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Expression of *GFP* can influence aging and climbing ability in *Drosophila*

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ABSTRACT. *Green fluorescent protein* (*GFP*) is widely used as a reporter transgene in a variety of organisms. Some of the advantages of using *GFP* include non-invasive visualization of biological events and/or tissues in live specimens and its benign nature. When *GFP* is expressed throughout the organism, in neurons and eyes, lifespan and climbing ability of flies are significantly decreased compared to similar crosses with a *lacZ* reporter. Also, *GFP* expression can have subtle effects on eye morphology, with neural and ubiquitous expression. Since *GAL4/UAS* expression of *GFP* can influence aging and climbing ability in the *Drosophila* system of directed gene expression, we found that the latter of these advantages, namely its harmless, non-toxic nature, can be conditional, depending upon the mode of expression and the biological endpoint. We suggest that caution should be used when using *GFP* to visualize cellular events, especially in long-term assays.

Key words: *Drosophila melanogaster*; Green fluorescent protein (GFP); Longevity; Climbing

INTRODUCTION

The *green fluorescent protein* (*GFP*) gene, originally isolated from the jellyfish *Aequorea victoria*, has been a vital tool in biological research, particularly for genetic screening and analyses of gene expression in *Drosophila melanogaster*. Initial studies used *GFP* included in P-element constructs, to facilitate localization of proteins and to visualize cell events, such as movement of ribonucleoprotein particles in *Drosophila* embryos (reviewed by Wang and Hazelrigg, 1994 and Misteli and Spector, 1997). Another gene used in P-element fusions to visualize cell and genomic components is *lacZ*, a gene from *Escherichia coli*, which encodes ß-galactosidase (O'Kane and Gehring, 1987). Creation of both *UAS-GFP* (Yeh et al., 1995; Dickson, 1996) and *UAS-lacZ* reporter lines (Manseau et al., 1997) presented novel ways of investigating gene expression in a variety of animals by using the *GAL4/UAS* system to direct expression in specific cell or tissue types (reviewed by Chalfie et al., 1994; Rosochacki and Matejczyk, 2002, and Zhan and Gong, 2010). These transgenes have been used as controls to examine effects of gene expression.

Advantages of using the *GAL4/UAS* system with the *GFP* reporter include being able to work with living cells with little or no need for sample preparation (reviewed by Misteli and Spector, 1997 and Duffy, 2002). While either *GFP* or *lacZ* can be chosen as the reporter gene in certain assays, *GFP* allows researchers to visualize expression patterns non-invasively and continuously in live specimens, unlike when *lacZ*/X-Gal staining is used (Yeh et al., 1995; Phelps and Brand, 1998). It is believed that both *GFP* and *lacZ* expressions are non-toxic and do not influence endogenous gene expression at any stage during fly development (Bier et al., 1989; Chalfie et al., 1994). Despite this, some studies have suggested that *GFP* affects cell biology. Cells with *GFP*-containing constructs that localize *GFP* in the nucleus tend to be more damaged, possibly due to free-radical generation and damage to DNA (Misteli and Spector, 1997). In analysis of zebrafish development, expression of *GFP* is delayed and a longer recovery period is required before target genes are activated (Zhan and Gong, 2010). The subtle consequences of *GFP* expression are of considerable interest to a wide range of biologists.

In addition to these effects of *GFP*, *GAL4* has been shown to affect cell biology in flies. Effects on ommatidia development have been observed and it was established that these altered phenotypes are caused by cell death in the eye (Freeman, 1996; Kramer and Staveley, 2003). In addition, *elav-GAL4* reduces longevity (Todd AM and Staveley BE, unpublished results). Examination of transgenes used for driving reporter expression is another route for research.

We find that *GFP* expression promoted by select *GAL4* transgenes can have adverse effects upon morphology and behavior in flies, in particular lifespan and climbing ability, when compared to similar population of flies expressing the *lacZ* transgene.

MATERIAL AND METHODS

Fly stocks and crosses

All *Drosophila* stocks were obtained from the Bloomington *Drosophila* Stock Centre (University of Indiana, Bloomington). The two responder fly lines used were *w; UAS-lacZ*$^{4\text{-}1\text{-}2}$ (Brand et al., 1994) and *UAS-GFP* (Dickson, 1996), each crossed with one of the following *Gal4* transgenes, *da-GAL4* (Wodarz et al., 1995), *GMR-GAL4*^{12} (Freeman, 1996), *y w; act-GAL4/CyO, y*$^{+}$ (Brand and Perrimon, 1993) and *w; P[GawB]*$^{l(3)31\text{-}1}$*/TM6C* (Ito et al., 1997).

Drosophila media and culture

Crosses were set up using four to five virgin females each of *UAS-lacZ* and *UAS-GFP*, mated to each of the drivers (*da-GAL4*, *GMR-GAL4*, *act-GAL4/CyO*, y^+ and *P[GawB]*$^{l(3)31-1}$*/TM6C*). All flies were maintained on standard cornmeal-yeast-agar media at 25°C. Parental generation was transferred to new food after two days, and again two days after to increase the number of progeny. Critical class males were determined as those not presenting the balancer marker phenotypes of *Tubby* (*TM6C*), *Stubble* (*TM6C*), and *Curly* (*CyO*).

Scanning electron microscopy

Critical class male flies were isolated under CO_2 and aged on standard cornmeal-yeast-molasses-agar media for three days, placed in 1.5-mL microcentrifuge tubes and frozen at -80°C.

These animals were mounted on aluminum scanning electron microscope (SEM) studs using double-sided sticky tape, with left eye facing up for eye SEMs and ventral side down with legs removed and wings spread to the sides for dorsal notum SEMs. Progeny that inherit the transgenes *da-GAL4* and *P[GawB]*$^{l(3)31-1}$ underwent dorsal notum analysis. The mounted flies were desiccated overnight and gold-coated using Electron Microscopy Science 500 Sputter Coater. Eyes were photographed at 150X magnification and dorsal nota were photographed at 80X magnification, using a Hitachi S-570 SEM.

Micrographs were analyzed using the ImageJ digital image analysis software (Abramoff et al., 2004). For the eye SEMs, the number of ommatidia was counted in each image (N = 10 per cross). The area (μm^2) of seven ommatidia (in a florette pattern) was determined for 10 sets, and the average area per ommatidium was calculated. Finally, overall eye area was measured. For dorsal notum analysis, the number of microchaetae on the dorsal notum was counted in each image (N = 25 to 30 counts per genotype). The total area (mm^2) of the dorsal notum was also measured. Microchaete counts and total area were used to calculate the bristle density, expressed as number of microchaetae per mm^2. All SEM data were exported into GraphPad Prism 5 and mean ± standard error of means was plotted for each characteristic for each genotype. Statistical analyses were conducted using ANOVA, followed by Newman-Keuls post-tests to determine significance between pairs. Significance was considered at $P < 0.05$.

Longevity assay

Two hundred critical class males of each genotype were collected using gaseous carbon dioxide and kept on standard cornmeal-yeast-molasses-agar media at 25°C. Flies were maintained in non-crowded conditions of 1 to 20 flies per vial. Flies were transferred to fresh media every two days (Staveley et al., 1990) and scored for the presence of dead adults. Survival data was transferred to the GraphPad Prism 5 program and were compared using ANOVA, followed by Newman-Keuls post-tests. Significance was considered to be 95%, with $P < 0.05$.

Time to eclosion (developmental assay)

Virgin females *UAS-lacZ* and *UAS-GFP* and males of each driver line were collected and aged on standard cornmeal-yeast-molasses-agar media for three to four days before mating.

Each cross was set up as described above in *Drosophila* culture, using three to four virgin females and two to three males. Flies were kept on the fresh food media for 6 h before being transferred to fresh media and kept another 6 h. This was repeated four times, with several vials per cross.

The number of eclosed adults for each mating was recorded and the percentage eclosion calculated using:

$$\% \text{ eclosed} = n/N * 100\%$$

where *n* is the number of eclosed adults for a cross on a given day, and *N* is the total number of adults eclosed. Both male and female critical class flies were counted. Data was exported to and analyzed in GraphPad Prism 5.

Climbing assay

Approximately 200 critical class males of each cross were assayed for climbing ability according to a protocol developed in our laboratory (Todd and Staveley, 2004). Flies were assayed and maintained in groups of 10. Flies were kept on standard cornmeal-yeast-molasses-agar media at 25°C and assayed every seven days. Climbing ability was determined using a glass tube apparatus, 30 cm in length, 1.5 cm in diameter, marked with five 2-cm sections with a buffer section at the top to limit interference between climbing flies. Climbing ability was scored after 10 s based on which of the five marked sections had been reached. Flies were scored 10 times per trial, and the climbing index calculated using:

$$\text{Climbing index} = \Sigma (nm)/N$$

where *n* is the number of flies at a given section, *m* is the section number (1-5) and *N* is the total number of flies that climbed in that trial. Data were exported to GraphPad Prism 5 (GraphPad Software, Inc.) and each climbing index was subtracted from five and a non-linear regression curve was fit to each set of data. Slopes of curves were compared with 95% confidence intervals.

RESULTS

Biometric analyses

The development of ommatidia in the fly eye and microchaetae on the dorsal notum are two tightly controlled processes. Any interruption in these can produce phenotypic changes, which can be examined using biometric analysis. Scanning electron micrographs of the eyes and dorsal nota of flies expressing *lacZ* and *GFP* in different patterns revealed alterations in development.

Ubiquitous expression of *GFP* affects ommatidium size and microchaete density

Examination of ubiquitous expression of *lacZ* and *GFP* was conducted using the transgenes *da-GAL4* and *act-GAL4*, which have been characterized to drive expression at low and high levels, respectively. Analyses of eye SEMs show that *GFP* expression does not greatly affect overall eye development. When directed in the pattern of *da-GAL4*, ommatidium number

(747 ± 4) and eye area (115,154 ± 1714 µm^2) were not unlike that seen for *lacZ*-expressing flies (745 ± 10; 114,234 ± 2532 µm^2) (Figure 1A). Ommatidium area was significantly larger in *UAS-GFP*; *da-GAL4* flies (216.6 ± 2.62 µm^2) compared to *UAS-lacZ*; *da-GAL4* (198.5 ± 1.17 µm^2) (Figure 1A, iv; Table 1). When directed by the *act-GAL4* driver, *lacZ* and *GFP* expression did not affect eye area or ommatidium number or size (Figure 1B; Table 1). Ubiquitous expression of these two reporters had no significant effect on eye development. However, in the dorsal notum, low-level ubiquitous expression did affect microchaete density. The density of small bristles in *UAS-GFP*; *da-GAL4* flies was significantly less (583.6 ± 10.9 mm^2) than *UAS-lacZ*; *da-GAL4* flies (667.1 ± 7.96 mm^2) (Figure 2A; Table 2). The expression of *lacZ* and *GFP* genes had variable effects on the morphology of flies, as observed in the eye and dorsal notum.

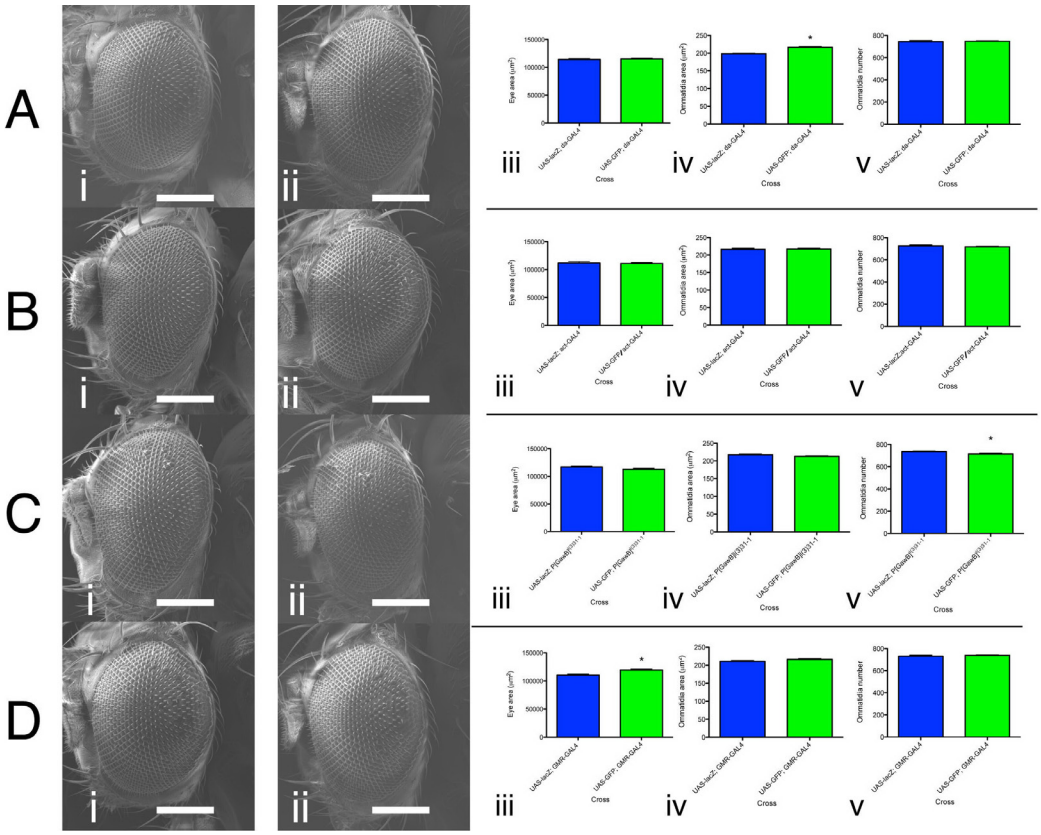

Figure 1. Biometric analyses of eye development influenced by *GFP* expression under the control of various transgenes. Scanning electron micrographs of left eyes from male flies. Scale bar (white) represents 160 µm. Genotypes are as follows: **A.** i = *UAS-lacZ/da-GAL4*; ii = *UAS-GFP/da-GAL4*. **B.** i = *UAS-lacZ/act-GAL4*; ii = *UAS-GFP/act-GAL4*; **C.** i = *UAS-lacZ;P[GawB]$^{l(3)31\text{-}1}$*; ii = *UAS-GFP*; *P[GawB]$^{l(3)31\text{-}1}$*. **D.** i = *UAS-lacZ/GMR-GAL4*; ii = *UAS-GFP/GMR-GAL4*. Graphic representations of scanning electron micrograph eye analyses from *da-GAL4* flies (A, iii-v), *act-GAL4* flies (B, iii-v), *P[GawB]$^{l(3)31\text{-}1}$* flies (C, iii-v), and *GMR-GAL4* flies (D, iii-v). *UAS-lacZ* flies are presented in blue and *UAS-GFP* flies are presented in green. Number of eyes analyzed from each cross (N = 10). Graphic representations of eye area, ommatidium area and number are shown in iii, iv and v, respectively. Values are reported as means ± SEM. *Represents significant difference calculated by Newman-Keuls post-tests.

Table 1. Summary of eye scanning electron micrograph analyses: effect of *GFP vs lacZ* expression upon morphology.

Genotype	Eye area (μm²)	Ommatida area (μm²)	Ommatida #
Low-level ubiquitous			
UAS-GFP; *da-GAL4*	115,154 ± 1714	217 ± 3 ↑	748 ± 4
UAS-lacZ; *da-GAL4*	111,966 ± 2400	198 ± 1	745 ± 10
High-level			
UAS-GFP; *act-GAL4*	111,078 ± 1716	217 ± 2	739 ± 5
UAS-lacZ; *act-GAL4*	111,873 ± 2582	216 ± 3	726 ± 8
Neurons and neuroblasts			
UAS-GFP; *P[GawB]*$^{l(3)31\text{-}1}$	113,069 ± 1579	213 ± 2	715 ± 7 ↓
UAS-lacZ; *P[GawB]*$^{l(3)31\text{-}1}$	117,111 ± 1594	217 ± 2	736 ± 5
Eye			
UAS-GFP; *GMR-GAL4*	111,935 ± 1854 ↑	216 ± 2	718 ± 4
UAS-lacZ; *GMR-GAL4*	110,469 ± 1723	211 ± 2	731 ± 10

Values are reported as means ± SEM. Arrows indicate increase (↑) or decrease (↓) in treatment, and significance is denoted by the P value. Unless otherwise noted, there is no significant difference.

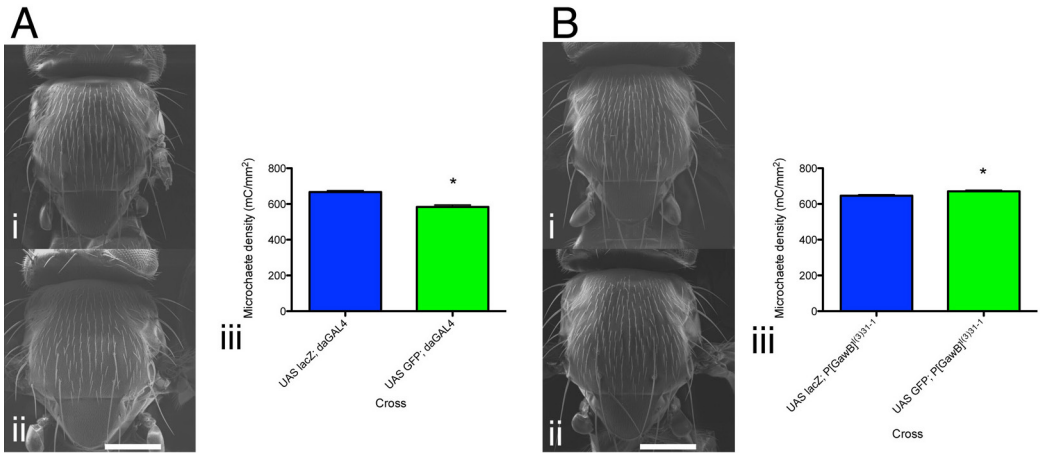

Figure 2. Biometric analysis of bristle density: *GFP vs lacZ* expression under the control of the ubiquitous transgene *da-GAL4* and the neural transgene *P[GawB]*$^{l(3)31\text{-}1}$. **A.** i-iii = Ubiquitous *GFP* expression (N = 27) significantly decreases microchaete density compared to the expression of *lacZ* (N = 29). **B.** i-iii = Neural *GFP* expression under *P[GawB]*$^{l(3)31\text{-}1}$ (N = 30) significantly increases microchaete density compared to *lacZ* (N = 29). *UAS-lacZ* flies are presented in blue and *UAS-GFP* is presented in green. Scanning electron micrographs are shown in A (i and ii) and B (i and ii). Scale bar (white) represents 0.30 mm. Graphic representation of microchaete density is shown in iii. Values are reported as means ± SEM. *Represents significant difference by Newman-Keuls post-tests. Genotypes are as follows: **A.** i = *UAS-lacZ/da-GAL4*; ii = *UAS-GFP/da-GAL4*. **B.** i = *UAS-lacZ*; *P[GawB]*$^{l(3)31\text{-}1}$; ii = *UAS-GFP*; *P[GawB]*$^{l(3)31\text{-}1}$.

Table 2. Summary of microchaete density on dorsal notum of flies with ubiquitous or neural *GFP* expression.

Genotype	Microchaete density (mC/mm²)	P value (compared with UAS-lacZ cross)	Significant
Low-level ubiquitous			
UAS-GFP; *da-GAL4*	584 ± 11	<0.0001	Yes ↓
UAS-lacZ; *da-GAL4*	667 ± 8		
Neurons and neuroblasts			
UAS-GFP; *P[GawB]*$^{l(3)31\text{-}1}$	671 ± 6	<0.0001	Yes ↑
UAS-lacZ; *P[GawB]*$^{l(3)31\text{-}1}$	646 ± 5		

Values are reported as means ± SEM. Arrows indicate increase (↑) or decrease (↓) in treatment.

Neural *GFP* expression affects ommatidium number and microchaete density

Analyses of neural expression of *lacZ* and *GFP* were accomplished by using *P[GawB]*$^{l(3)31-1}$, which drives gene expression in the neurons and neuroblasts. Expression of *GFP* via this driver did not greatly affect eye morphology compared to *lacZ*-expressing flies. The ommatidium number in *UAS-GFP*; *P[GawB]*$^{l(3)31-1}$ flies (714 ± 7) was significantly less than in *UAS-lacZ*; *P[GawB]*$^{l(3)31-1}$ flies (736 ± 5) (Figure 1C, v); however, ommatidium and eye areas were not different (Figure 1C, iii-iv). Microchaete density varied with neural expression of *GFP* compared to *lacZ*. Expression via the *P[GawB]*$^{l(3)31-1}$ driver produced a higher microchaete density in *GFP*-expressing flies (671.0 ± 5.70 mm^2) versus *lacZ*-expressing flies (646.5 ± 5.18 mm^2) (Figure 2B; Table 2). Neural driver coupled with *GFP* expression altered some aspects of morphology compared to *lacZ* expression.

GFP expression in the eye increases overall eye area

Directed expression of *lacZ* and *GFP* was done using the *GMR-GAL4* driver. This driver pushes gene expression posterior to the morphogenetic furrow, post-cell determination (Baker, 2001). *GFP* expression did not affect ommatidium number or size but did significantly increase overall eye area (111,935 ± 1854 mm^2) compared to *lacZ* (110,469 ± 1723 mm^2) (Figure 1D, Table 1). *GFP* expression seemed to affect eye development slightly when expression was driven in the eye, specifically.

Behavioral analyses

Behavioral analyses can show wide-ranging effects of gene expression. We performed a few assays that measured various aspects of development, including longevity, development time and climbing ability. We find that *GFP* expression significantly alters fly behavior compared to *lacZ* counterparts.

Ubiquitous *GFP* expression affects longevity and climbing ability and can delay development time

Ubiquitous expression of *GFP* was again completed using the transgenes *da-GAL4* and *act-GAL4*, driving low and high expression, respectively. In both cases, longevity of *UAS-GFP* flies was decreased (Figure 3A and B). The median life span of flies from these crosses was 58 days, 10-12 days shorter than *lacZ* flies, and the maximum life span varied from 78 (*UAS-GF*; *da-GAL4*) to 82 days (*UAS-GFP/act-GAL4*) (Table 3). Although flies with the *da-GAL4* driver eclosed at the same time as the control (Figure 4A), development time differed significantly when *GFP* was expressed with the *act-GAL4* driver (Figure 4B). About 50% of flies from this cross eclosed one day earlier than the *lacZ* flies (at 10 days versus 11). Climbing tests were conducted over several weeks and analyses were stopped when 75% of the initial number of flies had died. Expression of *UAS-GFP* using *act-GAL4* decreases climbing ability over time. The climbing indices for *UAS-GFP*; *da-GAL4* flies remain similar to *UAS-lacZ*; *da-GAL4* flies (Figure 5A and B). The climbing ability of *UAS-GFP/act-GAL4* flies, however, was significantly lower than that of the *lacZ* control (Figure 5B). Overall, *GFP* expression

throughout the organism had variable effects on eclosion time and climbing ability, and severely decreased longevity.

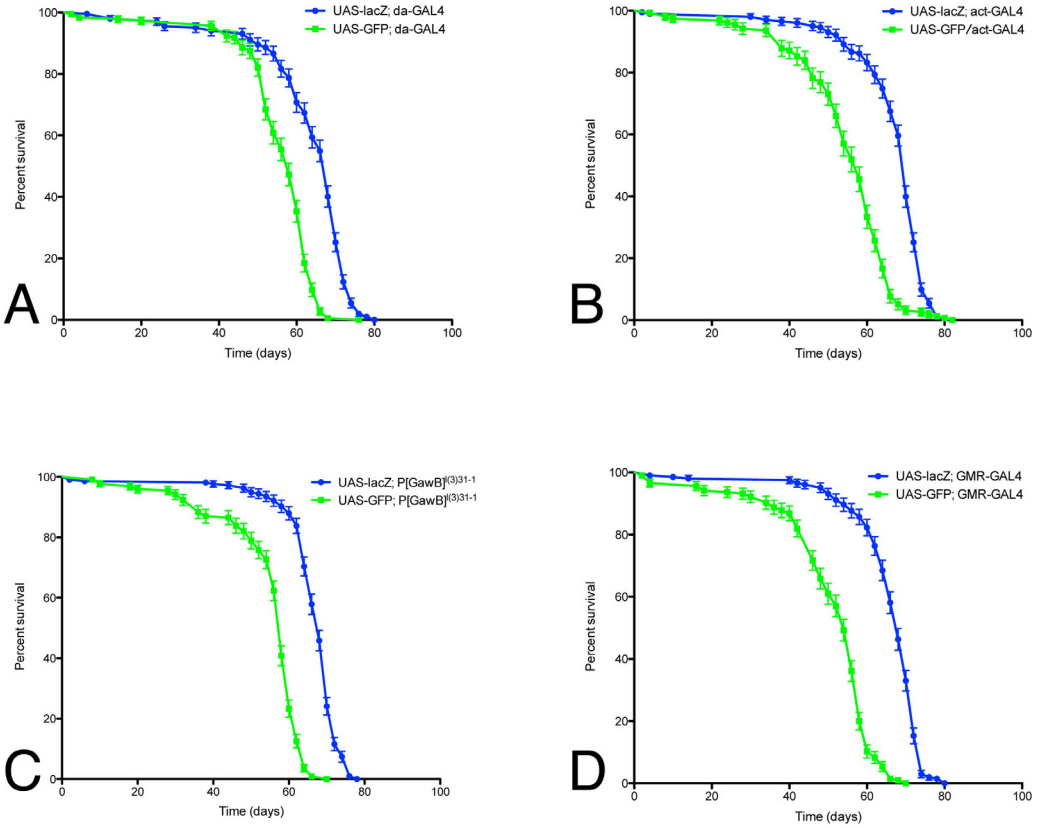

Figure 3. Behavioral analysis: effect of *GFP* expression throughout the organism, in the neurons and in the eye upon longevity. *GFP* expression significantly decreases lifespan in flies. Graphic representations of longevity for ubiquitous expression: **A.** *da-GAL4* and **B.** *act-GAL4* drivers; neural expression: **C.** *P[GawB]*$^{l(3)31-1}$, and eye expression: **D.** *GMR-GAL4*. Longevity is shown as percent survival ($P < 0.05$, determined by log-rank). Error bars = SEM.

Table 3. Analysis of longevity of flies with directed expression of *GFP* compared to *lacZ*.

Genotype	Initial number of flies analyzed (N)	Median survival day (50%)	Maximum lifespan (day)	P value (compared with *UAS-lacZ* cross)	Significant
Low-level					
UAS-GFP; *da-GAL4*	184	58	78	<0.0001	Yes ↓
UAS-lacZ; *da-GAL4*	202	68	80		
High-level					
UAS-GFP; *act-GAL4*	156	58	82	<0.0001	Yes ↓
UAS-lacZ; *act-GAL4*	203	70	82		
Neurons and neuroblasts					
UAS-GFP; *P[GawB]*$^{l(3)31-1}$	223	58	70	<0.0001	Yes ↓
UAS-lacZ; *P[GawB]*$^{l(3)31-1}$	216	68	78		
Eye					
UAS-GFP; *GMR-GAL4*	205	54	70	<0.0001	Yes ↓
UAS-lacZ; *GMR-GAL4*	203	68	80		

Survival curves analyzed using the log-rank test. Arrows indicate decrease (↓) in treatment.

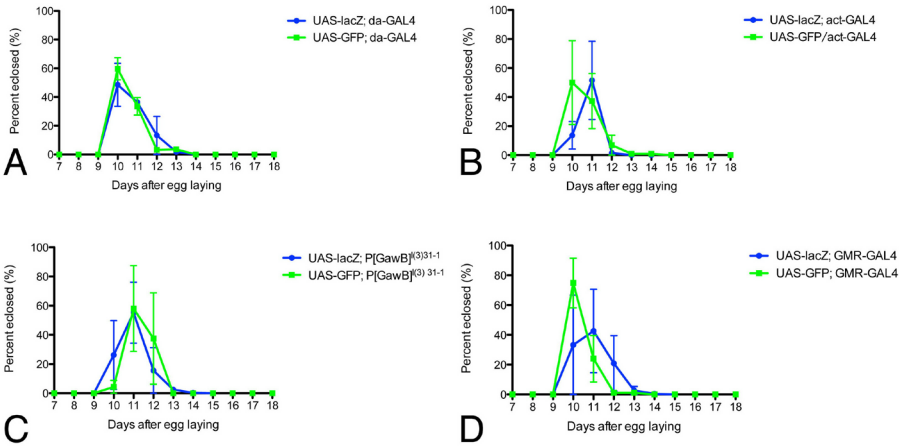

Figure 4. Behavioral analysis: effect of *GFP* expression throughout the organism, in the neurons and in the eye upon development (eclosion) time. *GFP* expression significantly does not have a significant effect on eclosion time in flies. Graphic representations of eclosion time (development time) for ubiquitous expression: **A.** *da-GAL4* and **B.** *act-GAL4* drivers; neural expression: **C.** *P[GawB]$^{l(3)31-1}$*, and eye expression: **D.** *GMR-GAL4*. Percentage of total flies eclosed each day is shown. Error bars = SEM.

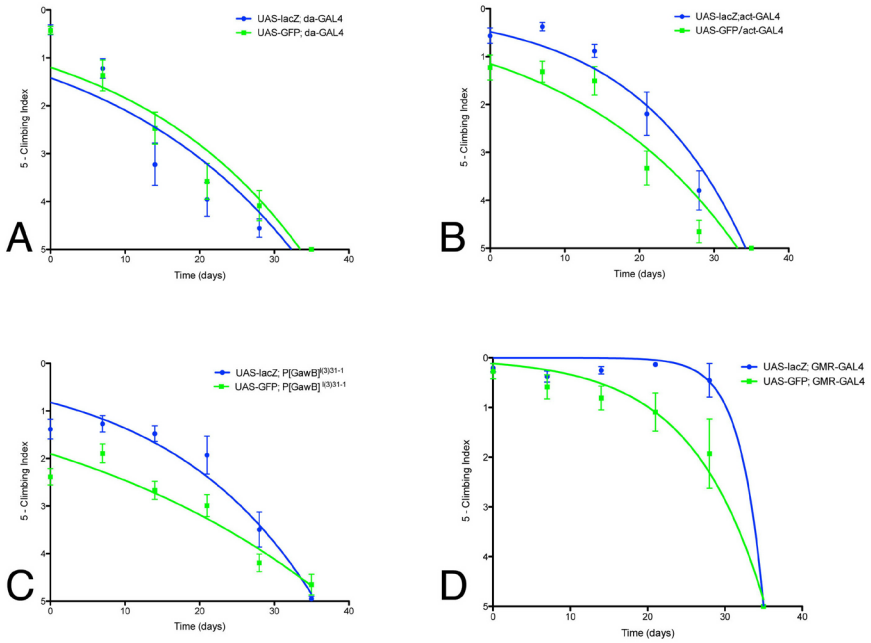

Figure 5. Behavioral analysis: effect of *GFP* expression throughout the organism, in the neurons and in the eye upon climbing ability. *GFP* expression produces flies with significantly decreased climbing ability when driven by the ubiquitous transgene *act-GAL4*, the neural transgene *P[GawB]$^{l(3)31-1}$* and eye-specific transgene *GMR-GAL4*. Graphic representations of longevity for ubiquitous expression: **A.** *da-GAL4* and **B.** *act-GAL4* drivers; neural expression: **C.** *P[GawB]$^{l(3)31-1}$*, and eye expression: **D.** *GMR-GAL4*. Climbing ability was determined via non-linear curve fit (CI = 95%). Error bars = SEM.

Expression of *GFP* in the brain and neurons affects longevity and climbing ability

We found that neural *GFP* expression can affect longevity and locomotor ability in flies. Neural expression was achieved using the *P[GawB]*$^{l(3)31\text{-}1}$ driver. Longevity studies of these flies showed significantly decreased life span in *GFP*-expressing flies (Figure 3C). The median life span was 58 days for *UAS-GFP*; *P[GawB]*$^{l(3)31\text{-}1}$ flies compared to *UAS-lacZ*; *P[GawB]*$^{l(3)31\text{-}1}$ flies with 68 days (Table 3). Maximum life spans for *GFP* and *lacZ* crosses did not differ significantly. Neural *GFP* expression did not affect eclosion time as *GFP* flies eclosed on the same day as *lacZ* flies - 12 days (Figure 4C). Expression of *GFP* produced flies with poor climbing ability when expressed in the neurons and neuroblasts (Figure 5C). Clearly, the directed neural expression of *GFP* affected the behavior of flies when compared to controls expressing *UAS-lacZ*.

GFP expression in the eye affects longevity, development time and climbing ability

Directed *GFP* expression in the eye was accomplished using the *GMR-GAL4* driver. Longevity of *UAS-GFP*; *GMR-GAL4* flies was significantly decreased compared to the control (Figure 3D). The median life span was shorter, 54 days compared to *UAS-lacZ*; *GMR-GAL4* with 68 days, and the maximum life span 70 to 80 days (Table 3). Development time of *UAS-GFP*; *GMR-GAL4* flies was shorter by one day (10 days) compared to the control (11 days) (Figure 4D). *GFP* expression in the eye also produced poor climbing ability (Figure 5D). *GFP* expression in the eye significantly affected behavior in these fruit flies.

DISCUSSION

The *GFP* gene is commonly used as a marker that allows visualization of tissues and biological events during development. The use of *GFP* has expanded into other species, including mice and zebrafish (Nowotschin et al., 2009; Zhan and Gong, 2010). Siegmund and Korge (2001) used *GFP* expression to visualize the ring gland in the *Drosophila* brain. *UAS-GFP*-bearing flies have been used in various assays, as it is believed that *GFP* expression has no physiological effect in flies. We demonstrated that this is not always the case. While *GFP* expression is not shown to affect physical characteristics significantly, such as in the eye and dorsal notum, *GFP* expression can affect physiological phenotypes.

Scanning electron micrograph analyses of the eye did not reveal many significant differences when compared to the *lacZ*-expressing flies. Slight variations in eye area, ommatidium area or count were observed with some of the trangenes. However, the nature of these differences seems minor, suggesting there is no serious effect of *GFP* expression on development or neurogenesis in the eye. Examination of bristle density can be a good indicator of neurogenesis. A recent study, which investigated the role of *Hip1* in neurogenesis (Moores et al., 2008), found that analysis of microchaete density is a sensitive assay that can assess subtle changes to cell signaling. Here we found that *GFP* expression under the influence of *da-GAL4* and *P[GawB]*$^{l(3)31\text{-}1}$ transgenes can affect bristle density, indicating that *GFP* may influence the cell signaling patterns important in bristle development and neurogenesis. These SEM data indicate that use of *GFP* in assays with certain transgenes may need to be carefully evaluated to ensure that *GFP* expression has not affected events during development, producing altered phenotypes.

Expression of *GFP* may only slightly affect neuronal development and eye and dorsal notum morphology. However, its expression significantly decreases longevity and climbing ability when directed in neural tissues and throughout the organism. The influence upon longevity may greatly influence continual developmental experiments, by introducing bias. Expression of *GFP* affects the condition of the flies over the long-term, resulting in poor climbing ability. This suggests that analyses of long-term locomotor assays (reviewed by Iliadi and Boulianne, 2010) or muscle development in flies may be affected when using *GFP* as a marker (Soler et al., 2004). If *GFP* influences fly health enough over time to not only decrease longevity but also locomotor (climbing) ability, its use in long-term development assays may be problematic, when expression is directed with the drivers that we used here.

Perhaps controlling the initiation of *GFP* expression, as with heat-shock promoters, would reduce the possible effects on development, allowing analysis in longitudinal assays (Grover et al., 2008). Caution should be taken when *GFP* is used in long-term experiments with any drivers.

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