Research

Roles for *Drosophila* mushroom body neurons in olfactory learning and memory

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Olfactory learning assays in *Drosophila* have revealed that distinct brain structures known as mushroom bodies (MBs) are critical for the associative learning and memory of olfactory stimuli. However, the precise roles of the different neurons comprising the MBs are still under debate. The confusion surrounding the roles of the different neurons may be due, in part, to the use of different odors as conditioned stimuli in previous studies. We investigated the requirements for the different MB neurons, specifically the α/β versus the γ neurons, and whether olfactory learning is supported by different subsets of MB neurons irrespective of the odors used as conditioned stimuli. We expressed the *rutabaga* (*rut*)-encoded adenylyl cyclase in either the γ or α/β neurons and examined the effects on restoring olfactory associative learning and memory of *rut* mutant flies. We also expressed a temperature-sensitive *shibire* (*shi*) transgene in these neuron sets and examined the effects of disrupting synaptic vesicle recycling on *Drosophila* olfactory learning. Our results indicate that although we did not detect odor-pair-specific learning using GAL4 drivers that primarily express in γ neurons, expression of the transgenes in a subset of α/β neurons resulted in both odor-pair-specific rescue of the *rut* defect as well as odor-pair-specific disruption of learning using *shi*^{ts1}.

Numerous studies indicate that the *Drosophila* mushroom bodies (MBs), a paired neuropil structure in the central brain, are critical for associative learning and memory (for review, see Roman and Davis 2001; Waddell and Quinn 2001; Dubnau et al. 2003; Heisenberg 2003; Davis 2005). The MBs are composed of three distinct classes of neurons—alpha/beta (α/β), alpha'/beta' (α'/β'), and gamma (γ)—which differentiate in a strict developmental order (Lee et al. 1999) and exhibit varied expression levels for several genes preferentially expressed in the MBs (Crittenden et al. 1998), suggesting different functional roles. The axons of the α/β and the α'/β' MB neurons split into two separate branches, while the axons of the γ MB neurons are unbranched.

Genetic and pharmacological disruptions of the MB neurons that result in anatomical defects or even complete absence of these neurons impair or abolish the learning of odors paired with electric shock reinforcement without grossly perturbing sensorimotor responses to the stimuli (de Belle and Heisenberg 1994; Connolly et al. 1996; Davis 1996; Heisenberg 1998; Wolf et al. 1998). Additionally, various genes associated with learning- and memory-related biochemical cascades have elevated levels of expression in MB neurons (Nighorn et al. 1991; Han et al. 1992; Skoulakis et al. 1993; Han et al. 1996; Skoulakis and Davis 1996; Muller 1997; Grotewiel et al. 1998; Roman and Davis 2001). Some of these genes encode molecules that are involved in cyclic AMP (cAMP) signaling: *rutabaga* (*rut*) encodes a Ca²⁺/calmodulin-dependent adenylyl cyclase (AC) that converts ATP to cAMP; *dunce* (*dnc*) encodes a phosphodiesterase involved in degrading cAMP; and DCO codes for a protein kinase A catalytic subunit. The importance of the cAMP signaling pathway for learning and memory is further supported by disruptions of genes encoding

other molecules involved in this pathway to produce memory-defective flies; the list of these disruptions has grown to include G-protein subunits, regulatory subunits of protein kinase, and cAMP-responsive transcription factor CREB (Waddell and Quinn 2001; Davis 2005). Other genes that are preferentially expressed in *Drosophila* MBs include *leonardo* (*leo*, 14–3–3), *Volado* (*vol*, codes for an α -integrin), and *fasciclin II* (*fasII*), whereas *amnesiac* (*amn*, homologous to a pituitary peptide that activates AC) is expressed preferentially in dorsal paired medial (DPM) neurons that project axons to the MB (Waddell et al. 2000). Mutations in these genes result in learning and memory defects (for review, see Roman and Davis 2001; Davis 2005).

Although the convincing picture that emerges suggests that the *Drosophila* MBs are an important center for associative learning and memory of olfactory stimuli, the issue of the precise roles for the different neurons comprising the MBs is controversial. Zars et al. (2000a) claimed that restoring *rut* AC expression specifically in the γ neurons of an otherwise *rut* mutant is sufficient to support normal olfactory learning. In contrast, McGuire et al. (2003) reported that expression of *rut* in both α/β and γ neurons is required to obtain complete rescue of the *rut* learning defect. They further reported that the synaptic output of α/β neurons is required for olfactory memory retrieval, but not acquisition or consolidation (McGuire et al. 2001). For the latter experiments, they specifically expressed in these neurons the temperature-sensitive *shibire* (*shi*) gene, which encodes a defective dynamin GTPase that dominantly disrupts synaptic transmission at temperatures above 29°C quickly and reversibly (Kitamoto 2001). In other studies involving *Drosophila* long-term olfactory memory, mutants with malformed MBs that were missing α/α' axon collaterals have no detectable long-term memory using a spaced training protocol (Pascual and Preat 2001), suggesting that long-term memory forms specifically in the α/α' collaterals or that it is retrieved specifically from these branches.

Olfactory learning assays in *Drosophila* are typically performed by exposing the flies to two odors in succession, one odor

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(the conditioned stimulus, CS+) paired with electric shock pulses (unconditioned stimulus; US) followed by a counter-odor (the CS-) without electric shock. Following training, the flies are presented with the two odors in a T-maze, and their avoidance of the CS+ is computed as a performance index (P.I.). Several variations of this assay have been developed (for review, see Davis 2005), but one variation that may have added to the confusion surrounding the roles of the different neurons of the MBs is the use of different odorants. Studies that have examined the contributions of the different MB neurons for olfactory learning have used different combinations of odors in the assays. Although most studies have used 4-methylcyclohexanol (MCH) and 3-octanol (OCT) as the CS+ and CS- (Connolly et al. 1996; McGuire et al. 2003; Isabel et al. 2004), some studies have used OCT and benzaldehyde (BEN) (McGuire et al. 2001), or MCH and BEN (Zars et al. 2000a). A recent paper by Keene et al. (2004) describes that flies sense BEN both by the classical olfactory pathway as well as a noncanonical route that involves bitter-sensitive gustatory neurons and *pox-neuro* (*poxn*)-affected neurons that are likely on the legs or mouthparts of *Drosophila*. The question posed by these variations in the use of odorants for olfactory training is whether olfactory associative learning is equally supported irrespective of the choice of odors, or whether the combination of odors used has an effect on the learning that takes place. It is possible, for instance, that different odors are represented differentially among the three classes of MB neurons. If the different odors are not represented equally by the MB neurons, either because of wiring specificities or differences in activation properties, this could explain some of the discrepancies that are found in the literature regarding the relative importance of the different MB neurons in olfactory learning.

Expression of *rut* in the MBs during adulthood has been shown to be sufficient for the rescue of the *rut* memory impairment, while expression during development is inconsequential to olfactory memory formation (McGuire et al. 2003; Mao et al. 2004). In our study, we examined the effects of restoring *rut*-encoded AC specifically to different neurons of the MBs of *rut* learning-deficient mutants. In parallel experiments, we drove the MB neuron-specific expression of a *shi* transgene to disrupt synaptic transmission at temperatures above 29°C and examined the effects on *Drosophila* learning. Both sets of experiments were performed using the three different odor combinations (MCH vs. OCT, MCH vs. BEN, and OCT vs. BEN). To our knowledge, this is the first time that experiments using all three odor combinations and using two different experimental manipulations have been used to investigate the roles of the different MB neurons. Our results demonstrate that although we did not detect any odor-specificity using drivers that express in *Drosophila* γ neurons, a thin core of α/β neurons may be differentially involved in learning to specific combinations of odors.

Results

GAL4 enhancer detector lines express in distinct subsets of *Drosophila* MB neurons

Expression patterns of different enhancer detector lines were examined by GAL4-dependent β -gal expression in adult *Drosophila* brain. We selected lines that have been identified in previous experiments to have enriched expression within subsets of MB neurons (Yang et al. 1995; Armstrong et al. 1998; Martin et al. 1998; Zars et al. 2000a,b; McGuire et al. 2001). In addition, we screened numerous new GAL4 lines and identified a driver with enriched expression in the γ neurons (*NP1131-GAL4*). Comparison of GAL4 lines 17d and c739 that were stained for the same amount of time (Fig. 1A,C) showed that although both have

Figure 1. Spatial expression patterns in the brains of flies carrying different GAL4 enhancer detector elements and a *UAS-lacZ* transposon. Frontal sections through the adult brain were stained for 18 h in a humidified chamber at 37°C for β -galactosidase activity and examined. Sections at the level of the mushroom body α/β and γ lobes are shown. (A,C) GAL4 lines 17d and c739 showed expression in the mushroom body α/β lobes, although the expression level of 17d-GAL4 was less because this line marks only a subset of α/β neurons when compared to c739-GAL4, which stained the α/β lobes more completely. (B,D) GAL4 lines NP1131 and H24 showed common expression in the γ lobes of the mushroom bodies with H24-GAL4 exhibiting robust expression in the antennal lobes (AL) as well. (E,F) Double GAL4 line c739;H24 showed expression in both the α/β lobes and the γ lobes.

expression in the MB α/β lobes, the expression level of 17d-GAL4 is less, and it stains only a subset of α/β neurons when compared to c739-GAL4, which appears to stain the α/β lobes more completely. GAL4 lines NP1131 and H24 (Fig. 1B,D) showed common expression in the γ neurons of the MBs with line H24-GAL4 additionally exhibiting robust expression in the antennal lobes (ALs) and ellipsoid bodies. Higher expression of the reporter is likewise observed for H24-GAL4, with NP1131-GAL4 showing modest levels of expression in the γ neurons but no expression in the ALs. In addition, NP1131-GAL4 expression is observed in a subset of α'/β' neurons that is better detected using GFP (Ito et al. 2003; N.K. Tanaka and K. Ito, unpubl.). For the double GAL4 line c739;H24 (Fig. 1E,F), two representative sections are shown that confirm expression in both the α/β and γ neurons. Compared to the single GAL4 drivers c739 and H24, the expression levels do not appear to be significantly altered when a combination of the two GAL4 drivers is present in the same fly.

Driving *rut* expression in γ neurons results in partial rescue of the *rut* learning defect for all odor combinations tested

Since it is well-established that the MBs are critical for olfactory learning and memory (for review, see Roman and Davis 2001; Waddell and Quinn 2001; Dubnau et al. 2003; Heisenberg 2003; Davis 2005), we wanted to determine whether the neurons comprising the different lobes played specific roles in olfactory learning and memory. We therefore examined the effects of restoring

rut-encoded AC specifically to different subsets of MB neurons of *rut* learning-deficient mutants. GAL4 drivers that express in the γ neurons are not numerous; a commonly used line has been *H24-GAL4* (Zars et al. 2000a; McGuire et al. 2003). However, in addition to the γ MB neurons, *H24-GAL4* also has robust expression in the ALs (Fig. 1D), and this extraneous expression might affect odor perception that would produce defects in olfactory learning. In fact, the expression of a constitutively activated stimulatory heterotrimeric GTP-binding protein α -subunit (Connolly et al. 1996) by *H24-GAL4* impairs olfactory avoidance and produces a severe wing phenotype (data not shown). We thus used *NP1131-GAL4*, which shows enriched expression in γ neurons (Fig. 1B), in addition to *H24-GAL4*, to verify the role of the γ neurons in olfactory learning.

We first assessed the responses of the different genotypes used in our studies by obtaining 2–5-d-old flies and testing each genotype for normal odor and shock avoidance (Table 1). Odor avoidance was assayed by measuring the choice of naive flies between odor and fresh air at odor concentrations used during training and testing. For each odorant, a one-way ANOVA with genotype as the main effect was performed, and experiments were comprised of $n \geq 6$ per group. Electric shock avoidance was assayed by measuring the avoidance response of naive flies to 90 V DC shock. A one-way ANOVA with genotype as the main effect was per-

Figure 2. Restoring *rut* in γ MB neurons produces a partial rescue of the learning-deficiency of *rut* mutants for all odors tested. Three-minute memory for flies carrying GAL4 drivers that express in γ neurons. (A,B,C) Flies bearing *H24-GAL4* in combination with the *UAS-rut* transgene demonstrated significant rescue of *rut* memory defect over the mutant control group *rut*²⁰⁸⁰; *H24-GAL4* ($P \leq 0.0006$), but were significantly impaired compared to control flies that were CS; *H24-GAL4* for all odor combinations tested ($P \leq 0.0006$). $n = 18$ per group except $n = 12$ for the MCH-BEN experiment. (D,E,F) Flies of the genotype *rut*²⁰⁸⁰; *NP1131-GAL4*; *UAS-rut* performed significantly better than control flies that were *rut*²⁰⁸⁰; *NP1131-GAL4* ($P \leq 0.0049$), but were significantly impaired compared to control flies that were CS; *NP1131-GAL4* for all odor combinations tested ($P \leq 0.0001$). $n = 18$ per group except $n = 17$ for MCH-BEN experiment. This suggests that driving *rut* in γ neurons results in partial rescue of the *rut* learning defect irrespective of odor combination used. In all experiments, the P.I.'s were subjected to a one-way ANOVA with genotype as the main effect, followed by post hoc analysis with the Bonferroni/Dunn test. Statistical significance is indicated by asterisks (**).

formed, and experiments were comprised of $n = 6$ per group. No significant differences were observed for odor and shock avoidance for the genotypes used in the *rut* rescue experiments (Table 1).

Table 1. Odor and shock avoidance scores for the different fly stocks used in the *rut* rescue experiments

Genotype	Odor avoidance			Shock avoidance
	MCH	OCT	BEN	
CS; <i>H24-GAL4</i>	0.84 ± 0.06	0.78 ± 0.04	0.92 ± 0.02	0.90 ± 0.03
<i>rut</i> ²⁰⁸⁰ ; <i>H24-GAL4/UAS-rut</i>	0.90 ± 0.01	0.74 ± 0.03	0.90 ± 0.04	0.86 ± 0.04
<i>rut</i> ²⁰⁸⁰ ; <i>H24-GAL4</i>	0.89 ± 0.02	0.78 ± 0.05	0.94 ± 0.02	0.84 ± 0.04
CS; <i>NP1131-GAL4</i>	0.73 ± 0.08	0.78 ± 0.04	0.73 ± 0.08	0.84 ± 0.04
<i>rut</i> ²⁰⁸⁰ ; <i>NP1131-GAL4</i> ; <i>UAS-rut</i>	0.76 ± 0.06	0.68 ± 0.06	0.76 ± 0.06	0.84 ± 0.03
<i>rut</i> ²⁰⁸⁰ ; <i>NP1131-GAL4</i>	0.75 ± 0.04	0.73 ± 0.04	0.75 ± 0.04	0.87 ± 0.04
CS; <i>c739-GAL4</i>	0.62 ± 0.03	0.74 ± 0.03	0.82 ± 0.03	0.80 ± 0.08
<i>rut</i> ²⁰⁸⁰ ; <i>c739-GAL4</i> ; <i>UAS-rut</i>	0.66 ± 0.07	0.76 ± 0.06	0.80 ± 0.04	0.76 ± 0.05
<i>rut</i> ²⁰⁸⁰ ; <i>c739-GAL4</i>	0.68 ± 0.06	0.75 ± 0.04	0.82 ± 0.03	0.75 ± 0.04
CS; <i>17d-GAL4</i>	0.66 ± 0.03	0.66 ± 0.05	0.78 ± 0.07	0.82 ± 0.08
<i>rut</i> ²⁰⁸⁰ ; <i>17d-GAL4</i> ; <i>UAS-rut</i>	0.67 ± 0.04	0.55 ± 0.07	0.76 ± 0.04	0.62 ± 0.06
<i>rut</i> ²⁰⁸⁰ ; <i>17d-GAL4</i>	0.70 ± 0.05	0.60 ± 0.06	0.74 ± 0.03	0.72 ± 0.08
CS; <i>c739-GAL4</i> ; <i>H24-GAL4</i>	0.74 ± 0.07	0.69 ± 0.07	0.85 ± 0.05	0.84 ± 0.04
<i>rut</i> ²⁰⁸⁰ ; <i>c739-GAL4</i> ; <i>H24-GAL4/UAS-rut</i>	0.75 ± 0.07	0.74 ± 0.03	0.82 ± 0.04	0.82 ± 0.04
<i>rut</i> ²⁰⁸⁰ ; <i>c739-GAL4</i> ; <i>H24-GAL4</i>	0.78 ± 0.03	0.68 ± 0.02	0.82 ± 0.05	0.80 ± 0.02

Odor avoidance was assayed by measuring the choice of naive flies between odor and fresh air at odor concentrations used during training and testing. Air was bubbled through an odorant:mineral oil mixture. Odor avoidance scores were compared based on the GAL4 driver used and compared with control groups that were comprised of the GAL4 driver crossed with wild-type *w* flies or the GAL4 driver crossed with the *UAS-rut* transgene. For each odorant, a one-way ANOVA with genotype as the main effect was performed, and experiments were comprised of $n \geq 6$ per group. No significant differences for odor avoidance were observed ($P \geq 0.1749$). Electric shock avoidance was assayed by measuring the avoidance response of naive flies to 90 V DC shock. A one-way ANOVA with genotype as the main effect did not detect any significant differences ($P \geq 0.0759$, $n = 6$ per group).

The results in Figure 2 demonstrate that restoring *rut*-encoded AC specifically to the different neurons defined by *H24-GAL4* or *NP1131-GAL4* results in a partial rescue of the learning deficiency in *rut* mutants. This partial rescue was observed for the different odor combinations tested: MCH-BEN (Fig. 2A,D), MCH-OCT (Fig. 2B,E), and OCT-BEN (Fig. 2C,F). In each case, flies of the genotype *rut*²⁰⁸⁰; *H24-GAL4/UAS-rut* performed significantly better than control flies that were *rut*²⁰⁸⁰; *H24-GAL4*, but significantly worse than control flies that were CS; *H24-GAL4* (Fig. 2A,B,C). Likewise, flies of the genotype *rut*²⁰⁸⁰; *NP1131-GAL4/UAS-rut* performed significantly better for all odor pairs than control flies that were *rut*²⁰⁸⁰; *NP1131-GAL4*, but significantly worse than control flies that were CS; *NP1131-GAL4* (Fig. 2D,E,F). It has been shown that expression of *rut* in the MBs during adulthood is sufficient for the rescue of the *rut* memory impairment, while expression during development is inconsequential to olfactory memory formation (McGuire et al. 2003; Mao et al. 2004). It has also been established using an inducible gene-switch system that 24 h of time is sufficient to increase levels of *rut* so that memory scores are indistinguishable from those of wild-type flies (Mao et al. 2004). Since we used flies that are at least 2 d old, *rut* should be at sufficient levels within the spatial expression pattern defined by the GAL4 driver to promote rescue of the *rut* memory impairment. Our results, therefore, establish that expression of *rut* in the γ MB neurons defined by either *H24-GAL4* or *NP1131-GAL4* is insufficient to promote complete behavioral rescue, irrespective of the odor combination used. However, the partial

rescue observed suggests that AC expression in the γ neurons is part of the neuroanatomical template on which memories are formed.

Expression of *rut* and *shi*^{ts1} in a subset of α/β neurons reveals an odor-pair-specific effect on olfactory learning

Expression of *shi*^{ts1} using *c739-GAL4* revealed a requirement for the α/β neurons during memory retrieval, but not during acquisition or consolidation (McGuire et al. 2001). In contrast, mutant *rut* flies with *c739-GAL4* in combination with the *UAS-rut* transgene had *rut* mutant-like learning scores (McGuire et al. 2003). A notable difference between these two experiments was the use of different combinations of odorants for the learning assay: McGuire et al. (2001) used OCT and BEN, whereas McGuire et al. (2003) used MCH and OCT. Furthermore, *rut* mutant flies carrying the *17d-GAL4* driver along with *UAS-rut* also exhibit impaired olfactory learning (Zars et al. 2000a) with MCH and BEN as odorants. To test whether these apparent discrepancies were due to the odor combinations used, we performed *rut* rescue experiments using these two drivers and the three different odor pairs.

Our results demonstrate that restoring *rut*-encoded AC specifically to the neurons defined by *c739-GAL4* fails to rescue the learning deficiency in *rut* mutants for all odor combinations tested (Fig. 3A,B,C). In these experiments, flies of the genotype *rut*²⁰⁸⁰; *c739-GAL4/UAS-rut* exhibited performance that was indistinguishable from that of *rut*²⁰⁸⁰; *c739-GAL4* flies. Surprisingly, driving expression with *17d-GAL4*, which targets a subset

of α/β neurons, conferred partial rescue with the MCH-BEN and OCT-BEN combinations (Fig. 3D,F), but not with the MCH-OCT combination (Fig. 3E).

To extend these odor-pair-specific behavioral studies, we used the GAL4 drivers to inactivate synaptic transmission from α/β neurons using the dominant and temperature-sensitive *shi*^{ts1} transgene (Kitamoto 2001). We first extended the aforementioned experiments of McGuire et al. (2001) by examining the effects of driving *UAS-shi*^{ts1} using *c739-GAL4* for all three odor combinations. Training and testing for 3-min memory was performed entirely at the permissive (25°C) or restrictive (32°C) temperatures. In each experiment, we compared the performance of *c739-GAL4/UAS-shi*^{ts1} flies to control flies bearing just the GAL4 element (*w*; *c739-GAL4*) or the *UAS-shi*^{ts1} element alone (*w*; *UAS-shi*^{ts1}) at the permissive (25°C) and restrictive (32°C) temperatures. For each odor pair, performance of *c739-GAL4/UAS-shi*^{ts1} flies at 25°C was indistinguishable from that of control flies that were *w*; *c739-GAL4* and *w*; *UAS-shi*^{ts1}. The *c739-GAL4/UAS-shi*^{ts1} flies exhibited a partially impaired performance at the restrictive temperature irrespective of the odor pair used for training and testing (Fig. 4A,B,C). This deficit is consistent with previous experiments demonstrating a disruption of 3-min memory when *c739-GAL4* drives the expression of *UAS-shi*^{ts1} (McGuire et al. 2001). However, it stands in contrast with our previous results demonstrating

Figure 3. Restoring *rut* in a subset of MB α/β neurons results in partial rescue of the learning-deficiency of *rut* mutants for two odor pairs. Three-minute memory rescue in lines carrying GAL4 drivers that express in α/β neurons. (A,B,C) *Rut* rescue with *c739-GAL4* using different odor combinations. Flies bearing *c739-GAL4* in combination with the *UAS-rut* transgene demonstrated no significant rescue of *rut* memory defect over the mutant control group *rut*²⁰⁸⁰; *c739-GAL4* for odor combinations MCH-BEN ($P = 0.0886$, $n = 12$), MCH-OCT ($P = 0.7554$, $n = 10$), and OCT-BEN ($P = 0.6218$, $n = 12$). For all odor combinations tested, *rut*²⁰⁸⁰; *c739-GAL4/UAS-rut* flies performed significantly differently from control flies that were CS; *c739-GAL4* ($P < 0.0001$), but were indistinguishable from control flies that were *rut*²⁰⁸⁰; *c739-GAL4*. (D–F) *Rut* rescue with *17d-GAL4*, a line that expresses in a subset of α/β neurons, using different odor combinations. Flies bearing the GAL4 driver 17d in combination with the *UAS-rut* transgene demonstrated significant rescue of the *rut* memory defect over the mutant control group *rut*²⁰⁸⁰; *17d-GAL4* for odor combinations MCH-BEN ($P < 0.0001$, $n = 24$) and OCT-BEN ($P = 0.0047$, $n = 12$), but not MCH-OCT ($P = 0.2577$, $n = 12$). For all odor combinations tested, *rut*²⁰⁸⁰; *17d-GAL4/UAS-rut* flies performed significantly more poorly than control flies that were CS; *17d-GAL4* ($P < 0.0001$). In all experiments, the P.I.'s were subjected to a one-way ANOVA with genotype as the main effect, followed by post hoc analysis with the Bonferroni/Dunn test. Statistical significance (**) or nonsignificance (N.S.) is indicated.

Figure 4. Odor-pair-specific disruption of 3-min memory when MB signaling from α/β neurons is blocked. Three-minute memory performance of flies carrying $UAS-shi^{ts1}$ in combination with the GAL4 drivers $c739$ or $17d$ with training and testing performed under permissive (25°C) and restrictive (32°C) conditions. The different odor combinations used for the experiments are indicated above the graphs: MCH-BEN (A,D), MCH-OCT (B,E), and OCT-BEN (C,F). (A,B,C) Performance of flies of the genotype $c739-GAL4; UAS-shi^{ts1}$ were indistinguishable from controls $w; c739-GAL4$ and $w; UAS-shi^{ts1}$ at the permissive temperature for MCH-BEN ($P \geq 0.2820$, $n = 6$), MCH-OCT ($P \geq 0.4691$, $n = 10$), and OCT-BEN ($P \geq 0.2569$, $n = 6$), but showed a significant disruption of memory at the restrictive temperature for MCH-BEN ($P \leq 0.0127$, $n = 6$), MCH-OCT ($P \leq 0.0014$, $n = 6$), and OCT-BEN ($P \leq 0.0002$, $n = 9$). (D–F) Performance of flies of the genotype $17d-GAL4; UAS-shi^{ts1}$ were indistinguishable from controls $w; 17d-GAL4$ and $w; UAS-shi^{ts1}$ at the permissive temperature for MCH-BEN ($P \geq 0.0981$, $n = 6$), MCH-OCT ($P \geq 0.7679$, $n = 10$), and OCT-BEN ($P \geq 0.0404$, $n = 13$). Significant disruptions of memory were observed for $17d-GAL4; UAS-shi^{ts1}$ flies at the restrictive temperature when compared to controls for MCH-BEN ($P \leq 0.0008$) and OCT-BEN ($P \leq 0.0111$) but not for MCH-OCT ($P \geq 0.2310$, $n = 10$ for all). In all experiments, the P.I.'s were subjected to a one-way ANOVA with genotype as the main effect, followed by post hoc analysis with the Bonferroni/Dunn test. Comparisons among the three different genotypes were performed, producing three pairwise planned comparisons per experiment at each of the temperatures. Comparisons were not significant unless indicated by asterisks (**).

no rescue of the *rut* memory defect using *c739-GAL4* (Fig. 3A,B,C). This may be caused simply by the nature of each experiment. The *rut* rescue experiments involve restoring some level of *rut* specifically to the neurons defined by the GAL4 drivers. On the other hand, the *shi^{ts1}* experiments involve blocking neurotransmission from these neurons. It is possible that under the conditions described for the experiments (see Materials and Methods), the levels of *rut* restored to the neurons defined by *c739-GAL4* are insufficient to restore performance to levels that are indistinguishable from controls, but that inactivating neurotransmission from these neurons is sufficient to produce significant deficits in performance.

We also assayed 3-min memory for flies bearing the GAL4 element *17d* along with the *UAS-shi^{ts1}* transgene. In each experiment, we compared the performance of *17d-GAL4; UAS-shi^{ts1}* flies to control flies bearing only the GAL4 element ($w; 17d-GAL4$) or the *UAS-shi^{ts1}* element alone ($w; UAS-shi^{ts1}$) at the permissive and restrictive temperatures. We observed the same odor-pair-specific effects in these experiments that were observed in the *rut* rescue experiments involving *17d-GAL4* (Fig. 3). The *17d-GAL4; UAS-shi^{ts1}* flies showed disruption of 3-min memory at the restrictive temperature using MCH-BEN (Fig. 4D) and OCT-BEN (Fig. 4F) when compared to the $w; 17d-GAL4$ and $w; UAS-shi^{ts1}$ controls. In contrast, no disruption at the restrictive temperature was observed using the odorants MCH and OCT (Fig. 4E). This is consistent with our previous results with *17d-GAL4* that showed no

rescue of the *rut* defect when MCH and OCT are used as odorants (Fig. 3E). In each case, the performance of *17d-GAL4; UAS-shi^{ts1}* flies at the permissive temperature was indistinguishable from that of the control flies $w; 17d-GAL4$ and $w; UAS-shi^{ts1}$, regardless of the odor combination used. Odor and shock avoidance for each genotype was tested at the permissive and restrictive temperatures (Table 2).

Expression of *rut* in both α/β and γ neurons rescues completely the *rut* learning defect for all odor combinations

Previous studies have shown that the α/β neurons are involved in the retrieval of short-term memory (McGuire et al. 2001), yet *rut* expression in these neurons provides no detectable rescue of the *rut* mutant learning defect (Fig. 3). Additionally, *rut* expression with a GAL4 driver that specifically labels γ neurons results in partial rescue of this *rut* mutant learning phenotype (Fig. 2; Zars et al. 2000a). To determine whether full rescue could be observed with expression in both α/β and γ MB neurons, McGuire et al. (2001) introduced two GAL4 drivers (*c739* and *H24*) into the same fly (McGuire et al. 2003). They found that *rut* mutant flies carrying both drivers in combination with the *UAS-rut* transgene had memory performance that was indistinguishable from that of wild-type control flies.

To determine if this complete rescue of the *rut* mutant phenotype holds true for all odor combinations, we repeated these experiments using all three odor combinations. Flies of the genotype *rut²⁰⁸⁰; c739-GAL4; H24-GAL4/UAS-rut* performed significantly better than control flies that were *rut²⁰⁸⁰; c739-GAL4; H24-GAL4* and had performance scores that were indistinguishable from control flies that were *CS; c739-GAL4; H24-GAL4* (Fig. 5). This complete rescue was observed for all odor combinations tested. We cannot determine from these experiments whether this complete rescue occurs by completing a spatial circuit that involves odorant representation in both α/β and γ neurons, or whether it represents the massed effect of expression in more MB neurons.

17d-GAL4 expresses in less than half the number of neurons compared to *c739-GAL4*

To extend our examination of the expression patterns of the different GAL4 drivers, we visualized the expression patterns at the level of the MB cell body region using antibodies against nuclear-localized GFP and the pan-neural marker ELAV (Robinson et al. 1988). Whole-mount brains were examined under a confocal microscope, and serial sections were taken at $0.5\text{-}\mu\text{m}$ intervals. Representative Z-projections of confocal stacks that were generated using ImageJ (Abramoff et al. 2004) are shown in Figure 6A. Only one brain hemisphere is shown since the brains for each group showed overall stereotypy and bilateral symmetry. Each panel was from an individual fly, and expression of GAL4

Table 2. Odor and shock avoidance scores for the different fly stocks used in the *shi^{ts1}* experiments

Genotype	Temperature	Odor avoidance			Shock avoidance
		MCH	OCT	BEN	
<i>w; c739-GAL4</i>	25°C	0.77 ± 0.04	0.82 ± 0.04	0.92 ± 0.02	0.90 ± 0.02
	32°C	0.79 ± 0.03	0.68 ± 0.03	0.88 ± 0.05	0.78 ± 0.04
<i>w; UAS-shi^{ts1}</i>	25°C	0.64 ± 0.05	0.88 ± 0.03	0.91 ± 0.03	0.82 ± 0.03
	32°C	0.65 ± 0.05	0.76 ± 0.03	0.94 ± 0.02	0.84 ± 0.02
<i>c739-GAL4; UAS-shi^{ts1}</i>	25°C	0.60 ± 0.05	0.74 ± 0.05	0.90 ± 0.03	0.88 ± 0.02
	32°C	0.65 ± 0.04	0.71 ± 0.04	0.84 ± 0.06	0.72 ± 0.05
<i>w; 17d-GAL4</i>	25°C	0.72 ± 0.04	0.91 ± 0.04	0.88 ± 0.05	0.80 ± 0.03
	32°C	0.58 ± 0.07	0.76 ± 0.02	0.81 ± 0.06	0.56 ± 0.08
<i>w; UAS-shi^{ts1}</i>	25°C	0.68 ± 0.06	0.82 ± 0.04	0.84 ± 0.04	0.87 ± 0.03
	32°C	0.62 ± 0.05	0.76 ± 0.02	0.90 ± 0.05	0.62 ± 0.06
<i>17d-GAL4; UAS-shi^{ts1}</i>	25°C	0.75 ± 0.05	0.87 ± 0.04	0.83 ± 0.02	0.79 ± 0.04
	32°C	0.44 ± 0.05	0.74 ± 0.05	0.87 ± 0.05	0.59 ± 0.06

Odor avoidance was assayed by measuring the choice of naive flies between odor and fresh air at odor concentrations used during training and testing. Air was bubbled through an odorant:mineral oil mixture. For each odorant, a one-way ANOVA with genotype as the main effect was performed for groups tested under similar conditions (25°C or 32°C; $n \geq 6$ per group). Post hoc analysis using the Bonferroni/Dunn test indicated that MCH odor avoidance of flies carrying the *UAS-shi^{ts1}* transgene with *GAL4* driver *c739* was lower than the control line *w; c739-GAL4* for both temperatures tested ($P \leq 0.0166$). However, MCH odor avoidance of these flies was not significantly different from the avoidance of *w; UAS-shi^{ts1}*, another control strain used in our experiments ($P \geq 0.5653$). No other significant differences were found in odor avoidance ($P \geq 0.0266$). Electric shock avoidance was assayed by measuring the avoidance response of naive flies to 90 V DC shock. A one-way ANOVA with genotype as the main effect followed by post hoc analysis using the Bonferroni/Dunn test was performed for groups tested under similar conditions (25°C or 32°C; $n = 6$ per group). No significant differences for shock avoidance were observed ($P \geq 0.0459$).

drivers was conserved across flies. We counted the number of neurons expressing the GFP reporter after image segmentation and identification of nuclei (Ponomarev and Davis 2003) and found no significant differences ($P \geq 0.4052$, $n = 5$) between the neuron counts for the left and right MB Kenyon cell (KC) soma region for the different *GAL4* drivers examined (Fig. 6B). From our neuron counts, we estimate that *17d-GAL4* is expressed in less than half (40%) the number of neurons that express GFP with *c739-GAL4* ($n = 10$ KC soma regions from five flies). Further analyses of the confocal Z-series for the two drivers reveal the presence of four clusters of neurons (arrowheads), which probably correspond to their separate lineages. MB cells are derived from four identical neuroblasts via asymmetric divisions through the embryonic, larval, and pupal stages with the γ neurons being born before the mid-third instar stage, the α'/β' neurons born between the mid-third instar stage and puparium formation, and the α/β neurons born starting at puparium formation (Lee et al. 1999). Comparison of the neuron counts for *H24-GAL4* and

NP1131-GAL4 did not reveal any significant difference in the number of labeled neurons ($n = 10$ KC soma regions from five flies).

Discussion

Drosophila olfactory learning is typically assayed using olfactory classical conditioning (Tully and Quinn 1985). Using this assay, several memory mutants and the genes involved in olfactory memory formation have been identified (Waddell and Quinn 2001; Dubnau et al. 2003; Heisenberg 2003; Davis 2005). This assay has also been used to investigate the roles of the different MB lobes (Connolly et al. 1996; Zars et al. 2000a; McGuire et al. 2001, 2003; Isabel et al. 2004). In each case, *GAL4* drivers that express in distinct lobes of the MBs were used. When G-protein signaling is disrupted using pan-MB *GAL4* drivers (*238y*, *c747*, and *c309*) to express a constitutively activated stimulatory heterotrimeric GTP-binding protein α -subunit ($G\alpha_s^*$), associative olfactory learning was reported to be completely abolished (Connolly et al. 1996). Using *201y-GAL4*, a line that expresses extensively in the γ lobes but only in the narrow core elements of the α/β lobes, learning was only reduced by ~50% (Connolly et al. 1996). In a study that investigated the effects of expressing *shi^{ts1}* using *c739-GAL4*, an α/β driver, and *201y-GAL4*, a significant impairment of performance was observed when neurotransmission was transiently inactivated through the α/β lobes with only a slight, but nonsignificant, decrease in memory performance observed using *201y-GAL4*, suggesting a greater role for MB α/β lobes in olfactory memory (McGuire et al. 2001). Other experiments involving rescue of the *rut* mutant defect by expressing a wild-type *rut* cDNA showed that memory was restored to wild-type levels using broad-MB *GAL4* drivers (*247*, *c772*, and *30y*) and the γ lobe driver

Figure 5. Complete rescue of the *rut* learning defect for all odor combinations is achieved when *rut* is expressed in both α/β and γ neurons. (A,B,C) Flies of the genotype *rut*²⁰⁸⁰; *c739-GAL4*; *H24-GAL4*/*UAS-rut* exhibited significantly elevated performance scores compared to the mutant control group *rut*²⁰⁸⁰; *c739-GAL4*; *H24-GAL4* for all odor combinations ($P < 0.0001$, $n = 6$ for all groups). In each case, the performance levels were indistinguishable from control flies of the genotype CS; *c739-GAL4*; *H24-GAL4*, indicating complete rescue of the *rut* learning defect ($P \geq 0.2853$, $n = 6$ for all groups). In all experiments, the P.I.'s were subjected to a one-way ANOVA with genotype as the main effect, followed by post hoc analysis with the Bonferroni/Dunn test. Statistical significance (**) or nonsignificance (N.S.) is indicated.

Figure 6. Neuron counts and GAL4 expression patterns of the different drivers at the level of the MB cell body region. (A) Expression patterns of 17d-GAL4, c739-GAL4, H24-GAL4, and NP1131-GAL4 in the MB cell body region visualized by crossing the drivers with *UAS-GFP::lacZ.nls 30.1* flies and doubly stained with anti-ELAV and anti-GFP. Whole-mount brains were examined under a confocal microscope, and serial sections were taken at 0.5- μ m intervals. Shown are representative projection images of confocal Z-series that were generated using ImageJ (Abramoff et al. 2004). Brains from five flies per GAL4 line were examined with a representative MB cell body region from individual flies shown for each driver. (B) The number of neurons expressing the GFP reporter was counted after image segmentation and identification of neuronal nuclei (Ponomarev and Davis 2003). Neuron counts for 17d-GAL4 were less than half the number of GFP-positive neurons for c739-GAL4 flies ($n = 10$ Kenyon cell soma regions from five flies). Neuron counts for H24-GAL4 and NP1131-GAL4 were not significantly different from one another ($P = 0.5407$, $n = 10$ Kenyon cell soma regions from five flies). Brains for each group showed overall stereotypy and bilateral symmetry with no significant differences in neuron counts between the left and right hemispheres ($P \geq 0.4052$, $n = 5$ for each line).

H24-GAL4. However, memory was only partially rescued using 201y-GAL4, and no rescue was observed using the GAL4 drivers 189y and 17d, which both express primarily in the MB α/β lobes. This, therefore, suggested a greater role of MB γ lobes in olfactory learning (Zars et al. 2000a). The apparent contradictions among these studies could be due to the fact that in each of these experiments different combinations of odors were used for the olfactory learning assay: MCH-OCT (Connolly et al. 1996), MCH-BEN (Zars et al. 2000a), and OCT-BEN (McGuire et al. 2001). In

addition, each experiment used a different means of addressing the issue: Connolly et al. (1996) disrupted G-protein signaling by expressing $G\alpha_s^*$ to abolish learning, McGuire et al. (2001) inactivated neurotransmission using *shits¹*, and Zars et al. (2000) expressed a *rut* cDNA in a *rut* mutant background to rescue the *rut* learning defect. When McGuire et al. (2003) tried to rescue the *rut* mutant defect by driving *UAS-rut* using either c739-GAL4 or H24-GAL4 alone, the performance of the flies was not significantly different from the *rut* mutants, but full rescue was observed when a double GAL4 driver c739; H24 was used (McGuire et al. 2003). To resolve the apparent discrepancies and inconsistencies among the different studies, we used all three commonly used odor combinations (MCH-OCT, MCH-BEN, and OCT-BEN) and two different assays, *shits¹* inactivation of neurotransmission as well as rescue of the *rut* memory defect, to examine the roles of the different neurons comprising the MB lobes.

Expressing transgenes using the two γ lobe GAL4 drivers NP1131 and H24 did not produce any odor-pair-specific learning effects. Using these γ drivers to express a *rut* cDNA in a *rut* mutant background results in a partial rescue of the learning defect for each of the odor combinations tested (Fig. 2). The γ driver of choice has typically been H24-GAL4, but the expression pattern of this driver is not limited to the γ neurons. In fact, it expresses very robustly in the ALs (Fig. 1D), and one concern is that this might affect performance scores since the learning assay is based on olfactory cues. The use of NP1131-GAL4 that expresses primarily in the γ neurons and a small subset of α'/β' neurons (Fig. 1B) is important to validate the results for H24-GAL4. A prior study using H24-GAL4 to rescue the *rut* learning defect resulted in performance scores that were statistically indistinguishable from wild-type flies using MCH and BEN (Zars et al. 2000a). However, among the different lines that were observed to rescue the *rut* defect, H24-GAL4 yielded the lowest scores, and another γ driver, 201y-GAL4, used in the same study failed to rescue the defect (Zars et al. 2000a). Yet another study that used H24-GAL4 to restore a *rut* cDNA in a *rut* mutant background failed to produce rescue of the defect using MCH and OCT as odorants (McGuire et al. 2003). Our data suggest that driving a *rut* cDNA in γ neurons results in *rut* levels that partially rescue the defect. Although we do not detect any odor-pair-specific learning using the two γ drivers we tested, this does not preclude the possibility that when γ drivers that express in other subsets of γ neurons are discovered, this would remain the case. It is likewise important to note that the three odorants used in our study do not represent the whole repertoire of odors that a fly responds and learns to. Thus, it remains possible that the γ neurons labeled by NP1131-GAL4 and H24-GAL4 may still be involved in the odor-pair-specific associative learning of other smells.

Rut rescue using GAL4 drivers that express in the α/β neurons reveals some odor-pair-specific effects (Fig. 3). Performance scores for flies carrying c739-GAL4 together with the *UAS-rut* transgene in a *rut* mutant background show no rescue of the *rut* defect for all odor combinations tested. In contrast, driving a *rut* cDNA using 17d-GAL4 shows partial rescue of the *rut* defect when MCH-BEN and OCT-BEN are used for the assay but not for MCH-OCT. To address the observed contrast in the rescue using the different odor combinations for these two lines, we compared the expression patterns for the two α/β drivers. As shown in Figures 1 and 6, the expression level for c739-GAL4 is greater than 17d-GAL4, and it appears that only a subset of neurons, a thin core, is labeled for 17d-GAL4. Using antibodies raised against glutamate, a further subdivision of the lobes has been described as a slender core of glutamatergic neurons, the α and β core neurons (α_c and β_c), that lie posteriorly and are partly enclosed by the α and β neurons (Strausfeld et al. 2003). Whether the core neurons that are marked by 17d-GAL4 correspond to the glutamatergic

subdivision of the α/β lobes remains to be determined. One explanation for the observed odor-pair-specific effects is that *17d-GAL4* and *c739-GAL4* express in nonoverlapping regions of the MB α/β neurons. Our behavioral experiments were performed on flies that were 2–5 d old, and although our neuron counts make clear that the expression of *c739-GAL4* is broader than *17d-GAL4*, we have been unable to confirm whether there is nonoverlapping expression at the α/β core. Preliminary data suggest, however, that during this time period, the spatial expression of *17d-GAL4* is in the core region, while *c739-GAL4* expression is more peripheral with a slight overlap of expression as a ring around this core (I. Sinakevitch, pers. comm.). Additional experiments are needed to extend these observations.

What is the significance of organizing the *Drosophila* MBs into different lobes, and is there differential representation of odors in the different lobes? Our findings indicate that to answer this question one must take into account the choice of odor pairs used in the olfactory assay. The differential effects we see when using different odor combinations for *17d-GAL4* suggest that even though this driver expresses in a subset of α/β neurons, some of these neurons must be important in MCH-BEN and OCT-BEN learning since driving *rut* using *c739-GAL4*, a line that expresses in a greater number of α/β neurons, is insufficient to see a partial rescue of the *rut* learning defect. Clearly, this partial rescue is more than a mass effect of *rut*-expressing MB neurons. This raises an important point: It may not be accurate to describe GAL4 driver patterns based solely on lobe-specificity, since different GAL4 drivers may highlight subsets of the neurons comprising individual lobes. The significance of achieving complete rescue when we used the double GAL4 *c739;H24* to express *UAS-rut* in a *rut* mutant background is still unclear. Although it is tempting to speculate that this complete rescue occurs by completing a spatial circuit that involves odorant representation in both α/β and γ neurons, more experiments need to be performed to investigate whether it may just represent the massed effect of expression in more MB neurons.

Examination of the odor-evoked activity in *Drosophila* MB neurons by expressing a green fluorescent protein-based Ca^{2+} indicator, G-CaMP, have revealed remarkable spatial stereotypy (Wang et al. 2004). In fact, several studies have shown that stereotypical anatomical and functional organization can be found at the different levels of the insect olfactory pathway. Each olfactory receptor neuron (ORN) likely expresses a single olfactory receptor (OR) gene, and ORNs that express the same OR genes converge on a common glomerulus in the AL, resulting in a stereotyped projection pattern (Gao et al. 2000; Vosshall et al. 2000; Scott et al. 2001). A near complete map of ORN connectivity constructed through a systematic survey of *Drosophila* OR expression has validated the principles of “one neuron–one receptor” and “one glomerulus–one receptor” (Couto et al. 2005). Different odors activate different combinations of ORs (de Bruyne et al. 2001), and individual receptors can mediate both excitatory and inhibitory responses to different odors in the same cell (Hallem et al. 2004). In addition, a topographic organization of the AL has been described wherein ORNs in distinct sensilla types project into distinct regions of the AL (Couto et al. 2005). At the level of the MB neuron cell bodies and the calyx, different odors evoked distribution patterns of fluorescence that were odor-specific and conserved across flies, resulting in stereotyped responses for BEN-, MCH-, and OCT-evoked fluorescence activity at both the wide-field and single-neuronal level (Wang et al. 2004). Our results demonstrate an odor-pair-specific effect with *17d-GAL4* using two odor combinations that have BEN as one of the odors. Several studies have indicated that *Drosophila* processes BEN differently from other odors (Helfand and Carlson 1989; Ayer Jr. and Carlson 1992; Kalidas and Smith 2002). In fact, although

surgical removal of the antennae and palps of wild-type flies results in the abolishment of MCH and OCT avoidance, BEN avoidance is only partially affected in both T-maze and arena paradigms, suggesting that BEN is sensed through other nonolfactory pathways (Keene et al. 2004). To eliminate naive odor bias, experiments are usually performed in a counterbalanced design, with half of the flies used in the calculation of the performance index being trained to the first odor and the other half to the second odor. An examination of the half P.I. scores for our experiments does not reveal obvious asymmetries between BEN and the two counter-odors (data not shown). Moreover, the fact that we see the odor-pair-specific effects in both rescue as well as disruption of memory experiments suggests that it is the expression of transgenes in the subset of neurons defined by *17d-GAL4* that confers the odor-pair-specific behavioral phenotypes we observed in this study.

The existence of such stereotypical anatomical and functional organization at the various levels of the *Drosophila* olfactory pathway may explain the odor-pair-specific *rut* rescue and *shi^{ts1}*-mediated disruption of learning that we observe in our study. The spatial pattern of odor-evoked fluorescence activity for BEN has been reported to occur mostly in the center of the calyx, OCT-evoked activities distribute more laterally and medially, while MB neurons that displayed fluorescence transients in response to MCH occur primarily in the top and middle portions of the soma layer (Wang et al. 2004). To investigate whether different lobes of the MBs receive olfactory information from different subsets of AL glomeruli, Tanaka et al. (2004) examined the spatial correlation between MB neurons and projection neurons (PNs). The MB dendrites of γ neurons were found to preferentially occupy the center of the calyx, and although the dendrites of the α'/β' and α/β core and surface neurons were more widespread across the calyx, their distribution was slightly nuanced (Tanaka et al. 2004). Based on these observations, we speculate that olfactory learning using different odors is, in part, a function of the relationships between the expression pattern of the GAL4 driver and the degree and pattern of overlap and nonoverlap in the populations of MB neurons that respond to the odor combinations chosen. This is a more complex picture than just having the different odors mapping to distinct lobes of the MBs, and, in fact, a shift toward describing GAL4 drivers based on actual patterns of expression may be more useful than describing them solely on MB lobe-specificity. Ultimately, determining the precise manner by which odors are encoded in the *Drosophila* brain and how this links to specific behavioral outputs will require careful analyses of the expression patterns of the GAL4 drivers and the representation of the odors in the different MB neurons.

Materials and Methods

Fly stocks and culture

Flies were cultured on standard medium at 25°C unless the flies were used in the *shi^{ts1}* experiments, in which case they were cultured at 19°C to minimize expression of the transgene under the control of the different drivers. Prior to behavioral training, flies were collected in vials and placed overnight in a 25°C incubator. Heat shock treatment of adult flies was carried out in vials placed in a 32°C water bath for 20–30 min before training for the *shi^{ts1}* experiments. Canton-S (CS) flies and *w(CS10)* flies (CS flies carrying the *w1118* mutation) served as wild-type controls. In this text, “*w*” refers to the *w(CS10)* allele. The *UAS-shi^{ts1}* flies were those previously used by McGuire et al. (2001) and described in Kitamoto (2001). The *UAS-rut* flies were those previously used by us (McGuire et al. 2003; Mao et al. 2004) and Zars et al. (2000a,b). These flies have a wild-type *rut⁺* cDNA under control of the UAS promoter that is inserted onto the third chromosome (Zars et al.

2000b). The GAL4 lines H24 and 17d were obtained from T. Zars (Theodor Boveri-Institut für Biowissenschaften, Würzburg, Germany), and *c739-GAL4* was from K. Kaiser (Division of Molecular Genetics, University of Glasgow, Scotland). The double GAL4 line *c739; H24* was described previously (McGuire et al. 2003).

Task-relevant sensory functions

Odor avoidance was assayed by measuring the distribution of naive flies between odor and fresh air at the odor concentrations used during training and testing. Air was bubbled through an odorant:mineral oil mixture, and for each odorant, a one-way ANOVA with genotype as the main effect was performed for groups tested under similar conditions. Electric shock avoidance was assayed by measuring the avoidance response of naive flies to 90 V DC shock. Statistical analyses were performed using STATVIEW. Avoidance indices were subjected to a one-way ANOVA followed by planned comparisons among the relevant groups with a Bonferroni/Dunn post-hoc test. Unless stated otherwise, all data points denoted as “statistically significant” are $P < 0.05$.

Memory assay

Flies were assayed by presenting one of two odors (CS+) with 12 pulses of 90 V electric shock (US) over 1 min, followed by presentation of the second odor (CS−) with the absence of shock. The three odorants—MCH, OCT, and BEN—were used two at a time (MCH-OCT, MCH-BEN, and OCT-BEN) to represent the CS+ and the CS− in a counterbalanced design, with half of the flies used in the calculation of the performance index being trained to the first odor and the other half to the second odor. Training was performed under dim red light at either 21°–25°C or 32°–35°C (for *sh1^{ts1}* experiments) and at 60%–68% relative humidity. The electric shock was delivered to the flies through an electrifiable copper grid that served as the floor of the plastic training tubes.

Trained flies were tested in a T-maze in which the two odors were simultaneously presented to the flies. Flies start at the center of the maze and are given 2 min to choose between the two arms of the maze that contained the different odors. Memory was indicated by approaching the CS− and avoiding the CS+. After the 2-min testing period, flies were anesthetized, collected, and counted. Odor concentrations were chosen that yielded ~50:50 distributions between the two odors for naive flies. To eliminate odor bias, experiments were performed in a counterbalanced design, with half of the flies used in the calculation of the P.I. being trained to the first odor and the other half to the second odor. The P.I. was calculated by adding the two half P.I.'s and represents the percentage of flies that correctly avoided the CS+ minus the percentage that made the incorrect decision by approaching the CS+. Statistical analyses were performed using STATVIEW. P.I.'s were subjected to a one-way ANOVA followed by planned comparisons among the relevant groups with a Bonferroni/Dunn post hoc test. Unless stated otherwise, all data points denoted as “statistically significant” are $P < 0.05$.

Fly histology and microscopy

To determine the expression pattern of different GAL4 drivers, enhancer detector lines were crossed with a line carrying *UAS-lacZ* line and the progeny analyzed. For immunohistochemistry or histochemical staining, 12-μm frontal and horizontal sections through the adult fly brain were obtained and examined using a light microscope.

Brains of female progeny flies carrying *UAS-GFP::lacZ.nls 30.1* and a GAL4 driver were dissected and double-stained with mouse anti-ELAV and rabbit anti-GFP. This was followed by incubation with Alexa 488 goat anti-mouse and Alexa 594 goat anti-rabbit antibodies. The brain samples were then mounted in 50% glycerol in 0.1% PBS. Signals were visualized using a Leica TCS confocal microscope with 488-nm and 594-nm excitation lines with serial sections taken at 0.5-μm intervals.

The GFP expression patterns at the level of the MB soma region were examined, and Z-stacks were generated using ImageJ (Abramoff et al. 2004). The number of neurons expressing the GFP reporter was counted after segmentation using an adjust-

able-threshold algorithm that segments fluorescently labeled objects contained within three-dimensional images (Ponomarev and Davis 2003). Z-sections were analyzed by “2D segmentation of one population.” Using this process, neuron counts for each Z-section separated by the average nuclear size were obtained and added up to arrive at an estimate for the total number of neurons per MB body region.

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