LETTER

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Layered reward signalling through octopamine and dopamine in *Drosophila*

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Dopamine is synonymous with reward and motivation in mammals[1,2]. However, only recently has dopamine been linked to motivated behaviour and rewarding reinforcement in fruitflies[3,4]. Instead, octopamine has historically been considered to be 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 octopamine. Moreover, octopamine-dependent memory formation requires signalling through dopamine neurons. Part of the octopamine signal requires the α-adrenergic-like OAMB receptor in an identified subset of mushroom-body-targeted dopamine neurons. Octopamine triggers an increase in intracellular calcium in these dopamine neurons, and their direct activation can substitute for sugar to form appetitive memory, even in flies lacking octopamine. Analysis of the β-adrenergic-like OCTβ2R receptor reveals that octopamine-dependent reinforcement also requires an interaction with dopamine neurons that control appetitive motivation. These data indicate that sweet taste engages a distributed octopamine signal that reinforces memory through discrete subsets of mushroom-body-targeted dopamine neurons. In addition, they reconcile previous findings with octopamine and dopamine and suggest that reinforcement systems in flies are more similar to mammals than previously thought.

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

We tested whether octopamine 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}*, where the *shi^{ts1}* sequence is cloned downstream of the upstream activation sequence (*UAS*))[10]. The *UAS-shi^{ts1}* transgene allows temporary blockade of synaptic transmission from specific neurons by shifting flies from the permissive temperature <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 octopamine neurons for 30 min before 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 octopamine blockade might lack consequence if octopamine 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 octopamine neurons while flies were conditioned with arabinose supplemented with nutritious sorbitol[11]. No differences were apparent (Fig. 1d), similar to blocking octopamine

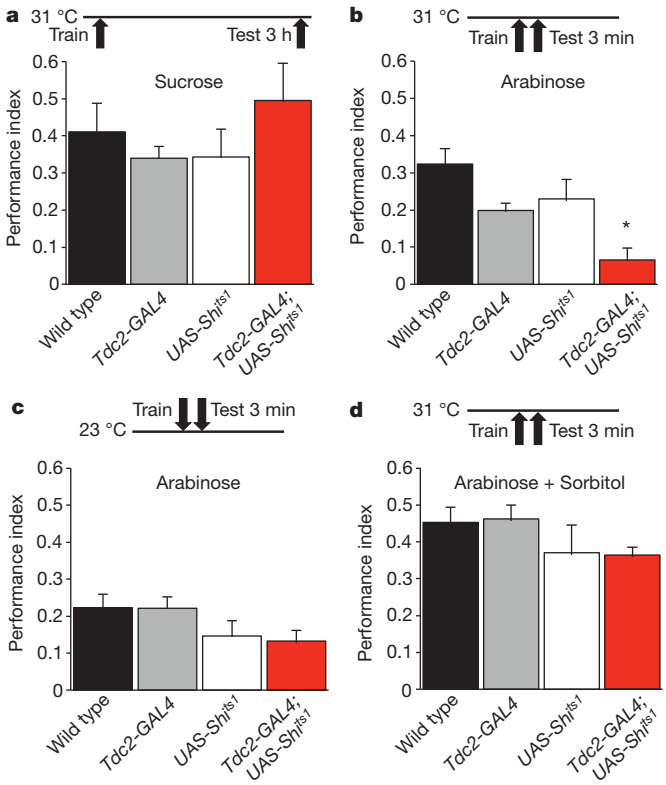

Figure 1 | Octopamine mediates the short-term reinforcing effects of sweet taste. **a**, Blocking octopamine neurons with *Tdc2-GAL4/UAS-shi^{ts1}* during conditioning with sucrose has no effect (all $P > 0.4$, $n \geq 6$). **b**, Blocking octopamine neurons during conditioning with arabinose significantly impairs appetitive learning ($P < 0.05$, $n \geq 10$). **c**, No significant defect is observed when flies are conditioned with arabinose at 23 °C (all $P > 0.1$, $n \geq 8$). **d**, Blocking octopamine 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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neurons in flies conditioned with sweet and nutritious sucrose (Fig. 1a). These data are consistent with octopamine only conveying the reinforcing effects of sweet taste and with nutrient value being sufficient for appetitive learning[12].

To determine whether octopamine provides instructive reinforcement we conditioned flies with odour presentation paired with artificial octopamine neuron activation, achieved by expressing *UAS-dTrpA1* with *Tdc2-GAL4*. *dTrpA1* (also known as *TrpA1*) 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 octopamine neuron activation is short-lived. *Tdc2-GAL4* is expressed in neurons that contain and could release tyramine, either alone or together with octopamine (Supplementary Fig. 1). To confirm that artificial learning requires octopamine we stimulated *Tdc2* neurons in *Tbh* mutant flies[9] that cannot synthesize octopamine from tyramine (Fig. 2e). No learning was observed, indicating that octopamine release is required for artificial learning.

Although octopamine neuron innervation of the mushroom body (MB) is relatively sparse in the γ lobe, heel and calyx[14] (Supplementary Fig. 1), previous work suggests that MB neurons are probably the eventual destination of appetitive reinforcement signals[4,7,15,16]. We therefore used the *NP7088*, *0665* and *0891-GAL4* lines to investigate the role of the four individual classes of octopamine (OA) neurons that innervate the MB[14]: OA-VUMa2, OA-VPM3, OA-VPM4 and OA-VPM5 (Fig. 2f). *NP7088* neurons visualized using *UAS-mCD8::GFP* (that is, expressing the green fluorescent protein (GFP)) broadly overlap with *Tdc2-GAL4* neurons in the brain (Fig. 2g), but *NP7088-GAL4* does not label OA-VPM5 neurons[14]. *0665-GAL4* (ref. 17) driven GFP expression is even more restricted and labels the OA-VPM3 and OA-VPM4 neurons (Fig. 2h). Finally, *0891-GAL4* (ref. 17) only labels OA-VPM4 (Fig. 2i).

Activating these subpopulations of MB-innervating octopamine neurons during odour presentation did not form appetitive memory (Fig. 2j). Similarly, blocking them (*NP7088*, *0665* or *0891-GAL4*) using *UAS-shi^{ts1}* did not significantly impair arabinose-reinforced memory (Supplementary Fig. 2). Importantly, these data indicate that the fly equivalent of the bee VUMmx1 neuron[5], OA-VUMa2 (ref. 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 indicate that either the calyx-innervating OA-VPM5 neurons are critical, or a more distributed octopamine signal involving other non-MB-innervating octopamine 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 octopamine neuron activation required DopR (Fig. 3a). No significant memory was observed in any group carrying *dumb^1*. Therefore a functional dopamine system is required to form appetitive memory with octopamine, suggesting that dopamine is downstream of octopamine in appetitive memory processes.

A recent study implicated dopamine neurons in the PAM (paired anterior medial) cluster in appetitive reinforcement[4]. We independently identified the *0273-GAL4* and *0104-GAL4* lines in the InSITE collection[17] that drive *UAS-mCD8::GFP* in subsets of PAM dopamine neurons that innervate the MB (Fig. 3b–e and Supplementary Fig. 3a). Co-labelling brains with *UAS-mCD8::GFP* and anti-tyrosine hydroxylase

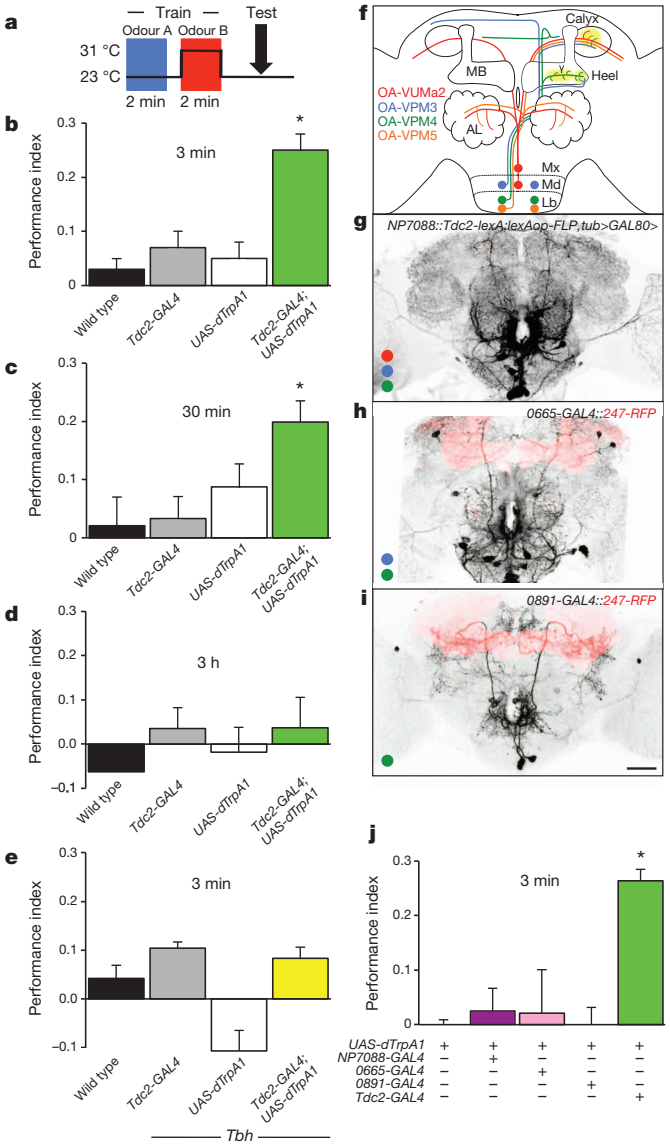

Figure 2 | Octopamine neuron stimulation can replace sugar presentation during conditioning to form short-term appetitive memory. **a**, Conditioning protocol pairing a 2 min odour presentation with heat-activation (red) of *UAS-dTrpA1* expressing neurons. **b**, *Tdc2-GAL4*/*UAS-dTrpA1* driven octopamine neuron activation contingent with odour presentation forms appetitive olfactory memory in satiated flies ($P < 0.001$, $n \geq 14$). **c**, Implanted memory remains significant 30 min after training in satiated flies ($P < 0.05$, $n \geq 8$). **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 octopamine. Artificial conditioning does not form significant memory in hungry *Tbh* flies ($P > 0.05$, $n \geq 8$). **f**, Schematic of all four octopamine 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 octopamine neurons. Projection of octopamine neurons common to *Tdc2-GAL4*/*Tdc2-lexA* and *NP7088-GAL4* revealed by genetic intersection. MB-octopamine cell types in each GAL4 are colour-coded. **h**, 0665-GAL4 labels MB-innervating OA-VPM3 and OA-VPM4 neurons; 247-RFP labelled MB (red). **i**, 0891-GAL4 specifically labels MB-innervating OA-VPM4 neurons. Scale bar, 50 μm. **j**, Stimulating octopamine neuron subsets cannot replace sugar presentation in appetitive conditioning. ($P > 0.05$, $n \geq 6$).

(TH) antibody revealed that *0273-GAL4* is expressed in all of the ~130 dopamine neurons in the PAM cluster (Fig. 3c), whereas *0104-GAL4* labels a subset of 40 PAM dopamine neurons (Fig. 3e). Importantly,

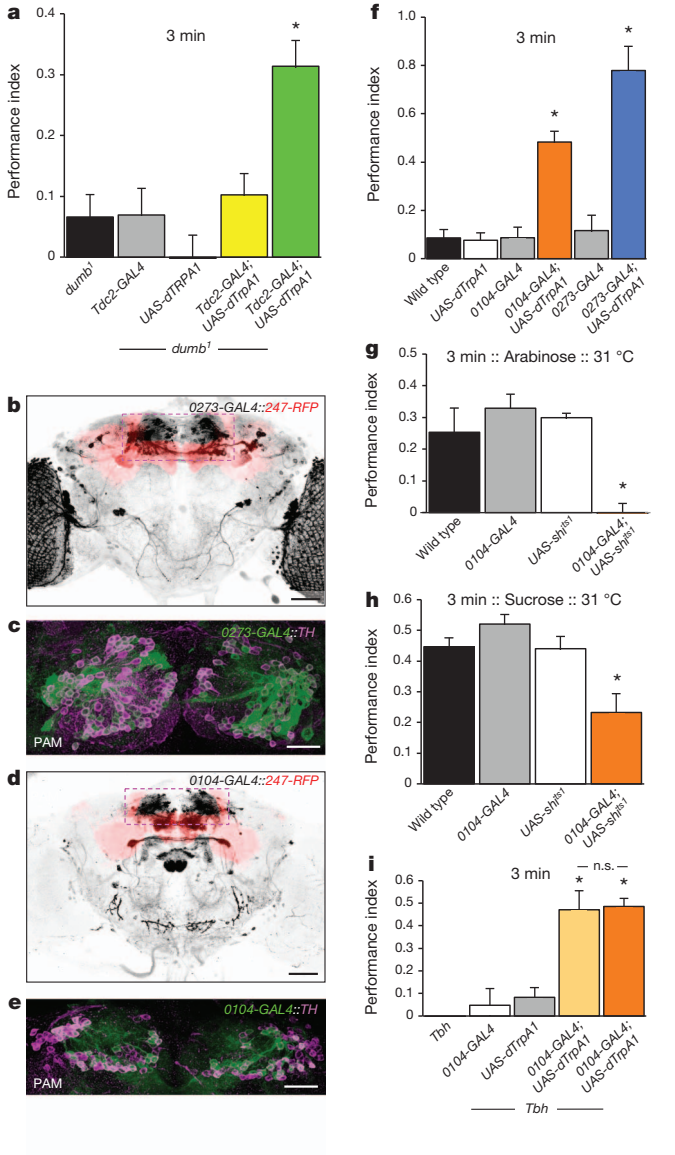

Figure 3 | Reinforcing dopamine neurons are functionally downstream of octopamine-dependent reinforcement. **a**, Memory cannot be implanted with *Tdc2* neuron stimulation in *dumb*^{1} (*DopR*) flies (all $P > 0.05$ except *Tdc2-GAL4/UAS-dTrpA1* control, $P < 0.001$, $n \geq 8$). **b**, 0273-GAL4 labels PAM dopamine neurons (dashed box) that innervate the MB (red). **c**, 0273-GAL4 labels all ~130 TH-positive PAM dopamine neurons. **d**, 0104-GAL4 labels PAM dopamine neurons (dashed box). **e**, 0104-GAL4 labels ~40 TH-positive PAM dopamine 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 ($P < 0.01$, $n \geq 4$). **g**, Blocking dopamine neurons with 0104-GAL4/*UAS-shi*^{ts1} during arabinose conditioning abolishes appetitive learning ($P < 0.001$, $n \geq 8$). **h**, Blocking 0104-GAL4 dopamine neurons during sucrose conditioning significantly impairs learning ($P < 0.05$, $n \geq 6$). **i**, 0104-GAL4 neuron stimulation forms appetitive memory in satiated *Tbh* flies ($P < 0.001$, $n \geq 6$).

neither line labels dopamine neurons in the paired posterior lateral 1 (PPL1) cluster that convey negative value[18–20] (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 octopamine neurons (Figs 2 and 3a).

Because *0104-GAL4* more precisely labels reinforcing PAM dopamine neurons than *0273-GAL4*, we used *0104-GAL4* with *UAS-shi*^{ts1} to test whether output from PAM dopamine 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 dopamine neurons, like octopamine neurons, are critical for conditioning with arabinose but, unlike octopamine neurons, they also contribute towards the reinforcing effects of nutritious sucrose.

We artificially conditioned *Tbh* mutant flies that lack octopamine to investigate further whether dopamine reinforcement is downstream of octopamine. Appetitive memory formed in *Tbh* 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 dopamine-mediated reinforcement is downstream, and can function independently, of octopamine.

To investigate a plausible direct link between octopamine and dopamine neurons we tested whether octopamine neuron activation could form memory in octopamine receptor mutant flies. Artificial learning worked effectively in satiated *octβ1R* (also known as *oa2*) 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 dopamine neurons we expressed *UAS-Oamb*^{RNAi} (Supplementary Fig. 5a) with *0104-GAL4* and conditioned flies with arabinose (Fig. 4b). The 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 previous experiments with octopamine manipulation, no effect was observed with this nutritious sugar (Supplementary Fig. 5b). Therefore, the OAMB receptor is required in PAM dopamine neurons for octopamine-dependent memory.

OAMB couples to calcium release from intracellular stores[21,22]. We therefore expressed GCaMP3.0 (ref. 23) in PAM dopamine 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 dopamine neurons that was abolished by pre-exposing the brain to the octopamine receptor antagonist mianserin[24,25] (Fig. 4c and Supplementary Fig. 6). Therefore behavioural, anatomical and physiological data are consistent with octopamine-dependent reinforcement involving OAMB-directed modulation of PAM dopamine neurons.

Studies in *octβ2R* (ref. 24) mutant flies revealed a more nuanced picture for octopamine-mediated reinforcement. Artificial learning with octopamine neuron activation was impaired in satiated *octβ2R/+* heterozygous flies (Fig. 4d), but was restored in *octβ2R/+* flies by food-deprivation (Fig. 4e; note that homozygous *octβ2R* mutations are lethal). These data suggest that octopamine also integrates with systems that are responsive to hunger to provide instructive reinforcement. Such a role for octopamine is also highlighted by the observation of memory performance in all previous experiments using satiated flies (Figs 2, 3 and Supplementary Fig. 4).

Our previous work demonstrated that fly neuropeptide F (dNPF) modulates the MB–heel-innervating MB-MP1 dopamine neurons to limit retrieval of appetitive memory performance to hungry flies[3]. Artificial learning with octopamine worked effectively in dNPF receptor mutant flies, indicating that octopamine functions independently of dNPF (Supplementary Fig. 7). We therefore tested for a role of *Octβ2R* in MB-MP1 dopamine neurons. We used a new *Tdc2-LexA* (Supplementary Fig. 8) to simultaneously express *lexAop-dTrpA1* in

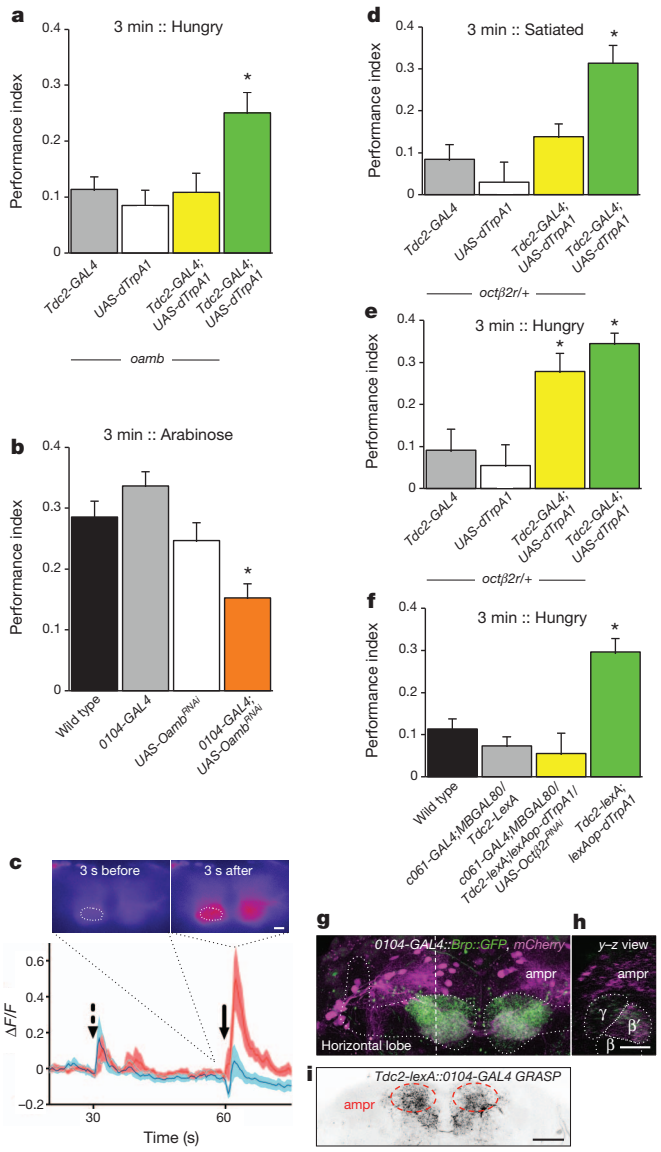

Figure 4 | Octopamine-dependent reinforcement functions through discrete groups of dopamine neurons. **a**, Memory cannot be implanted with *Tdc2* neuron stimulation in hungry *oamb* flies. Only *Tdc2-GAL4/UAS-dTrpA1* flies display significant learning ($P < 0.01$, $n \geq 8$). **b**, Memory formation with arabinose requires *Oamb* in 0104-GAL4 neurons. Memory of 0104-GAL4;*UAS-Oamb*^{RNAi} flies is significantly different to control groups ($P < 0.05$, $n \geq 18$). *UAS-Oamb*^{RNAi} causes ~40% decrease in *Oamb* transcript (Supplementary Fig. 5A). **c**, Applying 5 mM octopamine to the exposed fly brain drives an increase in intracellular Ca^{2+}, measured using *UAS*-GCaMP3.0, in 0104-GAL4 dopamine neurons ($\Delta F/F$ represents the evoked fluorescence change from baseline). Octopamine-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; octopamine or octopamine with mianserin application. Traces averaged (each $n = 11$ flies); solid line represents mean and shaded areas s.e.m. Panels, representative pseudo-coloured images of fluorescence intensity 3 s before (left) and 3 s after (right) octopamine 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 ($P < 0.05$, $n \geq 6$). **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, $P < 0.001$, $n \geq 8$). *UAS-Octβ2R*^{RNAi} efficacy has been reported[30]. **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 to be PAM dopamine 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 octopamine and 0104-GAL4 dopamine neurons in the ampr (dashed circles). Scale bar, 50 µm.

octopamine neurons and *UAS-Octβ2R*^{RNAi} in MB-MP1 neurons using *c061-GAL4;MBGAL80* (ref. 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 tested independently the role of MB-MP1 neurons in octopamine-mediated reinforcement by simultaneously stimulating octopamine 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. Because MB-MP1 neurons can provide aversive reinforcement if artificially engaged during odour presentation[19], they probably provide a negative influence to the system[26]. Therefore, our data indicate that octopamine-dependent appetitive reinforcement requires OCTβ2R modulation of negative dopamine signals from MB-MP1 neurons in addition to OAMB signalling in positive PAM dopamine neurons.

The *0104-GAL4* dopamine 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 green fluorescent protein reconstituted across synaptic partners (GRASP)[27,28] to investigate plausible sites of synaptic contact between octopamine neurons and PAM and MB-MP1 dopamine 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 labelling in the ampr (Fig. 4i and Supplementary Fig. 10). In addition, MB-MP1 dopamine neuron:octopamine GRASP labelled 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 labelled in *0891-GAL4* (Fig. 2i) and included in the *0665-GAL4* (Fig. 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*-labelled population[14]. Finally, the MB-calyx-innervating OA-VPM5 neurons that are in *Tdc2-GAL4* but not *NP7088* do not have arborizations in the ampr or MB heel[14], so cannot provide direct modulation of PAM or MB-MP1 dopamine neurons. Therefore, reinforcing octopamine in the fly is provided by a distributed set of neurons, some of which have arborizations in the ampr where they modulate reinforcing PAM and MB-MP1 dopamine neurons. We speculate that octopamine reinforcement may also require simultaneous regulation of other unidentified dopamine neurons, or involvement of additional parallel modes of octopamine action.

METHODS SUMMARY

Flies. All behavioural or anatomical experiments using transgenic expression were carried out on the F_1 progeny obtained from crossing *GAL4* driver flies to those harbouring reporter or effector transgenes.

Behaviour. Appetitive olfactory memory was measured as described[11] with modifications: for neural blockade using *UAS-shi*^{ts1}, flies were incubated at 31 °C for 30 min before and during training and testing 3 min memory. For memory implantation using *UAS-dTrpA1*, flies in filter-paper-lined tubes were presented with one odour for 2 min at 23 °C. They were then transferred into a new pre-warmed filter-paper-lined tube and presented with a second odour for 2 min at 31 °C. Flies were returned to 23 °C and tested for memory. Flies tested in the satiated condition were food-deprived 14–16 h then transferred onto fresh food for 4 h before training. Odours were 3-octanol and 4-methylcyclohexanol. 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 honestly significant difference post-hoc test.

Immunohistochemistry. Brains were analysed for native GFP, mRFP (red fluorescent protein), mCherry, anti-TH staining, BRP::GFP and GRASP as described[29]. Overnight preincubation in 10% NGS at 4 °C was followed by primary antiserum incubation for 3 days at 4 °C. After washing, secondary antibodies were applied for 2 days at 4 °C before brains were embedded and imaged.

***In vivo* calcium imaging.** Fly brains expressing UAS-GCaMP3.0 (ref. 23) were imaged in saline under a SliceScope microscope (Scientifica) using a Pike CCD camera (Allied). 30 s of baseline fluorescence was recorded, followed by bath application of either saline or mianserin (Sigma-Aldrich). After another 30 s, octopamine (Sigma-Aldrich) or octopamine plus mianserin were added. Image processing, data analysis and presentation were performed with Fiji/ImageJ 1.4, Excel and Prism 6 (GraphPad Software).

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

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.W. (scott.waddell@cncb.ox.ac.uk).

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 ref. 29. The *Tbh^{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 (P. Garrity) into the pLOT transformation vector[32]. Transgenic flies were commercially generated (BestGene). The *UAS-Oamb^{RNAi}* (strain number 2861GD) and *UAS-Octβ2R^{RNAi}* (strain number 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[34–36]. The *UAS-GCaMP3.0* flies are described[23].

Behavioural analysis. To generate flies to block or stimulate octopamine 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 octopamine 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 *Tbh* mutant background, *Tbh^{nM18}/FM7i-GFP; UAS-dTrpA1* females were crossed with *Tbh^{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 dopamine neurons, *UAS-dTrpA1* female flies were crossed to *0273-GAL4* or *0104-GAL4/TM6b* male flies. To block 0104-GAL4 dopamine neurons *UAS-shibire^{ts1}* females were crossed to *0104-GAL4/TM6b* males. To stimulate 0273-GAL4 or 0104-GAL4 neurons in the *Tbh* mutant background, *Tbh^{nM18}/FM7i;UAS-dTrpA1* females were crossed with *Tbh^{nM18};0273-GAL4/TM6b or Tbh^{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 behaviour 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 before 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 pre-warmed 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 was calculated as the number of flies running towards the conditioned odour minus the number of flies running towards the unconditioned odour divided by the total number of flies in the experiment. A single performance index 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 honestly significant difference 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 three times 10 min with PBS containing 0.1% Triton-X100 (PBT), and twice 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 min. Samples were washed three times 10 min with PBS, then twice 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 2 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 3 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 anaesthetized 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 three images per second 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 centred 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 $\Delta F/F$ 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* (Applied Biosystems primer set annotation no. Dm02150048_m1). *Gpdh* (Applied Biosystems primer set annotation no. Dm01841185_m1) was used

as endogenous control for normalization (ΔC_t value). The decrease in expression ($\Delta\Delta C_t$ value) was calculated and transformed to the exponential scale.

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