

Competing dopamine neurons drive oviposition choice for ethanol in *Drosophila*

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The neural circuits that mediate behavioral choice evaluate and integrate information from the environment with internal demands and then initiate a behavioral response. Even circuits that support simple decisions remain poorly understood. In *Drosophila melanogaster*, oviposition on a substrate containing ethanol enhances fitness; however, little is known about the neural mechanisms mediating this important choice behavior. Here, we characterize the neural modulation of this simple choice and show that distinct subsets of dopaminergic neurons compete to either enhance or inhibit egg-laying preference for ethanol-containing food. Moreover, activity in $\alpha'\beta'$ neurons of the mushroom body and a subset of ellipsoid body ring neurons (R2) is required for this choice. We propose a model where competing dopaminergic systems modulate oviposition preference to adjust to changes in natural oviposition substrates.

In nature, rotting fruit is the social hub for the fruit fly *Drosophila melanogaster*. Flies use fermenting fruit as a food source (1) and a site for oviposition (2). The choice of a suitable oviposition substrate is an ecologically important decision with a direct impact on species fitness. However, other than having a clear preference for fermenting fruit, how females choose oviposition sites in nature is largely unknown.

One of the main metabolites of fermentation is ethanol, which is present in ripe fleshy fruits (3). Although ethanol concentrations in the fruit are rather low [$\leq 5\%$ (vol/vol)] (4), plumes containing ethanol vapor can act as long-distance signals to attract flies to rotting fruit (3, 5). When given the choice, female flies prefer to lay their eggs on media containing low concentrations of ethanol (up to 5%) (6), which leads to enhanced fitness of the developing larva and the adult fly.

D. melanogaster's resistance to ethanol toxicity may have evolved to allow inhabitation of ethanol-containing environments (7). For example, adult flies allowed to mate on ethanol-containing media improve mating success and fecundity (8). Although rearing larvae on food containing relatively high ethanol concentrations delays development and decreases survival (9–11), larvae reared on low concentrations of ethanol develop into heavier adults (7, 12). This weight increase may be a result of *D. melanogaster* larvae metabolizing ethanol and using it as a food source (12). Ingestion of ethanol during the larval stage has additional benefits, such as protection from natural parasites such as endoparasitoid wasps (13).

Studies on the neural circuits underlying the oviposition program and choice of oviposition substrates have been initiated only recently in *D. melanogaster* (14, 15). Ethanol is a particularly intriguing stimulus for oviposition preference, because it has, depending on concentration, both beneficial and detrimental effects on developing larvae. In flies, the function of dopaminergic neurons has been implicated in responses to both rewarding and aversive stimuli (16–19), making it a candidate neuromodulator to signal the beneficial and detrimental effects of ethanol. Dopamine signaling has also been implicated in other innate behaviors required for survival, such as food consumption, sex, and social interaction (20–22). Although the circuitry for ethanol oviposition preference is unknown, both dopaminergic and mushroom body (MB) signaling is required for flies to remember ethanol as a reward (23).

We report that females show concentration-dependent preference for oviposition on food containing ethanol, with highest preference for food substrates containing the most ecologically beneficial concentrations of ethanol. This simple behavior relies on functional dopamine neurons, with different subsets regulating oviposition site preference in opposite ways. Our data also suggest that dopaminergic innervation of higher-order brain regions, including specific subsets of the MB and ellipsoid body (EB) neurons modulate the decision to lay eggs on ethanol.

Results

Flies Show Oviposition Preference for Ethanol-Containing Media. To measure oviposition preference, we used a simple assay in which flies choose between ethanol-containing food and regular food (Fig. 1A). We tested preference for concentrations of ethanol ranging from 0% to 10% and found that flies preferred substrates containing ethanol at all concentrations tested (Fig. 1B). There was a trend toward lower total numbers of eggs laid on plates containing higher concentrations of ethanol (Fig. 1C) known to be detrimental to larval development. Although high concentrations of ethanol (>5%) have negative effects on larval development (11) and decrease fecundity (8), females still preferred to lay eggs on food containing high ethanol concentrations over food lacking ethanol. To determine the optimal ethanol concentration for egg-laying, we presented flies with an arena containing six different concentrations of ethanol ranging from 0% to 10% (Fig. S1A). Similar to our two-choice assay, flies preferred to lay eggs on higher ethanol concentrations (Fig. S1B).

Oviposition Preference Depends on the Context of the Egg-Laying Substrate. The above results show that *Drosophila* females prefer to lay their eggs on ethanol concentrations that could potentially be toxic to developing larvae. However, oviposition decisions in *Drosophila* are also dependent on the surrounding

Significance

Flies use fermenting fruit as a food source and a site for oviposition. One of the main metabolites of fermentation is ethanol. When provided with different choices in the laboratory, female flies prefer to lay their eggs on food supplemented with ecologically relevant concentrations of ethanol. We show that different subsets of dopaminergic neurons have opposing effects on oviposition preference. We propose that in the wild, this may be a mechanism by which flies choose oviposition sites that are optimal for offspring fitness and survival and that this choice is highly dependent on context.

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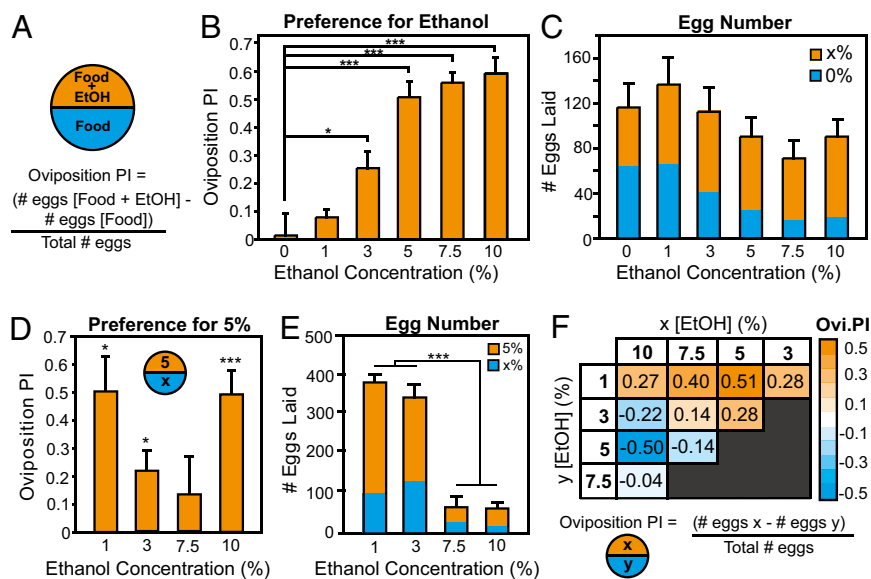


Fig. 1. Flies prefer to lay their eggs on ethanol-containing food. (A) Cartoon of egg-laying assay. (B) Given the choice between food with and without ethanol, flies preferred to lay eggs on ethanol [ANOVA: $F_{(5,71)} = 25.36$, $P < 0.0001$; $n = 13-16$; Tukey's post hoc comparisons: for 0% vs. 3%, $P = 0.04$; for 0% vs. 5%, 7.5%, or 10%, $P < 0.0001$]. (C) The total number of eggs laid is not significantly affected by the presence of ethanol [ANOVA: $F_{(5,66)} = 1.46$, $P = 0.22$; $n = 11-13$]. (D) Flies preferred to lay their eggs on 5% ethanol over 1% [Wilcoxon: $\chi^2_{(1,8)} = 6.05$, $P = 0.01$], 3% [$\chi^2_{(1,8)} = 6.05$, $P = 0.01$], or 10% [$\chi^2_{(1,18)} = 16.31$, $P < 0.0001$] ethanol. There was no preference between 5% and 7.5% ethanol [$\chi^2_{(1,10)} = 0.48$, $P = 0.49$]. (E) The number of eggs laid on ethanol concentrations lower than 5% was significantly greater than those laid on concentrations higher than 5% [ANOVA: $F_{(3,26)} = 249.45$]. (F) Oviposition preference depends on the context of the oviposition substrate. Two-choice tests between 1%, 3%, 5%, 7.5%, and 10% ethanol suggest that concentrations near 5% ethanol are preferred. Preference indices for all pairwise comparisons are summarized. Detailed data are shown in Fig. S2. Bars on graphs represent means \pm SEM. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

environment onto which first-instar larvae can migrate (24). Thus, flies may lay eggs on potentially toxic ethanol concentrations only when low concentrations of ethanol beneficial to young larvae are found nearby. To test this, we provided flies with two-choice tests between various combinations of ethanol concentrations. Given a choice between 5% and 1% or 5% and 10%, flies showed a strong preference for 5% ethanol (Fig. 1D). Weaker preference for 5% ethanol was obtained when flies were given choices of 3% and 5% or 7.5% and 5% (Fig. 1D). In addition, flies reduced overall egg-laying when given choices of 5% and 7.5% ethanol or 5% and 10% ethanol (Fig. 1E). Given a choice between various ethanol concentrations ranging from 3% to 10% and 1% ethanol, flies always preferred the higher concentrations (Fig. S2 A–C). However, when presented with choices between either 3% and 10% or 5% and 10% (Fig. 1D and Fig. S2 D–F), flies chose the lower ethanol concentrations. Thus, it seems that 3–5% ethanol may be an optimal oviposition substrate. When choosing between 3% and 5%, however, flies preferred 5% (Fig. 1D and Fig. S2 D–F), suggesting that 5% is the highest concentration of ethanol that may be beneficial.

Taken together, these results suggest that flies evaluate the context of the egg-laying environment to define their preference, which is highest at concentrations that are beneficial to larvae. However, their preference is highly sensitive to ethanol concentrations present in nearby substrates. These results also show that flies can clearly discriminate between subtle differences in ecologically beneficial ethanol concentration. For example, flies show a significant preference for 3% over 1% and 5% over 3% ethanol food (Fig. 1F and Fig. S2 A–F), but no significant preference for 5% over 7.5% or 7.5% over 10% ethanol-containing food (Fig. 1F and Fig. S2 G–L).

Flies Do Not Show Positional Preference for Ethanol. Despite the strong drive to lay eggs on a suitable substrate, the flies' choice to dwell on this substrate is uncorrelated. For example, whereas flies prefer to lay eggs on a food containing either acetic acid or lobeline, they show positional avoidance of the same substrates (15, 25). To test whether this was also the case for ethanol, we tested the relationship between oviposition and positional preference (0% vs. 5%; Fig. S3A). Flies preferred to be on ethanol-containing food only while laying most of their eggs, within the first 50 min of the assay (Fig. S3B), but showed no positional preference afterward (Fig. S3C). Thus, flies show neither positional preference nor avoidance for ethanol-containing food,

and the drive to lay eggs on ethanol can be dissociated from positional preference.

Flies Use Smell and Taste to Determine Oviposition Preference. Flies show preference for the odor of ethanol (Fig. S4A). However, although removing antennae eliminated olfactory preference, it did not eliminate oviposition preference for 5% ethanol, suggesting that other sensory modalities are involved (Fig. S4 B–D). Surprisingly, abolishing the ability to taste altogether or in subsets of chemoreceptor neurons (using *pox neuro* alleles) (26) resulted in oviposition aversion for ethanol-containing food (Fig. S4E). Removing the ability to smell and taste decreased, but did not abolish, this aversion, suggesting that another sensory modality may also be involved (Fig. S4F). Clearly, the sensory input leading to ethanol preference is complex and requires more study.

Dopamine Affects Oviposition Preference for Ethanol. In the *Drosophila* central brain, subsets of dopaminergic cell bodies form discrete clusters (Fig. 2A). Each dopaminergic neuron sends extensive arborizations throughout the central brain, suggesting a complex modulatory function (27, 28) (Fig. S5A). To ask whether dopamine neurons play a role in oviposition preference for 5% ethanol, we used the GAL4/UAS system (29) to express tetanus toxin light chain (*UAS-TeTx*) to block synaptic transmission (30) in tyrosine hydroxylase (TH)- and Dopa decarboxylase (*Ddc*)-expressing neurons. Expression was targeted with either *Ddc-GAL4* (31) (expressed in most dopaminergic and serotonergic neurons) or *TH-GAL4* (32) (expressed in most dopaminergic neurons; Fig. S5B). *Ddc-GAL4* and *TH-GAL4* share expression with the exception of a subset of neurons termed the PAM neurons (33) (Fig. 2 B and C).

Intriguingly, we found that blocking neurotransmission in *Ddc-GAL4* neurons decreased preference (Fig. 2B), whereas the same manipulation in *TH-GAL4* cells increased preference for 5% ethanol as an egg-laying substrate (Fig. 2C). The differences in the expression patterns of these GAL4 drivers are in (i) neurons producing serotonin (but not dopamine) and (ii) in most of the dopaminergic PAM neurons, which express *Ddc-GAL4* but not *TH-GAL4* (31, 32). Thus, our data lead to two alternate hypotheses: (i) dopamine suppresses oviposition preference, whereas serotonin enhances it; or (ii) nonoverlapping sets of dopaminergic neurons within the *Ddc*- and *TH-GAL4* patterns affect oviposition preference in opposite ways. To discriminate between these possibilities, we expressed *TeTx* in serotonergic neurons using the tryptophan hydroxylase GAL4 driver (*TRH-GAL4*, 34).

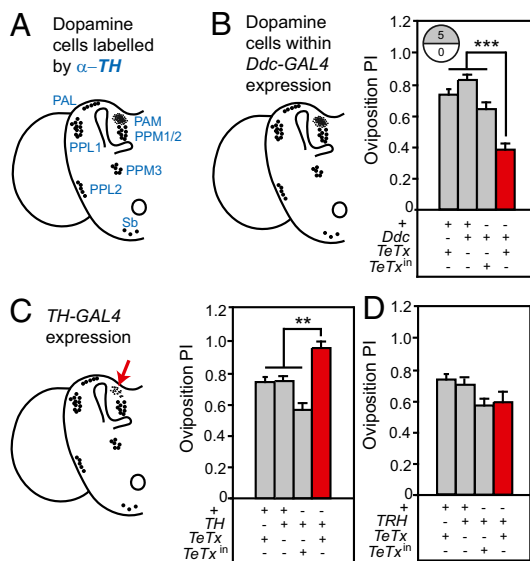


Fig. 2. Activity of dopamine and not serotonin neurons affects oviposition preference. (A) Dopaminergic neuron cell body positions in one hemisphere of the adult central brain are marked based on anti-TH immunohistochemistry (27, 28, 31, 32). PAM cell number is underrepresented in the schematic (33). (B) Cells colabeled with anti-TH antibody and *Ddc-GAL4* in the central brain (33). Disrupting neurotransmission in *Ddc* cells decreased oviposition preference [$n = 17$ –21 per strain; ANOVA: $F_{(3,73)} = 47.51$, $P < 0.0001$]. (C) Schematic of the *TH-GAL4* expression pattern in the central brain (33). See Fig. S5 for more details. Arrow highlights that most PAM neurons do not express *TH-GAL4*. Disrupting neurotransmission in *TH* cells increased oviposition preference [$n = 9$ –16 per strain; ANOVA: $F_{(3,51)} = 9.62$, $P < 0.0001$]. (D) Disruption of transmission in serotonergic neurons with *TRH-GAL4* did not disrupt oviposition preference [$n = 19$ –21 per strain; ANOVA: $F_{(2,76)} = 4.23$, $P = 0.08$; all Tukey's comparisons to *TRH/TeTx*: $P > 0.05$]. Bars on graphs represent means \pm SEM. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$. Clusters of dopaminergic neurons are named based on their location in the brain: PAM, protocerebral anterior median; PPL, protocerebral posterior lateral; PPM, protocerebral posterior median; PAL, protocerebral anterior lateral; Sb, subesophageal ganglion.

TRH-GAL4 expresses in nearly all serotonergic neurons including those expressing *Ddc-GAL4*, but not in dopaminergic neurons defined by *TH-GAL4* expression (27, 31, 34). If serotonin were required for oviposition preference for ethanol, we expected that blocking TRH neurons would suppress the response. However, it did not affect oviposition preference (Fig. 2D), supporting the hypothesis that the reduced oviposition preference observed upon silencing *Ddc-GAL4* neurons is attributable to a subset of dopaminergic neurons that do not express *TH-GAL4*.

Different Subsets of Dopamine Neurons Drive Competing Behavioral Responses. Although *Ddc-GAL4* and *TH-GAL4* are coexpressed in most dopaminergic neurons, a large subset of dopaminergic neurons within the PAM neuronal cluster expresses *Ddc-GAL4* but not *TH-GAL4* (31–34) (Fig. 2B and C; for definition of dopaminergic cell clusters see legend). These PAM neurons may thus function to enhance oviposition preference, while other dopaminergic neurons coexpressing *Ddc-GAL4* and *TH-GAL4* antagonize this effect. If so, blocking the activity of PAM neurons should suppress oviposition preference for 5% ethanol.

To test this, we blocked neurotransmission with *TeTx* in patterns specified by three GAL4 lines that drive expression in various subsets of *Ddc*-expressing cells including the PAM neurons (transgenes *HL5*, *HL7*, and *HL9*) (33, 35). Blocking neurotransmission in PAM, PAL, PPM1/2, PPL2, and Sb neurons (*HL5*) decreased oviposition preference for 5% ethanol (Fig. S6A and B). The same effect was observed upon silencing PAM neurons and subsets of PAL, PPM1/2, PPM3, PPL2, PPL1, and Sb neurons (*HL7* and *HL9*) (Fig. S6C and D). Because these

drivers are expressed in PAM neurons, which is not the case for *TH-GAL4*, these results provide additional evidence that PAM neuron activity normally enhances oviposition preference.

To confirm this, we blocked activity specifically in PAM neurons by combining *HL7-GAL4* (expressed in PAM neurons and some *TH-GAL4*-positive neurons) with the TH-specific GAL4-repressor line *TH-GAL80* (36). This led to decreased oviposition preference (Fig. 3A and Fig. S7), indicating that the PAM neurons normally enhance oviposition preference for 5% ethanol.

To identify which *TH*-expressing neurons normally suppress oviposition preference, we blocked neurotransmission with drivers that show expression in subsets of *TH*-expressing neurons. Indeed, blocking transmission in the PPM3 cluster (with GAL4 line *c346*) decreased oviposition preference (Fig. 3B). Thus, the PPM3 neurons normally enhance preference. In contrast, blocking transmission in a small subset of PPL1 neurons, the MB-MP1 neurons that project to the medial lobe and pedunculus of the MB [with GAL4 lines *NP2758* and *krasavietz (kra)*], (17) increased oviposition preference for 5% ethanol (Fig. 3C and D). Note that both *NP2758* and *kra* are expressed in other *TH*-negative neurons, but the overlap in expression is believed to be limited to the MB-MP1 PPL1 neurons (17).

To confirm that PPL1 neurons suppress oviposition preference, we expressed the temperature-sensitive cation channel *dTrpA1* (37) in the MB-MP1 PPL1 cells. This led to a significant decrease in oviposition preference for 5% ethanol (Fig. 3E and F). Taken together, these results suggest that PAM and PPM3 neurons compete with PPL1 neurons by promoting and suppressing, respectively, oviposition preference for ethanol (Table S1). Notably, we also blocked neurotransmission in PAM neurons using an alternate GAL4 driver [R58E02 (19)] and did not find an effect on behavior suggesting that PAM cluster neurons that affect oviposition preference for ethanol may not be present within the R58E02 expression pattern.

The MB and EB Mediate Oviposition Preference for Ethanol. To identify higher brain regions regulating oviposition preference, we surveyed brain structures innervated by the PAM, PPL1, and PPM3 dopaminergic neurons. PAM and PPL1 neurons innervate the MB (27). The MB consists of three major classes of neurons whose axonal branches occupy distinct lobes: the $\alpha\beta$, $\alpha\beta'$, and γ neurons (Fig. 4A) (38). PAM cluster neurons innervate different portions of the horizontal lobes, whereas the MB-MP1 neurons innervate an area of the MB peduncle occupied by the γ and $\alpha\beta$ lobes (17, 27, 39).

To determine whether the MB mediates oviposition preference, we used *TeTx* to block neurotransmission in specific subsets of MB neurons using the GAL4 drivers *MB247* (γ and $\alpha\beta$ neurons), *NP65* ($\alpha\beta$ and $\alpha\beta'$ neurons), and *4-59* ($\alpha\beta'$ neurons) (23, 38). Blocking transmission using *NP65* and *4-59*, but not *MB247*, decreased oviposition preference (Fig. 4B). Because we observed no change in preference when blocking γ and $\alpha\beta$ neurons, but saw a decrease in preference when silencing $\alpha\beta'$ neurons, we suggest that neurotransmission from these neurons is required for oviposition preference.

The remaining cluster of dopaminergic cells that affected oviposition preference, the PPM3 neurons, innervates the EB, a substructure of the central complex. *c346-GAL4* expresses in PPM3 cells that project to the EB (33). The EB is organized in a concentric pattern of glomeruli with major contributions from the projections of large-field ring or R neurons (Fig. 4C) (40, 41). A subgroup of these, the R2/R4 EB neurons, mediate both ethanol-induced hyperactivity (33) and ethanol tolerance (42, 43).

We used *TeTx* to block synaptic transmission in different subsets of EB neurons and measured oviposition preference for ethanol. Different subsets of R neurons were targeted using the *2-72* (R2, R3, and R4), *11-27* (R2, R3), *4-67a* (R2, R4), and *c232* (R3, R4) (33, 41) GAL4 drivers. Blocking synaptic transmission in the *2-72*, *11-27*, *4-67a*, but not *c232* patterns decreased oviposition preference (Fig. 4D). Because *2-72*, *11-27*, and *4-67* all express in the R2 EB neurons (33), we deduce that

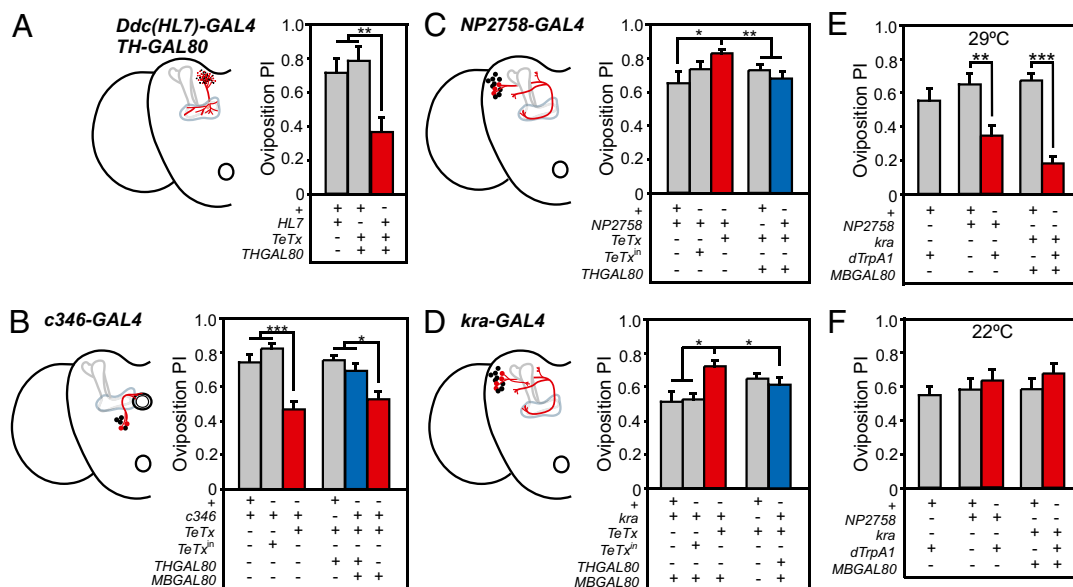


Fig. 3. Dopaminergic neurons compete to affect oviposition preference. (A–D) Schematics represent dopamine-expressing cell bodies (black dots) and a subset expressing the particular GAL4 pattern (red dots) with their respective innervation patterns (red lines) for each GAL4 line; MB is drawn in gray (A, C, and D) and EB in black (B). Nondopaminergic expression is omitted in schematics. (A) Disrupting transmission in the *HL7*-expressing PAM neurons decreased preference [$n = 9–19$ per strain; ANOVA: $F_{(2,42)} = 9.33$, $P = 0.0004$]. (B) Disrupting transmission in the PPM3 neurons (33) decreased preference [$n = 12–18$ per strain; ANOVA: $F_{(2,49)} = 20.65$, $P < 0.0001$]. Eliminating expression of TH within the *c346* expression pattern using *THGAL80* reverted the behavior to control levels, whereas eliminating MB expression using *MB-GAL80* did not. (C and D) Disrupting transmission in subsets of PPL1 neurons (17) increased preference [*NP2758*: $n = 10–12$ per strain; ANOVA: $F_{(2,30)} = 5.30$, $P = 0.01$; *kra*: $n = 20–21$ per strain; ANOVA: $F_{(2,59)} = 3.98$, $P = 0.02$]. Eliminating expression of TH within the *NP2758* and *kra* expression patterns using *TH-GAL80* reverted the behavior to control levels. (E and F) Activating the PPL1 neurons using *dTrpA1* decreases oviposition preference at 29°C [$n = 8–12$ per strain; ANOVA: $F_{(4,41)} = 20.48$, $P < 0.0001$] but not 22°C [$n = 6–15$ per strain; ANOVA: $F_{(4,49)} = 1.07$, $P = 0.38$]. Bars on graphs represent means \pm SEM. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

these neurons are necessary for oviposition preference for ethanol and propose that the PPM3 dopaminergic neurons activate R2 EB neurons to promote oviposition preference for ethanol-containing food.

To test whether the MB and EB are downstream targets of dopamine signaling, we decreased expression of either D1-type dopamine receptors, *DopR1* or *DopR2*, in the MB and EB. Decreasing expression of *DopR2*, but not *DopR1*, in the MB using RNA interference increased oviposition preference (44) (Fig. 4E). Decreasing expression of both *DopR1* and *DopR2* in the EB increased oviposition preference (Fig. 4F). This suggests that although the MB and EB are likely downstream targets of dopaminergic signaling, the role of dopamine signaling in mediating oviposition preference for ethanol is complex.

Discussion

This study characterizes *D. melanogaster*'s preferred concentration of ethanol for oviposition and uses this information to investigate neural circuits required for modulating this preference. We found that female flies are sensitive to subtle changes in ethanol concentration in the food substrate. Presumably, flies test the concentration of ethanol present in the food and then decide which substrate is most suitable for progeny fitness and survival. When flies are given a choice of food with or without ethanol, they always prefer to lay eggs on the ethanol substrates, including those containing potentially toxic concentrations. We speculate that this occurs because flies are able to evaluate the environment such that they only lay eggs on deleterious substrates when harmless alternatives are available for their mobile progeny to move toward. However, when the choice involves two different ethanol concentrations, flies prefer to lay eggs on the concentration of ethanol that is most beneficial (~5%).

Our data suggest that ethanol is sensed through multiple sensory systems, and this information is relayed through distinct sets of dopaminergic neurons (Fig. 5A). The PAM and PPM3

neurons promote oviposition preference for 5% ethanol, whereas the MB-MP1 PPL1 neurons suppress the response (Fig. 5B). A combination of anatomical and behavioral data has shown that the PAM and PPL1 dopaminergic inputs are relayed to the MB, whereas the PPM3 inputs to the EB. These signals are then transmitted by the MB $\alpha'\beta'$ neurons and the EB R2 neurons (Fig. 5B). We propose that 5% ethanol activates PAM and PPM3 neurons which, in turn, activate MB $\alpha'\beta'$ and EB R2 neurons, respectively, leading to increased preference for ethanol (Fig. 5B). PPL1 neurons, on the other hand, may inhibit MB $\alpha'\beta'$ neurons, thus resulting in reduced oviposition preference. The latter model is supported by the fact that activation of PPL1 neurons leads to strongly reduced preference.

In *Drosophila*, the innervation of the MB by dopaminergic neurons is crucial for choice behavior (16). This innervation is complex and can convey information regarding both aversive and appetitive stimuli. The PPL1 neurons signal punishment in the context of shock-conditioned odor memory (35, 45) but also play a role in modulating sugar-conditioned odor memory (17). The PAM neurons strongly signal reward for odor memory (19), although a small subset of these neurons have been reported to signal punishment (39, 45). Our results concur with studies in which the MB-MP1 PPL1 neurons signal an aversive stimulus, whereas the PPM3 and PAM neurons signal an appetitive stimulus. Unlike electric shock or sugar, however, ethanol acts as both an aversive and appetitive stimulus (23). We propose that recognition of ecologically beneficial ethanol concentrations would induce an appetitive response through activation of PAM and PPM3 neurons (Fig. 5B), whereas detrimentally high ethanol concentrations would induce an aversive response through preferential activation of PPL1 neurons, inhibition of MB function, and ultimately decreased oviposition preference for ethanol (Fig. 5C). Thus, the balance between appetitive and aversive responses would be dependent on ethanol concentration and context to optimize progeny fitness and survival (see the legend to Fig. 5 for more details).

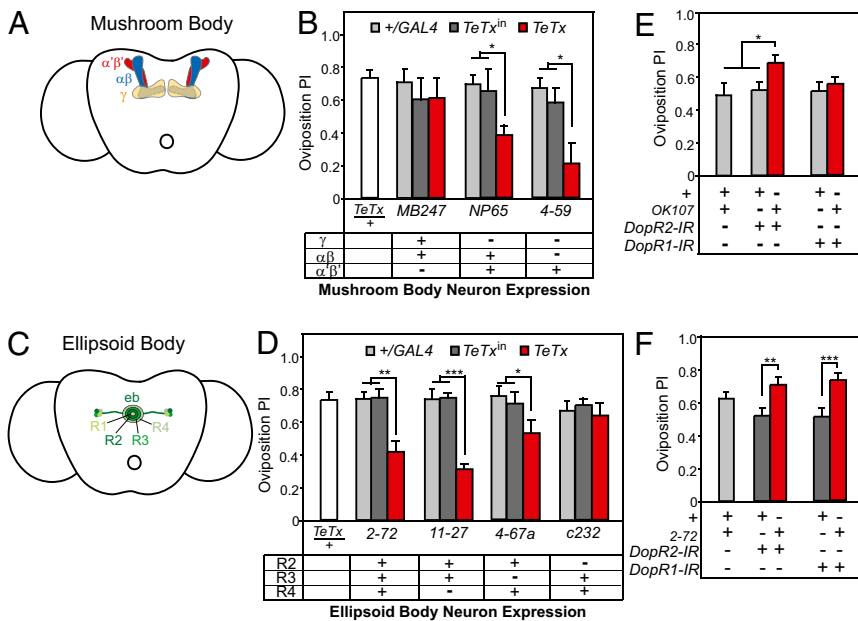


Fig. 4. The MB and EB are required for oviposition preference for ethanol. (A) The MB consists of three major classes of neurons whose axonal branches occupy distinct lobes elaborated by the $\alpha\beta$, $\alpha\beta'$, and γ neurons. (B) Disrupting neurotransmission in distinct subsets of MB neurons revealed that the $\alpha\beta'$ neurons mediate oviposition preference for ethanol [MB247: $n = 10$ –11 per strain; ANOVA: $F_{(2,30)} = 0.52$, $P = 0.60$; NP65: $n = 9$ –11 per strain; ANOVA: $F_{(2,28)} = 7.61$, $P = 0.003$; 4-59: $n = 10$ –11 per strain; ANOVA: $F_{(2,30)} = 6.00$, $P = 0.007$]. (C) The major contributions to the EB are the projections of R neurons that arborize as a ring. (D) Disrupting neurotransmission in R2 neurons decreased oviposition preference [2-72: $n = 7$ –11 per strain; ANOVA: $F_{(2,26)} = 28.56$, $P < 0.0001$; 11-27: $n = 10$ –12 per strain; ANOVA: $F_{(2,32)} = 52.03$, $P < 0.0001$; 4-67a: $n = 12$ –15 per strain; ANOVA: $F_{(2,32)} = 52.03$, $P < 0.0001$; c232: $n = 10$ –14 per strain; ANOVA: $F_{(2,35)} = 1.62$, $P = 0.21$]. (E) Decreasing *DopR2* expression in the MB increases oviposition preference for ethanol [$n = 18$ per strain; ANOVA: $F_{(2,51)} = 7.63$, $P = 0.001$]. (F) Decreasing *DopR1* and *DopR2* expression in the EB increases oviposition preference for ethanol [*DopR1*: $n = 18$ per strain; ANOVA: $F_{(2,50)} = 9.17$, $P = 0.0004$; *DopR2*: $n = 18$ per strain; ANOVA: $F_{(2,50)} = 10.60$, $P = 0.001$]. Bars on graphs represent means \pm SEM. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

Unexpectedly, we found that decreasing *DopR2* levels in the MB or decreasing *DopR1* or *DopR2* levels in the EB increased oviposition preference for ethanol. This may be attributable to an inhibitory role of the D1-type receptors in MB and EB neurons, compensatory responses to other dopamine receptors, or dopamine receptors having different behavioral effects within subpopulations of MB and EB neurons. Clearly, the role of dopamine receptors in regulating ethanol preference is complex.

How varying concentrations of ethanol activate or inhibit dopaminergic neurons remains to be investigated. Because both olfaction and taste affect oviposition preference for ethanol, integration of multiple sensory inputs likely plays a role. Because innate olfactory preference for ethanol is octopamine-dependent, and a subset of octopaminergic neurons mediates PAM neuron activation required for appetitive memory, octopamine may play a role in the olfactory response (46, 47). Neurons expressing neuropeptide F (NPF) or its receptor may also be involved. NPF signaling shifts oviposition preference to higher ethanol concentrations in the presence of parasitic wasps (48), resulting in higher survival (48). It would be intriguing to test whether NPF signaling acts through dopaminergic neurons to affect oviposition preference similarly to how it affects expression of food-associated memory (17).

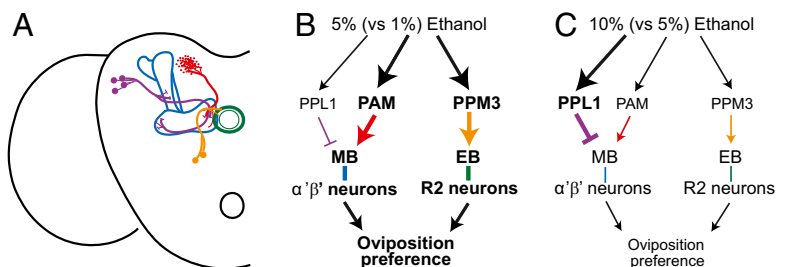
Our data suggest that integration of multiple sensory inputs may happen in the MB and EB, both of which have known roles

in decision-making. Output from the MB $\alpha\beta'$ neurons and EB neurons have been implicated in olfactory memory consolidation and visual pattern memory (23, 49–53). Because activity of dopamine neurons and MB $\alpha\beta'$ neurons are required for both oviposition preference for ethanol and ethanol-reward memory in the fly (23), our results raise the possibility that competing dopamine responses may also modulate memory for ethanol reward. Thus, a simple oviposition preference assay might be useful to identify circuits involved in more complex ethanol preference behaviors such as ethanol-reward memory. We speculate that circuits that evolved for female flies to evaluate an oviposition substrate as beneficial to the fitness of their progeny may be more generally required to evaluate appetitive and aversive stimuli. Interestingly, similar neural principles can be applied to motivational control of behavior in mammals, where dopamine neurons can also transmit signals related to rewarding and nonrewarding experiences (54).

Materials and Methods

Fly Stocks and Growth. Flies were grown and maintained on standard cornmeal/molasses/yeast/agar medium in an incubator at 25 °C and 70% humidity. All experiments used wild-type *Berlin* flies that were collected 1 d after eclosion and tested 4–5 d after eclosion. All transgenic strains used were backcrossed for at least five generations to a w^{1118} ; *Berlin* strain. *UAS-TeTx*

Fig. 5. A model for the neural circuitry mediating oviposition preference for ethanol. (A) A schematic representation of one hemisphere of the *Drosophila* central brain depicting innervation of the MB (blue) and EB (green) by dopaminergic neurons of the PAM (red), PPL1 (purple), and PPM3 (yellow) clusters. (B) Sensory information about ethanol is gated through dopaminergic neurons. The PAM (red) and PPM3 (yellow) neurons promote oviposition preference, whereas the PPL1 neurons (purple) inhibit preference. Dopaminergic input is relayed to the MB via the PAM and PPL1 neurons and to the central complex EB via the PPM3 neurons. The output response is relayed through the MB $\alpha\beta'$ neurons and EB R2 neurons. Sensory information about 5% ethanol activates the PAM and PPM3 neurons, which activate the MB $\alpha\beta'$ neurons and EB R2 neurons, respectively, leading to an increase in oviposition on ethanol. Sensory information about 5% ethanol simultaneously activates PPL1 neurons, which inhibit the MB $\alpha\beta'$ neurons, resulting in a decrease in oviposition on ethanol. When given choices of low ethanol concentrations (for example 5% vs. 0%) activation of the PAM neurons would have a stronger effect on oviposition behavior than activation of PPL1 neurons, resulting in increased preference for ethanol 5%. (C) When given choices that include detrimental ethanol concentrations (for example 10% vs. 5%), we hypothesize that activation of the PPL1 neurons has a stronger effect on oviposition preference relative to PAM neurons, resulting in a decrease in oviposition preference for the higher ethanol concentration. See Table S1 for detailed expression data.



(active) and *UAS-TeTx^{dn}* (inactive) were provided by S. Sweeney (University of York, York, UK) and *UAS-dTrpA1* by P. Garrity (Brandeis University, Waltham, MA). *NP65* and *c739* were provided by I. King (University of North Carolina at Chapel Hill, Chapel Hill, NC); *201Y*, *MB247*, *c232*, *OK348*, *Ddc-GAL4*, *TH-GAL4*, *TH-GAL80*, *HL5*, *HL7*, and *HL9* by F. Wolf (University of California, Merced, CA); *dTRH* by E. Kravitz (Harvard Medical School, Boston); *NP2758* and *kra-MBGAL80* by S. Waddell (University of Oxford, Oxford); *2-72*, *4-59*, *4-64*, *4-67*, and *11-27* are from the P[GAL4^{GawB1}] collection of U.H. (23, 33). Dopamine receptor *UAS-RNAi*-carrying flies were obtained from the Vienna *Drosophila* RNAi Center.

Two-Choice Oviposition Assay. The oviposition assay was performed similarly to that described by Joseph et al. (15). Groups of 30 mated females and 7 males were placed (without anesthesia) in a 6-ounce round-bottom plastic bottle, topped with a two-choice food plate, and inverted. Flies were left in the dark at 25 °C and 70% humidity to lay eggs for 3 h. The food plate was removed and photographed for later analysis. The number of eggs laid for each choice test is listed in Table S2.

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Statistical Analysis. All data are presented as means ± SEM. Statistical analyses were performed using JMP 8.0. 2 (SAS Institute). Two-sample *t* tests and one-way ANOVAs, followed by Tukey's two-sample post hoc comparisons, were performed when data were parametric. Post hoc comparisons are included in Table S3. Levene and Brown–Forsythe tests were used to confirm homogeneity of variance. Wilcoxon or Kruskal–Wallis tests were performed when data were nonparametric. Significance level was set to $P < 0.05$.

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