Chem. Senses 31: 627–639, 2006

doi:10.1093/chemse/bjl002
Advance Access publication June 8, 2006

Behavioral Responses to Odorants in *Drosophila* Require Nervous System Expression of the β Integrin Gene *Myospheroid*

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Abstract

Integrins are cell adhesion molecules that mediate numerous developmental processes in addition to a variety of acute physiological events. Two reports implicate a *Drosophila* β integrin, βPS, in olfactory behavior. To further investigate the role of integrins in *Drosophila* olfaction, we used Gal4-driven expression of RNA interference (RNAi) transgenes to knock down expression of *myospheroid* (*mys*), the gene that encodes βPS. Expression of *mys*-RNAi transgenes in the wing reduced βPS immunostaining and produced morphological defects associated with loss-of-function mutations in *mys*, demonstrating that this strategy knocked down *mys* function. Expression of *mys*-RNAi transgenes in the antennae, antennal lobes, and mushroom bodies via two Gal4 lines, H24 and MT14, disrupted olfactory behavior but did not alter locomotor abilities or central nervous system structure. Olfactory behavior was normal in flies that expressed *mys*-RNAi transgenes via other Gal4 lines that specifically targeted the antennae, the projection neurons, the mushroom bodies, bitter and sweet gustatory neurons, or *Pox neuro* neurons. Our studies confirm that *mys* is important for the development or function of the *Drosophila* olfactory system. Additionally, our studies demonstrate that *mys* is required for normal behavioral responses to both aversive and attractive odorants. Our results are consistent with a model in which βPS mediates events within the antennal lobes that influence odorant sensitivity.

Key words: behavior, odor attraction, odor avoidance, olfaction, RNA interference

Introduction

Olfaction is a key sensory modality that animals use for finding food, identifying mates, and avoiding predators (Vosshall, 2000; Firestein, 2001). A better understanding of olfactory system function is central for a more comprehensive view of how animals extract information from the environment and then respond appropriately via behavioral outputs. A wealth of studies describe the similarities in functional organization of the olfactory system in mammals and the fruit fly, *Drosophila melanogaster*, as well as other insects (Vosshall, 2000; Firestein, 2001; Eisthen, 2002). These similarities, combined with powerful genetic tools available in *Drosophila*, make fruit flies an attractive model system for investigating the molecular genetic basis for olfactory system function and development (Warr *et al.*, 2001).

Many of the main neural components of the olfactory system in *Drosophila* are well described (Stocker, 1994). Most of the primary olfactory sensory neurons are housed within the antennae. These neurons project in a stereotypical pattern to glomeruli within the antennal lobe, a region of the central insect brain that is functionally analogous to the vertebrate olfactory bulb. Olfactory information is processed in antennal lobe glomeruli composed of synapses between olfactory receptor neurons, local interneurons of the antennal lobes, and projection neurons. Projection neurons in turn synapse with neurons in the lateral protocerebrum as well as neurons that form the mushroom bodies. Olfactory information, therefore, proceeds from the olfactory receptor neurons in the antennae through the antennal lobe glomeruli and projection neurons to higher brain centers where it ultimately elicits a variety of behaviors.

The molecular genetic basis for olfactory system development is being vigorously investigated, leading to the identification of numerous genes important for proper formation of this key sensory system. In contrast, the influence of genes on olfactory behavior has not been as extensively studied. Thus, there is a large need to connect individual genes to

many aspects of olfactory behavior in numerous species including *Drosophila*.

In *Drosophila*, an abrupt presentation of odorant causes flies to jump. *Drosophila olfC* mutants are defective in this jump response to some but not all odorants (Ayyub *et al.*, 1990). Genetic complementation with standard loss-of-function mutations and other tests indicate that *olfC* is likely allelic to the *myospheroid* (*mys*) gene (Ayyub *et al.*, 2000) that encodes βPS, a β integrin (MacKrell *et al.*, 1988). Integrins are a major class of cell adhesion molecules involved in numerous developmental processes as well as a variety of acute physiological events (Hynes, 1992). The studies by Ayyub and coworkers strongly implicate integrins in the development or function of the olfactory system in *Drosophila*.

Some of the data from studies using standard loss-of-function alleles (Ayyub *et al.*, 2000; Ayyub and Paranjape, 2002), however, are difficult to reconcile with a model in which all the olfactory defects described map to the *mys* locus. To independently address whether *mys* plays a role in *Drosophila* olfactory behavior, we used a strategy based on RNA interference (RNAi) to knock down *mys* expression. We find that expression of *mys*-RNAi in the central nervous system causes deficits in avoidance of aversive odorants as well as attraction to appetitive odorants. The deficits in behavioral responses are associated with a reduction in odorant sensitivity but not with altered locomotor behavior or deranged central nervous system structure. Our studies confirm that *mys* is important for behavioral responses to aversive odorants and establish that *mys* also has a role in behavioral responses to attractive odorants. Our data are consistent with a model in which *mys* functions within the local interneurons of the antennal lobes to influence odor sensitivity.

Materials and methods

Fly husbandry and genetics

Flies were reared on standard food medium (10% sucrose, 2% yeast, 3.3% cornmeal, 1% agar) at 25°C/65% relative humidity under a 12-h day/night cycle. Flies were generously supplied by the following sources: *decapentapalegic*-Gal4 (*dpp*-Gal4) and *engrailed*-Gal4, L.S. Shashidhara, CCMB, Hyderabad; H24, Martin Heisenberg, Universitat Wurzburg; MT14, Reinhard Stocker, University of Fribourg; 107, Patrick Callaerts, University of Houston; 72Y, C739, C133, 59Y, 16Y, 125Y, Doug Armstrong, University System of Taiwan; *Pox neuro*-Gal4 (*Poxn*-Gal4), Gr66a, Gr5a, Scott Waddell, University of Massachusetts and Kristen Scott, University of California, Berkeley; Or83b, John Carlson, Yale University; mys^{nj42} flies, Danny Brower, University of Arizona; GH146, Liqun Luo, Stanford University; and tubulin-Gal4, actin-Gal4, and UAS-lacZ, *Drosophila* Stock Center at Indiana University (Bloomington, Indiana).

The UAS-*mys*-RNAi (UMR) transgene was constructed by cloning a 1.2-kb *Xho*I–*Bgl*II trigger sequence from expressed sequence tag clone RE55238 (Berkeley *Drosophila* Genome Project, GeneBank accession no. AY113499) into the SympUAST vector (Giordano *et al.*, 2002) (kindly provided by Ennio Giordano, Universita di Napoli). Trigger sequences in this vector are flanked by two UAS sites that drive both sense and antisense expression (Figure 2A), thereby producing a double-stranded RNA species that elicits RNAi-mediated knockdown of gene expression (Giordano *et al.*, 2002). For UAS-mys^{+} transgenic animals, a 2.5-kb *Not*I–*Spe*I fragment from the *mys* cDNA (generously provided by Danny Brower, University of Arizona) was cloned into pUAST (Brand and Perrimon, 1993). Transgenic flies carrying both UAS constructs were generated using standard methods (Rubin and Spradling, 1982) in a cantonized-w^{1118} (w[CS]) genetic background used previously for behavioral analyses (Cook-Wiens and Grotewiel, 2002; Goddeeris *et al.*, 2003; Stoltzfus *et al.*, 2003). Flies harboring independent insertions of the UMR transgene are designated as UMR1, UMR2, and UMR3 (a.k.a. PDM5i, PDM5g, and PDM3, respectively). The integrity of the UMR and UAS-mys^{+} transgenes was confirmed by polymerase chain reaction on genomic DNA isolated from the respective transgenic flies (data not shown).

Flies used in these studies were either generated in the w[CS] genetic background (UAS lines) or were moved into this background via backcrossing for at least six generations (Gal4 lines). The exceptions to this strategy were Or83b, Gr5a, Gr66a, and GH146, Gal4 drivers that did not affect olfactory behavior when crossed to UMR flies. In studies with these four Gal4 drivers, all flies tested were in a hybrid genetic background derived from w[CS] and the Gal4 driver. For all studies, control flies containing a single copy of a UAS transgene without a Gal4 driver or a Gal4 driver without a UAS transgene were generated by crossing flies with either transgene construct to w[CS] animals. Flies with Gal4-driven expression of UAS transgenes were generated by crossing flies containing either construct. For determining the viability of adult flies expressing *mys*-RNAi, balanced UMR flies were crossed to various Gal4 lines. Viability was calculated as the number of nonbalanced adult progeny divided by the number of balanced adult progeny × 100% from these crosses. Viability greater than 100% indicates that more nonbalanced progeny were observed than balanced progeny (Table 1).

Behavioral assays

Flies for behavioral studies were reared using standard conditions (Cook-Wiens and Grotewiel, 2002; Goddeeris *et al.*, 2003; Stoltzfus *et al.*, 2003; Gargano *et al.*, 2005). All flies for individual experiments were grown, collected, and handled in parallel. Adult flies (4- to 7-days old) were briefly anesthetized with CO_2, sorted into fresh food vials in groups of 25,

Table 1 Viability of flies expressing *mys*-RNAi via various Gal4 drivers

	Actin	Tubulin	72Y	C739	H24	MT14	107	C309	59Y	C133	Or83b
UMR1	0	0	0	0	116	22	120	71	75	100	106
UMR2	0	0	0	13	153	42	128	81	78	121	115
UMR3	0	0	0	10	118	30	112	58	118	115	76

Survival to adulthood was assessed in flies harboring a single UMR transgene (column 1) and a single Gal4 driver (row 1). Data are percent survival determined from crosses using balanced UMR strains.

and then allowed to recover for at least 18 h before being used in behavioral tests.

Olfactory behavior was assessed in T-mazes under dim red light at 22–25°C and 55–65% relative humidity as previously described (Cook-Wiens and Grotewiel, 2002; Stoltzfus *et al.*, 2003). Groups of 25 flies were lowered to a choice-point in a T-maze and allowed to choose for 2 min between a maze arm containing an odorant and a maze arm containing no odorant. Odorants were diluted in mineral oil and supplied to the arms of the maze in an air stream at 750 ml/min. At the conclusion of individual tests, the flies in each maze arm were anesthetized, collected, and counted. Avoidance indices were calculated as the percentage of flies that moved into the arm without odorant minus the percentage of flies that moved into the arm with odorant (Connolly and Tully, 1998). The odorants used were 4-methylcyclohexanol (MCH), benzaldehyde (BNZ), linalool (LIN), ethyl acetate (EA), 2-heptanone (HEP), and isoamyl acetate (IAA). Odorants and mineral oil were obtained from Sigma–Aldrich (St Louis, MO).

Negative geotaxis was assessed in rapid iterative negative geotaxis assays as described (Gargano *et al.*, 2005). In brief, groups of 25 flies from multiple genotypes were tested simultaneously for negative geotaxis. Data were captured with digital photography and extracted via computer-aided data analysis using Scion Image (PC version of NIH Image, Scion Corporation, Frederick, MD).

All behavioral data are presented as mean ± SEM. Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA). One-way ANOVAs, Bonferroni's multiple comparisons, and *t*-tests were used as indicated in the figure legends. *P* values >0.05 were considered not significant (NS).

Histology

Wing discs were dissected from third instar larvae using standard procedures. For immunostaining, discs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20–30 min, rinsed in PBS, and then blocked in PBSTB (1× PBS, 0.1% Triton X-100, 2% bovine serum albumin) for 60 min. Fixed and blocked wing discs were incubated overnight at 4°C with a 1:10 dilution of anti-βPS monoclonal CF.6G11 (generously provided by Danny Brower, University of Arizona) in PBSTB, rinsed, and then incubated with an FITC-conjugated anti-mouse secondary antibody (Sigma, St Louis, MO) for 60 min at 25°C. All incubations were carried out in a rotating tube holder. For β-galactosidase staining, wing discs were incubated in X-gal at room temperature and monitored until prominent LacZ reaction product was observed.

For analysis of adult brain, fly heads were loaded into a fly collar, embedded in Tissue Tek M (VWR Scientific Products, West Chester, PA), frozen at −20°C, and sectioned at 14 μm on a Microm HM550 cryostat (provided by Paul Ratz, Virginia Commonwealth University). Sections were fixed in 2% or 4% paraformaldehyde in PBS for 5–10 min, rinsed in PBS, and then blocked with PBSTB. Blocked sections were incubated with monoclonal nc82 (provided by Alois Hofbauer, University of Regensburg) or anti-Discs Large (DLG) monoclonal 4F3 (Developmental Studies Hybridoma Bank at the University of Iowa) for 12–16 h at 4°C, rinsed, and then incubated with FITC-conjugated anti-mouse antiserum (Sigma). Coverslips were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Digital images were obtained using a Zeiss Axioplan-2 microscope, Axiocam CCD camera, and Axiovision software (Carl Zeiss, Germany).

Results

βPS, encoded by the *mys* locus (MacKrell *et al.*, 1988; Leptin *et al.*, 1989), is the major β integrin in flies (Devenport and Brown, 2004). To better define the role of integrins in *Drosophila* adult behavior, we initially assessed the innate aversion of BNZ in flies harboring the mys^{nj42} allele, a partial loss-of-function mutation (Bunch *et al.*, 1992). BNZ avoidance was blunted in mys^{nj42} animals (Figure 1), consistent with previous reports indicating that *mys* is important for normal behavioral responses to odorants in flies (Ayyub *et al.*, 2000; Ayyub and Paranjape, 2002). Unfortunately, stronger combinations of *mys* alleles [mys^{nj42} *in trans* to a null (mys^{XG43}) or an antimorph (mys^{XR04})] had very low viability in our laboratory as described by others (Bunch *et al.*, 1992), precluding behavioral testing of animals with greater reductions in *mys* function. Weaker alleles at the *mys* locus (mys^{ts1}, mys^{ts2}) (Bunch *et al.*, 1992) alone or *in trans* to mys^{nj42} were viable but had normal avoidance of odors (data not shown), making such mutants uninformative. Finally, neither heat shock induced nor Gal4-driven expression of wild-type

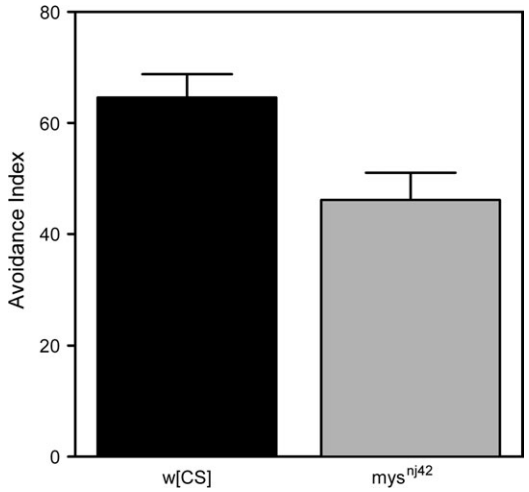

Figure 1 Odor avoidance in *mys* hypomorphic mutants. Avoidance of BNZ was reduced in mys^{nj42} flies compared to w[CS] controls (two-tailed *t*-test, $P = 0.0069$, $n = 19$). Data are compiled from four independent experiments.

βPS rescued the BNZ avoidance defects in mys^{nj42} flies. Thus, our studies on loss-of-function mutants added little to our understanding of *mys* in *Drosophila* olfactory behavior.

Given these issues, we adopted a strategy based on targeted expression of *mys*-RNAi via the Gal4/UAS system (Brand and Perrimon, 1993) for investigating the role of *mys* in olfaction. RNAi is a type of gene silencing that relies on the expression of a double-stranded RNA species to elicit degradation of homologous mRNA and consequently reduce expression of the corresponding translation product (Cerutti, 2003). We predicted that such a strategy would circumvent the problems associated with pleiotropic effects of strong loss-of-function *mys* mutants as well as problems with the lack of phenotype in weak *mys* mutants. We also predicted that targeted expression of *mys*-RNAi would allow us to begin to identify regions of the olfactory system that require expression of *mys* to function properly.

Construction and characterization of *mys*-RNAi flies

To generate *mys*-RNAi flies, we subcloned a 1.2-kb region of the *mys* cDNA (corresponding to exons 4 and 5 and a portion of exon 6) into the SympUAST vector (Giordano *et al.*, 2002) (Figure 2A) and then introduced this construct into w[CS], a control strain, via P-element transformation (Rubin and Spradling, 1982). The SympUAST vector contains two opposing UAS sites that drive sense and antisense transcription of the trigger in response to ectopically supplied Gal4 (Giordano *et al.*, 2002). The SympUAST vector eliminates the need to clone inverted repeat sequences for the trigger. The advantages of using this vector are that the trigger is substantially easier to clone and that the transgene is more stable once integrated into the genome (Giordano *et al.*, 2002). Several independent UMR transformants were generated. Two lines with insertions on the second chromosome (UMR1, UMR3) and one on the X-chromosome (UMR2) were chosen for further study.

Strong loss-of-function mutations in *mys* are lethal (MacKrell *et al.*, 1988; Leptin *et al.*, 1989; Zusman *et al.*, 1990; Brown, 1994). To determine whether widespread expression of *mys*-RNAi was similarly lethal, we crossed UMR1, UMR2, and UMR3 flies to a series of Gal4 drivers and then assessed the viability of the resulting F1 progeny. Ubiquitous expression of *mys*-RNAi driven by tubulin-Gal4 or actin-Gal4 resulted in complete lethality (Table 1). Two other Gal4 lines, 72Y and C739, also strongly reduced viability when driving UMR transgenes (Table 1). Like strong loss-of-function *mys* alleles, ubiquitous expression of *mys*-RNAi is lethal.

The normal adult wing of *Drosophila* is a highly ordered structure with tight adhesion between the dorsal and ventral layers in addition to an essentially invariant vein pattern (Figure 2B). Strong loss-of-function mutations in *mys* disrupt adhesion between the dorsal and ventral layers of the adult wing, resulting in wing blisters (Zusman *et al.*, 1990; Bunch *et al.*, 1992). Additionally, *mys* loss-of-function mutations disrupt normal vein formation in the adult wing (Zusman *et al.*, 1990). We expressed *mys*-RNAi in the developing wing to determine whether it would phenocopy the defects in wing structure caused by strong loss-of-function *mys* alleles. *dpp*-Gal4 expresses in a central stripe in the developing wing (Figure 2F). Consistent with this expression pattern, adult wings from all flies that expressed *mys*-RNAi via *dpp*-Gal4 had a substantial wrinkled area containing vein defects (Figure 2C). Additionally, all flies with *mys*-RNAi expression driven by *dpp*-Gal4 had wing blisters within the presumptive wrinkled area (data not shown). Expression of *mys*-RNAi via *engrailed*-Gal4 in the wing caused similar wrinkling, blistering, and vein defects (data not shown). These data demonstrate that expression of *mys*-RNAi phenocopies the wing defects found in severe *mys* loss-of-function mutants and strongly suggest that *mys*-RNAi reduces function of the *mys* locus.

We evaluated expression of βPS, the *mys* gene product (MacKrell *et al.*, 1988; Leptin *et al.*, 1989), in third instar larval discs to determine whether *mys*-RNAi expression knocked down βPS levels. Consistent with previous studies (Brower *et al.*, 1984), wild-type larvae expressed βPS in a fairly uniform pattern throughout the wing disc (Figure 2D). Larvae with expression of *mys*-RNAi driven by *dpp*-Gal4 had reduced βPS immunostaining in a central region of the disc (Figure 2E). The reduction in βPS immunostaining in larvae expressing *mys*-RNAi corresponded to the expression pattern of *dpp*-Gal4 (Figure 2F). These data show that expression of *mys*-RNAi knocks down βPS expression. Together, our studies on viability, wing structure, and βPS immunostaining provide strong evidence that expression of *mys*-RNAi causes loss-of-function at the *mys* locus by knocking down expression of βPS.

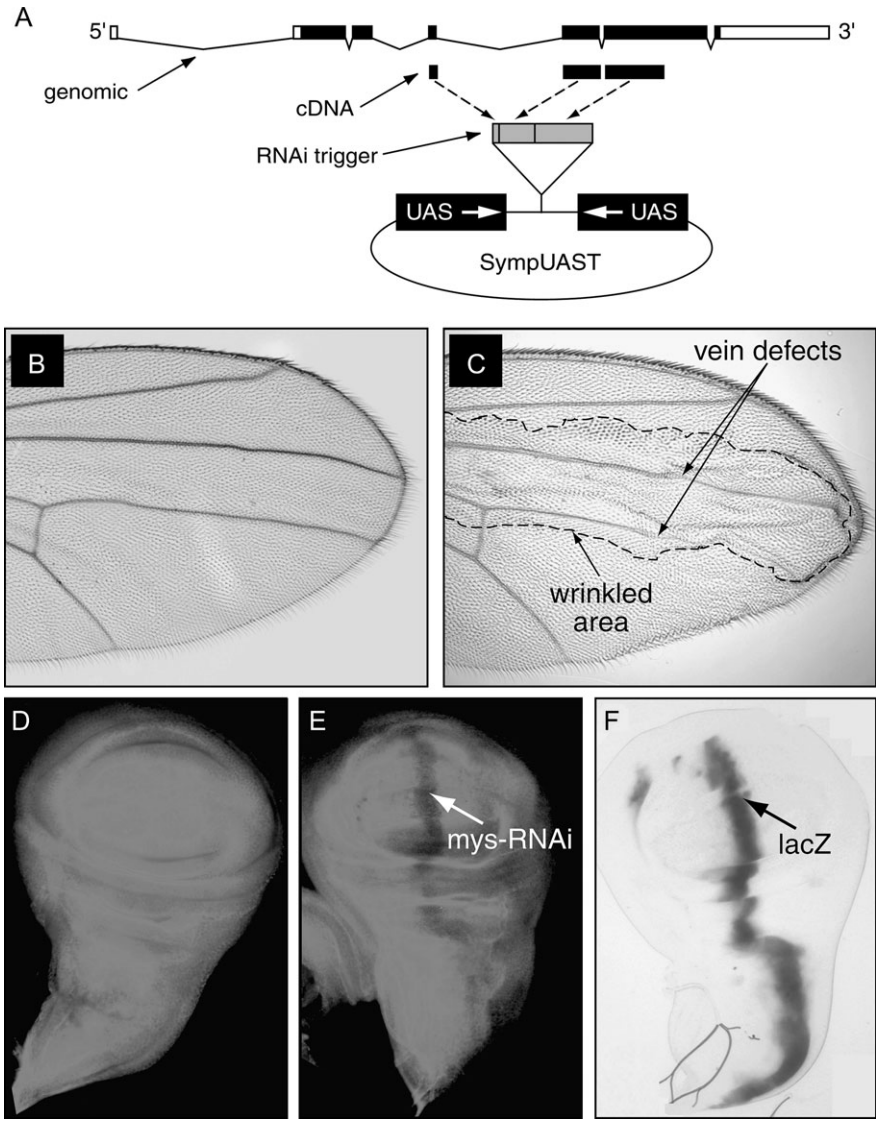

Figure 2 Expression of *mys*-RNAi causes wing defects and knocks down βPS expression. **(A)** Construction of *mys*-RNAi trigger. Upper schematic represents the structure of the *mys* locus. Open and closed boxes correspond to untranslated and translated regions of exons, respectively. Single lines represent introns. Middle schematic indicates cDNA derived from exons 4 to 6 cloned into SympUAST to generate the *mys*-RNAi trigger (gray box). The *mys*-RNAi trigger resides between two opposing UAS in SympUAST. The *mys*-RNAi-SympUAST construct was injected into w[CS] flies to generate UMR transgenics. **(B)** Wild-type adult wing. **(C)** Adult wing from *dpp*-Gal4/+;UMR1/+ fly with *mys*-RNAi expressed in a central stripe. Wrinkled surface is encircled with a dashed line. Truncated wing veins within the wrinkled area are indicated. **(D)** Wild-type third instar larval wing disc with widespread βPS expression detected via immunostaining with anti-βPS monoclonal antibody CF6.G11 (Brower *et al.*, 1984) and an FITC-labeled secondary antibody. **(E)** *dpp*-Gal4/+;UMR1/+ wing disc with reduced βPS expression in a stripe along the disc midline. **(F)** Expression pattern of *dpp*-Gal4 in third larval wing discs visualized by a UAS-lacZ reporter and β-galactosidase staining. Anterior and ventral are to the left and top, respectively, of the images in D–F.

Expression of *mys*-RNAi disrupts olfactory behavior

The two main external components of the insect olfactory system are the antenna, a structure that houses most olfactory receptor neurons, and the maxillary palp, a structure that contains most other olfactory receptor neurons. Neurons from these two structures project to the antennal lobe, a region of the central nervous system that is functionally and structurally analogous to the vertebrate olfactory bulb (Stocker, 1994). The antennal lobes are organized into glomeruli composed of synapses between olfactory receptor neurons, local interneurons, and projection neurons. Projection neurons are the output neurons from the antennal lobes and thereby convey olfactory information to a higher brain center, the lateral protocerebrum, either directly or indirectly via the mushroom bodies. The insect mushroom body is intimately involved in associative olfactory memory (Dubnau *et al.*, 2001; McGuire *et al.*, 2001; Pascual and Preat, 2001) and more recently was implicated in the innate responses to

odorants in *Drosophila* (Wang *et al.*, 2003). Additionally, a recent report identified bitter sensing gustatory neurons and *Pox neuro* (*Poxn*) chemosensory neurons as important for behavioral responses to BNZ in flies (Keene *et al.*, 2004). Toward characterizing the spatial requirement for βPS in olfactory behavior, we evaluated BNZ avoidance in flies with Gal4-driven expression of *mys*-RNAi in the antennae, antennal lobes, mushroom bodies, sweet sensing gustatory neurons, bitter sensing gustatory neurons, and *Poxn* chemosensory neurons. Flies that expressed *mys*-RNAi via the Gal4 drivers used in our studies (H24, MT14, 59Y, C133, 16Y, 125Y, 107, C309, Or83b, GH146, Gr66a, Gr5a, and *Poxn*-Gal4) were viable (Table 1 and data not shown), exhibited no obvious locomotor defects, and seemed generally healthy, making them suitable for behavioral analyses. H24 drives expression in the antennal lobes, ellipsoid body of the central complex, and mushroom bodies (Figure 3A,B) as reported (Martin *et al.*, 1998; Zars *et al.*, 2000). MT14 drives expression in the antennal lobes (Figure 3C) as found previously (Tissot *et al.*, 1997). This line also expresses Gal4 in the mushroom bodies (Figure 3D). As reported by others (Chiang *et al.*, 2004) (Doug Armstrong, http://www.fly-trap.org/), 59Y (Figure 3E), C133 (Figure 3F), 16Y, and 125Y (data not shown) express in the antennal lobes but not in mushroom bodies. Conversely, 107 and C309 drive expression in the mushroom bodies (Figure 3G,H) but not in the antennal lobes, consistent with other data (Connolly *et al.*, 1996). Or83b drives expression in the antenna but not in the central brain (Vosshall *et al.*, 2000; Larsson *et al.*, 2004). GH146, Gr5a, Gr66a, and *Poxn*-Gal4 express in the projection neurons (Heimbeck *et al.*, 2001), sweet sensing gustatory neurons, bitter sensing gustatory neurons, and *Poxn* chemosensory neurons, respectively (Keene *et al.*, 2004). H24, MT14, 59Y, C133, and C309 drive expression in the antennae and maxillary palps (data not shown).

Avoidance of BNZ was substantially reduced in flies with H24- and MT14-driven expression of *mys*-RNAi (Figure 4A). Avoidance of BNZ was not significantly altered in flies with *mys*-RNAi expressed via Or83b (Figure 4B), 107 or C309 (Figure 4D), GH146, Gr5a, Gr66a, or *Poxn*-Gal4 (data not shown). BNZ avoidance was not significantly reduced in flies with *mys*-RNAi expression driven by C133, 59Y (Figure 4C), 16Y, or 125Y (data not shown), although there was a trend toward decreased BNZ avoidance that was not statistically significant when C133, 59Y, and 16Y were used to drive *mys*-RNAi. Thus, within our experiments, avoidance of BNZ is significantly disrupted by expression of *mys*-RNAi only when driven by H24 and MT14. This suggests that these two Gal4 lines define the tissues that require *mys* expression within the context of BNZ avoidance. Since BNZ avoidance defects or trends toward such defects were associated with *mys*-RNAi expression in the antennal lobes but not in other regions of the nervous system, our current model is that *mys*-RNAi expression in local interneurons of the antennal lobes disrupts odor avoidance.

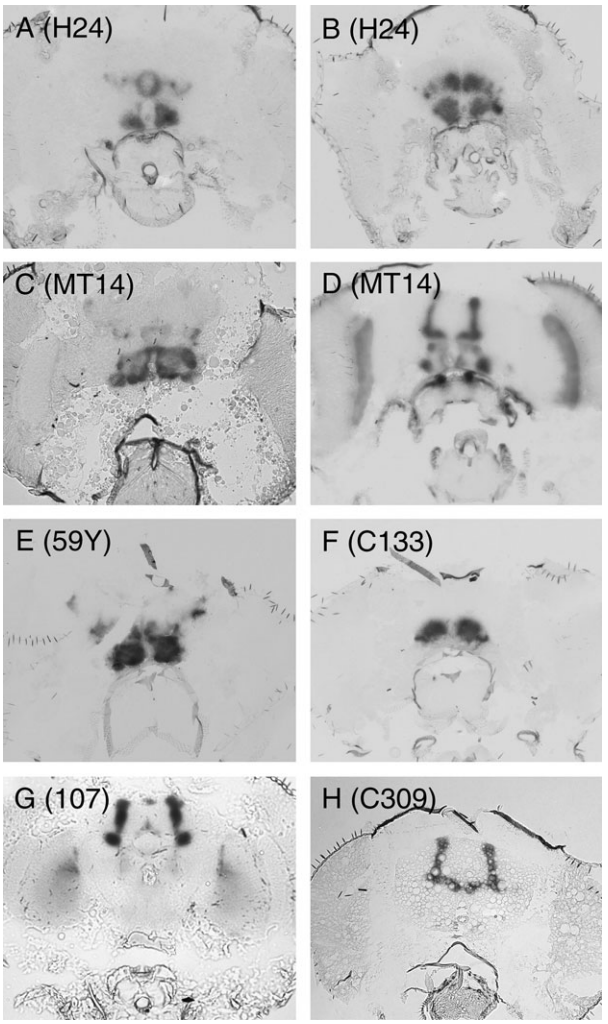

Figure 3 Adult brain expression patterns of Gal4 drivers. Frontal sections through the central brain showing expression of H24 **(A, B)**, MT14 **(C, D)**, 59Y **(E)**, C133 **(F)**, 107 **(G)**, and C309 **(H)**. H24 and MT14 are expressed in the antennal lobes (A, C) and the mushroom bodies (B, D). H24 is also expressed in the ellipsoid body and noduli of the central complex (A). 59Y and C133 are expressed in the antennal lobes (E, F) but not in the mushroom bodies. 107 and C309 are expressed in the mushroom bodies (G, H) but not in the antennal lobes. In all sections, Gal4 expression was determined by β-galactosidase staining in flies harboring a single copy of the indicated Gal4 driver and a UAS-lacZ reporter.

To determine whether expression of *mys*-RNAi disrupted behavioral responses to all odorants and whether *mys*-RNAi expression perturbed avoidance of and attraction to odorants, we assessed the behavioral responses of flies expressing *mys*-RNAi to a series of olfactory stimuli. Representative odorants from different chemical classes (aldehydes, esters, and alcohols) were tested. Consistent with the data in Figure 4A, expression of *mys*-RNAi via H24 reduced avoidance of BNZ (Figure 5A). Avoidance of MCH (Figure 5B) and attraction to EA (Figure 5C) were also disrupted in flies with H24-driven expression of *mys*-RNAi. Expression of *mys*-RNAi via H24 marginally reduced avoidance of HEP

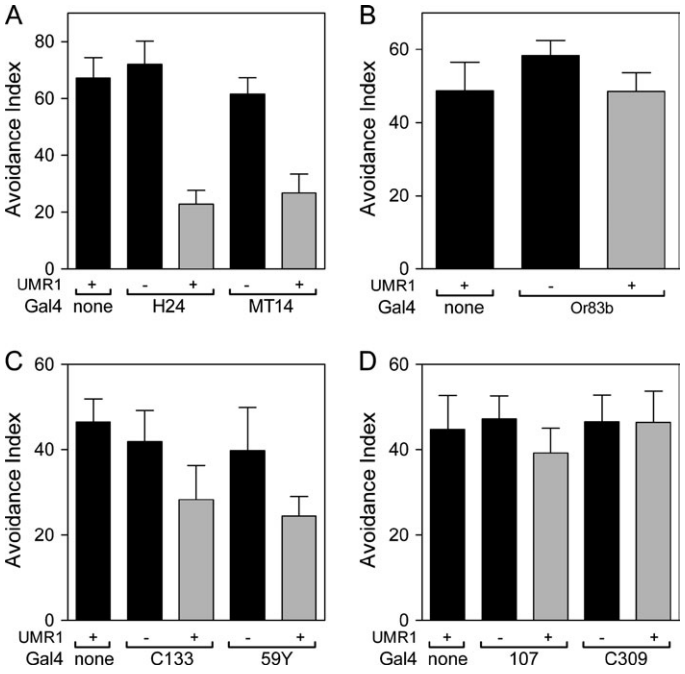

Figure 4 BNZ avoidance in flies expressing *mys*-RNAi in different regions of the nervous system. Data from control genotypes are shown in black, and data from flies that expressed *mys*-RNAi are shown in gray. **(A)** Effect of *mys*-RNAi expression via H24 and MT14. There was a significant effect of genotype on BNZ avoidance (one-way ANOVA, $P < 0.0001$, $n = 10$). BNZ avoidance was significantly reduced in H24/+;UMR1/+ and in MT14/+;UMR1/+ flies compared their controls harboring a single copy of either the Gal4 driver or UMR transgene (Bonferroni's multiple comparison, $P < 0.01$). BNZ avoidance in flies with *mys*-RNAi expression via Or83b **(B)**, C133 and 59Y **(C)**, or 107 and C309 **(D)**. There was no significant effect of genotype on BNZ avoidance in B–D (individual one-way ANOVAs, NS, $n = 10$). Data are representative of at least two independent experiments for all genotypes.

(Figure 5D) but did not significantly affect avoidance of LIN (Figure 5E) or IAA (Figure 5F). Expression of *mys*-RNAi, therefore, disrupts behavioral responses to particular odorants but not particular classes of compounds. Additionally, *mys*-RNAi expression causes defects in both odor avoidance and odor attraction.

To examine whether odor potency was altered in flies expressing *mys*-RNAi, we performed dose-response studies with BNZ. This odorant is attractive at relatively low concentrations and becomes aversive at higher concentrations (Devaud *et al.*, 2003; Wang *et al.*, 2003). As expected, control flies exhibited a strong attraction to BNZ at low concentrations and a robust aversion to this odorant at higher concentrations (Figure 6A). Attraction to low concentrations of BNZ was undetectable in flies expressing *mys*-RNAi via H24. These flies also had a rightward shift in their dose response to BNZ that is consistent with a decreased aversiveness of this odorant. The maximal avoidance response to BNZ, however, was unaffected by *mys*-RNAi expression. These studies confirm that odor avoidance as well as attraction are disrupted by expression of *mys*-RNAi and suggest that expression of *mys*-RNAi decreases odor potency.

Confirmation that expression of *mys*-RNAi disrupts olfactory behavior

We tested BNZ avoidance in two additional *mys*-RNAi lines, UMR2 and UMR3, to determine whether the defects in olfactory behavior were *bona fide* outcomes of *mys*-RNAi expression. As expected, flies harboring either UMR2 (Figure 6B) or UMR3 (Figure 6C) in conjunction with the H24 driver had significant defects in BNZ avoidance as compared to control flies with either the Gal4 driver or the *mys*-RNAi transgene alone. These data confirm that Gal4-driven expression of *mys*-RNAi disrupts BNZ avoidance.

The simplest interpretation of the data in Figures 4, 5, and 6A–C is that *mys*-RNAi expression knocks down βPS in the brain as it does in the developing wing (Figure 2) and that this reduction in βPS expression causes defects in olfactory behavior. Unfortunately, despite extensive efforts using multiple anti-βPS antibodies to immunostain adult head sections, we were unable to unequivocally demonstrate βPS expression in the central brain and its knockdown by *mys*-RNAi. Thus, we used an alternative strategy to address the connection between reduced βPS expression and olfactory defects.

We postulated that if the odor avoidance and attraction defects in flies with H24-driven expression of *mys*-RNAi were due to reduced expression of βPS, then expression of wild-type βPS should rescue the olfactory deficits in these flies. We therefore assessed olfactory behavior in flies expressing *mys*-RNAi alone, flies overexpressing wild-type *mys* (i.e., βPS) alone, and flies expressing *mys*-RNAi and wild-type *mys* together using the H24 driver. BNZ avoidance (Figure 6D) and attraction (Figure 6C) were disrupted in flies expressing *mys*-RNAi alone as expected. Overexpression of wild-type *mys* via H24 also disrupted BNZ avoidance and attraction. This dominant negative-like activity of overexpressed *mys* is consistent with other studies showing that overexpressed wild-type integrin can phenocopy integrin loss-of-function (Brabant *et al.*, 1996). More importantly for our studies, flies that expressed *mys*-RNAi and wild-type *mys* together had normal BNZ avoidance and attraction. Expression of wild-type *mys*, therefore, rescued the olfactory defects associated with *mys*-RNAi expression. These studies indicate that the behavioral changes in flies that express *mys*-RNAi are due to knockdown of βPS expression.

Locomotion and central brain morphology are normal in *mys*-RNAi flies

We assessed negative geotaxis to explore whether decreased locomotion could explain the olfactory defects in flies expressing *mys*-RNAi. In negative geotaxis assays, flies are tapped to the bottom of a container to elicit an escape response that manifests as walking up the container wall

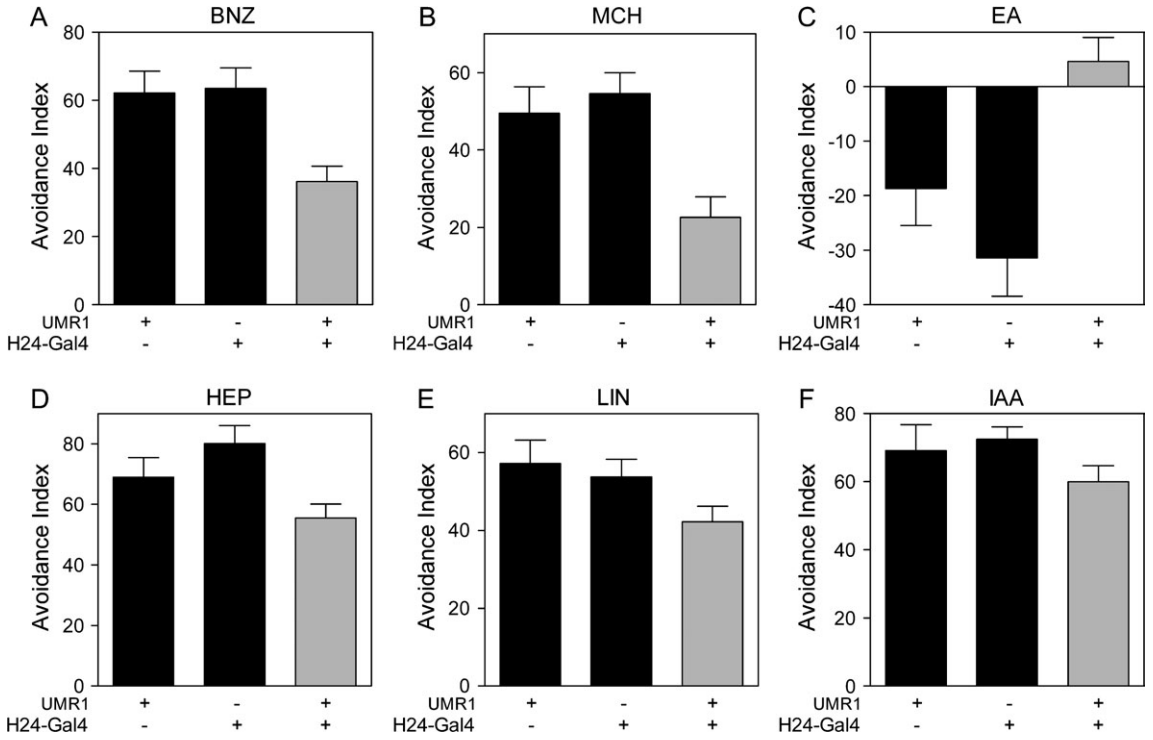

Figure 5 Innate responses to different odorants in flies with H24-driven expression of *mys*-RNAi. Odor avoidance **(A, B, D–F)** and attraction **(C)** in H24/+;UMR1/+ (gray bars) and controls containing either transgene alone (black bars). Avoidance of BNZ (A) and MCH (B) and attraction to EA (C) were reduced relative to control strains (individual one-way ANOVAs, $P \leq 0.003$, $n = 10$; Bonferroni's multiple comparison test, $P \leq 0.05$). (D) There was a significant effect of genotype on avoidance of HEP (one-way ANOVA, $P = 0.0188$, $n = 10$). Bonferroni's multiple comparison revealed a significant difference between H24/+;UMR1/+ and H24/+ animals ($P < 0.05$) but not between UMR1/+ and H24/+;UMR1/+ flies (NS). Avoidance of LIN (E) and IAA (F) was not significantly altered in H24/+;UMR1/+ flies (individual one-way ANOVAs, NS, $n = 10$). Data are representative of two independent experiments.

(Gargano *et al.*, 2005). The distance walked is a measure of locomotor ability. Expression of *mys*-RNAi via H24 had no effect on negative geotaxis (Figure 6F), indicating that the odor avoidance and attraction defects in these flies are not linked to altered locomotor ability. Additionally, since *mys*-RNAi flies respond normally to some odorants (Figure 5D–F) and have normal maximal avoidance of BNZ (Figure 6A), their locomotor skills are wholly sufficient for good performance within the T-maze.

βPS is involved in a number of developmental processes, including axon guidance (Hoang and Chiba, 1998) and synapse maturation (Beumer *et al.*, 1999). To investigate whether developmental abnormalities were associated with the olfactory defects in flies expressing *mys*-RNAi, we evaluated central brain morphology via light microscopy and immunostaining for DLG, the *discs large 1* gene product, in adult head cryosections. DLG is expressed in neuropil regions throughout the *Drosophila* brain (Ruiz-Canada *et al.*, 2002), making it a good marker for central brain anatomy. Careful examination of the morphology as revealed by DLG immunostaining in antennal lobes (Figure 7A,C), mushroom bodies and central complex (Figure 7B,D), and other regions of the central brain revealed no consistent differences between flies expressing *mys*-RNAi and controls. We also examined central brain anatomy in *mys*-RNAi flies via immunostaining with another neuropil marker, monoclonal antibody nc82 (Laissue *et al.*, 1999). Consistent with the anti-DLG immunostaining, no reproducible effect of *mys*-RNAi expression on brain morphology was observed in head sections stained with nc82 (data not shown). The absence of obvious developmental defects as assessed at the light microscope level in *mys*-RNAi flies raises the possibility that *mys* plays a role in the acute function of neurons within the *Drosophila* olfactory system.

Discussion

Integrins mediate a variety of key biological processes (Hynes, 1992). In *Drosophila*, integrins are essential for proper migration of the dorsal epithelium during development (Bunch *et al.*, 1992) and for the normal development of the eye (Zusman *et al.*, 1990), the wing (Brower and Jaffe, 1989), the midgut (Brabant and Brower, 1993), the nervous system (Hoang and Chiba, 1998), and the musculature (Volk *et al.*, 1990). Integrins are also important for development in *Caenorhabditis elegans* (Gettner *et al.*, 1995) and mice (Kil *et al.*, 1996; Cachaco *et al.*, 2003; Blaess *et al.*, 2004). In addition to their well-established roles in development, integrins also participate in a number of acute physiological processes.

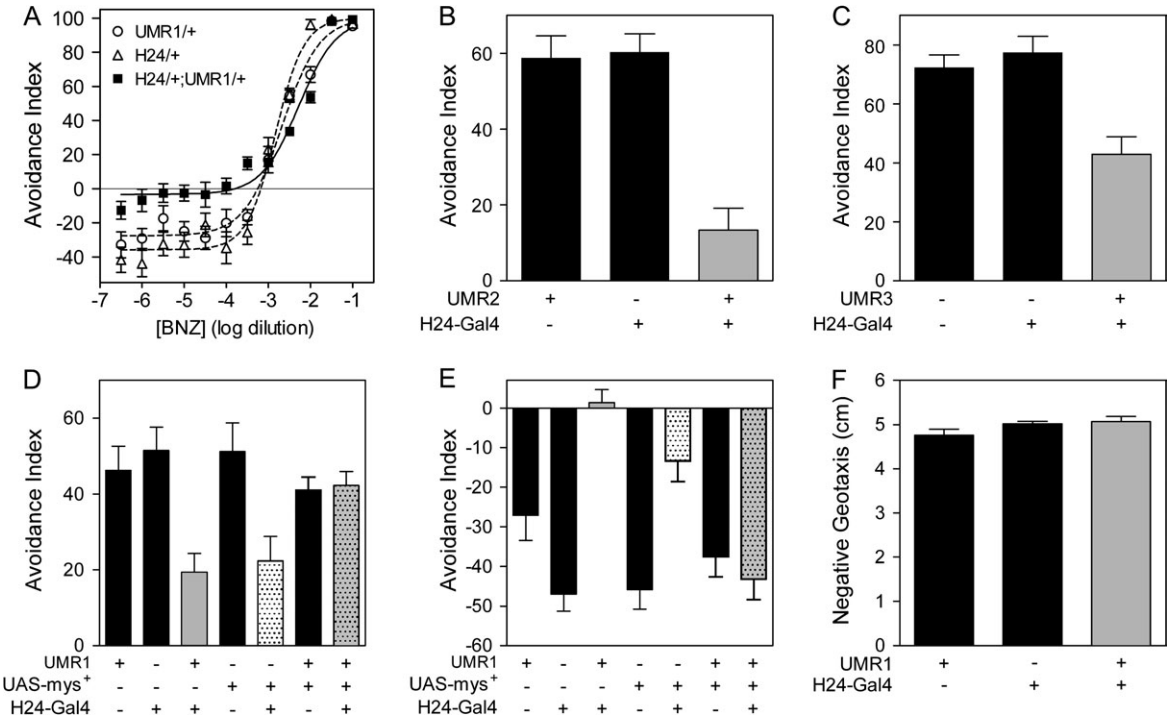

Figure 6 **(A)** BNZ avoidance dose response in *mys*-RNAi flies. Odor avoidance in H24/+;UMR1/+ (filled squares, solid line) and controls (H24/+, open triangles, dashed line and UMR1/+, open circles, dashed line) flies with increasing concentrations of BNZ (*x*-axis). Overall, BNZ avoidance was significantly affected by genotype (nonlinear regression, sigmoidal dose response, *F*-test, $P < 0.0001$). Attraction to low concentrations of BNZ was significantly blunted and the effective concentration producing 50% of the maximal response (EC_{50}) was significantly greater in H24/+;UMR1/+ flies relative to control strains ($P < 0.0001$). Each symbol represents $n = 10$. Avoidance of BNZ in flies expressing *mys*-RNAi from two additional UMR transgenic strains, UMR2 **(B)** and UMR3 **(C)**. Individual one-way ANOVAs indicated a significant effect of genotype in B and C ($P \leq 0.0002$, $n = 5$–10). BNZ avoidance was significantly reduced in H24/+;UMR2/+ and H24/+;UMR3/+ (gray bars) compared to controls (black bars) (Bonferroni's multiple comparison, $P < 0.01$). **(D, E)** Expression of βPS rescues BNZ avoidance and attraction defects in *mys*-RNAi flies. Aversion to a high concentration (dilution factor 1:500; D) and attraction to a low concentration of BNZ (dilution factor 1:10,000; E) in flies with H24-driven expression of *mys*-RNAi (UMR1, gray bars), βPS (UAS-*mys*$^+$, white dotted bar), or *mys*-RNAi and βPS together (gray spotted bar) in addition to controls harboring the Gal4 driver alone or the UMR1 and UAS-*mys*$^+$ transgenes without H24 (black bars). Individual one-way ANOVAs indicated a significant effect of genotype on BNZ avoidance (D; $P = 0.0002$, $n = 10$) and attraction (E; $P < 0.0001$, $n = 15$). BNZ avoidance and attraction were reduced in flies expressing *mys*-RNAi or *mys*$^+$ compared to their respective controls (Bonferroni's multiple comparison, $P < 0.05$). BNZ avoidance and attraction in flies expressing *mys*-RNAi and *mys*$^+$ simultaneously were indistinguishable from control flies (Bonferroni's multiple comparison, NS). **(F)** Negative geotaxis in flies expressing *mys*-RNAi. Negative geotaxis in H24/+;UMR1/+ flies (gray bar) and controls (black bars) was indistinguishable (one-way ANOVA, NS, $n = 5$).

Integrins are essential for proper olfactory memory in *Drosophila* (Grotewiel *et al.*, 1998) and spatial memory in the mouse (Chan *et al.*, 2003). Integrins also play a role in synaptic plasticity in rodents and *Drosophila* (Staubli *et al.*, 1998; Rohrbough *et al.*, 2000; Chan *et al.*, 2003), functions that might underlie their role in various forms of memory. Additionally, integrins mediate the acute stretch-induced increase in neurotransmitter release at the frog neuromuscular junction (Chen and Grinnell, 1995) and are involved in the increase in neurotransmitter release elicited by hypertonic solutions at the neuromuscular junction in *Drosophila* (Suzuki *et al.*, 2002). Thus, integrins have important roles in a variety of acute physiological processes.

βPS is encoded by the *mys* locus and is the major β integrin in flies. A series of *olfC* mutants was previously identified that have defects in their behavioral responses to a subset of odorants (Ayyub *et al.*, 1990). Using a classical genetic approach, Siddiqi and coworkers showed that three independent alleles of *mys* fail to complement four different *olfC* mutations for an innate jump response elicited by IAA. This group also found that a wild-type *mys* transgene rescues the IAA-induced jump defects in two *olfC* alleles (Ayyub *et al.*, 2000). Additional experiments revealed that flies with two different alleles *olfC* (presumably *mys*) *in trans* to *olfE* (an allele of "swisscheese") have defects in IAA- and BNZ-induced jumping (Ayyub and Paranjape, 2002). These data support the hypothesis that the *olfC* and *mys* mutations are allelic and that *mys* is involved in the behavioral response to IAA and possibly other odorants.

There are several complexities, however, within the data reported on *mys* in olfactory behavior. First, although multiple alleles of *mys* fail to complement two *olfC* mutations for IAA-induced behavior, they fully complement both *olfC* alleles for behavioral responses to EA (Ayyub *et al.*, 2000). Similarly, flies with two different *mys* alleles *in trans* have defects in IAA behavior but normal responses to EA (Ayyub

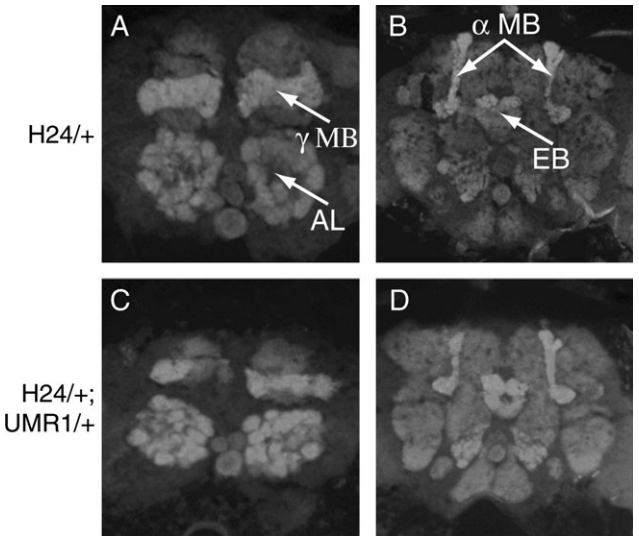

Figure 7 DLG expression in the central brain of *mys*-RNAi flies. Representative images from frontal sections of adult fly heads incubated with anti-DLG monoclonal 4F3 and an FITC-labeled secondary antibody. **(A, C)** Sections at the level of the antennal lobes (AL) and mushroom body γ lobes (γMB). **(B, D)** Sections at the level of the mushroom body α lobes (αMB) and the ellipsoid body of the central complex (EB). No consistent differences in the structure of the antennal lobes, mushroom bodies, or other regions of the central brain were observed between H24/+ (A, B) and H24/+;UMR1/+ flies (C, D). Structural differences between genotypes in the images presented here are consistent with normal variations found within genotypes.

et al., 2000). These data suggest that *mys* is important for IAA-induced behavior, while behavioral responses to EA might be independent of the *mys* locus. Second, the behavioral response to BNZ is normal in the *olfC* mutants (Ayyub and Paranjape, 2002), making it unclear whether *mys* is involved in detecting or responding to this or other odorants. Third, some of the chromosomes harboring *mys* mutations in these studies carried phenotypic markers that could, in principle, account for some of the olfactory behavioral defects observed (Ayyub *et al.*, 2000; Ayyub and Paranjape, 2002). Finally, data from control groups in some experiments were not reported (Ayyub *et al.*, 2000; Ayyub and Paranjape, 2002), making it difficult to directly assess the effects of individual mutations and transgenes on olfactory behavior in all cases. Together, these complexities undermine the strength of the connection between olfactory behavior and *mys*.

To further investigate the relationship between *mys* and olfactory behavior in *Drosophila*, we initially evaluated *mys* loss-of-function mutants in odor avoidance assays performed in T-mazes. We found that mys^{nj42} partial loss-of-function mutants have defects in avoidance of BNZ. Additional experiments in our laboratory on these and other *mys* loss-of-function mutants, however, were inconclusive. Transgenic wild-type *mys* failed to rescue the olfactory behavior defect in mys^{nj42} flies [possibly due to a dominant-negative effect of integrin overexpression (Brabant *et al.*, 1996)], stronger combinations of *mys* alleles were essentially lethal, and a series of weaker alleles or combinations of weaker alleles had no reproducible effect on odor avoidance. Thus, our experiments on *mys* loss-of-function mutants provided little if any additional insight into the role of integrins in *Drosophila* olfactory behavior.

To circumvent limitations associated with the *mys* loss-of-function mutations that we studied, we generated transgenic flies that expressed *mys*-RNAi. Ubiquitous expression of *mys*-RNAi is lethal. When expressed in the wing, *mys*-RNAi produces wing blisters and vein defects. Furthermore, expression of *mys*-RNAi reduces expression of βPS, the *mys* gene product. All these phenotypes are characteristic of strong *mys* loss-of-function alleles (Zusman *et al.*, 1990; Bunch *et al.*, 1992), demonstrating that expression of *mys*-RNAi reduces *mys* expression.

We assessed olfactory behavior in flies that expressed *mys*-RNAi via a variety of Gal4 drivers that targeted various components of the nervous system associated with olfaction. Expression of *mys*-RNAi by H24 and MT14, Gal4 lines that express in the antennae, the antennal lobe, and regions of the mushroom bodies, disrupts avoidance of BNZ. Similarly, expression of *mys*-RNAi by three antennal lobe drivers (C133, 59Y, and 16Y) results in slight decreases in BNZ avoidance that did not reach statistical significance. In contrast, BNZ avoidance is normal in flies that express *mys*-RNAi via all other Gal4 lines tested (Or83b, 125Y, 107, C309, GH146, *Poxn*-Gal4, Gr66a, and Gr5a). Of the Gal4 lines we have tested, therefore, only H24 and MT14 significantly disrupt BNZ avoidance when driving *mys*-RNAi expression. These studies confirm that *mys* plays a role in odor-induced behavior in *Drosophila* as suggested previously (Ayyub *et al.*, 2000; Ayyub and Paranjape, 2002).

Our studies are a first step in defining the spatial requirements for *mys* in olfactory behavior. Since H24, MT14, 59Y, C133, and Or83b express Gal4 in the antennae and maxillary palps in addition to other tissues, yet defects in BNZ avoidance were found only in flies that expressed *mys*-RNAi via H24 and MT14, our data indicate that expression of *mys*-RNAi in the antennae and maxillary palps is likely insufficient to disrupt BNZ avoidance. Additionally, since flies that express *mys*-RNAi via GH146, *Poxn*-Gal4, Gr66a, and Gr5a do not have defects in BNZ avoidance, the data indicate that projection neurons, *Poxn* chemosensory neurons, and neurons that detect sweet and bitter gustatory stimuli, respectively, are also not likely to be key sites for *mys* function within the context of BNZ avoidance. Expression of *mys*-RNAi in the mushroom bodies via 107 and C309 did not disrupt olfactory behavior, consistent with previous reports indicating that this region of the nervous system is not involved in naive responses to aversive odorants (de Belle and Heisenberg, 1994; McGuire *et al.*, 2001). Based on the overlapping expression patterns of H24, MT14, C133, 59Y, and 16Y, our current model is that expression of *mys*-RNAi in local interneurons of the antennal lobes disrupts avoidance of BNZ. We note, though, that BNZ avoidance was not

statistically reduced in flies with *mys*-RNAi expression driven in the antennal lobe by C133, 59Y, 125Y, and 16Y. One possible explanation for this apparent discrepancy is that the antennal lobe Gal4 lines we used do not express at high levels in the key interneurons involved in behavioral responses to the odorants we tested. Another possible explanation is that H24- and MT14-driven expression of *mys*-RNAi disrupts olfactory behavior because these two Gal4 lines drive expression in the antennal lobe interneurons at critical times during development, whereas C133, 59Y, 125Y, and 16Y do not. Our model of *mys* functioning within local interneurons of the antennal lobes, however, requires additional testing. For example, our studies do not exclude the possibility that H24 and MT14 drive expression in an as yet unidentified component of the olfactory system of *Drosophila*.

Previous studies suggest that *mys* is involved in behavioral responses to IAA and possibly BNZ and EA (Ayyub *et al.*, 2000). To further address whether *mys* is important for detection of all odorants, we assessed behavioral responses to a panel of odorants in flies that express *mys*-RNAi. H24-driven expression of *mys*-RNAi disrupted avoidance of BNZ and MCH as well as attraction to EA. In contrast, H24/*mys*-RNAi flies had only marginal or no defects in avoidance of HEP, LIN, and IAA. Our data indicate that *mys* is important for behavioral responses to aversive as well as attractive odorants and that behavioral responses to some, but not all, odorants might depend on expression of *mys*. It is interesting that flies expressing *mys*-RNAi had no detectable change in IAA, whereas several *mys* loss-of-function mutants have defects in their behavioral response to this odorant (Ayyub *et al.*, 2000). Additional experiments will be required to determine whether this apparent discrepancy is due to differences between using T-maze and odor jump assays, tissue-limited knockdown of *mys* versus ubiquitous reduction in *mys* function, or some other reason. Nevertheless, our data confirm that *mys* is important for olfactory behavior in *Drosophila*.

To more rigorously map the defect in olfactory behavior to the *mys* locus, we performed two additional genetic studies. In the first study, we assessed BNZ avoidance in flies harboring H24 and either of two independently derived *mys*-RNAi transgenes. As found in all our other experiments, flies expressing *mys*-RNAi from these additional transgenes have defects in BNZ avoidance. Thus, expression of multiple *mys*-RNAi transgenes produces the same behavioral phenotype. In the second study, we evaluated the effect of expressing wild-type *mys* on the olfactory behavior defects in *mys*-RNAi flies. Overexpression of wild-type *mys* in an otherwise normal fly disrupts attraction and aversion to BNZ, consistent with a dominant-negative effect of overexpressing integrins found by others (Brabant *et al.*, 1996). Nevertheless, expression of wild-type *mys* completely rescues the defects in BNZ attraction and aversion. These data are consistent with transgenic wild-type *mys* compensating for the knockdown of endogenous *mys* by *mys*-RNAi. Together, these two studies confirm that olfactory behavior in *Drosophila* requires expression of *mys*.

The olfactory behavioral deficits in flies that express *mys*-RNAi could be caused by developmental abnormalities or a disruption in the acute physiological events involved in the behavior. To begin to address this question, we evaluated brain morphology in *mys*-RNAi flies using two different neuropil markers, DLG and nc82, on cryosections. At the level of resolution possible with light microscopy, expression of *mys*-RNAi via H24 had no obvious effect on the morphology of the antennal lobes, mushroom bodies, central complex, or other central brain regions. These data demonstrate that H24/*mys*-RNAi flies do not have grossly deranged central nervous system development and are consistent with an acute role for *mys* in olfactory behavior. Additional studies will be required, however, to formally resolve whether *mys* mediates developmental or acute processes that underlie olfactory behavior in *Drosophila*.

Acknowledgements

The authors thank Danny Brower (University of Arizona), Ennio Giordano (Universita di Napoli), L. S. Shashidhara (CCMB, Hyderabad), Alois Hofbauer (University of Regensburg), Ron Davis (Baylor College of Medicine), Douglas Armstrong (University System of Taiwan), Martin Heisenberg (Universitat Wurzburg), Reinhard Stocker (University of Fribourg), Patrick Callaerts (University of Houston), Liqun Luo (Stanford University), Scott Waddell (University of Massachusetts), Kristen Scott (University of California, Berkeley), John Carlson (Yale University), and Danny Brower (University of Arizona) for providing fly stocks and other reagents. The authors appreciate the technical assistance of Ryan Lewandowski and Jessica Howell. This work was supported by grants to M.S.G. from the National Institutes of Health.

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Accepted May 17, 2006