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ARTICLES

Select *Drosophila* glomeruli mediate innate olfactory attraction and aversion

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Fruitflies show robust attraction to food odours, which usually excite several glomeruli. To understand how the representation of such odours leads to behaviour, we used genetic tools to dissect the contribution of each activated glomerulus. Apple cider vinegar triggers robust innate attraction at a relatively low concentration, which activates six glomeruli. By silencing individual glomeruli, here we show that the absence of activity in two glomeruli, DM1 and VA2, markedly reduces attraction. Conversely, when each of these two glomeruli was selectively activated, flies showed as robust an attraction to vinegar as wild-type flies. Notably, a higher concentration of vinegar excites an additional glomerulus and is less attractive to flies. We show that activation of the extra glomerulus is necessary and sufficient to mediate the behavioural switch. Together, these results indicate that individual glomeruli, rather than the entire pattern of active glomeruli, mediate innate behavioural output.

The olfactory systems of phylogenetically diverse species have several common features[1,2], many of which are found in *Drosophila*. For example, each olfactory receptor neuron (ORN) expresses one or a few receptor genes that determine its odorant response profile[3–6], all ORNs expressing the same receptor genes project to the same glomerulus[5–8], and most output neurons send dendrites to a single glomerulus[9–11]. Thus, each glomerulus can be considered a functional unit. A single odorant typically activates several receptor types[12,13], and therefore elicits a distinct spatial pattern of activated glomeruli in the antennal lobe[14–16]. However, the mechanism by which these patterns are actually used to drive behavioural responses remains to be determined. It is possible that the whole pattern is necessary to elicit behavioural output. Alternatively, parts of the pattern, or even individual glomeruli, could be important for olfactory behaviours. This information from the antennal lobe can then be read out by higher brain centres, which probably integrate information from several sensory modalities to generate motor responses.

In contrast to the patterns of several glomeruli activated by most odorants, recent studies have identified two odorants that activate single glomeruli—CO_2 and the male-specific pheromone *cis*-vaccenyl acetate (cVA)—and trigger innate avoidance and female courtship receptivity, respectively[17–21]. By manipulating activity in the cognate receptor neurons, the activation of these single ORN channels was shown to be necessary and sufficient to produce the behaviour, suggesting that these receptors are hardwired to specific behavioural outputs[17,18,22]. These examples could be special cases because these odorants activate only one glomerulus, whereas most odorants excite several glomeruli. Furthermore, food odours contain many individual odorants[23], thus activating multiple glomeruli. Here we set out to study innate attraction to cider vinegar, a complex and highly attractive food odour, and to determine the role of individual glomeruli within the odour-evoked pattern.

Behavioural assay

Fruitflies are highly attracted to vinegar, which is associated with their favourite food source, rotting fruit[24]. To observe this innate attraction behaviour in individual flies, we used a four-field olfactometer design, which was recently applied to *Drosophila*[21]. By recording the outcome of several decisions in each fly, we were able to obtain a robust and reliable score even when using a relatively small number of flies. We measured attraction by observing single flies walking in a four-field arena, in which each quadrant received a separate air stream. When vinegar was added to one of the air streams, the fly spent most of its time in the corresponding quadrant (Fig. 1a). We recorded the location of the fly at 1-s intervals, and calculated a performance index by measuring the time spent in the odour quadrant. A fly that remained in the odour quadrant for the length of the assay scored 100%, whereas a fly that distributed its time equally among the four quadrants scored 0%, and a fly that spent no time in the odour quadrant scored −100%.

Using a concentration of 3 p.p.m. (isobutylene equivalents) vinegar, we saw an average performance index (PI) of 75% (Fig. 1b), which is consistent with previous results[21]. To verify that the behaviour is mediated by the olfactory system, we measured attraction in flies whose antennae had been amputated, and found that they were indifferent to vinegar ($PI = -6.7\%$, $n = 20$). Furthermore, we tested flies with a targeted mutation of *Or83b*. *Or83b* is expressed in 80% of all ORNs[8], and acts together with other olfactory receptors to generate responses to odorants[25–27]. We found that attraction was virtually abolished in *Or83b* mutant flies (Fig. 1b, c), with the distribution of control w^{1118} flies almost entirely separated from the *Or83b* mutant animals (Fig. 1d and Supplementary Fig. 1). In the absence of odours, control and mutant flies are distributed equally in all four quadrants (Supplementary Fig. 2), and *Or83b* mutant flies showed no impairment in CO_2 avoidance ($PI = -87 \pm 9\%$, mean ± s.e.m., $n = 12$), suggesting that their locomotion capability is normal. Thus, attraction in this assay requires ORNs, and the *Or83b* mutation provides a useful tool to link ORN activity with behavioural output.

Visualizing glomerular activity

We next determined which glomeruli are activated by vinegar. We used the genetically encoded calcium sensor G-CaMP to monitor activity in the antennal lobe using two-photon microscopy[15]. We imaged flies bearing the *GH146-Gal4* (also known as *P{GAL4}GH146*) and *UAS-GCaMP* (*P{UAS-G-CaMP}*) transgenes, which have G-CaMP expression in 83 out of 150 projection neurons[9,28,29]. Projection neurons are the output neurons of the antennal lobe; thus their responses to

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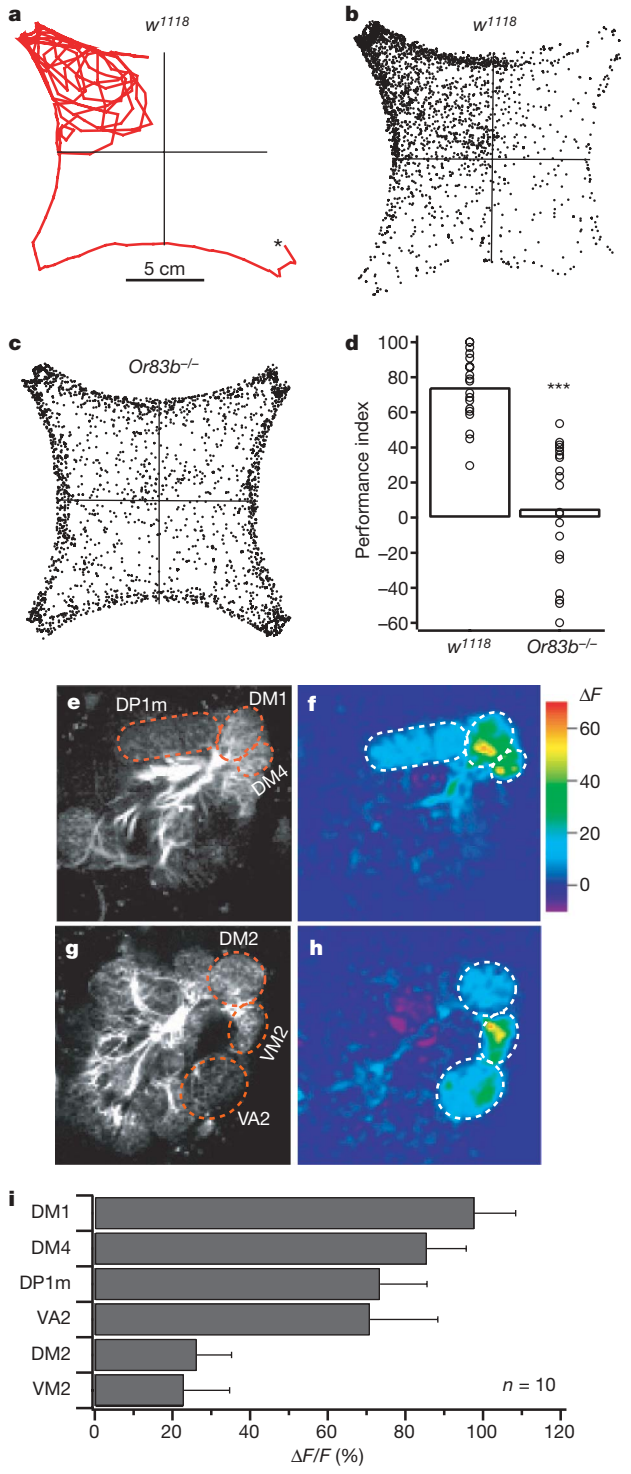

Figure 1 | Flies are robustly attracted to apple cider vinegar, which excites six glomeruli. **a**, Path of a single fly with 3 p.p.m. vinegar in the top left quadrant. **b**, **c**, Density plot of 20 w^{1118} (**b**) and $Or83b^{-/-}$ (**c**) flies. **d**, Performance index of w^{1118} and $Or83b^{-/-}$ flies. ***$P < 0.001$; *t*-test. **e**, **g**, Pre-stimulation images showing glomerular structure. The antennal lobe is roughly 65 μm in diameter. **f**, **h**, Responses to 3 p.p.m. vinegar in flies bearing the *GH146-Gal4* and *UAS-GCaMP* transgenes. **i**, Quantification of the change in fluorescence ($\Delta F/F$) for all six glomeruli over ten flies. Error bars indicate s.e.m.

odorants contain the information that is important for the behavioural response. We also imaged ORNs in flies bearing *Or83b-Gal4* and *UAS-GCaMP*, and found that the projection neuron response pattern is similar to the response of the ORNs (Supplementary Fig. 3), a result that is consistent with previous studies[14,15]. Although excitatory interglomerular connections do exist[30], recent studies have found that ORN input is the main determinant of projection neuron output[31,32].

From projection neuron and ORN imaging, we found that at 3 p.p.m. (the concentration used for the behavioural assay) vinegar elicited a response in 6 out of 34 glomeruli labelled by *GH146-Gal4*. In the most posterior plane of the antennal lobe, three glomeruli—DM1, DM4 and DP1M—responded quite robustly (Fig. 1e, f, i). On a more anterior plane, three more glomeruli—DM2, VA2 and VM2—also responded to varying degrees (Fig. 1g–i). Thus, at this behaviourally relevant concentration, vinegar excites six glomeruli. Although vinegar is a complex stimulus with many volatile components, previous studies have shown that several natural stimuli also elicit a surprisingly sparse response in the rodent olfactory bulb[33].

Two glomeruli relevant for attraction

To determine the role each activated glomerulus has in mediating the attraction to vinegar, we silenced each ORN channel in turn and addressed how that affected the attraction behaviour. Recently, a nearly complete map of ORN to glomerulus targeting was generated[5,6], so we were able to match five of the six activated glomeruli with their corresponding olfactory receptors (the receptor for DP1m remains unknown). *shibire*^{ts} is a temperature-sensitive mutant dynamin, which reversibly prevents neurotransmitter release at the non-permissive temperature (32 °C) by blocking endocytosis[34]. By generating flies bearing the *UAS-shi*^{ts} transgene and selective Or-Gal4 drivers, we should be able to silence five of the six glomeruli. Indeed, silencing individual ORN types resulted in a marked reduction in the activity of their cognate projection neurons, without affecting the non-cognate projection neuron response (Supplementary Fig. 4).

We found that when the Or42b neurons, which innervate the DM1 glomerulus, were silenced the attraction to vinegar was virtually eliminated (Fig. 2b, g). At the non-permissive temperature, the performance index for these flies was −4%, compared to 69% at the permissive temperature. To independently confirm this result, we have measured attraction behaviour in an *Or42b* mutant[35] and found a similar attraction deficit (PI = −18 ± 14%, $n = 18$). Silencing the Or92a neurons, which innervate the VA2 glomerulus, also had a marked effect on the behaviour, with the performance index declining to 50% at 32 °C (Fig. 2c, g). Flies with silenced DM4 and VM2 glomeruli showed normal attraction, as did all the genetic background controls (Fig. 2 and Supplementary Fig. 5). The deficits we observed when DM1 or VA2 were silenced suggest that these receptor neuron channels are required for the innate attraction behaviour, and could function as labelled lines for attraction. However, a model in which DM1 and VA2 are necessary for attraction in conjunction with other ORNs would also be consistent with these data.

We next asked whether individual receptor neuron channels could elicit attraction when activated alone. Because *Or83b* mutant flies lack a vital component of the olfactory signalling pathway and are non-responsive to vinegar, we reasoned that by restoring Or83b expression in specific ORNs, we could force vinegar to selectively activate a single Or83b-expressing glomerulus. Thus, we can determine what type of behavioural output each glomerulus would produce. We used *Or-Gal4* lines to drive expression of a *UAS-Or83b* transgene in *Or83b* mutant flies. Calcium imaging experiments confirmed that the rescue flies had normal olfactory responses in the corresponding ORNs (Supplementary Fig. 6). Notably, when the receptor neurons for either DM1 or VA2 were rescued, attraction was restored to normal levels (Fig. 3). These results indicate that it is activity in DM1 or VA2, and not the pattern of the six glomeruli, which is read out by higher brain centres to signal the attractiveness of the odour. The finding that VA2 activity is sufficient for attraction may seem inconsistent with the fact that DM1-silenced flies show no attraction to vinegar. However, VA2 may be more robustly activated in the rescue flies, because in the silencing experiments, activation of several remaining ORN channels could result in inhibition of VA2. Indeed, a recent study has shown that adding receptor channel inputs

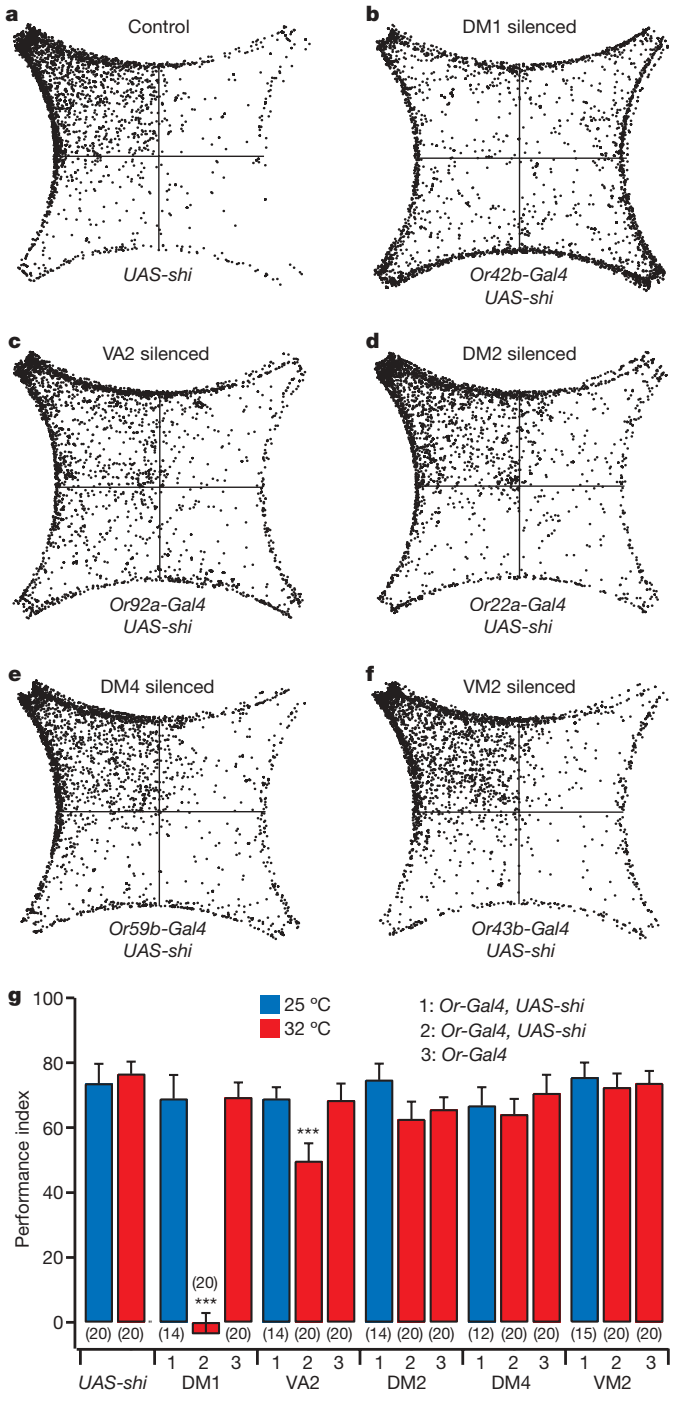

Figure 2 | Silencing DM1 or VA2 reduces attraction to 3 p.p.m. vinegar. **a–f**, Density plots composed of 20 flies each. **g**, Performance indices for flies bearing the *OrX-Gal4* and *UAS-shi*^{ts} transgenes at permissive and non-permissive temperatures. Analysis of variance (ANOVA) followed by Tukey's test was performed on PI values from flies of the experimental group at the permissive and non-permissive temperatures, and the corresponding genetic background controls at the non-permissive temperature. The number of flies is shown in parentheses. ***$P < 0.001$. Error bars indicate s.e.m.

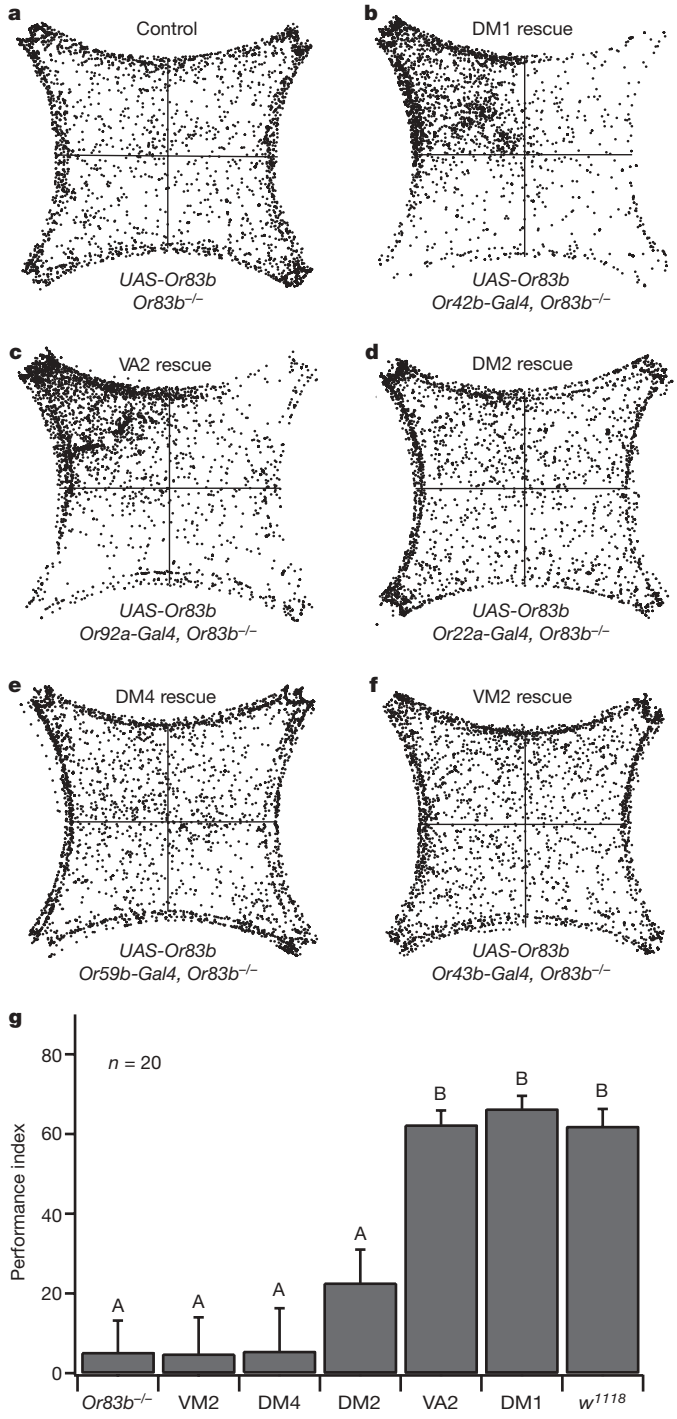

Figure 3 | Restoring *Or83b* in DM1 and VA2 ORNs returns attraction to control levels. **a–f**, Density plots of 20 flies responding to 3 p.p.m. vinegar. **g**, Performance indices of flies in which *Or83b* is selectively restored in individual ORN types. Comparisons between groups were made using ANOVA followed by Tukey's test. Significant differences ($P < 0.05$) are denoted by different letters. Error bars indicate s.e.m.

increases lateral inhibition, leading to a reduction in the projection neuron response[36].

Concentration-dependent behavioural switch

As odour concentration is increased, odours that are attractive at low concentrations often become less attractive or even repulsive[37]. Increasing the odorant concentration often recruits extra receptor neurons, and thus it has been proposed that the change in behaviour is mediated by the addition of these glomeruli to the ensemble of activated glomeruli[38], but this hypothesis has not been tested directly. It is also possible that the increased activation of the glomeruli that were active at the low concentration could mediate the change in behavioural output[39]. Alternatively, the new glomeruli could independently induce aversion.

We first measured the olfactory behaviour over a range of vinegar concentrations. As we increased the concentration, we observed a slight increase in attractiveness at 12 p.p.m. (Fig. 4a, b), but then a marked decrease in attractiveness at 32 p.p.m. with the performance index dropping to 9% (Fig. 4a, c). We wondered whether the change could

be due to the recruitment of additional glomeruli, and used calcium imaging to determine the difference in the pattern of glomeruli activated in response to 12 and 32 p.p.m. vinegar. We observed that the DM5 glomerulus, which showed no response at 12 p.p.m., was strongly activated at 32 p.p.m. (Fig. 4d, bottom row and 4e). All other glomeruli that were activated at 12 p.p.m. (DM1, DM4, DP1m, DM2, DM3, VM2 and VA2) showed small to moderate increases in response to 32 p.p.m. vinegar.

We next addressed whether DM5 could be responsible for the decrease in attraction to vinegar observed at 32 p.p.m. Therefore, we silenced the DM5 glomerulus by expressing *shibire*^{ts} in its cognate ORNs, which express Or85a. At the non-permissive temperature, we found that the performance index for 32 p.p.m. vinegar increased to 87% (Fig. 5a, c). In contrast, silencing DM1 resulted in repulsion towards 32 p.p.m. vinegar (Fig. 5c). Thus, the activation of DM5 is responsible for the decrease in attractiveness towards 32 p.p.m. vinegar.

In light of the above result, it is possible that the activation of DM5 alone mediates aversion, or that the activation of DM5 together with other specific glomeruli could mediate aversion. To distinguish between these models, we forced the stimulus to activate only DM5 by expressing Or83b in Or85a ORNs in the *Or83b* mutant background. We found that these flies were repulsed by 32 p.p.m. vinegar, whereas the *Or83b* mutant flies showed no preference or aversion to the odorant (Fig. 5b, c). In contrast, when DM1 was selectively activated by expression of *Or83b* in Or42b ORNs, flies were attracted to 32 p.p.m. vinegar (Fig. 5c and Supplementary Fig. 8). These findings suggest that the higher concentration of vinegar recruits an extra glomerulus that independently mediates aversion. When wild-type flies are exposed to 32 p.p.m. vinegar, the activation of an aversive glomerulus may counterbalance the activation of the two attractive glomeruli, resulting in a PI near zero.

If attraction and aversion are mediated by the activation of specific glomeruli, other odours that activate these glomeruli should give the same behavioural output. For example, an odour that excites DM1 should be attractive to flies in which DM1 ORNs are selectively activated, whereas an odour that selectively excites DM5 should be repulsive. We have identified an odorant, ethyl butyrate, that excites the DM1, DM2, VM2 and DM5 glomeruli (Supplementary Fig. 7), but has not been detected by gas chromatography in cider vinegar[40]. When we selectively restored function in DM1 ORNs, we found that ethyl butyrate triggered attraction behaviour, with a PI of 65%. Conversely, when we selectively restored function in the DM5 ORNs, the result was an aversion to ethyl butyrate, with a PI of −34% (Fig. 6a). These results indicate that the activation of DM1 or DM5 by any odour should be sufficient for attraction and aversion, respectively.

If specific glomeruli are hardwired to generate attraction and aversion behaviour, activation of ectopically expressed receptors should give a similar behavioural output. We predict that expression of the *Or22a* receptor in Or85a ORNs, which project to DM5, should make these neurons sensitive to lower concentrations of vinegar, and bias the behaviour towards aversion. Indeed, these flies show a marked reduction in the PI value in response to 12 p.p.m. vinegar (Fig. 6b), indicating that it is activity in the DM5 ORNs, rather than activation of a particular receptor, that biases the behaviour towards aversion.

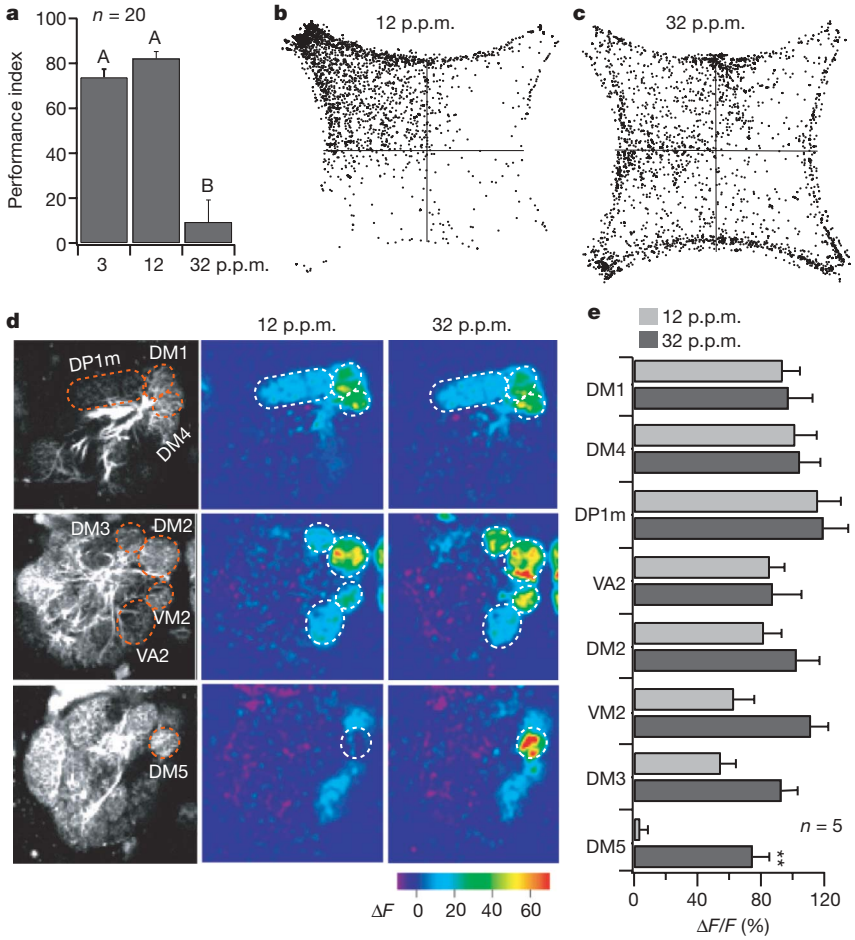

Figure 4 | Vinegar becomes less attractive and activates an additional glomerulus at high concentrations. **a**, Performance indices of w^{1118} flies at various concentrations of vinegar. PI values were compared using ANOVA followed by Tukey's test. Significant differences ($P < 0.05$) are denoted by different letters. **b**, **c**, Density plots of w^{1118} behaviour in response to 12 p.p.m. (**b**) and 32 p.p.m. (**c**) vinegar. **d**, Responses to 12 p.p.m. and 32 p.p.m. vinegar in flies bearing the *GH146-Gal4* and *UAS-GCaMP* transgenes. The antennal lobe is roughly 65 μm in diameter. **e**, The average change in fluorescence ($\Delta F/F$) is shown. $^{**}P < 0.01$; *t*-test. Error bars indicate s.e.m.

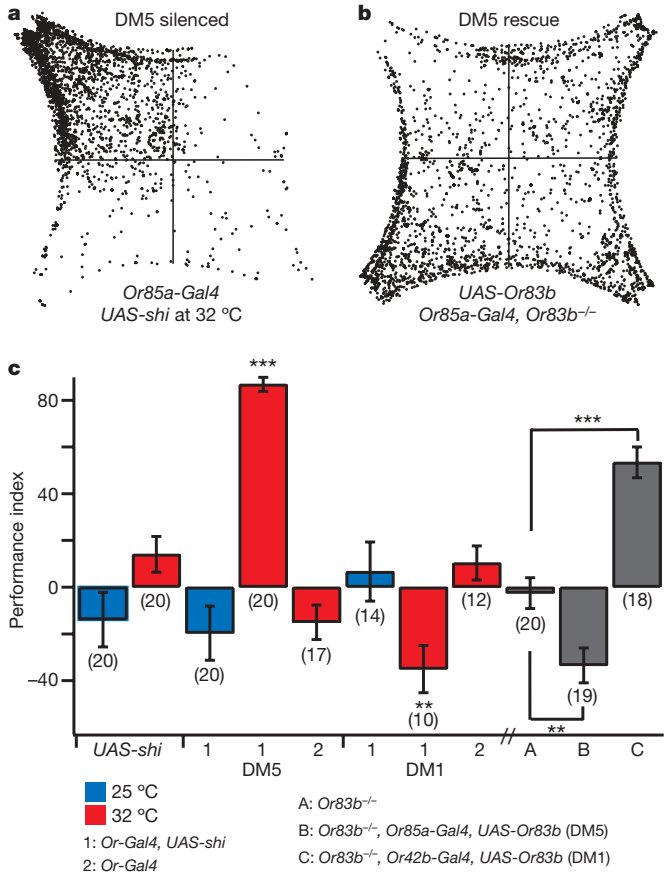

Figure 5 | DM5 mediates the decrease in attraction in response to 32 p.p.m. vinegar. a, Density plot of 20 flies in which the DM5 ORNs are silenced. **b**, Density plot of 20 DM5 rescue flies. **c**, Behavioural responses to 32 p.p.m. vinegar for flies in which DM5 and DM1 are silenced and selectively rescued. For silencing experiments, we performed the same statistical analysis as in Fig. 2. DM5 rescue and DM1 rescue flies were compared to $Or83b^{-/-}$ flies by *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$.

Discussion

Previous studies have shown that in certain cases, olfactory behaviours are elicited by dedicated receptor channels or labelled lines[17,18]. In this study, we demonstrate that innate attraction to a complex food odour is similarly mediated by a few of the activated glomeruli. However, it is possible that other glomeruli not activated by cider vinegar could also mediate innate attraction to other food odours. A recent study of olfactory behaviour in *Drosophila* larvae also addressed how receptor activation leads to behavioural output, and found that the responses of five ORNs to a panel of odorants can be used to generate a model that accounts for 81% of the variation in olfactory behaviour[39]. Selective activation of these ORNs should generate robust innate attraction or aversion. In fact, the Or42a ORN, one of the five critical ORNs, has been shown to be sufficient for attraction behaviour[41].

Furthermore, we show that the decrease in attractiveness in response to a higher concentration of vinegar is due to the activation of an additional glomerulus. It is a common feature of olfactory perception that most odours become less pleasant and eventually repellent as their intensity is increased[37], a phenomenon that has also been observed in *Drosophila*[23,42,43]. The recruitment of further glomeruli has been proposed as a mechanism to mediate this change in behavioural output[38]. A recent paper has suggested that different levels of activation in the same ORNs could generate qualitatively different behavioural responses[39]. Here we found that a glomerulus recruited by a high concentration of vinegar, DM5, has an important role in the behavioural switch. Silencing and selective activation experiments show that DM5 is necessary and sufficient for the behavioural switch.

The present results indicate that certain olfactory receptor neurons in *Drosophila* are genetically hardwired to generate robust innate olfactory attraction or avoidance behaviour, an organizing principle that has been observed in several chemosensory systems[17,18,44,45]. In the fly, projection neurons receive input from ORNs and send axons to the mushroom body and lateral horn. Further studies should shed light on the mechanism by which these centres generate the behaviours we observe.

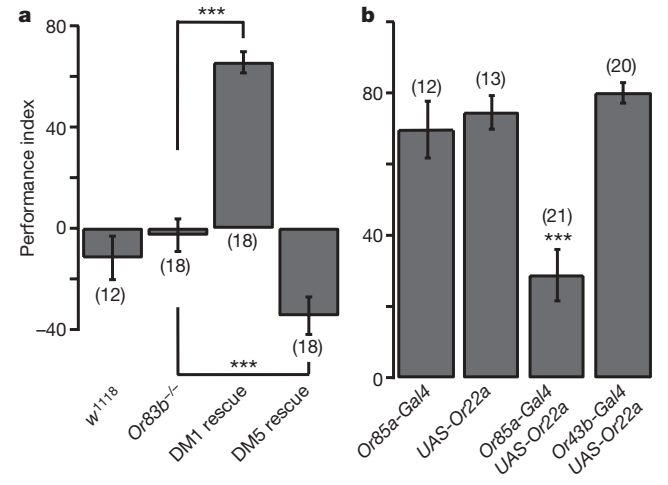

Figure 6 | DM1 and DM5 mediate attraction and aversion in response to ethyl butyrate. a, Performance indices in response to 7 p.p.m. ethyl butyrate for DM1 and DM5 rescue flies. $^{***}P < 0.001$; *t*-test. **b**, Ectopic expression of Or22a in Or85a ORNs reduced attraction to 12 p.p.m. vinegar. PI values were compared using ANOVA followed by Tukey's test. $^{***}P < 0.001$.

METHODS SUMMARY

Behavioural assay. An existing behavioural model was modified to measure the response of single flies to odours[21]. The four-field olfactometer consisted of a four-pointed star-shaped arena. Air flow was maintained by vacuum suction, such that air entered each quadrant at a rate of 200 ml min^{-1}, after passing through a 100-ml bottle. Female flies that had been starved for 50 h were used. After the addition of an odorant to one quadrant, the fly's location was measured once per second. The performance index is defined as $(2p^{1/2} - 1) \times 100\%$, in which p is the fraction of time the fly spends in the odour quadrant between 50 and 250 s after odour application.

Odour stimuli. Odour concentration was measured using a photoionization detector (Rae Systems, MiniRAE 2000) and an air flow of 200 ml min^{-1} through a 100-ml bottle containing the odorant. As the conversion factor to determine the exact concentration of cider vinegar volatiles is unknown, we express the concentration in isobutylene equivalents. The 3 p.p.m. concentration of vinegar corresponds to 40 µl of a 1:2 dilution of apple cider vinegar with water on filter paper. Twelve parts per million is 80 µl vinegar, 32 p.p.m. is 1 ml vinegar, and 7 p.p.m. ethyl butyrate came from 40 µl of a 1:1,000 dilution of ethyl butyrate in mineral oil. The odour source was replenished for each experiment. Odour concentrations stayed constant over the time course of an experiment.

G-CaMP imaging experiments. Calcium imaging was performed as described[15,46] except that the air-flow rate was 200 ml min^{-1}. Odorants were administered from 100-ml bottles as described earlier, and stimuli were given for 2 s.

Transgenic flies. The following fly stocks were used: *Or42b-Gal4*, *Or43b-Gal4*, *Or92a-Gal4*, *Or22a-Gal4* and *Or92a-Gal4* (ref. 5), *Or59b-Gal4* (ref. 6), *UAS-Or22a*, *UAS-Or83b*, *Or83b*-targeted deletion ($Or83b^2$)[26], *UAS-shibire^{ts}* (ref. 34), *UAS-GCaMP* (ref. 15), *GH146-Gal4* (ref. 29), *GH146-LexAGAD*[47] and *LexAop-GCaMP-IRES-GCaMP*[46].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Behavioural assay. An existing behavioural model was modified to measure the response of single flies to odours[21]. As previously described, the four-field olfactometer consisted of a four-pointed star-shaped arena 30 cm across diagonally and 1 cm deep, covered by a glass plate. Air flow was maintained by vacuum suction such that air entered each quadrant at a rate of 200 ml min^{-1}, after passing through a 100-ml bottle. Only female flies were used, and at the time of the assay the flies were 4-days-old and had been starved for 50 h in a vial with a wet kimwipe. After a single fly was introduced into the chamber, its speed was measured for 100 s, and only flies with an average speed between 0.5 and 1.0 cm s^{-1} were used. At the start of the assay, one of the empty 100-ml bottles was replaced with an odour-containing bottle. The fly's location was measured once per second using a Logitech quickcam and Labview software (National Instruments). The chamber was illuminated by a panel of light-emitting diodes (660 nm). Light reflected from the glass plate was eliminated by polarizing optics. The performance index is defined as $(2p^{1/2} - 1) \times 100\%$, in which p is the fraction of time the fly spends in the odour quadrant during the period between 50 and 250 s after odour application. Thus, if the fly is in the odour quadrant for the entire time window, $P = 1$ and the performance index is 100%, whereas if the fly avoids the odour quadrant entirely, $P = 0$ and the performance index will be -100%. Except for the *shibire*^{ts} non-permissive temperature experiments (which were performed at 32 °C), all behavioural experiments were performed at 25 °C and 70% humidity. Data were analysed using Igor Pro (Wavemetrics) and a custom macro. The Jarqe-Bera test was used to verify that the data were normally distributed. Density plots show data collected between 50 and 250 s for 20 flies. Each dot indicates one fly spending one second at that location. Odour application was alternated among the four quadrants, and the density plots were created by rotating the positional data so that the odour quadrant becomes the top left quadrant.