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Effects of *α-synuclein* expression in the developing *Drosophila* eye.

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Introduction

As the fly brain has over 300,000 neurons and is organized into specialized areas for learning, olfaction, vision and memory (Wolf and Herbelein, 2003; Cauchi and Heuvel, 2006; Hardaway, 2010), *Drosophila* has become an important organism in which to model human neurodegenerative disorders. Furthermore, the *Drosophila* eye is tolerant to genetic manipulations and is dispensable for the survival of the fly (Chan and Bonini, 2000; Celotto and Palladino, 2005; Jeibman and Paulus, 2009). The directed expression of *α-synuclein* results in flies that are viable, accumulate aggregated α-synuclein in perinuclear and neuritic filamentous inclusions similar to Lewy bodies and Lewy neurites, age–dependent loss of dorsomedial DA neurons, neuronal degeneration, age-dependent loss of climbing ability, retinal degeneration (Feany and Bender, 2000; Auluck *et al.*, 2002), and ommatidial degeneration (Todd and Staveley, 2008). Using the bipartite UAS/GAL4 system (Brand and Perrimon, 1993) to overexpress *α-synuclein* in eyes of *Drosophila melanogaster* and performed biometric analysis, we investigated the possibility that developmental phenotypes become more severe with increased expression of α-synuclein.

Materials and Methods

Drosophila *stock and culture*

Dr. M. Feany of Harvard Medical School generously provided *UAS-α-synuclein* flies (Feany and Bender, 2000). The *GMR-GAL4*^{12} (Freeman, 1996) and *UAS-lacZ* were obtained from the Bloomington Drosophila Stock Center at Indiana University. The *GMR-GAL4 UAS-α-synuclein/CyO* line was generated using standard recombination, tested via PCR, and used to overexpress *α-synuclein* in the developing eye in the Glass Multiple Reporter (*GMR*) pattern. Stocks and crosses were maintained on standard medium containing cornmeal, molasses, yeast, and agar. Stocks were

kept at room temperature (22 ± 2°C) while crosses and experiments were carried out at 29°C.

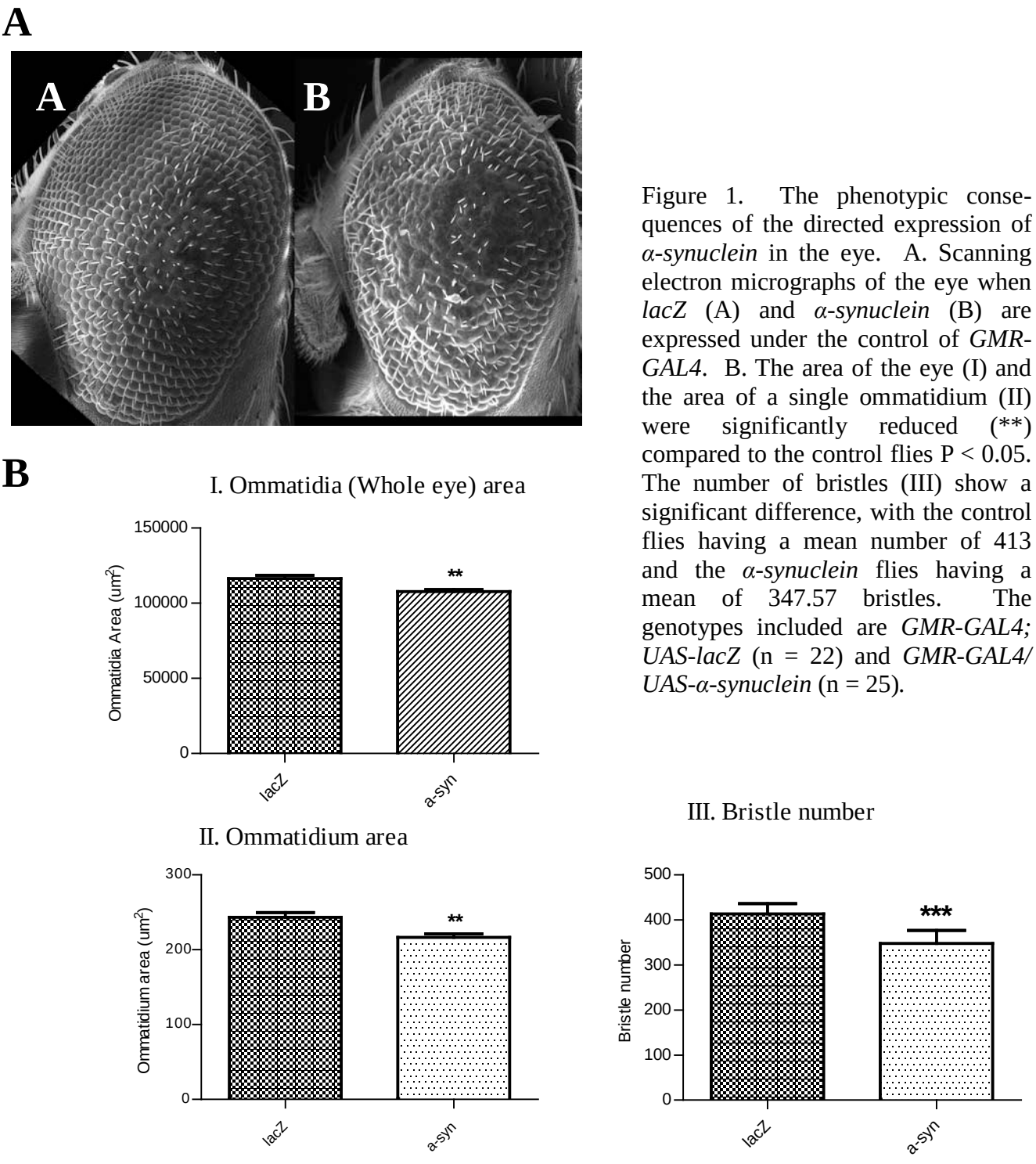


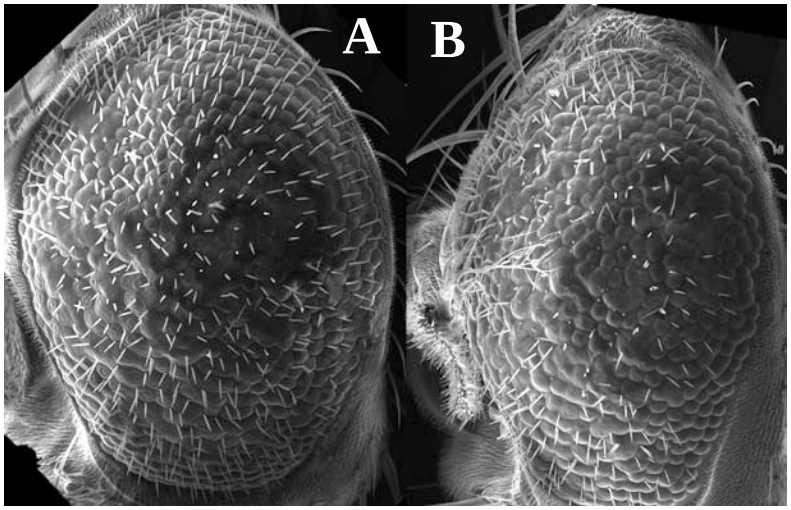
Figure 1. The phenotypic consequences of the directed expression of *α-synuclein* in the eye. A. Scanning electron micrographs of the eye when *lacZ* (A) and *α-synuclein* (B) are expressed under the control of *GMR-GAL4*. B. The area of the eye (I) and the area of a single ommatidium (II) were significantly reduced (**) compared to the control flies $P < 0.05$. The number of bristles (III) show a significant difference, with the control flies having a mean number of 413 and the *α-synuclein* flies having a mean of 347.57 bristles. The genotypes included are *GMR-GAL4; UAS-lacZ* (n = 22) and *GMR-GAL4/ UAS-α-synuclein* (n = 25).

Biometric analysis of the Drosophila *eye*

Several single vial matings of three to five females plus three to five males were made of each genotype at 29° C and a cohort of adult heterozygous male flies collected upon eclosion and aged for three days on standard cornmeal-yeast-molasses-agar before being frozen at -80° C. Whole flies were

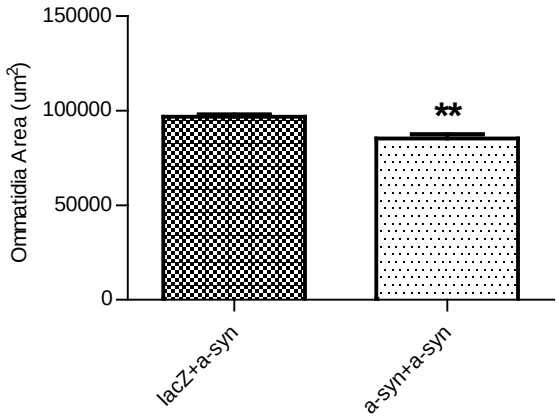
mounted on SEM studs, desiccated overnight and coated in gold prior to photography at 170× magnification with a Hitachi S-570 scanning electron microscope was done. For each cross at least 20 eye images were analysed using the NIH ImageJ software (Abramoff *et al.*, 2004) and biometric analysis performed. The ratio of the area of disruption was calculated from the total area of the eye divided by the total disrupted area. Disrupted area was considered as an area occupying two to three fused ommatidia.

A

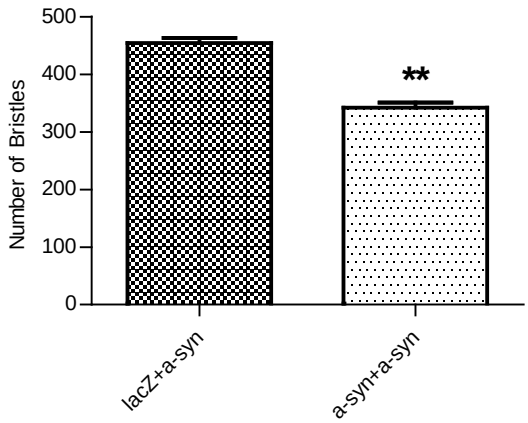


B

I. Ommatidia (Whole eye) area



II. Bristle number



III. Ratio of disrupted area of eye

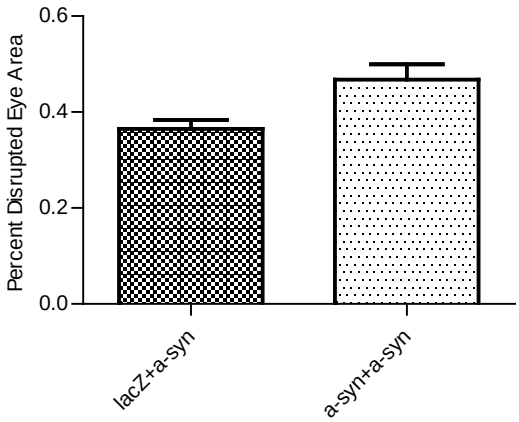


Figure 2. The consequences of the directed expression of an additional copy of *α-synuclein* in the eye. A. Scanning electron micrographs of both the control flies *lacZ+a-syn* (A), overexpressing a single copy of *α-synuclein* plus a copy of *lacZ* (as a control) and *α-synuclein* flies (B), overexpressing two copies of *α-synuclein (a-syn+a-syn).* Panel B; Biometric analysis of the eyes showing significance (*) for the whole area of the eye (I), the bristle number (II), and the ratio of disrupted eye area (III) when compared to the control flies ($P < 0.05$). The genotypes were *GMR-GAL4 UAS-α-synuclein/UAS-lacZ* (n = 24) and *GMR-GAL4 UAS-α-synuclein/UAS-α-synuclein* (n = 23).

Results and Discussion

Eye development in *Drosophila* is very precise with the maturation of each ommatidium and the organization of the ommatidial array being tightly controlled (Thomas and Wassarman, 1999). We expressed *α-synuclein* in the eyes using the eye specific driver *GMR-GAL4* crossed to the responding transgene, and secondly we expressed higher levels of *α-synuclein* in the eye using the *GMR-GAL4 UAS-α-synuclein* recombinant line. Analysis of SEMs of flies overexpressing a single copy of *α-synuclein* (n = 25) compared to the control flies (n = 22) overexpressing *lacZ* revealed differences in eye development, but notable was a slight decrease in the overall area of the eye of *α-synuclein* flies. The area of the eye (107802 ± 1311; 116459 ± 2153) (Figure 1-BI) and the area of a single ommatidium (216.6 ± 4.826; 243.2 ± 6.332) (Figure 1-BII) were slightly reduced for *α-synuclein* flies when compared to the control flies $P < 0.05$. The number of interommatidial bristles were significantly reduced (Figure 1-BIII), with the control flies having a mean number of 413 ± 22.92 and the *α-synuclein* flies having a mean of 347.57 ± 28.99 bristles.

We compared the SEMs of flies that developed while expressing one copy of *α-synuclein* plus one copy of *lacZ* (n = 24) to those of flies that were expressing two copies of *α-synuclein* (n = 23). We found that elevated levels of *α-synuclein* slightly altered overall eye development. The whole eye area (85346.4 ± 2250) (Figure 2-BI), bristle number (341.7 ± 9.276) (Figure 2-BII), and the ratio of disrupted area (0.4673 ± 0.0322) (Figure 2-BIII) for *α-synuclein* were significantly different from that of the control flies with whole eye area (96791 ± 1288), bristle number (454.4 ± 8.871), and ratio of disrupted area (0.3152 ± 0.0187). This suggests that elevated expression of *α-synuclein* alters the development of the eye.

In the pathology of Parkinson disease, the accumulation of *α-synuclein* is implicated with the progression of PD, and the intra-cytoplasmic inclusions or Lewy bodies have been shown to contain aggregates of *α-synuclein*, ubiquitin, and other proteins (Forno, 1996; Polymeropoulos *et al*, 1997; Leroy *et al*, 1998). The accumulation of these proteins is believed to result in cellular toxicity and pathogenesis. The *Drosophila α-synuclein*-induced models display retinal degeneration and other disease-like symptoms (Feany and Bender, 2000). We further investigated the overexpression of *α-synuclein* in eye development. The directed expression of *α-synuclein* in the eye of flies with *GMR-GAL4* revealed significant differences in the morphology of the eye when compared to the *lacZ*-expressing flies. The area of the whole eye and ommatidium was slightly decreased in *α-synuclein* flies, and the interommatidial bristle number was reduced. This may suggest that expressing *α-synuclein* in the eye of flies alters neurogenesis and might be attributed to the loss or death of the neurons due to *α-synuclein*-induced toxicity. Expression of *α-synuclein* in flies that were overexpressing a second copy of *α-synuclein* in the *GMR-GAL4* pattern slightly affected the development of the eye and in particular, 1) the overall area of the eye was reduced, 2) the interommatidial bristles were reduced in number, and 3) the ratio of disrupted area of the eye was slightly greater when compared to the control flies overexpressing *lacZ* and a single copy of *α-synuclein.* It is possible that the elevated levels of *α-synuclein* result in greater biological protein toxicity that causes the system for clearing malformed proteins to be stressed and lead to more neuronal cell death.

Recent studies have suggested that *α-synuclein* toxicity results in chaperone-mediated autophagy and lysosomal dysfunction by interfering with its ability to degrade *α-synuclein* and other products and seems to lead to the up-regulation of autophagy (Auluck *et al.,* 2002; Martinez-Vicente *et al.,* 2008; Winslow *et al.,* 2010; Xilouri and Stefanis, 2010). Indeed, neuronal death has been attributed to mitochondrial damage resulting from stress-induced by *α-synuclein* and causing an age-dependent decrease in substrate specific respiration along with an increase in mitophagy (Chinta *et al.,* 2010). It, therefore, seems that accumulation of *α-synuclein* promotes mitochondrial depletion

and neuronal death.

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Exposure to heat stress modulates DNA methyltransferase activity in the embryonic S2 cell line of *Drosophila melanogaster*.

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Summary

D. melanogaster is a dipteran model system for many diverse phenomena including animal development. The first report on the presence of 5-methylcytosine in the genomic DNA was by Deobagkar nee' Achwal (Achwal *et al*, 1984), where by use of sensitive and specific immunochemical staining and photoacoustic spectroscopy, the amount of 5mC was shown to be of