Non-synaptic inhibition between grouped neurons in an olfactory circuit

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Diverse sensory organs, including mammalian taste buds and insect chemosensory sensilla, show a marked compartmentalization of receptor cells; however, the functional impact of this organization remains unclear. Here we show that compartmentalized Drosophila olfactory receptor neurons (ORNs) communicate with each other directly. The sustained response of one ORN is inhibited by the transient activation of a neighbouring ORN. Mechanistically, such lateral inhibition does not depend on synapses and is probably mediated by ephaptic coupling. Moreover, lateral inhibition in the periphery can modulate olfactory behaviour. Together, the results show that integration of olfactory information can occur via lateral interactions between ORNs. Inhibition of a sustained response by a transient response may provide a means of encoding salience. Finally, a CO2-sensitive ORN in the malaria mosquito Anopheles can also be inhibited by excitation of an adjacent ORN, suggesting a broad occurrence of lateral inhibition in insects and possible applications in insect control.

An intriguing feature of a number of sensory systems is the compartmentalization of their primary sensory cells. These cells are housed together in specialized structures such as the taste buds of vertebrates and the chemosensory sensilla of invertebrates. The compartmentalized primary sensory cells often respond to diverse stimuli. The functional consequence of such organization is unknown.

Olfactory receptor neurons (ORNs) are the primary units of odour perception1. ORNs are widely believed to function as autonomous units, each responding to odors independent of other ORNs. In some organisms, such as insects, ORNs are compartmentalized into sensilla (Fig. 1a). An individual sensillum encapsulates the dendrites of neurons2–4. The neighbouring ORNs exhibit differing spike amplitudes and odorant sensitivities5. In Drosophila melanogaster, each ORN is assigned a designation indicating the type of sensillum in which it is housed and its relative spike amplitude among the ORNs of the sensillum. Thus, the ab3A neuron is located in antennal basiconic sensilla of type 3, and the ‘A’ indicates that its spike amplitude is greater than that of the neighbouring ‘B’ neuron. In fruitflies, moths and mosquitoes, ORNs are grouped in stereotyped combinations5–9.

The functional significance of this widespread pattern of ORN organization is unknown. In Drosophila, neighbouring ORNs do not have obvious functional relationships10, and they do not project to adjacent regions in the brain11. In certain sensilla of flies, moths and beetles, the activation of neighbouring ORNs elicits opposing behaviours6,8,9,12–16. There are theoretical predictions based on electrical circuit modelling that the transient activation of one ORN may interfere with the signalling of a neighbouring ORN17, and there is precedent for olfactory stimuli that activate one neuron and inhibit its neighbour15,16, but in the absence of molecular genetic analysis it is difficult to determine whether such stimuli act uniquely on one ORN or directly on both. Similar examples can also be found in insect taste sensilla18–22, but in Drosophila some bitter compounds have been shown to act directly both on a sugar neuron and on a bitter neuron, inhibiting one and exciting the other23.

Here we use the molecular genetics of Drosophila to examine the coding of pairs of odours by the ORNs of olfactory sensilla. We find that the prolonged activation of one ORN is inhibited by the transient excitation of its neighbour. This lateral inhibition is observed within diverse types of Drosophila sensilla, and the activation of a mosquito ORN laterally inhibits the response of a neighbouring ORN to CO2, a key cue used by mosquitoes to find their human hosts. The communication between neurons does not require a synapse, and probably proceeds via ephaptic coupling. Finally, we find that this lateral inhibition at the periphery of the olfactory circuit can modulate olfactory behaviour. Together, our results indicate that ORNs do not signal cell-autonomously in all circumstances, but rather their responses can be regulated by the activity of their ORN neighbours in a sensillum.

Activation of an ORN inhibits its neighbour

To analyse the relationship between two ORNs in a sensillum, we used a paradigm that allows us to deliver two odours, one for each neuron (Fig. 1b, c). One odorant, the ‘background odorant’, is provided continuously via an airstream and elicits the sustained firing of one ORN, the A neuron in most experiments. Superimposed on this background stimulus, a short pulse of a second odorant is delivered to activate the other ORN, usually the B neuron. This paradigm of odour presentation is distinct from the single-odorant paradigm used commonly in many studies5,10,24, but it simulates a coding problem that the system encounters in its natural environment, for example when a fly receiving sustained olfactory input from a local source receives a superimposed, transient stimulus from a distant source delivered by a gust of wind.

When the ab3 sensillum is stimulated with a prolonged dose of methyl hexanoate, the ab3A neuron responds with a sustained train of action potentials (large action potentials in Fig. 1d). When a pulse of 2-heptanone is superimposed on this background, not only does ab3B fire (small action potentials) but there is a marked reduction in the firing of ab3A (Fig. 1d).

This inhibitory effect could, in principle, be due to direct inhibition of OR22A, the receptor of ab3A, by 2-heptanone. However, ablation of ab3B by expression of the cell death gene reaper (rpr) completely abolished the inhibition of ab3A (Fig. 1d, bottom). This result indicates that the inhibition of the A neuron depends on the excitation of the B neuron.

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When H134R-ChR2 was expressed in ab3A, a blue-light stimulus increased ab3B activity of ab3B (Fig. 1f, top). Genetic ablation of ab3A demonstrated that ab3B was not lacking H134R-ChR2 (Fig. 1e, bottom), indicating that it does not inhibit ab3A directly. The simplest interpretation of these results is that lateral inhibition is observed broadly in the Drosophila antenna.

To test further the possibility that activation of the ab3B neuron can inhibit the ab3A neuron, we expressed Channelrhodopsin2 (H134R-ChR2) in ab3B. As expected, blue light elicited an excitatory response in ab3B of these engineered flies (Fig. 1e). Activation of ab3B by light also inhibited the tonic firing of ab3A elicited by methyl hexanoate. Right, averaged responses. Grey traces indicate responses in the absence of a neighbouring ORN (Supplementary Fig. 1). We note also that the pulsed odorant alone did not directly inhibit the spontaneous firing of the A neuron (Supplementary Fig. 2). These results indicate that lateral inhibition is observed broadly in the Drosophila antenna.

Lateral inhibition in other sensilla

There are four morphological types of antennal sensilla: large basiconic sensillum, small basiconic sensillum, coeloconic sensillum and trichoid sensilla. ab3 is a large basiconic sensillum containing two ORNs. We analysed four other sensilla, chosen for their morphological diversity and their functional specificities. Their ORNs express receptors that have been functionally characterized, and odorants have been identified that at certain concentrations selectively activate the receptor of only one ORN in each sensillum.

Lateral inhibition between ORNs was observed in all sensillar types examined: a large basiconic sensillum containing four ORNs (ab1); a large basiconic sensillum with two ORNs (ab2); a small basiconic sensillum (ab5); and a coeloconic sensillum (ac3). In each case, a short odorant pulse that activated one target ORN inhibited the tonic firing of a neighbouring ORN (Fig. 2a–d). When the targeted ORN was ablated or non-functional, the short odorant pulse showed no inhibition of the neighbouring ORN (Supplementary Fig. 1). We note also that the pulsed odorant alone did not directly inhibit the spontaneous firing of the A neuron (Supplementary Fig. 2). These results indicate that lateral inhibition is observed broadly in the Drosophila antenna.

Inhibition is dose–dependent

When ab3A was tonically excited with a constant concentration of methyl hexanoate, increasing doses of 2-heptanone produced increasing inhibition of ab3B to 2-heptanone (−38 spikes s−1, 5 × 10−7). Bottom: genetic ablation of ab3A prevented inhibition. Inset: orange dots indicate ab3B spikes. Very large spikes represent the coincidence of A and B spikes. g, ChR2 expressed in ab3A. A pulse of blue light (−25 mW mm−2) excited ab3A, inhibiting the response of ab3B to 2-heptanone (−35 spikes s−1, 5 × 10−7). n = 12 in d–g.
When the background odorant, methyl hexanoate, was delivered at increasing concentrations, the rate of ab3A tonic firing increased across a range of ~15 spikes s⁻¹ to ~50 spikes s⁻¹ (Fig. 3c, d, and Supplementary Table 2). Inhibition by a strong ab3B stimulus was potent across all these concentrations; in all of these cases the rate of firing was reduced to approximately the same level. A genetic ablation experiment confirmed that these reductions depended on ab3B (Supplementary Fig. 3). We note that 2-heptanone alone did not directly inhibit ab3A spontaneous activity (Fig. 3c, d, ‘no bkg’).

Transmission without a synapse
Next we asked whether the intrasensillar communication is mediated by synapses. First we used tetanus toxin (TNT)²⁹ to block synaptic transmission. We expressed TNT in ORNs using the Orco promoter and the GAL4/UAS system, which is expected to drive expression in all basiconic ORNs³⁰ except the CO2-sensitive ab1C neuron¹,¹². Activation of ab3B inhibited the tonic excitation of ab3A in these TNT-expressing flies (Fig. 3a, top). Moreover, the degree of inhibition was comparable to that in control flies (Fig. 3a, bottom). T-maze behavioural tests confirmed that synaptic transmission was blocked in the targeted ORNs (Fig. 4b).

Second, we performed single-unit recordings from isolated antennae, severed from the heads of flies. Activation of ab3B again inhibited the tonic excitation of ab3A (Fig. 4c), supporting the conclusion that lateral inhibition between neighbouring ORNs occurs in the periphery without involvement of central synapses.

Third, we tested the possibility of axo-axonic synapses between ORNs with a cross-correlation analysis³⁷. Analysis of ab3A and ab3B spontaneous spikes did not reveal coordinated spiking patterns and thus provided no evidence for axo-axonic synaptic interactions (Fig. 4d), similar to what has been found between homotypic ORNs in Drosophila³⁴.

Finally, we used Cd²⁺ to block synaptic neurotransmission⁴⁵. We included a high concentration of Cd²⁺ in the recording pipette so as to allow Cd²⁺ to diffuse into the sensillum lymph and block any peripheral dendro-dendritic synapses in sensilla of Orco-GALA/UAS-TNT flies. We observed little if any effect on the inhibition of ab3A firing after ab3B excitation (Fig. 4e; compare with Fig. 4a). To verify the efficacy of our drug delivery method, we applied the Orco agonist VUAA1 (ref. 36) via the recording pipette and observed elevated ORN spike activities, as expected (Fig. 4f). Together, these results indicate that lateral inhibition does not depend on chemical synapses.

Intrasensillar communication could, in principle, be mediated via gap junctions; however, the activation of one ORN would then probably lead to the activation, rather than the inhibition, of its neighbour. Moreover, we found that nitric oxide signalling inhibitors had
no effect on lateral inhibition (not shown). In summary, conventional forms of neuronal communication are unlikely to mediate lateral inhibition in a sensillum.

Lateral inhibition modulates behaviour

To determine whether intra-sensillar neuronal inhibition can modulate olfactory behaviour, we examined a pair of neighbouring ORNs, the activation of which leads to opposing behavioural outputs (Fig. 5). ab1A mediates attraction to apple cider vinegar (ACV)\(^1\), whereas its neighbour ab1C mediates aversion to low concentrations of CO\(_2\) (refs 12, 32, 37, 38). We confirmed that in a T-maze assay, when given a choice between CO\(_2\) and air alone, flies avoid CO\(_2\), whereas when faced with a choice between ACV and water, they are attracted to ACV (Fig. 5a, black bars). We note that in these flies, physiological recordings confirmed that ab1A neurons respond to ACV (not shown). When given a choice between two arms, one with CO\(_2\) and one with CO\(_2\) and ACV, these flies preferred the arm with ACV (Fig. 5b). Because synaptic transmission from ab1A neurons is blocked and the flies have no attraction to ACV, the simplest interpretation of these results is that activation of ab1A attenuated the response of ab1C to CO\(_2\) via lateral inhibition: the reduced CO\(_2\) response decreased the avoidance of the arm containing CO\(_2\) and ACV relative to the arm containing CO\(_2\) alone, and this decreased avoidance is seen as an attraction to the arm containing CO\(_2\) and ACV.

If this interpretation is correct, and the preference for the arm containing CO\(_2\) and ACV depends on the activation of ab1A, then the preference should be abolished in Orco mutants, which lack a co-receptor required for the response of ab1A but not ab1C. Consistent with this prediction, Orco mutants showed no preference between the arm containing CO\(_2\) and the arm containing CO\(_2\) and ACV (Fig. 5c). We note that ACV does not inhibit ab1C directly (Supplementary Fig. 4). Taken together, these results provide evidence that lateral inhibition within a sensillum can modulate behaviour.

Discussion

Integration of olfactory information has long been known to occur in the CNS, and has more recently been shown to occur in individual ORNs\(^8\). We have demonstrated that integration also occurs at a third
Lateral inhibition of a prolonged signal by a transient signal may strongly by stronger transient pulses. This graded pattern of lateral inhibition may give rise to a potent form of contrast enhancement in the dendrites, and the haemolymph, which surrounds the somata of an ORN can influence the LFP in a neighbouring sensillum46, we find that the magnitude of the LFP change in nearby unstimulated sensilla is small (Supplementary Fig. 7). Consistent with this observation, lateral inhibition does not spread among homotypic sensilla that are in close proximity to one another (Supplementary Fig. 8). These results further support the conclusion that the lateral inhibition is due to local electrical interactions between neighbouring ORNs within a sensillum.

The two-odour paradigm used in this analysis, in which a transient odour is superimposed upon a sustained odour, differs from the classic one-odour paradigm in which a transient pulse of a single odour is delivered. A priori one might expect to observe ephaptic effects in the one-odour paradigm if one ORN were excited sufficiently strongly, but the effects may be expected to be less pronounced than in the two-odour paradigm. ORN spike frequency is determined not only by the somatic transmembrane potential \( V_m \), but also by its rate of change, \( dV_m/dt \) (ref. 46). According to the model, transient activation of ORN\(_2\) reduces the depolarizing current of ORN\(_1\) (Supplementary Fig. 6). In the two-odour paradigm, activation of ORN\(_2\) has a marked effect on the value of \( dV_m/dt \), which changes from 0 to a negative value (\( dV_m/dt < 0 \); Supplementary Fig. 6). By contrast, in the one-odour paradigm, the activation of ORN\(_2\) has a more subtle effect on \( dV_m/dt \) when the sensillum is stimulated with an odour that activates both neurons; \( dV_m/dt \) is positive either in the presence or absence of ORN\(_2\) activation, only somewhat less positive when ORN\(_2\) is activated. The more subtle influence of ORN\(_2\) activation on \( dV_m/dt \) in the one-odour paradigm may explain why in the one-odour paradigm, the excitatory responses of an ORN containing an ectopically expressed receptor were markedly similar to those of the ORN that endogenously expresses the same receptor27, despite major differences in the response profiles of their neighbours.

We note finally that our results indicate the possibility of a new approach to insect control: the inhibition of key insect ORNs by activation of their neighbours with odours.

### METHODS SUMMARY

Fly antennal preparations and single-unit recordings were performed essentially as described26, except for the isolated antennal preparation in which the stabilized antenna was severed from the head using the broken tip of a tapered glass micropipette tube. Recordings were performed on adult female flies 5–7 days after eclosion, except that flies 24–36 h after eclosion were used in UAS-TNT experiments because TNT-expressing ORNs began to lose spike activities in older flies. Supplementary Table 1 lists fly genotypes for all experiments. Female mosquito larvae47. AC signals (300–2,000 Hz) were recorded, except for local field potential recordings where DC signals (low-pass filtered at 2 kHz) were recorded. ORN spikes were detected and sorted based on spike amplitude using routines in Igor Pro 6.01 and binned at 50-ms intervals.

For optogenetic experiments, flies were reared in constant darkness on fly food supplemented with \(-100 \mu M\) all trans-retinal37. Recordings were performed on adult females 7 days after eclosion using an established optics set-up36. For pharmacological experiments, chemicals were delivered inside the sensillum via the recording glass electrode.

T-maze behavioural tests were performed essentially as described27. For experiments shown in Fig. 4b, flies were given 1 min to choose between the two arms; air versus CO\(_2\) (0.67%) or H\(_2\)O versus ACV (25%). For experiments shown in Fig. 5, four experimental conditions were used: (1) air versus CO\(_2\) (0.13%); (2) H\(_2\)O versus ACV (100%, pH 7.5); (3) CO\(_2\) (0.13%) versus CO\(_2\) (0.13%); (4) CO\(_2\) (0.13%) plus H\(_2\)O versus CO\(_2\) (0.13%) plus ACV (100%, pH 7.5). Preference

![Figure 5](https://example.com/figure5.png)
index was calculated as the fraction of flies entering the test arm minus the fraction of flies entering the control arm.

**Full Methods** and any associated references are available in the online version of the paper.

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METHODS

**Drosophila stocks.** Recordings were performed on adult female flies 5 days after eclosion, except that 7-day flies were used in UAS-prp experiments, and flies 24–36 h after eclosion were used in UAS-TNT experiments because TNT-expressing ORNs began to lose spike activities in older flies. Flies were reared at 25 °C in an incubator with a 12-h light/dark cycle. The following fly stocks were used: (1) UAS-prp (ref. 49), (2) w^1118 and P{Bac[WH]Or35}^20653, (3) UAS-TNT (ref. 29), (4) UAS-H134R-ChR2 (ref. 25), (5) Or-GAL4 lines (Bloomington stock centre), (6) Gr21a-GAL4 (ref. 31). Supplementary Table 1 lists genotypes for all experiments.

**Mosquitoes.** Female *Anopheles gambiae* mosquitoes were used ~4 days after eclosion. Extracellular recordings from the capitate-peg sensilla on the maxillary palps were performed as described.47

**Electrophysiology and data analysis.** For the standard antennal preparation, a fly was wedged into the narrow end of a truncated plastic pipette tip to expose the antenna, which was subsequently stabilized between a tapered glass microcapillary tube and a coverslip covered with double-sided type. For the isolated antennal preparation, a standard antennal preparation was made first and the stabilized antenna was gently severed from the head using the broken tip of a tapered glass microcapillary tube. Extracellular single-unit recordings were performed essentially as described.47 Briefly, electrical activity of the ORNs was recorded extracellularly by placing a sharp electrode filled with Ringer solution into a sensillum and the reference electrode filled with the same Ringer solution was placed in the eye (standard antennal preparation) or in the first antennal segment (severed antennal preparation). No more than four sensilla from the same antenna were recorded in the standard preparation, and no more than two sensilla from the same antenna were recorded in the severed preparation. For each sensillum, one electrodogram concentration was presented. AC signals (300–2000 Hz) were recorded on an Iso-DAM amplifier (World Precision Instruments), except for local field potential recordings where DC signals (low-pass-filtered at 2 kHz) were recorded and digitized at 5 kHz with Axoscope 10.2 (Molecular Devices). ORN spikes were detected and sorted based on spike amplitude using routines in Igor Pro 6.01 (Wavemetrics). Peri-stimulus time histograms (PSTHs) were obtained by averaging spike activities in 50-ms bins and smoothed using a binomial algorithm (Igor Pro 6.01, Wavemetrics).

**Odour stimuli.** Odorants were diluted in paraffin oil (v/v). For short odour pulses, odour stimuli (50 μl applied to a filter disc) were delivered from a Pasteur pipette via a pulse of air (200 ml min⁻¹) into the main air stream (2000 ml min⁻¹) as described previously47. In Fig. 2a, stimulation with CO₂ was by filling the Pasteur pipette with pure CO₂, which was subsequently puffed into the main air stream. On the basis of the published dose–response relationship in *D. virilis*, we expect a concentration of CO₂ estimated to be ~1% (mean ab1C response shown in Fig. 2a: 163 Hz). Background odour stimuli were delivered from a 125-ml flask containing 3 ml of odour dilutions (or 25 ml of carbonated water for background CO₂) directly downstream of the main air stream (2000 ml min⁻¹).

**Optogenetic stimulation.** Flies expressing H134R-ChR2 in targeted ORNs and control flies (UAS-H134R-ChR2) were reared in constant darkness on fly food supplemented with ~100 μM all trans-retinal (Sigma) as described.95 Recordings were performed on adult females 7 days after eclosion using an established optics setup48. Briefly, a light stimulus was generated via a blue laser (MBL-III-473/30 mW, Opto Engine LLC) and delivered by an optical fibre (200-μm core diameter, BFH22-200, Thorlabs). The tip of the optical fibre was positioned above the antenna. Light pulses (500-ms duration) were controlled by an isolated pulse stimulator (Model 2100, A-M Systems). Light output at the tip of the optical fibre was measured with an optical power meter (Model 1916-C, Newport).

**Cross-correlation analysis.** The basal spike activity was investigated using 30 sweeps of 10-s duration. Action potentials of the ab3A and ab3B neuron were identified based on size and their triphasic (ab3A neuron) or more biphasic (ab3B neuron) shape using Origin software (OriginLab Corporation). Spike times of ab3A and ab3B neurons of individual sweeps were cross-correlated using Matlab software (MathWorks). Interspike times were accumulated across all recorded sweeps and binned in 10-ms intervals. Such an analysis can reveal coordinated spiking patterns and was used to identify axo-axonic synapses between neighbouring scorpion ORNs.10

**Pharmacology.** Drugs were prepared as concentrated stock solutions and diluted in Ringer solution before experiments. Chemicals were delivered inside the sensillum via the recording glass electrode. Recordings were performed in flies expressing TNT in the ORNs ~15 min after drug introduction, except for the experiments with VUAA1, where recordings were performed within minutes after electrode insertion. The electrode stayed inside the sensillum throughout the 15-min period. VUAA1 (Chemical Diversity Research Institute, Joint Stock Company) was used at 1 mM (stock: 100 mM in DMSO). CdCl₂ (Aldrich) was used at 1 mM (stock: 100 mM in Ringer solution).

**T-maze assay.** Flies were collected within ~8 h after eclosion without using CO₂ anaesthesia. Flies were tested 24–32 h after eclosion after ~24 h starvation. For starvation, flies were gently tapped into empty vials with moistened foam plugs and kept at 25 °C in an incubator.

Behavioural tests were performed as described previously12 at room temperature in a dark room. About 40–60 flies were transferred by an aspirator into a 15-ml centrifuge tube (Corning 430791), which was subsequently connected to the sliding chamber (elevator) of the T-maze apparatus. Flies were gently tapped into the elevator, which was then lowered to the opening where the test arm and the control arm were connected. A 16-inch 15-W fluorescent bulb was placed horizontally behind the test and control arms, and the light was on only for the duration of the assay. Phototaxis drew flies out of the elevator. Flies were given 1 min to choose between the two arms, after which the elevator was partially lifted to block any further choices. Preference index was calculated as the fraction of the flies entering the test arm minus the fraction of the flies entering the control arm. The total number of flies used in calculation of the preference index included flies in both arms and in the elevator.

For the experiment shown in Fig. 4b, 10 μl of apple cider vinegar solution or 10 μl of water was added to a Whatman filter disc (1/2 inch diameter) that was positioned around the 1.5-ml mark of the 15-ml centrifuge tube. Twenty-five per cent apple cider vinegar was used because it attracted flies in a T-maze assay without triggering the acid-mediated avoidance pathway. Twenty minutes of equilibrium time was allowed before the tubes were connected to the T-maze apparatus immediately before the assay. For the experiment in Fig. 4b using CO₂, 0.1 μl of pure CO₂ (UN1013, Airgas) was injected into the tube immediately before the assay. The positions of the test and control tubes were alternated for each trial. New groups of flies and new tubes were used for each test. The air inside the 15-ml tube was equilibrated with the air in the room for at least 4 h before use.

For experiments shown in Fig. 5 to address the behavioural relevance of lateral inhibition, we used four experimental conditions: (1) air versus CO₂; (2) H₂O versus ACV; (3) CO₂ versus CO₂; and (4) CO₂ plus H₂O versus CO₂ plus ACV. Thirty microlitres of neutralized apple cider vinegar (100%, pH 7.5) or water was added to a Whatman filter disc that was positioned horizontally via permanent double-sided tape (Scotch, 3M) around the 10-ml mark of the centrifuge tube. When CO₂ was used, 0.1 μl of pure CO₂ (UN1013, Airgas) was injected into the tube(s) near the 5-ml mark immediately before the assay. When CO₂ was used in both arms, the CO₂ was injected, the two tubes were connected to the T-maze apparatus, and then the apparatus was inverted gently ~10 times and allowed to equilibrate for an additional minute to ensure that CO₂ was distributed evenly between the two arms. The elevator was then lowered to release the flies. The positions of the test and control tubes were alternated for each trial.

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