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A *Drosophila* model for alcohol reward

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The rewarding properties of drugs contribute to the development of abuse and addiction. We developed a new assay for investigating the motivational properties of ethanol in the genetically tractable model *Drosophila melanogaster*. Flies learned to associate cues with ethanol intoxication and, although transiently aversive, the experience led to a long-lasting attraction for the ethanol-paired cue, implying that intoxication is rewarding. Temporally blocking transmission in dopaminergic neurons revealed that flies require activation of these neurons to express, but not develop, conditioned preference for ethanol-associated cues. Moreover, flies acquired, consolidated and retrieved these rewarding memories using distinct sets of neurons in the mushroom body. Finally, mutations in *scabrous*, encoding a fibrinogen-related peptide that regulates Notch signaling, disrupted the formation of memories for ethanol reward. Our results thus establish that *Drosophila* can be useful for understanding the molecular, genetic and neural mechanisms underling the rewarding properties of ethanol.

The rewarding properties of abused drugs engage neural and molecular mechanisms that have evolved to promote the pursuit of natural rewards. However, drug rewards become overvalued at the expense of other rewards and, unlike natural rewards, do not serve a beneficial homeostatic or reproductive purpose[1]. Elucidating the mechanisms underlying the intense rewarding properties of abused drugs is therefore critical for understanding reward-seeking behaviors related to addiction. Despite recent progress, much remains to be learned about drug reward at the molecular and cellular level.

Humans and animals rapidly learn cues and contexts that predict the availability of highly rewarding drugs. One of the most popular rodent models for drug reward, conditioned place preference (CPP), measures preference for a cue that was previously associated with a drug[2]. We developed and characterized a CPP-like assay to measure the rewarding properties of ethanol in the genetically tractable model organism *D. melanogaster*.

Drosophila are similar to mammals in both their behavioral responses to acute ethanol exposure and the molecular pathways shown to regulate this response, including the cAMP, neuropeptide F (neuropeptide Y in mammals), epidermal growth factor (EGF) receptor and dopamine pathways[3]. *Drosophila* also exhibit a natural preference for low, non-intoxicating concentrations of ethanol: they lay their eggs on ethanol-containing substrate, move toward the smell of ethanol and show a preference for consuming ethanol-containing food[3]. It has not been determined, however, whether flies experience ethanol intoxication, which is only achieved by unnaturally high doses of ethanol, as rewarding. To investigate this, we developed an assay that assesses choice between two neutral cues following administration of an acute, intoxicating dose of ethanol associated with one of the cues. Thus, similar to CPP models in mammals, our assay ascertains the rewarding properties of ethanol intoxication by measuring preference for an associated cue.

In addition to establishing that flies perceive intoxicating levels of ethanol as rewarding, we investigated whether neurochemical systems important in reward perception in mammals, such as dopamine, have a similar role in flies. By temporally blocking neurotransmission in dopaminergic neurons, we found that dopamine release was required for expression, but not acquisition or consolidation, of conditioned preference. Moreover, the sequential activation of distinct neuronal subsets of the mushroom body, which is a target of dopaminergic neurons, was required for acquisition, consolidation and retrieval of memory for ethanol reward.

Finally, in a screen for molecules required for memories of ethanol reward, we identified *scabrous* (*sca*), a gene related to mammalian microfibrillar-associated glycoproteins (MPAGs) that regulates the Notch signaling pathway[4–7]. Although Notch signaling is best known for roles in development of the nervous system[8], its activation also influences structural and functional plasticity in the adult mouse CNS[9], long-term spatial memory in mice[10] and shock memory in flies[11,12]. Our results suggest that regulation of Notch signaling by *sca* may also be important for memories of ethanol reward.

RESULTS

Ethanol is both aversive and rewarding to flies

To establish whether flies experience ethanol intoxication as rewarding, we exposed flies to two attractive odor cues (isoamyl alcohol and an ethyl acetate mixture), one of which was associated with a moderately intoxicating dose of ethanol vapor. We then determined which odor the flies preferred to move toward in a Y maze (**Supplementary Fig. 1a**). Although naive flies had no significant preference for the odors used ($P = 1.00$; **Supplementary Fig. 1b**), we used a reciprocal training procedure to control for any inherent odor preferences (**Fig. 1a**). Preference for the ethanol-associated odor was expressed as a conditioned preference index (CPI), where a positive CPI indicates attraction and a negative CPI implies repulsion to the ethanol-paired odor (control odor tests, ethanol absorption assays performed for each fly strain and detailed statistical analyses are listed in **Supplementary Tables 1–5**).

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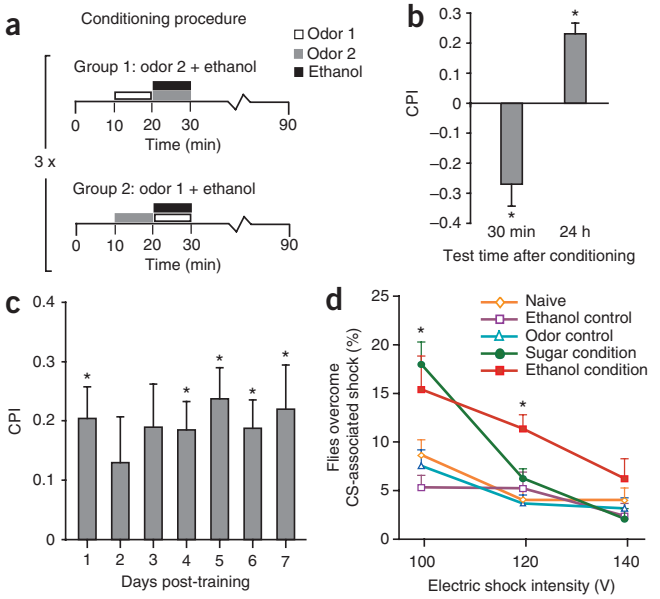


Figure 1 Ethanol is both aversive and rewarding to flies. (**a**) Flies were trained by three spaced training sessions of a 10-min exposure to one odor followed by a 10-min exposure to the second odor paired with 53% ethanol vapor. During the test, flies were given the choice of the two odors and a preference index was calculated by subtracting the number of flies entering the vial with the odor not previously associated with ethanol (odor–) from the vial with the odor previously associated with ethanol (odor+) and dividing this number by the total number of flies. CPI was calculated by averaging the preference indexes of the two reciprocal groups. (**b**) Flies showed conditioned aversion when tested 30 min after training ($n = 8$, $P = 0.006$) and conditioned preference 24 h after training ($n = 8$, $P = 0.02$) compared with an unpaired control (Wilcoxon two sample). $^*P < 0.05$. (**c**) Conditioned preference lasted for up to 7 d if flies were left undisturbed (Wilcoxon one way, $n = 8$, $P = 0.007$ on day 7). (**d**) Compared with flies that received either odor or ethanol alone, flies conditioned with ethanol or sucrose (Dunnett's, $n = 11$ flies per group, $P = 0.05$ and $P = 0.006$, respectively) walked over a 2-cm, 100-V electric grid to attain the conditioned odor, whereas only flies conditioned with ethanol walked over a 120-V electric grid to attain the conditioned odor ($P = 0.0004$). Exposures to either odor alone or ethanol alone ($P = 0.99$ and $P = 0.87$, respectively) did not affect the likelihood of walking over an electric grid. CS, conditioned stimulus. All values are reported as mean ± s.e.m.

After an extensive search for ethanol doses and training procedures that produced an optimal conditioned response, we chose a protocol in which flies were given three training sessions spaced by 1 h. Each training session consisted of a 10-min exposure to odor 1 followed by a 10-min exposure to odor 2 together with 53% ethanol vapor (and vice versa; **Fig. 1a**). We observed conditioned aversion when flies were tested 30 min after training, but conditioned preference 24 h later (**Fig. 1b**). The transition from aversion to preference occurred between 12 and 15 h post-training (**Supplementary Fig. 1c**). Notably, if the flies were left undisturbed after training, conditioned preference was maintained for at least 7 d (**Fig. 1c**). These results indicate that ethanol intoxication, although initially aversive, is associated with a long-lasting expression of reward.

Conditioned aversion or preference did not result from either repeated odor exposure (**Supplementary Fig. 2a**), ethanol exposure alone (**Supplementary Fig. 2b**) or from repeated trials in which odors were presented 20 min after ethanol exposure (**Supplementary Fig. 2c,d**). Thus, both aversion and preference are contingent on the temporal association between odor and ethanol exposure.

Flies overcome a shock barrier to attain conditioned cue

A hallmark of addiction is continued drug use despite negative consequences. To examine whether our assay could be used to test addiction-like behavior, we asked whether flies would overcome an aversive stimulus to gain access to the odor previously associated with ethanol. Indeed, conditioned flies showed reduced aversion to both 100-V and 120-V electric shocks, an effect that was not observed in flies exposed to either odor or ethanol alone (**Fig. 1d** and **Supplementary Fig. 3a**).

We also trained flies to associate odors with sucrose (**Fig. 1d** and **Supplementary Fig. 3b**), a substance that is known to be rewarding to flies. Sucrose-conditioned flies showed reduced aversion to the 100 V, but not the 120 V, grid (**Fig. 1d**). Thus, flies tolerated punishment (electrical shock) to approach an odor cue predictive of ethanol or sucrose reward. However, ethanol-conditioned flies tolerated a larger electric shock than sucrose-conditioned flies.

Pharmacological properties of ethanol induce preference

Although previous studies have shown that flies display innate preference for low concentrations of ethanol, such as those found in fermenting fruit[3], it is unlikely that flies become intoxicated under these conditions. In our assay, however, we aimed to use a moderately intoxicating concentration of ethanol that would elicit hyperactivity, a behavior thought to model the disinhibiting and euphoric effects of ethanol in humans[13].

To determine whether conditioned odor preference could be attributed to the pharmacological effects of ethanol, we first measured internal ethanol levels in conditioned flies. Indeed, extracts of conditioned flies contained substantial levels of ethanol (**Fig. 2a** and **Supplementary Fig. 3c**), which were sufficient to stimulate locomotion (**Fig. 2b**). We next asked whether flies needed to be 'under the influence' to form associations with odors by delivering the odor either immediately before (**Fig. 2c**) or after (**Fig. 2d**) the ethanol exposure. We found that conditioned preference was elicited only when the odor was presented immediately after the ethanol exposure, when flies still contained substantial levels of ethanol (**Fig. 2d**). Thus, the formation of conditioned preference required the temporal coincidence of internal ethanol levels and the odor cue.

Dopamine is required for expression of ethanol reward

In mammals, investigations using diverse methods have converged on the conclusion that natural rewards and addictive drugs both influence behavior as a result of their ability to increase synaptic dopamine in the nucleus accumbens[1,14]. Although dopamine is involved in the acute locomotor effects of cocaine and ethanol in flies[15,16], it is unknown whether it also has a role in ethanol reward. We therefore blocked dopaminergic neurotransmission with spatial and temporal resolution in behaving flies to determine dopamine's role in the aversive and rewarding effects of ethanol. To do this, we expressed the dominant-negative and temperature-sensitive variant of dynamin, *shibire* temperature sensitive (*shi*^{ts}), in dopaminergic neurons using the *UAS-GAL4* binary expression system[17]. At temperatures at or above 29 °C, *shi*^{ts} rapidly impairs synaptic transmission in targeted neurons by inhibiting neurotransmitter vesicle recycling[17]. We silenced dopaminergic neurons using two different *GAL4* drivers: one expressed under the control of the *tyrosine hydroxylase* (*TH*) promoter (*TH-GAL4*, expressed in most dopaminergic neurons[18]) and one under the control of *dopamine decarboxylase* (*Ddc-GAL4*, expressed in most dopaminergic and serotonergic neurons[19]). Perturbing neurotransmission in *Ddc-* or *TH*-expressing neurons using *UAS-shi*^{ts} at the restrictive temperature (30 °C) during both

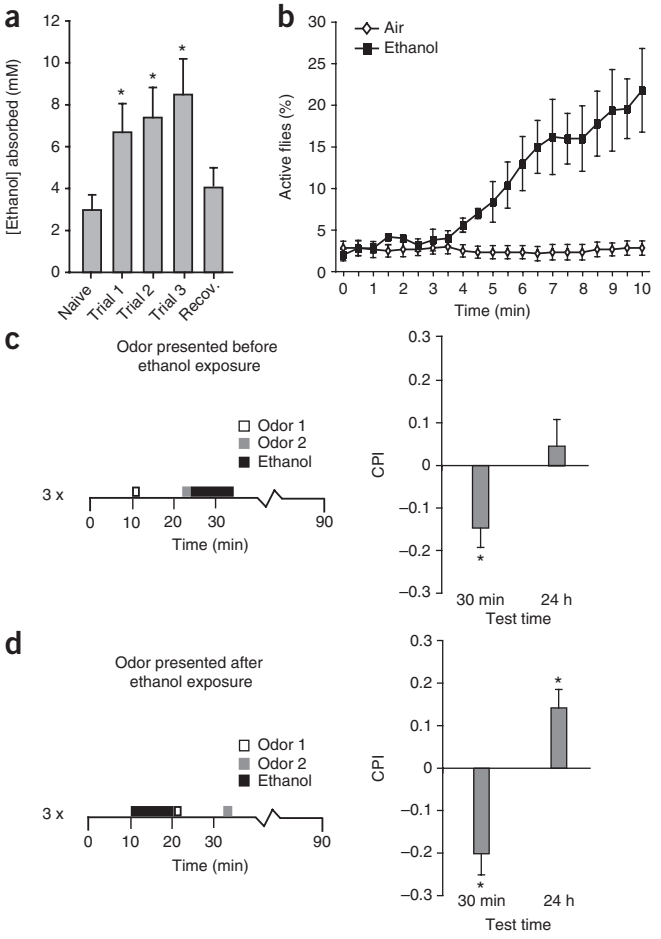
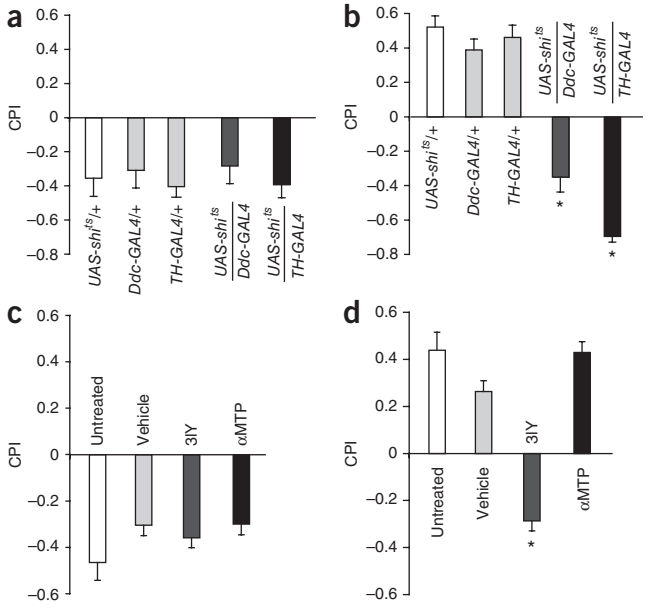


Figure 2 Pharmacological properties of ethanol induce preference. (**a**) Flies absorbed significant amounts of ethanol during conditioning (Student's *t post hoc* test, $n = 11$ flies per group, $P = 0.04$, $P = 0.02$ and $P = 0.004$ for trials 1, 2 and 3, respectively) and recovered within 30 min ($P = 0.54$). $^*P < 0.05$. (**b**) Ethanol absorbed during training induced a significant increase in locomotor activity characteristic of acute intoxication (repeated-measures ANOVA, $n = 11$ flies per group, $P = 0.002$). (**c**) An odor presented before ethanol resulted in significant conditioned aversion (Wilcoxon one way, $n = 8$ flies per group, $P = 0.007$), but not conditioned preference ($P = 1.00$), suggesting that an odor can predict onset of the aversive effects of ethanol. (**d**) An odor presented directly after ethanol resulted in significant conditioned aversion and significant conditioned preference (Wilcoxon one way, $n = 8$, $P = 0.007$ for both behaviors), suggesting that ethanol intoxication is required for conditioned preference to form. All data are shown as mean ± s.e.m.

training and testing did not affect conditioned aversion (**Fig. 3a**), but blocked the formation of conditioned preference (**Fig. 3b**). No effects were observed at the permissive temperature (24 °C; **Supplementary Fig. 4a**,**b**). Note that testing flies at 29 °C compared with 24 °C generally increased their conditioned responses (**Fig. 3a**,**b** and **Supplementary Fig. 4a**,**b**), possibly as a result of enhanced locomotion or stronger perception of the training odors. These results indicate that distinct neuronal populations mediate conditioned aversion and preference for ethanol, where the activity of dopaminergic neurons is required for flies to form and/or express the association between the attractive properties of ethanol and an odor, but is not required for flies to form aversive memories.

Serotonin is involved in the formation of memories of cues associated with abused drugs and in modulating the behavioral effects induced by these drugs[20]. To distinguish between the contributions of dopamine and serotonin neurons that expressed *Ddc-GAL4*, we tested conditioned preference in flies that had been fed the tyrosine hydroxylase inhibitor 3-iodo-tyrosine (3IY) or the serotonin synthesis inhibitor α-methoxytryptophan (αMTP). As expected, 3IY and αMTP substantially reduced dopamine and serotonin levels, respectively, in the flies' brain (**Supplementary Fig. 4c**,**d**). Neither drug had an effect on conditioned aversion (**Fig. 3c**). However, 3IY, but not αMTP, blocked the formation of conditioned preference (**Fig. 3d**), thus supporting a role for dopamine in the memory of the attractive properties of ethanol.



In mammals, drugs of abuse, including ethanol, amplify dopaminergic responses to natural rewards and reward-related environmental cues[1,14]. Consequently, reward-related cues become associated with the reinforcing effects induced by dopamine release in specific brain regions. To test whether activity of dopaminergic neurons was required during acquisition of conditioned preference, we used *UAS-shi*^{ts} to impair transmission of tyrosine hydroxylase–expressing neurons with temporal specificity. We shifted the temperature such that neural activity was silenced only during training, when ethanol was presented simultaneously with the odor cue (**Fig. 4a**). We found that this manipulation had no effect on the development of conditioned preference (**Fig. 4a**). This result suggests that dopaminergic neurons might be recruited into the relevant neural circuit during the processes of memory consolidation and/or retrieval. We therefore suppressed neurotransmission in the 24 h between training and testing (presumed consolidation phase) or during preference testing (retrieval phase).

Figure 3 Dopamine is required for conditioned preference. (**a**,**b**) Blocking synaptic transmission in dopaminergic neurons during both training and testing did not affect conditioned aversion tested 30 min after training (Kruskal-Wallis, $n = 8$ flies per group, $P = 0.84$, **a**), but blocked the formation of preference in both *TH*- and *Ddc*-expressing neurons tested 24 h later (Wilcoxon one way, $n = 8$, $P = 0.0003$ and $P = 0.007$, respectively, **b**). $^*P < 0.05$. (**c**) Conditioned aversion was not affected by decreasing serotonin levels in the brain using αMTP or dopamine levels using 3IY (Kruskal-Wallis, $n = 8$ flies per group, $P = 0.07$). (**d**) Conditioned preference was not affected by αMTP (Student's *t post hoc*, $n = 8$ flies per group, $P = 0.21$), but was blocked by decreasing dopamine levels in the brain using 3IY ($P = 0.0002$). All data are shown as mean ± s.e.m.

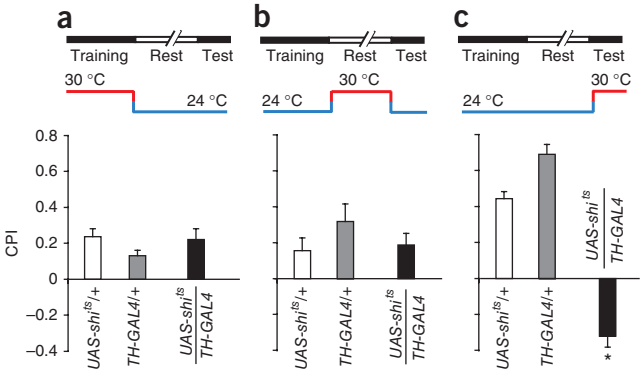


Figure 4 Dopamine is required for expression of ethanol reward. (**a**,**b**) Transiently blocking neurotransmission of *TH*-expressing cells during acquisition (**a**) or consolidation (**b**) did not affect conditioned preference (Kruskal-Wallis, $n = 8$ flies per group, $P = 0.06$ and $P = 0.27$, respectively). (**c**) Activity of *TH*-expressing cells was required for the retrieval or expression of conditioned preference (Kruskal-Wallis, $n = 8$ flies per group, $P = 0.0005$). $^*P < 0.05$. All data are shown as mean ± s.e.m.

Although the activity of dopaminergic neurons during consolidation was dispensable for conditioned preference to form (**Fig. 4b**), their activity was essential for expression of the memory (**Fig. 4c**).

The mushroom body is required for ethanol reward memory

In mammals, discrete brain regions mediate the conditioned response to drug reward, including the nucleus accumbens, prefrontal cortex, amygdala and dorsal striatum[1]. In *Drosophila*, the regions of the brain mediating ethanol preference or reward are unknown. However, it is well established that discrete subsets of neurons in the fly mushroom body are involved in odor memory processing, including the conditioned response to sugar reward[21]. In addition, dopaminergic neurons known to be involved in modulating memories for sucrose reward project to the mushroom body[22]. Given that the mushroom body is important for memories of sugar reward and that dopamine is required for expression of memories of ethanol reward, we hypothesized that the mushroom body may be required for conditioned preference.

The mushroom body consists of three major classes of neurons whose axonal branches occupy distinct subsets of lobes: the αβ, α′β′ and γ neurons. We investigated the role of different mushroom body neurons in the conditioned response to ethanol by suppressing synaptic transmission using *UAS-shi*^{ts} in combination with a series of mushroom body GAL4 drivers (**Fig. 5a** and **Supplementary Fig. 5a–f**). Impairing neurotransmission during training and testing in the entire mushroom body (OK107; **Supplementary Fig. 5b**), in a combination of γ and αβ neurons (MB247 and 201Y; **Supplementary Fig. 5c**,**d**) or in just αβ neurons (5-66a; **Supplementary Fig. 5e**) disrupted both conditioned aversion (**Fig. 5b**) and preference (**Fig. 5c**). There were no differences in conditioned responses in these groups at the permissive temperature with the exception of the highly expressed OK107 driver (**Supplementary Fig. 5g**,**h**), an effect that is likely a result of low levels of expression at the permissive temperature. In addition, olfactory control tests revealed that manipulating mushroom body neurotransmission did not significantly impair odor attraction (**Supplementary Table 1**). The odor acuity showed more within-group variation than conditioned preference (**Supplementary Table 1**), suggesting that it may be sensitive to small fluctuations in ambient humidity, temperature and fly activity.

To determine when each subset of mushroom body neurons was required for memories of ethanol reward, we blocked transmission specifically during acquisition, consolidation or retrieval of conditioned preference. We found that silencing a combination of γ and αβ neurons (OK107, MB247, 201Y) during acquisition blocked conditioned preference (**Fig. 6a**). Acquisition was not affected by silencing of αβ neurons alone (5-66a), suggesting that the γ neurons, rather than αβ neurons, are important for acquisition (**Fig. 6a** and **Supplementary Fig. 5a–e**). In contrast, silencing the α′β′ neurons (OK107, 4–59) during consolidation or the αβ neurons (5-66a) during testing blocked conditioned preference (**Fig. 6b**,**c**). Thus, the function of different subsets of mushroom body neurons is necessary for the distinct phases involved in forming conditioned preference. Our results thus reveal sequential use of the γ, α′β′ and αβ neurons. These results indicate that neurotransmission of both dopaminergic and αβ neurons specifically affect conditioned preference expression and we

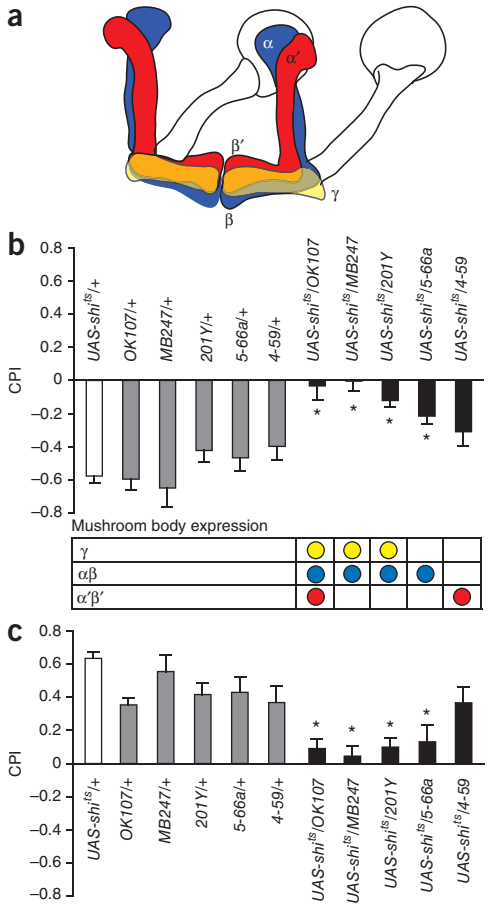


Figure 5 The mushroom body is required for aversion and preference. (**a**) Schematic of the subsets of mushroom body neurons: yellow, γ neurons; blue, αβ neurons; red, α′β′ neurons. (**b**) We transiently inactivated neurotransmission in selected sets of mushroom body neurons using the GAL4 drivers *OK107*, *201Y*, *MB247*, *5-66a* and *4-59*. Blocking synaptic transmission of specific mushroom body neurons using the GAL4 drivers *OK107* (Student's *t post hoc*, $n = 8$ per group, $P < 0.0001$), *201Y* ($P = 0.003$), *MB247* ($P < 0.0001$) and *5-66a* ($P = 0.01$) during training and testing disrupted conditioned aversion tested 30 min after training. Colored circles represent mushroom body neurons in which GAL4 drivers are expressed as defined above. $^*P < 0.05$. (**c**) Inactivation of mushroom body using *OK107* ($P < 0.0001$), *201Y* ($P = 0.003$), *MB247* ($P < 0.0001$) and *5-66a* ($P = 0.01$) during both training and test disrupted conditioned preference 24 h after training. All data are shown as mean ± s.e.m.

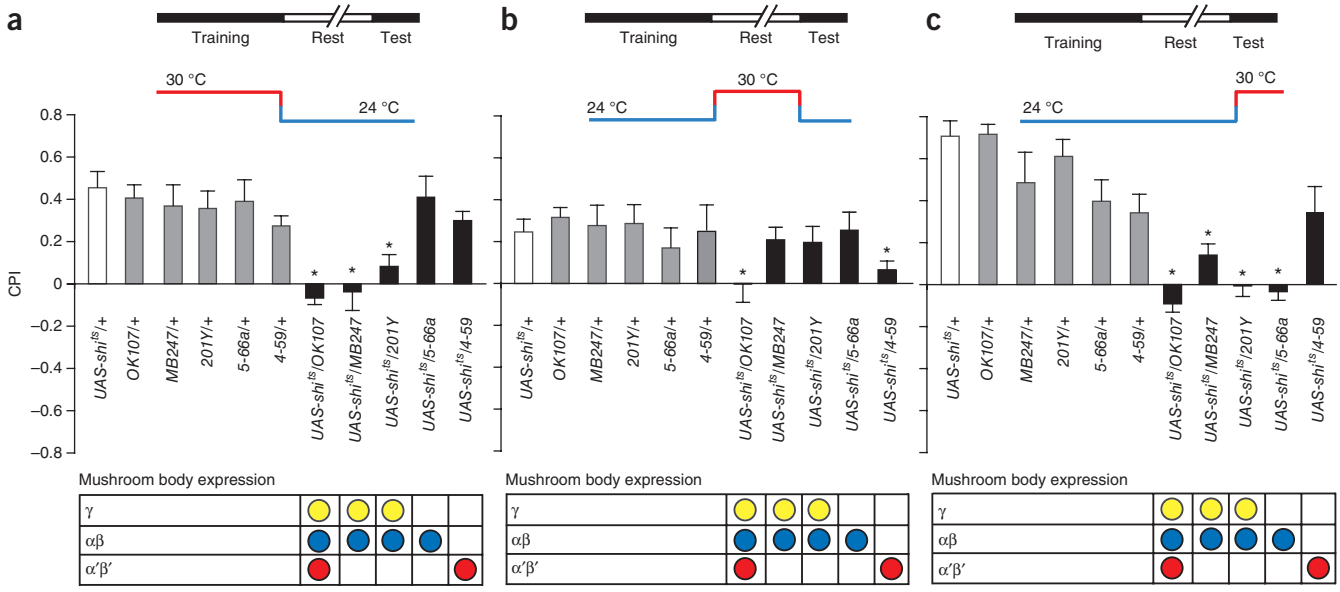


Figure 6 Sequential use of mushroom body neurons. (**a**) Inactivation using drivers *OK107* (Student's *t post hoc*, $n = 8$ flies per group $P < 0.0001$), *201Y* ($P < 0.0001$) and *MB247* ($P = 0.006$), but not *5-66a*, during training disrupted acquisition, implicating the γ neurons in acquisition of conditioned preference. $^*P < 0.05$. (**b**) Inactivation using drivers *OK107* ($P = 0.004$) and *4-59* ($P = 0.02$) disrupted stabilization, implicating the α′β′ neurons in consolidation of conditioned preference. (**c**) Inactivation using drivers *OK107* ($P < 0.0001$), *201Y* ($P = 0.003$), *MB247* ($P < 0.0001$) and *5-66a* ($P < 0.0001$) disrupted retrieval, implicating the αβ neurons in retrieval or expression of conditioned preference. All data are shown as mean ± s.e.m.

hypothesize that the manifestation of ethanol reward memory may be mediated by dopaminergic innervation of the αβ neurons. This is consistent with recent studies showing that dopaminergic innervation of the α lobe is important for conditioned response to shock and the effect of satiety on the conditioned response to sucrose[22,23].

scabrous is required for ethanol reward memory

Given that we found that activity of mushroom body neurons was required for the memory of ethanol reward, we were interested in identifying genes that may act in the mushroom body to regulate these memories. To investigate this, we tested memory for ethanol reward

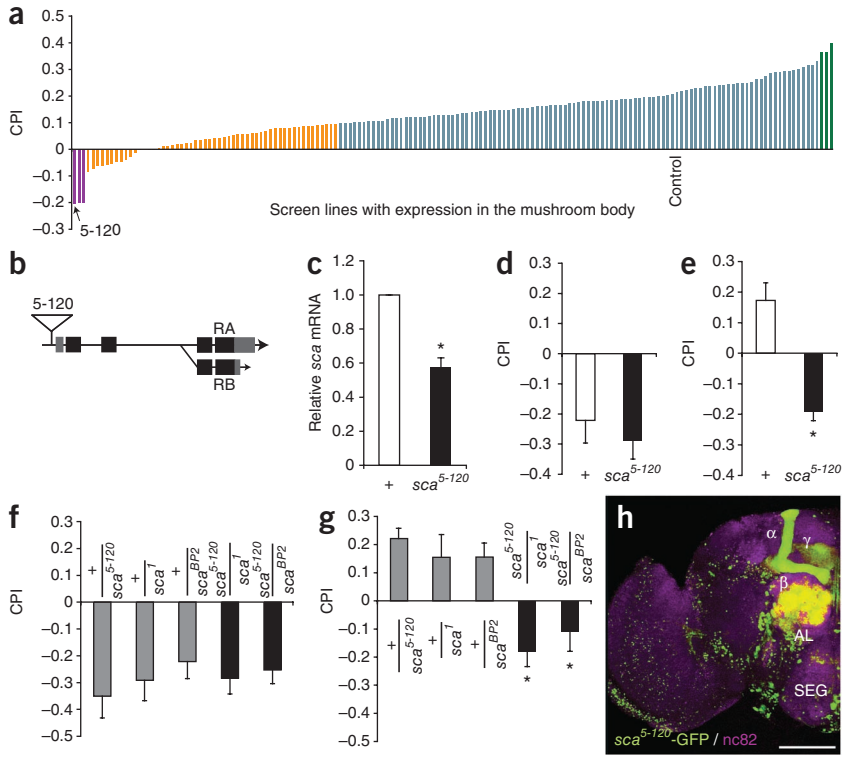


Figure 7 *sca* affects memories for ethanol reward. (**a**) A screen for conditioned ethanol preference of 160 *P{GawB}*-containing strains with known expression in the mushroom body identified three mutations in which conditioned aversion persisted 24 h after training (magenta), 54 mutations in which conditioned preference was not expressed (orange) and three mutations in which conditioned preference was enhanced (green). Strains that showed CPI that was not significantly different from control are shown in gray. Values represent mean ($n = 8$ per strain). (**b**) In the $sca^{5\text{-}120}$ mutant, the *P{GawB}* element was inserted 125 bp 5′ of exon 1 of *sca*. (**c**) Quantitative PCR revealed that the $sca^{5\text{-}120}$ mutation decreased *sca* mRNA expression to 55% that of wild-type controls (mean ± s.e.m., $n = 6$ independent samples). (**d**) $sca^{5\text{-}120}$ did not affect conditioned aversion for ethanol 30 min after training. (**e**) $sca^{5\text{-}120}$ affected conditioned preference for ethanol 24 h after training. (**f**) Complementation analysis of conditioned preference 30 min after training with two independent *sca* alleles confirmed that *sca* did not affect conditioned aversion. (**g**) $sca^{5\text{-}120}$ failed to complement the sca^{1} and sca^{BP2} alleles for 24-h conditioned preference. CPI values represent mean ± s.e.m. (**h**) The $sca^{5\text{-}120}$-GAL4 expression pattern suggests that *sca* is expressed in the mushroom body αβ and γ neurons, the antennal lobe (AL), eye and a number of cell bodies near the ventrolateral protocerebrum and subesophageal ganglia (SEG) (see also **Supplementary Fig. 7**). Scale bar represents 50 μm.

24 h after training in 160 strains with GAL4 reporter expression in the mushroom body. We found 3 mutations that resulted in persistent conditioned aversion, 54 mutations that resulted in lack of conditioned preference and 3 mutations that resulted in enhanced conditioned preference (**Fig. 7a**).

Of particular interest were the three mutants in which conditioned aversion persisted, as this phenotype resembled what was observed when dopaminergic neurotransmission was blocked. The mutant with the most severe phenotype, 5–120, carries a *P{GawB}* (*GAL4*-containing P-element) insertion 125 bp 5′ of exon 1 of *scabrous* (*sca*)[24] (**Fig. 7b**). Quantitative RT-PCR showed that the $sca^{5\text{-}120}$ mutation significantly decreased *sca* expression to 55% that of wild-type controls ($P = 0.01$; **Fig. 7c**). Although $sca^{5\text{-}120}$ mutants showed conditioned aversion to ethanol when tested 30 min after training (**Fig. 7d**), they failed to switch from conditioned aversion to preference (**Fig. 7e**). $sca^{5\text{-}120}$ mutants showed normal sensitivity to acute ethanol exposure, place-memory performance and olfactory-shock memory performance[24], indicating that their failure to form associations between odorants and the rewarding effects of ethanol is not a result of a general inability to learn. $sca^{5\text{-}120}$ mutants also showed normal olfactory acuity to the odorants used in this assay (**Supplementary Table 5**) and did not show any gross morphological abnormalities of the mushroom body (**Supplementary Fig. 6**). An attempt to rescue the $sca^{5\text{-}120}$ phenotype by overexpressing *UAS-sca* in $sca^{5\text{-}120}$ mutant flies resulted in lethality. However, complementation analysis with two independent amorphic *sca* alleles[25], sca^{1} and sca^{BP2}, revealed that both failed to complement the behavioral deficit of $sca^{5\text{-}120}$ (**Fig. 7f,g**). This confirmed that disruption of *sca* results in a failure to switch from conditioned aversion to conditioned preference. Expression of *UAS-GFP* in the $sca^{5\text{-}120}$*-GAL4* pattern revealed that, in the adult brain, *sca* was expressed in the mushroom body αβ and γ neurons, antennal lobe, eye and a number of cell bodies near the ventrolateral protocerebrum and subesophageal ganglia (**Fig. 7h** and **Supplementary Fig. 7**).

DISCUSSION

Here we provide evidence that ethanol intoxication is rewarding to flies, map this response to a discrete location in the fly brain and describe a previously unknown gene important for the formation of reward memory. Our model reveals that, similar to mammals, flies show both conditioned aversion and preference to ethanol, show long-lasting preference for a cue associated with ethanol intoxication, will overcome an aversive stimulus to obtain a cue associated with ethanol, use dopaminergic systems to express memories for a cue associated with ethanol intoxication, and rely on sequential use of distinct brain circuits to acquire, consolidate and express memories of reward. These similarities suggest that the neural and molecular pathways mediating ethanol's rewarding properties are evolutionarily conserved.

A model for ethanol reward in *Drosophila*

Previous studies using *Drosophila* to model alcohol-related behaviors have concentrated either on the acute locomotor stimulatory and sedative effects of ethanol or preference for low concentrations of alcohol[3]. Our findings indicate that flies develop long-lasting preference for an intoxication-associated cue and that ethanol is rewarding as a result of its pharmacological properties. This strengthens previous findings that flies will develop preference for ethanol consumption even in the presence of an aversive tastant[26]. In addition, because the concentrations of ethanol required for conditioned preference to form are considerably higher than what *Drosophila* would encounter in nature, it is unlikely that our assay measures an innate adaptive attraction to ethanol that is specific to fruit flies.

The conditioned preference that we investigated focuses specifically on the acute rewarding properties of ethanol. Our hope is that this assay will spur the development of more complex tests that model more advanced stages in the addiction cycle, such as withdrawal after chronic exposure and reinstatement of drug seeking after abstinence.

Conditioned aversion to conditioned preference

We found that the preference for a cue associated with ethanol spontaneously switched from negative (aversion) to positive (attraction) 12–15 h after conditioning. Opposing behavioral responses to an identical dose of a drug of abuse are surprisingly common. For example, acute exposure to nicotine in rats results in short-term conditioned place aversion that switches to longer-lasting conditioned place preference[27]. These opposing responses may result from conflicting responses to the sensory and pharmacological effects of the drug. For example, infant rats exhibit aversive learning to ethanol's orosensory effects, but show positive reinforcement to its post-ingestive effects[28]. We speculate that the initial aversive properties of ethanol in flies are not dependent on its intoxicating effects, but rather are a result of a negative physiological reaction or sensory response, similar to that observed in rats.

It is also possible that, in addition to an associative process, aversion may also be dependent on non-associative mechanisms such as sensitization. This is supported by the observation that flies still develop conditioned aversion when an odor is presented immediately before ethanol exposure. Future genetic and neuroanatomical studies should allow us to clearly discern whether conditioned aversion and preference are interdependent or parallel processes.

Dopamine and ethanol reward

Our finding that blocking dopaminergic systems resulted in a lack of conditioned preference is consistent with a large body of existing data demonstrating the requirement for dopamine in reward. Despite extensive investigation on the role of dopamine in reward, however, little is known about the dopaminergic mechanisms mediating ethanol reward. In fact, much of the evidence investigating the role of dopamine in ethanol reward in mammals is conflicting. We speculate that much of the variation between the results of these studies potentially derives from compensatory mechanisms that occur throughout development in mutant animals and nonspecific or ineffective pharmacological manipulations. The genetic tools available in *Drosophila* allowed us to avoid these caveats by permitting precise temporal control over neurotransmission of dopaminergic cells. Our data are consistent with the findings that mice lacking the D2 receptor[29] or DARPP-32 (ref. 30) show decreased CPP and that both fluphenazine administration[31] and dopamine receptor blockade[32] prevent expression of memory for ethanol reward. This suggests a conserved role for dopamine in expression of conditioned preference in flies and mice.

Our finding that dopamine was involved specifically in expression of ethanol reward can be interpreted in two ways: that dopamine is required for retrieval of the conditioned odor-ethanol association or that dopamine is involved with the conditioned motivational effects of alcohol cues without affecting cue memory retrieval. Although our results cannot directly distinguish between these hypotheses, they are consistent with the prediction-error hypothesis that supports involvement of dopamine in the motivational effects of alcohol predictive cues. In mammals, it has been proposed that dopamine is released when the actual value of a reward differs from its predicted value[14,33]. We found that activation of dopamine systems is required only when the conditioned odors are presented in the absence of the ethanol reward; that is, when the flies may expect ethanol, but receive only

odor cues. We therefore speculate that dopamine may be involved in the assessment of differences in actual and predicted reward in flies.

Dissociating mechanisms underlying natural and drug reward

The molecular and cellular mechanisms that lead to compulsive drug use are believed to be similar to those normally involved in reward-related learning[1]. We found that, although some of the neural substrates underlying both are conserved in flies, such as the mushroom body αβ neurons, there are distinct mechanisms that underlie the motivational properties of ethanol and sucrose as rewards. For example, although blocking output of dopaminergic neurotransmission does not directly affect 3-min memory for sucrose[34], it does disrupt 24-h memory for ethanol reward. However, blocking output of the PPL1 cluster of tyrosine hydroxylase–positive neurons enhances 3-h memory for sucrose reward in satiated flies[22]. This suggests that the neural substrates for memory of ethanol reward may overlap with the neural substrates that modify, but are not required for, memory of sucrose reward.

Comparisons between these studies need to be interpreted with caution, however, as the odors used and time of test differed for each of these experiments. Our data, however, in combination with other evidence showing the dopamine receptor dDA1 mediates formation of memories associated with sucrose reward in *Drosophila*[35], suggest that dopamine has a more complex role in mediating reward in the fly than was previously thought.

Sequential use of mushroom body neurons

Experiments conducted with flies defective in learning and memory have led to the prevailing model in which mushroom body neurons associate the odor-conditioned stimulus with a shock or sugar unconditioned stimulus using potential coincidence detecting molecules, such as the adenylyl cyclase rutabaga[36], and store the associations in the specific neurons that are activated by a particular odor[37,38]. Similarly to previous studies, we found that the αβ and α′β′ neurons were required for retrieval and stabilization of memory, respectively[21,37,38]. Our results differ from previous work on memory that used pairings of aversive odors and electric shock, as we found a potential role for γ neurons in acquisition of the memory for ethanol reward. These results suggest that either the odors that we employed (which are attractive) and/or the ethanol stimulus require γ neuron activation. Indeed, some combination of γ and/or αβ neuron activation is required for the acute locomotor stimulatory effects of ethanol[39], suggesting that the acute response to the ethanol stimulus requires mushroom body neuron activation. Our results, however, must be interpreted with caution, as we did not use a γ neuron–specific driver. It is possible that acquisition of memory may require non-overlapping subsets of αβ neurons defined by the 5-66a, 201Y and MB247 GAL4 drivers.

Our results also differ from recent work describing a role for the α′β′ neurons in acquisition of memory for both aversive (shock) and appetitive (sucrose) conditioning[21]. This model suggests that olfactory information received from the second-order olfactory projection neurons is first processed in parallel by the αβ and α′β′ neurons during acquisition[21]. Subsequently, memories are stored in αβ neurons, whose activity is required during recall[21]. Our data suggest that, for ethanol reward memories, olfactory information received from the second-order olfactory projection neurons is first processed in parallel by the αβ and γ neurons before activity of α′β′ neurons, establishing consolidation of memory in the αβ neurons. Then, as with memories for shock and sucrose, memories for ethanol reward are stored in αβ neurons, whose activity is required during recall.

An important difference between these studies is the time at which memory was tested. Shock memory was tested 3, 5 or 30 min after training[37,38], sucrose memory was tested 3 h after training[21], and ethanol memory was tested 24 h after training. It is possible that the output of distinct subsets of mushroom body neurons is required for different stages of memory. For example, recent work suggests that animals that receive multiple spaced odor-shock pairings form a memory trace in the αβ neurons between 9 and 24 h after training and in the γ neurons between 18 and 48 h after training[40].

scabrous is required for ethanol reward memory

Because activation of the mushroom body was required for the development of conditioned preference for ethanol, we assayed a collection of fly strains with known expression in the mushroom body to search for genes involved in ethanol reward. We found that *sca* is required for the formation of memories of ethanol reward. *sca* is a fibrinogen-related secreted peptide that is involved in neurogenesis, spacing differentiation and boundary formation via a Notch-dependent pathway[4,5]. During development, Sca associates with Notch and can stabilize Notch protein at the cell surface, thus sharpening proneural cluster boundaries and ensuring the establishment of single pioneer neurons[5].

Notch is required for memory formation in both mice and flies. Mice heterozygous for *Notch1* and the downstream cofactor *Rbpj* have deficits in spatial learning and memory[10]. Temperature-sensitive *Notch* mutant flies show deficits in 24-h memory for an odor associated with shock[11,12] and RNAi-mediated *Notch* silencing in the mushroom body reduces this memory[12]. We speculate that *sca* may regulate *Notch* signaling to mediate memories of ethanol reward. This may occur in the mushroom body αβ neurons, where $sca^{5\text{-}120}$-*GAL4* is expressed, and that are required for long-term memory[41].

Mammalian microfibril-associated glycoprotein (MAGP) shows peptide homology to *sca* and interacts with the Notch signaling pathway via direct interactions with the Notch1 receptor, as well as the Jagged1, Jagged2 and Dll-1 Notch ligands[6,7]. This suggests potential conservation of *sca-Notch* interactions between flies and mammals. It would be interesting to investigate whether MAGP mediates memory of reward in mammals as it does in flies.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

K.R.K. conceived, conducted and interpreted the experiments, performed data analysis, and co-wrote the paper. R.A. assisted with the behavior experiments. Z.M. conducted control experiments. J.H. performed high-performance liquid chromatography experiments. U.H. conceived and interpreted experiments and co-wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Strains. Flies were grown and maintained on standard cornmeal/molasses/yeast/agar media at 25 °C and 70% humidity on a 12-h:12-h light:dark cycle with lights-on at 9:00 a.m. Behavioral characterization was performed on wild-type Canton-S flies recently isogenized for the second and third chromosomes. All GAL4 and UAS lines[42] were backcrossed for at least five generations to a w^- Berlin strain, recently isogenized for the second and third chromosome. No significant differences were found between Canton-S and Berlin backgrounds in conditioned preference (Wilcoxon two-sample: 30-min memory $Z_{1,16} = -0.05$, $P = 0.96$, 24-h memory $Z_{1,16} = -0.17$, $P = 0.86$). Lines 4–59 and 5-66a were obtained from our *P{GAL4*^{GawB}*}* screen collection (**Supplementary Fig. 5b**,**c**). *UAS-shi*^{ts} was obtained from S. Sweeney. *sca*^{1} and *sca*^{BP2} were obtained from Bloomington Stock Center and *UAS-sca* from N. Baker. The screen was carried out using 160 *P{GAL4}* homozygous viable strains in a w^- Berlin genetic background (carrying the *GawB* element)[42] with previously characterized expression in the mushroom body. *sca*$^{5\text{-}120}$ was backcrossed for five generations onto a newly isogenized w^- Berlin background and re-tested.

Conditioned preference for an odor associated with ethanol. Groups of 50 male flies were collected 0–1 d after eclosion and trained at 3–5 d of age. Flies were trained in vials (2.5-cm diameter and 9.5-cm height) containing 1% agar. Vials contained 64 evenly spaced perforations and a mesh lid to allow even distribution of ethanol. Vials were placed into a holder in a 30-cm-long, 15-cm-high. 15-cm-wide training chamber. The training chamber (Aladin Enterprises) had three nozzles to allow for air/odorants/ethanol to stream in and one nozzle for waste to stream out.

Humidified air was bubbled through 95% ethanol to vaporize ethanol with a combined flow rate of 80 U vaporized ethanol and 70 U humidified air (where 100 U is equal to 1.7 l min^{-1} at 22 °C). Humidified air was streamed over odors placed in a 2.5-cm diameter and 13-cm height cylinder at a flow rate of 130 U for training and 100 U for tests. The odors that we used were 3 ml iso-amyl alcohol (1:36 in mineral oil) and a mixture of 2 ml ethyl acetate (1:36 in mineral oil) and 1 ml acetic acid (1:400 in mineral oil). Odors were replaced daily.

Reciprocal training was performed to ensure that inherent preference for either odor did not affect conditioning. Training consisted of three repetitions (spaced by 50 min) of 10 min of odor 1, then 10 min of odor 2 plus ethanol. A separate group of flies was simultaneously trained with 10 min of odor 2, then 10 min of odor 1 plus ethanol. Vials of flies from group 1 and group 2 were paired according to placement in the training chamber and tested simultaneously.

The testing chamber was a 6-cm cube with a mesh Y maze in the middle (Aladin Enterprises). Odors were streamed in through opposite arms of the Y (each 6 cm). Vials of flies were placed at the lower arm and flies climbed up the mesh cylinder and chose between opposing arms of the Y to 2.5-cm diameter, 9.5-cm height vials. After 2 min, vials were removed and capped. The numbers of flies that moved into the odor 1 and odor 2 vials were counted. A preference index for the odor paired with ethanol was calculated as (number of flies in paired odor vial – number of flies in unpaired odor vial) / total number of flies. A performance index for conditioned odor preference or aversion (CPI) was calculated by averaging the preference indexes for reciprocally trained groups of flies.

Memory was tested 30 min or 24 h post-training. Immediately after training, yeast was added to the training vials to ensure flies did not become food deprived before test. For experiments lasting several days, flies were trained on food containing 10 g of yeast, 10 g of sugar and 4 g of agar boiled in 200 ml of water. All training and tests were performed in a dark room under red light. The temperature was controlled with an oil-filled radiator (DeLonghi TRD0715T) and humidity controlled with a warm-mist humidifier (Vicks V745A, Proctor and Gamble).

Odor controls. Odor controls were performed exactly described above, except that instead of choosing between two different odors, flies chose between each single odor and air streamed through mineral oil. Preference index was calculated by (number of flies in odor vial – number of flies in air vial) / total number of flies. Odor control data for all UAS-GAL4 experiments are presented in **Supplementary Table 1** and data for all *sca*-related experiments are presented in **Supplementary Table 5.**

Sucrose conditioning. Groups of 50 4–5-d-old males were trained using odor training boxes described in the conditioned preference protocol above, and tested in the Y-maze apparatus described above. Flies were food deprived for 16–20 h before training. Reciprocal training was performed to ensure that inherent preference for either odor did not affect conditioning scored. Training consisted of 10-min habituation to the training chamber with air (flow rate 130), 10-min presentation of odor 1 with plain filter paper pre-soaked in water and dried, then 10 min of odor 2 with filter paper pre-soaked in 2 M sucrose and dried. Simultaneously, a separate group of flies was trained using odor 1 as the sucrose-paired odor. Vials of flies from Group 1 and Group 2 were paired according to placement in the training chamber and tested simultaneously 10 min following training. Preference and performance indices for conditioned sucrose preference were calculated in the same way as for conditioned ethanol preference (described above).

Electric shock. Electric shock was delivered via a Grass S88 Stimulator (Grass Technologies) to a small cylindrical copper grid 2 cm in height and 2.5 cm in diameter placed at the opening of the test vial from which the odor previously associated with ethanol or sucrose flowed. Electric shocks were applied every 50 ms for 1 s each throughout the test. The percentage of flies willing to overcome shock was measured by calculating percent of flies within each vial that walked over shock to obtain the ethanol- or sucrose-associated odor. The percentage of flies from one reciprocal pair of vials was averaged for each $n = 1$.

Ethanol concentrations. Fly internal ethanol concentrations were determined from whole-fly homogenates of 50 flies per sample. Flies were exposed to vaporized ethanol or air as outlined in training protocol. Flies were frozen immediately in liquid nitrogen and stored at −80 °C. Flies were homogenized in 500 µl of cold 50 mM Tris-HCl (pH 7.5, Sigma) and the homogenate was centrifuged at 14,000 r.p.m. for 20 min at 4 °C. Ethanol concentrations in supernatants were measured using an alcohol dehydrogenase–based spectrophotometric assay (Ethanol Assay Kit 229-29, Diagnostic Chemicals). To calculate fly internal ethanol concentration, the volume of one fly was estimated to be ~2 µl[43]. Ethanol concentration control data is presented in **Supplementary Table 2**.

Pharmacology. Pharmacological treatment with 3IY (Sigma) and αMTP (Sigma) was carried out as described previously[15]. We dissolved 10 mg ml^{-1} 3IY or 20 mM αMTP in a heated aqueous 5% sucrose, 5% yeast solution. 2 ml of cooled drug solution was added to a Kimwipe (Kimtech) in a training vial. We kept 1-d-old males in these vials for 40–48 h at 25 °C and 70% relative humidity before training.

High-performance liquid chromatography. High-performance liquid chromatography was performed as published previously[44] on a Jasco model PU-2080 isocratic pump (Jasco) with changes as outlined below. Four replicates of ten brains per sample were run for each condition. Ten 3-d-old adult *Drosophila* brains were hand dissected on dry ice between 1 and 3 p.m. (during the middle of the light phase) with as much precision as possible to remove all trace of eye pigmentation. Brains were placed directly into 50 µl of ice-cold 50 mM citrate acetate, pH 4.5, and quickly homogenized with a Teflon pestle. Homogenates were run through a 0.22-µm spin filter (Millipore) and frozen at −80 °C. The typical injection volume was 10 µl, or one brain equivalent. Standard mixes of dopamine and serotonin (Sigma-Aldrich) were injected at a concentration of 10 ng ml^{-1} and a calibration curve was generated based on injections containing 1, 5, 10, 50 and 100 pg.

Ethanol hyperactivity. Male flies ($n = 50$) were transferred to perforated vials containing 1% agar capped with a mesh lid as described previously. The flies were allowed to habituate to the environment 15 min before exposure to air or 53% ethanol vapor for 10 min under light conditions. Activity was filmed using a Sony HDR-SR12 Digital HD Video Camera Recorder (Sony). Recorded activity was hand scored as the percent of flies showing active locomotion during each 30-s interval for 10 min.

Immunohistochemistry and imaging. We dissected 4-d-old adult male brains in a phosphate-buffered saline solution and fixed them for 45 min at 22 °C with 4% formaldehyde (vol/vol, Ted Pella). Tissue was left overnight at 4 °C with 1:200 goat rabbit antibody to GFP (Invitrogen Molecular Probes) and 1:50 goat antibody

to mouse nc82 (Jackson Laboratories), washed four times and left overnight in 1:500 Alexa-Fluor 594 goat antibody to mouse (Invitrogen Molecular Probes) and 1:200 Cy2 goat antibody to rabbit (Jackson Laboratories). Brains were mounted in Fluoromount-G (SouthernBiotech).

Samples were imaged using a Nikon C1si Spectral Confocal (Nikon Imaging Center at Q3B, University of California at San Francisco). Images were acquired using a 20× objective and scanned at a resolution of 1,024 × 1,024 pixels. Images were prepared using Nikon EZ-C1, NIS C for C1 analysis software (http://www.nikon-instruments.jp/). Adobe Photoshop CS was used to tile images and to enhance contrast on whole images. Mushroom body expression was analyzed similarly to previous MB-GAL4 characterizations[45].

Real-time quantitative PCR. RNA was extracted from 4-d-old adult flies by homogenization in Trizol (Invitrogen) and stored in −80 °C. Samples were treated with DNase (Promega RQ1 RNase-free DNAse M6101) and cDNA was synthesized using Applied Biosystems TaqMan reverse transcription reagents (ABS N808-0234). Quantitative RT-PCR was performed using TaqMan Universal PCR Master Mix (no AmpErase UNG, ABS432018) on an Applied Biosystems 7900HT Fast Real-Time PCR System. Primers and probes recognizing *sca* (Dm01793316_g1) and *Rpl32* (Dm02151827_g1) were designed by and obtained from Applied Biosystems.

Statistics. Statistics were performed using JMP 7.0.1 (2007, SAS Institute). Shapiro-Wilk tests were used to establish normality and Levene's test to establish homogeneity of variance. Nonparametric tests were used primarily because the small sample size ($n = 8$ per strain per condition for conditioned preference experiments) often resulted in non-normality and lack of homogeneity of variance in the data. In addition, we chose to nonparametric tests because they tend to be more conservative than their parametric equivalents. Wilcoxon tests were used to compare performance indices to zero. Kruskal-Wallis tests followed by Student's *t*, Tukey's or Dunnet's *post hoc* tests were used to compare the performance indices between groups. For ethanol hyperactivity assays, repeated-measures ANOVA was used to compare activity between groups. Significance was marked at $P < 0.05$. All statistics reported are outlined in detail in **Supplementary Tables 3** and **4**.

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