Plasticity of local GABAergic interneurons drives olfactory habituation

Sudeshna Das1,2, Madhumala K. Sadanandappa1,2, Adrian Dervan2, Aoife Larkin2, John Anthony Lee2, Indulekha P. Sudhakaran2, Rashii Priya2, Raheleh Heidari2, Eimear E. Holohan2, Angel Pimentel3, Avni Gandhi3, Kei Ito3, Subhabrata Sanyal1, Jing W. Wang3, Veronica Rodrigues1,2, and Mani Ramaswami1,2,3

1National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore 560065, India; 2School of Genetics and Microbiology, School of Natural Sciences, and Trinity College Institute of Neuroscience, Trinity College, Dublin 2, Ireland; 3Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721; 4Department of Biological Sciences, Tata Institute of Fundamental Research Mumbai 400005, India; 5Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 1130032, Japan; 6Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322; 7Neurobiology Section, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093

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Despite its ubiquity and significance, behavioral habituation is poorly understood in terms of the underlying neural circuit mechanisms. Here, we present evidence that habituation arises from potentiation of inhibitory transmission within a circuit motif commonly repeated in the nervous system. In Drosohila, prior odorant exposure results in a selective reduction of response to this odorant. Both short-term (STH) and long-term (LTH) forms of olfactory habituation require function of the rutabaga-encoded adenylate cyclase in multiglomerular local interneurons (LNs) that mediate GABAergic inhibition in the antennal lobe; LTH additionally requires function of the cAMP response element-binding protein (CREB2) transcription factor in LNs. The odorant selectivity of STH and LTH is mirrored by requirement for NMDA receptors and GABA2 receptors in odorant-selective, glomerulus-specific projection neurons (PNs). The need for the vesicular glutamate transporter in LNs indicates that a subset of these GABAergic neurons also releases glutamate. LTH is associated with a reduction of odorant-evoked calcium fluxes in PNs as well as growth of the respective odorant-responsive glomeruli. These cellular changes use similar mechanisms to those required for behavioral habituation. Taken together with the observation that enhancement of GABAergic transmission is sufficient to attenuate olfactory behavior, these data indicate that habituation arises from glomerulus-selective potentiation of inhibitory synapses in the antennal lobe. We suggest that similar circuit mechanisms may operate in other species and sensory systems.

Habituation is a specific form of implicit learning in which repeated exposure to an unreinforced stimulus results in a decreased behavioral response (1–3). By filtering out such constant sensory input, habituation enhances an animal’s ability to focus its cognitive resources on novel or salient features of the environment. Thus, habituation serves as a building block for normal cognition (2, 4). Although it has been studied in many different contexts, causal connections between mechanisms, neuronal changes, and behavioral habituation have not been clearly identified (2). Given our current understanding, it remains unclear whether similar or distinct mechanisms underlie different forms of habituation; also unclear is how these mechanisms compare with those mechanisms used in consolidation or extinction of associative memory (1, 5).

The olfactory system provides an experimentally accessible circuit in which to analyze mechanisms that underlie different timescales of behavioral habituation (4, 6, 7). Particularly useful is the adult Drosohila olfactory system, which has organization similar to that of mammals (8, 9). Here, olfactory sensory neurons (OSNs) expressing a single type of functional odorant receptor molecules (ORs) send axons to the antennal lobe and synapse onto (i) glomerulus-specific projection neurons (PNs) that project to the mushroom body and lateral horn, (ii) multiglomerular local interneurons (LNs) that mediate both intraglomerular and transglomerular inhibition (10–12), and (iii) other PN and LN types in early stages of characterization (13–17). The antennal lobe is also innervated by neuromodulatory cells, which could regulate both olfactory responses and olfactory plasticity (18–22). Different odor-induced behaviors show robust habituation in Drosohila (23). Larval short-term olfactory habituation (STH), which is dependent on the rutabaga (rut) -encoded type I adenylate cyclase and central N-Methyl-D-aspartate (NMDA) receptors, shows significant selectivity for the exposed odorant (24–28). In adults, transient habituation of the odor-evoked, reflexive jump response requires rut as well as other cAMP signaling components (29, 30). In addition, a long-term olfactory habituation (LTH) of the avoidance response to benzaldehyde, a chemical potentially sensed by nonolfactory mechanisms, requires rut and persists for several days (31–33). A more recent study characterized LTH of the CO2 response, better understood in terms of the relevant, Gr21a-expressing sensory neurons and their target projection neurons (VPN) in the V glomerulus (34, 35), which is associated with a reduced olfactory startle response, selective growth of the CO2-responsive V glomerulus, decreased CO2-evoked calcium influx in VPN terminals in the lateral horn, and an increased CO2-induced calcium influx into processes of a subclass of LNs within the V glomerulus (35). However, mechanisms that underlie these phenomena as well as whether they cause habituation remain unknown.

To understand the mechanisms of odorant-selective STH and LTH in terms of (i) the necessary genes, (ii) the cell types in which they function, and (iii) consequent neuronal plasticity necessary for habituation, we standardized behavioral assays for STH and LTH to selected odorants and combined them with genetic, behavioral, anatomical, and live imaging methods feasible in Drosophila. Our results indicate that olfactory habituation arises because of plasticity in the antennal lobe. In addition, they suggest a simple and theoretically generalizable model for habituation in other systems.


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Results

STH and LTH in Drosophila. After a 30-min exposure either to 15% CO\textsubscript{2} or 5% ethyl butyrate (EB), Drosophila show a diminished olfactory avoidance response as measured in a Y-maze test (Fig. 1 A and B and raw data in Tables S1). This STH is selective for the odorant used during training and recovers with a half-life of about 20 min (Fig. 1 B and C).

Because CO\textsubscript{2} and EB are sensed by nonoverlapping odorant receptors expressed in distinct sets of OSNs expressing Gr21a and Or83b, respectively, odor-selective habituation could, in principle, arise from adaptation of odorant-receptor signaling pathways in distinct groups of OSNs. However, several observations suggest that STH can be induced without receptor activation and thus, cannot be explained by adaptation of the odorant-receptor signaling pathway.

Thirty minutes of direct depolarization of CO\textsubscript{2}-sensing, Gr21a-positive OSNs expressing the heat-activated Transient Receptor Potential ion channel A1 (TRPA1) channel (36, 37) results in a reduced response to CO\textsubscript{2} but not to EB (Fig. 1D Left and Figs. S1 and S2). Similar 30-min activation of OSNs expressing Or83b results in selectively reduced avoidance of EB but not of CO\textsubscript{2} (Fig. 1 D Right and Figs. S1 and S2). Thus, as reported for Drosophila larvae (28), odorant-selective STH (i) does not require odorant receptor activation and (ii) can arise from a process downstream of action potential firing in OSNs.

After a more prolonged exposure period, where Drosophila adults are maintained in the presence of an odorant (e.g., 5% CO\textsubscript{2}) for 4 d, flies tested in a Y-maze (Fig. 1A) show a diminished avoidance response (Fig. 1E) that lasts for several days (Fig. 1F (31, 35)). LTH to CO\textsubscript{2} causes minimal change in response to EB (Fig. 1E Left). Similar LTH can be observed to EB after 4-d exposure to 20% EB. Like LTH to CO\textsubscript{2}, LTH to EB is selective, and the decreased response to EB occurs with little effect on CO\textsubscript{2} sensitivity (Fig. 1E Right). LTH to CO\textsubscript{2} and EB are both reversible. When habituated animals are restored to normal rearing environment, normal olfactory sensitivity returns within a few days (Fig. 1F). As shown for STH, a “phantom” LTH to CO\textsubscript{2} or EB can be induced by prolonged (4-d) depolarization of appropriate OSNs. Thus, LTH can also occur without odorant receptor activation through a process downstream of action potential firing in OSNs (Fig. 1G).

A biochemical similarity in the neuronal signaling cascades that underlie STH and LTH is indicated by the observation that both STH and LTH are greatly diminished in rutabaga (ry\textsuperscript{ru2000}) mutants (Fig. 2 and Fig. S3A) deficient in an adenylate cyclase activated by the coincidence of increased cytosolic calcium and G protein-coupled receptor (GPCR) activation (38–41). Measurements of basal odor-evoked behavior in naïve flies show that the observed defects in habituation cannot be ascribed to altered odorant sensitivity in rut mutants (Fig. S3B and Tables S1 and S3).

Fig. 1. Properties of STH and LTH in Drosophila. (A) Y-maze used to measure aversive odorant response of adult flies. (B) White bars, response to odorants (5% CO\textsubscript{2} or 10\textsuperscript{-3} dilution EB) before exposure; black bars, after 30 min exposure to either 15% CO\textsubscript{2} (Left) or 5% EB (Right). (C) Recovery profile of STH to CO\textsubscript{2} and EB. White bars indicate the percent response of naïve flies, and the black bars indicate the same for the 0- and 30-min recovered flies after odor exposure. ***, comparisons between preexposed and exposed animals at t = 0; ++, comparisons between exposed animals after 0 and 30 min of recovery. One-way repeated measure ANOVA shows significant difference between naïve and CO\textsubscript{2} (F = 28.237, P < 0.001) and naïve and EB treatments (F = 15.564, P < 0.001). Posthoc testing showed a significant difference between naïve and 0-min recovered flies (t = 10.507, P < 0.001; Student-Newman-Keuls (SNK) test) and 0- and 30-min recovered flies (t = 6.364, P < 0.001; SNK) for CO\textsubscript{2} treatment. The same test for EB treatment showed significant difference between naïve and 0-min recovered flies (t = 7.813, P < 0.001) and 0- and 30-min recovered flies (t = 4.993, P = 0.003). (D) TRP1-mediated direct activation (29 °C) of OSN subsets for 30 min reduces behavioral responses mediated by these OSNs. White bars, control responses at 22 °C. (E) Four-day exposure to 5% CO\textsubscript{2} (Left) or 20% EB (Right) causes odorant selective LTH. (F) Recovery profile of LTH to CO\textsubscript{2} and EB. White bars indicate the percent response of naïve flies, and the black bars indicate the same for the 0- and 6-d recovered flies after odor exposure. ***, comparisons between mock-exposed and 4-d odor-exposed animals 1 d after exposure; ++, comparisons between exposed animals after 1 and 6 d of recovery. One-way repeated measure ANOVA shows significant difference between mock and CO\textsubscript{2} (F = 239.598, P < 0.001) and mock and EB treatments (F = 82.124, P < 0.001). Posthoc testing showed a significant difference between naïve and 0-d recovered flies (t = 30.958, P < 0.001) and 0- and 6-d recovered flies (t = 15.422, P < 0.001) for CO\textsubscript{2} treatment. The same test for EB treatment showed significant difference between naïve and 0-d recovered flies (t = 14.467, P < 0.001). (G) Forced activation of TRPA1-expressing OSNs for 4 d causes odorant-selective LTH. At least eight sets of flies (n) were used for each measurement (exact n and P values are shown in Table S1). Control data showing WT behavior in various Gal4 and Upstream Activation Sequence (UAS) transgene stocks examined in isolation are shown in Fig. S2. Bars show the mean ± SEM. **P < 0.01; ***P < 0.001. The student’s t test was used for all statistical comparisons except for Fig. 1C and F.
Fig. 2. The rut-encoded adenylate cyclase is required in GABAergic LN1 neurons for habituation. (A) Schematic showing the major classes of olfactory neurons and Gal4 lines that mark them. GAD1 predominantly marks GABAergic neurons and 95% of LN1-expressing interneurons (45–47). The LN1 promoter is highly restricted to a subgroup of antennal lobe interneurons, is distinct from a different set of LN2-positive inhibitory interneuron class, and shows minimal overlap with kra-Gal4–marked excitatory LN1s (45) (Fig. S2 F and G). (B and C) Histograms show the efficiency of STH (B) and LTH (C) to EB of rut2080 flies expressing a WT rut+ transgene (44) in different classes of olfactory neurons. White bars indicate olfactory response behavior of mock-treated siblings; dark bars indicate response after the relevant period of odorant exposure. (D) RNAi-mediated knockdown of rut using a validated transgenic RNAi construct (VDRCS569) (43) in LN1 or GAD1 neurons blocks STH and LTH to EB. Similar results obtained with an independent rut RNAi line, VDRC101759, are not shown. Additional Gal4 and UAS transgene alone controls are shown in Fig. S2. Bars show the mean ± SEM determined through the Student t test [n > 8 (Tables S1 and S3); ***P < 0.001].

cAMP Signaling Is Required in GABAergic LN1s for Habituation. The often unique, anatomical location where rut is required for a given learning task defines cell types in which neuronal plasticity is required for the specific form of memory (42–44). To identify neurons whose intrinsic properties change during olfactory habituation, we looked to identify the cell type(s) in which rut is required (Fig. 2, Figs. S2 and S3, and Tables S1 and S3).

Tissue-restricted expression of a WT rut+ transgene in either the LN1 class of antennal lobe local interneurons (45) (Fig. S2 F and G) or GABA-expressing glutamate decarboxylase (GAD1)–positive neurons was sufficient to rescue rut2080 defects in STH or LTH (Fig. 2A–C and Fig. S3 C and D). Arguing against a developmental requirement in these cells, experiments using the Gal80p conditional expression system show that rut+ expression in adult LN1 neurons is sufficient to rescue the habituation defects in rut2080 (Fig. 2 A–C and Figs. S2 F and G and S3 C and D). In contrast, rut+ expression in OSNs, PNs, or MB247-positive mushroom-body neurons had no effect on the rut2080 mutant phenotype (Fig. 2 A–C and Fig. S3 C and D). This finding indicates that rutabaga function is necessary for both STH and LTH and resides principally in adult inhibitory local interneurons in the antennal lobe.

The conclusion that rut is necessary in LN1 neurons is also supported by the observation that LN1 or GAD1 promoter-driven expression of either of two previously validated transgenic RNAi constructs against rut (43) also blocked habituation to EB (Fig. 2 D and Fig. S3F). Together, these observations indicate that rut-dependent changes in the intrinsic properties of inhibitory local interneurons are necessary for behavioral habituation.

LTH Is Accompanied by a rut-Dependent Reduction in PN Responses to Odorant. The observed requirement for rut in LN1s suggests that behavioral habituation arises from rut-dependent strengthening of GABAergic LN1 synapses in the antennal lobe. This observation predicts first, that odor-evoked responses in PN dendrites will be reduced after habituation and second, that the reduction in PN response will be dependent on rut function. We tested these predictions by measuring the effects of 4-d EB exposure on EB-evoked responses in dorso-medial (DM)2 and DM5 glomeruli, which respond to EB under the conditions used here (48).

EB-evoked responses of PN dendrites in DM2 and DM5 were significantly reduced after habituation. We imaged odor-evoked calcium fluxes in GH146-Gal4, UAS GCaMP3/+ flies (also termed GH146>GCaMP3/+), which carry one copy each of GH146-Gal4 and UAS-GCaMP transgenes heterozygous to a WT + chromosome. Two-second pulses of 0.5% EB induced strong calcium fluorescence induced by odor stimulation (Fig. 3 A and B and Table S4). However, 4-d exposure to 5% CO2, previously shown to reduce CO2-evoked fluorescence induced by odor stimulation (Fig. 3 A and B and Table S4). However, 4-d exposure to EB caused a significant reduction in EB-evoked GCaMP responses compared with paraffin-exposed controls (DM2 P = 0.004, DM5 P < 0.001). In contrast, 4-d exposure to 5% CO2, previously shown to reduce CO2-evoked responses in the V projection neuron (35), did not reduce the EB response (Fig. 3B). Furthermore, EB exposure had no effect on the 3-octanol (3-Oct) response in the dorsal-central (DC)2 glo- merulus (Fig. 3B Right). DC2 has previously been shown to respond to 3-Oct (49) and in our experiments, was unresponsive to 0.5% EB. These data indicate that LTH is accompanied by a selective reduction of PN responses to the habituated odor.

If the observed reduction in PN response causes behavioral habituation, then it should not occur in nonhabituating mutants. Consistent with this premise, 4-d EB exposure had no significant effect on EB-evoked PN responses of nonhabituating, rut2080/Y; GH146 > GCaMP3/UAS rut+ flies (Fig. 3B). This observation tightens the relationship between the observed physiological plasticity and behavioral LTH.
GABAergic Transmission from LNs Is Necessary and Its Enhancement Is Sufficient for Habituation. If the reduced PN response arises from enhanced GABAergic transmission onto PNs, then synaptic output from LN1 neurons should be necessary for the reduced olfactory aversion in habituated animals, which was previously observed for larval STH (28). To test this prediction for adult STH and LTH, we expressed a dominant, conditional dynamin (Shits1) in local interneurons and used a rapid temperature shift, from 22 °C (permissive) to 32 °C (restrictive) to conditionally reduce transmitter release from LNs during any specified time interval (50). At permissive temperatures (22 °C), LN1 > Shits1 Drosophila showed normal STH to EB and CO2. However, unlike control flies that show robust habituation at 32 °C (Fig. S4A and Tables S2 and S3), LN1 > Shits1 flies behaved like naive unhabituated animals when tested at temperatures (32 °C) for dynamin function (Fig. 4A and Fig. S4B). Therefore, synaptic output from LN1 neurons is necessary for display of STH (Fig. 4A). We also found that transmitter release from LN1 neurons was necessary during the exposure period (Fig. 4A), an observation that we consider later in this paper.

Transmitter release from LN1 interneurons is also required for the expression of LTH to CO2 and EB. Thus, although both control and LN1 > Shits1 adults exposed to EB or CO2 at permissive temperatures for 4 d showed habituated behavior when tested at room temperature, LN1 > Shits1 flies alone behave like naive unhabituated animals when tested at temperatures nonpermissive for Shits1 function (Fig. 4B and Fig. S4C).

To test if GABAergic transmission from LNs to PNs mediates the enhancement of habituation, we asked whether habituation defects were also evident when the function of GABA receptors was knocked down in odorant-responsive PNs through the transgenic RNAi line (UAS-Rdl) previously shown to knockdown ionotropic GABA_A (Rdl) receptors (51) (Fig. S4F and Tables S1 and S3). Knockdown of GABA_A receptors in defined subsets of PNs selectively blocked habituation to their respective odors without affecting the basal olfactory response (Fig. 4C and D and Fig. S4D and E). Thus, Rdl RNAi expression in GH146-positive neurons that communicate the odor of EB (48) blocked STH and LTH to EB without affecting habituation to CO2. In contrast, Rdl RNAi expression in the CO2-responsive projection neuron (VPN) blocks STH and LTH to CO2 without affecting STH or LTH to EB (Fig. 4C and D). Taken together with the observation that synaptic output from predominantly GABAergic LN1 neurons is required for display of habituation, these data point to a model in which PN inhibition by LN1 neurons is necessary for the decrease in olfactory avoidance behavior observed after habituation.

To determine whether increased transmission from LN1 neurons could be potentially sufficient to attenuate olfactory behavior, we expressed the heat-activated, cation-permeable TRPA1 channel in LN1 cells and compared olfactory response indices for these LN1 > TRPA1 flies at 22 °C, when TRPA1 channels would be silent, with responses measured just after the flies were shifted to 29 °C, when LN1 neurons showed increased activity (Fig. S1). Activation of LN1 neurons was sufficient to substantially reduce version of naïve LN1 > TRPA1 flies to CO2 or EB (Fig. 4E). Thus, transmitter release from LN1 neurons is necessary (Fig. 4A and B), and the potentiation of LN1 activity is sufficient (Fig. 4E) for the attenuated behavioral response observed in habituation.

PN-Specific NMDA Receptors May Explain Odorant Selectivity in Habituation. Forced activation of LN1 neurons results in a nonspecific reduction in the behavioral response to both CO2 and EB (Fig. 4E). This finding is easily explained by the fact that most LN1 local interneurons are multiglomerular and would be expected to inhibit most, if not all, PNs (45). However, the observation that behavioral habituation is odorant-selective possibly posits the need for a mechanism to ensure glomerulus-selective potentiation of LN1 synapses in vivo (Fig. 5A). How may synapse-selective potentiation of GABAergic outputs be controlled?

Previous studies have shown that presynaptic LTP of GABAergic synapses usually arises from a heterosynaptic mechanism that requires retrograde signaling, which is often mediated through postsynaptic NMDA receptor activation (52, 53). Although glutamate is not the main excitatory transmitter in insect brains, it is likely that NMDA receptors, which typically mediate synaptic plasticity rather than synaptic depolarization (54), serve evolutionarily conserved functions in synaptic plasticity (55). We, therefore, asked whether postsynaptic NMDA-type glutamate receptors on PNs could contribute to such glomerular selectivity of LN potentiation.

The Drosophila NMDA receptor, a complex of NR1 and NR2 subunits, is expressed in many neurons of the adult antennal lobe (55). Levels of NR1 are substantially reduced by panneural expression in
expression of an RNAi transgene, dsNR1 (Fig. S5A). Expression of dsNR1 in CO2-responsive VPN-positive PNs blocked STH and LTH to CO2 without affecting habituation to EB (Fig. 5 B and C). In contrast, NMDAR knockdown in EB-responsive GH146-expressing PNs efficiently blocked STH and LTH to EB without affecting habituation to CO2 (Fig. 5 B and C and Fig. S5C). Thus, the NMDA receptor is required in a PN-specific manner for odorant selective habituation.

In mammals and Drosophila, NMDA receptors respond to the coincidence of postsynaptic depolarization and glutamate release (54, 55). PN depolarization is driven by OSN input. To determine the source of glutamate in the Drosophila antennal lobe, we examined the distribution of the vesicular glutamatergic transporter (DVGlut) in vivo. DVGlut is expressed widely in the antennal lobe, including a significant subset of LN1 local interneurons (Fig. S5B). RNAi-based knockdown of either GAD1 or DVGlut in LN1 blocks STH and LTH, indicating that LN1 terminals are an important source of both GABA and glutamate required for habituation (Fig. 5D). Disruption of STH and LTH after knockdown of DVGlut in GAD1-expressing, GABAAergic cells (47) further suggests that habituation requires glutamate corelease from GABAAergic neurons, a phenomenon previously observed in mammalian nervous systems and recently linked to inhibitory synapse plasticity (56–58) (Fig. 5E and F). In contrast, mushroom-body expression of the VGLUT RNAi construct in MB247-expressing neurons had no effect on STH (Fig. S5D).

The postulated need for glutamate release from inhibitory local interneurons in the lobe can also explain why STH requires shi function in LN1 cells during initial odorant exposure (Fig. 4D).

Together, the data can explain glomerulus-selective potentiation of LN–PN synapses by postulating that (i) odor stimulation causes glutamate release onto PNs in multiple glomeruli, (ii) glomerular specificity arises because glutamate activates NMDA receptors only on PNs that show coincident, odorant-induced depolarization, and (iii) NMDAR signaling in selected PNs then mediates, possibly through retrograde signaling (52, 53), the potentiation of GABAAergic transmission onto these PNs.

Additional observations (Fig. 2 B and C and Fig. S3F and Fig. S5E) showing that the key signaling components (rut, NMDAR, and VGLUT) are required in adult-stage neurons provide additional data consistent with this model.

**Glomerulus-Selective Structural Plasticity Requires the Same Mechanisms Required for Odorant-Selective LTH.** Previous work has shown that 4-d exposure to CO2 or EB causes selective increase in the volume of the V and DM2 glomeruli, respectively (35). Our experiments confirmed these observations (Fig. S6) and additionally showed that 4-d EB exposure, which causes EB-selective LTH (Fig. 1), also causes particularly robust growth of the EB-responsive DM5 glomerulus that mediates the behavioral response to EB (48) (Fig. S6 A and B). We tested whether mechanisms required for odorant-selective habituation were also required for glomerulus-selective structural plasticity.

rutmutants that do not show LTH to either CO2 or EB do not show associated increases in glomerular volume. Remarkably, expression of a WT rut+ transgene in LN1 interneurons or GAD1-expressing neurons not only restores normal LTH to rut mutants but also restores respective glomerulus-selective growth (Fig. 6A and Fig. S6C). Thus, rut is required in LN1/GAD1 cells for both behavioral LTH and LTH-associated structural change.

Similar to their role in behavioral habituation, NMDA receptors are required in odorant-responsive PNs for glomerulus-selective structural plasticity. Consistent with synapse-specific requirement for NMDARs, knockdown of NR1 in VPN neurons blocked not only LTH to CO2 but also the CO2-induced increase in V glomerular volume. However, these flies (which showed normal LTH to EB) showed normal EB-evoked growth of the DM5 glomerulus (Fig. 6B and Fig. S6D). In contrast, knockdown of NR1 in GH146 neurons blocked LTH to EB as well as the associated increase in DM5 glomerular volume without affecting LTH to CO2 or associated growth of the V glomerulus. Thus, postsynaptic NMDARs in PNs contribute not only to odorant-selective habituation but also to glomerulus-selective structural plasticity.

Knockdown of DVGlut in GAD1 neurons again showed effects analogous to observations in behavioral habituation. Consistent with the need for glutamate secretion from multiglomerular LN1s, knockdown of DVGlut in GAD1-expressing neurons blocked both CO2- and EB-induced structural plasticity (Fig. S6F).

It is interesting that the GABA receptor (Rdl), which is essential for expression of habituated behavior (Fig. 4 C and D), is not necessary for LTH-associated structural plasticity (Fig. S6F). This finding is probably because the stable expression of GABA receptors is necessary for expressing the physiological consequence of plasticity in the antennal lobe, namely for the increased GABAAergic transmission onto PNs. However, they are not required for the changes per se.
Fig. 5. NMDA receptors are required in PNs and VGLUT in LNs for odorant-selective habituation. (A) Diagram showing a single multiglomerular LN that forms synapses with two different PNs. (B and C) Effects of PN-specific NMDAR knockdown on STH and LTH to CO2 and EB. VPN > dsNR1 selectively blocks CO2 habituation; GH146 > dsNR1 selectively blocks EB. (D–F) Behavioral effect of GAD1 or DVGLUT knockdown in LNs and GAD1 expressing neurons. Expression of an RNAi against GAD1 (VDRC32344) or DVGLUT (VDR104324) in LN1–LN7 or GAD1-expressing neurons (E and F) blocks STH and LTH to EB and CO2. Bars indicate mean ± SEM calculated using the Student t test. ***P < 0.001; n > 7 (Tables S1–S3) show specific n values, and Fig. S2 has additional Gal4 and UAS transgene alone controls).

Discussion

Circuit Mechanism of Olfactory Habituation. A key observation is that rut function is uniquely required in adult-stage GABAergic local interneurons for STH and LTH (Fig. 2). This observation contrasts with the rut requirement in mushroom-body neurons for olfactory aversive memory (42, 44). The demonstration of fundamentally different neural mechanisms used in olfactory habituation and olfactory-associative memory elegantly refutes a proposal of the Rescorla–Wagner model that habituation (and extinction) may be no more than associations made with an unrelated, irrelevant stimulus (40). First, it refers to inhibitory synapses, with potentiation that may involve a specific heterosynaptic mechanism similar to that used for inhibitory Long Term Potentiation (iLTP) in the rodent ventral tegmentum (52, 53). Second, by presenting evidence for necessary glutamate corelease from GABAergic neurons, it proposes the involvement of a relatively recently discovered synaptic mechanism for plasticity (56). Third, it posits an in vivo mechanism to enable glomerulus-specific plasticity of LN terminals.

It is pleasing that, in all instances tested, physiological and structural plasticity induced by 4-d odorant exposure requires the same mechanisms required for behavioral LTH (Figs. 3–6 and Figs. S6 and S7). When taken together, these different lines of experimental evidence come close to establishing a causal connection between behavioral habituation and accompanying synaptic plasticity in the antennal lobe.

It is important to acknowledge that, although our experiments show that plasticity of LN–PN synapses contributes substantially to the process of behavioral habituation, it remains possible that plasticity of other synapses, such as of recently identified excitatory inputs made onto inhibitory LNs (16, 17, 59), also accompany and contribute to olfactory habituation (60).

Potentially General Mechanism for Habituation? The conserved organization of olfactory systems suggests that mechanisms of olfactory STH and LTH could be conserved across species (8, 9). Although this prediction remains poorly tested, early observations indicate that a form of pheromonal habituation in rodents, termed the Bruce effect, may arise from enhanced inhibitory feedback onto mitral cells in the vomeronasal organ (61–63).
Less obviously, two features of the circuit mechanism that we describe suggest that it is scalable and generalizable. First, selective strengthening of inhibitory transmission onto active glomeruli can be used to selectively dampen either uniglomerular (CO$_2$) or multiglomerular (EB) responses; thus, the mechanism is scalable. Second, the antennal lobe/olfactory bulb uses a circuit motif commonly repeated throughout the brain, in which an excitatory principal cell activates not only a downstream neuron but also local inhibitory interneurons, which among other things, limit principal cell excitation (64–66).

It is possible that, in nonolfactory regions of the brains, a sustained pattern of principal neuron activity induced by a prolonged, unreinforced stimulus could similarly result in the specific potentiation of local inhibition onto these principal neurons. Subsequently, the pattern of principal cell activity induced by a second exposure to a now familiar stimulus would be selectively gated such that it would create only weak activation of downstream neurons. In this manner, the circuit model that we propose for olfactory habituation could be theoretically generalized. We expect more studies to test the biological validity of this observation.

**Experimental Procedures**

*Drosophila* Stocks. Unless otherwise stated, all flies were raised at 25 °C on standard cornmeal agar medium. The stocks were obtained from stock centers or through the generosity of *Drosophila* colleagues as listed in SI Experimental Procedures.

Measuring Olfactory Responses and Habituation. Olfactory avoidance was measured using an upright Y-Maze apparatus (Fig. 1). Odorant was drawn through one arm of the maze, and control air was drawn through the other arm. Flies starved overnight were allowed into the entry tube, and their preference for the arm with the odorant (O) vs. the control (C) arm with air was quantified as a response index [RI; the difference in the number of flies in the odorant and control arms as a fraction of the total flies $R=\frac{(O-C)}{(O+C)}$]. At least eight batches were assayed for each data point, RI values were normalized to the appropriate control (e.g., response of naive flies for STH, paraffin-exposed flies for LTH to EB, and air exposed for LTH to CO$_2$), and the data were plotted as percent control response. Except where otherwise indicated, comparisons were carried out by the unpaired Student t test. All batches were coded, and the experimenter was blind to the genotype being tested. STH. Two-day-old flies (unless otherwise stated) starved overnight were pretested to 5% CO$_2$ or 10$^{-3}$ dilution EB to measure the naive RI. RIs were again determined after the same set of flies was exposed to either 15% CO$_2$ or 5% EB for 30 min.

**LTH.** The protocol was adapted from refs. 31 and 35. Flies aged between 0 and 12 h were collected; 5% CO$_2$ habituation involved 4-d exposure to CO$_2$ (air control). EB habituation involved 4-d incubation with a perforated tube with odorant 1:5 dilution of EB in light liquid parafin oil (paraffin control). For TrpA1-mediated “phantom” habituation, control flies were maintained and tested at room temperature (22 °C); experimental flies were tested at room temperature after exposure to 29 °C for 30 min or 4 d.
Brain Dissection, Immunohistochemistry, and Volume Measurements. Brains were dissected and stained using standard conditions (67). For measurements of glomerular volume, 3D confocal stacks (Vt000, Olympus) were analyzed using Amira 5.2. Dissection was made using the unpaired Student’s t test. Except for the rut rescue analysis where male flies were used, 10–15 female flies from each genotype, and at least 10 glomeruli were analyzed to determine the mean and SEM. Investigators performing the measurements were blind with respect to the genotype and experimental treatment.

Imaging Calcium Dynamics in Vivo. The procedure was as described previously with minor modifications (12) (SI Experimental Procedures). Analysis of changes in fluorescence was performed (with user blind to genotype) using a custom Matlab script. WT and rutabaga flies (mock or EB-exposed) were raised in the dark away ANOVA. A Student Newman-Keuls posthoc test was used where appropriate. A Student t test was used when only comparing two groups of data.

ACKNOWLEDGMENTS. In acknowledgement of her formidable biological insight, enduring friendship, insightful mentorship, and generous friendship that guided this work, MR, SD, MK5, IPS, RP, AG, and SS dedicate this paper to the memory of our co-author, Veronica Rodrigues. We thank Alberto Ferrus for introducing us to long-term habituation. We acknowledge A. Fiala, E. Buchner, M. Heisenberg, R. Davis, G. Norman, J. Jayaraman, L. Locrover, L. Liu, A.-S. Chang, G. Miesenbök, L. Voshall, and S. Sache for fly stocks and reagents, L. Voshall for sharing data on CO2 habituation before publication, C. Root and S. Kim for advice on anteronal/nerve stimulation and imaging, J. Truman for drawing our attention to the Bruce effect, and F. Chee and V. Kumar for early help with brain imaging. We thank K. VijayRaghavan, B. Keverne, S. O’Mara, T. Weinert, R. Parker, J. Joseph, J. P. Labador, P. Paranjpe, and A. Chang for useful discussions and/or comments on the manuscript. I.P.S. was supported by a Council of Scientific and Industrial Research (Government of India) postgraduate fellowship. This work was funded by support from the Fogarty International Research Collaboration Award and National Scientist Student Award from the National Institute on Drug Abuse/National Institutes of Health, was funded by grants from Department of Biotechnology (DBT, India) and Science Foundation Ireland (to M.R.).
**Supporting Information**

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**SI Experimental Procedures**

**Drosophila Stocks.** The *Drosophila* stocks were generously provided by the following sources. The olfactory sensory neuron (OSN) Gal4 lines *Otr83b-Gal4* and *Gr21a-Gal4* (1, 2) and the projection neuron (PN) Gal4 line *VPN-Gal4* (3) were provided by Leslie Vosshall (Rockefeller University, New York, NY). *GHI46-Gal4*, which labels about 90 PNs, was a gift from Reinhard Stocker (University of Fribourg, Fribourg, Switzerland); *GAD1-Gal4* was from Gero Miesenbrook (Oxford University, Oxford, UK) (4). The RNAi lines were provided by the following sources. *UAS-RdlRNAi* (Rdl) lines were obtained from Ron Davis (Baylor College of Medicine, Houston, TX) (5, 6). *UAS-dsNR2, UAS-dsNR2* was from Tim Tully (Cold Spring Harbor Laboratory, New York, NY) (7, 8). *UAS-sirt1* was from Toshi Kitamoto (University of Iowa, Iowa City, IA). *UAS-VGLUT RNAi* stock CG9887(KK) and the *UAS-RutRNAi* (Ruti) lines (VDRCS569 and VDRC101759) (9) were from the Vienna Drosophila Research Centre. The *nubabunga* allele *rut*°5000 and the *UAS-rut*° transgenes were obtained from Martin Heisenberg (University of Würzburg, Würzburg, Germany) (10). *UAS-TRPA1* strains were generated by Paul Garrity (Brandeis University, Waltham/ Boston, MA) (11, 12). *UAS-GCaMP3* flies were from Vivek Jayaraman (Janelia Farm Research Campus, Ashburn, VA) and Loren Looger (13), and balancer and other strains were obtained from the Bloomington Stock Centre.

**Olfactory Behavior.** Flies were starved overnight in a vial containing water-soaked filter paper before testing. In experiments using ethyl butyrate (EB), air was bubbled through water for the control arm or through appropriately diluted EB solution in water; in the case of CO₂, a 5% dilution was bubbled through distilled water. About 30–40 flies were placed in the entry tube and allowed to move up against gravity. At the junction of the two arms, the flies move either into the arm with odorant or into the control arm. The test was run for 1 min, and flies were counted in each arm. At the end of the test, flies were tapped back into the entry tube, and the Y maze was flipped such that the positions of the odorant and control arms were switched. This cycle was repeated one more time, making a total of four runs per set of flies. The sum of the numbers over four runs was used to calculate the response index (RI), which is the difference between the number of flies in the odorant (O) and control (C) arms as a fraction of the total number of flies participating in the test [RI = (O - C)/ (O + C)]. At least eight such batches were assayed for each data point to calculate the mean and SEM. Comparison of two sets of data was carried out by the unpaired Student t test. All batches were coded, and the experimenter was blind to the genotype being tested.

**Inducing Short-Term Habituation.** Newly eclosed 0- to 0.5-d-old flies were collected, aged for 2 d, and starved overnight on a moist filter paper in an empty vial. Flies were pretreated to 5% CO₂ or 10⁻³ dilution of EB to measure the naïve olfactory chemotactic response. Then, the same set of flies was exposed to either 15% CO₂ or 5% EB for 30 min, which was followed by posttest to 5% CO₂ or 10⁻³ EB, respectively. CO₂ exposures were performed in a CO₂ incubator (flies in an empty vial that was covered with cheese cloth). For EB exposure, an Eppendorf tube containing 1-mL dilution of odorant in paraffin oil was suspended in a 100-mL glass bottle. Perforated holes were made on the plastic trap for odorant diffusion. Responses of the postexposure test were compared as a percentage of the pretest.

**Inducing Long-Term Habituation.** A 4-d odor-exposure protocol was adapted from a procedure used by Devaud et al. (14). About 50–60 flies aged between 0 and 12 h were collected in each media bottle with a tightened cotton stopper. For CO₂ habituation, either air or 5% CO₂ was bubbled through distilled water before being allowed to enter the exposure bottles. Calibrated flow meters (Cole-Parmer) were used to mix CO₂ and air to make up the 5% concentration of CO₂ used for exposure. For some experiments, we used an alternative method in which flies in a media bottle covered with cheesecloth were placed in a tissue culture incubator that maintained a 5% CO₂ concentration at 25 °C for 4 d. Mock-treated animals were exposed to air under similar conditions. For EB habituation, 1 mL odorant (20%) diluted in light liquid paraffin oil was placed in an Eppendorf tube and covered with perforated plastic clean wrap. The tube was suspended with wire in the bottle containing the flies. In the case of mock-treated control flies, 1 mL liquid paraffin oil was placed in the Eppendorf tube as described. Responses of the animals exposed to odorant were compared as a percentage of the mock-treated (air for CO₂ experiments and paraffin oil for EB experiments) animals.

For experiments to assess long-term habituation (LTH) induced by direct Trp1-mediated stimulation of OSNs, flies are exposed for 4 d to 22 °C instead of paraffin oil/air or 29 °C instead of EB/CO₂.

**Testing Responses of TRPA1 Flies at 22 °C and 29 °C.** Chemotactic responses of the flies at 22 °C were determined as usual. For testing at 29 °C, flies were taken into a behavior/testing room maintained at 29–30 °C just before the test. Each batch of flies was tested four times as described above.

**Brain Dissection, Immunohistochemistry, and Imaging.** Brains were dissected in PBS and fixed in 4% paraformaldehyde diluted in PBS containing 0.3% Triton-X100 (PTX) for 30 min at room temperature. Fixed samples were washed three times for 20 min each in PTX and then incubated in primary antibody diluted in 0.3% PTX for 48 h at 4 °C on a shaker. The primary antibodies used were rabbit anti-GFP (1:10,000; Molecular Probes), mouse anti-Bruce (mAbnc82, 1:20; DSHB), anti-NR1 (1:5,000) (8), and *Drosophila* vesicular glutamate transporter (DVGLUT, 1:500; a gift from Aaron DiAntonio). After incubation, brains were washed repeatedly in 0.3% PTX and incubated in Alexa-488, Alexa-568, or Alexa-647 coupled secondary antibody (Molecular Probes) diluted at 1:400 in PTX. Samples were washed in PTX, mounted in 70% glycerol on a spacer slide, and imaged with Olympus Fluoview (FV1000).

**Measurement of Glomerular Volumes.** The 3D confocal stacks were imported into the segmentation software Amira 5.2.0 (Mercury Computer Systems). The glomerulus of interest was identified based on mAb nc82 staining and traced on the three-axis view (xy, yz, and xz axes) to reconstruct the glomerular structure and calculate its volume. Comparisons between conditions were done using the unpaired Student t test. Except for the rut rescue analysis, where only males were used, only female flies were used, and for each genotype, 10–15 flies and at least 10 glomeruli (unless other indicated) were analyzed to determine the mean and SEM. All volume measurements were carried out with coded flies such that the investigators were blind with respect to the genotype and experimental treatment. The volumes of glomeruli are represented as a percentage of the volume of the mock-treated animals.
Imaging Calcium Dynamics in Vivo. The procedure was essentially as previously described (15). For two-photon imaging of the olfactory lobes, the isolated brain preparation was used (15, 16). Briefly, cold-anesthetized flies were decapitated and placed in chilled calcium-free adult haemolymph (AHL) solution (108 mM NaCl, 5 mM KCl, 5.2 mM MgCl₂, 4 mM NaHCO₃, 1 mM NaH₂PO₄, 5 mM trehalose, 10 mM sucrose, 5 mM Hepes, pH 7.4). The brain was carefully dissected by first pinning the proboscis and removing the surrounding cuticle (except for the frontal region containing the antennae) and all connective tissues and trachea. Thereafter, the preparation was rotated, and the ventral nerves, esophagus, and all underlying connective tissues were removed except for the olfactory nerves. The preparation was then transferred in AHL to a small slab of sylgard on a microscope slide and pinned through the proboscis; then, the buffer solution changed to calcium-containing AHL (2 mM CaCl₂, pH 7.4; kept at room temperature), and the brain was gently maneuvered away from the cuticle to expose the olfactory lobes and secured using two tungsten pins. The entire preparation was covered in a thin layer of 2.5% low melting point agarose (dissolved in AHL containing 2 mM CaCl₂ and kept at 38.5 °C). After it was set, the agarose surrounding the antennae were gently removed, dried using soft tissue, and finally, topped with a coverslip to facilitate imaging.

For odor stimulation, each preparation was placed on a metal plate specially designed to slot into the microscope stage and holding a pipe of 12 mm in diameter that permitted a constant flow of air of 1 L/min to the antennae. Solenoids controlled division of 50% of the air stream through a 100-mL bottle containing 50 μL 10 × 10⁻³ EB [or in separate experiments involving the DC2 responses, 50 μL 60 × 10⁻³ 3-octanol (3-Oct)] on a piece of filter paper to deliver a final odorant concentration of 5 × 10⁻³ (30 × 10⁻³ 3-Oct) to the antennae. Repeated odor pulses of 2 s were delivered 120 s apart. Imaging was carried out using a Zeiss 510 confocal microscope coupled to a Chameleon ultrafast laser (Coherent). The excitation wavelength was set at 930 nm, and emission was collected between 500 and 550 nm (peak GCaMP emission is 509 nm). Images were collected at 4 frames/s (250 ms/frame), with a resolution of 512 × 512 pixels. Glomeruli were identified anatomically using the glomerular atlas of Laisne et al. (17).

Analysis of changes in fluorescence was performed (user blinded) using a custom Matlab script. Background fluorescence, subtracted from subsequent pixel intensity measurements, was measured by finding the mean pixel intensity within a user-defined region between the antennal lobes. \( \Delta F/F \% \) plots were calculated using the formula \( \Delta F/F \% = (F_1 - F_0)/F_0 \times 100 \), where \( F_1 \) is the fluorescence at time point \( t \) and \( F_0 \) is the mean of the fluorescence in the 10 time points preceding the odor-evoked response. The values calculated (\( \Delta F/F \cdot dT \)) represent the average of the areas under the curve in the 0- to 5.5-s time windows of each odor response (where \( t = 0 \) is the point when the curve rises 4 SDs above the baseline). For comparisons between only two groups, Student t tests were used. For comparisons of multiple groups, two-way ANOVAs were used followed by Student Newman–Keuls posthoc testing where appropriate.

Pseudocolored images were constructed by subtracting the mean of five baseline images from the mean of two adjoining images at peak fluorescence.

Fig. S2. Responses of P(Gal4) and P(UAS) transgene lines. LTH and STH to EB and CO occur normally in flies carrying either P(UAS) (A and B) or P(Gal4) (D and E) transgenes as homozygotes. In addition, UAS TRPA1 alone flies exposed to elevated temperatures show no detectable decrement in olfactory response (C). These control data serve to confirm that defects presented in other figures are attributable to a Gal4-mediated expression of the respective transgenes rather than genetic background effects. (F and G) Conditional expression of UASmCD8GFP driven by LNV expressing neurons at 29°C and 18°C, respectively. Bars indicate mean ± SEM. ***P < 0.001.

Fig. S1. Exposure at 28 °C induces neural activity in OSNs and local interneurons (LNs) expressing the heat-activated TRPA1 channel. (A) Basal fluorescence of GCaMP in sensory neuron terminals in the antennal lobe of GCaMP3;Or83b > TrpA1 flies at 22 °C. (A′) In the same flies, a temperature shift from 22 °C to 28 °C, which should activate the TrpA1 channel, results in enhanced neuronal activity in the antennal lobe and increased GCaMP3 fluorescence. (B) Quantified δF/F value [(F28 - F22)/F22] for GCaMP3;Or83b compared with GCaMP3;Or83b > TrpA1 at 22 °C and 28 °C. (C and C′) Fluorescence of GCaMP coexpressed with TRPA1 in local interneurons of GCaMP3; LN1 > TrpA flies at 22 °C and 28 °C. (D) Histograms showing that elevated temperatures cause a significant, TRPA1-dependent increase in δF/F. (Scale bar: 10 μm.)

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rutabaga is required in local interneurons for STH and LTH to EB and CO$_2$. (A) rut$_{2080}^{1080}$ mutants do not show STH or LTH. (B) rut$_{2080}^{1080}$/UAS-rut$_{*}$ flies, which do not habituate to EB, show normal (like controls) behavioral sensitivity to EB. (C) STH to CO$_2$ requires rut function in LN1- or GAD1-expressing neurons. (D) LTH to CO$_2$ requires rut function in LN1- or GAD1-expressing neurons. (E) STH and LTH to CO$_2$ does not require function of rutabaga in CO$_2$ specific PNs (V PN). (F) Conditional knock down of rutabaga in LNT neurons blocks STH, but not the LTH to EB. Bars indicate mean ± SEM; n values are given in Tables S1–S3. ***p < 0.001.
Fig. S4. GABAergic transmission from LN1 neurons and onto PNs is required for expression of habituation memory. (A) Control flies exposed or tested at elevated temperatures show normal STH and LTH. (B and C) LN1>shi<sup>ts1</sup> flies exposed to 5% CO<sub>2</sub> do not display STH (C) or LTH (D) if exposed or tested at 32 °C. (D and E) Dose–response assays of transgenic lines to CO<sub>2</sub> (D) and EB (E). White bars show the response of UAS-RdlRNAi (Rdli) heterozygotes, and dark bars show Gal4 lines crossed to Rdli. (F) A previously validated transgenic Rdli RNAi construct (UAS Rdli<sub>1-10</sub>) against GABA<sub>A</sub> receptors in PNs increases baseline electrical activity in projection neurons. In GC56/+; GH146/ messenger RNA (mRNA), and coexpression of an RNAi construct that targets the Drosophila GABA<sub>A</sub> receptor (ii) increases substantially levels of live GCaMP signal without any change in levels of GCaMP protein (iii and iv). This finding indicates higher levels of spontaneous activity, which is consistent with reduced basal GABA inhibition. (v) Fluorescence intensities of images. Bars indicate mean ± SEM. ***P < 0.001.
Fig. S5. RNAi-mediated knockdown of the NMDA receptor or vesicular Glutamate transporter. (A) Tub:Gal4-driven expression of the dsNR1 transgene causes a large reduction in the level of NMDA receptor subunits in the antennal lobe detected by respective antibodies (1). (B) DVGLUT expression in the antennal lobe detected by anti-DVGLUT staining (2) is reduced when the transgenic RNAi construct is expressed in LN1 neurons. (C) Expression of an RNAi line specific to the second NMDA subunit dsNR2 in PNs leads block in both the forms of olfactory habituation. (D) Knock down of DVGLUT in mushroom body neurons marked by MB247Gal4, has no effect on habituation. Adult specific knock down of NMDA receptors in GH146 expressing PNs or DVGLUT in LN1 neurons, blocks olfactory short-term habituation to EB (E). Bars indicate mean ± SEM; n values are given in Tables S1–S3. ***P < 0.001.

Fig. S6. Glomerular volume changes that accompany LTH induced by 4-d exposure to CO$_2$ (5%) or EB (20%). (A and B) Volumes of several glomeruli measured in the antennal lobe of mock- or odor-exposed Drosophila. Consistent with prior work (1), 4-d CO$_2$ exposure results in a glomerular-specific 30% in the volume of the CO$_2$-responsive V glomerulus; 4-d exposure to 20% EB has no effect on the volume of V. Instead, it results in a 20% increase in the volume of DM2 (1) and an often larger increase in the volume of DM5, recently shown to be the glomerulus that drives behavioral aversion to EB, which is assayed in our behavioral experiments. (C) Effect of CO$_2$ and EB exposure on glomeruli not shown in Fig. 6; significantly, 4-d EB exposure does not evoke DM2 growth in $\text{rut}^{2080}$; UAS $\text{rut}$+ flies but does in flies expressing the WT $\text{rut}$ transgene in LN1- or GAD1-expressing neurons. (D) Effect of 4-d EB exposure on DM2 growth after NMDAR knockdown in PNs. (E) Effect of VGLUT knockdown in GAD1-expressing neurons on 4-d CO$_2$- or EB-evoked glomerular growth. (F) Effect of knocking down the GABA$_A$ receptor (Rdl) in PNs on 4-d odor-evoked glomerular growth. Although essential for the expression of LTH (Fig. 4 C and D), Rdl is not necessary for LTH-associated structural plasticity. A simple model to explain these data (Fig. 8) proposes that habituation arises from cell biological changes that eventually allow increased GABA release from LNs. GABA$_A$ receptors are required to sense and transduce the physiological consequence of these changes, namely the increased strength of GABAergic transmission onto PNs, but they are dispensable for processes that underlie the changes themselves. Bars indicate mean ± SEM. ***$P < 0.001$; **$P \geq 0.01$; *$P \geq 0.05$.

Fig. S7.  cAMP response element-binding protein 2 (CREB2) is required for odor-induced structural plasticity. In (A) whole flies or (B) LN1 local interneurons, transient expression of CREB2b, an inhibitory isoform of the CREB2 transcription factor, blocks 4-d EB-induced growth of DM2 and DM5 glomeruli. (A) Within 10 h of eclosion, hsCREB2b adult flies were exposed to 37 °C for 1 h before 4-d EB exposure. (B) Within 6 h of eclosion, LN1Gal4; TubGal80ts > UAS dCREB2b adult flies were shifted from 18 °C to 29 °C for 8 h before 4-d EB exposure. **P ≤ 0.01; *P ≤ 0.05.
**Table S1. Olfactory response indices (negative RI values) for genotypes tested to different habituation paradigms: STH**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Odorant tested</th>
<th>Odorant exposed</th>
<th>Naive</th>
<th>Postexposure</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (+)</td>
<td>Odorant tested 5% CO₂ (n)</td>
<td>15% CO₂</td>
<td>0.53 ± 0.02 (12)</td>
<td>0.26 ± 0.02 (12)</td>
<td>3.55E-08</td>
</tr>
<tr>
<td>WT (+)</td>
<td>5% EB</td>
<td>0.63 ± 0.02 (8)</td>
<td>0.62 ± 0.02 (9)</td>
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<td></td>
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<tr>
<td>WT (+)</td>
<td>Odorant tested 10⁻³ EB (n)</td>
<td>15% CO₂</td>
<td>0.69 ± 0.02 (9)</td>
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<td></td>
</tr>
<tr>
<td>WT (+)</td>
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<td>0.72 ± 0.01 (21)</td>
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<tr>
<td>+/- TRPA1</td>
<td>CO₂</td>
<td>0.47 ± 0.02 (7)</td>
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<tr>
<td>Gr21a &gt; TRPA1</td>
<td>EB</td>
<td>0.48 ± 0.02 (8)</td>
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<td>Or83b &gt; TRPA1</td>
<td>EB</td>
<td>0.55 ± 0.01 (7)</td>
<td>0.55 ± 0.02 (8)</td>
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<table>
<thead>
<tr>
<th>Odorant tested</th>
<th>Odorant exposed</th>
<th>Naive</th>
<th>Postexposure</th>
<th>P value</th>
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<tr>
<td>+/- rut2080; UASrut+ (♂)</td>
<td>15% CO₂</td>
<td>0.50 ± 0.02 (10)</td>
<td>0.47 ± 0.02 (9)</td>
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<tr>
<td>+/- rut2080; UASrut+ (♂)</td>
<td>15% CO₂</td>
<td>0.57 ± 0.03 (10)</td>
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<td>+/- rut2080; UASrut+ (♂)</td>
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<td>+/- rut2080; UASrut+ (♂)</td>
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<tr>
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<tr>
<td>+/- dsNR1</td>
<td>15% CO₂</td>
<td>0.64 ± 0.03 (9)</td>
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<td>+/- dsNR1</td>
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<td>0.62 ± 0.01 (7)</td>
<td>0.66 ± 0.03 (7)</td>
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<td>+/- dsNR1</td>
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<td>0.55 ± 0.03 (8)</td>
<td>0.23 ± 0.02 (8)</td>
<td>1.96134E-06</td>
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<tr>
<td>+/- DVGGLUT RNAI</td>
<td>15% CO₂</td>
<td>0.52 ± 0.02 (8)</td>
<td>0.56 ± 0.03 (8)</td>
<td></td>
</tr>
<tr>
<td>Odorant tested 5% EB (n)</td>
<td>Odorant exposed</td>
<td>Naive</td>
<td>Postexposure</td>
<td>P value</td>
</tr>
<tr>
<td>-------------------------</td>
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<tr>
<td>CS (37 °C)</td>
<td>5% EB</td>
<td>0.65 ± 0.03 (10)</td>
<td>0.34 ± 0.01 (10)</td>
<td>1.89714E-07</td>
</tr>
<tr>
<td>hs-dCREb2b (Hs–)</td>
<td>5% EB</td>
<td>0.75 ± 0.01 (9)</td>
<td>0.31 ± 0.02 (9)</td>
<td>4.10946E-10</td>
</tr>
<tr>
<td>hs-dCREb2b (Hs+)</td>
<td>5% EB</td>
<td>0.75 ± 0.03 (11)</td>
<td>0.31 ± 0.01 (11)</td>
<td>8.66776E-10</td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS dCREB2b –18 °C</td>
<td>5% EB</td>
<td>0.74 ± 0.01 (16)</td>
<td>0.33 ± 0.03 (16)</td>
<td>3.31054E-13</td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS dCREB2b –29 °C pulse</td>
<td>5% EB</td>
<td>0.69 ± 0.03 (4)</td>
<td>0.25 ± 0.07 (4)</td>
<td>0.000434</td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS rutRNAi –18 °C</td>
<td>5% EB</td>
<td>0.74 ± 0.02 (6)</td>
<td>0.29 ± 0.02 (6)</td>
<td>1.44999E-08</td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS rutRNAi –29 °C</td>
<td>5% EB</td>
<td>0.81 ± 0.01 (7)</td>
<td>0.80 ± 0.01 (7)</td>
<td></td>
</tr>
<tr>
<td>GH146GAL4, TubGAL80ts &gt; UAS dsNR1 –18 °C</td>
<td>5% EB</td>
<td>0.75 ± 0.04 (8)</td>
<td>0.31 ± 0.02 (8)</td>
<td>5.21944E-05</td>
</tr>
<tr>
<td>GH146GAL4, TubGAL80ts &gt; UAS dsNR1 –29 °C</td>
<td>5% EB</td>
<td>0.82 ± 0.03 (9)</td>
<td>0.82 ± 0.02 (9)</td>
<td></td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS DVGLUT RNAi –18 °C</td>
<td>5% EB</td>
<td>0.64 ± 0.05 (4)</td>
<td>0.2 ± 0.02 (4)</td>
<td>0.043881</td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS DVGLUT RNAi –29 °C</td>
<td>5% EB</td>
<td>0.63 ± 0.01 (4)</td>
<td>0.72 ± 0.01 (4)</td>
<td></td>
</tr>
<tr>
<td>Odorant tested Temperature exposed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22 °C (n)</td>
<td>29 °C (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN1 &gt; TRPA1</td>
<td>CO₂</td>
<td>0.44 ± 0.02 (8)</td>
<td>0.13 ± 0.01(8)</td>
<td>4.2E-07</td>
</tr>
<tr>
<td>LN1 &gt; TRPA1</td>
<td>EB</td>
<td>0.58 ± 0.03 (9)</td>
<td>0.14 ± 0.02 (9)</td>
<td>1.98397E-08</td>
</tr>
</tbody>
</table>

Table S2. Olfactory response indices (negative RI values) for genotypes tested to different habituation paradigms

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Odorant exposed (15% CO₂)</th>
<th>naïve</th>
<th>Exposure at RT, testing at RT (n); postexposure</th>
<th>Exposure at 32 °C, testing at RT (n); postexposure</th>
<th>Exposure at RT, testing at 32 °C (n); postexposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/- Shi ts1</td>
<td>0.69 ± 0.03 (7)</td>
<td>0.35 ± 0.03 (3); 0.003</td>
<td>0.39 ± 0.02(3) 0.002164</td>
<td>0.33 ± 0.04 (3); 0.007362</td>
<td></td>
</tr>
<tr>
<td>LN1 &gt; Shi ts1</td>
<td>0.64 ± 0.02 (5)</td>
<td>0.3 ± 0.01 (5); 8.0628E-05</td>
<td>0.62 ± 0.02 (10)</td>
<td>0.7 ± 0.02 (9)</td>
<td></td>
</tr>
<tr>
<td>+/- Shi ts1</td>
<td>5% EB</td>
<td>0.65 ± 0.04 (4)</td>
<td>0.35 ± 0.04 (5); 3.457E-04</td>
<td>0.31 ± 0.04 (4); 6.157E-06</td>
<td>0.36 ± 0.03 (8); 1.33564E-05</td>
</tr>
<tr>
<td>LN1 &gt; Shi ts1</td>
<td>0.76 ± 0.06 (4)</td>
<td>0.43 ± 0.02 (4); 0.000475</td>
<td>0.78 ± 0.02 (6)</td>
<td>0.60 ± 0.02 (10)</td>
<td></td>
</tr>
</tbody>
</table>
Table S3. Olfactory response indices (negative RI values) for genotypes tested to different habituation paradigms: LTH

<table>
<thead>
<tr>
<th>Genotype Odorant exposed</th>
<th>Odorant tested (n)</th>
<th>Odorant tested (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5% CO₂</td>
<td>0.83 ± 0.2 (11)</td>
</tr>
<tr>
<td>WT</td>
<td>20% EB</td>
<td>0.75 ± 0.02 (7)</td>
</tr>
<tr>
<td>Odorant tested 10⁻³ EB (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>5% CO₂</td>
<td>0.75 ± 0.02 (7)</td>
</tr>
<tr>
<td>WT</td>
<td>20% EB</td>
<td>0.73 ± 0.04 (6)</td>
</tr>
<tr>
<td>Odorant tested 10⁻³ EB (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/- TRPA1</td>
<td>5% CO₂</td>
<td>0.52 ± 0.04 (8)</td>
</tr>
<tr>
<td>Gr21a &gt; TRPA1</td>
<td>5% CO₂</td>
<td>0.51 ± 0.01 (16)</td>
</tr>
<tr>
<td>Or83b &gt; TRPA1</td>
<td>5% CO₂</td>
<td>0.53 ± 0.04 (10)</td>
</tr>
<tr>
<td>+/- TRPA1</td>
<td>EB 10⁻³</td>
<td>0.66 ± 0.02 (8)</td>
</tr>
<tr>
<td>Gr21a &gt; TRPA1</td>
<td>EB 10⁻³</td>
<td>0.65 ± 0.04 (9)</td>
</tr>
<tr>
<td>Or83b &gt; TRPA1</td>
<td>EB 10⁻³</td>
<td>0.54 ± 0.01 (8)</td>
</tr>
</tbody>
</table>

Das et al. www.pnas.org/cgi/content/short/1106411108
Table S3. Cont.

<table>
<thead>
<tr>
<th>Odorant tested (n)</th>
<th>Genotype</th>
<th>Odorant exposed</th>
<th>Mock</th>
<th>Exposed</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-3}$ EB (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS &gt; dsNR1</td>
<td>20% EB</td>
<td>0.61 ± 0.04 (7)</td>
<td>0.20 ± 0.02 (7)</td>
<td>1.50828E-05</td>
<td></td>
</tr>
<tr>
<td>V PN &gt; dsNR1</td>
<td>20% EB</td>
<td>0.68 ± 0.04 (7)</td>
<td>0.23 ± 0.02 (7)</td>
<td>0.001092</td>
<td></td>
</tr>
<tr>
<td>GH146 &gt; dsNR1</td>
<td>20% EB</td>
<td>0.97 ± 0.01 (9)</td>
<td>0.97 ± 0.01 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS &gt; dsNR2</td>
<td>20% EB</td>
<td>0.68 ± 0.02 (9)</td>
<td>0.25 ± 0.03 (9)</td>
<td>1.41889E-07</td>
<td></td>
</tr>
<tr>
<td>GH146 &gt; dsNR2</td>
<td>20% EB</td>
<td>0.64 ± 0.01 (8)</td>
<td>0.60 ± 0.05 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% CO$_2$ (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS &gt; DVGLUT RNAi</td>
<td>5% CO$_2$</td>
<td>0.68 ± 0.01(8)</td>
<td>0.30 ± 0.02 (8)</td>
<td>7.28518E-06</td>
<td></td>
</tr>
<tr>
<td>GAD1 &gt; DVGLUT RNAi</td>
<td>5% CO$_2$</td>
<td>0.53 ± 0.02 (8)</td>
<td>0.45 ± 0.01 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Odorant tested $10^{-3}$ EB (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS &gt; DVGLUT RNAi</td>
<td>20% EB</td>
<td>0.74 ± 0.02 (9)</td>
<td>0.14 ± 0.02 (8)</td>
<td>3.80811E-11</td>
<td></td>
</tr>
<tr>
<td>LN1 &gt; DVGLUT RNAi</td>
<td>20% EB</td>
<td>0.62 ± 0.02 (8)</td>
<td>0.62 ± 0.02 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAD1 &gt; DVGLUT RNAi</td>
<td>20% EB</td>
<td>0.49 ± 0.03 (8)</td>
<td>0.53 ± 0.02 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB247 &gt; DVGLUT RNAi</td>
<td>20% EB</td>
<td>0.73 ± 0.03 (7)</td>
<td>0.30 ± 0.03 (11)</td>
<td>2.295E-07</td>
<td></td>
</tr>
<tr>
<td>CS (37 °C)</td>
<td>20% EB</td>
<td>0.69 ± 0.02 (10)</td>
<td>0.37 ± 0.02 (10)</td>
<td>2.31495E-10</td>
<td></td>
</tr>
<tr>
<td>hs-dCREb2b (Hs-)</td>
<td>20% EB</td>
<td>0.76 ± 0.01(9)</td>
<td>0.32 ± 0.02 (9)</td>
<td>6.28112E-10</td>
<td></td>
</tr>
<tr>
<td>hs-dCREb2b (Hs+)</td>
<td>20% EB</td>
<td>0.79 ± 0.02 (10)</td>
<td>0.82 ± 0.02 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS dCREB2b –18 °C</td>
<td>20% EB</td>
<td>0.72 ± 0.01 (10)</td>
<td>0.33 ± 0.03 (9)</td>
<td>2.71237E-08</td>
<td></td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS dCREB2b –29 °C pulse</td>
<td>20% EB</td>
<td>0.64 ± 0.03 (5)</td>
<td>0.6 ± 0.03 (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS rutRNAi –18 °C</td>
<td>20% EB</td>
<td>0.73 ± 0.02 (3)</td>
<td>0.26 ± 0.03 (5)</td>
<td>1.1592E-05</td>
<td></td>
</tr>
<tr>
<td>LN1GAL4; TubGAL80ts &gt; UAS rutRNAi –29 °C</td>
<td>20% EB</td>
<td>0.79 ± 0.03 (9)</td>
<td>0.5 ± 0.02 (11)</td>
<td>9.44566E-08</td>
<td></td>
</tr>
</tbody>
</table>

Table S4. Raw $\int \Delta F/F$ dT values

<table>
<thead>
<tr>
<th>Genotype and glomerulus</th>
<th>Odorant exposed</th>
<th>Odorant tested</th>
<th>Mock exposed</th>
<th>N</th>
<th>Odor exposed</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH146 Gal4, UAS GCaMP3/+ DM2</td>
<td>20% EB</td>
<td>0.5% EB</td>
<td>878 ± 106</td>
<td>6</td>
<td>410 ± 91</td>
<td>4</td>
</tr>
<tr>
<td>GH146 Gal4, UAS GCaMP3/+ DM5</td>
<td>20% EB</td>
<td>0.5% EB</td>
<td>928 ± 115</td>
<td>6</td>
<td>456 ± 53</td>
<td>7</td>
</tr>
<tr>
<td>rut$_{-g^{10}Y}$/GH146 Gal4, UAS GCaMP3/UAS rut$^+$ DM2</td>
<td>20% EB</td>
<td>0.5% EB</td>
<td>963 ± 93</td>
<td>12</td>
<td>783 ± 63</td>
<td>8</td>
</tr>
<tr>
<td>rut$_{-g^{10}Y}$/GH146 Gal4, UAS GCaMP3/UAS rut$^+$ DM5</td>
<td>20% EB</td>
<td>0.5% EB</td>
<td>905 ± 60</td>
<td>8</td>
<td>845 ± 80</td>
<td>8</td>
</tr>
<tr>
<td>GH146 Gal4, UAS GCaMP3/+ DM2</td>
<td>5% CO$_2$</td>
<td>0.5% EB</td>
<td>940 ± 85</td>
<td>8</td>
<td>1,265 ± 82</td>
<td>7</td>
</tr>
<tr>
<td>GH146 Gal4, UAS GCaMP3/+ DM5</td>
<td>5% CO$_2$</td>
<td>0.5% EB</td>
<td>638 ± 68</td>
<td>6</td>
<td>730 ± 99</td>
<td>7</td>
</tr>
<tr>
<td>GH146 Gal4, UAS GCaMP3/+ DC2</td>
<td>20% EB</td>
<td>3% 3-Oct</td>
<td>718 ± 117</td>
<td>11</td>
<td>923 ± 131</td>
<td>12</td>
</tr>
</tbody>
</table>

Represented are the mean values (of area under the curve 0–5.5 s) ± SEM and numbers of flies (N) for each genotype used for imaging.