Drosophila Learn Opposing Components of a Compound Food Stimulus

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Summary

Dopaminergic neurons provide value signals in mammals and insects [1–3]. During Drosophila olfactory learning, distinct subsets of dopaminergic neurons appear to assign either positive or negative value to odor representations in mushroom body neurons [4–9]. However, it is not known how flies evaluate substances that have mixed valence. Here we show that flies form short-lived aversive olfactory memories when trained with odors and sugars that are contaminated with the common insect repellent DEET. This DEET-aversive learning required the MB-MP1 dopaminergic neurons that are also required for shock learning [7]. Moreover, differential conditioning with DEET versus shock suggests that formation of these distinct aversive olfactory memories relies on a common negatively reinforcing dopaminergic mechanism. Surprisingly, as time passed after training, the behavior of DEET-sugar-trained flies reversed from conditioned odor avoidance into odor approach. In addition, flies that were compromised for reward learning exhibited a more robust and longer-lived aversive-DEET memory. These data demonstrate that flies independently process the DEET and sugar components to form parallel aversive and appetitive olfactory memories, with distinct kinetics, that compete to guide learned behavior.

Results and Discussion

DEET has been reported to drive aversive behavior in flies through olfactory [10–12] and gustatory [13] pathways. We therefore used a low concentration presented in solid medium (1% agar) to decrease the effects of volatile DEET and increase the chance that flies would taste and perhaps ingest it. To further encourage flies to sample DEET, we increased its palatability by adding it to a mixture of sweet sugars—3 M xylose and 100 mM sucrose (from here on referred to as “carrier”). Xylose is detected by sweet-sensitive gustatory neurons and is palatable to flies, but it contributes no measurable nutrient value [14]. The low concentration of sweet and nutritious sucrose was added to further increase palatability [14]. We first determined the optimum DEET concentration by adding increasing amounts to sugar carrier and conditioning hungry flies by pairing the exposure of the second of two odors with DEET presentation.

Flies trained with only the sugar carrier showed a significant appetitive memory (Figure 1A). In contrast, those trained with increasing amounts of DEET formed aversive memory, with the score rising in line with the increase in DEET concentration, up to 0.4%. Surprisingly, flies trained with 0.8% DEET did not exhibit significantly negative aversive memory scores, suggesting a change in the flies’ perception of DEET at this concentration. We therefore tested the effect of 0.4% and 0.8% DEET on fly feeding by measuring ingestion marked with blue food dye (Figure 1B). Whereas flies ate a significant amount of dye with 0.4% DEET, ingestion was abolished with 0.8% DEET. These data suggest that the failure to train flies with 0.8% DEET reflects an inhibition of sampling by the proboscis and perhaps ingestion of DEET and sugar. To further test a requirement for feeding in learning, we attempted to train flies that were not hungry or with 0.4% DEET without sugar carrier (Figure 1C). Both of these conditions significantly impaired aversive learning when compared to hungry flies trained with 0.4% DEET in sugar carrier. We also observed a similar concentration-dependent aversive memory formation when flies were trained with bitter-tasting quinine that was mixed with sugar carrier (Figure S1A available online). Furthermore, flies that were defective in the Ir40a olfactory route of DEET detection displayed normal DEET learning (Figure S1B). We therefore conclude that robust learning with 0.4% DEET-laced sugar requires the flies to attempt to eat DEET and that low DEET concentrations convert the conditioned approach that is formed when flies are trained with the sugar carrier into a conditioned aversion.

We next measured the persistence of DEET memory by conditioning flies and testing their odor preference at extended times after training (Figure 1D). Whereas aversive memory performance was robust immediately after training, no statistically significant performance was evident 15 min later. Aversive memory formed with 0.4% DEET is therefore surprisingly labile. DEET and quinine can be sensed by bitter-taste neurons [13, 15, 16], and ablation of bitter-sensing neurons with Gr66a-GAL4-directed expression of cell-death genes [17] partially impaired DEET, but not sugar, learning (Figures 1E and S1D). We therefore tested whether flies could be aversively conditioned by pairing odor presentation with artificial bitter-taste neuron [16, 18] activation, achieved by expression of UAS-dTrpA1 (Figure 1F). The dTrpA1 gene encodes a transient receptor potential (TRP) channel that conducts Ca2+ and depolarizes neurons when flies are exposed to temperature >25°C [19]. Gr66a-GAL4, UAS-dTrpA1, and Gr66a-GAL4; UAS-dTrpA1 flies were conditioned by presentation of the first odor with activating 32°C and were immediately tested for memory. Gr66a-GAL4; UAS-dTrpA1 flies exhibited aversive memory that was statistically different from that of all other groups (Figure 1F). However, unlike flies conditioned with DEET (Figure 1D), significant memory remained 3 hr after training (Figure 1F). The differing persistence could result from artificial stimulation of bitter neurons being stronger than DEET activation, in addition to lacking plausible competition from a copresented sugar stimulus.

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Octopamine is required to convey the reinforcing effects of sweet taste [9]. We therefore tested DEET learning in Tbhm18 mutant flies that cannot synthesize octopamine [20] (Figure 2A). Whereas appetitive conditioning with 1 M sucrose was significantly impaired in Tbhm18 flies, aversive learning with 0.4% DEET was indistinguishable from that of wild-type flies. Therefore, octopamine is not required for DEET learning.

Electric-shock-reinforced aversive memory formation also requires specific dopaminergic neurons and the DopR1 dopamine receptor [5–7, 21]. We therefore first determined whether DEET learning required the DopR1 receptor (Figure 2B). Mutant dumb1 flies that are defective for the DopR1 dopamine receptor did not display aversive learning with DEET. Similarly, aversive learning with artificial activation of bitter-taste neurons was abolished in dumb1 flies (Figure 2C).
Figure 2. DEET Reinforcement Involves Specific Dopaminergic Neurons

(A) DEET learning does not require octopamine. Appetitive memory formation with 1 M sucrose was significantly impaired in \( Tbh^{MD6} \) flies (\( p < 0.05, t \) test, \( n = 4 \)), whereas aversive memory with DEET was statistically indistinguishable from that of control flies (\( p > 0.5, t \) test, \( n = 6 \)).

(B) DEET memory formation was significantly impaired in \( dumb^1 \) mutant flies (\( p < 0.001, t \) test, \( n = 10 \)).

(legend continued on next page)
The MB-MP1, MB-MV1, and MB-M3 classes of dopamine neuron have been previously implicated in shock learning [6, 7] (Figure 2D). To test whether either of these neurons were required for DEET learning, we expressed the dominant temperature-sensitive UAS-shibirets1 transgene [22] in MP1, MV1, and M3 neurons using the c061; MBGAL80, R73F07, and NP5272 and NP1528 GAL4 drivers [7, 23, 24], respectively. The shi ts1 transgene permitted blockade of the respective neurons by performing DEET conditioning experiments at the restrictive temperature of 31°C. This analysis revealed significantly impaired DEET learning performance when MP1 neurons were blocked (Figure 2E) but nonsignificant effects when either MV1 (Figure 2F) or M3 (Figure 2G) neurons were compromised. Blockade of MP1 neurons, however, did not significantly affect DEET avoidance in naive flies (Figure S2).

To further support a role for the dopaminergic MP1 neurons in c061; MBGAL80, we removed them from the expression pattern by including a TH-GAL80 transgene [25]. When the remaining cells were blocked during conditioning, flies exhibited levels of DEET learning that were indistinguishable from those of wild-type flies (Figure 2H). We therefore conclude that MP1 neurons are critical for DEET learning, whereas MV1 and M3 neurons contribute a lesser role. We note that prior work implicated the MV1 and M3 neurons in the formation of more persistent forms of shock-reinforced aversive memory [8, 7].

Next we used live imaging to determine whether DEET ingestion activated the MP1 dopamine neurons. We expressed UAS-GCaMP3 [26] in dopaminergic neurons with TH-GAL4 [27] and imaged DEET-evoked changes in fluorescence in the dopaminergic neuron processes on the mushroom body (Figure 2). These analyses revealed strong activation of the MP1 innervated heel and MV1 innervated junction regions of the mushroom body while presenting flies with 0.4% DEET in sugar carrier, sugar carrier alone, and DEET alone. In comparison, water presentation did not activate the MP1 and MV1 neurons. Therefore, functional imaging does not reveal obvious valence specificity of MP1 and MV1 signals, being activated by both sugar and DEET. It should be noted that the MP1 neurons have been previously implicated in shock- and sugar-reinforced learning and memory expression [8, 7, 9, 23]. Since we observed a strong requirement for MP1 neurons in behavioral DEET learning (Figures 2E and 2H), we conclude that MP1 activity is likely to represent aversive reinforcement signals to mushroom body neurons. As expected, transmission from mushroom body neurons is required for the expression of DEET memory (Figure S3).

Finding a role in DEET learning for dopamine neurons that are also required for shock learning [6, 7] suggests a common reinforcement process, despite the different nature of the external unconditioned stimulus. We therefore designed a differential conditioning paradigm to further test this model. Flies were trained by pairing of one odor with DEET and the other odor with a varying intensity of electric shock. These experiments revealed an avoidance of the previously DEET-associated odor when countered with 30 or 60 V but an avoidance of the shock-paired odor when countered with 80 or 90 V (Figure 3A). Extrapolation of a curve fit between the tested points predicted 70 V as being equivalent to 0.4% DEET—which was subsequently confirmed in direct experiments (Figure 3B). Having established the point of reinforcer equivalence, we reasoned that if the shock and DEET reinforcement processes were common, blocking some of the responsible dopamine neurons would equally impair shock and DEET learning and therefore not alter equivalence. If, on the other hand, MP1 neurons contribute differently to DEET and shock reinforcement, we expected to see that blocking them would unevenly affect learned behavior and would skew performance toward one or the other, reflecting the imbalance. Strikingly, differential learning remained balanced in c061; MBGAL80; UAS-shi ts1 flies in which MP1 neurons were blocked. Importantly, this balanced valuation does not reflect a “zero versus zero” learning because the same c061; MBGAL80; UAS-shi ts1 flies only display a partial defect if they were trained with 70 V shock alone (Figure 3C). Therefore, these experiments support a model in which the reinforcing systems for 0.4% DEET and 70 V shock are similar, with MP1 being part of the system for both. In addition, it is notable that despite the relative magnitude of immediate memory scores (~0.6 for 70 V shock and <0.3 for DEET) and the difference in respective memory persistence (hours for shock and minutes for DEET), the immediate learned value of these two aversive stimuli is comparable.

We next investigated whether the apparent fragility of aversive DEET memory could be explained by the coformation of a more persistent sugar memory. Reasoning that these analyses would benefit from the induction of a more robust sugar memory, we first established optimal conditions for aversive memory formation with DEET-laced 1 M sucrose. Flies trained with
DEET in 1 M sucrose showed a similar dose-dependent aversive learning to those trained in prior experiments with DEET in xylose and sucrose carrier, although the optimal DEET concentration for learning shifted from 0.4% to 0.6% (Figure 4A). We next tested the DEET memory performance of *TbhM18* mutant flies that are impaired in appetitive learning. Strikingly, whereas the behavior of wild-type flies became conditioned approach within 30 min, *TbhM18* flies showed a more persistent aversive memory performance, with scores remaining significantly negative 30 and 60 min after training (Figure 4B). However, the performance still converted from odor avoidance to approach by 24 hr. Since octopamine only provides short-term sweet-taste reinforcement [9], we hypothesized that persistent nutrient-dependent memory must be independently formed in *TbhM18* flies. Indeed, *TbhM18* flies trained with 1 M sucrose did not display immediate memory, but significant avoidance (Figure 4E). In contrast, blockade of the negatively reinforcing MB-MP1 dopaminergic neurons with c061; MBGAL80; UAS-sh*t1* uncovered significant conditioned odor approach performance (Figure 4F). We therefore conclude that training with the compound DEET and sugar stimulus leads to the independent formation of aversive and appetitive memories. The differing stability of these competing memories subsequently determines which one of them guides learned behavior after training.

The extent to which rewarding and aversive stimuli are coded in mammalian dopaminergic neurons is hotly debated [2, 28, 29]. Recordings in the monkey have shown that some dopaminergic neurons respond to either bitter taste or an aversive air puff [30], suggesting that the quality of an aversive reinforcer may be represented. Work in flies has functionally split dopaminergic neurons into groups that are critical for reward performance emerged 1 hr after training and remained for at least 24 hr (Figure 4C). These data support the prior model of octopamine specifically conveying short-term appetitive reinforcement and not the nutrient-dependent long-term signal [9]. In addition, they suggest that our DEET learning protocols form parallel aversive and appetitive memories. To further test a parallel memory trace model, we trained flies with 0.3% DEET and 1 M sucrose, a combination with which no immediate odor avoidance or approach performance is evident, and blocked either the rewarding or aversive dopaminergic neurons during training (Figures 4D–4F). Strikingly, blockade of the rewarding dopaminergic neurons with 0104; UAS-sh*t1* revealed significant conditioned avoidance (Figure 4E). In contrast, blockade of the negatively reinforcing MB-MP1 dopaminergic neurons with c061; MBGAL80; UAS-sh*t1* uncovered significant conditioned odor approach performance (Figure 4F).
learning and others for aversive learning [3, 5–9]. However, recent studies suggested a requirement for modulation of the aversive system in appetitive learning [9] and demonstrated a role for rewarding dopaminergic neurons in relative aversive learning [31]. In addition, imaging activity in negatively reinforcing MB-MP1 neurons revealed responses to both sweet sugar and bitter DEET. Nevertheless, the DEET reinforcement data presented here, when taken with published knowledge of shock reinforcement [7], imply that flies utilize the same, or at least an overlapping, evaluation system to convey the reinforcing effects of discrete aversive stimuli. It will be interesting to determine the respective input pathways to the negatively reinforcing dopaminergic neurons. These experiments also highlight the importance of being able to both record from and control recognizable subpopulations of dopaminergic neurons. Without intervention, it is difficult to understand whether a given dopaminergic neuron provides a reinforcement or motivational salience [2] signal.

Perhaps most surprisingly, our data demonstrate that during learning flies independently assign the value of individual components of a compound food stimulus to an odor. Rather than forming a single memory of the relative quality of the tainted sugar, they learn the bitter and sugar components in parallel. This multiplexing is further illustrated by sugars in which octopamine distinguishes between memories of sweet taste and nutrient components [9]. These results suggest that despite the integration of tastant information that occurs within the first layers of the gustatory system [15, 16, 32, 33] and provides control over food ingestion, each component also gains unprocessed access to the negative and positive arms of the reinforcement system. The fly therefore appears to retain as much information of foraging history as possible,
Experimental Procedures

Details of all experiments are provided in the Supplemental Experimental Procedures.

Fly Strains

Flies were raised on cornmeal food at 25°C and 40%–50% relative humidity. The wild-type Canton-S [23], c061; MBGAL80 [23], food sources that happen to be unpalatable but remember Learning Components of Tainted Food Flies were raised on cornmeal food at 25°C.

Behavioral Analysis

Mixed-sex populations of 6- to 9-day-old flies were tested together in all experiments. For DEET training, groups of ~100 flies were food deprived for 22–28 hr in vials containing 2–3 ml 1% agar and filter paper. Liquid DEET was diluted to the appropriate final concentration with a given sugar solution in 1% molten agar (in water) and shaken to create a suspension and spread onto filter paper. Dried papers were rolled into training tubes. Training paradigms are indicated in the figure legends.

Two-Photon Imaging was performed with a custom-tical genotype flies are trained with the reciprocal reinforced/non-reinforced number going the other direction, divided by the total number of flies in the experiment. A single PI is the average of two experiments in which identical genotype flies are trained with the reciprocal reinforced/non-reinforced odor combination. Odors were 3-octanol and 4-methylcyclohexanol.

Statistical Analysis

Statistical analyses were performed with PRISM (GraphPad Software) and are specifically referenced in the figure legends.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.05.076.

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