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Representations of Novelty and Familiarity in a Mushroom Body Compartment

Graphical Abstract



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In Brief

The Drosophila mushroom body, a brain region that functions in classical learning, also helps flies identify novel stimuli and become accustomed to them.

Highlights

- α'3 MBONs respond to novel odors but suppress their activity to familiar odors
- MBON suppression requires odor-evoked excitation of the a'3 dopaminergic neuron
- Flies elicit alerting behavior to novel odors that disappears upon familiarization
- The alerting response to novel odors requires the activity of α'3 MBONs





Representations of Novelty and Familiarity in a Mushroom Body Compartment

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SUMMARY

Animals exhibit a behavioral response to novel sensory stimuli about which they have no prior knowledge. We have examined the neural and behavioral correlates of novelty and familiarity in the olfactory system of Drosophila. Novel odors elicit strong activity in output neurons (MBONs) of the $\alpha'3$ compartment of the mushroom body that is rapidly suppressed upon repeated exposure to the same odor. This transition in neural activity upon familiarization requires odor-evoked activity in the dopaminergic neuron innervating this compartment. Moreover, exposure of a fly to novel odors evokes an alerting response that can also be elicited by optogenetic activation of $\alpha'3$ MBONs. Silencing these MBONs eliminates the alerting behavior. These data suggest that the $\alpha'3$ compartment plays a causal role in the behavioral response to novel and familiar stimuli as a consequence of dopamine-mediated plasticity at the Kenyon cell-MBON $\alpha'3$ synapse.

INTRODUCTION

Novel stimuli can elicit a behavioral response that alerts an organism to unexpected, potentially salient events. An alerting response to sensory stimuli not previously encountered by an animal, such as the orienting response described by Pavlov (So-kolov, 1963), provides an organism the opportunity to explore the potential significance of the novel stimulus. A behavioral response to novelty is elicited by sensory cues about which an animal has no prior knowledge. Most behaviors, in contrast, are based upon past experience acquired either over long periods of evolutionary time (innate behaviors) or by learning over the life of an animal. The observation that sensory cues can be identified as novel and can evoke a behavioral response in the absence of prior knowledge poses an interesting problem.

A neural circuit encoding novelty should respond to all novel stimuli, but this response should be suppressed upon familiarization. The memory of a familiar sensory cue should be stimulus specific and long-lasting, distinguishing it from sensory adaptation. Neural responses that correlate with novelty and familiarity are seen in a number of mammalian brain regions. The transition from novelty to familiarity is associated with suppression of neural responses in higher brain centers that appears distinct from intrinsic or sensory adaptation. Electrophysiologic recordings along the visual and auditory pathways reveal that neurons exhibit activity in response to novel or unexpected cues that diminish upon repeated exposure (Brown and Banks, 2015; Desimone, 1996; Khouri and Nelken, 2015; Malmierca et al., 2015). In the auditory pathway, neurons in the inferior colliculus and the auditory cortex exhibit responses to novel or unexpected tones that attenuate upon repetition (Khouri and Nelken, 2015; Malmierca et al., 2015). Similarly, neurons in the perirhinal and inferior temporal cortices respond to novel visual stimuli, and this response attenuates rapidly upon repetition (Brown and Banks, 2015; Desimone, 1996), a phenomenon known as repetition suppression.

Dopaminergic neurons in the substantia nigra (SN) pars compacta and ventral tegmental area (VTA) also exhibit phasic bursting activity in response to novel or unexpected sensory events. Unexpected flashes of light or auditory tones evoke burst firing in 60%–70% of the dopaminergic neurons that attenuates as the novel stimulus becomes familiar (Bromberg-Martin et al., 2010; Ljungberg et al., 1992). Related neural events may underlie attenuation in the BOLD signal observed in extra striate cortex as well as SN and VTA in fMRI studies of humans upon repeated exposure to sensory stimuli (Buckner et al., 1998; Bunzeck and Düzel, 2006). Thus, mammals have evolved neural systems that distinguish novel from familiar sensory stimuli that may facilitate the determination of the potential salience of unfamiliar environmental events.

We have analyzed the behavioral and neural correlates of novelty and familiarity in the olfactory system of Drosophila. Olfactory perception in the fly is initiated by the binding of an odor to an ensemble of olfactory sensory neurons in the antennae that results in the activation of a unique and topographically fixed combination of glomeruli in the antennal lobe (reviewed in Vosshall and Stocker, 2007). Each antennal lobe projection neuron extends dendrites into one of the 54 glomeruli and extends axons that bifurcate to innervate two distinct brain regions, the lateral horn, and the mushroom body (MB). The invariant circuitry of the lateral horn is thought to mediate innate behaviors, whereas the unstructured projections to the MB translate olfactory sensory information into learned behavioral responses (Heisenberg, 2003; Fişek and Wilson, 2014; and references within Aso et al., 2014a). In the MB, each odor activates a sparse representation (5%-10%) of principal neurons, the Kenyon cells (KCs) (Honegger et al., 2011; Turner et al., 2008). KCs extend axons that



form en passant synapses in the compartments of the MB lobes (Aso et al., 2014a; Ito et al., 1998; Tanaka et al., 2008). The KCs synapse on the MB output neurons (MBONs), which have distinct spatially stereotyped dendritic arbors within compartments that collectively tile the lobes (Aso et al., 2014a). MBONs provide the only output of the MB and the activity of different MBON combinations biases behavior (Aso et al., 2014b; Owald et al., 2015; Perisse et al., 2016). Each of the 15 compartments is also innervated by the axons of one to three of 20 dopaminergic cell types (dopaminergic neurons, or DANs) (Aso et al., 2014a; Mao and Davis, 2009; Tanaka et al., 2008). Distinct DANs respond to different unconditioned stimuli and dopamine release elicits plasticity in the synapses between the KCs and MBONs (Cohn et al., 2015; Galili et al., 2014; Hige et al., 2015a; Kirkhart and Scott, 2015; Lin et al., 2014; Liu et al., 2012; Mao and Davis, 2009; Waddell, 2013). The alignment of DAN arbors with compartmentalized KC-MBON synapses creates a unit for learning that transforms the disordered KC representation into ordered MBON output to collectively bias behavioral responses to sensory stimuli.

The transition from novelty to familiarity involves memory formation, and learning and memory in the fly are accomplished by the circuitry of the MB (Heisenberg, 2003). We have identified a neural circuit in the MB that appears to encode a representation of novelty and familiarity (Figure 1A). We observed that MBONs innervating the $\alpha'3$ compartment respond to novel odors and that their activity is rapidly suppressed upon repeated exposure to the same stimulus. This suppression upon familiarization is observed for all novel odors tested regardless of innate valence, is stimulus specific, lasts for more than 20 min, and recovers in 1 hr. Repetition suppression of MBON- $\alpha'3$ is distinct from sensory adaptation and requires odor-evoked activity in the DAN innervating the $\alpha'3$ compartment. Our data suggest that repeated exposure to an odor mediates dopamine-dependent plasticity at the KC synapses onto MBON- $\alpha'3$ that suppresses MBON output. Moreover, behavioral experiments demonstrate that the α' 3 MBONs mediate an alerting response to novel odors. Exposure of a fly to novel olfactory stimuli evokes an alerting behavior. This behavioral response can be elicited by optogenetic activation of $\alpha'3$ MBONs and eliminated by $\alpha'3$ MBON silencing. These observations suggest that the behavioral response to novelty and the transition to familiarity is mediated by the circuitry of KCs, DANs, and MBONs within the α '3 compartment.

RESULTS

Repetition Suppression in the α' 3 Compartment

MBONs in the fly and in other insects respond to odor, and this response is modulated by experience and internal state (Owald et al., 2015; Cassenaer and Laurent, 2007; Cohn et al., 2015; Hige et al., 2015a, 2015b; Okada et al., 2007; Séjourné et al., 2011). If novelty is encoded in a compartment of the MB, we would expect that a novel odor would activate an MBON, but repeated exposure should lead to response suppression. Flies were presented with a 1-s odor stimulus, 4-methyl-cyclohexanol (MCH), repeated ten times with a fixed inter-stimulus interval (ISI) that ranged from 6 to 60 s in different experiments. Odor-evoked calcium transients were imaged at the soma of multiple MBONs that define different compartments, using the genetically en-

coded calcium indicator GCaMP6f (Chen et al., 2013). An initial odor pulse elicits a strong response in the $\alpha'3$ MBONs that diminishes monotonically upon repetition (Figures 1B and S1A). Over 50% suppression is apparent by the second exposure to odor, and MBON activity declines more than 80% within three to seven repetitions (Figure 1C). This decrease in odor-evoked activity is observed when monitoring calcium transients in either the soma or the dendrites of MBON- $\alpha'3$ and is similar for all ISIs tested between 6 and 60 s (Figure 1C). In 40% of the flies, the calcium response upon repetition exhibits negative deflections below baseline, suggesting that the suppression due to repeated exposure may be unmasking an inhibitory component of the odor response (Figures 1B, 1D, S1B, and S1C). These data suggest that the MBON- $\alpha'3$ exhibits strong repetition suppression. We did not observe consistent response suppression in the six other MBONs that collectively innervate seven different compartments (see below). Thus, we identified $\alpha'3$ as a candidate compartment encoding novelty and familiarity.

We examined the response to six additional novel odors, including neutral, innately attractive, and innately aversive odors, and similar suppression in MBON- α '3 activity was observed upon repetition (Figures 2 and S1C–S1E). Repetition suppression is therefore independent of the identity of the odor or its valence. Moreover, suppression in MBON activity is only observed upon repeated exposure to the same odor. Ten trials with MCH, for example, results in 80% suppression of the calcium signal, but subsequent exposure to a different odor, benzaldehyde, elicits a strong transient activation of MBON- α '3 (Figure 2). Similar results were obtained when the order of exposure to MCH and benzaldehyde was reversed. The specificity of repetition suppression was further demonstrated for five additional odor pairs (Figure 2). Thus, repetition suppression in the α '3 compartment was observed for all odors tested and is stimulus specific.

The suppression of MBON- $\alpha'3$ observed upon the transition from odor novelty to odor familiarity may reveal a form of shortterm memory. We therefore examined the persistence of repetition suppression. Flies were exposed to 15 pulses of an odor to suppress the activity of MBON- $\alpha'3$, and the response to the same odor was then examined after recovery times ranging from 5 min to 1 hr (Figure 3A). Suppression of the response to a familiar odor is still observed after 20 min of recovery, but activity returns to levels observed with a novel odor after 1 hr (Figures 3B-3D and S1F). Exposure to a different novel odor at any time during the recovery period evokes a strong response in the MBON- $\alpha'3$ (Figures 3B, 3C, and S1F). These data suggest that the transition from novelty to familiarity involves a form of short-term memory within the $\alpha'3$ compartment that lasts from 20 min to 1 hr.

We examined the response to repetitive exposure to a novel odor for MBONs innervating seven additional compartments and did not observe the consistently strong decrease in odor-evoked activity seen in MBON- $\alpha'3$ (Figures 4A, 4B, and S2A–S2C). Analysis of repetition suppression in eight compartments reveals three clusters, $\alpha'3$ that exhibits the strongest suppression, a number of compartments that exhibit more modest suppression, and β 1 that exhibits strong facilitation (Figure 4B). Thus, the strong suppression of the response to novel odors upon familiarization appears specific to the $\alpha'3$ compartment. Although all of the $\alpha'3$ MBONs respond maximally to novel odors, strong suppression is observed

Α



Bottom 50%

n

1s

Figure 1. Repetition Suppression of Odor-Evoked MBON-α'3 Activity

(A) Neurons in the α '3 compartment. Left: confocal micrographs of α'/β' KCs, MBON- α' 3 (also known as MB-V2 α') and the α' 3 DAN, PPL1- α' 3 (white). All KCs are labeled in blue. Right: a simplified schematic of the circuit and the α' 3 compartment. α'/β' KCs receive olfactory input from antennal lobe (AL) projection neurons and synapse with the α' 3 MBONs. The α' 3 MBONs project to the lateral horn (LH), superior intermediate protocerebrum (SIP), superior medial protocerebrum (SMP), and posterior lateral protocerebrum (PLP). The compartment is also innervated by a single DAN, PPL1- α' 3. The genotypes of the flies used in all figures are shown in Table S1.

(B) Two-photon calcium imaging of the soma of MBON-α'3 upon repeated odor presentations (gray bars). Odor delivery measured with a photoionization detector (PID) is shown below.

(C) Calcium signals in MBON- $\alpha'3$ measured in soma (left) or dendrite (middle) after repeated presentation of MCH. Integrated $\Delta F/F_0$ signal and PID signal (right) was normalized for each cell to the maximum integral over the ten trials. The error bars represent ± 1 SEM in all figures unless otherwise indicated. Soma: n = 27 cells (20 flies) for 6-s ISI, 6 (5) for 12-s ISI, 72 (59) for 30-s ISI, 9 (7) for 60-s ISI; dendrite: n = 6 flies: PID; n = 58, 21, 57, and 22 flies for 6-, 12-, 30-, and 60-s ISI. Data for 30-s ISI from experiments described in Figures 3 and 6G are shown.

(D) Average traces of odor-evoked MBON- $\alpha'3$ responses for the first three trials and the last three trials (trials 8–10) are plotted as Z scores. Left panels: cells are sorted according to the response magnitude in trials 8–10. Odor onset is at time 0, and the odor was presented for 1 s. n = 72 cells (59 flies). Right panels: average Z score traces of all cells, as well as average of highest and lowest 50% of cells based on response magnitude in trials 8–10. Black lines indicate odor presentation. Shaded areas indicate ±1 SD. Data acquired from VT037580-positive MBON- $\alpha'3$ for Figures 3 and 6G were used for this analysis.



in only one of the two classes of α '3 MBONs (Figures S2D–S2I). The Split-GAL4 line, *MB027B*, labels all of the α '3 MBONs (Figures 1A, S2D, and S3; Aso et al., 2014a). A second line, *VT037580-GAL4*, used in imaging experiments, labels only two MBONs that define one class of the α '3 MBONs (Figures S2D and S3). The VT037580-positive and -negative MBONs are anatomically and functionally distinct (Figures S2E–S2I). Strong response suppression is observed for only the two VT037580-positive neurons (hereafter, simply MBON- α '3).

Repetition suppression does not appear to arise from KC adaptation because MBONs in the $\alpha'2$ and $\beta'2$ compartments receive input from the same set of KCs as MBON- $\alpha'3$ (Aso et al., 2014a), but they do not exhibit repetition suppression (Figures 4A, 4B, and S2C). Nonetheless, we examined the activity of the α'/β' KCs upon repeated odor presentation. The response of α'/β' KCs persists over the course of repetitive stimulation with an

Figure 2. Repetition Suppression of MBON- $\alpha'3$ Is Observed for All Odors Tested and Is Odor Specific

MBON-o'3 responses upon ten presentations of one odor (odor 1) followed by three presentations of a different odor (odor 2). The darkest line in each panel indicates the first trial of the three trials presented. Left, example cells; right, normalized population data. Odors and valence (Parnas et al., 2013): 4-methylcyclohexanol (MCH, aversive), benzaldehyde (BEN, aversive), 3-octanol (OCT, aversive), acetophenone (ACP, neutral), 3-methyl-1-butanol (MBL, neutral), isopentyl acetate (IPA, attractive), hexyl acetate (HXA, attractive). n = 6 cells (5 files) for MCH-MCH, 7 (7) for MCH-BEN, 7 (6) for BEN-MCH, 6 (6) for OCT-MCH, 8 (6) for ACP-MCH, 10 (7) for MBL-MCH, 9 (6) for IPA-MCH, 7 (7) for HXA-MCH. See Figure S1E for PID data.

average reduction of 30%-40% in contrast to over 80% suppression in MBON-a'3 (Figures 4B, 4C, S2J, and S2K). This reduction in KC activity may result from adaptation at early stages of the olfactory processing (Stopfer and Laurent, 1999) and is unlikely to account for the complete suppression observed in the MBON- $\alpha'3$. Moreover, our data indicate that the suppression of MBON activity is not due to intrinsic adaptation of the MBONs, since the suppression is specific to the repeated odor (Figure 2). Taken together, these results suggest that repetition suppression of MBON- $\alpha'3$ arises from plasticity at the synapse between α'/β' KCs and MBON- $\alpha'3$.

Dopaminergic Input to the $\alpha'3$ Compartment Is Required for Repetition Suppression

In associative learning paradigms, the KC to MBON synapse is modified by compart-

ment-specific dopaminergic input (Cohn et al., 2015; Hige et al., 2015a). We therefore examined the role of PPL1- $\alpha'3$, the only dopaminergic neuron (DAN) innervating the a'3 compartment (Figures 1A and S3; Aso et al., 2014a), in repetition suppression. We silenced PPL1- $\alpha'3$ using Kir2.1, an inward rectifying potassium channel (Baines et al., 2001). Kir2.1 was specifically expressed in PPL1- $\alpha'3$ using MB304B-SplitGAL4 (that drives GAL4 expression in this single DAN [Aso et al., 2014a]) and UAS-Kir2.1 transgenes. The activity of MBON-a'3 was monitored by calcium imaging in flies expressing GCaMP6f under control of the VT037580-LexA driver (Figure S3). When PPL1- $\alpha'3$ is silenced, MBON- $\alpha'3$ no longer exhibits repetition suppression (Figures 5A, 5B, and S4A). In contrast, repeated presentation of an odor suppresses the response of MBON- $\alpha'3$ in flies carrying either MB304B-SplitGAL4 or UAS-Kir2.1 alone. These observations demonstrate that the activity of PPL1- α '3 is necessary for



Figure 3. Persistence of MBON- α' 3 Repetition Suppression

(A) A schematic of the experiments to examine the persistence of repetition suppression.

(B) Example cells.

(C) Normalized population data. Green, responses to MCH; red, responses to BEN. n = 10 cells (8 flies) for 0-min recovery, 13 (8) for 5 min, 9 (9) for 10 min, 7 (6) for 20 min, 11 (10) for 60 min.

(D) Time course of recovery. The response magnitudes were normalized to that of the first trial, and the difference in response between trials before and after the recovery period (i.e., trial 15 and 16) is plotted. **p < 0.01, ***p < 0.001, Kruskal-Wallis test with post hoc Tukey's HSD test. Boxplot: boxes, data within the first and third quartiles; horizontal lines inside boxes, median; whiskers, data within 1.5 interquartile range; crosses, outliers.

the suppression of the response of MBON- $\alpha^\prime 3$ upon repeated odor stimulation.

We also blocked synaptic release from PPL1- α '3 conditionally using Shibire^{ts1}, a temperature-sensitive dominant-negative form of dynamin (Kitamoto, 2001). In flies bearing both *MB304B*-

SplitGAL4 and *UAS-Shibire^{ts1}*, repeated exposure to odor at the restrictive temperature elicits a 40% decrease in MBON- α '3 activity (Figures 5C and S4C). In contrast, flies bearing either of the transgene components alone exhibit over 80% suppression. We note that the kinetics of suppression is slowed at the elevated



Figure 4. Responses of α'/β' KCs and Multiple Different MBONs upon Repeated Odor Presentation

(A) Example calcium traces of MBONs innervating different compartments upon repeated presentation of MCH. See Figures S2A–S2C for normalized population data.

(B) The average of normalized integrated $\Delta F/F_0$ over the first and last three of ten trials is plotted. Each point represents the average of multiple cells of a cell type, and the error bars represent ±1 SEM. Sample numbers in Figures S2A–S2C and S2J.

(C) Response of α'/β' KCs upon repeated presentations of MCH. Top: example calcium traces. Bottom panels: population data of 79 cells from 6 flies. Left two panels: the response magnitude across trials and summed activity of all cells over the trials. Right three panels: analysis of cells with significant response to MCH. sig.; significant (*Z* score >1.64), n.s.; not significant. n = 47 cells. See STAR Methods and Figure S2K.

temperature, and therefore more odor pulse repetitions are required to observe full suppression of the response. Flies expressing Shibire^{ts1} in PPL1- α '3 were also tested at the permissive temperature, and strong suppression of activity in MBON- α '3 is observed after repeated odor exposure (Figures 5C and S4D). These observations support the conclusion that the activity of PPL1- α '3 is necessary for the repetition suppression in MBON- α '3 that accompanies the transition from novelty to familiarity.

The requirement of PPL1- $\alpha'3$ for repetition suppression suggests that dopamine release within the $\alpha'3$ compartment mediates the observed suppression. We employed recombinasemediated cassette exchange (MiMIC) (Diao et al., 2015; Venken et al., 2011) to demonstrate the presence of the two fly D1 dopamine receptors (DAMB and dDA1) in MBON- $\alpha'3$ and the KCs (Figure S4G and data not shown; see also Han et al., 1996; Kim et al., 2003). We therefore manipulated the levels of these two dopamine receptors using RNA interference. We expressed a short-hairpin RNA (shRNA) that targets the DAMB mRNA (Cohn et al., 2015; Perkins et al., 2015) in the MBON- $\alpha'3$. Flies bearing two transgenes, *VT037580-GAL4* and *UAS-DAMB-shRNA*, express the interfering RNA in MBON- $\alpha'3$ and show 50% suppression to repeated odor stimulation (Figures 5D and S4E). In the presence of either transgene alone, we observe over 80% suppression of the MBON- $\alpha'3$ activity. Experiments targeting dDA1 with shRNA were difficult to interpret because flies bearing the *UAS-dDA1-shRNA* transgene alone exhibit a significant defect in repetition suppression (data not shown). These results indicate that dopamine release from PPL1- $\alpha'3$ activates the



Figure 5. The Activity of the PPL1-a'3 Dopaminergic Neuron Is Required for Repetition Suppression of MBON-a'3

(A and B) Odor-evoked MBON- α '3 responses to repeated presentation of MCH (60-s ISI) upon silencing PPL1- α '3 with overexpression of Kir2.1 (see STAR Methods). Example cells (A) and normalized population data (B) are shown. n = 11 cells (10 flies) for PPL1 α '3-SplitGAL4, 13 (10) for UAS-Kir2.1, and 10 (9) for PPL1 α '3 > Kir2.1. See Figure S4A for statistics. We note that silencing PPL1- α '3 reduces the magnitude of the initial response to MCH (Figure S4B).

(C) Odor-evoked MBON- $\alpha'3$ responses to repeated presentation of MCH (12-s ISI) upon silencing PPL1- $\alpha'3$ with Shibire^{1s1}. MCH was presented 30 times (12-s ISI) at the restrictive temperature (left). After a recovery period (see STAR Methods), MCH was presented ten times (12-s ISI) at the permissive temperature (right). Restrictive temperature: n = 8 cells (8 flies) for PPL1 $\alpha'3$ -SplitGAL4, 14 (10) for UAS-Shibire^{1s1}, and 13 (12) for PPL1 $\alpha'3$ -Shibire^{1s1}. Permissive temperature: n = 5 cells (5 flies) for PPL1 $\alpha'3$ -SplitGAL4, 10 (8) for UAS-Shibire^{1s1}, and 11 (10) for PPL1 $\alpha'3$ -Shibire^{1s1}. See Figures S4C and S4D for statistics.

(D) MBON- α '3 odor-evoked responses in flies in which shRNA targeting DAMB is expressed in MBON- α '3. MCH was presented ten times (60-s ISI). n = 11 cells (11 flies) for VT037580-GAL4, 9 (9) for UAS-DAMB-shRNA, and 10 (10) for VT037580 > DAMB-shRNA. See Figure S4E for statistics. UAS-shRNA control is the same as in (E).

(E) MBON- $\alpha'3$ odor-evoked responses in flies in which shRNA targeting DAMB is expressed in α'/β' KCs. MCH was presented ten times (60-s ISI). n = 11 cells (11 flies) for α'/β' KC-SplitGAL4, 9 (9) for UAS-DAMB-shRNA, and 8 (8) for α'/β' KC > DAMB-shRNA. See Figure S4F for statistics.

DAMB receptor in MBON- $\alpha'3$, and this modulation contributes to repetition suppression.

We performed similar experiments in which the shRNA targeting DAMB is expressed in the α'/β' KCs rather than the MBON- $\alpha'3$. Flies expressing dopamine receptor shRNA in the KCs exhibit repetition suppression indistinguishable from controls (Figures 5E and S4F). Although the DAMB receptor is not required in the KCs for repetition suppression, other dopamine receptors in KCs may contribute to this process.

PPL1- α '3 Responds to Odors

The observation that dopamine release from PPL1- $\alpha'3$ is essential for repetition suppression in MBON- $\alpha'3$ led us to explore the activity of PPL1- $\alpha'3$ during the transition from novelty to familiarity. Calcium imaging of PPL1- $\alpha'3$ reveals that this DAN responds to all odors tested (Figures 6A, 6B, S5A, S5B; see also Mao and Davis, 2009). This response is severely attenuated in flies deficient in the sensory neuron coreceptor, ORCO (Larsson et al., 2004), demonstrating that the activity of the DAN is dependent on the olfactory sensory neurons (Figure S5C). The activity of PPL1- α' 3 is high in response to a novel odor, and this response suppresses about 50% upon repeated odor presentation (Figure 6B). As observed with the MBONs, this suppression is specific to the repeated odor; exposure to a different novel odor elicits a strong activation of the DAN. A novel odor may activate MBON- $\alpha'3$ that, in turn, stimulates PPL1- $\alpha'3$. We have tested this possibility by optogenetically activating MBON- $\alpha'3$ while recording PPL1- $\alpha'3$ activity by calcium imaging, but we do not observe excitation of the DAN upon optical activation of the MBONs (data not shown). The pathway of DAN activation notwithstanding, these data indicate that the presentation of a novel odor coordinately activates a KC ensemble and the PPL1-a'3 neuron. Coincident dopamine release and KC activation may depress active KC-MBONa'3 synapses (Cohn et al., 2015; Hige et al., 2015a), resulting in stimulus-specific suppression of the MBON response.

We have obtained additional support for the role of PPL1- $\alpha'3$ in repetition suppression by coordinating exogenous activation of PPL1-a'3 with odor presentation. The red-shifted channelrhodopsin CsChrimson (Hoopfer et al., 2015; Klapoetke et al., 2014) and GCaMP6f were expressed in PPL1- α '3 under the control of the MB304B-SplitGAL4 driver. The response to photoactivation was monitored by calcium imaging, and this permitted us to calibrate the light intensity required to evoke PPL1-a'3 activity in the physiological range observed with odors (Figures S5D-S5F). We then asked whether pairing odor exposure with photoactivation of PPL1- $\alpha'3$ accelerates the suppression of MBON- $\alpha'3$ upon repeated odor exposure. GCaMP6f was expressed in MBON- $\alpha'3$ in flies expressing CsChrimson in PPL1- $\alpha'3$. We then paired odor exposure with photoactivation and observed 80% suppression of MBON- $\alpha'3$ activity upon the second exposure to odor compared to 20% suppression with odor alone (Figures 6C and 6D). We note that the odor-evoked PPL1- α '3 activity is reduced in CsChrimson-expressing flies (Figure S5G), explaining the relatively low suppression in response to odor alone in early trials. These data further support the suggestion that odor-evoked dopamine release from PPL1-a'3 depresses active KC-MBON synapses, suppressing MBON output upon familiarization.

Dopamine-Dependent Memory Decay in the $\alpha'3$ Compartment

MBON- $\alpha'3$ activity is suppressed for at least 20 min after repeated odor exposure and returns to values observed with a novel odor after 1 hr (Figure 3). This suppression in MBON- $\alpha'3$ activity reflects a form of short-term memory. We therefore examined the neural events that contribute to memory decay and the restoration of a novel odor response. Behavioral experiments have implicated dopamine in memory decay (Aso and Rubin, 2016; Berry et al., 2012; Plaçais et al., 2012). Recent physiologic experiments have demonstrated that coincident activation of KCs by odor and dopamine release within a compartment can lead to synaptic depression, but dopamine release in the absence of specific KC activity can lead to synaptic facilitation and the restoration of MBON output (Cohn et al., 2015; Hige et al., 2015a). Our data indicate that coincident KC activation and dopamine release within the $\alpha'3$ compartment upon novel odor exposure suppresses MBON output. We therefore asked whether dopamine release in the absence of odor (i.e., in the absence of KC activation) may have an opposite effect and promote the recovery of MBON responses to familiar odors. CsChrimson was expressed in PPL1- $\alpha'3$ using *MB304B-SplitGAL4* and *UAS-CsChrimson*. We suppressed the activity of MBON- $\alpha'3$ by repeated exposure to odor and then photoactivated PPL1- $\alpha'3$ in the absence of odor (Figure 6E). Recovery of the suppressed MBON- $\alpha'3$ odor response is observed after six to ten light stimulations in flies expressing CsChrimson in PPL1- $\alpha'3$ (Figures 6F and S5H). Control flies bearing either transgene alone do not express CsChrimson and do not exhibit recovery following the same protocol. These observations suggest that dopamine release in the absence of odor following repetition suppression enhances memory decay and restores the novelty response.

We next examined the persistence of MBON suppression in more natural conditions. Repeated exposure to an odor elicits significant suppression of MBON- $\alpha'3$ output, presumably as a consequence of the depression of active KC-MBON synapses. Exposure to a second novel odor will activate a different ensemble of KCs. Dopamine release due to the second odor should then facilitate the synapses depressed by repeated exposure to the first odor (see Figure 7D). This should lead to restoration of the novelty response to the first odor. We tested this hypothesis by suppressing MBON- $\alpha'3$ activity by repeated odor exposure to MCH. We then presented these flies with 15 interleaved pulses of four novel odors over the course of 5 min. The response to MCH recovered from 100% to 70% suppression after exposure to this limited set of novel odors (Figures 6G and S5I). Little recovery from suppression was observed in control experiments in which flies were presented with mineral oil solvent rather than novel odors. Thus, the suppression of MBON- $\alpha'3$ activity in response to repeated exposure to a specific odor can be reversed through exposure to novel odors. These observations suggest that dopamine release upon repeated encounters with an odor depresses active KC-MBON synapses, whereas subsequent exposure to other odors facilitates these synapses, restoring novelty (see Figure 7D and Discussion).



Figure 6. Odor and Optogenetic Activation of PPL1- $\alpha'3$

(A) Example calcium traces of PPL1- α '3 odor responses measured at the soma. Lighter lines represent individual trials (ten total), whereas darker lines represent the average. Mineral oil (OIL) was the solvent for all odors tested. See Figure S5A for normalized population data. See Figure S5B for correlation between the activity of PPL1- α '3 and flies' spontaneous movement.

(B) Normalized PPL1- α '3 odor responses across repetition (12-s ISI). n = 8 flies.

(C) Examples of MBON- $\alpha'3$ odor-evoked response with or without optogenetic activation of PPL1- $\alpha'3$ (see STAR Methods).

(D) MBON- α '3 activity upon repeated presentation of MCH with or without optogenetic activation of PPL1- α '3. n = 9 cells (7 flies) for "Odor+Light" and 10 (10) for "Odor only." p < 0.001 for interaction between trial types and trial numbers, two-way ANOVA. Post hoc tests with Wilcoxon's rank-sum test with Bonferroni correction (*p < 0.05, **p < 0.01, ***p < 0.001, n.s. p > 0.05).

(E) Effects of optogenetic activation of PPL1- $\alpha'3$ in the absence of odor upon recovery of MBON- $\alpha'3$ responses. MCH was presented 15 times (30-s ISI) to suppress MBON- $\alpha'3$ activity. MBON- $\alpha'3$ responses to MCH were then tested after one, three, six, and ten light stimulations. Left, example calcium traces of MBON- $\alpha'3$ responses to MCH. Trials 25-27 are after ten light stimulations. Right, normalized population data. n = 10 cells (7 flies) for PPL1 $\alpha'3$ -SplitGAL4, 10 (9) for UAS-CsChrimson, and 10 (10) for PPL1 $\alpha'3$ > CsChrimson.

(F) Recovery of the MBON- $\alpha'3$ responses upon optogenetic activation of PPL1- $\alpha'3$ in the absence of odor. Differences between the average responses of trials 13–15 (before light stimulation) and those after light stimulations are plotted. p < 0.01 for interaction between genotypes and number of light stimulation, two-way ANOVA. *p < 0.05, **p < 0.01 by post hoc Kruskal-Wallis test followed by Bonferroni correction (see Figure S5H).

(G) Recovery of MBON- α '3 responses upon novel odor presentations. MCH was presented 15 times (30-s ISI) to suppress MBON- α '3 activity, followed by 60 presentations (5-s ISI) of either four novel odors (15 presentations each, interleaved) or the solvent, mineral oil, over the course of 5 min. Novel odors used were benzaldehyde, 3-octanol, hexyl acetate, and isopentyl acetate. n = 12 cells (10 flies) for oil and 10 (8) for novel odor presentations. **p < 0.01, n.s. p > 0.05, Wilcoxon's signed-rank test. See Figure S5I for statistics comparing the degree of recovery between odor and oil presentations.



Figure 7. MBON- α' 3 Mediates a Behavioral Response to Novelty

(A) Behavioral responses of dusted grooming flies upon alternating presentations of mineral oil (solvent) or odor (see Movie S1 and STAR Methods). Left, grooming ethograms of different exposure trials (see Figure S7A for all trials). Time is relative to the valve (oil/odor) onset. The 2-s odor/oil exposure periods are indicated by red/gray lines, respectively. Flies were sorted based on the total time not grooming per exposure trial. Right, fraction of flies that stop grooming upon exposures to oil (gray) or odor (red). Interruption of grooming is defined here valve onset. See Figures S6A and S6B for analyses using different parameters. n = 41 flies (five odors, see Figure S6A).

(B) Effects of optogenetic activation of the a'3 MBONs upon interruption of grooming. Left, ethograms for the first stimulus exposure trial (see Figure S7B for all trials). Right, time course of percentage of flies grooming. Time is relative to stimulus onset. Shaded areas indicate 95% confidence interval as determined by bootstrapping. Bottom, fraction of flies that exhibit grooming interruption in the first stimulus exposure trial. See Figures S6C and S6D for analyses using different parameters and for statistics. "Light only"; n = 20 flies for MBONa'3-SplitGAL4, 21 for UAS-CsChrimson, and 37 for MBON α '3 > CsChrimson: "Odor + Light"; n = 25 flies for MBONa'3-SplitGAL4, 31 for UAS-CsChrimson, and 30 for MBON $\alpha'3 >$ CsChrimson: n = 19 for "Odor only" (MBON $\alpha'3$ > CsChrimson). (C) Effects of silencing the a'3 MBONs upon odorinduced interruption of grooming. Top, ethograms for the first three odor (benzaldehyde) exposure trials (see Figure S7C for all trials). Bottom, fraction of flies that exhibit grooming interruption in different exposure trials. See Figures S6E and S6F for analyses using different parameters. n = 14 flies for MBONa'3-SplitGAL4, 21 for UAS-Kir2.1, and 19 for MBONa'3>Kir2.1.*p<0.05, **p<0.01, Fisher's exact test followed by Bonferroni correction.

(D) Model of representation of novelty and familiarity in the $\alpha'3$ compartment.

revealed that short pulses of a novel odor elicit an apparent alerting response that disrupts ongoing behavior. The variability in the activity of freely moving flies, however, precluded a quantitative analysis of this behavior. We therefore developed a single fly behavioral paradigm that elicits a stereotyped and persistent initial behavioral state, grooming, and we then

The $\alpha'3$ MBONs Mediate a Behavioral Response to Novelty

The imaging experiments demonstrate strong activity in MBON- $\alpha'3$ upon exposure to novel odors that is suppressed upon familiarization. We therefore examined whether behavioral response of flies to novel odors correlates with the activity of MBON- $\alpha'3$. Casual observation of freely behaving flies

measured the disruption of grooming, an alerting response, upon exposure to a novel odor. When dusted with fine particles of organic dye, flies initiate stereotyped grooming behaviors that often persist for more than 10 min (Seeds et al., 2014). An odor stimulus was delivered after the fly initiated grooming, and the behavioral response was monitored by video recording (see Movie S1 and STAR Methods). Flies were exposed to interleaved presentations of either a novel odor or mineral oil solvent to control for non-olfactory events associated with odor presentation. Upon exposure to a novel odor, 50% of the flies stop grooming (Figures 7A, S6A, S6B, and S7A, see STAR Methods). Further pulses of the same odor interrupt grooming in only 10%–20% of the flies, a value similar to that observed upon exposure to the mineral oil solvent (Figures 7A, S6A, S6B, and S7A). These data demonstrate that the presentation of a novel odor elicits an alerting response that disrupts grooming, and this response diminishes significantly upon repeated odor presentation.

The development of a quantitative behavioral assay that distinguishes the response to a novel odor from the response after repeated exposures (a familiar odor) permitted us to ask whether MBON- $\alpha'3$ contributes to the response to novelty. We expressed the red-shifted channelrhodopsin CsChrimson in all $\alpha'3$ MBONs using the MB027B-SplitGAL4 driver and asked whether photoactivation of the $\alpha'3$ MBONs elicits an alerting response. Disruption of grooming was examined upon photoactivation of $\alpha'3$ MBONs in the presence and absence of odor. Exposure to light alone disrupts grooming in 41% of the flies expressing CsChrimson, whereas the presentation of odor alone results in disruption of grooming in 42% of the flies (Figures 7B, S6C, S6D, and S7B). Flies that do not express CsChrimson but carry either of the transgene components alone (MB027B-SplitGAL4 or UAS-CsChrimson) do not exhibit cessation of arooming upon light exposure. Photoactivation of flies expressing CsChrimson paired with the presentation of a novel odor disrupts grooming in 83% of the flies (Figures 7B, S6C, S6D, and S7B). This value approximates the sum of percentages for odor and light alone (42% + 41%). These data suggest that light or odor alone elicits submaximal activation of the MBONs, but this is significantly enhanced upon pairing, resulting in alerting responses in the vast majority of the flies. We note that photoactivation of $\alpha'3$ MBONs only evokes the alerting response on the first photoactivation trial but not on subsequent trials (Figures S6C, S6D, and S7B). This may be due to adaptation downstream of the MBONs. Nonetheless, optogenetic activation of the $\alpha'3$ MBONs elicits an alerting response in the absence of odor and significantly enhances alerting to novel odor.

The observation that optogenetic activation of the α '3 MBONs elicits an alerting response does not preclude a contribution from other compartments to the behavioral response to novel odors. Novel odors are likely to activate most, if not all, MBONs. Exposure to a familiar odor is also likely to activate all MBONs (Hige et al., 2015b, and Figures 4 and S2) except α '3. However, familiar odors fail to elicit an alerting response, suggesting an essential role for this compartment in the response to novelty.

We therefore performed genetic silencing to determine whether the activity of $\alpha'3$ MBONs is necessary for the alerting behavior to novel odors. We expressed the inward rectifying potassium channel, Kir2.1, using the *MB027B-SplitGAL4* driver, to silence the $\alpha'3$ MBONs. Grooming behavior in these flies, in the absence of odor, mirrored that of control flies, indicating that silencing $\alpha'3$ MBONs does not lead to changes in the motivation to groom (Figures S6E–S6G, and S7C). Presentation of a novel odor, however, disrupts grooming in only 10% of the flies in which the $\alpha'3$ MBONs are silenced, a value close to the levels of disruption observed upon exposure to mineral oil (Figures 7C, S6E, S6F, and S7C). This is in sharp contrast to disruption of grooming in 50% of the control flies carrying either of the transgene components alone (*MB027B-SplitGAL4* or *UAS-Kir2.1*) upon exposure to a novel odor. These behavioral experiments indicate that the activity of the $\alpha'3$ MBONs mediates an alerting response to novelty.

DISCUSSION

Sensory stimuli elicit adaptive behavioral responses that reflect experience acquired through either evolution (innate behaviors) or learning. Novel stimuli, however, can elicit alerting behavior that is contingent on the absence of prior experience. These responses are extinguished upon repeated exposure, reflecting a transition from novelty to familiarity. The behavioral response to novelty, the response to a stimulus about which an organism has no prior knowledge, poses an interesting problem.

We have examined the neural and behavioral correlates of novelty and familiarity in the olfactory system of Drosophila. Exposure of flies to a novel odor interrupts grooming. This alerting response is dependent upon the activity of output neurons of the a'3 compartment of the MB. Optogenetic activation of the $\alpha'3$ MBONs elicits an alerting response, whereas silencing these neurons eliminates the behavioral response to novel odors. A neural correlate of this behavioral response is observed in the activity of MBON- $\alpha'3$. Novel odors elicit strong activity in these neurons that is rapidly suppressed upon repeated exposure to the same odor. This transition in MBON- $\alpha'3$ response upon familiarization requires the activity of PPL1- α '3, the DAN innervating the $\alpha'3$ compartment, and dopamine receptors in the MBONs. These data suggest that the $\alpha'3$ compartment may play a causal role in the behavioral response to novel and familiar stimuli as a consequence of dopamine-mediated plasticity at the KC-MBONa'3 synapse. Although the circuitry of the $\alpha'3$ compartment is central to the behavioral response to novel and familiar odors, our data do not exclude a contribution from other compartments.

Plasticity at the KC-MBON synapses has been invoked to explain olfactory learning and memory (Heisenberg, 2003). In associative learning, exposure to a conditioned stimulus (CS), when paired with an unconditioned stimulus (US), imposes an associative memory upon the CS. The identity of the CS is represented by activity in a specific ensemble of KCs, whereas USs of different valence activate distinct DANs. Dopamine input depresses the KC-MBON synapse in specific compartments to transform the unstructured KC representation of an odor into an ordered MBON representation encoding behavioral bias. The neural mechanism governing the novelty response differs from this classical model of associative learning. In the $\alpha'3$ compartment, a novel odor elicits strong MBON output and also activates the DAN. Dopamine release from PPL1- α '3 depresses the KC-MBONa'3 synapse, suppressing MBON output on further exposure to this odor. In this manner, a novel odor effectively serves as both a CS and a US to drive the transition from novelty to familiarity.

A second distinction between the novelty response and associative learning emerges from the observation that the response to novelty is suppressed by learning whereas the conditioned response depends on learning. The stereotyped alerting behavior in response to novel odor does not require learning and is therefore innate. In associative learning models, exposure to odor prior to learning activates all MBONs examined, but this combinatorial of MBONs does not elicit a behavior. Rather, behavioral bias is imposed by the suppression of specific MBON output after learning (Cohn et al., 2015; Hige et al., 2015a; Owald et al., 2015; Perisse et al., 2016; Séjourné et al., 2011). In the novelty response, innate alerting behavior is elicited by strong output from MBON- $\alpha'3$ in response to novel odors prior to learning. Learning that accompanies the transition to familiarity suppresses MBON- α '3 activity and the behavioral response to novelty. If, however, the novel odor is accompanied by salient events in the environment, this will result in activation of additional DANs, leading to the formation of associations in other compartments. The alerting response evoked by MBON-a'3 may enhance the awareness of these environmental events. Thus, $\alpha'3$ output may elicit an immediate and stereotyped response to an odor independent of its salience, which is then assessed by the remaining MB compartments to mediate more measured associative responses.

Odor-evoked dopamine release by PPL1- α '3 appears to be essential to modulate MBON output in the transition from novelty to familiarity. Dopamine release also contributes to decay in the memory of familiar odors. MBON- α '3 activity in response to novel odors is suppressed upon repeated exposure, but activity is restored after 1 hr. Dopamine release in the absence of odor, following repetition suppression, accelerates this recovery process. These observations are consistent with recent experiments demonstrating that dopamine release within a compartment in the absence of odor can lead to synaptic facilitation and the restoration of MBON output (Cohn et al., 2015). In this manner, familiarity in the fly is a transient phenomenon and the restoration of the perception of novelty may be accelerated by dopamine.

We suggest that the neural events responsible for the transition from novelty to familiarity involve depression of only those KC-MBON synapses activated by the novel odor. In this manner, novelty and familiarity can be both universal and odor specific. A given odor activates about 5%-10% of the KCs in the MB (Honegger et al., 2011; Turner et al., 2008) and by inference 5%-10% of the KC-MBON synapses. An organism is likely to encounter multiple odors that may be construed as novel in the course of hours. If a single novel odor suppresses 5%-10% of the KC-MBON synapses and this synaptic depression is long term, all KC-MBON synapses within the $\alpha'3$ compartment would be depressed after exposure to roughly 20 novel odors. Depression of all the synapses would prevent subsequent response to novel odors. Our data suggest this problem may be obviated in two ways. First, the depression is relatively short lived. Second, although novel odors result in the depression of active KC-MBON synapses, they also enhance the recovery of previously suppressed but currently inactive synapse (Figure 7D). This implies that the rate of synaptic recovery is proportional to the rate of exposure to novel odors. Thus, the $\alpha'3$ compartment has evolved a mechanism to assure that novelty responses can be generated in both dense and sparse odor environments without saturation.

The MB is an associative center in invertebrate brains thought to impose valence on sensory representations. Our data suggest that the MB not only functions in classical learning paradigms, but also supports novelty detection and the transition to familiarity. An organism can have no knowledge of a novel stimulus, and hence it exhibits an indiscriminate alerting response. The MB also integrates information about the organism's internal state (hunger, satiety, sleep, wakefulness, roaming, dwelling) (Aso et al., 2014b; Bräcker et al., 2013; Cohn et al., 2015; Joiner et al., 2006; Krashes et al., 2009; Perisse et al., 2016; Pitman et al., 2006) allowing the fly to more comprehensively contextualize the diverse sensory experiences it may encounter throughout its life. Thus, the MB may afford the fly an "individuality" (Hige et al., 2015b) allowing different flies to respond differently to the same stimuli in accord with its unique history and current state.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cell. 2017.04.028.

AUTHOR CONTRIBUTIONS

D.H., Y.A., and K.J.S. conducted experiments. All authors analyzed data and contributed to writing the manuscript.

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STAR*METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
chicken anti-GFP	Aves Labs	GFP-1020; RRID: AB_10000240
rabbit anti-DsRed	Clontech	632496; RRID: AB_10013483
mouse mAb anti-bruchpilot (nc82)	Developmental Studies Hybridoma Bank	nc82; RRID: AB_2314865
rat mAb anti-FLAG	Novus Biologicals	NBP1-06712; RRID: AB_1625981
rabbit anti-HA	abcam	ab9110; RRID: AB_307019
mouse anti-V5-TAG	AbD Serotec	MCA2892GA; RRID: AB_1658039
DyLight550-conjugated mouse anti-V5	BIO-RAD	MCA1360D550GA
AlexaFluor488-conjugated goat anti-rabbit IgG	Life Technologies	A11008; RRID: AB_143165
AlexaFluor488-conjugated goat anti-chicken IgY	Life Technologies	A11039; RRID: AB_142924
AlexaFluor568-conjugated goat anti-rabbit IgG	Life Technologies	A11036; RRID: AB_143011
AlexaFluor568-conjugated goat anti-mouse IgG	Life Technologies	A11004; RRID: AB_141371
AlexaFluor633-conjugated goat anti-mouse IgG	Life Technologies	A21052; RRID: AB_141459
AlexaFluor633-conjugated goat anti-rat IgG	Life Technologies	A21094; RRID: AB_141553
Chemicals, Peptides, and Recombinant Proteins		
mineral oil, heavy	Fisher Scientific	O122-1
4-methyl-cyclohexanol	Fluka	66360
benzaldehyde	Sigma	418099
3-octanol	Sigma	218405
acetophenone	Sigma	00790
3-methyl-1-butanol	Sigma	309435
isopentyl acetate	Fluka	79857
hexyl acetate	Sigma	108154
all trans-Retinal	Sigma	R2500
reactive yellow 86	Organic Dyes and Pigments	N/A
Experimental Models: Organisms/Strains		
Drosophila, MB002B-SplitGAL4	Aso et al., 2014a	N/A
Drosophila, MB005B-SplitGAL4	Aso et al., 2014a	N/A
Drosophila, MB018B-SplitGAL4	Aso et al., 2014a	N/A
Drosophila, MB027B-SplitGAL4	Aso et al., 2014a	N/A
Drosophila, MB083C-SplitGAL4	Aso et al., 2014a	N/A
Drosophila, MB112C-SplitGAL4	Aso et al., 2014a	N/A
Drosophila, MB304B-SplitGAL4	Aso et al., 2014a	N/A
Drosophila, MB399B-SplitGAL4	Aso et al., 2014a	N/A
Drosophila, <i>MB005C-SplitGAL4</i> (R13F02-ZpG4DBD in attP2 and R34A03-p65ADZp in VK00027)	This paper	N/A
Drosophila, NP100-GAL4	Kyoto DGRC (Ito et al., 1998)	112043
Drosophila, VT037580-GAL4 in attP2	VDRC	208122
Drosophila, R82C10-LexAp65 in attP40	Bloomington (Janelia Research Campus)	54981
Drosophila, UAS-GCaMP6f in attP40	Bloomington (Chen et al., 2013)	42747
Drosophila, UAS-GCaMP6f in VK00005	This paper	N/A
Drosophila, UAS-DAMB-shRNA (TRiP: HMC02893)	Bloomington (Perkins et al., 2015)	51423
Drosophila, LexAop-GCaMP6f in su(Hw)attP5	Bloomington (Janelia Research Campus)	44277
Drosophila, LexAop-myr-tdTomato in su(Hw)attP5	Gift from B. Pfeiffer (Janelia Research Campus)	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Drosophila, MB247-DsRed	Gift from A. Fiala (Riemensperger et al., 2005)	N/A
Drosophila, MCFO-4	Gift from A. Nern (Nern et al., 2015)	N/A
Drosophila, UAS-EGFP-Kir2.1	Gift from A. Devineni (Baines et al., 2001)	N/A
Drosophila, 2U (w ¹¹¹⁸ isoCJ1)	Gift from J. Dubnau (Dubnau et al., 2001)	N/A
Drosophila, UAS-Shibire ^{ts1}	Gift from J. Dubnau (Dubnau et al., 2001)	N/A
Drosophila, UAS-CsChrimson-tdTomato in VK00005	Gift from D. Anderson (Hoopfer et al., 2015)	N/A
Drosophila, UAS-CsChrimson-mVenus in attP18	Aso et al., 2014b	N/A
Drosophila, Empty-SplitGAL4 (Empty-p65ADZp in attP40 and Empty-ZpG4DBD in attP2)	Gift from J. Simpson (Hampel et al., 2015)	N/A
Drosophila, VT037580-LexAp65 in VK00031	This paper	N/A
Drosophila, VT037580-LexAp65 in attP18	This paper	N/A
Drosophila, <i>Mi{MIC}DAMB^{MI08664}</i>	Bloomington (Venken et al., 2011)	
Drosophila, DAMB-MiMIC-GAL4	This paper	N/A
Drosophila, DAMB-MiMIC-inv-GAL4	This paper	N/A
Recombinant DNA		
pBS-KS-attB1-2-GT-SA-GAL4-Hsp70pA	Drosophila Genomics Resource Center (Venken et al., 2011)	Stock number: 1325
pJFRC7-20xUAS-IVS-mCD8GFP	Addgene (Pfeiffer et al., 2010)	26220
pBPLexAp65Uw	Addgene (Pfeiffer et al., 2010)	26231
20xUAS-IVS-Syn21-GCaMP6f (in pJFRC7 backbone)	This paper	N/A
VT037580-LexAp65 (in pBPLexAp65Uw)	This paper	N/A
Software and Algorithms		
MATLAB	Mathworks (http://www.mathworks.com/)	N/A
ImageJ	NIH (https://imagej.nih.gov/ij/)	N/A
FluoRender	Wan et al., 2012 (http://www.sci.utah.edu/ software/fluorender.html)	N/A
subpixel registration algorithm	Mathworks File Exchange (Guizar-Sicairos et al., 2008)	N/A
Other		
5 min epoxy	Devcon	N/A
tape for calcium imaging preparation	Duck Brand	EZ Start Packaging Tape
syringe filter for odor delivery	Whatman	6888-2527

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Richard Axel (ra27@columbia.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Flies

SplitGAL4 lines; *MB002B*, *MB005B*, *MB018B*, *MB027B*, *MB083C*, *MB112C*, *MB304B*, and *MB399B* were described in Aso et al. 2014 (Aso et al., 2014a). *MB005C* is a new SplitGAL4 line carrying *R13F02-ZpG4DBD* in *attP2* and *R34A03-p65ADZp* in *VK00027*. *NP100-GAL4* line was described in Ito 1998 (Ito et al., 1998) and is from DGRC. *VT037580-GAL4* is from VDRC. *R82C10-LexA*, *UAS-GCaMP6f* in *attP40*, *UAS-DAMB-shRNA* (TRIP collection; HMC02893; (Perkins et al., 2015)), *LexAop-GCaMP6f* in *su(Hw)attP5* is from Bloomington Stock Center. *LexAop-myr-tdTomato* in *su(Hw)attP5* is a gift from Barret Pfeiffer. *MB247-DsRed* was described in Riemensperger 2005 (Riemensperger et al., 2005) and is a gift from Andre Fiala. *MCFO-4* was described in Nern 2015 (Nern et al., 2015) and is a gift from Aljoscha Nern. *UAS-EGFP-Kir2.1* was described in Baines 2001 (Baines et al., 2001) and is a gift from Josh Dubnau. *UAS-CsChrimson-tdTomato* was described in Hoopfer 2015 (Hoopfer et al., 2001) and are gift from Josh Dubnau. *UAS-CsChrimson-tdTomato* was described in Aso 2014

(Aso et al., 2014b). *Empty-SplitGAL4 (Empty-p65ADZp* in *attP40* and *Empty-ZpG4DBD* in *attP2*) was described in Hampel 2015 (Hampel et al., 2015) and is a gift from Julie Simpson.

UAS-GCaMP6f inserted in VK00005 was generated by PCR amplification of Syn21-GCaMP6f from genomic DNA of flies with UAS-GCaMP6f in attP40 (Chen et al., 2013) and subsequent cloning into the NotI site of a modified pJFRC7 (20xUAS-IVS-mCD8GFP, (Pfeiffer et al., 2010)) without mCD8GFP.

VT037580-LexA was generated by PCR amplification of VT037580 enhancer from genomic DNA of the VT037580-GAL4 line and subsequent Gateway cloning into pBPLexAp65Uw (Pfeiffer et al., 2010), and is inserted into attP18 and VK00031.

DAMB-MiMIC-GAL4 and *DAMB-MiMIC-inv-GAL4* were generated by injecting pBS-KS-attB1-2-GT-SA-GAL4-Hsp70pA plasmid into the *DAMB^{MI08644}* line (Venken et al., 2011). The orientation of the insertion was determined by genomic PCR. All new transgenic lines were generated by BestGene.

METHOD DETAILS

Immunostaining and Confocal Microscopy

Brains were dissected in 1xPBS, fixed for 1.5 hr at room temperature with 2%PFA/PBL (2% paraformaldehyde in 75mM lysine, 37mM sodium phosphate buffer, pH7.4), washed multiple times with PBS containing 0.3% Triton X-100 (PBST), blocked with 10% normal goat serum diluted in PBST over 30 minutes at room temperature (RT), incubated in primary antibody mix at 4°C overnight, washed multiple times in PBST, and incubated in secondary antibody mix at 4°C overnight, or at RT for more than 3hr, before final washes with PBST. For MCFO experiments, brains were dissected, fixed, and immunostained up to the wash after incubation with secondary antibody mix as above, and then blocked with 5% normal mouse serum diluted in PBST for 1.5 hours at RT, and incubated with a fluorophore-conjugated antibody at 4°C overnight before the final wash. Brains were mounted using SlowFade Gold (Life Technologies) and imaged using an LSM510 system with a Plan-APOCHROMAT 20x/0.75 (or 20x/0.8), a C-APOCHROMAT 40x/1.5W, a Plan-Neofluar 40x/1.3, or a Plan-APOCHROMAT 63x/1.4 objective (Zeiss). Primary antibodies used are chicken anti-GFP (1:1000, Aves Labs), rabbit anti-DsRed (1:1000, Clontech), mAb anti-bruchpilot (nc82, 1:10, Developmental Studies Hybridoma Bank), rat mAb anti-FLAG (1:200, Novus Biologicals), rabbit anti-HA (1:1000, abcam), mouse anti-V5-TAG (1:1000, AbD Serotec), and DyLight550-conjugated mouse anti-V5 (1:500, BIO-RAD); secondary antibodies are Alexa Fluor 488 goat anti-rabbit (1:400, Life Technologies), Alexa Fluor 488 goat anti-chicken (1:200, Life Technologies), Alexa Fluor 568 goat anti-chicken (1:200, Life Technologies), Alexa Fluor 568 goat anti-chicken (1:200, Life Technologies), Alexa Fluor 568 goat anti-rabbit (1:200, Life Technologies), Alexa Fluor 568 goat anti-chicken (1:200, Life Technologies), Alexa Fluor 568 goat anti-rabbit (1:200, Life Technologies), Alexa Fluor 568 goat anti-chicken (1:200, Life Technologies), Alexa Fluor 568 goat anti-chicken (1:200, Life Technologies), Alexa Fluor 568 goat anti-chicken (1:200,

Calcium Imaging

Calcium imaging experiments were performed using a two-photon laser scanning microscope (Ultima, Bruker) equipped with an ultra-fast Ti:S laser (Chameleon Vision, Coherent) that is modulated by Pockels Cells (Conoptics). 60x/1.0NA water immersion objective (Olympus) was used and emitted photons were detected by a GaAsP detector (Hamamatsu Photonics) for GCaMP fluorescence and a photomultiplier tube for red fluorescence. The laser was tuned at 925nm and the power measured after objective was 2-4 mW. Pixel size was 0.38 µm and pixel dwell time was either 2 or 4 µs. Except for 6-second ISI experiments for α'/β' KCs, galvanometer was used for scanning with a frame rate of approximately 8 (for neurite imaging) or 13-14 Hz (for soma imaging). 6-second ISI experiments for α'/β' KCs were performed using an acousto-optic deflector with frame rate of approximately 9.5Hz with up to 50% more laser power. All data were acquired at 12-bit using the microscope software (PrairieView, Bruker). For a subset of MBON soma imaging experiments, two somas were simultaneously imaged when both were in the imaging plane.

Flies were reared at 25°C (except for flies for experiments involving Shibire^{ts1}, which were reared at 21°C) with 12-hour/12-hour light/dark cycle, genotyped under CO₂ anesthesia within one day after eclosion, and housed as a pair of one male and one female until the day of imaging. Only 3- to 5-day old females were used for experiments. Preparation of the flies for in vivo imaging was performed in a similar way with that described in Ruta 2010 (Ruta et al., 2010) with modifications. Each fly was cold anesthetized, and the head was fixated against a piece of tape (Duck Brand) covering a hole made in 35mm petri dish with a thin strip of tape across cervical connective. The proboscis was then extended and glued to the thorax using epoxy (Devcon). Another thin strip of tape was applied between maxillary palp and proboscis such that the proboscis stays in an extended position and the maxillary palp stays clear of the epoxy and tape. Orientation of the head was adjusted so that the dorsal side is perpendicularly against the tape covering the hole, and two more pieces of tapes were used to fixate the thin strips of tape on each side of the fly. These procedures were done on a cold plate cooled with ice (approximately five minutes or less) and then the preparation was moved to room temperature. A small rectangular hole was cut open in the tape above the head with a thin hypodermic needle, extending more anteriorly so that the antennae would not be damaged, and another piece of tape was used to cover the anterior part of the hole, leaving only the head cuticles dorsal of the antennae above the tape. The dish was filled with external saline described in Kazama and Wilson 2008 (Kazama and Wilson, 2008) (103mM NaCl, 3mM KCl, 5mM TES, 26mM NaHCO₃, 1mM NaH₂PO₄, 1.5mM CaCl₂, 4mM MgCl₂, 8mM trehalose, 10mM glucose, adjusted to 270-275 mOsm, bubbled with 95%O₂/5%CO₂, pH7.3), the cuticles were cut open using another thin hypodermic needle, and fat tissues and trachea were removed with fine forceps for optical access to the brain. The muscle of frontal pulsatile organ, muscle 16, was cut by pinching it with fine forceps. External saline was perfused throughout experiments by a gravity-mediated perfusion system with a variable flow rate of 0.5-1.5mL per minute.

Odors were delivered using a computer-controlled olfactometer (Island Motion). Odor delivery line was split into two, one directed to the fly and the other directed to the photoionization detector (mini-PID, Aurora Scientific) to monitor odor delivery for each trial. Different mixing ratios of carrier and odor streams were tested for consistency of the odor delivery (Figure S1A). For experiments using 10L/min carrier stream and 500mL/min odor stream, the flow was split into two downstream of the mixing manifold, with one end connecting to a mass flow controller so that the flow delivered to the fly and the PID was within the range of flow rate for the other mixing ratios (1.5L/min total). We chose to use 1L/min carrier stream with 500mL/min odor stream (i.e., 750mL/min to the fly and 750mL/min to the PID) for all the experiments. Odors were diluted in mineral oil (O122-1, Fisher Scientific) at the following concentrations: 1:250 for MCH (4-methyl-cyclohexanol, Fluka, 66360), 1:2500 for 0.1x conc. MCH (Figure S1D), 1:25 for 10x conc. MCH (Figure S1D), 1:333 for BEN (benzaldehyde, Sigma, 418099), 1:167 for OCT (3-octanol, Sigma, 218405), 1:333 for ACP (acetophenone, Sigma, 00790), 1:333 for MBL (3-methyl-1-butanol, Sigma, 309435), 1:333 for IPA (isopentyl acetate, Fluka, 79857), and 1:333 for HXA (hexyl acetate, Sigma, 108154). 500 µL of these diluted odors were absorbed into syringe filters (Whatman, 6888-2527) for a minimum of 30 minutes before to interface with a mixing manifold of the olfactometer. Each odor was loaded in one port of the manifold, except for the experiments described in the top panels of Figure 2, in which the same odor (MCH) was loaded in two different ports to mimic delivery of two different odors in the rest of the experiments. The odor delivery line consists of the 0.036 inch ID Teflon tubing, and the ends of the odor delivery line were connected to Teflon-lined tubing (ID 1/8 inch) and a cut P200 pipet tip with the end diameter of about 1/4 inch. The flies were positioned about 3-5mm away from this end. Positioning the flies and finding the target for imaging were done without airflow. Flies were given at least one minute upon start of the airflow before beginning the experiments. Odor delivery schedule was programmed using a custom graphical user interface written in MATLAB (Mathworks), which triggers both image acquisition as well as odor delivery and issues timestamps for all the trigger events for analysis. PID signals were collected using the imaging software at 100Hz.

For experiments involving Shibire^{ts1}, imaging preparation was made at room temperature ($20-22^{\circ}C$), and the saline was then exchanged to heated saline ($32^{\circ}C$). The imaging chamber was then held in a temperature-controlled platform (Warner Instruments) mounted on the microscope. The perfusion line was also heated by an in-line solution heater (Warner Instruments). The temperature was constantly monitored using a thermistor placed in the saline right by the objective, and maintained within $30-32^{\circ}C$ range by adjusting the set temperature of in-line solution heater. The temperature of the saline measured for the restrictive condition was $31.1+/-0.3^{\circ}C$ for SplitGAL4 only, $31.2+/-0.3^{\circ}C$ for UAS-Shi^{1s1} only, and $31.2+/-0.4^{\circ}C$ for GAL4/UAS. Once the temperature control systems are turned off, the temperature of the saline returned to room temperature within 10-15 minutes.

For experiments involving Kir2.1, the MBON- $\alpha'3$ somas were distinguished from the PPL1- $\alpha'3$ soma, which expressed EGFP (because the transgene encoding Kir2.1 was tagged with EGFP), based on the size of the soma (PPL1- $\alpha'3$ is larger than MBON- $\alpha'3$) as well as an increase in calcium signals in the MBON- $\alpha'3$ upon starting airflow.

For optogenetic imaging experiments, flies were reared in the dark with regular cornmeal-agar food, genotyped under CO_2 anesthesia within one day after eclosion, housed as a pair of one male and one female with food containing all trans-Retinal (0.4mM), and kept in the dark for 3- to 4-day until the day of imaging. One 617nm high power LED (Luxeon Star) with a collimating lens was used to deliver light. The light onset and offset were triggered using the imaging software's voltage output function through an LED controller (BuckPuck 700mA, Luxeon Star). The light intensity was measured below the objective using a power meter (Coherent). In order to avoid the light interfering with the imaging and to protect the GaAsP detector, a high-speed shutter (Bruker) was installed in the emission path, and the light stimulus patterns were designed such that light was on for 50ms every other frame in which the shutter is closed. With 13-14 frames per second scan speed, this leads to \sim 7Hz stimulation with 50ms pulse duration. This was repeated 10 times leading to a total of 500ms stimulation over an approximately 1.5-second period. Imaging data were linearly interpolated for these light stimulation periods (see below). The light intensity was calibrated so that the level of PPL1- $\alpha'3$ optogenetic activation is within the physiological range observed with odor (Figures S5D–S5F). Except for the calibration experiments, we used 4.8 μ W/mm² light for optogenetic activation.

To monitor fly's movement while imaging, a USB microscope (veho) was used with a MATLAB graphical user interface set up in a different computer. Video recording was performed at 30 frames per second for a duration blanketing the imaging period, such that the imaging onset and offset are detected by extracting laser onset and offset (see below).

The imaging experiments were not performed blinded.

Behavior

The behavior chamber is made of acrylic with a rectangular arena (with rounded edges) of dimensions 50mm (width), 7mm (depth), and 3.5mm (height), which is sealed by a lid with an elastic o-ring and screw clamps at two ends. The experiments were done in the dark with infrared (IR) LED panels lighting the chamber from both sides. Each of the longer ends of the chamber is connected to tubings through a small hole (< 1mm), allowing a unidirectional airflow to be generated. The olfactometer was made with mass flow controllers (Aarlborg) and solenoid valves (Lee Company), which are controlled by a USB-DAQ (Measurement Computing) with solid state relays (Omron). The carrier stream was set at 800mL/min and the odor stream 200mL/min. The airflow was delivered to the chamber from a T-connector by a vacuum line set at 150mL/min, with most of the flow to be exhausted to an open end of the T, which were collected by an independent vacuum line. The length of the exhaust tubing past the T-connector on this open end was adjusted so that there is < 100mL/min flow going through the chamber without the vacuum line connected to the chamber. Odors were diluted in mineral oil at the following concentrations: BEN low (1:100), BEN high (1:50), ACP (1:50), IPA (1:50), and MCH (1:50), and 500 µl of

these diluted odors were absorbed in syringe filters at least for 30 minutes and these were used for odor delivery. PID recording of the odor delivery at the vacuum end after the chamber showed fast (<300ms for onset) and consistent odor delivery across trials (data not shown). We used 2-second odor stimulation for all experiments.

For optogenetic experiments, light stimulus was delivered from a strip of five 655nm high power LEDs (Luxeon Star) positioned below the chamber. The chamber was positioned on a diffuser so that the arena was directly above the LED strip. The light intensity measured at the chamber was $\sim 170 \mu$ W/mm². We used constant 2-second light stimulation for all experiments. The light onset and offset were triggered using a digital output from Arduino UNO (Arduino) through an LED controller (BuckPuck 350mA, Luxeon Star). For "Odor + Light" conditions, light onset was delayed by 0.3-second from the valve onset to account for the delay in odor delivery.

Video recording was performed from above the chamber using an IR-sensitive CMOS camera (Basler) with a 16mm/F1.8 lens (Edmunds Optics) at 20 frames per second. For optogenetic experiments, we mounted two 780nm longpass filters (Thorlabs) directly in front of the lens. We confirmed light delivery in the video frames by a 940nm low power LED positioned outside the arena. A custom MATLAB graphical user interface was used to control the timing of video recording and stimulus presentations, which issued timestamps for each trigger event. These timestamps were subsequently used for data analysis.

Flies used for all behavior experiments except those for optogenetic experiments were reared at 25°C with 12-hour/12-hour light/ dark cycle, genotyped under CO₂ anesthesia within one day after eclosion, and housed as a pair of one male and one female until the day of experiment. For optogenetics experiments, flies were reared in the dark with regular cornmeal-agar food, genotyped under CO₂ anesthesia within one day after eclosion, housed as a pair of one male and one female with food containing all trans-Retinal (0.4mM), and kept in the dark for 3- to 4-day until the day of experiment. Only 3- to 5-day old females were used for experiments. Each fly was introduced into a plastic vial with a small amount (~50mg) of powdered organic dye (reactive yellow 86, Organic Dyes and Pigments), tossed 20 times to be coated with the dye powder, and then transferred to the chamber using a foot pump. This procedure was performed with no anesthesia applied to the fly. A flow meter was used to check whether the chamber was properly sealed upon connecting to the vacuum line each time a fly was loaded into the chamber. Flies were given at least one minute after the start of the airflow before odor/oil exposure trials were started. The fly's behavior was constantly monitored using the video camera, and exposure trials were started only after it exhibited grooming behavior using either its forelegs or hindlegs. Timeout between trials was < 3-second for Oil/Odor alternating experiments with median interexposure interval of 9 seconds, (i.e., ~18 seconds between odor presentations) and 15-20 seconds for the MBON activation and silencing experiments. Each exposure trial consists of 6-second of video recording, with approximately one second of baseline period, two seconds of stimulus period, and approximately three seconds after stimulus offset. Timestamps were issued at each of the trigger events, which allowed us to align video data to these events (stimulus onset/offset) post hoc. The experiments were aborted if the fly was sucked into the vacuum hole during the course of experiments - only flies that completed the entire sequence of exposure trials were analyzed.

The behavior experiments were not performed blinded.

QUANTIFICATION AND STATISTICAL ANALYSIS

Calcium Imaging Data Analysis

Data acquired for calcium imaging were analyzed by a custom code/graphical user interface written in MATLAB. Images representing each frame were first registered across frames by a subpixel registration algorithm (Guizar-Sicairos et al., 2008) using a visually verified baseline average image (typically 2-second before stimulus presentation) as a template, and the quality of registration was visually confirmed by comparing averaged images as well as maximum intensity projection images across total frames of a trial before and after registration. Regions of interest (ROIs) were drawn manually based on the averaged image after registration, and mean pixel intensity within the ROI was extracted as raw fluorescence value representing signal in each frame. The timing information was extracted from a metadata file of the imaging software, and the timestamps issued from the MATLAB code controlling image acquisition and olfactometer. The raw fluorescence traces were converted into $\Delta F/F_0$ traces using a baseline period 2 seconds immediately before the odor or light onset for odor/light presentation experiments or 1 second immediately before movement onset/offset for experiments examining correlation of activity with flies' spontaneous movement (Figure S5B). Example $\Delta F/F_0$ traces shown in Figures were smoothed by 5-point moving average. Z score traces shown in Figures 1D and S1B-S1C were generated per cell using signals 2-second before odor onset as a baseline. For light stimulation experiments shown in Figures 6C, 6D, and S5D-S5G, the data during the stimulation period were linearly interpolated to replace signals of those frames for which no data were acquired (i.e., light ON frames, see above, optogenetic imaging experiments). This interpolation was performed for all trials of the experiments, regardless of whether the light stimulus was presented, for consistency. To quantify changes in response magnitudes across trials, and compare them across flies, the Δ F/F₀ signals were integrated over a 2-second period after stimulus onset, and normalized to the maximum response of the cell across trials and stimuli (e.g., different odors, or odors and light). PID signals were processed in the same manner using baseline-subtracted traces, but normalized to the maximum signal per odor. These generated the line plots shown, for example, in Figure 1C.

We analyzed the α'/β' KC response in two ways. First, we summed the integrated $\Delta F/F_0$ signals across all cells (Figure 4C bottom, left two panels). Second, because KCs are narrowly tuned unlike MBONs (Hige et al., 2015b), we analyzed only those cells that exhibited significant response to MCH in any of the 10 trials (Figure 4C bottom, right three panels, and Figure S2K). Significance was determined by calculating z scores of the average calcium signal over a 2-second period after stimulus onset using 2-second

baseline. Two different thresholds for significance were used: z score >1.64, equivalent to one-tailed p < 0.05 (Figure 4C bottom, right three panels), or z score >2.57, equivalent to one tailed p < 0.005 (i.e., p < 0.05 after Bonferroni correction for 10 trials) (Figure S2K). Number of significantly responding cells over repetition (Figures 4C bottom and S2K, top right) and normalized integrated $\Delta F/F_0$ signals of significantly responding cells (Figures 4C bottom and S2K, bottom right) are shown.

To compare the degrees of repetition suppression (or recovery from suppression, Figure S5I) across conditions, cell types, or genotypes, normalized responses averaged across the initial three trials (i.e., trials 1-3) and across later three trials (e.g., trials 8-10) were plotted per cell, generating the scatter plots shown, for example, in the right panel of Figure 5B. Significance of the difference in the degrees of suppression or recovery across conditions was assessed using permutation test in a pairwise manner by shuffling the labels of two conditions 20000 times to generate a null distribution of the distance between centroids of the data points for the two conditions. The actual observed ("obs" in Figures) distance was then compared to this distribution to obtain p values. Uncorrected p values are shown in the Figures.

For analysis of flies' movement (movement of legs and abdomen) during imaging trials (Figure S5B), we first identified the frames corresponding to imaging onset and offset to align the video data to imaging data. We then calculated the pixel variance across a 9-frame sliding window (i.e., corresponding to 300ms), and used this value for movement proxy of the middle (i.e., the fifth) frame. This operation was done across the length of the movie to generate movement traces for the trial. Successful detection of movement was confirmed visually for a subset of movies. We then applied an arbitrary threshold for the pixel variance to binarize the data into "moving" and "not moving", and only those events that last more than one second in each state were further processed. Imaging data were then aligned to the movement onset and offset based on the time extracted from the binarized movement data.

Behavior Data Analysis

Each video was annotated frame-by-frame using a custom MATLAB graphical user interface. Each frame was assigned with one of the three behavioral states, grooming with forelegs, grooming with hindlegs, or not grooming. Onset of grooming was assigned to the frame when the fly first exhibited movement immediately leading to grooming (e.g., when it raises both forelegs toward its head, or when it raises both hindlegs toward the abdomen). The foreleg grooming behavior usually persisted for many seconds without resting the legs back on the floor. If the fly, however, maintains the same position of the forelegs midair for more than 3-4 frames (\geq 150-200ms), these events were considered to be a pause, and thus these frames were annotated as not grooming. The offset of foreleg grooming was assigned to the frame when the legs no longer touch the body or each other for at least 2 immediately preceding frames. Pausing of hindleg grooming midair was rare, but hindleg grooming was often interrupted with resting of the legs on the floor. Each of these resting events was annotated as not grooming although in many cases grooming resumed after a short delay (~200ms). The offset of hindleg grooming was assigned to the frame one frame one frame after both of the hindlegs touch the floor.

Annotation data were aligned to the timestamps of stimulus onset (i.e., valve (odor/oil) or light onset), and those exposure trials in which the fly was not annotated to be grooming in the frame immediately preceding the stimulus onset time were eliminated from analysis (e.g., light gray rectangles in Figures S6A, S6C, and S6E). These trials mostly represent those in which flies stopped grooming within the baseline period before stimulus onset.

For binary classification, flies were considered to have "stopped grooming" if they did not exhibit grooming for one second or more during 2.5-second period after stimulus onset (Figure 7). We examined two additional conditions to define interruption of grooming (≥ 0.5 second not grooming during 2.5-second period, and ≥ 1.5 second not grooming during 3.5-second period) and observed qualitatively similar results (see Figures S6A, S6C, and S6E). Statistical analysis was performed by Fisher's exact test followed by Bonferroni correction.

Additionally, we analyzed the data without binary classifications by using "time not grooming" during either 2.5- or 3.5-second period after stimulus onset, and observed essentially the same trends (see Figures S6B, S6D, and S6F). For these analyses, "time not grooming" was simply a sum of frames annotated as "not grooming" throughout the time window (i.e., either 2.5- or 3.5-second) after stimulus onset, thus whether the fly exhibited continued suppression of grooming or intermittent suppression of grooming is not distinguished. Kruskal-Wallis test with posthoc Tukey's HSD test was used for comparison across genotypes or conditions, and Wilcoxon's signed rank test was used for oil/odor alternating exposure experiments (Figure S6B).

Bootstrapping to determine confidence interval (Figure 7B, right panels) was done with 20000 iterations. To obtain linear sum of "Odor only" and "Light only" (Figure 7B, right panels), the trace for "Light only" was shifted by +300ms to account for the delay for light onset that we introduced in "Odor + Light" conditions (see above).

Supplemental Figures



Figure S1. Related to Figures 1, 2, and 3

(A) Consistency of odor delivery and MBON- $\alpha'3$ responses upon ten presentations of MCH (green) followed by three presentations of benzaldehyde (BEN, red). Left, the consistency of odor delivery was examined using PID for different mixing ratios of carrier and odor streams (see STAR Methods). Normalized population

data for integrated PID signal. PID signals were normalized per odor. Right, normalized population data for GCaMP signal using odor delivery with different mixing ratios. We chose to use 1000mL/min carrier mixed with 500mL/min odor for all other experiments. Top panel is the same data as the second row of Figure 2. n = 7 cells (7 flies) for 1000mL/500mL, 7(5) for 950/50, 9(6) for 1000/100, 8(6) for 10L/500mL.

⁽B) Z score traces of MBON-a'3 activity upon repeated presentations of MCH with 30 s ISI. Time is relative to odor onset and the odor was presented for one second. More repetition (trials 13-15, right panel) leads to more cells exhibiting negative deflections. The left two panels are the same as those presented in Figure 1D. Color scale is shown in (C).

⁽C) Z score traces of MBON-v/3 activity upon repeated presentations of different odor with 60 s ISI. Top panels show responses to MCH (1:250 conc.), and bottom panels show responses to all odors shown in Figures 2 and S1D.

⁽D) MBON- α '3 responses to repeated presentation of MCH at different concentrations (60 s ISI). Concentrations are relative to that used in all other experiments. Left, example cells. Right, normalized population data for MBON- α '3 activity and PID signals. n = 6 cells (5 flies) for 0.1x conc., and 7(5) for 10x conc. PID signals were normalized per odor.

⁽E) Normalized population PID data for different odors presented in Figure 2. PID signals were normalized per odor.

⁽F) Time course of recovery upon repetition suppression (related to Figure 3). Left panels show comparison of the initial responses to MCH (average of trials 1-3) and the responses in the test trials after recovery periods (average of trials 16, 18, and 20), and right panels show comparison of the initial responses to MCH and the responses to BEN in the test trials (average of trials 17, 19, and 21). *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon's signed-rank test.



Figure S2. Related to Figure 4

(A–C) Normalized population data of those presented in Figures 4A and 4B. MCH was presented 10 times with various ISIs as indicated. Scatterplots: each point represents a cell and crosses represent population mean \pm 1SEM. (A) MBON- $\alpha'3$; n = 27 cells (20 flies) for 6 s ISI, 6(5) for 12 s ISI, 72(59) for 30 s ISI, 9(7) for 60 s ISI: (B) MBON- β 1> α ; 18(18): (C) MBON- $\alpha'2$; 9(9) for 6 s ISI, 10(10) for 12 s ISI: MBON- γ 1pedc> α/β ; 5(5): MBON- γ 3/ γ 3 β' 1; 9(6): MBON- $\beta'2$ mp; 5(5): MBON- β 2 $\beta'2$; 5(5).

(D–I) Anatomic and functional distinction between the two classes of α '3 MBONs. (D) VT037580 enhancer expression distinguishes two classes of α '3 MBONs. Left, confocal micrographs of a brain labeled with *MB027B-SplitGAL4* (green) and *VT037580-LexA* (red) drivers. Neuropil is visualized with nc82 staining (blue). Right, fractions of brain hemispheres containing indicated numbers of VT037580-positive (VT+) and VT037580-negative (VT-) α '3 MBONs. Most brains contain two VT037580-positive α '3 MBONs, whereas the number of VT037580-negative α '3 MBONs varied from one to two. "Other" indicates hemispheres in which only one VT037580-positive α '3 MBON was observed. In no case did we detect VT037580-positive/MB027B-negative cells in the area where the somas of α '3 MBONs reside.

(E) Single cell morphologies of VT037580-positive and VT037580-negative α '3 MBONs. Multicolor flpout (Nern et al., 2015) was used to achieve single cell labeling. 18 out of 18 single cells obtained from VT037580-GAL4 driver exhibit little innervation in the superior intermediate protocerebrum (SIP; red arrows), whereas six out of 22 single cells obtained from MB027B-SplitGAL4 driver densely innervate the SIP. Red arrowheads indicate soma and blue arrowheads indicate dendritic elaboration of the α '3 compartment.

(F) Odor-evoked responses of VT037580-positive and VT037580-negative $\alpha'3$ MBONs. MCH was presented 10 times with 60 s ISI. Left, example cells; right, normalized population data. n = 7 cells (7 flies) for VT037580-positive and 7(7) for VT037580-negative $\alpha'3$ MBONs.

⁽G) The magnitude of odor-evoked responses in VT037580-positive (VT+) and -negative (VT-) α '3 MBONs upon the initial exposure to MCH. ***p < 0.001, Wilcoxon's rank-sum test.

⁽H) Comparison of the initial (trials 1-3) and later (trials 8-10) responses to repeated odor presentations between VT037580-positive (VT+) and -negative (VT-) α '3 MBONs. Each point represents a cell, and each cross represents mean \pm 1SEM of a cell type.

⁽I) Graphical representation of the results of permutation test that examined the significance of the difference in response suppression observed between VT037580-positive and -negative α/3 MBONs. The observed Euclidean distance of the population mean between these two cell types (the distance between orange and gray crosses in panel (E); red line, "obs," in this panel) is compared with a null distribution generated by randomly shuffling the cell type labels 20000 times. The resulting p value is indicated. See STAR Methods.

⁽J and K) Responses of α'/β' KCs upon repeated odor presentation. (J) Individual data for those presented in Figure 4B. Each point represents a cell. Only significantly responding cells are included (z score > 1.64, n = 64 cells (out of 103 cells total, 6 flies) for 6 s ISI, and 47(6) for 12 s ISI). (K) Analysis of cells with significant response to MCH (12 s ISI) using more stringent threshold than in Figure 4C (sig, z score > 2.57; n.s., not significant). n = 43 cells (6 flies). See STAR Methods.



Figure S3. Expression of Different Drivers Used in This Study, Related to Figures 1, 2, 3, 4, 5, 6, and 7

Maximum intensity projection images of confocal micrographs. The brains of flies of indicated genotypes were immunostained with an anti-GFP antibody (detecting mCD8GFP or GCaMP6f; white or green) and/or an anti-DsRed antibody (detecting tdTomato; white or red), along with the nc82 antibody (neuropil; blue).



Figure S4. Related to Figure 5

(A) Results of permutation test for PPL1-a'3 silencing experiments described in Figures 5A and 5B. The observed distance (red line, "obs") between centroids of data plotted in the right panel of Figure 5B was compared to a null distribution resulting from shuffling genotype-labels 20000 times. Shuffling was performed in a pairwise manner. Resulting uncorrected p values are shown. See STAR Methods.

(B) Comparison of MBON- α '3 response magnitudes across genotypes and trials (related to Figures 5A and 5B). Left, response to the initial presentation of odor across genotypes. *p < 0.05, Kruskal-Wallis test with post hoc Tukey's HSD test. Middle, comparison of the initial responses of MBON- α '3 in control flies and the last response (i.e., trial 10) in flies in which PPL1- α '3 was silenced. n.s., p > 0.05, Kruskal-Wallis test. Right, comparison between the initial and last responses of MBON- α '3 in flies in which PPL1- α '3 was silenced. *p < 0.01, Wilcoxon's signed-rank test.

(C–F) Results of permutation test for Figures 5C–5E. The observed distance (red line, "obs") between centroids of data plotted in Figures 5C (C and D), 5D (E), and 5E (F) was compared to a null distribution resulting from shuffling genotype-labels 20000 times. Shibire^{1s1} is abbreviated as Shi^{1s1}. Shuffling was performed in a pairwise manner. Resulting uncorrected p values are shown. See STAR Methods.

(G) Expression of DAMB in the MBON- α '3 and the KCs. Top, the expression patterns of the *DAMB* gene examined with a line in which GAL4 is inserted into the *DAMB* locus (the first non-coding intron) by recombinase-mediated cassette exchange using MiMIC (Venken et al., 2011). A line with GAL4 inserted in the inverted orientation (DAMB-MiMIC-inv-GAL4) was used as a control. Bottom, expression of DAMB-MiMIC-GAL4 in the MBON- α '3 was assessed by double labeling.



Figure S5. Related to Figure 6

(A) Normalized population data for PPL1- α '3 odor responses measured at the soma. n = 8 flies. **p < 0.01, Kruskal-Wallis test with post hoc Tukey's HSD test. (B) Correlations between flies' movement and calcium signals in PPL1- α '3 and PPL1- γ 2 α '1 (also known as MB-MV1; (Tanaka et al., 2008)). Calcium signal was measured at the axon termini in the α '3 compartment and γ 2 compartment, while monitoring flies' spontaneous movement with video recording (see STAR (legend continued on next page)

(F) PPL1-α'3 responses to 4.8 μW/mm² light in flies with or without CsChrimson. **p < 0.01, Wilcoxon's rank-sum test.

(G) PPL1- α '3 responses to MCH in flies with or without CsChrimson. **p < 0.01, Wilcoxon's rank-sum test.

(I) Comparison of the degree of MBON-a'3 response recovery between novel odor and oil stimulations. Related with Figure 6G. Permutation test.

Methods). Left, example traces of GCaMP signal and the movement of the fly. The movement of the fly was extracted as pixel variance across a sliding window from the video recording. Right, movement onset or offset triggered GCaMP traces (top panels) for PPL1- $\alpha'3$ and PPL1- $\gamma2\alpha'1$ neurons. Bottom panels show the pixel variance used to detect movement. Traces were averaged per fly across onset/offset events (5-26 events per fly) and then across flies (n = 5). Shaded areas indicate \pm 1SEM across flies. Time is relative to the beginning of the movement onset/offset events. The activity of PPL1- $\gamma2\alpha'1$ correlates with the spontaneous movement of the fly as shown in Berry et al. (2015) and Cohn et al. (2015), whereas the activity of PPL1- $\alpha'3$ shows little correlation with movement.

⁽C) Comparison of PPL1- α '3 odor responses between flies heterozygous or homozygous for a null mutation of ORCO ($orco^2$), a coreceptor expressed in a majority of the olfactory sensory neurons (Larsson et al., 2004). Calcium imaging was performed at the axon termini of PPL1- α '3 in the α '3 compartment. Odors were presented with 30 s ISI. n = 7 flies for each genotype. ***p < 0.001, Wilcoxon's rank-sum test.

⁽D) Example traces of odor- and light-evoked activity of PPL1- α '3 with or without expression of CsChrimson. Calcium signal was measured at the axon termini in the α '3 compartment. Lighter lines represent individual trials, whereas darker lines represent the average. 617nm light of different intensities was delivered as 10 times 50 ms pulses at approximately 7Hz (see STAR Methods).

⁽E) Normalized population data for odor- and light-evoked PPL1- α '3 activity. Light responses are averaged across three trials. Odor responses are the maximum response to each odor. We used 4.8 μ W/mm² for all subsequent imaging experiments. n = 7 flies for PPL1 α '3 without CsChrimson, and n = 6 flies for PPL1 α '3 > CsChrimson.

⁽H) Post hoc tests for Figures 6E and 6F. Significant differences were observed after six and 10 light stimulations (*p < 0.05, **p < 0.01, Kruskal-Wallis test with Bonferroni correction).



Figure S6. Related to Figure 7

(A, C, and E) Behavioral response of grooming flies to stimulus presentations analyzed by binarizing the data (i.e., "stopped grooming" or "continued grooming"). (A) Oil/odor alternating exposure experiments, (C) α'3 MBON optogenetic activation experiments, and (E) α'3 MBON silencing experiments. Interruption of

grooming is defined either as ≥ 1 s not grooming during the 2.5 s period after valve (i.e., oil/odor) or light (for light only trials) onset (left panels, and Figure 7), ≥ 0.5 s not grooming during 2.5 s period (middle panels), or ≥ 1.5 s not grooming during 3.5 s period (right panels). Top panels (i.e., matrices) show fly-by-fly data with each row representing an individual fly, and each column, an exposure trial. Flies are sorted based on the responses to the first presentation of odor and/or light. Trials filled with colors indicate those in which flies stopped grooming. For oil/odor alternating exposure experiments, data are presented in two separate panels for oil and odor exposure trials for the purpose of clarity. Odors used for (A) ACP (n = 10), IPA (5), MCH (4), BEN low (12), BEN high (10); (B) BEN low; (C) BEN low. Line plots show fraction of flies that exhibit interruption of grooming in different exposure trials. Definition (i.e., threshold) of grooming interruption for each panel is indicated column-wise. Statistics: ***p < 0.001, **p < 0.05, Fisher's exact test followed by Bonferroni correction across genotypes or conditions. (B, D, and F) Behavioral response of grooming flies to stimulus presentations analyzed without binarizing the data (i.e., "time not grooming"). (B) Oil/odor alternating exposure experiments, (D) $\alpha'3$ MBON optogenetic activation experiments, and (F) $\alpha'3$ MBON silencing experiments. Data were analyzed using either 2.5 s, or 3.5 s time window. Light gray boxplots show the distribution of data per exposure trial. Thick lines connecting different exposure trials are median. Statistics: ***p < 0.001, **p < 0.05, Nilcoxon's signed-rank test for (B), and Kruskal-Wallis test with post hoc Tukey's HSD test for (D and F). (G) Mean interexposure interval before the odor exposure trials (i.e., three intervals of oil exposure trials leading to the first odor exposure trial) was analyzed or $\alpha'3$ MBON silencing experiments. Because the exposure trials (i.e., three intervals of oil



Figure S7. Related to Figure 7

Grooming ethograms. Time is relative to stimulus onset, and the stimulus (i.e., mineral oil, odor, light, or odor + light) was presented for a 2 s period. Flies are sorted based on "time not grooming" per exposure trial, and flies not grooming at the stimulus onset are not shown.

(A) Oil-odor alternating exposure trials. The order of exposures was one exposure to mineral oil, then one exposure to an odor. This was repeated 10 times, thus a total of 20 exposure trials.

(B) $\alpha'3$ MBON activation experiments. The experiment consists of three trials of oil exposures followed by ten trials of stimulus exposures.

(C) $\alpha'3$ MBON silencing experiments. The experiment consists of three trials of oil exposures followed by ten trials of odor exposures.