to generate flies overexpressing either the catalytic (GCLc) or modulatory (GCLm) subunit of this enzyme, in a global or neuronally targeted pattern. The GCL protein content of the central nervous system was elevated dramatically in the presence of either global or neuronal drivers. GCL activity was increased in the whole body or in heads, respectively, of GCLc transgenic flies containing global or neuronal drivers. The glutathione content of fly homogenates was increased by overexpression of GCLc or GCLm, particularly in flies overexpressing either subunit globally, or in the heads of GCLc flies possessing neuronal drivers. Neuronal overexpression of GCLc in a long-lived background extended mean and maximum life spans up to 50%, without affecting the rate of oxygen consumption by the flies. In contrast, global overexpression of GCLm extended the mean life span only up to 24%. These results demonstrate that enhancement of the glutathione biosynthetic capability, particularly in neuronal tissues, can extend the life span of flies, and thus support the oxidative stress hypothesis of aging.

The tripeptide, γ-glutamylcysteinylglycine, i.e. glutathione (GSH), is a versatile biological reductant, which is often present in millimolar amounts in tissues (1, 2). It serves multiple physiological functions, including acting as a substrate in the enzymatic reduction of peroxides, as a conjugant to xenobiotics to facilitate their export from cells, in transport of amino acids, thiolation/dethiolation of proteins, and maintenance of cellular redox state (3–6). GSH is synthesized de novo by the consecutive action of two enzymes: glutamate-cysteine ligase (GCL, EC 6.3.2.2), which catalyzes the first and rate-limiting step in de novo synthesis (Reaction 1), and GSH synthase (GS, EC 6.3.2.3), which links glycine to γ-glutamylcysteine (γ-GC) to form GSH (Reaction 2) (4). The primary determinants of the rate of GSH synthesis are the availability of substrates, activity of GCL, and feedback inhibition of GCL by GSH (7).

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\begin{align*}
\text{L-Glutamate + L-cysteine} & \quad + \text{ATP} \rightleftharpoons \text{L-γ-glutamyl-L-cysteine + ADP + P}_i \\
\text{(REACTION 1)}
\end{align*}
\]

\[
\begin{align*}
\text{L-γ-Glutamyl-L-cysteine} & \quad + \text{glycine} + \text{ATP} \rightleftharpoons \text{GSH + ADP + P}_i \\
\text{(REACTION 2)}
\end{align*}
\]

Mammalian GCL is a heterodimeric enzyme, consisting of a catalytic subunit, GCLc, and a regulatory or modulatory subunit, GCLm, which are the products of two distinct genes (8). The GCLc subunit can catalyze the formation of γ-GC in the absence of GCLm, but its activity is increased substantially by covalent interactions with GCLm. GCL activity is increased under oxidative stress, i.e., an imbalance between antioxidants and pro-oxidants, by the formation of disulfide bonds between the GCLm and GCLc subunits (7, 9). Oxidants can also differentially up-regulate the transcription of GCL genes, thereby providing a protective mechanism against oxidative stress-induced cellular dysfunction. A considerable body of evidence indicates that GSH provides protection against oxidative stress in vivo. For instance, experimental depletion of GSH by L-buthionine-SR-sulfoximine, an inhibitor of GCL activity, results in enhanced vulnerability to oxidative challenges (10). Transfection of cells with GCLc cDNAs elevates GSH levels as well as resistance to oxidative stress (11). The amount of GSH and the ability to maintain its steady-state level under oxidative stress tend to decrease in some tissues with increasing age, which is hypothesized to be reflective of an impairment of the mechanisms of GSH synthesis (12). Indeed, a recent study of the housefly indicates that GCL has significantly higher affinities for its substrates in young than in old flies, suggesting the occurrence of a catalytic inefficiency of the rate-limiting step of glutathione biosynthesis (13).

Several lines of evidence suggest that the level of oxidative stress is enhanced during the aging process (14). For instance, rates of mitochondrial reactive oxygen species generation and steady-state amounts of the products of reactions between reactive oxygen species and macromolecules such as nucleic acids, proteins, and lipids are relatively high in aged animals. Furthermore, aged organisms are more susceptible to induced oxidative damage than the young. In this context, the principal objective of this study was to examine the role of GSH and oxidative stress in the aging process, using Drosophila melanogaster as a model organism. Molecular cloning of both a ~31-kDa regulatory subunit and a ~80-kDa catalytic subunit has been accomplished in Drosophila (15). Accordingly, the specific hypothesis was that overexpression of Drosophila GCL catalytic and modulatory subunits, using global and tissue-specific promoters, would enhance the ability to synthesize GSH and prolong the life span.
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MATERIALS AND METHODS

Chemicals and Reagents—HPLC calibration standards (γ-GC and GSH) were obtained from Sigma. α-Phosphoric acid was purchased from EMD Science (Gibbstown, NJ). Milli-Q grade water was prepared by reverse osmosis on a Millipore® water-purification system. All chemicals were either analytical grade or of the highest purity commercially available.

Construction of pP[UAST]-GCLc and pP[UAST]-GCLm—The EcoRI-BamHI fragment of the GCLc gene was digested from AT05811 cDNA clone (BDGP) plasmid DNA and subcloned into corresponding sites of the pBluescript SKI(−) vector. The 5′-EcoRI site was derived from the polylinker of the POT7 vector of the AT05811 clone, whereas the BamHI site was located in the 3′-untranslated region of the GCLc mRNA. The resulting construct contained 42 bp of 5′-untranslated sequence of the GCLc gene, the entire coding region (2157 bp) and 374 bp downstream from the GCLc gene stop codon. This 2573-bp GCLc fragment was subsequently excised from the EcoRI and Xbal sites, which flank the sequence, and introduced into corresponding sites in the polylinker of the p[UAST] vector.

For the construction of pP[UAST]-GCLm, the EcoRI-XhoI fragment of the GCLm gene was extracted from GH01757 cDNA clone (BDGP) and subcloned into the corresponding EcoRI-XhoI sites of the polylinker of p[UAST] vector. The 5′’-EcoRI and the 3′′-XhoI sites were both provided by the polylinker of pOT2A vector of the GH01757 clone. The resulting construct contained 77 bp of 5′′-untranslated sequence of the GCLm mRNA, the entire coding domain (857 bp) and 202 bp downstream from the stop codon.

Drosophila Strains, P-mediated Transformation, and Life Span Studies—The y w strain used in these studies contains the X chromosome markers yellow (y) and white (w) and has been maintained in this laboratory for >12 years. The Tub-GAL4, elav-GAL4, Appl-GAL4, and D42-GAL4 driver lines were kindly supplied by Blanka Rogina (University of Connecticut Health Science Center).

DNA preparations of pP[UAST]-GCLc and pP[UAST]-GCLm constructs were injected along with pTurbo plasmid (16) carrying transposase into Drosophila embryos of the y w recipient strain. Southern analysis and chromosome mapping were used to confirm the presence of single individual transgenes at distinct loci after microinjection. The collection of transgenic lines was further expanded using transient mobilization by the Δ2–3 element (17) following crosses with CyO/Spr, Sb Δ2–3/TM6, Ubx flies. All transgenic lines, including the driver lines, were back-crossed a minimum of six times to the same y w isolate, to ensure that the genetic backgrounds were equivalent. Experimental flies were then obtained from crosses between virgin females containing the GAL4 driver and males containing the UAS-GCLc or UAS-GCLm responder transgene. Control flies containing the GCLc transgene alone or the GAL4 driver alone were obtained by substituting the parental y w strain for either the driver or responder strain in parallel crosses. Life-span studies were performed as described previously (18).

Immunoblot Analysis—Antibodies were raised against recombinant GCLc and GCLm proteins. Fragments of GCLc and GCLm genes comprising the whole-length coding sequences were inserted in pProEx vector (Invitrogen) in-frame with histidine-6x sequence. Recombinant proteins were expressed in Escherichia coli strain DH5-α and purified by nickel-agarose chromatography. Purified proteins were sent to Covance Research Products (Denver, PA) for the preparation of rabbit antisera.

Immunoblot analysis was performed as described previously (19). Primary antibodies (0.2–0.5 mg/ml) were diluted 1:5,000 (GCLc) or 1:20,000 (GCLm). Anti-actin antibodies used as a loading control were purchased from MP Biomedicals (Aurora, OH). The secondary anti-bodies were horseradish peroxidase anti-rabbit and anti-mouse conjugates (Invitrogen and Amersham Biosciences, respectively).

HPLC-based Assays of GCL Enzyme Activity and Glutathione Content in Fly Homogenates—GCL enzyme activity was measured as described previously for houseflies (13). GSH present in the fly homogenates at the time of sacrifice was also quantified by HPLC, using a Shimadzu Class VP solvent delivery system and CoulArray 6500 electrochemical detector, as described previously (20).

Oxygen Consumption—Respiration rates were determined for three to five groups of 25 male flies per genotype, at ages 13–20 days, using an Oxzilla Dual Absolute and Differential Oxygen Analyzer (Sable Systems International, Las Vegas, NV). Flies in each group were placed in a respiration chamber with nearly the same dimensions as standard vials used for life-span studies. Air was passed through the chamber and baselining system in alternating 10-min intervals, with a flow rate of 50 ml/min and sampling interval of 2 s. The calculated rate of oxygen consumption for each vial of flies was the average of five to six consecutive chamber minus baseline estimations, after subtraction from oxygen concentration in a parallel reference channel.

Statistical Analysis—Enzyme activities (overexpressor versus control) were compared by unpaired Student’s t tests using Microsoft Excel software. Rates of oxygen consumption were compared by analysis of variance, with post hoc pairwise comparisons based on Tukey tests. The significance of differences between Kaplan-Meier survival curves was determined separately for each experimental combination and its corresponding driver-only and responder-only controls, using semiparametric log rank tests (SYSTAT 10 software). Additionally, the mean life spans and 90% mortality times of all genotypes were compared by analysis of variance, with planned, pairwise comparisons between GCLc overexpressors and the corresponding driver-only and responder-only controls.

RESULTS

Overexpression of GCLc and GCLm—Microinjection of the recombinant P element plasmids yielded 14 independent transformants carrying UAS-GCLc and 5 transformants carrying UAS-GCLm transgenes. Southern analysis was performed to select fly lines carrying unique insertions, which were mapped to specific chromosomes by segregation.
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For each UAS construct, three to four different transgenic lines with a single insertion were selected and back-crossed a minimum of six times into a reference y w background to ensure genetic homogeneity. The responder strains carrying the UAS-GCL transgenes were mated to the following driver strains: Tub-GAL4, which drives constitutive, broad-range expression, and elav-Gal4, Appl-GAL4, and D42-GAL4, which drive distinct patterns of neuronal expression.

Expression of GCLc and GCLm driven by Tub-GAL4 was increased by 4–6-fold and 60–100%, respectively, in comparison with controls, as revealed by immunoblot analysis (data not shown) and the GCL enzyme activity assay (Fig. 1). When neuronal drivers were used, only slight increases in GCL activity were noted in whole body homogenates (Fig. 1A); the same was true when GCL protein levels were determined by Western analysis. More pronounced differences in GCL content were detected in proteins extracted from heads, using either global or neuronal drivers of GCLc overexpression (Fig. 2). GCL enzyme activity was increased significantly in heads but not in the thoraces and abdomens of Appl/GCLc flies (Fig. 3).

Neuronal Overexpression of GCLc Extends Life Span—Comparisons of life spans between experimental and control groups were made using ~225 male flies per group. Experimental flies contained one of four distinct target UAS-GCLc transgenes and either the Tub-GAL4, Appl-GAL4, D42-GAL4, or elav-GAL4 driver. Controls for each driver/UAS-GCLc transgene combination included the UAS-GCLc transgene without the driver and the driver without its transgene UAS target.

The experimental groups overexpressing GCLc globally (UAS-GCLc with the ‘Tub-GAL4 driver) exhibited no significant increases in life span, in comparison with their respective controls. In the presence of Appl-GAL4 and D42-GAL4 neuronal drivers, however, there was a substantial, reproducible, and statistically significant increase in longevity (Fig. 4) (p < 0.01, based on log rank tests or comparisons of mean life spans). Positive effects were also observed with the elav-GAL4 driver, but these effects were consistently significant (p < 0.05) only for the elav/GCLc5 genotype. Both mean and maximum life spans (90% mortality) were increased by up to 50% (TABLES ONE and TWO); however, the effects on maximum life span were consistently significant only for two out of four genotypes for the Appl-GAL4 and D42-GAL4 drivers. Overall, the rank order of impact on life span was Appl-GAL4 > D42-GAL4 > elav-GAL4.

In contrast to the results obtained for GCLc, neuronal overexpression of the regulatory subunit of glutamate-cysteine ligase, GCLm, had no impact on longevity. However, when the Tub-GAL4 driver was used, a small but reproducible extension of life span was noted (up to 24% for mean life span; Fig. 5).

GSH Content—To determine whether or not overexpression of GCLc or GCLm resulted in an increased content of GSH, lysates from a subset of the transgenic flies were analyzed by HPLC. There was a statistically significant, 85–104% increase in GSH content in experimental flies containing the target, UAS-GCLc, together with the global driver, Tub-GAL4 (Fig. 6). Flies with the elav-GAL4/UAS-GCLc transgene combinations had small but significant increases in GSH levels (10–23%), reflecting the fact that neuronal tissue constitutes only a small fraction of the body weight. Consistent with this observation, GSH content was increased 70–118% in the heads but only 16–29% in the thoraces plus abdomens of Appl/GCLc flies (Fig. 7). In GCLm overexpressors containing the global Tub-GAL4 driver, the increases in GSH content ranged from 25–50%.
Oxygen Consumption and Resistance to Experimental Oxidative Stress—Rates of oxygen consumption were compared between flies containing both the Appl-GAL4 or elav-GAL4 driver and the UAS-GCLc6 responder transgene in combination, and controls containing either the driver or responder transgene alone. There was no significant difference between experimental and control groups for either driver (Fig. 8), suggesting that the extension of life span was not an indirect effect resulting from decreased respiration rates in the long-lived flies. Additionally, although there were no beneficial effects on stress resistance for any of the drivers at 10 days of age, 40-day-old Appl/GCLc and D42/GCLc flies exhibited increased resistance to hydrogen peroxide and paraquat (Figs. 9 and 10).

**DISCUSSION**

The main finding of this study was that overexpression of the catalytic subunit of glutamate-cysteine ligase, GCLc, in tissues of the CNS, extended the mean and maximum life spans of *D. melanogaster* by up to 50%, whereas global overexpression had no positive effect on survival times. In contrast, GCLm overexpression in the CNS had no effect on
The main issue raised by these findings is how overexpression of GCLc, specifically in the CNS, extends the life span of flies. The brain of aging Drosophila is known to undergo a number of degenerative changes, including vacuolization, decreased amounts of neuronal cytoplasm and ribosomes, and a progressive accumulation of age pigments, which may be products of mitochondrial degradation (21). Additionally, the level of oxidative stress in flies, indicated by rates of mitochondrial O$_2$/H$_2$O$_2$ generation, GSH:glutathione disulfide ratios, and amounts of macromolecular oxidative damage, increases with age (14, 20). In the present study, GCLc overexpression was found to enhance the amount of GSH, which has multiple functions in cells, acting as a reductant or a nucleophile for the detoxification of reactive intermediates. It can react directly with a variety of radical species, generating glutathionyl radical, which after subsequent reactions produces O$_2$, a substrate of superoxide dismutase (SOD) (22). Glutathione and SOD, acting in concert, are hypothesized to provide a crucial mechanism for the elimination of various reactive oxygen species, with O$_2$ acting as an intracellular radiocal “sink” (22). The existence of such a sink could explain why the results for GCL are similar to those obtained by overexpressing Cu,Zn-SOD in the nervous system, which also extends the life span of Drosophila (14).

### Table One

| Genotype                | Mean | % vs. +/responder | Mantel $p$ | % vs. Driver/+ | Mantel $p$ |
|------------------------|------|-------------------|------------|----------------|------------|
| yw;+/+                 | 48.3 |                   |            |                |            |
| yw;+/GCLc3            | 54.2 |                   |            |                |            |
| yw;+/GCLc5            | 53.3 |                   |            |                |            |
| yw;+/GCLc6            | 55.9 |                   |            |                |            |
| Tubulin/+              | 63.4 |                   |            |                |            |
| Tubulin/GCLc3         | 64.8 | 19.6              | <0.0005    | 2.3            | 0.579      |
| Tubulin/GCLc5         | 48.7 | −8.6              | 0.035      | −23.2          | <0.0005    |
| Tubulin/GCLc6         | 48.6 | −13.2             | <0.0005    | −23.4          | <0.0005    |
| elav/+                | 51.9 |                   |            |                |            |
| elav/GCLc3           | 64.4 | 18.8              | <0.0005    | 24.0           | <0.0005    |
| elav/GCLc5           | 74.5 | 39.8              | <0.0005    | 43.4           | <0.0005    |
| elav/GCLc6           | 49.2 | −12.0             | <0.0005    | −5.2           | 0.589      |
| D42/+                 | 54.8 |                   |            |                |            |
| D42/GCLc3            | 72.2 | 33.2              | <0.0005    | 31.8           | <0.0005    |
| D42/GCLc5            | 73.1 | 37.3              | <0.0005    | 33.5           | <0.0005    |
| D42/GCLc6            | 74.9 | 34.0              | <0.0005    | 36.7           | <0.0005    |
| Appl/+               | 61.1 |                   |            |                |            |
| Appl/GCLc3          | 74.5 | 37.3              | <0.0005    | 22.0           | <0.0005    |
| Appl/GCLc5          | 79.1 | 48.5              | <0.0005    | 29.5           | <0.0005    |
| Appl/GCLc6          | 83.5 | 49.3              | <0.0005    | 36.8           | <0.0005    |

### Table Two

| Parental Female Genotype | Parental Male Genotype | Mean Life Span |
|-------------------------|------------------------|----------------|
| yw;+/+                  | yw;+/+                 | 48.3           |
| yw;+/GCLc3             | yw;+/GCLc3             | 54.2           |
| yw;+/GCLc5             | yw;+/GCLc5             | 53.3           |
| yw;+/GCLc6             | yw;+/GCLc6             | 55.9           |
| Tubulin/+               | Tubulin/+               | 63.4           |
| Tubulin/GCLc3          | Tubulin/GCLc3          | 64.8           |
| Tubulin/GCLc5          | Tubulin/GCLc5          | 48.7           |
| Tubulin/GCLc6          | Tubulin/GCLc6          | 48.6           |
| elav/+                 | elav/+                 | 51.9           |
| elav/GCLc3            | elav/GCLc3            | 64.4           |
| elav/GCLc5            | elav/GCLc5            | 74.5           |
| elav/GCLc6            | elav/GCLc6            | 49.2           |
| D42/+                  | D42/+                  | 54.8           |
| D42/GCLc3             | D42/GCLc3             | 72.2           |
| D42/GCLc5             | D42/GCLc5             | 73.1           |
| D42/GCLc6             | D42/GCLc6             | 74.9           |
| Appl/+                 | Appl/+                 | 61.1           |
| Appl/GCLc3            | Appl/GCLc3            | 74.5           |
| Appl/GCLc5            | Appl/GCLc5            | 79.1           |
| Appl/GCLc6            | Appl/GCLc6            | 83.5           |

Life span, whereas global overexpression resulted in a moderate increase in longevity.

The mean life spans of GCLc transgenic flies is shown in Table One. The data indicate that overexpression of GCLc in the CNS significantly increases the life span of flies. The table shows the mean life spans of different genotypes and the percent changes in relation to the corresponding responder-only controls. The significance probabilities (p values) of the associated log rank tests are also provided.

The mean life spans of GCLc transgenic flies in replicate experiments are shown in Table Two. The table includes data from replicate experiments for each group, and the mean life span was determined for 189–230 flies in each replicate experiment.
The CNS of *D. melanogaster* may be relatively vulnerable to age-associated increases in oxidative stress, because it has a low content of Cu,Zn-SOD in comparison with other tissues (24). The absence of life extension resulting from global elevation of GSH content in GCLc transgenic flies raises the possibility that antioxidative defenses in other tissues are adequate and that adverse effects of overexpression outside the CNS may offset the beneficial effect in the CNS. Alternatively, the effect of GCL overexpression may be dose-dependent, with moderate increases in activity counteracting age-related oxidative stress, while dramatic increases interfere with redox-related signal transduction or protein folding. In support of this idea, the global increases in GCL activity and GSH content were much greater in Tub/GCLc than in elav/GCLc flies. Another possible explanation, that Tub/GCLc flies failed to overexpress GCLc sufficiently in the nervous system, is inconsistent with the finding that GCL protein content was similar in the heads of GCLc transgenic flies possessing global versus neuronal drivers. Thus, the greatest extension of life span appears to be achieved by overexpressing GCL at moderate rather than extremely high levels, and by targeting the overexpression of GCL to the CNS.

The differential, tissue-specific, life-extending effects of GCLc and GCLm overexpression may be due to differences in their roles in GSH biosynthesis, and variations in substrate abundance among tissues. The isolated *Drosophila* GCLc monomer exhibits catalytic activity (15), but the formation of reversible intermolecular disulfide bridges with GCLm decreases the $K_m$ for glutamate from 2.88 mM to 0.45 mM, and diminishes feedback inhibition by GSH. This difference in $K_m$ could account for the extension of life span by global overexpression of GCLm, because the concentration of glutamate in the *Drosophila* hemolymph is 0.9 mM (25), implying that the monomeric GCLc subunit functions less efficiently in GSH synthesis outside the CNS than the heterodimeric holoenzyme. Consistent with the hypothesis that maximal increases in...
GCL activity have adverse effects, the difference in $K_m$ for glutamate could also explain the lethal effect of simultaneous, global overexpression of both GCLc and GCLm during metamorphosis. Conversely, the absence of life extension resulting from neuronal overexpression of GCLm might be explained by tissue-specific differences in the abundance of glutamate or in the regulation of GCLc (e.g. GCLc mRNA is stabilized by nerve growth factor during oxidative stress (26)).

A noteworthy feature of the physiology of poikilotherms, such as insects, is the existence of an inverse relationship between longevity and metabolic rate (14). Experimental regimens that lower the rate of oxygen consumption frequently extend the life span. Thus, in the present study, it was deemed necessary to rule out such an indirect mechanism for life-span extension by the overexpression of GCL. Additionally, oxygen consumption was measured in vials with dimensions similar to those used for life-span studies, to ensure comparable levels of physical activity during the experiments measuring life span and metabolic rate. GCLc overexpression driven by Appl-GAL4 or elav-GAL4 had no effect on the rate of oxygen consumption. This finding excludes a simple metabolic trade-off as the cause of the increase in longevity. Furthermore, the extension of life span was observed in long-lived genetic backgrounds (control life spans were 50–63 days for both the neuronal driver and UAS-GCLc control flies), which strengthens the conclusion that an increased GSH biosynthetic capacity in the CNS has a robust effect on aging.

The present finding, that enhancement of the capacity for de novo GSH synthesis prolongs the life span of flies by up to 50%, has important ramifications for the oxidative stress hypothesis of aging. Although the validity of this hypothesis is not strictly dependent upon the relationship between antioxidant levels and longevity, an intuitive prediction is that bolstering antioxidant levels would increase longevity. Results of this study indicate that overexpression of GCLc in a long-lived background can extend the life span and increase resistance to oxidative stress, without affecting the metabolic rate. Notably, such benefit was observed only when tissues of the central nervous system exhibited an increased glutathione biosynthetic capability. This tissue-specific effect underscores the susceptibility of the nervous system to oxidative stress and strengthens the possibility that the spatially restricted overexpression of antioxidants could delay the aging process.

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