LETTERS

A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone

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Insects, like many other animals, use sex pheromones to coordinate their reproductive behaviours[1]. Volatile pheromones are detected by odorant receptors expressed in olfactory receptor neurons (ORNs). Whereas fruit odours typically activate multiple ORN classes[2], pheromones are thought to act through single dedicated classes of ORN[3]. This model predicts that activation of such an ORN class should be sufficient to trigger the appropriate behavioural response. Here we show that the *Drosophila melanogaster* male-specific pheromone 11-*cis*-vaccenyl acetate (cVA) acts through the receptor Or67d to regulate both male and female mating behaviour. Mutant males that lack *Or67d* inappropriately court other males, whereas mutant females are less receptive to courting males. These data suggest that cVA has opposite effects in the two sexes: inhibiting mating behaviour in males but promoting mating behaviour in females. Replacing Or67d with moth pheromone receptors renders these ORNs sensitive to the corresponding moth pheromones. In such flies, moth pheromones elicit behavioural responses that mimic the normal response to cVA. Thus, activation of a single ORN class is both necessary and sufficient to mediate behavioural responses to the *Drosophila* sex pheromone cVA.

Several lines of evidence initially suggested that *Drosophila* mating behaviours might be guided in part by pheromones detected by the class of ORNs that expresses the odorant receptor Or67d. First, the Or67d ORNs innervate a sexually dimorphic glomerulus (DA1) in the antennal lobe[4,5]. Second, these neurons constitute one of only three ORN classes that express the sex-specific transcripts of the behavioural sex determination gene *fruitless* (*fru*)[6,7]. Third, blocking the activity of all three classes of *fru*-positive ORNs impairs male courtship activity[5].

To assess the role of Or67d and the Or67d ORNs in *Drosophila* mating behaviour, we generated mutant alleles in which the open reading frame of *Or67d* was replaced with that of the yeast transcriptional activator *GAL4* (Fig. 1a). These mutant knock-in alleles allowed us to assess the function of *Or67d* itself, and also to use GAL4-responsive transgenes to study the function of the Or67d ORNs. We initially used ends-in homologous recombination[8] to produce a tandem duplication at the *Or67d* locus, consisting of one copy of the wild-type locus and one copy with the *GAL4* replacement (Supplementary Fig. S1). By resolving this duplication, we recovered two independent mutant alleles that carried only the *GAL4* replacement (*Or67d*^GAL4[1] and *Or67d*^GAL4[2]), and two independent control alleles in which the original intact locus was restored (*Or67d*^+[1] and *Or67d*^+[2]).

Using a *UAS–mCD8–GFP* reporter to label these cells with green fluorescent protein (GFP), we confirmed that the *Or67d*^GAL4 knock-in drives transgene expression exclusively in the ORNs that also express *Or67d* (Fig. 1b). Previous studies using an *Or67d* promoter fragment to drive GAL4 expression did not fully clarify whether Or67d ORNs project to DA1 exclusively[6] or to both the DA1 and VA6 glomeruli[7]. Using the *Or67d*^GAL4 knock-in, we confirmed that the DA1 glomerulus alone is targeted by Or67d ORNs, in both males (Fig. 1c) and females. We did not detect reporter expression anywhere else in adults, nor in embryos or larvae.

The Or67d receptor is thought to mediate the detection of the male pheromone cVA ([Z]-11-octadecenyl acetate): cVA activates the T1 class of trichoid sensilla[9] in which the Or67d neurons alone are housed[6], and ectopic expression of Or67d in other trichoid ORNs confers sensitivity to cVA[10]. Indeed, using single-sensillum recordings, we found that cVA elicits a rapid and robust firing response in the T1 sensilla of control *Or67d*^+ males but not in those of *Or67d*^GAL4 mutants (Fig. 1d). Spontaneous activity was also greatly reduced in these mutants. Restoring *Or67d* function with a *UAS–Or67d* transgene fully rescued both the spontaneous and evoked responses (Fig. 1d). The responses to cVA were quantitatively indistinguishable in males and females (Fig. 1e) across a 1,000-fold range of concentrations (Fig. 1f). To assess whether odorant receptors in other ORNs might also detect cVA, we used electroantennograms to simultaneously measure the responses of a large number of ORNs on the third antennal segment. Neither *Or67d*^GAL4 males nor females produced a detectable response to cVA, although both responded normally to ethanol (Fig. 1g, h). These genetic data confirm that Or67d mediates physiological responses to cVA, and show further that males and females respond equally to cVA and that Or67d is likely to be the only receptor for cVA.

If cVA acts as a sex pheromone and Or67d is its sole receptor, then *Or67d*^GAL4 males or females should be impaired in their performance of one or more mating behaviours. To test this, we first monitored male courtship behaviour in single-pair courtship assays, using the courtship index (CI, the percentage of time for which the male courts during a 10-min assay) as a simple measure of overall courtship activity. Typically, wild-type males court with a CI of about 80% when paired with a virgin female (Fig. 2a), but only about 10% when paired with another male (Fig. 2b). When paired with virgin females, *Or67d*^GAL4 mutant males courted at levels comparable to those of the control *Or67d*^+ males (Fig. 2a). In contrast, when paired with wild-type males, *Or67d*^GAL4 mutant males displayed a roughly threefold higher courtship activity than the *Or67d*^+ controls (P < 0.0001, permutation test; Fig. 2b and Supplementary Fig. S2). To confirm that this increased male–male courtship was indeed due to the loss of *Or67d* function, we introduced independent *UAS–Or67d* transgene insertions into each of the two *Or67d*^GAL4 lines. For both alleles, restoring *Or67d* function in this way suppressed male–male courtship back to its normal low levels (Fig. 2b).

To assess whether *Or67d* also functions in female mating behaviour, we paired individual mutant or control virgin females with

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naive wild-type males in a series of small chambers and measured their latency to copulation. About 50% of the control $Or67d^+$ females copulated within 18 min, and about 60% within 30 min (Fig. 2c). In contrast, only about 20% of the $Or67d^{GAL4}$ females copulated within 18 min, and about 30% within 30 min ($P<$ 0.0001 at both time points, χ^2 test; Fig. 2c and Supplementary Fig. S2). When $Or67d$ expression was restored with the $UAS–Or67d$ transgenes, the mutant females copulated as rapidly as the control females (Fig. 2c). The reduced receptivity of $Or67d^{GAL4}$ females was evidently not due to a lower attractiveness of these females to males, because they were courted as vigorously as $Or67d^+$ females (Fig. 2d).

These behavioural data imply that cVA is a dual-purpose sex pheromone, acting in males to inhibit mating[11] (Fig. 2b) and in females to promote mating (Fig. 2c). To test directly whether cVA inhibits male courtship behaviour, and if so whether this requires Or67d, we applied cVA to the abdomens of virgin females and offered these females to mutant or control males in single-pair courtship assays. Indeed, application of cVA suppressed courtship by $Or67d^+$ control males but not by $Or67d^{GAL4}$ mutant males ($P<0.0001$, permutation test; Fig. 3). cVA also suppressed courtship by $Or67d^{GAL4}$ males carrying the $UAS–Or67d$ transgene (Fig. 3). Thus, cVA acts through Or67d to inhibit male courtship behaviour. However, it should be noted that cVA is not the sole mediator of sex discrimination
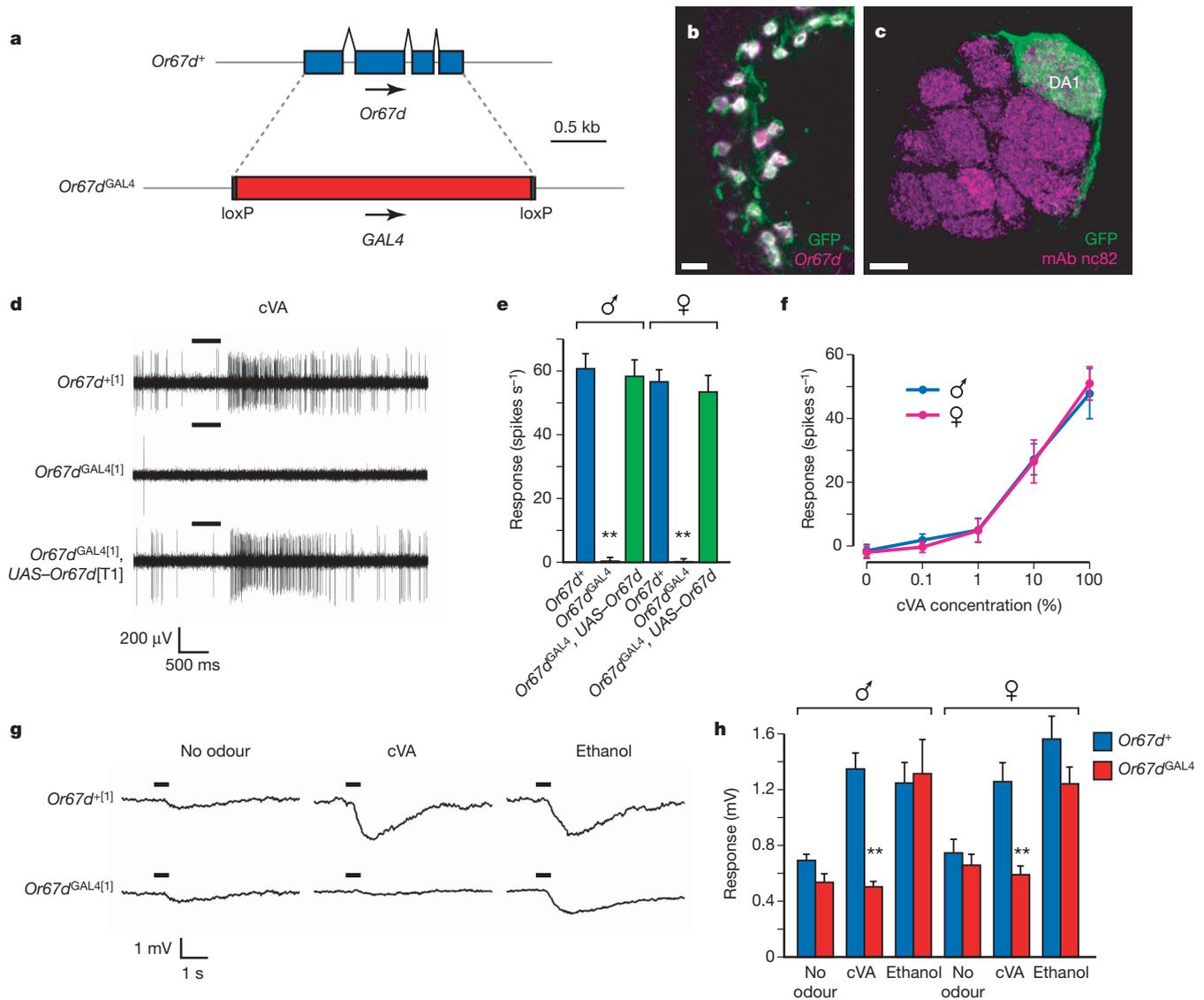

Figure 1 | Or67d mediates physiological responses to cVA. a, Diagram showing the organization of the $Or67d$ locus in the wild-type control ($Or67d^+$) and GAL4 knock-in ($Or67d^{GAL4}$) alleles. kb, kilobase. **b,** Expression of mCD8–GFP (anti-GFP, green) and $Or67d$ mRNA (magenta) in the third antennal segment of an $Or67d^{GAL4}/UAS–mCD8–GFP$ male. Scale bar, 10 μm. **c,** Confocal section of the antennal lobe of a $Or67d^{GAL4}/UAS–mCD8–GFP$ male, stained with anti-GFP (green) to visualize the projections of the Or67d ORNs and the synaptic marker mAb nc82 (magenta) to visualize the glomerular organization. Scale bar, 10 μm. **d,** Recordings from T1 sensilla in $Or67d$ control, mutant, and rescue males stimulated with cVA. The horizontal bar indicates the duration of stimulus delivery. The delay in the response is consistent with the time required for the odour to reach the antenna. **e,** Summary of single-sensillum recording data, plotted as means and s.e.m.; $n = 10$ for each control and rescue, and

$n = 6$ for mutants. No significant difference was observed between the two independent alleles for each genotype; data for each genotype were therefore pooled. Two asterisks, $P < 0.0001$ compared with controls; Kruskal–Wallis analysis of variance (ANOVA) test. **f,** Dose–response curve for T1 sensilla from $Or67d^+$ males and females, plotted as means ± s.e.m.; $n = 6$ for each data point. cVA was serially diluted in paraffin oil. **g,** Electroantennogram plots from control and mutant males, stimulated with cVA, ethanol or an empty odorant cartridge. The horizontal bar indicates the duration of stimulus delivery. **h,** Summary of electroantennogram data, plotted as means and s.e.m.; $n = 16$ and 13 for $Or67d^+$ and $Or67d^{GAL4}$ males, respectively, and $n = 10$ for females for both genotypes. Data from the two independent alleles were pooled. Two asterisks, $P < 0.002$ for $Or67d^{GAL4}$ compared with corresponding $Or67d^+$ control; Kruskal–Wallis ANOVA test.
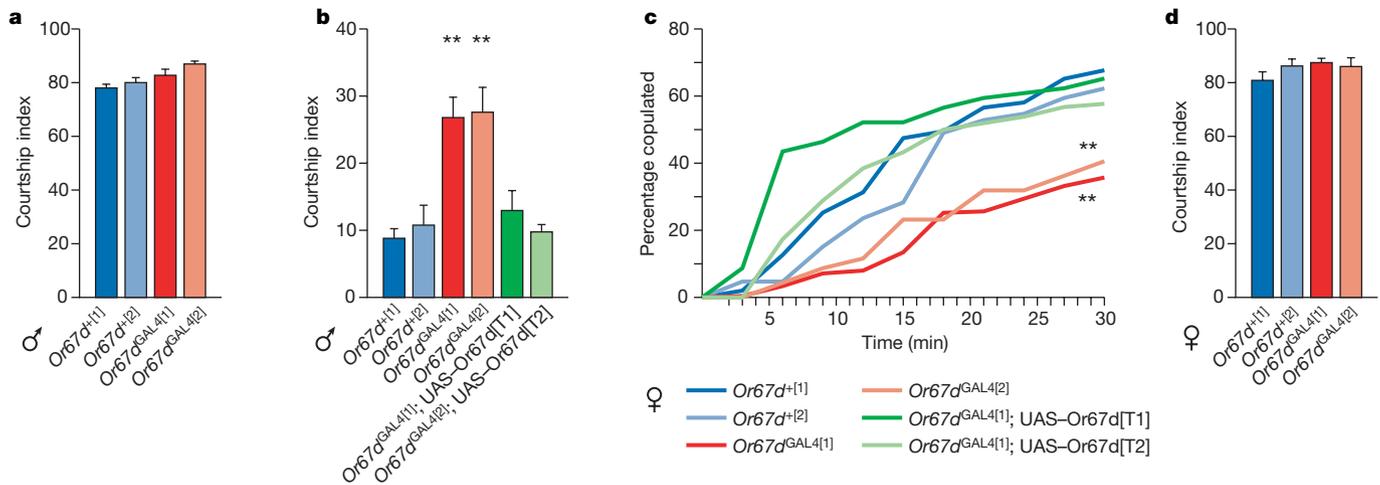
Figure 2 | Or67d functions in male and female mating behaviours.
a, Courtship indices for males of the indicated genotypes paired with wild-type virgin females. $n = 37, 57, 47$ and 61, respectively. **b**, Courtship indices for males of the indicated genotypes paired with wild-type males. $n = 103, 42, 92, 60, 30$ and 57, respectively. Two asterisks, $P < 0.0001$ compared with both $Or67d^+$ controls; permutation test. **c**, Copulation latency for females of the indicated genotypes paired with wild-type males. $n = 198, 106, 238, 69, 69$ and 104, respectively. Two asterisks, $P < 0.0001$ compared with both $Or67d^+$ controls at both the 18-min and 30-min time points; χ^2 test. **d**, Courtship indices for wild-type males paired with virgin females of the indicated genotypes. $n = 28, 18, 35$ and 24, respectively. Error bars indicate s.e.m. throughout.

in *Drosophila*, because *Or67d* mutant males still courted females much more avidly than they courted other males (Fig. 2a, b). This implies the existence of either additional inhibitory cues from the male, stimulatory cues from the female, or both.

General odorants are thought to activate many different receptors, with odour identity encoded by the specific combination of receptors that are activated[2,12]. In this model, no single ORN class encodes a specific odour, and odour perception is thought to arise through spatial[13] and temporal[14] integration of ORN signals in higher-order olfactory circuits. In contrast, odours of particular biological significance, such as pheromones, may activate only a single class of ORN, such that this ORN class alone communicates an unambiguous signal to the brain through a dedicated 'labelled line'[3]. We have shown that *Or67d* is required for physiological and behavioural responses to cVA, but this does not distinguish between the combinatorial and labelled-line models for signal processing. For this, we required a method of activating the Or67d ORNs artificially. In a combinatorial model, stimulation of Or67d ORNs alone is not predicted to induce a behavioural response, but in the labelled-line model it should.
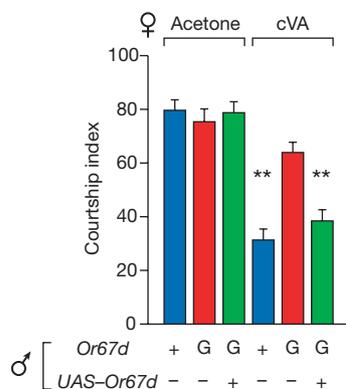
We sought to stimulate Or67d ORNs artificially with a heterologous ligand–receptor pair. For this, we turned to the sex pheromones of moths. The female silkmoth *Bombyx mori* emits the pheromone bombykol ([*E,Z*]-10,12-hexadecadien-1-ol) to attract and stimulate the male[15]. To the same effect, the female tobacco budworm *Heliothis virescens* produces a sex pheromone blend, one component of which is (*Z*)-11-hexadecenal[16]. The receptor BmOR1 has recently been identified as a receptor for bombykol, on the basis of its ability to confer cellular responses to bombykol in *Xenopus* oocytes and *Drosophila* ORNs[17–20]. Similarly, using single-sensillum recordings we found that replacing Or67d with BmOR1 conferred a new response to bombykol in the Or67d ORNs (Fig. 4a, b). The *Heliothis* receptors HR13-16 have been identified as candidate pheromone receptors[21], and by the same strategy we found that HR13 conferred sensitivity to (*Z*)-11-hexadecenal in the Or67d ORNs (Fig. 4a, b). Thus, despite more than 300 million years of evolutionary divergence, these moth pheromone receptors are fully functional when expressed in *Drosophila* ORNs.

To test whether artificial activation of the Or67d ORNs suppressed courtship, we applied bombykol or hexadecenal to virgin females and offered these females to naive males expressing either BmOR1 or HR13, respectively, in the Or67d ORNs. Courtship activity of these males was suppressed to a similar degree to that observed when cVA-treated females were offered to wild-type males (Figs 3 and 4c). In contrast, both $Or67d^+$ and $Or67d^{GAL4}$ males vigorously courted the bombykol-treated or hexadecenal-treated females, and the receptor replacement males vigorously courted females treated with solvent alone (Fig. 4c). Thus, courtship suppression is strictly dependent on both the presence of the moth pheromone on the female and the corresponding pheromone receptor in the Or67d ORNs in the males. We infer that the activation of the Or67d ORNs alone is sufficient to inhibit male courtship behaviour, in accordance with the labelled-line hypothesis for pheromone detection.

Tracing this labelled line into higher olfactory centres should help to reveal how the activation of Or67d ORNs suppresses male mating behaviour, and perhaps also how the same signal might have the opposite effect in females. These neurons connect in the DA1 glomerulus to two distinct classes of second-order projection neurons (PNs): GABAergic vPNs and cholinergic iPNs[22–27]. Both the DA1 vPNs and iPNs project their axons to a putative sex-pheromone processing centre in the lateral horn of the protocerebrum, specifically targeting two sexually dimorphic regions, one enlarged in males
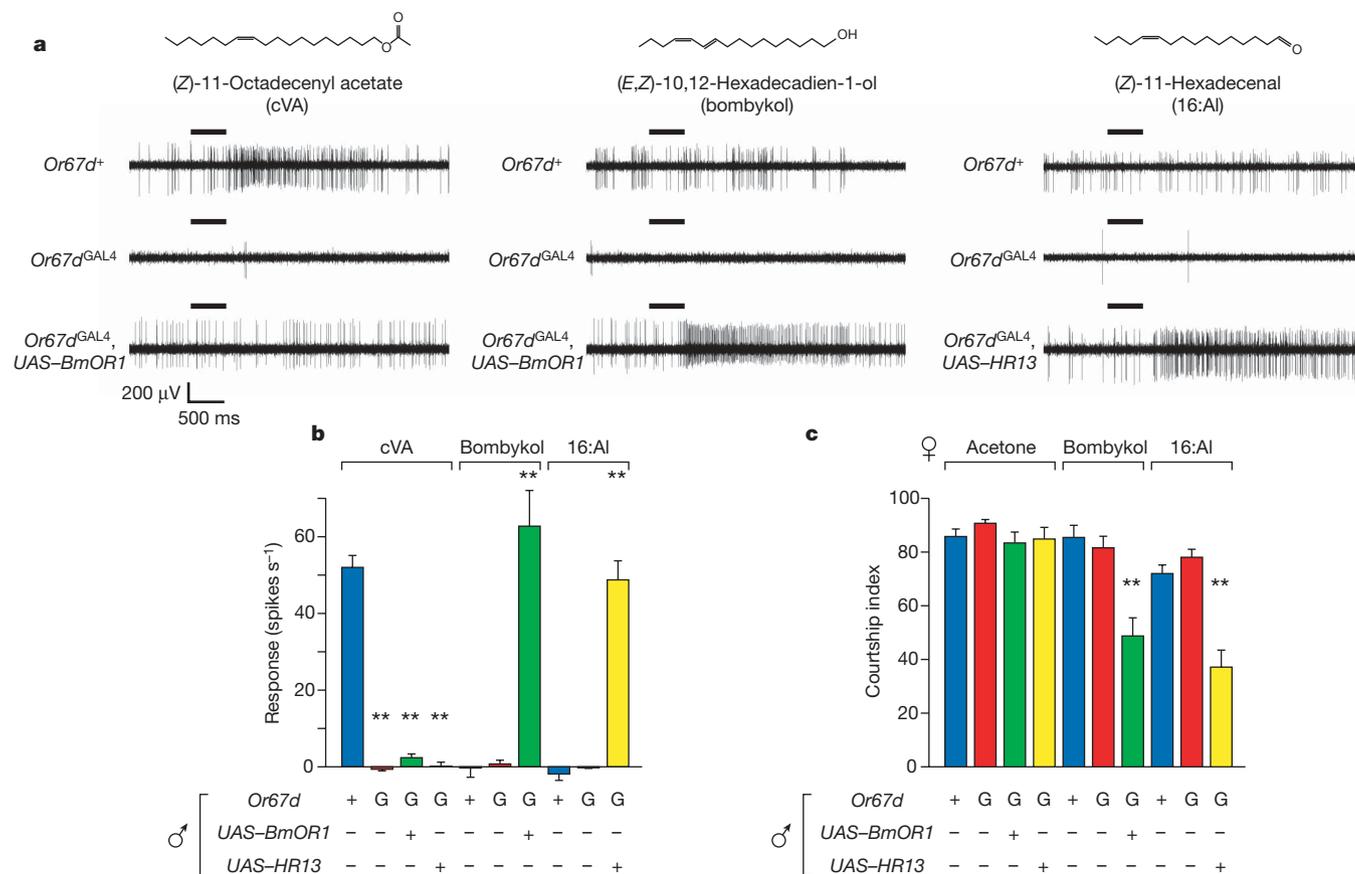

Figure 3 | Or67d mediates cVA-induced courtship suppression. Courtship indices for homozygous males of the indicated genotypes paired with wild-type virgin females treated either with acetone solvent alone or with cVA. Error bars indicate s.e.m.; $n = 14, 15, 23, 62, 58$ and 55, respectively. Two asterisks, $P < 0.0001$ compared with the corresponding solvent control; permutation test. For the male genotypes, '+' and 'G' indicate the $Or67d^+$ and $Or67d^{GAL4}$ alleles, respectively, and '+' and '−' the presence and absence of the *UAS–Or67d* transgene.

Figure 4 | Artificial activation of Or67d ORNs mimics cVA responses.
a, Recordings from T1 sensilla in $Or67d^+$, $Or67d^{GAL4}$, and $Or67d^{GAL4}$
$UAS–BmOR1$ or $Or67d^{GAL4}$ $UAS–HR13$ males stimulated with either cVA,
bombykol or (Z)-11-hexadecenal (16:Al). The horizontal bar indicates the
duration of stimulus delivery. The variation in the response latency most
probably reflects slight variations in the settings of the stimulus controller
and the position of the preparation. **b**, Summary of single-sensillum
recording data, plotted as mean and s.e.m.; $n = 8$–18 for each experiment.

Two asterisks, $P < 0.0001$ compared with $Or67d^+$ controls stimulated with
the same pheromone; Kruskal–Wallis ANOVA test. **c**, Courtship indices for
males of the indicated genotypes paired with wild-type virgin females treated
either with acetone solvent alone, with bombykol or with 16-al. Error bars
indicate s.e.m.; $n = 30, 40, 24, 12, 17, 21, 18, 34, 28$ and 32, respectively. Two
asterisks, $P < 0.0001$ compared with the corresponding $Or67d^+$ control;
permutation test.

and the other enlarged in females[24]. It will be interesting to test
whether the DA1 PNs make sexually dimorphic patterns of inhibitory
or excitatory connections in these regions, and if so, to what extent
such dimorphic circuitry might shape distinct male and female
responses to the cVA signal.

METHODS

Genetic manipulations. Targeting of the *Or67d* locus was performed essentially
according to the ends-in method of homologous recombination[8] and is illu-
strated in Supplementary Fig. S1. Each of the two final $Or67d^{GAL4}$ and $Or67d^+$
lines was verified by polymerase chain reaction (PCR) amplification and
sequencing of the entire *GAL4* or *Or67d* coding region. These lines were then
backcrossed into an endogenous *white*+ background for behavioural and
physiological studies.

UAS–Or67d was prepared by PCR amplification of the *Or67d* coding region
from *Drosophila melanogaster* genomic DNA. *UAS–BmOR1* was prepared by
amplifying each of the six coding exons of *BmOR1* from genomic DNA from
the *B. mori* Daizo strain, and then using overlap-extension PCR to combine all
six fragments into a single intronless product. *UAS–HR13* was prepared by
subcloning an *HR13* cDNA.

Electrophysiology. Single-sensillum recordings[28] and electroantennograms[29]
were performed as described previously and in Supplementary Methods. cVA
(99% purity), bombykol (95% purity) and (Z)-11-hexadecenal (93% purity)
were obtained from Pherobank. Ethanol, of the highest purity available, was
obtained from Sigma-Aldrich.

Behavioural assays and histochemistry. Behavioural assays[30], *in situ* hybridiza-
tion[6] and immunohistochemistry[5] were performed as described previously. For
the application of cVA, bombykol or (Z)-11-hexadecenal, the pheromones were
diluted 1:10, 1:100 or 1:50, respectively, in acetone, and 0.2 μl was applied to the

dorsal abdomen of flies lightly anaesthetized with CO_2. Flies were allowed to
recover for 1 h in food vials before behavioural assays were performed. All beha-
vioural assays were scored blind to the genotype. For permutation tests, CIs for
control and experimental animals were pooled and then randomly assorted into
simulated data sets of the same size as in the original data. The *P* value was
determined as the fraction of 10,000 such randomly permuted data sets that
produced a CI difference at least as large as that observed in the actual data.

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