Segregation of Odor Identity and Intensity during Odor Discrimination in *Drosophila* Mushroom Body

Shouzhen Xia, Tim Tully

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, United States of America

Molecular and cellular studies have begun to unravel a neurobiological basis of olfactory processing, which appears conserved among vertebrate and invertebrate species. Studies have shown clearly that experience-dependent coding of odor identity occurs in “associative” olfactory centers (the piriform cortex in mammals and the mushroom body [MB] in insects). What remains unclear, however, is whether associative centers also mediate innate (spontaneous) odor discrimination and how ongoing experience modifies odor discrimination. Here we show in naïve flies that G_{as}-mediated signaling in MB modulates spontaneous discrimination of odor identity but not odor intensity (concentration). In contrast, experience-dependent modification (conditioning) of both odor identity and intensity occurs in MB exclusively via G_{as}-mediated signaling. Our data suggest that spontaneous responses to odor identity and odor intensity discrimination are segregated at the MB level, and neural activity from MB further modulates olfactory processing by experience-independent G_{as}-dependent encoding of odor identity and by experience-induced G_{as}-dependent encoding of odor intensity and identity.

**Introduction**

Naturally, most odors are experienced as complex mixtures in the environment. Consequently, an animal’s ability to discriminate between two odor cues reflects the interplay between spontaneous and experience-dependent processes [1–3]. Odor discrimination traditionally has been assessed either by using conditioning procedures to reveal that an animal can learn a conditioned response for one odor over another and therefore can discriminate them (e.g., [1,4,5]) or by demonstrating experience-dependent olfactory adaptation (e.g., [6–9]). When trying to identify the underlying cellular and molecular mechanisms for odor discrimination and associative learning, however, this approach becomes confounding. The cAMP signaling pathway, for instance, has been shown to be involved in both olfactory processing and associative learning in mammals and in *Drosophila* [10–14]. Similarly, experience-dependent changes in odor discrimination have been shown to occur in the piriform cortex of vertebrates [1,5,15–18] and in the mushroom body (MB) of *Drosophila* [19–22], both of which are activated by odor stimulation in naïve animals [23–28].

Odors elicit a variety of behavioral responses in *Drosophila* via a relatively simple but sensitive olfactory system readily accessible to genetics [29]. A number of behavioral assays have been reported for screening single-gene mutations that cause defects in olfactory function and in olfactory associative learning. The predominant learning assay employs Pavlovian conditioned discrimination between two odors [30]. A behavioral mutant might display an abnormal behavioral response in this assay because of (i) a sensorimotor deficit to odor(s), (ii) a sensorimotor deficit to footshock, (iii) a sensorimotor deficit to odor discrimination, or (iv) a deficit in the association of odor and footshock. Traditionally, sensorimotor deficits to odors and to footshock have been assessed in a T-maze using an olfactory acuity assay ([31]; also see below) and a shock reactivity assay [32]. For the former, flies are given a choice between odor and air. For the latter, flies are given a choice between footshock versus no footshock. To date, mutants are considered to be associative learning/memory mutants if they behave normally in these two sensorimotor assays. Significantly, no assay for sensorimotor deficits in odor discrimination per se has yet been developed.

Ultimately to address this issue, we have developed two novel assays to measure discrimination of odor intensity and odor identity with a T-maze [30] in naïve flies. Work on odor intensity discrimination in naïve flies then prompted us to develop a third novel assay to measure conditioned discrimination of odor intensity (cf. [33,34]). Importantly, genetic manipulations in *Drosophila* can be used to link these behavioral responses to specific molecular mechanisms. By disrupting MB function in four distinct ways (i.e., developmental lesion of MB, silencing of synaptic transmission from MB, RNA interference [RNAi]–mediated suppression of G_{as}-mediated signaling, and disruption of G_{as}-mediated signaling in MB) and assessing the effects with each of our

* To whom correspondence should be addressed. E-mail: tully@cshl.edu
Author Summary

Considerable progress has been made in understanding how olfaction works as the receptor proteins, sensory neurons, and brain circuitry responsible have become increasingly well-characterized. However, olfactory processing in higher brain centers, where neuronal activity is assembled into the perception of odor quality, is poorly understood. Here, we have addressed how the mushroom body (MB)—a secondary olfactory center—is involved in olfactory discrimination. We manipulated the MB by ablation, disruption of synaptic transmission, and interruption of key cellular signaling molecules in naïve flies and in flies trained to discriminate odors. We first show that although both odor identity and intensity are encoded in the MB, only the former requires Goa-dependent signaling and is necessary for naïve flies to spontaneously discriminate different odors. We then show that training flies to alter their olfactory response requires Goa-mediated signaling in MB for both odor intensity and odor identity. We have thus identified (i) segregation of odor identity and odor intensity at the MB level in naïve flies and (ii) different G-protein-dependent signaling pathways for spontaneous versus experience-dependent olfactory discrimination.

Results

To explore how Drosophila MB participates in spontaneous versus conditioned odor discrimination, we designed three novel odor discrimination assays—a spontaneous odor intensity assay, a spontaneous odor identity assay, and a conditioned odor intensity assay (see Protocol S1 for details)—to use along with our original conditioned odor identity assay [30]. With these four behavioral protocols, we were able to quantify (i) discrimination of odor intensity in naïve flies (Figure S1A), (ii) discrimination of odor identity in naïve flies (Figures S1B, S2, and S3), (iii) discrimination of odor intensity in conditioned flies (Figure S1C), and (iv) discrimination of odor identity in conditioned flies (Figure S1D).

We then disrupted MB structure or function in four distinct ways and assessed their effects with each of these four behavioral assays. First, we lesioned MB using chemical ablation [20]. When hydroxyurea feeding is restricted to the first few hours after hatching, this method kills proliferating cells during development and results in a dramatic reduction of adult MB and loss of a portion of the antennal lobes (ALs; Figure S4). Second, we acutely blocked dynamin-dependent synaptic transmission from MB of transgenic flies by using three different PGAL4 enhancer-trap drivers to express UAS-shi1(Flp) (shi1) preferentially in MB [35]. Third, we disrupted expression of Gαq in MB of transgenic flies by using the same PGAL4 drivers to express a UAS-dsGαq (RNAi) construct [36]. We then duplicated the essential experiment with a second RNAi (UAS-dGαq1F1) transgene [37]. Both UAS-dsGαq and UAS-dGαq1F1 produced severe knockdowns of Gαq expression (Figure S5; see also [36,37]). Fourth, we disrupted Gαq signaling in MB of transgenic flies by using the same PGAL4 drivers to express UAS-Gαq1Q215L (Gαq1i), a constitutively active stimulatory subunit of guanosine triphosphate-binding protein [19]. As a genetic control, wild-type Gαq transgene (UAS-Gαq1i) was overexpressed using the same PGAL4 drivers.

Spontaneous Odor Intensity Discrimination Does Not Require the Normal Function of MB

MB ablation had no effect on flies’ spontaneous odor intensity discrimination (Figure 1A) and avoidance of individual odors (Table S1). To check whether neuronal activity from MB is acutely involved with the spontaneous odor response, synaptic transmission from MB was transiently silenced by driving transgenic expression of shi1 with several MB-specific PGAL4 drivers. C309 and 247 drive transgenic expression in all lobes of MB, while 201Y drives transgenic expression preferentially in γ-lobes ([19,38]; see also Figure S4). Consistent with MB ablation, transient silencing of synaptic transmission from MB left spontaneous odor intensity discrimination (Figure 1B) and avoidance of individual odors (Table S1) unchanged. Similarly, neither disruption of Gαq by overexpression of UAS-dsGαq nor disruption of Gαq by overexpression of Gαq1i with the same PGAL4 drivers interfered with flies’ spontaneous odor intensity discrimination (Figure 1C and 1D) or avoidance of individual odors (Table S1). These observations indicate that MB is not required for discrimination of odor intensity or perception of individual odors in naïve flies. Such olfactory processing perhaps is accomplished in ALs or other brain regions, such as the lateral horn [39].

Spontaneous Odor Identity Discrimination Requires Synaptic Transmission from MB and Depends on Gαq Signaling

MB ablation (Figure 2A) and transient silencing of synaptic transmission from MB (Figure 2B) disrupted flies’ spontaneous odor identity discrimination, suggesting that MB is acutely involved with the process. Some spontaneous response remained, in particular after MB ablation, suggesting other brain regions may also be involved in the discrimination of odor identity. This is consistent with the fact that projection neurons do not form synaptic collaterals with the calyx of MB after hydroxyurea-based ablation, whereas their arborizations in the lateral horn remain [40].

RNAi-mediated disruption of Gαq in MB with the same PGAL4 drivers was sufficient to interfere with spontaneous odor identity discrimination (Figure 2C). To rule out the possibility that a developmental defect underlay this adult defect in the spontaneous odor identity response, the tub·GAL80α transgene was used along with the binary PGAL4/UAS system [21]. Groups of flies, raised at 18°C, were kept for 3 d either at 18°C (permissive for inhibition of PGAL4 by GAL80* function) or 30°C (restrictive for GAL80* function) before testing of spontaneous odor identity discrimination. The performance was significantly disrupted in transgenic C309i+; dsGαq1i, GAL80*/+ flies at the restrictive but not at the permissive temperature (Figure 2D), suggesting an adult-specific physiological role for Gαq during spontaneous odor identity discrimination. To rule out the possibility of an off-
target RNAi effect (cf. [41, 42]), a second RNAi transgene construct, UAS-dGql fluor [37], was evaluated. Again, we saw significant disruption of spontaneous odor identity discrimination (Figure 2E). As a further control for a more general “poisoning” of neuronal function by RNAi, we showed that expression of a UAS-drl RNAi transgene in MB had no effect on this odor response (Figure 2F). Collectively, these results demonstrate, to our knowledge for the first time, an adult-specific role in the central nervous system for Ga q signaling during discrimination of odor identity in naïve flies.

In contrast to the effect of Ga q, disruption of Ga s signaling in MB had no effect on spontaneous odor identity discrimination (Figure 2G). Consistent with this observation, the rutabaga and dunce mutants, in which two other components of the cAMP signaling pathway (i.e., adenylyl cyclase and cAMP-specific phosphodiesterase, respectively) are defective [43, 44], showed normal spontaneous odor identity discrimination (Figure 2H). Taken together, these observations suggest that (i) odor identity discrimination in naïve flies occurs independently of the cAMP signaling pathway and (ii) Ga s-dependent signaling in MB specifically contributes to this response.

Conditioned Odor Intensity Discrimination Requires Synaptic Transmission from MB and Depends on Ga s Signaling

MB ablation completely abolished conditioned odor intensity discrimination (Figure 3A), suggesting that conditioned odor intensity discrimination requires an intact MB. Similarly, transient block of synaptic transmission from MB severely disrupted conditioned odor intensity discrimination (Figure 3B), suggesting an acute role for neuronal activity in MB for this conditioned response. Finally, disruption of Ga s but not Ga q signaling also severely disrupted conditioned odor intensity discrimination, and the effect was adult-specific and physiological (Figure 3C–3E). Considering that spontaneous odor intensity discrimination occurs independent of MB (Figure 1), these observations reveal that (i) there is an “anatomical dissection” for odor intensity discrimination between naïve and conditioned flies and (ii) Ga s-mediated neural plasticity in MB modulates odor intensity discrimination in conditioned but not in naïve flies.

Conditioned Odor Identity Discrimination Requires Synaptic Transmission from MB and Depends on Ga s Signaling

MB ablation abolished conditioned odor identity discrimination (Figure 4A), confirming a previous report [20]. Conditioned odor identity discrimination with a different odor pair, 3-octanol and 4-methyl-cyclohexanol (MCH), has been reported to be abolished by transient silencing of synaptic transmission from MB in C309/shits flies [22] and mildly disrupted even at the permissive temperature in 247/shits flies [21]. Here, we confirmed and extended such observations by showing that conditioned odor identity discrimination between MCH and benzaldehyde (BA) was severely disrupted by transient silencing of synaptic transmission from MB in C309/shits and 247/shits flies and mildly disrupted in 201Y/shits flies at restrictive temperature (Figure 4B).
Disruption of Gαq in MB had only a very mild effect on conditioned odor identity discrimination (Figure 4C). Considering that the same flies were defective for spontaneous odor identity discrimination, this mild effect may be indirect.

Conditioned odor identity discrimination between 3-octanol and MCH has been reported to be disrupted when Gαs but not Gαq is overexpressed in MB [19]. Here we again confirmed our previous finding with 3-octanol and MCH (data not shown) and extended it by showing a similar effect with a different odor pair, MCH and BA (Figure 4D). Again, to rule out the possibility that a developmental defect underlay this effect on conditioned odor identity discrimination, the tub-GAL80ts transgene was used to restrict expression of Gαs* in adults. Conditioned odor identity discrimination was abolished in transgenic C309/+; Gαs*/GAL80ts flies when kept at the restrictive but not at the...
permissive temperature (Figure 4E), suggesting an adult-specific physiological role for Gs during conditioned odor identity discrimination. Consistent with this notion, rutabaga and dunce mutants showed disrupted conditioned odor identity discrimination (Figure 4F), as demonstrated long ago [30]. These results are strikingly different from those for spontaneous odor identity discrimination, revealing a ‘‘genetic dissection’’ for odor identity discrimination between naïve and conditioned flies. In MB, Gq signaling mediates odor identity discrimination in naïve flies, whileGs signaling mediates odor identity discrimination in conditioned flies.

**Discussion**

The use of a Pavlovian conditioned odor discrimination assay [30] over the past 20 y has helped to establish the view that G-protein-mediated cAMP signaling in MB subserves this form of associative learning [45]. Now, by developing additional behavioral measures, we are able to dissect odor discrimination into four functionally distinct components: the spontaneous response to odor intensity, the spontaneous response to odor identity, the conditioned response to odor intensity, and the conditioned response to odor identity. Our results refine our view of olfactory behavior as it relates to MB function, revealing an anatomical and molecular (genetic) dissection between odor identity and odor intensity in this higher brain center (Table 1). We have established MB as one important anatomical site for odor identity discrimination in naïve flies (Figure 2). Odor intensity discrimination, in contrast, does not require MB at all in naïve flies (Figure 1). Within MB, different G-protein-mediated signaling pathways distinguish odor identity discrimination between naïve and conditioned flies. Gq-dependent signaling regulates spontaneous responses to odor identity, while Gs-dependent signaling is involved in conditioned responses to odor identity (Figures 2 and 4). Strikingly, disruption of Gq-dependent signaling produces similar effects on conditioned responses to both odor identity and odor intensity, while disruption of Gs has little or no effect on these behavioral responses (Figures 3 and 4).
Segregation of Odor Identity and Odor Intensity in Naïve Flies

Odor discrimination is usually assayed in the context of olfactory adaptation (perceptual learning) or conditioning (associative learning) because of the difficulty in directly evaluating the process in naïve animals (e.g., [1,4–9]). Our spontaneous odor identity discrimination assay, where naïve flies are allowed to recognize and discriminate a second odor from a saturated odor as the background (Figures S1–S3), is analogous to a saturation discrimination paradigm in Caenorhabditis elegans, the only other known discrimination assay developed for naïve animals [46]. When an odor is saturated in intensity, higher concentrations produce no further change in response (Figure S2A and S2C). Against a saturated background odor, a “foreground” odor may be represented in two possible ways. First, the odor may activate additional receptors in the sensory neurons, leading to activation of additional subsets of glomeruli and the corresponding MB neurons (cf. [47]). Second, the foreground odor may activate additional neural processes or signaling pathways, leading to activation of additional subsets of MB neurons. Either way, flies clearly recognize the presence of the foreground odor and discriminate it from a background odor (Figure S2C). More importantly, our saturation assay appears

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Conditioned Odor Identity Discrimination Requires Synaptic Transmission from MB and Depends on Gαs Signaling

Conditioned odor identity discrimination between MCH and BA was (A) abolished by MB ablation (+HU), (B) disrupted by transient block (30 °C) of synaptic transmission from MB in C309/shi15, 247/shi15, or 201Y/shi15 flies, (C) mildly affected by disruption of Gαq in MB of dsGαq/C309, dsGαq/247, or dsGαq/201Y flies, (D) severely diminished by developmental disruption of Gαq in MB of Gαq/C309, Gαq/247, or Gαq/201Y flies, (E) abolished by adult-specific, physiological disruption of Gαq in MB of Gαq*, GAL80ts/C309 flies at restrictive (30 °C) but not permissive (18 °C) temperature, and (F) disrupted in rut or dnc mutants. n = 6 PIs for each group.

doi:10.1371/journal.pbio.0050264.g004

### Table 1. Effects of Four Experimental Manipulations of MB Function on Four Behavioral Assays of Odor Discrimination

| Behavioral Assay       | Chemical Lesion of MB | Silencing of MB | Disruption of Gαq in MB | Disruption of Gαs in MB |
|------------------------|-----------------------|----------------|-------------------------|-------------------------|
| Spontaneous intensity  | −                     | −              | −                       | −                       |
| Spontaneous identity   | +                     | +              | +                       | +                       |
| Conditioned intensity  | +                     | +              | −*                      | +                       |
| Conditioned identity   | +                     | +              | −*                      | +                       |

Minus signs indicate no effect; plus signs indicate severe disruption or abolition of function.

*Very mild effect.

doi:10.1371/journal.pbio.0050264.t001
Segregation of Odor Identity and Intensity in Naïve versus Conditioned Flies

Little is known about Gₛₐᵦ and Gₛₐₜ mediated signaling pathways in secondary olfactory centers in spite of the facts that (i) Gₛₐᵦ appears to be involved in olfactory signal transduction in sensory neurons [36], (ii) Gₛₐᵦ is involved in conditioned odor identity discrimination in Drosophila [19], and (iii) these higher centers clearly are involved in experience-dependent modification of odor discrimination in vertebrates [1,5,15–18] and in Drosophila [19–22]. The fact that MB is required (i) for spontaneous responses to odor identity but not intensity (Table 1) and (ii) for conditioned responses to odor identity [19–22] prompted us to check Gₛₐᵦ versus Gₛₐₜ-mediated signaling pathways in MB.

Disruption of Gₛₐᵦ in MB affects the spontaneous response to odor identity but unexpectedly affects the conditioned response to odor identity only minimally. Disruption of Gₛₐᵦ in MB, on the other hand, affects the conditioned response to odor identity but does not affect the spontaneous response to odor identity (Table 1). Such (i) exclusion of Gₛₐᵦ from the conditioned response to odor identity and of Gₛₐᵦ from the spontaneous response to odor identity and (ii) specificity of Gₛₐᵦ for the spontaneous response to odor identity and of Gₛₐᵦ for the conditioned response to odor identity constitute a dual dissection of spontaneous and conditioned odor identity discrimination. Moreover, the conditioned response to odor intensity is abolished by disruption of Gₛₐᵦ in MB (Figure 3D), while the spontaneous response to odor intensity occurs independent of MB, suggesting different anatomical contributions to spontaneous and conditioned odor intensity discrimination. Such an anatomical/molecular dissection of spontaneous and conditioned odor responses suggests that the learned response occurs independently of the spontaneous response, questioning the traditional experiments where odor discrimination per se is evaluated in the context of olfactory learning.

Finally, the correspondence between the conditioned responses to odor intensity and odor identity when disrupted by MB ablation, MB silencing, or jamming of Gₛₐᵦ is simply striking (Table 1). This general observation argues that, in contrast to the fact that the spontaneous responses to odor intensity or odor identity are segregated at the MB level, Gₛₐᵦ-mediated neuronal plasticity in MB nonetheless underlies behavioral changes to both.

Our data collectively demonstrate that (i) odor identity encoded in MB contributes to olfactory discrimination through a Gₛₐᵦ-dependent signaling process, whereas odor intensity encoded in MB exists but is not necessary for behavioral responses in naïve flies and (ii) Gₛₐᵦ-mediated signaling in MB is exclusively involved in neural plasticity, which then modulates behavioral responses to both odor intensity and identity. Such segregation of odor identity and odor intensity at the MB level in naïve flies and identification of different G-protein-dependent signaling pathways for spontaneous and conditioned odor discrimination, combined with function imaging [23] and the recently established spatial map of olfactory representations in MB [47], make it possible to check how neuronal activity in MB is assembled into the perception of odor identity (cf. [48]).

Materials and Methods

Fly stocks. Fly stocks included wild-type Canton-S w¹¹¹⁸ (CS10); the PGAL4 insertions 247 [38], C309, and 201Y; and UAS constructs UAS-Gₛₐᵦ-Gₛₐᵦ [19], shi⁰ [53], UAS-dsGₛₐᵦ-RRai [36], and UAS-dsGₛₐᵦ [37]. UAS-Gₛₐᵦ-Gₛₐᵦ was obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/). All stocks were “Cantonized” by outcrossing the heterozygous virgins to w¹¹¹⁸ (CS10) males for six generations, selecting for the mini-white eye color associated with each P element transposant.

Odorants. The following odors were used: two alcohols, 3-octanol and MCH; one aromatic compound, BA; one ester, ethyl acetate; one ketone, diacetyl; one amine, triethylamine; and thiazole. Some chemically similar odors were also tested including (R)-(+)-carvone and (S)-(+)-carvone, (R)-(+)-octanol and (S)-(+)-octanol, pentanol, 2-hexanol, and hexanol. All odors were dissolved in heavy mineral oil (Fisher Scientific, https://www.fishersci.com/) and delivered to the training chamber or the two T-maze arms with a “test bubbler” [54].

For conditioned discrimination, the concentration of MCH was chosen to be 0.4 × 10⁻⁵ (in mineral oil [v/v]) [54] or alternatively 1.0 × 10⁻⁴. Then the relative concentrations of BA (0.4 × 10⁻⁵ or 0.4 × 10⁻⁴) were determined so that naïve flies distributed themselves 50:50 in the test chamber or the two T-maze arms with a “test bubbler” [54].

For conditioned discrimination, the concentration of MCH was chosen to be 0.4 × 10⁻⁵ (in mineral oil [v/v]) [54] or alternatively 1.0 × 10⁻⁴. Then the relative concentrations of BA (0.4 × 10⁻⁵ or 0.4 × 10⁻⁴) were determined so that naïve flies distributed themselves 50:50 in the T-maze when given a choice between MCH and BA. For odor avoidance, MCH and BA were tested at concentrations from 10⁻⁴ to 10⁻². For saturation discrimination, the concentrations were much higher and varied with odors as shown in Table S2.

Behavioral assays. To assess the flies’ ability to sense individual odors, “fresh” air was delivered in one T-maze arm and odor (MCH...
or BA at different concentrations (Table S1) was delivered in the other, with all other parameters identical to those used during the traditional Pavlovian conditioned discrimination assay (30); and see below). Groups of about 100 naive flies were lowered to the center of the T-maze, and their odor avoidance was quantified (31).

To assay spontaneous intensity discrimination, groups of about 100 naive flies were given a choice between a saturated background odor and a mixture of the same odors (i.e., MCH and BA) plus an “equivalent” concentration of a second odor (BA+) in the left T-maze arm and the background odor (MCH) only in the right T-maze arm. For the reciprocal group of naive flies, the background odor (MCH) alone was presented in the left T-maze arm, and the mixture of the background and the second odor (MCH+ BA+) presented in the right arm.

To assay conditioned intensity discrimination, groups of flies were first subjected to associative conditioning with different concentrations of the same odors before being tested for their acquired response to the different intensities. Again, two groups of flies were always tested in one complete run to produce a pure measure of spontaneous identity discrimination for naive flies (Figure S1B). The first group of naive flies was exposed to a saturating concentration of the background odor (MCH) plus an “equivalent” concentration of a second odor (BA+) in the left T-maze arm and the background odor (MCH) only in the right T-maze arm. For the reciprocal group of naive flies, the background odor (MCH) alone was presented in the left T-maze arm, and the mixture of the background and the second odor (MCH+ BA+) presented in the right arm.

We also assayed conditioned identity discrimination, which corresponds to the traditional conditioned discrimination assay of Tully and Quinn [30] with minor modifications [34]. Again, two groups of flies were always tested in one complete run to produce a pure measure of acquired intensity discrimination for conditioned flies (Figure S1C). The first group of about 100 flies was first exposed to the higher odor concentration (C1) of either MCH or BA; CS+) and subjected to foot shock (US) for 60 s and then presented with a dilution of BA (10^{-6}) of either BA or MCH; CS−) without foot shock for another 60 s. A second reciprocal group of flies was trained with the lower odor concentration as the CS+ and the higher odor concentration as the CS−.

Disruptions of MB structure or function. MB ablation was achieved by feeding newly hatched larvae with hydroxyurea for about 5 h [29]. As a control, newly hatched larvae were fed with yeast suspension for 6 h. Silencing of MB was achieved with the shi ts transgene, which reversibly interferes with neuronal transmission in a temperature-dependent, dominant-negative fashion when overexpressed in a wild-type background [33] and MB by combining an independent RNAi transgene, UAS-dGFP11, with C309 and 247 in transgenic flies (dGFP11/C309 and dGFP11/247). To disrupt G_qα, a constitutively activated stimulatory heterotrimetric guanosine triphosphate-binding protein, G_qα*, was overexpressed to “jam” G_qα signaling in MB by crossing G_qα* flies with the above PGAL4 drivers (G_qα*/C309, G_qα*/247, and G_qα*/201Y). As genetic controls, white-eyed, wild-type MB flies were overexpressed with the same PGAL4 drivers (G_qα*/C309, G_qα*/247, and G_qα*/201Y) and the higher odor concentration. The total molecules of each odor or odor mixture were measured in a paired manner for the four different behavioral tests of Figure S2C with a PID detector (miniPID-2; Aurora Scientific, http://www.aurorascientific.com/) in a 50 min period, and then the supernatant was saved. Lysate proteins were electrophoresed on 12% SDS-PAGE, then electroblotted onto PVDF membranes. Immobilized proteins were probed with rabbit polyclonal anti-Gq (1:500 dilution) antisera (SC-392; Santa Cruz Biotechnology, http://www.ncbi.nlm.nih.gov/) or, rabbit polyclonal anti-actin (1:5,000 dilution) antibody (A5060; Sigma-Aldrich, http://www.sigmaaldrich.com/) as the loading control, and the membrane was incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5,000 dilution). The positive signal was visualized with the ECL System (GE Healthcare, http://www.gehealthcare.com). Quantitation was performed by digital image analysis using an Epson (http://www.epson.com/) scanner and ImageJ (National Institute of Mental Health, http://www.nih.gov/); experiments were repeated six times for G_qα, UAS-dGFP11, and UAS-dGFP11/247 flies. The level of G_qα* was normalized to the actin control.

Whole-mount GFP expression. The protocol for whole-mount GFP expression was described before [55]. Briefly, homozygous PGAL4 females were crossed to homozygous UAS-GFP553 males. Three- to five-day-old heterozygous female progeny were examined for GFP expression patterns. The whole brains were carefully transferred to 3% paraformaldehyde for 30 min, then to 4% paraformaldehyde + 0.25% Triton X-100 (Fisher Scientific) for 30 min under mild vacuum. Brains were then soaked in FocusClear (CelExplorer Labs, http://www.celexplorer.com/) solution for 5 min and mounted in a drop of the same solution [55]. The whole-mount brains were imaged with a Zeiss (http://www.zeiss.com) LSM 510 confocal microscope, and stacks of confocal images were taken through the full thickness of the brain. The distance between successive images (z-axis distance) was adjusted for the refractive index mismatch of the air and mounting medium as described previously [55]. In some cases, front and rear projection reconstructions were performed using Adobe Photoshop. The expression patterns of MB were considered representative when the brain structures and MBs are better reveal internal structures.

Supporting Information

Figure S1. The Behavioral Protocols for Different Odor Discrimination Assays

To control for potential side bias in the T-maze, two reciprocal groups always were tested as one complete experiment. A PI for the complete experiment was defined as the average of the PIs (always calculated as the number of flies avoiding the measured variable [i.e., the high concentration for spontaneous odor intensity discrimination, the mixture of the saturated background and the foreground odor for spontaneous odor identity discrimination, or the CS− concentration or odor for conditioned odor intensity or odor identity discrimination assays] minus avoiding the other “control” variable [i.e., the low concentration, the saturated background alone, or the CS− concentration or odor in the relevant assays], divided by the total number of flies and finally multiplied by 100) from the two reciprocal groups.

(A) The spontaneous odor intensity discrimination assay for naive flies. Naive flies were allowed to choose between two different concentrations (C1 and C2) of the same odors (10^{-3} and 10^{-4} for MCH, or 0.4 × 10^{-5} and 0.4 × 10^{-4} for BA), with the higher concentration delivered to the left arm in one group and to the right arm in the reciprocal group. This protocol was set with the spontaneous odor identity discrimination assay for naive flies. Naive flies were allowed to make a choice between a saturated background odor (e.g., MCH) and a mixture consisting of the same saturated odor and a second foreground odor (e.g., BA). The mixture...
was delivered to the left arm in one group and to the right arm in the reciprocal group to produce a pure measure of spontaneous odor identity discrimination.

(C) The conditioned odor intensity discrimination assay for trained flies. The flies were conditioned to avoid one of the two concentrations (C1 or C2) of the satiated (10⁻³ and 10⁻²) for MCH, or 0.4 x 10⁻³ and 0.4 x 10⁻⁴ for BA), with US (marked with red electric volt symbol) associated with the higher concentration in one group and the lower concentration in the reciprocal group. The higher concentration was always delivered to the left arm during testing to cancel any spontaneous odor intensity response and side bias. (D) The conditioned odor intensity discrimination assay for trained flies. The flies were conditioned to avoid one of the two equivalent odors (i.e., both spontaneous identity and intensity responses are close to zero; 10⁻³ for MCH and 0.4 x 10⁻⁴ for BA), with US associated with MCH in one group and with BA in the reciprocal group. MCH was always delivered to the left arm for both reciprocal groups.

Found at doi:10.1371/journal.pbio.0050264.sg003 (406 KB TIF).

Figure S2. The Saturation Discrimination Assay for Measuring Spontaneous Odor Identity Discrimination in Naïve Flies

(A) Saturation of the background odor. To saturate MCH as the background odor, naïve flies were allowed to choose in the T-maze between 2 x [MCH] versus 1 x [MCH]. As the concentration of the MCH was increased, a threshold (i.e., above 10%) was reached at which flies would fail to recognize the identity difference, thereby yielding a PI of zero. At that concentration, MCH was considered saturated for intensity. (B) Determination of the equivalent intensity of the foreground odor. To determine the concentration of BA equivalent to that of the saturated MCH (MCH₁), i.e., 15% throughout the study), naïve flies were given a choice between [BA] and MCH₁. As the concentration of BA was increased, the distributions of flies in the T-maze approached 50:50, yielding a PI of zero. That concentration of BA, BA₁ (about 1.5%), was considered equivalent to saturated MCH. (C) The saturation discrimination assay was produced by presenting either MCH₁ + BA₁ versus MCH₂ (black bar, chosen as our standard assay throughout the study) or MCH₁ + BA₁ versus MCH₂ and MCH₃ (grey bar) in the T-maze. Because MCH₃ (15%) was saturated, flies produced a score close to zero when presented with 2 x MCH₃ versus MCH₄ (p = 0.18). Similarly, because BA₁ (1.5%) was equivalent to MCH₂, flies also produced a score close to zero when presented with BA₁ versus MCH₂ (p = 0.46). The non-zero scores (p < 0.0001) for the two different spontaneous groups indicate that naïve flies are able to recognize the presence of BA and discriminate it from the saturated background of MCH. Scores from these two discrimination assays were not different from each other (p = 0.79), further confirming that MCH was saturated for intensity. The actual vapor concentrations of the individual odors or the mixture of MCH₁ + BA were quantified and are shown in the lower panel. (D) Saturation discrimination is concentration-dependent. Naive flies were allowed to discriminate [BA] from the MCH₁ (15%) across a wide range of concentrations (0.4 x 10⁻³, 0.4 x 10⁻⁴, 0.4 x 10⁻⁵, 0.75 x 10⁻⁵, and 0.1 x 10⁻⁵). Odor discrimination improved as [BA] increased. (E) Saturation discrimination is influenced by exposure time to odors. Naive flies were given a discrimination assay testing MCH₁ versus BA₁ or MCH₂ versus BA₂ for different lengths of time (30, 45, 60, 90, and 120 s). Optimal scores were produced quickly (i.e., with 30 s). When the time was longer than 60 s discrimination scores dropped substantially.

n = 4 PIs for each group.

Found at doi:10.1371/journal.pbio.0050264.sg002 (572 KB TIF).

Figure S3. Spontaneous Discrimination between Chemically Different Odors

(A) Naïve flies were tested for their ability to discriminate 3-octanol, BA, ethyl acetate, diacetyl, triethylamine, and thiazole from MCH₁ (15%; grey columns); or MCH, 3-octanol, ethyl acetate, diacetyl, triethylamine, and thiazole from BA₂ (10%; black columns) as the saturated background odor (see Table S1 for concentrations of all odors). (B) Reciprocally, naïve flies were tested for their ability to discriminate diacetyl (grey columns) and BA₂ (black columns) from saturated 3-octanol, ethyl acetate, diacetyl, and thiazole as the background.

n = 4 PIs for each group.

Found at doi:10.1371/journal.pbio.0050264.sg003 (406 KB TIF).

Figure S4. Confirmation of Lesion of MB

To confirm the hydroxyurea-induced ablation of MB, we expressed UAS-GFPGW361 in MB (and a few other regions) using 201Y (201Y/UAS-GFP) PGAL4 drivers, or in projection neurons from ALs to MB using GH146 (GH146/UAS-GFP) PGAL4 drivers. Ten flies were sampled for the 201Y/UAS-GFP genotype with or without hydroxyurea treatment, and five flies were sampled for GH146/UAS-GFP with or without the treatment. In all cases, MB was ablated after hydroxyurea treatment, as indicated by the absence of UAS-GFP signal in MB calyces, consistent with previous reports [29,30]. (A) Without hydroxyurea treatment (−HU), UAS-GFP was expressed strongly in MB and a few scattered big neurons, and weakly in ALs with 201Y. (B) Without hydroxyurea treatment (−HU), UAS-GFP was targeted to MB and the projection neurons with GH146. Some weak GFP signal was also present in MB. (C) The 201Y-driven GFP signal was reduced in MB specifically by hydroxyurea treatment (+HU), but still was present in the scattered neurons and ALs, suggesting the specific ablation of MB. The residual GFP signal in MB might represent the embryonic Kenyon cell fibers, unaffected by the treatment [57]. (D) The GH146-driven GFP signal was completely removed in MB by hydroxyurea (+HU). The hydroxyurea treatment also reduced GFP expression in ALs and the projection neurons, resulting from ablation of one lateral neuroblast [57,58].

Found at doi:10.1371/journal.pbio.0050264.sg004 (3.4 MB TIF).

Figure S5. Knockdown of the Gαq Protein

There was a 50-bp overlap between the sequences used for creating UAS-dsGαq [56] and UAS-dsGαq₁ [57] transgenes. Therefore, Western blot analyses were done to confirm the RNAi-mediated disruption of Gαq protein with these two RNAi transgenes. Wild-type, UAS-Gαq or UAS-dsGαq (containing 10% hydroxyurea) flies were crossed with the Elav-PGAL4 line virgins. Adult heads from their progenies were used for Western blot analyses. The quantification of six repetitions is shown in lower panel. Gαq expression was greatly disrupted in flies carrying both Elav-PGAL4 and UAS-dsGαq (dsGαq/Elav) or UAS-dsGαq₁ (dsGαq₁/Elav) as compared with that in control flies carrying only Elav-PGAL4 driver.

Found at doi:10.1371/journal.pbio.0050264.sg005 (4.1 MB TIF).

Protocol S1. Detailed Description of Saturation Discrimination Assay in Drosophila and Supporting References

Found at doi:10.1371/journal.pbio.0050264.sd001 (76 KB DOC).

Table S1. Olfactory Acuity to MCH and BA after Chemical Lesion of MB, Silencing of MB with shi+, or Disruption of Gαq or Gαs Signaling in MB

Chemical ablation of MB with hydroxyurea treatment (+HU), silencing of MB, or disruptions of Gαq or Gαs do not affect olfactory acuity. No significant differences were detected between MB-ablated (+HU) and control (−HU) flies (p ≥ 0.29), between +shi+ control flies and those with synaptic transmission from MB blocked (p ≥ 0.21), between dsGαq/Elav control flies and those with dsGαq expressed in MB (p ≥ 0.39), or among genotypes overexpressing Gαq₁ or Gαs (p ≥ 0.23).

Found at doi:10.1371/journal.pbio.0050264.s001 (60 KB DOC).

Table S2. Chemical Odors and Their Concentrations Used in the Saturation Discrimination Assay

Found at doi:10.1371/journal.pbio.0050264.s002 (51 KB DOC).

Acknowledgments

We thank Drs. Savitha Kalidas, Dean P. Smith, Santanu Banerjee, Gaiti Hasan, and the Bloomington Drosophila Stock Center for fly stocks and reagents. We thank Drs. Zachary Mainen and Matthew Smear for the PIDs, and Dr. Matthew Smear and Mr. Joshua Sanders for technique support. We thank Drs. Santanu Banerjee and Gaiti Hasan for sharing unpublished observations. We also thank Drs. Martin Heisenberg, Josh Dubnau, Ann-Shyn Chiang, Yi Zhong, and Akira Mamiya for helpful discussions and comments.

Author contributions. SX conceived, designed, and performed the experiments. SX and TT analyzed the data and wrote the paper.

Funding. This work was supported by grants to TT from the National Institutes of Health and Dart Neurosciences.

Competing interests. The authors have declared that no competing interests exist.
References

1. Wilson DA, Stevenson RJ (2003) The fundamental role of memory in olfactory perception. Trends Neurosci 26: 243–247.
2. Ache BW, Young JM (2005) Olfaction: Diverse species, conserved principles. Neuron 48: 417–430.
3. Shepherd GM (2005) Outline of a theory of olfactory processing and its relevance to humans. Chem Senses 30: i5–i5.
4. Linster C, Johnson BA, Morse A, Yue E, Leon M (2002). Spontaneous versus reinforced olfactory discriminations. J Neurosci 22: 6842–6845.
5. Shimshek DR, Bus T, Kim J, Mihaljevic A, Mack V, et al. (2005) Enhanced odor discrimination and impaired olfactory memory by spatially controlled switch of AMPA receptors. PLoS Biol 3: e54.
6. L’etanche ND, Bargmann CI (2008) Olfaction and odor discrimination are mediated by the C. elegans guanylyl cyclase ODR-1. J Neurosci 25: 575–586.
7. Kellner KR, Ziemann J, Munger SD, Reed RR, Zuffal F (2003) Importance of the CNGA1 channel gene for odor discrimination and adaptation in behaving mice. Proc Natl Acad Sci U S A 100: 4299–4304.
8. Kadoumis M, Wilson DA (2006) Olfactory cortical adaptation facilitates detection of odors against background. J Neurophysiol 95: 1888–1896.
9. Devaud JM, Acebes A, Ferrus A (2001) Olfactome exposure causes central adaptation and morphological changes in selected olfactory glomeruli in Drosophila. J Neurosci 21: 6274–6282.
10. Hildebrand JG, Shepherd GM (1997) Mechanisms of olfactory discrimination: Converging evidence for common principles across phyla. Annu Rev Neurosci 20: 595–631.
11. Ronnett GV, Moon C (2002) G proteins and olfactory signal transduction. Annu Rev Physiol: 189–222.
12. Mayford M, Kandel ER (1999) Genetic approaches to memory storage. Trends Genet 15: 463–470.
13. Dubnau J, Tully T (1998) Gene discovery in Drosophila: New insights for learning and memory. Annu Rev Neurosci 21: 407–444.
14. Gomez-Diaz C, Martin F, Alcorta E (2004) The cAMP transduction cascade mediates olfactory reception in Drosophila melanogaster. Behav Brain 34: 395–406.
15. Gottfried JA, Winston JS, De Bell JS (1997) Neuroblast ablation in Drosophila P[GAL4] lines reveals origins of olfactory interneurons. J Neurobiol 32: 445–456.
16. Stocker RF, Stocker RS, Martin F, Alcorta E (2004) The structural gene for cAMP phosphodiesterase. Proc Natl Acad Sci U S A 98: 15356–1541.
17. Stocker RF, Stocker RS, Martin F, Alcorta E (2004) A central neural circuit for experience-independent olfactory and courtship behavior in Drosophila melanogaster. Proc Natl Acad Sci U S A 98: 15356–1541.
18. Stocker RS, Stocker RS, Martin F, Alcorta E (2004) Drosophila melanogaster, and memory in Drosophila. Curr Biol 14: R700–R713.
19. Bargmann CI, Hartweg E, Horvitz HR (1993) Olfactory-selective genes and neurons mediate olfactory c. elegans. Cell 74: 513–527.
20. De Belle JS, Heisenberg M (1994) Associative odor learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. Science 265: 692–695.
21. McGuire SE, Le PT, Davis RL (2001) The role of Drosophila mushroom body signaling in olfactory memory. Nature 411: 476–479.
22. De Belle JS, Heisenberg M (1994) Associative odor learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. Science 265: 692–695.
23. Wing Y, Guo HF, Pologruto TA, Haman F, Hakker I, et al. (2004) Stereotyped odor-evoked activity in the mushroom body of Drosophila revealed by green fluorescent protein-based Ca2+ imaging. J Neurosci 24: 6507–6514.
24. Wang Y, Wright NJ, Guo H, Xie Z, Svoboda K, et al. (2001) Genetic manipulation of the odor-evoked distributed neural activity in the Drosophila mushroom body. Neuron 29: 267–276.
25. Zou Z, Buck LB (2006) Combinatorial effects of odorant mixtures in olfactory cortex. Science 311: 1477–1479.
26. Zou Z, Li F, Buck LB (2005) Odor maps in the olfactory cortex. Proc Natl Acad Sci U S A 102: 7724–7729.
27. Illig KR, Haberly LB (2003) Odor-evoked activity is spatially distributed in piriform cortex. J Comp Neurol 457: 361–373.
28. Stopfer M, Jayaraman V, Laurent G (2003) Intensity versus identity coding in an olfactory system. Neuron 39: 991–1004.
29. Carlson JR (1996) Olfaction in Drosophila: From odor to behavior. Trends Genet 12: 175–180.
30. Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant Drosophila melanogaster. J Comp Physiol [A] 157: 265–277.
31. Boynton S, Tully T (1992) latho, a new gene involved in associative learning and memory in Drosophila melanogaster, identified from P element mutagenesis. Genetics 131: 655–672.
32. Dura JM, Preat T, Tully T (1995) Identification of liriope, a new gene affecting learning and memory in Drosophila melanogaster. J Neurogenet 9: 1–14.
33. Boro A (1985) Calculation of olfactory signals in Drosophila melanogaster. J Comp Physiol [A] 152: 573–583.
34. Duda Y (1977) Properties of learning and memory in Drosophila melanogaster. J Comp Physiol [A] 114: 69–89.
35. Kitamoto T (2001) Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. J Neurobiol 48: 71–92.
36. Kalidas S, Smith DP (2002) Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. Neuro 33: 177–184.
37. Chan DC, Lin C, Kennedy AM, Tully T, et al. (2002) Compensation of insositol 1,4,5-trisphosphate receptor function by altering sarco-endoplasmic reticulum calcium ATPase activity in the Drosophila mushroom body. J Neurosci 22: 353–361.
38. Zars T, Fischer M, Schulte R, Heisenberg M (2000) Localization of a short-term memory in Drosophila. Science 288: 672–675.
39. Heimbeck G, Bugnon V, Gendre N, Keller A, Stocker RF (2001) A central neural circuit for experience-independent olfactory and courtship behavior in Drosophila melanogaster. Proc Natl Acad Sci U S A 98: 15356–1541.
40. Levin LR, Han PL, Hwang PM, Feinstein PG, Davis RL, et al. (1992) The Drosophila learning and memory gene rutabaga encodes a CalC1.3(1)modulin-like protein and is required for normal learning and memory. Cell 69: 672–675.
41. Kalidas S, Smith DP (2002) Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. Neuro 33: 177–184.
42. Selvarathinamoorthy S, Arndt S, Miller MG, Tully T, et al. (2002) Genetic manipulation of the odor-evoked distributed neural activity in the Drosophila mushroom body. Neuron 29: 267–276.
43. Wang Y, Chiang AS, Liu YC, Chiu SL, Hu SH, Huang CY, et al. (2001) Three-dimensional mapping of brain neuropils in the cockroach, Diploptera punctata. J Comp Neurol 443: 359–363.