LETTER

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A subset of dopamine neurons signals reward for odour memory in *Drosophila*

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Animals approach stimuli that predict a pleasant outcome[1]. After the paired presentation of an odour and a reward, *Drosophila melanogaster* can develop a conditioned approach towards that odour[2,3]. Despite recent advances in understanding the neural circuits for associative memory and appetitive motivation[4], the cellular mechanisms for reward processing in the fly brain are unknown. Here we show that a group of dopamine neurons in the protocerebral anterior medial (PAM) cluster signals sugar reward by transient activation and inactivation of target neurons in intact behaving flies. These dopamine neurons are selectively required for the reinforcing property of, but not a reflexive response to, the sugar stimulus. *In vivo* calcium imaging revealed that these neurons are activated by sugar ingestion and the activation is increased on starvation. The output sites of the PAM neurons are mainly localized to the medial lobes of the mushroom bodies (MBs), where appetitive olfactory associative memory is formed[5,6]. We therefore propose that the PAM cluster neurons endow a positive predictive value to the odour in the MBs. Dopamine in insects is known to mediate aversive reinforcement signals[5,7–11]. Our results highlight the cellular specificity underlying the various roles of dopamine and the importance of spatially segregated local circuits within the MBs.

Reward is positive reinforcement and drives the formation of appetitive associative memory. In insects, octopamine was shown to be involved in reward[5,11–13] (see also ref. 14), whereas specific sets of dopamine neurons were identified to mediate aversive reinforcement[8,10]. Recent studies in *Drosophila* suggest that dopamine in the MBs is involved in appetitive odour memory[15–17], but the specific role of dopamine and the underlying circuit are unclear.

To examine whether the activation of dopamine neurons can substitute for a rewarding stimulus in the formation of an appetitive odour memory, we targeted the expression of a thermosensitive cation channel dTRPA1 (ref. 18) to different, but overlapping sets of, dopamine neurons by using two GAL4 drivers (Fig. 1a, b), *TH-GAL4* and *DDC-GAL4*. Activation of dTRPA1 in *DDC-GAL4* flies during the presentation of an odour (Fig. 1c)[10] resulted in a weak appetitive memory, but robust aversive memory in *TH-GAL4* flies (Fig. 1d)[8,10,13]. The same activation on starvation induced a much greater appetitive memory in *DDC-GAL4/UAS-dTrpA1* flies (Fig. 1d). Activation of dTRPA1 that was not paired with an odour did not induce appetitive memory (Supplementary Fig. 1a, b). Thermo-activation with the driver *HL9-GAL4*, a variant of *DDC-GAL4* (ref. 8), induced similar appetitive memory (Supplementary Fig. 1e, f). Furthermore, *TH-GAL80* (ref. 19) did not significantly suppress induced memory in *DDC-GAL4/UAS-dTrpA1* flies (Supplementary Fig. 1g, h), suggesting that the neurons labelled in *DDC-GAL4* but not in *TH-GAL4* flies are responsible for signalling reward. As in appetitive memory with sugar, a single thermo-activation using *DDC-GAL4* induced persistent appetitive memory, which lasted for up to 24 h (Fig. 1e). We therefore conclude that *DDC-GAL4* labels neurons signalling reward.

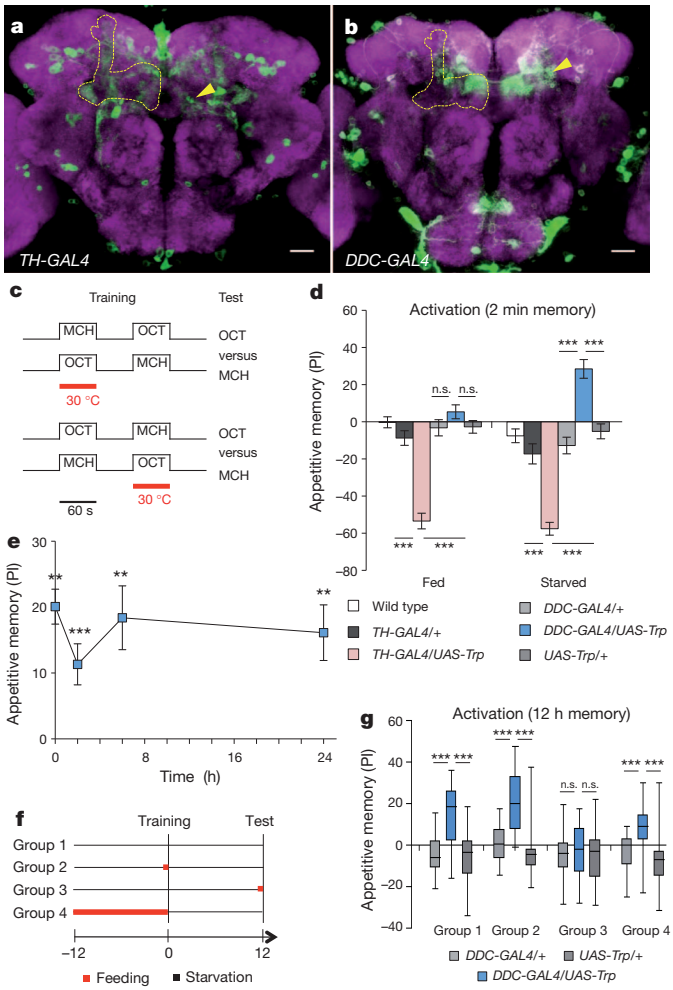

Figure 1 | Thermo-activation with *DDC-GAL4* induces appetitive memory. **a**, **b**, Expression patterns of *TH-GAL4* (**a**) and *DDC-GAL4* (**b**) in the brain with a neuropile counterstaining (magenta). Outline, MB; arrowheads, PAM neurons. Scale bars, 20 μm. **c**, Protocol for dTRPA1(Trp)-mediated reinforcement substitution. MCH, 4-methylcyclohexanol; OCT, octan-3-ol. **d**, Thermo-activation with *DDC-GAL4* and *TH-GAL4* with or without starvation. PI, performance index. $n = 16$. **e**, Retention of induced memory. $n = 10$–27. **f**, Protocol for feeding before the training or the test of 12-h memory. Flies were satiated with a short feed (30 min) or continuous feeding. **g**, Test of 12-h memory by thermo-activation with *DDC-GAL4*. $n = 16$. Midline, box boundaries and whiskers are median, quartiles and 10th and 90th centiles, respectively. Results in **d** and **e** are means ± s.e.m. Two asterisks, $P < 0.01$; three asterisks, $P < 0.001$; n.s., not significant.

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To address when starvation is required for the dTRPA1-induced memory performance, we examined the effect of changing motivational states before either training or test by a brief feeding (Fig. 1f). Appetitive memory was induced on thermo-activation despite feeding before training (groups 2 and 4 in Fig. 1f, g). If applied before the test, feeding fully suppressed the behavioural expression of 12-h memories (group 3 in Fig. 1f, g). These results suggest that starvation is required for the retrieval, but not the acquisition, of appetitive memory induced by thermo-activation.

To explore the role of *DDC-GAL4*-labelled neurons in mediating the sugar reward, we blocked the output of these neurons using Shi^{ts1}, which inhibits neuronal output at high temperature[20]. Unlike another known type of dopamine neurons that restricts appetitive memory retrieval[15], blocking the *DDC-GAL4*-labelled neurons did not release memory expression in fed flies (Supplementary Fig. 2). Instead, the blockade impaired the acquisition, but not the expression, of the sugar-induced memory (Supplementary Fig. 3a, b). Neither memory performance at the permissive temperature nor sugar preference at the restrictive temperature was impaired (Supplementary Fig. 3c, d).

Next we sought to identify the cells responsible for reward processing. *DDC-GAL4* heavily labels the PAM cluster neurons, whereas this cluster is sparsely labelled by *TH-GAL4* (Fig. 1a, b)[8]. For selective manipulation of the PAM cluster neurons, we screened a collection of GAL4 driver lines[21] and identified *R58E02-GAL4*. This driver strongly labels the PAM cluster neurons and glial cells in the optic lobes with little expression elsewhere (Fig. 2a, Supplementary Fig. 4a and Supplementary Movie 1). Arbours of the PAM neurons in the MBs are largely localized to the medial lobes (Fig. 2a and Supplementary Fig. 3b). The enhancer of *R58E02-GAL4* is derived from the first intron of the *Drosophila* dopamine transporter gene. Consistently, the PAM neurons labelled in *R58E02-GAL4* as well as in *DDC-GAL4* flies are dopamine immunoreactive (Fig. 2b–e) with no detectable serotonin labelling (Supplementary Fig. 5). Thermo-activation of the PAM neurons with the use of *R58E02-GAL4* induced robust appetitive odour memory in starved flies

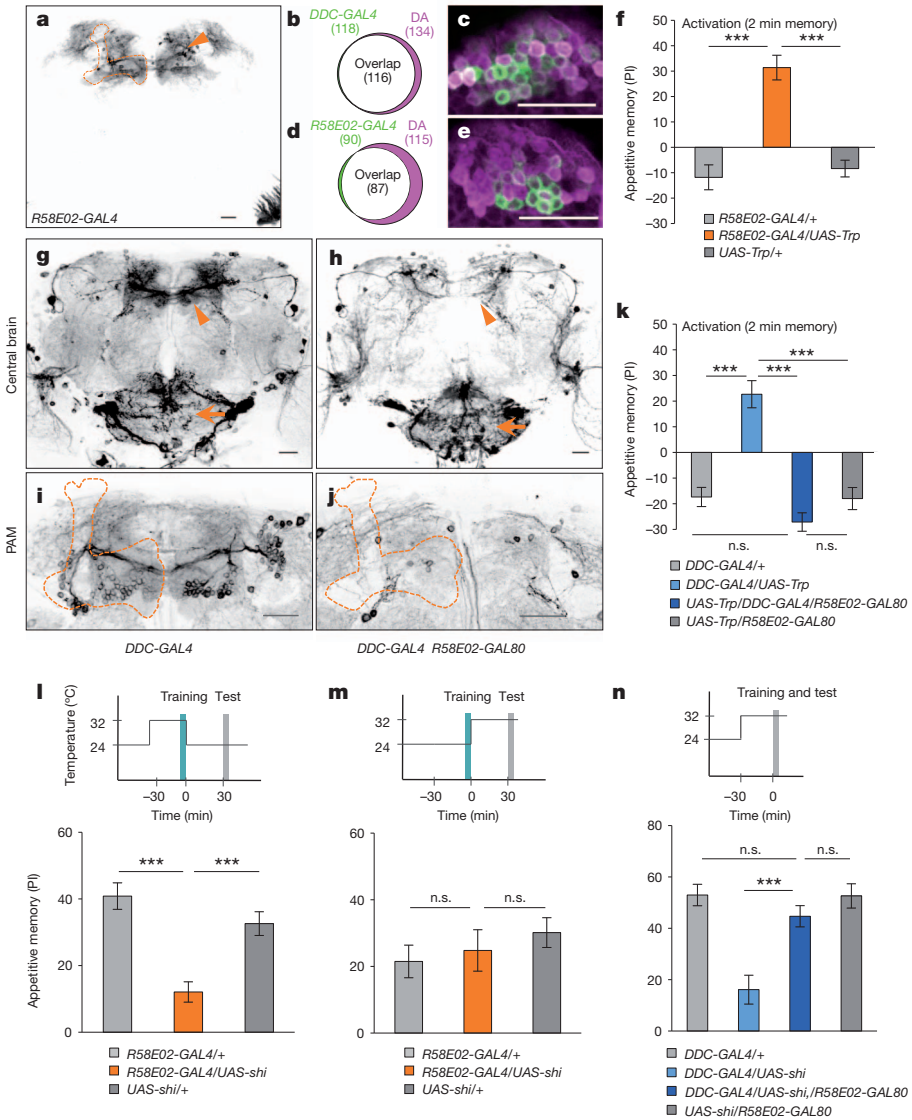

Figure 2 | The PAM cluster neurons signal reward for olfactory memory. **a**, Expression pattern of *R58E02-GAL4* in the central brain revealed with *UAS-mCD8::GFP*. **b**–**e**, Co-localization of dopamine (magenta) and GAL4-expressing cells (green) in the PAM cluster of *DDC-GAL4* (**b**, **c**, $n = 6$) and *R58E02-GAL4* (**d**, **e**, $n = 4$). **f**, Thermo-activation with *R58E02-GAL4/UAS-dTrpA1* induces significant appetitive memory. $n = 16$. **g**–**j**, *R58E02-GAL80* silences transgene expression in most PAM neurons of *DDC-GAL4* (arrowheads in **g** and **h**) without greatly affecting other neurons (such as the suboesophageal ganglion (arrows)). **k**, *R58E02-GAL80* suppresses the induction of appetitive memory by *DDC-GAL4/UAS-dTrpA1*. $n = 10$. **l**, **m**, Blockade of the PAM neurons in *R58E02-GAL4/UAS-*shi^{ts1} during memory acquisition (**l**) or applied after training (**m**). **n**, *R58E02-GAL80* rescues the memory impairment in *DDC-GAL4/UAS-*shi^{ts1}, $n = 14$–20. Upper panels in **l**–**n**, protocols; lower panels, sugar conditioning. Results with error bars are means ± s.e.m. Three asterisks, $P < 0.001$; n.s., not significant. Scale bars, 20 μm.

(Fig. 2f), whereas the activation itself did not cause any obvious reflexive appetitive behaviour (such as proboscis extension; data not shown).

DDC-GAL4 labels many neurons outside the PAM cluster, including those projecting to the suboesophageal ganglion, where sweet taste neurons terminate (Fig. 1b). To address the contribution of the non-PAM cells in *DDC-GAL4* flies, we generated *R58E02-GAL80*, a GAL80 line using the same enhancer integrated at the same genomic location as in *R58E02-GAL4*. Combination of *R58E02-GAL80* with *DDC-GAL4* suppressed transgene expression in most PAM neurons in *DDC-GAL4* flies (Fig. 2g–j and Supplementary Movies 2 and 3; see also Supplementary Fig. 6e). Thermo-activation with *DDC-GAL4/R58E02-GAL80* did not induce appetitive memory (Fig. 3k), demonstrating the importance of PAM neurons in reward signalling.

A transient Shi^{ts1} block of the PAM neurons by *R58E02-GAL4* impaired the acquisition, but not the expression, of sugar-induced memory (Fig. 2l, m). Furthermore, blocking the PAM neurons did not impair the reflexive choice of sugar (Supplementary Fig. 4c). Consistently, *R58E02-GAL80* rescued the memory impairment of *DDC-GAL4/UAS-shi^{ts1}* flies (Fig. 2n). Thus, the PAM neurons are necessary and sufficient for signalling the sugar reward.

Expression of a presynaptic marker using *R58E02-GAL4* demonstrated that input and output sites of the PAM neurons are highly segregated, with presynaptic terminals localized predominantly in the MBs (Fig. 3a–c). To address whether the signal from the PAM neurons is mediated by dopamine receptors, we activated these neurons in the background of *dumb^2*, a mutant for the *dDA1* gene (also known as *DopR*), which encodes a D1-type dopamine receptor. We first confirmed the previously reported role of dDA1 in the Kenyon cells of the MBs for sugar-induced appetitive memory (Fig. 3d)[16]. Because we wished to use a GAL4 driver to express dDA1 in Kenyon cells simultaneously with dTRPA1 in the PAM neurons, we generated a LexA driver *R58E02-LexA::p65*. It recapitulated the expression pattern in *R58E02-GAL4* and was able to induce appetitive memory using *LexAop2-dTrpA1* (Fig. 3e and Supplementary Fig. 6c, d). Activation of the PAM neurons failed to induce marked appetitive memory in flies lacking dDA1 (Fig. 3f). Driving wild-type dDA1 expression in α/β and γ Kenyon cells by using the driver *MB247-GAL4* restored appetitive memory in *R58E02-LexA/LexAop2-dTrpA1* flies (Fig. 3f). These results indicate the importance of dopamine signalling in the MBs for reward processing, but do not exclude a role for other possible co-transmitters released by the PAM neurons.

We previously identified that MB-M3 neurons in the PAM cluster are important for aversive memory formation[10]. We labelled both MB-M3 and the reward-signalling PAM neurons in the same brain and found no overlap (Fig. 3g–i, Supplementary Fig. 7 and Supplementary Movie 4). This highlights the functional heterogeneity of individual cell types in the PAM cluster.

Similarly, we made different populations of dopamine neurons that signal appetitive and aversive reinforcement visible by using *R58E02-LexA* and *TH-GAL4*, respectively, and examined the distribution of their projections in the MBs. The terminals of the PAM and protocerebral posterior lateral (PPL)1 clusters are largely non-overlapping in the MBs and together cover the entire lobes (Fig. 3j–l and Supplementary Movie 5) despite the simultaneous expression of *R58E02-LexA* and *TH-GAL4* in a few PAM cluster neurons (Supplementary Fig. 8a–d). Thus, axonal compartments of Kenyon cells are targeted by functionally different dopamine neurons.

Given the importance of octopamine signalling in reward processing[5,11–13], we activated the PAM cluster neurons in *TβH* mutants, which lack octopamine[22]. We found no marked effect of *TβH* on appetitive memory induced by activation of the PAM neurons (Fig. 4a), indicating that the PAM neurons act in parallel with or downstream of, but not upstream of, octopamine signalling. Consistently, double labelling of the octopamine and PAM cluster dopamine neurons revealed potential direct contacts of these arbours in the spur of the γ lobe and protocerebral regions (Fig. 4b–d), where

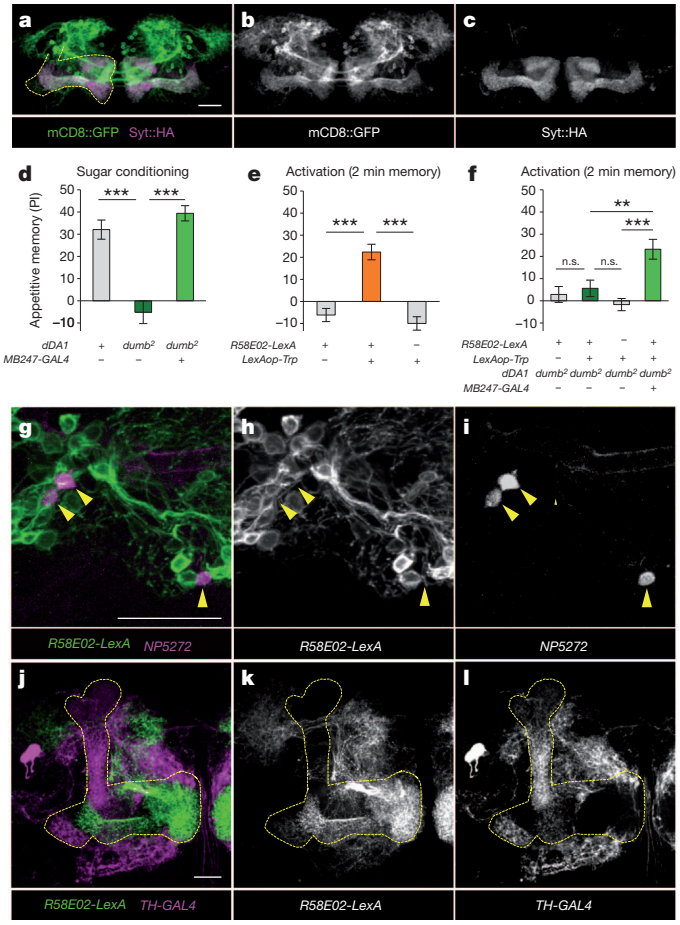

Figure 3 | The PAM neurons convey the reward signal to the MB. **a–c**, Presynaptic terminals (magenta) of the PAM neurons (green) in *R58E02-GAL4* are localized to the MB (outline). **d**, The defect in sugar-induced memory in *dumb^2*, a piggyBac insertion allowing GAL4-mediated dDA1 expression[16], is rescued by *MB247-GAL4*. $n = 16$. **e**, Appetitive memory induced by thermo-activation in *R58E02-LexA/LexAop2-dTrpA1*. $n = 16$. **f**, Thermo-activation of the PAM neurons in *dumb^2* induces memory by rescuing dDA1 in the MB. $n = 14–22$. **g–i**, No detectable overlap of *R58E02-LexA*-labelled neurons expressing mCD8::GFP (green) and MB-M3 neurons (magenta; arrowheads) visualized with mCD8::RFP. See also Supplementary Fig. 7. **j–l**, Differential labelling of *R58E02-LexA* (green) and *TH-GAL4* (magenta) in the MB lobe region. Results with error bars are means ± s.e.m. Two asterisks, $P < 0.01$; three asterisks, $P < 0.001$; n.s., not significant. Scale bars, 20 μm.

the putative input and output sites of the PAM and octopamine neurons, respectively, are located (Fig. 3a–c)[23]. This suggests that octopamine may regulate reward processing by directly modulating the activity of the PAM cluster neurons.

To test whether the PAM neurons respond to the sugar reward, we performed *in vivo* calcium imaging in starved flies expressing the fluorescent calcium reporter GCaMP3. We devised a gustatory stimulation protocol with the unrestrained proboscis that enabled confocal imaging of the PAM terminals in the MBs (Supplementary Fig. 9). Sugar ingestion caused stronger calcium responses than water or a bitter caffeine solution (Fig. 4e–g). We found that the calcium response of the PAM neurons on stimulation with sugar was greatly reduced when flies were fed (Fig. 4h). Flies can sense sweet taste with their tarsi, but stimulating tarsi with sugar barely activated the PAM neurons, suggesting that sweet substances need to be ingested to trigger the reward signal (Fig. 4h).

Our data suggest the existence of a reward circuit in which the PAM neurons integrate gustatory reward and other relevant regulatory inputs, and then convey the summed positive value signal to specific subdomains of the MBs (Fig. 4i). The MB lobes can be anatomically

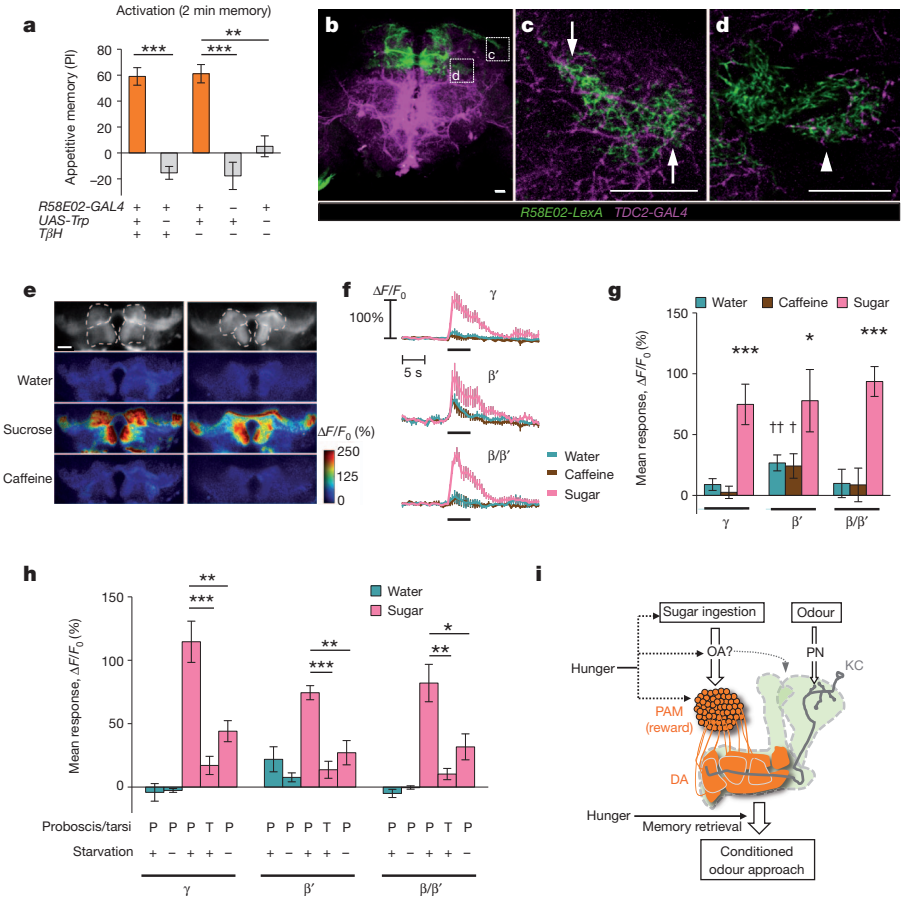

Figure 4 | The PAM neurons integrate reward and relevant signals. **a**, Thermo-activation of the PAM neurons induces appetitive memory without octopamine. $n = 16–22$. **b–d**, Octopamine and PAM neurons contact each other (arrows) in a protocerebral region (**c**) and the spur of the γ lobe (**d**). **e–g**, The PAM neurons respond selectively to the sugar reward. **e**, Representative images (anterior down) and calcium responses of the PAM neurons at different levels of the MB. **f**, Time course of responses. $n = 10$. Black bar: stimulus application. **g**, Average response to different gustatory substances. Terminals in the β′ lobe responded significantly to water and caffeine (dagger, $P < 0.05$; two daggers, $P < 0.01$ in one-sample *t*-test from zero). **h**, Response of the PAM neurons without starvation or with tarsal stimulation only. $n > 7$. **i**, Model of a reward circuit in the fly brain. Dashed arrows indicate hypothetical pathways. The reward value integrated by the PAM neurons is signalled by dopamine (DA) to the MB (green shading), thus they may drive associative plasticity in odour-representing Kenyon cells (KC, grey). OA, octopamine; PN, projection neurons. Results with error bars are means ± s.e.m. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. Scale bars, 20 μm.

divided into 35 subdomains that are defined by specific combinations of intrinsic and extrinsic neurons[24]. Distinct sets of dopamine neurons may provide functionally independent local circuits within the MBs, potentially allowing appetitive and aversive modulation of the same odour. The PAM neurons may drive positive associative modulation of concomitant olfactory signals of the Kenyon cells (Fig. 4i). The dual processing of appetitive and aversive stimuli may be a conserved function of dopamine, highlighting the physiological pleiotropy of a neurotransmitter[4,25,26].

METHODS SUMMARY

The genomic fragment *R58E02* was cloned into previously described vectors[21,27]. Sugar conditioning and thermo-activation with two odours (4-methylcyclohexanol and octan-3-ol) and fluorescent immunohistochemistry were performed as described previously[10]. Sugar preference was measured with a previously described setup[28], with slight modification. Most of the groups tested did not violate the assumption of the normal distribution and the homogeneity of variance. Performance indices were therefore subjected to parametric statistics. For data that significantly differed from the normal distribution (Fig. 1g), non-parametric statistics were applied. For *in vivo* calcium imaging, female flies expressing GCaMP3 (ref. 29) with *R58E02-GAL4* were singly prepared as described[30], with slight modifications. A droplet of 8 μl of mineral water, 1 M sucrose or 0.1 M caffeine solution in mineral water was delivered on a plastic plate controlled by a micromanipulator (Supplementary Fig. 9).

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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Author Contributions C.L., N.Y., Y.A. and H.T. designed and C.L. and N.Y. performed all the behavioural experiments in this study. P.Y.P., T.P. and H.T. designed *in vivo* imaging experiments, and P.Y.P. and T.P. devised a new gustatory stimulation method. P.Y.P. performed imaging experiments and analysed the data. B.D.P. and G.M.R. designed and generated the new transgenic flies (GAL4, GAL80, LexA and *LexAop2-dTrpA1* lines). Y.A. and H.T. identified *R58E02* by using a database of GAL4 expression patterns created by G.M.R. and the Janelia Farm Fly Light Project Team. A.B.F. and I.S. performed immunohistochemistry, and C.L., A.B.F. and H.T. analysed the microscopic data. C.L. and H.T. made the figures and wrote the paper with the help of all the other authors.

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METHODS

Flies. The 1.2 kb DNA enhancer fragment in *R58E02* was amplified by PCR (primer sequences 5′-cgaaggcgcaacagctccgattttg-3′ and 5′-ccttgacccaaaatgtggag atccc-3′) and originates from the *dopamine transporter* locus[31]. The fragment was cloned into the transformation vectors pBPGUw (ref. 21), pBPGAL80Uw-6 (ref. 27) and pBPLexA::p65Uw (ref. 27). *R58E02-GAL4* and *R58E02-GAL80* were integrated into *attP2* (3L), and *R58E02-LexA::p65* was integrated into *attP40* (2L) using PhiC31 integrase. *pJFRC26-13XLexAop2-IVS-dTrpA1-WPRE* and *pJFRC15-13XLexAop2-mCD8::GFP* were cloned using the modified LexA vector and integrated into *VK00005* (3L) and *su(Hw)attP8* (X), respectively, using PhiC31-mediated site-specific recombination.

Behavioural experiments (except Fig. 4d) used F_1 progenies of crosses between females of *w;UAS-dTrpA1* (ref. 18), *w;;UAS-shi^{ts1}* (one copy of *UAS-shi* from the Preat laboratory)[20], *w;;LexAop2-dTrpA1*, *w;UAS-dTrpA1;R58E02-GAL80*, *w;;UAS-shi^{ts1} R58E02-GAL80*, *w;UAS-dTrpA1;dumb^2*, *TβH^{M18}/FM7* (ref. 22), *TβH ^{M18}/FM7;UAS-dTrpA1*, *w;R58E02-LexA;dumb^2*, *w;R58E02-LexA;dumb^2 MB247-GAL4*, *w;;dumb^2* or *w* females and males of *w;;TH-GAL4* (ref. 32), *w;;DDC-GAL4* (ref. 33), *w;;HL9-GAL4* (ref. 8), *w;;R58E02-GAL4*, *w;R58E02-LexA::p65*, *w;;dumb^2*, *w;;LexAop-dTrpA1 dumb^2* or *w* males, raised 60% relative humidity with a 14 h/10 h light/dark cycle. Crosses with *UAS-dTrpA1* (*LexAop-dTrpA1*) and *UAS-shi^{ts1}* flies were raised at 23 and 17 °C, respectively. At experimentation, flies were aged 3–14 days after eclosion and starved according to mortality rate. For the experiments with *TβH*, the performance of the male progeny was used for calculating PI. For immunohistochemistry, female reporter strains *y w UAS-mCD8::GFP*[34], *w mCD8::GFP;TH-GAL80;mCD8::GFP*, *w UAS-Syt::HA;UAS-mCD8::GFP*[35], *w;UAS-mCD8::GFP;R58E02-GAL80* or *w pJFRC21-10XUAS-IVS-mCD8::RFP* (*attp18*) *pJFRC15-13XLexAop2-mCD8::GFP* (*su(Hw)attP8*) were crossed to male GAL4 drivers, *w;;TH-GAL4*, *w;;DDC-GAL4*, *w;;HL9-GAL4*, *w;;R58E02-GAL4*, *w;R58E02-LexA::p65*, *w;NP5272* or *w;TDC2-GAL4* (ref. 36). Flies used for whole-mount immunohistochemistry were aged to 5–10 days after eclosion.

Behavioural assays. The conditioning protocol was as described previously[10]. Except where noted, behavioural experiments were performed in dim red light for training phases and in complete darkness for tests. A group of about 50 flies in a training tube alternately received octan-3-ol (OCT; Merck) and 4-methylcyclohexanol (MCH; Sigma-Aldrich) for 1 min in a constant air stream (Fig. 1c and Supplementary Fig. 1c). OCT and MCH were diluted 1:10 in paraffin oil (Sigma-Aldrich) and presented in a cup with a diameter of 3 and 5 mm, respectively. For sugar-induced memory, filter paper was soaked in 2 M sucrose solution, dried, and presented together with one of the odours (Supplementary Fig. 1c). Alternately, the control odour was paired with dried filter paper (Supplementary Fig. 1c). For thermo-activation with dTRPA1, flies were trained by being transferred from a background temperature of 24 °C to a pre-warmed tube in a climate box (30 °C) and presented with the trained odorant (Fig. 1c)[10]. Temperature was measured with a VC-960 digital multimeter (Voltcraft). For unpaired thermo-activation with dTRPA1, flies were exposed to the pre-warmed tube either between or after the two odorants (Supplementary Fig. 1a). The restrictive temperature for the experiments with *UAS-shi^{ts1}* was 32 °C. For memory retention, trained flies were kept in a vial with moistened filter paper. After a given retention time, the trained flies were allowed to choose between MCH and OCT for 2 min in a T-maze. A learning index was then calculated by taking the mean preference of the two reciprocally trained groups[2]. Half of the trained groups received reinforcement together with the first presented odour and the other half with the second odour to cancel the effect of the order of reinforcement. To control feeding motivation, flies were kept in a food vial for 30 min before training or test. For 24-h memory, flies were fed for 1 h after training.

For testing sugar preference, flies were placed in a cylindrical, infrared-illuminated arena, and given a choice between two halves of dried filter paper, one of which was soaked in 2 M sucrose[28]. The arena was video-recorded from above with a CMOS video camera (Firefly MV; Point Grey) at one frame per second, and the first 120 s were analysed. The performance index represents the difference in the number of flies in the two halves, divided by the total number of flies.

Statistics. Statistical analyses were performed with Prism5 software (GraphPad). Most tested groups did not violate the assumption of normal distribution and homogeneity of variance. All the data except the experiment in Fig. 1g were therefore analysed with parametric statistics: one-sample *t*-test or one-way analysis of variance followed by the planned pairwise multiple comparisons (Bonferroni). Because the data in Fig. 1g were significantly different from the normal distribution, non-parametric statistics (that is, Kruskal–Wallis test followed by Dunn's multiple pairwise comparison test) were applied. The significance level of statistical tests was set to 0.05.

Histochemistry. Immunolabelling was performed with a standard protocol[10], with the exception of a fixation (0.6% glutaraldehyde in PBS for 30 min) and the following glutaraldehyde protocol for dopamine[23]. Fluorescence of mCD8::GFP and mCD8::RFP was detected without immunohistochemistry. Frontal optical sections of whole-mount brains were sampled with a confocal microscope (Olympus FV1000). To evaluate the effect of *R58E02-GAL80*, brains to be compared were scanned with comparable intensity and offset. Confocal stacks were analysed with Image-J (National Institutes of Health).

***In vivo* calcium imaging.** Female flies of the genotype *w^{1118};UAS-GCaMP3/+;R58E02-GAL4/+* aged 1–2 days after eclosion were prepared essentially as described[30], with the following modifications: the fly was briefly anaesthetized with CO_2; the proboscis was left free, to enable ingestion (except in tarsal stimulation experiments, in which the proboscis was occluded with the glue); sucrose was replaced with ribose in the solution for bathing the head capsule during surgery[37]. At the end of the surgery, the brain was covered with a drop of the same solution supplemented with 1% agarose, so that the brain was embedded in a gel to limit motion artefacts. Time courses of fluorescence changes were recorded in a transverse section of the brain showing the projections of PAM neurons in the different levels of both MB medial lobes at a rate of two images per second. The terminals on the γ and β′ lobes were clearly discernible, whereas the slice of the β terminals might contain some β′ terminals. We therefore here use the label 'β/β′ terminals' collectively. Three types of stimulation were performed: a droplet of 8 µl of mineral water, 1 M sucrose or 0.1 M caffeine solution in mineral water deposited on a plastic plate was brought within reachable distance of the fly for 5 s with the use of a micromanipulator. These concentrations were chosen because they elicit saturating responses in taste neurons in the suboesophageal ganglion (SOG)[37]. The solutions were delivered in random order, and when sucrose was not the last solution tested, an additional sucrose response was measured at the end of the experiment as a positive control, which was not included in analyses.

Image analysis was performed essentially as described[30]. For each region of interest, the baseline (F_0) was estimated as the mean fluorescence over the 10 s preceding the stimulus, and the mean response was calculated as the average of $\Delta F/F_0$ during the period when the drop of solution was available. Responses from both hemispheres were averaged to yield the mean response of each fly, but for time courses (Fig. 4f) only one hemisphere was considered for each animal (six right, four left).

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