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Layered reward signaling through octopamine and dopamine in *Drosophila*

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Abstract

Dopamine (DA) is synonymous with reward and motivation in mammals[1,2]. However, only recently has dopamine been linked to motivated behavior and rewarding reinforcement in fruit flies[3,4]. Instead octopamine (OA) has historically been considered the signal for reward in insects[5-7]. Here we show using temporal control of neural function in *Drosophila* that only short-term appetitive memory is reinforced by OA. Moreover, OA-dependent memory formation requires signaling through DA neurons. Part of the OA signal requires the α-adrenergic like OAMB receptor in an identified subset of mushroom body (MB)-targeted DA neurons. OA triggers an increase in intracellular calcium in these DA neurons and their direct activation can substitute for sugar to form appetitive memory, even in flies lacking OA. Analysis of the β-adrenergic like Octβ2R receptor reveals that OA-dependent reinforcement also requires an interaction with DA neurons that control appetitive motivation. These data suggest that sweet taste engages a distributed OA signal that reinforces memory through discrete subsets of MB-targeted DA neurons. In addition, they reconcile prior findings with OA and DA and suggest that reinforcement systems in flies are more similar to mammals than previously envisaged.

Fruit fly OA is synthesized from tyrosine via two-steps catalyzed by tyrosine decarboxylase (TDC) and tyramine β-hydroxylase (Tβh)[8,9]. The *Tdc2* gene encodes the neuronal TDC and a *Tdc2*-GAL4 can be used to label and manipulate many of the OA neurons[8]. Although *Tβh* mutant *Drosophila* that lack octopamine cannot form appetitive memory[7], the precise role of OA release is currently unknown.

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Author Contributions S.W., C.J.B. and W.H. conceived this project and designed all experiments. C.J.B. and W.H. constructed fly strains, C.J.B. performed most behavior, with some assistance from E.P. Anatomical data were produced by W.H and C.B. Live imaging was performed by D.O. and W.H. The study was initiated by the experiments of M.J.K. G.D. constructed lexAop-*dTrpA1*. The 0104-, 0273-, 0665-, and 0891-GAL4 flies were generated and initially characterized by D.G. and M.S. S.C. constructed and initially characterized *Tdc2*-LexA flies. S.W., W.H. and C.B. wrote the manuscript.

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We tested whether OA neurons were required for appetitive olfactory conditioning with sucrose reinforcement by blocking them throughout the experiment using *Tdc2*-GAL4 driven uas-*shibire*^{ts1} (uas-*shi*^{ts1})[10]. The uas-*shi*^{ts1} transgene allows temporary blockade of synaptic transmission from specific neurons by shifting flies from the permissive <25°C to the restrictive >29°C. We assayed *Tdc2*-GAL4;uas-*shi*^{ts1} flies in parallel with GAL4 driver, uas-*shi*^{ts1} transgene and wild-type flies for comparison (Fig. 1A). All flies were incubated at 31°C to disrupt output from OA neurons for 30 min prior to being trained and tested for 3 h appetitive memory at 31°C. Surprisingly, no defects were apparent.

Sweet taste and nutrient value both contribute to appetitive memory reinforcement in *Drosophila*[11,12]. We reasoned that OA blockade might lack consequence if OA only represents sweet taste and nutrient value provides sufficient reinforcement. We therefore blocked *Tdc2* neurons while training flies with arabinose, a sweet but non-nutritious sugar[11] (Fig. 1B). All flies were trained and tested for 3 min memory at 31°C. In this case, memory of *Tdc2*-GAL4;uas-*shi*^{ts1} flies was significantly impaired compared to all control groups. Importantly, no significant differences were apparent between groups trained and tested at 25°C (Fig. 1C). To further challenge a nutrient bypass model we blocked OA neurons while flies were conditioned with arabinose supplemented with nutritious sorbitol[11]. No differences were apparent (Fig. 1D), similar to blocking OA neurons in flies conditioned with sweet and nutritious sucrose (Fig. 1A). These data are consistent with OA only conveying the reinforcing effects of sweet taste and with nutrient value being sufficient for appetitive learning[12].

To determine whether OA provides instructive reinforcement we conditioned flies with odour presentation paired with artificial OA neuron activation, achieved by expressing uas-*dTrpA1* with *Tdc2*-GAL4. *dTrpA1* encodes a Transient Receptor Potential (TRP) channel that conducts Ca^{2+} and depolarizes neurons when flies are exposed to temperature >25°C[13]. *Ad libitum* fed wild-type, *Tdc2*-GAL4, uas-*dTrpA1* and *Tdc2*-GAL4;uas-*dTrpA1* flies were conditioned by presenting an odour with activating 31°C, and immediately tested for memory (Fig. 2A). *Tdc2*-GAL4;uas-*dTrpA1* flies exhibited robust appetitive memory that was statistically different from all other groups (Fig. 2B). Significant memory remained at 30 min (Fig. 2C) in satiated flies but was statistically indistinguishable from all other groups at 3 h, even in hungry flies (Fig. 2D). Therefore appetitive memory implanted with OA neuron activation is short-lived. *Tdc2*-GAL4 expresses in neurons that contain and could release tyramine, either alone or together with OA (Supplementary Fig. 1). To confirm that artificial learning requires OA we stimulated *Tdc2* neurons in *Tβh* mutant flies[9] that cannot synthesize OA from tyramine (Fig. 2E). No learning was observed suggesting that OA release is required for artificial learning.

Although OA neuron innervation of the MB is relatively sparse in the γ lobe, heel and calyx[14] (Supplementary Fig.1), prior work suggests that MB neurons are the likely eventual destination of appetitive reinforcement signals[4,7,15,16]. We therefore used the NP7088, 0665-GAL4 and 0891-GAL4 lines to investigate the role of the four individual classes of OA neurons that innervate the MB[14]: OA-VUMa2, OA-VPM3, OA-VPM4 and OA-VPM5 (Fig. 2F). NP7088 expression broadly overlaps with *Tdc2*-GAL4 neurons in the brain (Fig. 2G) but does not label OA-VPM5 neurons[14]. 0665-GAL4[17] expression is even more restricted and labels the OA-VPM3 and OA-VPM4 neurons (Fig. 2H). Finally, 0891-GAL4[17] only labels OA-VPM4 (Fig. 2I).

Activating these subpopulations of MB-innervating OA neurons during odour presentation did not form appetitive memory (Fig. 2J). Similarly, blocking them (NP7088, 0665-GAL4 or 0891-GAL4) using uas-*shi*^{ts1} did not significantly impair arabinose-reinforced memory (Supplementary Fig. 2). Importantly, these data suggest that the fly equivalent of the bee

VUMmx1 neuron[5], OA-VUMa2[14], and the other MB-innervating neurons covered by these drivers, are neither sufficient nor essential for conditioned olfactory approach behaviour in flies. Instead the data imply that either the calyx-innervating OA-VPM5 neurons are critical, or a more distributed OA signal involving other non-MB innervating OA neurons is required for appetitive reinforcement.

One study implicated the DopR dopamine receptor in appetitive memory. Flies with the *dumb*^{1} mutation have impaired appetitive memory that can be restored by expressing DopR in the MB[16]. We therefore tested whether memory formation with OA neuron activation required DopR (Fig. 3A). No significant memory was observed in any group carrying *dumb*^{1}. Therefore a functional DA system is required to form appetitive memory with OA, suggesting that DA is downstream of OA appetitive memory processes.

A recent study implicated DA neurons in the PAM (Paired Anterior Medial) cluster in appetitive reinforcement[4]. We independently identified the 0273- and 0104-GAL4-GAL4 lines in the InSITE collection[17] that express in subsets of PAM-DA neurons that innervate the MB (Fig. 3B-E, Supplementary Fig. 3A). Co-labeling brains with uas-mCD8::GFP and anti-tyrosine hydroxylase (TH) antibody revealed that 0273-GAL4 expresses in all thê130 DA neurons in the PAM cluster (Fig. 3C) whereas 0104-GAL4 labels a subset of 40 PAM-DA neurons (Fig. 3E). Importantly, neither line labels DA neurons in the Paired Posterior Lateral 1 (PPL1) cluster that convey negative value[18,19,20] (Supplementary Fig. 3A).

We tested whether 0104-GAL4 and 0273-GAL4 PAM neurons could provide appetitive reinforcement by activating them with uas-*dTrpA1* while presenting an odour in satiated flies. Both 0104-GAL4;uas-*dTrpA1* and 0273-GAL4;uas-*dTrpA1* flies exhibited robust appetitive memory that was statistically different from all control flies (Fig. 3F) and far greater than scores observed with a similar stimulation of OA neurons (Fig. 2, 3A).

Since 0104-GAL4 more precisely labels reinforcing PAM-DA neurons than 0273-GAL4, we used uas-*shi*^{ts1} to test whether output from PAM-DA neurons was required for appetitive learning with sugar reinforcement. The 0104-GAL4;uas-*shi*^{ts1} flies were tested in parallel with GAL4 driver, uas-*shi*^{ts1} transgene and wild-type flies for comparison. Blocking 0104-GAL4 neurons abolished memory in arabinose-conditioned flies (Fig. 3G). The initial memory performance of sucrose-conditioned flies was also significantly impaired (Fig. 3H). Moreover, sucrose-conditioned 24 h memory was abolished if 0104-GAL4 neurons were only blocked during training (Supplementary Fig. 3B). Importantly, training and testing the flies at the permissive temperature did not impair performance (Supplementary Fig. 3C). Therefore PAM-DA neurons, like OA neurons, are critical for conditioning with arabinose but, unlike OA neurons, they also contribute towards the reinforcing effects of nutritious sucrose.

We artificially conditioned *Tβh* mutant flies that lack OA to further challenge whether DA reinforcement is downstream of OA. Appetitive memory formed in *Tβh* flies with 0104-GAL4;uas-*dTrpA1* or 0273-GAL4;uas-*dTrpA1* was statistically indistinguishable from that formed in the wild-type background (Fig. 3I and Supplementary Fig. 3D) confirming that DA-mediated reinforcement is downstream, and can function independently, of OA.

To investigate a plausible direct link between OA and DA neurons we tested whether OA neuron activation could form memory in OA receptor mutant flies. Artificial learning worked effectively in satiated *octβ1R* mutant flies (Supplementary Fig. 4) but was impaired in hungry *oamb* mutant flies[21] (Fig. 4A) suggesting a key role for OAMB in reinforcement. To determine whether *oamb* is required in PAM-DA neurons we expressed uas-*oamb*^{RNAi} (Supplementary Fig. 5A) with 0104-GAL4 and conditioned flies with arabinose (Fig. 4B). Memory of 0104-GAL4;uas-*oamb*^{RNAi} flies was significantly different to that of both

control groups. We also tested the same flies conditioned with sucrose. Consistent with prior experiments with OA manipulation, no effect was observed with this nutritious sugar (Supplementary Fig 5B). Therefore the OAMB receptor is required in PAM-DA neurons for OA-dependent memory.

OAMB couples to calcium release from intracellular stores[21,22]. We therefore expressed GCaMP3.0[23] in PAM-DA neurons with 0104-GAL4 and assayed intracellular Ca^{2+} responses evoked by application of exogenous octopamine. Octopamine application drove a significant increase in Ca^{2+} signal in PAM-DA neurons that was abolished by pre-exposing the brain to the OA receptor antagonist mianserin[24,25] (Fig. 4C and Supplementary Fig. 6). Therefore behavioral, anatomical and physiological data are consistent with OA-dependent reinforcement involving OAMB-directed modulation of PAM-DA neurons.

Studies in *octβ2R*[24] flies revealed a more nuanced picture for OA-mediated reinforcement. Artificial learning with OA neuron activation was impaired in satiated *octβ2R/+* heterozygous flies (Fig. 4D) but was restored in *octβ2R/+* flies by food-deprivation (Fig. 4E)[Φ]. These data suggest that OA also integrates with systems that are responsive to hunger to provide instructive reinforcement. Such a role for OA is also highlighted by the observation of memory performance in all prior experiments using satiated flies (Fig. 2, 3 and Supplementary Fig. 4).

Our previous work demonstrated that fly neuropeptide F (dNPF) modulates the MB-heel innervating MB-MP1 DA neurons to limit retrieval of appetitive memory performance to hungry flies[3]. Artificial learning with OA worked effectively in dNPF receptor mutant flies suggesting that OA functions independently of dNPF (Supplementary Fig. 7). We therefore tested for a role of Octβ2R in MB-MP1 DA neurons. We used a new *Tdc2*-LexA (Supplementary Fig. 8) to simultaneously express lexAop-*dTrpA1* in OA neurons and uas-*octβ2R*^{RNAi} in MB-MP1 neurons using c061-GAL4;MBGAL80[3] (Fig. 4F). Hungry *Tdc2*-LexA;lexAop-*dTrpA1* flies formed robust appetitive memory. However, *Tdc2*-LexA;lexAop-*dTrpA1* flies that also carried c061;MBGAL80;uas-*octβ2R*^{RNAi} transgenes to knockdown *octβ2R* expression in MB-MP1 neurons did not display memory (Fig. 4F). We independently tested the role of MB-MP1 neurons in OA-mediated reinforcement by simultaneously stimulating OA neurons while disrupting output from MB-MP1 neurons with uas-*shi*^{ts1} (Supplementary Fig. 9). Flies in which MB-MP1 neurons were simultaneously blocked during artificial conditioning showed no significant memory. Since MB-MP1 neurons can provide aversive reinforcement if artificially engaged during odour presentation[19], they likely provide negative influence to the system[26]. Therefore our data indicate that OA-dependent appetitive reinforcement requires Octβ2R modulation of negative DA signals from MB-MP1 neurons in addition to OAMB signaling in positive PAM-DA neurons.

The 0104-GAL4 DA neurons have presynaptic terminals in the tip of MB β′ and γ lobes and presumed dendrites in the anterior medial protocerebrum (ampr, Fig. 4G,H). We used GFP-reconstituted across synaptic partners (GRASP)[27,28] to investigate plausible sites of synaptic contact between OA neurons and PAM and MB-MP1 DA neurons. We expressed lexAop-mCD4::spGFP11 with Tdc2-LexA and uas-mCD4::spGFP1-10 with 0104-GAL4 or c061-GAL4;MBGAL80. Both of these combinations revealed strong GFP labeling in the ampr (Fig. 4I and Supplementary Fig. 10). In addition, MB-MP1 DA neuron:OA GRASP labeled the MB-heel region. The best candidates to bridge these two regions are the OA-VPM4 neurons, which densely innervate the MB heel and γ lobes and the ampr[14]. However OA-VPM4 neurons are cleanly labeled in 0891-GAL4 (Fig. 2I) and included in the 0665 (Fig.

[Φ]Homozygous *octβ2R* mutant flies are lethal.

2H) and NP7088 (Fig. 2G) GAL4 lines, all of which were insufficient for appetitive conditioning (Fig. 2J). The rest of the ampr innervating neurons, OA-VUMa6, OA-VUMa7, OA-VUMa8 and OA-VPM3, are also included in the NP7088 GAL4 labeled population[14]. Lastly, the MB-calyx innervating OA-VPM5 neurons that are in *Tdc2*-GAL4 but not NP7088 do not have arbors in the ampr or MB-heel[14] so cannot provide direct modulation of PAM or MB-MP1 DA neurons. Therefore reinforcing OA in the fly is provided by a distributed set of neurons, some of which have arbors in the ampr where they modulate reinforcing PAM and MB-MP1 DA neurons. We speculate that OA reinforcement may also require simultaneous regulation of other unidentified DA neurons, or involvement of additional parallel modes of OA action.

Methods

Flies

Fly stocks were raised on standard cornmeal/agar food at 25°C and 60% relative humidity. The wild-type strain is Canton-S. The *Tdc2*-GAL4, NP7088, uas-*shi*^{ts1} (carrying insertions on the first and third chromosome) and uas-*dTrpA1* flies are described[8,10,13,14]. The uas-mCD8::GFP and uas-mCD8::mCherry strains are those in[29] . The *Tβh*^{nM18}, *oa2*^{f02819}, *octβ2r*^{f05679}, *oamb*^{584} and *dumb*^{1} mutant strains are described[9,16,30,31]. The MB-MP1 expressing c061-GAL4:MBGAL80 flies are described[3]. The *Tdc2-lexA:VP16* transgenic line was generated by cloning the same regulatory region as described previously[8] into the pBS_LexA::VP16_SV40 vector[32]. Transgenic flies were raised by standard procedures and lines were screened for those with appropriate expression. The GRASP reporters lexAop-mCD4::spGFP11 and uas-mCD4::spGFP1-10 are described[28]. The lexAop-*dTrpA1* was constructed by subcloning a *NotI* and *XhoI* restriction site- flanked *dTrpA1* cDNA from pOX-*dTrpA1* (Paul Garrity, Brandeis University) into the pLOT transformation vector[32]. Transgenic flies were commercially generated (BestGene Inc., CA). The uas-*oamb*^{RNAi}[2861GD] and uas-*octβ2R*^{RNAi}[104524KK] were obtained from the VDRC[33]. The 247-LexA::VP16 flies are described[29]. 0104-GAL4, 0665-GAL4, 0891-GAL4 and 0273-GAL4 flies refer to PBac{IT.GAL4}0104, PBac{IT.GAL4}0665, PBac{IT.GAL4}0891 and PBac{IT.GAL4}0273 and were generated within the framework of the InSITE project[17]. The lexAop-rCD2::mRFP, uas-Brp::GFP, and uas-DenMark flies are described[34,35,36]. The uas-GCaMP3.0 flies are described[23].

Behavioural analysis

To generate flies to block or stimulate OA neurons we crossed uas-*shibire*^{ts1} or uas-*dTrpA1* female flies to *Tdc2*-GAL4 males. 6-8 day old flies were tested. To block refined subsets of OA neurons we crossed uas-*shibire*^{ts1} female flies to male NP7088/CyO, 0665-GAL4 or 0891-GAL4/TM3 males, and only flies negative for CyO or TM3 were assayed. To stimulate *Tdc2* neurons in the *Tβh* mutant background, *Tβh* ^{nM18}/FM7i-GFP;uas-*dTrpA1* females were crossed with *Tβh* ^{nM18};*Tdc2*-GAL4 males and only progeny negative for FM7i-GFP were assayed. To stimulate *Tdc2* neurons in the *dumb*^{1} mutant background, uas-*dTrpA1*; *dumb*^{1}/TM3 females were crossed with *Tdc2*- GAL4;*dumb*^{1} males and only *dumb*^{1} homozygous flies were assayed. To stimulate 0273-GAL4 or 0104-GAL4 PAM-DA neurons, uas-*dTrpA1* female flies were crossed to 0273-GAL4 or 0104-GAL4/TM6b male flies. To block 0104-GAL4 DA neurons uas-*shibire*^{ts1} females were crossed to 0104-GAL4/TM6b males. To stimulate 0273-GAL4 or 0104-GAL4 neurons in the *Tβh* mutant background, *Tβh*^{nM18}/FM7i;uas-*dTrpA1* females were crossed with *Tβh*^{nM18};0273-GAL4/TM6b *or Tβh*^{nM18};0104-GAL4/TM6b males respectively and progeny negative for FM7i and TM6b were assayed. To stimulate *Tdc2* neurons in the *oa2*, *oamb* and *octβ2R* mutant backgrounds uas-*dTrpA1*; *oa2*^{f02819}/TM3 or uas-*dTrpA1*;*octβ2R*^{f05679}/TM6 or uas-*dTrpA1*;*oamb*^{584}/TM6 females were crossed to *Tdc2*- GAL4;*oa2*^{f02819}/TM3 or *Tdc2*-

GAL4;*octβ2R*^{f05679}/TM3 or *Tdc2*-GAL4;*oamb*^{584}/TM3 males, respectively. Only flies homozygous for *oa2* and *oamb* flies were assayed. The *octβ2R* insertion is homozygous lethal so only heterozygous *octβ2R*/+ flies were assayed. To express *oamb*^{RNAi} in 0104-GAL4 neurons, we crossed uas-*oamb*^{RNAi}[2861GD] females to 0104-GAL4/ TM6b males and only flies lacking TM6b were assayed. To express *octβ2R*^{RNAi} in MB-MP1 neurons while stimulating *Tdc2* neurons, we crossed c061-GAL4;MBGAL80;*Tdc2*-LexA/TM3 females to uas-*octβ2R*^{RNAi}[104524KK];lexAop-*dTrpA1*/TM3 males. To express *shibire*^{ts1} in MB-MP1 neurons while stimulating *Tdc2* neurons, we crossed c061-GAL4;MBGAL80;*Tdc2*-LexA/TM3 females to uas-*shi*^{ts1};lexAop-*dTrpA1*/TM3 males. To stimulate *Tdc2* neurons in the *npfr1*^{c01896} mutant background female uas-*dTrpA1*;*npfr1*^{c01896} flies were crossed to *Tdc2*-GAL4;*npfr1*^{c01896} males. Heterozygous control flies were generated by crossing the respective uas-transgene flies with wild-type flies. For the *oa2*, *octβ2R*, *oamb*, *dumb*^{1} and *npfr1* experiments, *Tdc2*-GAL4;[mutant] or uas-dTrpA1;[mutant] flies were flies mutant at the same locus to generate heterozygous transgene controls within the relevant homozygous mutant background. Controls for MB-MP1 neuron manipulation were generated by crossing c061-GAL4;MBGAL80;*Tdc2*-GAL4/ TM3 or uas-*shi*^{ts1};lexAop-*dTrpA1*/TM3 or uas-*octβ2R*^{RNAi}[104524KK];lexAop-*dTrpA1*/ TM3 females to wild-type flies.

Mixed sex populations were tested together in all behavior experiments unless genotype required sorting single sexes. Hungry state experiments involved food depriving flies for 18–20 h before training in milk bottles containing a damp filter paper. To test flies in the satiated state, flies were food-deprived 14-16 h, then transferred into fresh bottles containing food to satiate 4 h before training. The olfactory appetitive paradigm was performed as described[11] with the following modifications: For neural blockade experiments using uas-*shi*^{ts1}, flies were incubated at 31°C for 30 min prior to and during training and testing for 3 min memory. For permissive temperature experiments flies were kept at 23°C at all times. For memory implantation experiments using uas-*dTrpA1*, flies were presented with one odour at the permissive temperature 23°C for 2 min in filter paper-lined tubes. They were then transferred into a new prewarmed filter paper-lined tube and immediately presented with a second odour at the activating 31°C for 2 min. Flies were then returned to 23°C and tested for immediate memory. To test 3 h memory flies were trained as above and stored in plastic vials containing dampened filter paper until testing. For 24 h memory experiments, flies were trained as above and stored in food vials for 3 h followed by 21 h of food-deprivation before testing. Odours were 3-octanol (9.2 μl in 8 ml mineral oil) or 4-methylcyclohexanol (18 μl in 8 ml mineral oil). The performance index (PI) was calculated as the number of flies running toward the conditioned odour minus the number of flies running toward the unconditioned odour divided by the total number of flies in the experiment. A single PI value is the average score from flies of the identical genotype tested with each odour.

Statistical analyses were performed using PRISM (GraphPad Software). Overall analyses of variance (ANOVA) were followed by planned pairwise comparisons between the relevant groups with a Tukey HSD post-hoc test.

Imaging

To visualize native GFP, mRFP or mCherry adult female flies were collected 2-10 days after eclosion (1 day for GRASP flies) and brains were dissected in ice-cold 4% paraformaldehyde solution in PBS [1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, 175 mM NaCl] and fixed for an additional 60-120 min at room temperature under vacuum. Samples were washed 3×10 min with PBS containing 0.1% Triton-X100 (PBT), and 2X in PBS before mounting in Vectashield (Vector Labs).

For immunohistochemistry, brains were fixed and washed as described above, followed by overnight incubation on a shaker in 10% normal goat serum (NGS) at 4°C. For staining using the Tβh antiserum[30], brains were dissected in ice-cold PBS, then fixed in undiluted Bouin's solution for 20 minutes. Samples were washed 3×10 min with PBS, then 2×10 min with PBT and incubated in 10% NGS overnight as above. The anti-Tβh antiserum[30] was added to a final dilution of 1:300, together with anti-nc82 antibody (Mouse IgG1) and anti-GFP antibody (Mouse IgG2a) to a final dilution of 1:200 each and incubated for 3 days. After washing with PBT, Alexa594-coupled donkey anti-rat, Alexa488 goat anti-mouse IgG2a, and Alexa649 goat anti-mouse IgG1 antibodies were added 1:200 for two days, followed by washing and embedding as described above.. An anti-tyrosine hydroxylase (TH) antibody raised in rabbit (AB152, Millipore) was added to a final dilution of 1:200 and kept in same conditions for another three days. After washing with PBT, Alexa594-coupled goat anti-rabbit antibody (A-11037, Invitrogen) was added 1:200 for one more night, followed by washing and embedding as described before.

Imaging was performed on a Zeiss LSM 5 Pascal confocal microscope and a Leica TCS SP5 X. Images were processed in AMIRA 5.2 (Mercury Systems). In some cases, debris on the brain surface and/or antennal and gustatory nerves were manually deleted from the relevant confocal sections to permit construction of a clear projection view of the z-stack.

In vivo calcium imaging

Up to 7 day old uas-GCaMP3.0;0104-GAL4 flies were anaesthetised on ice and waxed to a custom imaging chamber. The head capsule was opened under 800 μl of sugar-free HL3-like saline[37], and the whole preparation transferred under a SliceScope microscope (Scientifica). Epifluorescence images were acquired using a Pike CCD camera (Allied) at a rate of 3 images/s at one set gain. The spontaneous baseline GCaMP3.0 response was imaged for 30 s, then either 100 μl saline or 100 μl mianserin (12.5 mM, Sigma-Aldrich, filtered) were added to the bath. After another 30 s, 100 μl octopamine (50 mM, Sigma-Aldrich) or 100 μl octopamine (50 mM) + mianserin (12 mM, filtered) were added as before, to reach a final bath concentration of 5 mM octopamine and 2.45 mM mianserin, respectively. After registration of images (StackReg plugin) a standardized region of interest (ROI) was centered within the area of the MB β′ lobe tip (Fig. 4C). Image processing and analysis was performed with Fiji / ImageJ 1.4. Intensity tables were exported to Excel and the ΔF / F was calculated, with an F consisting of the averaged first 24 images. Traces were generated in Prism 6 (GraphPad Software). Respective peak intensities within 5 s after saline/octopamine/mianserin application were selected and compared to other groups for significant differences.

Real-Time PCR

Total RNA from adult fly heads was isolated with Trizol (Invitrogen) and cleaned with RNeasy Micro Kit (Qiagen) with DNAse I treatment. RNA (200 ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and random primers. The cDNA was used for quantitative real-time PCR with ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) with standard cycling parameters (2 min at 50 °C, 10 min at 95 °C, and 45 alternate cycles of 15 s at 95 °C and 60 s at 60 °C). The PCR mixture contained TaqMan® Gene Expression Master Mix and the appropriate Gene Expression Assay (Applied Biosystems). TaqMan qPCR assays were ordered for OAMB (AB: Dm02150048m_1). GAPDH (AB: Dm01841185_m1) was used as endogenous control for normalization (ΔCT value). The decrease in expression (ΔΔCT value) was calculated and transformed to the exponential scale.

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Supplementary Material

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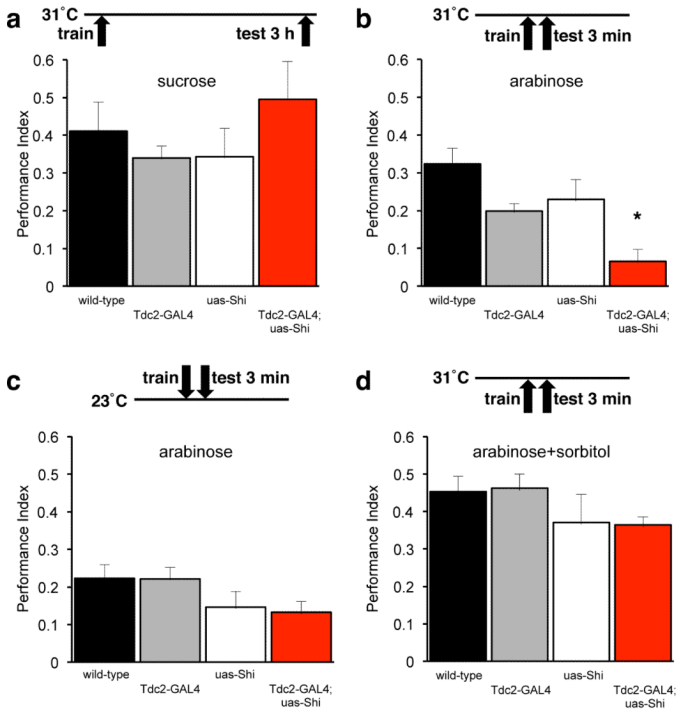

Figure 1. Octopamine mediates the short-term reinforcing effects of sweet taste

a, Blocking OA neurons with *Tdc2*-GAL4/ uas-*shi*^{ts1} during conditioning with sucrose lacks consequence (all $p>0.4$, $n\geq6$). **b**, Blocking OA neurons during conditioning with arabinose significantly impairs appetitive learning, ($p<0.05$, $n\geq10$). **c**, No significant defect is observed when flies are conditioned with arabinose at 23°C, (all $p>0.1$, $n\geq8$). **d**, Blocking OA neurons during conditioning with sorbitol supplemented arabinose has no significant effect (all $p>0.4$, $n=8$). Data are mean ± standard error of the mean (s.e.m.). Asterisks denote significant difference between marked groups and all others (all $p<0.05$, ANOVA).

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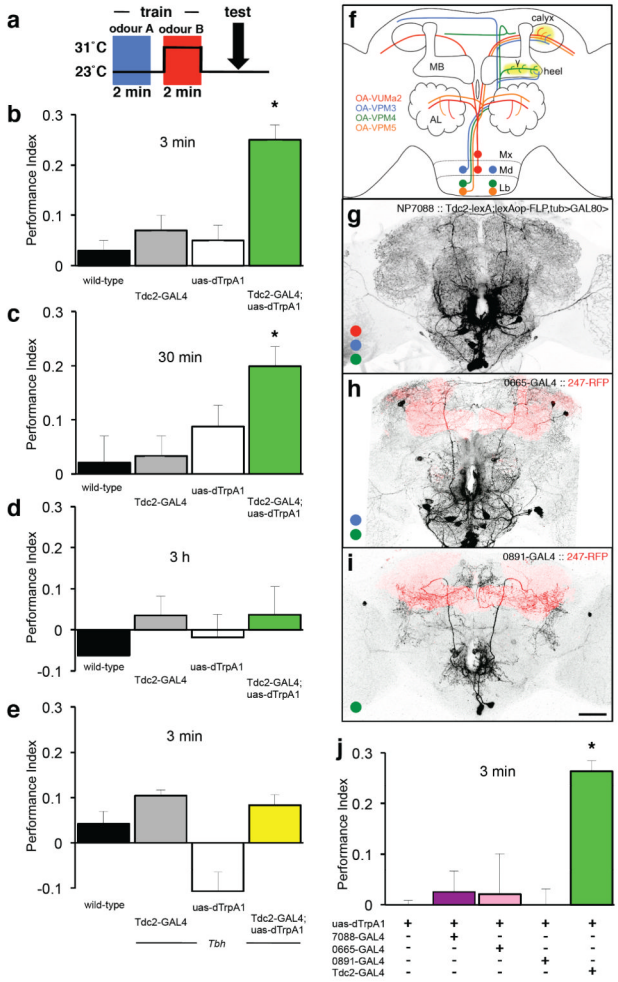

Figure 2. OA neuron stimulation can replace sugar presentation during conditioning to form short-term appetitive memory

a, Conditioning protocol pairing a 2 min odour presentation with heat-activation (red) of uas-*dTrpA1* expressing neurons. **b**, *Tdc2*-GAL4/uas-*dTrpA1* driven OA neuron activation contingent with odour presentation forms appetitive olfactory memory in satiated flies ($p<0.001$, $n\geq14$). **c**, Implanted memory remains significant 30 min after training in satiated flies ($p<0.05$, $n\geq8$). **d**, No memory is observed at 3 h, even in hungry flies ($p>0.5$, $n=6$). **e**, Implanting memory with *Tdc2*-GAL4 neuron stimulation requires OA. Artificial conditioning does not form significant memory in hungry *Tβh* flies ($p>0.05$, $n\geq8$). **f,** Schematic of all four OA neurons that innervate the MB calyx (OA-VUMa2, OA-VPM5, plus the antennal lobe, AL), heel (OA-VPM4, plus the MB γ lobe), or calyx and heel (OA-VPM3). Somata reside in the maxillary (Mx), mandibulary (Md), or labial (Lb) neuromere. **g**, NP7088-GAL4 expresses in many *Tdc2* positive OA neurons. Projection of OA neurons common to *Tdc2*-GAL4/*Tdc2*-LexA and NP7088-GAL4 revealed by genetic intersection. MB-OA cell types in each GAL4 are colour-coded. **h**, 0665-GAL4 labels MB-innervating OA-VPM3 and OA-VPM4 neurons; 247-RFP labeled MB (red). **i**, 0891-GAL4 specifically labels MB-innervating OA-VPM4 neurons. Scale bar 50 μm. **j**, Stimulating OA neuron subsets cannot replace sugar presentation in appetitive conditioning. ($p>0.05$, $n\geq6$).

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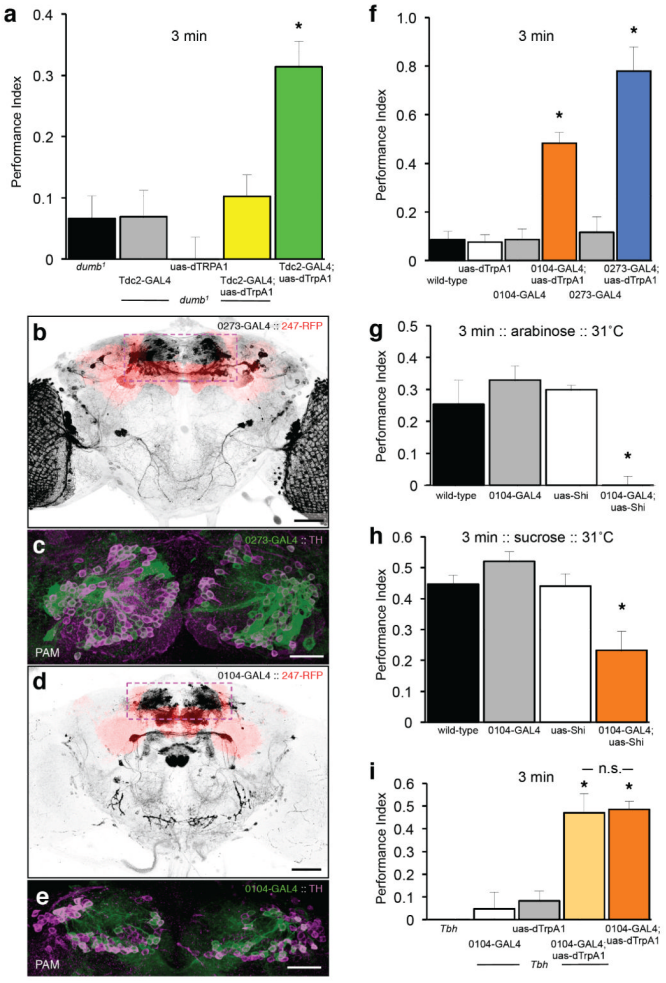

Figure 3. Reinforcing DA neurons are functionally downstream of OA-dependent reinforcement

a, Memory cannot be implanted with *Tdc2* neuron stimulation in *dumb*^{1} (DopR) flies (all p>0.05, except *Tdc2*-GAL4/uas-*dTrpA1* control p<0.001, n≥8). **b**, 0273-GAL4 labels PAM-DA neurons (dashed box) that innervate the MB (red). **c**, 0273-GAL4 labels all ~130 TH-positive PAM-DA neurons. **d**, 0104-GAL4 labels PAM-DA neurons (dashed box). **e**, 0104-GAL4 labels ~40 TH-positive PAM-DA neurons. Scale bar 50 μm (b and d), 20 μm (c and e). **f**, Robust appetitive memory implanted with 0104-GAL4 and 0273-GAL4 neuron activation contingent with odour presentation. Memory of 0104-GAL4;uas-*dTrpA1* and 0273-GAL4;uas-*dTrpA1* flies is significantly different from all others (p<0.01, n≥4). **g**, Blocking DA neurons with 0104-GAL4/uas-*shi*^{ts1} during arabinose conditioning abolishes appetitive learning (p<0.001, n≥8). **h**, Blocking 0104-GAL4 DA neurons during sucrose conditioning significantly impairs learning, (p<0.05, n≥6). **i**, 0104-GAL4 neuron stimulation forms appetitive memory in satiated *Tβh* flies (p<0.001, n≥6).

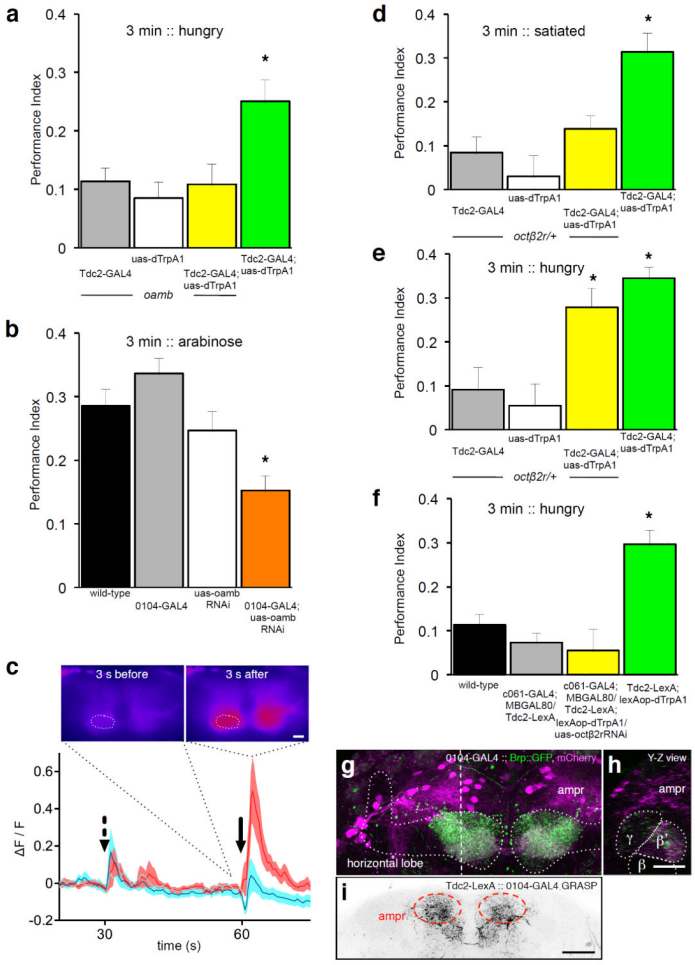

Figure 4. OA-dependent reinforcement functions through discrete groups of DA neurons

a, Memory cannot be implanted with *Tdc2* neuron stimulation in hungry *oamb* flies. Only *Tdc2*-GAL4/uas-*dTrpA1* flies display significant learning ($p<0.01$, $n\geq8$). **b,** Memory formation with arabinose requires *oamb* in 0104-GAL4 neurons. Memory of 0104-GAL4;uas-$oamb^{RNAi}$ flies is significantly different to control groups ($p<0.05$, $n\geq18$). Uas-$oamb^{RNAi}$ causes ~40% decrease in *oamb* transcript (Supplementary Fig. 5A). **c,** Applying 5 mM OA to the exposed fly brain drives an increase in intracellular Ca^{2+}, measured using GCaMP3.0, in 0104-DA neurons. OA-evoked response (red trace) is significantly decreased in brains treated with 2.45 mM mianserin (blue trace, see also Supplementary Fig. 6). First dotted arrow; time of mianserin or vehicle application. Solid arrow; OA or OA with mianserin application. Traces averaged (each $n = 11$ flies); solid line represents mean and shaded areas s.e.m. Panels, representative pseudocoloured images of fluorescence intensity 3 s before (left) and 3 s after (right) OA application. Dotted circle, analysed region of interest. Scale bar 10 μm. **d,** Memory cannot be implanted with *Tdc2* neuron stimulation in satiated *octβ2R*/+ heterozygous flies ($p>0.05$ all groups, except *Tdc2*-GAL4/uas-*dTrpA1*, $p<0.05$, $n=8$). **e** Memory implantation is restored in hungry *octβ2R*/+ flies. *Tdc2*-GAL4/uas-*dTrpA1* flies and *Tdc2*-GAL4/uas-*dTrpA1*; *octβ2R*/+ flies are significantly different to all other groups ($p<0.05$, $n\geq6$). **f**, Memory cannot be formed with *Tdc2* neuron stimulation in flies that express $octβ2R^{RNAi}$ in MB-MP1 neurons (all $p>0.05$ except *Tdc2*-GAL4/uas-*dTrpA1*

control, $p<0.001$, $n\geq8$). Uas-*octβ2R*^{RNAi} efficacy has been reported[42]. **g**, 0104-GAL4 co-expression of mCherry (magenta) and Bruchpilot::GFP (Brp::GFP, green) reveals presynaptic label in the horizontal MB lobe tips. Brp::GFP negative processes in ampr are presumed PAM-DA dendrites. **h**, Y-Z section (at the level of dashed line in **b**) reveals Brp::GFP expression only in β′ and γ lobe tips. Scale bar 20 μm. **i,** GRASP indicates contact between OA and 0104-DA neurons in the ampr (dashed circles). Scale bar 50 μm.