Sweet taste and nutrient value subdivide rewarding dopaminergic neurons in Drosophila

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Sweet Taste and Nutrient Value Subdivide Rewarding Dopaminergic Neurons in *Drosophila*

**Highlights**
- Sweet taste and nutrient value recruit different reinforcing dopaminergic neurons
- Sweetness and nutrient value separately reinforce short- and long-term memories
- Reinforcement of short-term memory is not dependent on the state of hunger
- Acquisition and retrieval of long-term memory are hunger state dependent

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**In Brief**
A small number of dopaminergic neurons in the fly brain are crucial for appetitive memory reinforcement. Huetteroth et al. further subdivide these rewarding neurons into those required for sweet taste reinforcement of short-term memory, nutrient-dependent long-term memory, and others that can artificially reinforce food-independent long-term memory.
Sweet Taste and Nutrient Value Subdivide Rewarding Dopaminergic Neurons in Drosophila

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Summary

Dopaminergic neurons provide reward learning signals in mammals and insects [1–4]. Recent work in Drosophila has demonstrated that water-reinforcing dopaminergic neurons are different to those for nutritious sugars [5]. Here, we tested whether the sweet taste and nutrient properties of sugar reinforcement further subdivide the fly reward system. We found that dopaminergic neurons expressing the OAMB octopamine receptor [6] specifically convey the short-term reinforcing effects of sweet taste [4]. These dopaminergic neurons project to the β2 and γ4 regions of the mushroom body lobes. In contrast, nutrient-dependent long-term memory requires different dopaminergic neurons that project to the γ5b regions, and it can be artificially reinforced by those projecting to the β lobe and adjacent α5 region. Surprisingly, whereas artificial implantation and expression of short-term memory occur in satiated flies, formation and expression of artificial long-term memory require flies to be hungry. These studies suggest that short-term and long-term sugar memories have different physiological constraints. They also demonstrate further functional heterogeneity within the rewarding dopaminergic neuron population.

Results and Discussion

Sweet taste and nutrient value of sugars reinforce learning in Drosophila [7, 8]. Octopaminergic neurons specifically convey sweet taste signals [4, 9]. Blocking them impaired short-term memory (STM) reinforced by the sweet but non-nutritious arabinose. In contrast, long-term memory (LTM) formed with sweet and nutritive sucrose was unaffected. Reinforcing octopamine activates a subpopulation of dopaminergic neurons via the Ca2+-coupled α2-adrenergic-like octopamine receptor OAMB. However, despite the evident separation of memory phases with octopamine [4, 10]. manipulating dopaminergic neurons has so far impacted sweet taste and nutrient-reinforced memory [3, 4]. We therefore investigated whether octopamine dependence separates rewarding dopaminergic neurons.

TbhM18 mutant flies, lacking octopamine, cannot form STM reinforced with 2 M sucrose [11]. However, a persistent memory slowly emerges after training Tbhm18 flies with odor and 1 M sucrose [10], suggesting that nutrient-dependent LTM is likely to be formed in parallel and independent of appetitive STM. Since nutrient-dependent memory can guide behavior as quickly as 2 min after training [7], we first determined whether nutrient memory could be observed in wild-type and Tbhm18 flies trained with saturated sucrose, ~ 5.8 M (Figure 1A). Strikingly, this analysis revealed performance at all times in Tbhm18 flies that was statistically different to wild-type immediately after training but indistinguishable from wild-type 30 min, 3 hr, and 24 hr after training. These data are consistent with Tbhm18 flies only lacking sweet-taste-reinforced STM [4, 10]. Moreover, they demonstrate that nutrient-dependent (octopamine-independent) memory is observable immediately after training with high sucrose concentrations.

Prior knowledge that octopamine activates rewarding dopaminergic neurons through the OAMB receptor [4] led us to identify R48B04-GAL4 in the FlyLight collection [19]. R48B04-GAL4 is driven by a promoter fragment from the oamb gene (although we acknowledge that this reagent is unlikely to label all oamb-expressing neurons, from here on, we will refer to it as oambP-GAL4). We verified the relevance of oambP-GAL4 neurons by knocking down OAMB expression with UAS-oambRNAi [4, 14]. As expected, these flies completely lacked STM when trained with the sweet and non-nutritious sugar arabinose (Figure 1B). The memory defect was more pronounced than when OAMB was knocked down in dopaminergic neurons with 0104-GAL4 [4], suggesting that oambP-GAL4 may more accurately label octopamine-responsive dopaminergic neurons than 0104-GAL4 (Figures S1A–S1E). Our initial examination of oambP-GAL4 revealed expression in approximately 55 rewarding dopaminergic neurons (and ~ 12 tyrosine hydroxylase (TH)-negative neurons) in the protocerebral anterior medial (PAM) cell cluster that innervates the horizontal mushroom body lobes (Figures 1C–1E, S1A, S1C, and S1D; [5]).

We next tested the contribution of oambP-GAL4 neurons to saturated sucrose-reinforced memory by blocking their output using the dominant temperature-sensitive UAS-shibirets1 (UAS-shibrets1) transgene [15]. Blocking oambP-GAL4 neurons significantly impaired STM (Figure 1F). However, LTM performance of oambP-GAL4;UAS-shibrets1 flies was indistinguishable from controls, demonstrating a specific loss of STM (Figure 1G), consistent with Tbhm18 flies trained with sucrose (Figure 1A).

We also tested a reinforcing role of oambP-GAL4 neurons by pairing their activation, using UAS-dTrpA1, with odor presentation (Figures 1H and 1I). The dTrpA1-encoded transient receptor potential (TRP) channel conducts Ca2+ and depolarizes neurons when temperature exceeds 25°C [16]. This protocol implanted STM that was statistically different from all controls in both starved and fed flies (Figures 1H and S1F). However, implanted memory did not persist. Performance of oambP-GAL4;UAS-dTrpA1 flies was indistinguishable from controls 24 hr after training (Figure 1I). Taken together, these
Figure 1. Sweet Taste Reinforces Short-Term Memory via Octopamine Signaling in oambP-GAL4 Dopaminergic Neurons

(A) TbhM18 flies exhibit defective STM following training with concentrated sucrose (compared to wild-type, p < 0.0001, t test). Residual memory of TbhM18 flies persists and is statistically indistinguishable from memory in wild-type flies 30 min, 3 hr, and 24 hr after training (all p > 0.7, t test). All n ≥ 12.

(B) Hungry oambP-GAL4;UAS-oambRNAi flies lack STM following training with arabinose (versus controls, p < 0.0001, ANOVA, n ≥ 8).

(C) R48B04 (referred to as oambP-GAL4) labels about 55 dopaminergic neurons that zonally innervate γ1, γ2, γ4, and γ5 of the γ lobe and β1, β2 of the β lobe. 1.5-μm frontal confocal sections at the level of the γ lobe and β lobes are shown; scale bars represent 20 μm. See Figure S1A for full brain expression.

(D) Schematic of the mushroom body lobes and additional zonal suborganization of the horizontal β (red), γ (green), and γ (magenta) lobes. The β1, β2 and γ1, γ2 border their respective α1 and α γ1 subregions on the base of the vertical lobes. The exclusively horizontal γ lobe can be split into γ1–γ5 [12].

(E) Illustration of the lobe subregions, highlighting those innervated by oambP-GAL4 dopaminergic neurons (gray).

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data suggest that oambP-GAL4 dopaminergic neurons specifically convey octopamine-dependent and hunger-state-independent sweet taste reinforcement, whereas other rewarding dopaminergic neurons contribute nutrient value signals.

0104-GAL4 labels octopamine-responsive dopaminergic neurons and some required for nutrient reinforcement [4]. We reasoned that intersecting 0104-GAL4 and oambP-GAL4 would separate sweet and nutrient reinforcement. Common neurons in 0104-GAL4 and oambP-GAL4 can be visualized by combining R48B04-LexA (i.e., oambP-LexA, which expresses in oambP-GAL4 dopaminergic neurons; Figure S1C) with 0104-GAL4-driven UAS-STOP-GFP (where > represents a FLP-recombinase target sequence) and lexAop-FLP. In these flies, GFP labels 10–20 dopaminergic neurons innervating the β/2m (and γa) regions of the mushroom body.

(F and G) Blocking oambP-GAL4 neuron output with UAS-shRNA significantly impairs STM in starved flies trained with sucrose (p < 0.0001, ANOVA, n = 8) (F), whereas it has no effect on 24-hr LTM, as compared to controls (p > 0.1, ANOVA, n = 8) (G).

(I) Summary of STM formation. Sweet taste requires βγ and γ2 Dopaminergic Neurons. (A–C) Positive intersection between 0104-GAL4 and oambP-lexA with lexAop-FLP,tub>Gal80-stop and UAS-mCD8::GFP labels about 20 neurons. (A) Projection of 20 1-μm confocal sections at the level of the γa lobe reveals processes in βa, β1, and γ2a. (B) Projection of ten 1-μm confocal sections at the level of the βa, β2a, and β2m neurons shows innervation in β2a and β2m and some posterior γ2a from the same cell type as in (A). (C) Projection of ten 1-μm confocal sections of the βa lobe reveals shared 0104-GAL4 and oambP-LexA innervation of β1.

(F and G) Removing oambP neurons from 0104-GAL4;0104-GAL4/0104-GAL4,UAS-6xGFP;0104-GAL4 leaves expression in about 15 neurons innervating γa, and β2m (G) neurons. Projection of five 2-μm confocal sections covering the γa lobe (F) and the β2m lobe (G). oambP-GAL80 also unexpectedly suppresses expression in the β2m (red arrow), which is otherwise labeled by 0104-GAL4 (Figure S1B). Scale bars represent 20 μm.

(H) Blocking output from the γa and β2m dopaminergic neurons, shown in illustration, during arabinose-reinforced training impairs STM. Performance of oambP-GAL80;0104-GAL4/UAS-shRNA flies was significantly different to controls (p < 0.0001, ANOVA, n = 8). See permissive temperature control in Figure S1H. (I) Summary of STM formation. Sweet taste engages octopaminergic neurons. Octopamine acts through the OAMB receptor to activate dopaminergic neurons. Octopamine gages octopaminergic neurons.

(J) Blocking output from the β2m and γa subset of 0104-GAL4 neurons during sucrose-reinforced training impairs 24-hr LTM. Performance of 0104-GAL4/UAS-shRNA and oambP-GAL80;0104-GAL4/UAS-shRNA flies was significantly different to controls (p < 0.0002, ANOVA, n = 5–12). See permissive temperature control in Figure S2B.
Both oambP-GAL4 and 0104-GAL4 contain γs-innervating neurons (Figures 1C and S1A–S1E), but the positive intersection does not label them, suggesting that each GAL4 includes unique γs neurons: γs \text{ narrow} (γsΔ) in oambP-GAL4 (Figures 1C, S1A, S1C, and S1D) and γs \text{ broad} (γs\text{b}) in 0104-GAL4 (Figures S1B and S1E).

To assess the role of subsets of oambP-labeled neurons, we removed expression in 0104 neurons by combining 0104-GAL4 with UAS-lexA\text{RNAi}oambP-LexA flies and a lex-Aop-shifactant transgene, thereby restricting expression to dopaminergic neurons innervating βs2m, γs, and γs5 [5]. These flies exhibited no STM following training with sweet-only arabinose at restrictive 33°C (Figure 2E). No significant defect was evident at permissive 23°C (Figure S1G). We also constructed 0104-GAL4:oambP-GAL80 flies in which GAL80 inhibits GAL4-driven gene expression [17] resulting in expression being restricted to βs2m and γs5 0104 neurons (Figures 2F–2H). These flies also displayed defective STM following conditioning at restrictive 33°C with arabinose (Figure 2H), while no significant defect was evident at permissive 23°C (Figure S1H). Since blocking γs5 and γs6 neurons with R15A04-GAL4 does not impair STM (Figure S2A), we conclude that sweet taste reinforcement is conveyed by octopaminergic signaling through the OAMB receptor in dopaminergic neurons that innervate the βs2m and γs4 zones of the mushroom body (Figure 2B).

0104-GAL4 also includes neurons required for nutrient-dependent LTM, which are not in oambP-GAL4. Indeed, blocking 0104-GAL4:oambP-GAL80 neurons with UAS-shifactant revealed a significant LTM defect (Figure 2J). No defects were apparent at the permissive temperature (Figure S2B). These data indicate that dopaminergic neurons in βs2m and/or γs5 are required for nutrient-dependent LTM formation.

We next visually screened for GAL4 lines with expression in PAM dopaminergic neurons that innervate the horizontal mushroom body lobes. We used these and three established PAM lines [3, 4, 18] to express UAS-shifactant and tested LTM following sucrose-reinforced learning. Blocking 0273, R58E02, or R15A04 neurons during training significantly impaired LTM performance compared to the relevant controls (Figure 3A). In contrast, blocking 0279, 0804, R87D06, or R56H09 neurons did not. No significant defects were apparent when R15A04;UAS-shifactant, R58E02;UAS-shifactant, or 0273;UAS-shifactant flies were trained and tested at permissive 23°C (Figure S2C).

0273 and R58E02 label ~130 and ~90 dopaminergic neurons, respectively, that broadly innervate the horizontal lobes (Figures 3B and 3C; [3, 4]). R15A04 expresses in ~26 dopaminergic neurons projecting to γs1, βs1, βs2, γs2m, and γs5 (Figures 3D, S2D, and S3K). These overlap with 0104 in βs2 and γs5. Ineffective GAL4 lines further refine necessary nutrient-reinforcing neurons (Figures S3 and S4A–S4C; Table S1). Briefly, 0279-GAL4 dopaminergic neurons innervate βs1 and βs2 (Figure 3E; [18]). 0804-GAL4 innervates βs1 and γs5 (Figures 3F, S2E, and S3L), R87D06-GAL4 project to γs1 and γs1 (Figures 3G, S2F, and S3M), and R56H09-GAL4 innervate βs2m and γs5 (Figures 3H, S2G, and S3J). These negative data indicate that βs2 and γs5 innervation is dispensable (Figures 3A, 3E–3H, and S4C). Therefore, we conclude that nutrient reinforcement requires dopaminergic neurons innervating γs5 (Figure 3I).

Artificially activating large groups of 0273 or R58E02 dopaminergic neurons paired with odor formed robust appetitive memory [3, 4]. We therefore tested for subsets that were sufficient to reinforce LTM (Figure 3J). We combined each GAL4 with UAS-dTrpA1 and paired dTrpA1-activating 33°C with odor. Surprisingly, 0273, R58E02, 0104, 0279, R15A04, 0804, and R87D06 produced LTM performance that was statistically different to their relevant control flies, whereas R56H09 did not (Figure 3J). Notably, 0279, 0804, and R87D06 neurons, which were not required for sucrose LTM, reinforced artificial 24-hr memory. These and all other LTM-competent lines (0273, R58E02, 0104, R15A04) include dopaminergic innervation of the βs1 or adjacent γs1 lobe (Figures 3B–3G, S1B, S1E, and S4C; Table S1), whereas those that cannot implant LTM lack projections to these regions (R4BD04, R56H09; Figures 1C–1E, 3H, S1A, S1C, S1D, S3J, and S4C; Table S1). Therefore, we conclude that artificial LTM can be formed by dopaminergic neurons innervating γs1, βs1, and βs2 (Figure 3K).

Given the discordance between the γs5 neurons required for sucrose LTM and those targeting βs1, βs2, and γs5 that can reinforce persistent memory, we tested the food relevance of implanted memories. Expression of sugar-reinforced memory, but not water-reinforced memory, can be suppressed by feeding flies after training [5]. Feeding suppressed LTM performance of 0104;UAS-dTrpA1, R15A04; UAS-dTrpA1, 0804;UAS-dTrpA1, and R87D06;UAS-dTrpA1 flies to levels that were statistically indistinguishable from their respective fed controls (Figure 4A). In contrast, significant performance remained in 0279;UAS-dTrpA1 and 0273;UAS-dTrpA1 flies. Therefore, memory reinforced in the βs1 and adjacent γs1 regions by 0104, R15A04, 0804, and R87D06 neural activation mimics sucrose-reinforced memory, whereas 0279-implanted memory has different properties.

Although flies ordinarily need to be hungry to form sugar-reinforced appetitive memory, prior experiments and those here demonstrate that appetitive STM can be formed in fed flies by pairing octopaminergic or dopaminergic neuron activation with odor presentation (Figure S1F; [3, 4]). We therefore tested whether nutrient-dependent LTM could also be formed artificially in food-satiated flies. We analyzed food-relevant 0104-formed and R87D06-formed memory and non-food satiable 0279-formed and 0273-formed memory in parallel. Strikingly, 0104, R87D06, and 0279 activation did not form LTM in satiated flies, whereas 0273;UAS-dTrpA1 flies exhibited robust LTM (Figure 4B), which was even evident following 7 days of ad libitum feeding after training (Figure 4C). Satiety therefore also constrains the artificial formation of appetitive LTM. We speculate that some 0273 dopaminergic neurons represent rewarding events other than food.

Taken with prior studies [4, 7, 10], results here demonstrate that the sweet taste and nutrient properties of sugars are independently processed and reinforce memories of different duration. Sweet taste is transduced through octopaminergic neurons whose released octopamine, via the OAMB receptor, activates dopaminergic neurons that project to the βs2m and γs5 regions of the mushroom body. Octopaminergic reinforcement also modulates the state dependence of STM via the
Figure 3. Dopaminergic Neurons Required for Nutrient-Dependent LTM Differ from Those that Can Artificially Implant LTM

(A) Blocking output from 0273, R58E02, and R15A04 neurons during sucrose training significantly disrupted LTM in starved flies (all $p < 0.001$, ANOVA, $n \geq 9$). See permissive temperature control in Figure S2C. LTM was not statistically impaired by blocking 0279, 0804, R87D06, or R56H09 neurons ($p > 0.7$, ANOVA, $n \geq 8$).

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OCTβ2R receptor that is required in the dopaminergic MB-MP1 neurons [4]. Nutrient-dependent LTM does not involve octopamine [4, 10] or sweet-taste-reinforcing dopaminergic neurons. Nutrient reinforcement instead requires dopaminergic neurons innervating γ5b of the mushroom body, whereas those going to β1, β2, and the adjacent α1 region are sufficient. More work will be required to understand this distributed process, which apparently has an immediate and delayed dynamic (Figure S4D; [7, 10]).

Whereas formation and expression of sweet-taste-reinforced STM is insensitive to satiety state, artificial formation and expression of nutrient-relevant memory require flies to be hungry. Even direct stimulation of the relevant rewarding dopaminergic neurons cannot implant appetitive LTM in food-satiated flies. These experiments suggest that hunger establishes an internal state that permits the nutrient-reinforcing signals to be effective. It will be interesting to understand what the permissive state involves and where it is required. Others have previously described a role for CRTC in enabling hunger-dependent LTM in the fly [24] and promoting persistent memory in the mouse [25]. It therefore seems plausible that such a mechanism might be required in the mushroom body neurons to permit nutrient-dependent reinforcement.

Experimental Procedures

Fly Strains

Fly stocks were raised on standard cornmeal food at 25°C and 50%–60% relative humidity. The wild-type Drosophila strain used in this study is Canton-S. The TbhM18 mutant is described [26]. The UAS-mCD8::GFP, the 20xUAS-6xGFP, and the 247-lexA,lexAop-RFP flies are described [17, 19, 27]. The UAS-oambRNAi (strain number 2861GD) was obtained from the Vienna Drosophila Resource Center (VDRC) [14]. The UAS-shyRNAi, on the first and third chromosome, and UAS-dTrpA1 transgenic strains are described [15, 16]. The R48B04, R15A04, R87D06, and R56H09 flies [13] were obtained from Bloomington. The R56H09-LexA, R56H02–GAL80, 0104, 0273, and 0279 flies are described [3, 4, 18]. The 0804 fly strain, more correctly named PBac(IT.GAL4)0804, was generated and initially characterized by Marion Sillies and Daryl Gohl as part of the InSITE collection [28]. The

(B–H) Mushroom body lobe (red) innervation of 0273-GAL4 (B) [4], R58E02-GAL4 (C) [3], R15A04-GAL4 (D), 0279-GAL4 (E) [18], 0804-GAL4 (F), R87D06-GAL4 (G), and R56H09-GAL4 (H) revealed with UAS-mCD8::GFP. The mushroom body (red) is labeled in each brain with 247-lexA::VP16-driven lexAop-rCD2::mRFP [19]. Scale bars represent 20 μm. Zonal innervation of each line is shown in the corresponding inset illustration.

(l) Summary. Dopaminergic neurons innervating γ5b are essential to reinforce nutrient-dependent LTM.

(J) Pairing odor with dTrpA1 activation of 0273, R58E02, 0104, 0279, R15A04, 0804, and R87D06 dopaminergic neurons forms significant LTM (all p < 0.0001, ANOVA, n ≥ 6). No significant memory was formed in R56H09-UAS-dTrpA1 flies (p > 0.3, ANOVA, n ≥ 10). All flies were food deprived before and after training.

(K) Summary. Dopaminergic neurons innervating α1, β1, or β2 (and perhaps β1 and γ5b) are sufficient to form LTM.
To visualize native GFP or mRFP, we collected adult flies 2–11 days after Imaging performed using PRISM (GraphPad Software). Overall ANOVA was followed by a ZIF Marie Curie 2-year fellowship. M.K. and S.L. were funded by a Wellcome Trust Senior Research Fellowship in the Basic Biomedical Sciences and by funds from the Gatsby Charitable Foundation and Oxford Martin School.

Behavior Experiments

Appetitive memory was assayed as described [22] with the following modifications. Mixed sex populations of 4- to 8-day-old flies raised at 25°C were tested together in all behavior experiments. Before training, groups of ~100 flies were food deprived for 18–22 hr in a 25-mL vial containing 1% agar and a 20 × 60 mm piece of filter paper. Training was performed with either saturated sucrose or 3 M arabinose as unconditioned stimulus. The odors used were 3-octanal (Sigma) and 4-methylcyclohex- anol (Sigma) at 1:1,000 in mineral oil. Artificial memory implantation experiments using UAS-dTrpA1-mediated neuronal activation were performed as described [1]. Briefly, 8- to 11-day-old flies raised at 20°C were either kept in food vials or starved for 18–22 hr before training. Flies were presented with one odor at the permissive 23°C for 2 min in filter paper-lined tubes and were then transferred into a prewarmed filter paper-lined tube and trained immediately with a second odor at dTrpA1-mediated neuronal activation was performed as described [1].

Imaging

To visualize native GFP or mRFP, we collected adult flies 2–11 days after eclosion, and brains were dissected in ice-cold 4% paraformaldehyde solution in PBS (1.86 mM NaH₂PO₄, 8.41 mM NaHPO₄, and 175 mM NaCl) and fixed for an additional 60 min at room temperature [19]. Samples were then washed 3 × 10 min with PBS containing 0.1% Triton X-100 (PBT) and 2 × 10 min in PBS before mounting in Vectashield (Vector Labs). Imaging of frontal brain views was performed on a Leica TCS SP5 X and a Zeiss LSM 510. The resolution of the image stacks was 1024 × 1024 with 0.5–1.5-μm step size and a frame average of 4. Images were processed in AMIRA 5.3 (Mercury Systems) and Fiji. The immunostaining against TH and GFP was performed as previously described [4].

Supplemental Information

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.01.036.

References


