

# The propensity for consuming ethanol in *Drosophila* requires *rutabaga* adenylyl cyclase expression within mushroom body neurons

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**Alcohol activates reward systems through an unknown mechanism, in some cases leading to alcohol abuse and dependence. Herein, we utilized a two-choice Capillary Feeder assay to address the neural and molecular basis for ethanol self-administration in *Drosophila melanogaster*. Wild-type *Drosophila* shows a significant preference for food containing between 5% and 15% ethanol. Preferred ethanol self-administration does not appear to be due to caloric advantage, nor due to perceptual biases, suggesting a hedonic bias for ethanol exists in *Drosophila*. Interestingly, *rutabaga* adenylyl cyclase expression within intrinsic mushroom body neurons is necessary for robust ethanol self-administration. The expression of *rutabaga* in mushroom bodies is also required for both appetitive and aversive olfactory associative memories, suggesting that reinforced behavior has an important role in the ethanol self-administration in *Drosophila*. However, *rutabaga* expression is required more broadly within the mushroom bodies for the preference for ethanol-containing food than for olfactory memories reinforced by sugar reward. Together these data implicate cAMP signaling and behavioral reinforcement for preferred ethanol self-administration in *D. melanogaster*.**

Keywords: cAMP, *Drosophila*, ethanol self-administration, learning, mushroom body

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Alcohol can act as a complex rewarding stimulus in humans, and positive reinforcement of alcohol consumption may ultimately lead to compulsive drinking and addiction.

Since alcohol broadly affects neural activity, both presynaptically and postsynaptically, by interacting with several classes of receptors, the underlying neurobiology of continued alcohol self-administration has been difficult to unravel.

The rodent two-bottle choice paradigm has been widely used to model alcohol reward during alcohol self-administration (Belknap *et al.* 1993; Cicero 1980). A similar two-choice assay has been developed for *Drosophila melanogaster*. In this paradigm, known as the Capillary Feeder (CAFE) assay, *Drosophila* are provided with a choice of drinking from two microcapillaries; one capillary tube contains liquid food and the other liquid food plus ethanol (Devineni & Heberlein 2009; Ja *et al.* 2007). In the CAFE assay, *Drosophila* display significant preference for food containing up to 15% ethanol over the non-ethanol food. Ethanol self-administration is independent of an olfactory or gustatory basis for ethanol within the liquid food (Devineni & Heberlein 2009). Moreover, altering the ratio of calories between the normal food and the ethanol-containing food in the CAFE assay did not significantly change the ethanol preference, suggesting ethanol self-administration is not due to the additional calories potentially provided by the ethanol (Devineni & Heberlein 2009). Interestingly, *Drosophila* also will prefer alcohol even when mixed with the bitter tasting quinine, and will rapidly display strong preference after deprivation, suggesting relapse (Devineni & Heberlein 2009). The continued preference despite the negative consequences of bitterness and the relapse after deprivation are features of addiction (Morse & Flavin 1992; Rodd *et al.* 2004). The CAFE assay is hence a capable behavioral model to genetically dissect the molecular mechanisms involved in ethanol self-administration.

Herein, we have used a modified CAFE assay to uncover a molecular mechanism and neural center involved in the preference for ethanol-containing food. We initially verified that the ethanol preference of wild-type flies is independent of a gustatory, olfactory or caloric bias for the ethanol-containing food. We further found that a mutation in the learning gene *rutabaga* has significant reduction in preference for ethanol-containing food. The targeted rescue of this mutant phenotype has identified intrinsic mushroom body neurons as important in the display of an ethanol preference. These neurons are central to positively reinforced olfactory conditioning with either sugar or ethanol as the rewarding stimulus (Kaun *et al.* 2011; Schwaerzel *et al.* 2003; Thum *et al.* 2007). Hence, *rutabaga*-dependent ethanol self-administration may function through alcohol's positive reinforcement of feeding behavior.

## Methods and materials

### Fly strains and husbandry

All flies were cultured on standard medium at 25°C, approximately 60% relative humidity and a 12-h light/dark cycle. With the exception of the Gal4 lines, mutations and transgenes used in this study were out-crossed into the Canton-S (CS) background for a minimum of six generations prior to behavioral analysis. All the Gal4 lines were out-crossed into *w<sup>1118</sup>* (CS10) background for at least seven generations. The *orco<sup>2</sup>* mutation is a loss-of-function disruption of the *orco* locus (Larsson et al. 2004). The *lush<sup>1</sup>* mutation has a deletion of *lush*-coding sequences (Laughlin et al. 2008). The *rut<sup>2080</sup>* mutation is severe hypomorphic allele caused by a P{IArB} insertion within *rutabaga* (Levin et al. 1992). The UAS-*rutabaga* line is capable of rescuing *rut<sup>2080</sup>* mutant phenotypes when expressed by Gal4 (Zars et al. 2000a). The *elav*-GeneSwitch transgene is an RU486-inducible UAS driver (Osterwalder et al. 2001). GH146-Gal4 drives expression within the projection neurons of the antennal lobe (Stocker et al. 1997). MB247-Gal4 is a Gal4 line driving expression in the  $\alpha$ ,  $\beta$  and  $\gamma$  lobes of mushroom body, and the c305a Gal4 transgene drives expression in  $\alpha'$ / $\beta'$  lobes (Aso et al. 2009; Zars et al. 2000a). The c305a and MB247 Gal4 lines were combined in a single genotype to drive expression in all the classes of mushroom body neurons. OK107, c772 and 238y express Gal4 in all classes of intrinsic mushroom body neurons (Aso et al. 2009). The MB-specific Gal80 represses Gal4 expression in the mushroom body (Krashes et al. 2007). For the genetic rescue of *rut<sup>2080</sup>*, the experimental genotype was generated by crossing virgin females of *rut<sup>2080</sup>; UAS-rut/CyO* (or *rut<sup>2080</sup>; +; UAS-rut*) with males of the indicated Gal4 lines. All Gal4 insertions were autosomal. F<sub>1</sub> male progeny was selected for analysis.

### Ethanol preference assay

The ethanol preference feeding assay is as described with some modification (Devineni & Heberlein 2009; Ja et al. 2007). The feeding apparatus is composed of two nested vials: an inner feeding vial and an outer humidity chamber. The feeding inner vial (1.5 cm diameter and 3.5 cm long) was plugged by a rubber stopper (standard 0#) with two holes inserted by two 200- $\mu$ l pipette tips that were cut to fit microcapillary pipettes. One of the tips was labeled. The bottom of inner vial was pierced to allow entry of water vapor and air from the outer vial, which was standard 25 mm *Drosophila* vial filled with 5 ml of water. Calibrated glass 5  $\mu$ l micropipettes (VWR, West Chester, PA, USA) filled with liquid food by capillary action were inserted through the 200- $\mu$ l pipette tips. A mineral oil (CAS: 8042-47-5; Sigma-Aldrich, St. Louis, MO, USA) overlay was applied in micropipette to minimize evaporation.

Liquid food was prepared with the designated amount of sucrose (CAS: 57-50-1; Sigma-Aldrich), yeast extract (Bacto yeast extract; BD Diagnostic Systems, Franklin Lakes, NJ, USA) and ethanol (CAS: 64-17-5; AAPER Alcohol and Chemical CO., Shelbyville, KY). All the solutions were made fresh each week and stored at 4°C.

Flies of the desired genotypes were collected at 0–1 day old. After 2 days, flies were anesthetized by CO<sub>2</sub>, and males were placed individually into the feeding chamber. They were habituated in the CAFE apparatus for 24 h, with *ad libitum* medium. After 24 h, the micropipettes which contain liquid food and liquid food plus ethanol were added to each feeding chamber. As a control, micropipettes were also applied in three empty vials, to measure the vaporized volume. The micropipettes were changed daily, while also altering the position of the ethanol-containing microcapillary pipette. The testing conditions were 4 days at 25°C, 60–70% humidity, with 12L:12D cycle.

To measure the liquid consumed, first the difference between the liquid surface present at the beginning and end of each 24 h was determined. The amount of liquid consumed was then determined as this difference minus the average evaporated liquid. Food consumption was defined as food with ethanol consumption + food without ethanol consumption. A preference index was defined as (food with ethanol consumption food without ethanol consumption)/food consumption. The caloric content of the medium was calculated on the basis of the following values: 4 kcal/g (sucrose),

1.58 kcal/g (yeast extract) and 7 kcal/g (ethanol) (Ja et al. 2007). Energy intake was defined as energy from ethanol-containing food + energy from non-ethanol food.

### Proboscis extension response assay

Our proboscis extension response (PER) assay was developed from a preceding assay (Kimura et al. 1986). Three-day-old flies were starved for 16–20 h to increase their gustatory sensitivity. A vial containing the flies was placed on ice for 2 min to briefly immobilize the flies. The flies were then transferred to a Petri dish. Small drops, each approximately 0.7  $\mu$ l of Loctite 404 (Henckel CO., Lewisville, TX, USA), were placed on a glass slide lined with labeling tape. The flies were then glued to the glass slide on their dorsal side with their legs free. Then the flies were covered with moist Kimwipes and allowed 15–30 minutes to recover from the cold stress. After the recovery period, a drop of water was applied to each fly's foreleg in order to habituate the PER to water. To measure the gustatory sensitivity, a drop of solution was briefly introduced to each fly's foreleg. The proboscis extension of the fly was recorded as an all-or-none event. For each trial, 10 flies were tested first at 5% sucrose + 5% yeast, or at 5% sucrose + 5% yeast + 5% (10% or 15%) ethanol. PER index was defined as the ratio between the flies showing proboscis extension and total flies tested.

### RU486 feeding

As previously described (Roman & Davis 2002; Roman et al. 2001), a 10 mM stock solution of RU486 (mifepristone; CAS: 84371-65-3; Sigma-Aldrich) dissolved in 80% ethanol was diluted to 500  $\mu$ M in 2% sucrose. Adult males were transferred to vials, each containing one Kimwipe wetted with 2 ml of diluted RU486 solution. Control flies were fed on 2% sucrose with 4% ethanol. Flies were kept in these vials at 25°C, 60–70% relative humidity, with 12L:12D cycle for 24 h and then transferred to the CAFE apparatus for a 24-h habitation period before beginning the ethanol preference assay.

### Survivorship assays

The survivorship assay was a modification from a previous protocol (Libert et al. 2007). In this assay, all flies were aged from synchronize embryos. Upon eclosion, they were collected for a 24-h period and allowed to mate for 2–3 days. After this time, females were collected under light CO<sub>2</sub> anesthesia and housed at 25 flies per group, and transferred to fresh food every other day. At 10 days, the flies were transferred into vials containing 1% agar, 1% agar mixed with 0.7% ethanol or 1% agar mixed with 0.89% sucrose. In the sucrose top experiments, the sucrose was layered on top of the agar to ensure the fly had full access to the sugar (no significance was seen in this treatment vs. mixing the sucrose with the agar). Deaths were recorded approximately every 3–5 h during the period of highest mortality until 100% mortality was reached.

### Immunohistochemistry

The methods for immunohistochemical detection of green fluorescent protein (GFP) were largely based on previously published techniques (Wu & Luo 2006). Adult flies between 2 and 7 days after eclosion, containing the indicated Gal4 driver and the UAS-GFP responder, were used. The brains were dissected in phosphate-buffered saline (PBS) solution, fixed with 4% paraformaldehyde (CAS: 30525-89-4; Sigma-Aldrich) in PBS for 20 min at room temperature and washed with PBS containing 0.4% Triton X-100 (PBT) (CAS: 9002-93-1; Sigma-Aldrich). The fixed brains were subsequently rinsed with PBT for 20 min three times. After being blocked with PBT containing 5% normal goat serum (Sigma-Aldrich) for 30 min at room temperature, the brains were incubated with the mouse polyclonal antibody against GFP (1:200; Roche, Penzberg, Germany) in PBT at 4°C for 48 h. The brains were then washed with PBT for 20 min three times. The prepared labeled brains were next incubated with Alexa Fluor® 488-conjugated goat anti-mouse (1:500; Invitrogen, Carlsbad, CA, USA) in blocking solution for 48 h at 4°C. Finally, the brains were rinsed with PBT (3  $\times$  20 min) and mounted in mounting

medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). The prepared brains were imaged with confocal microscopy on a Fluoview FV1000 microscope (Olympus, Tokyo, Japan).

### Statistical analysis

All statistical analysis was performed using STATVIEW v.5.0 software (SAS Institute, Inc., Cary, NC, USA). In the CAFE assay, energy intake, food consumption or preference index was analyzed using Student's *t*-test, or one-way analysis of variance (ANOVA), after tested by Kolmogorov–Smirnov normality test. Bonferroni correction was performed when more than two groups were compared. As described before (Devineni & Heberlein 2009; Masek & Scott 2010), PER score were analyzed using one-way ANOVA or Student's *t*-test. In the survivorship assay, Cox regression and log-rank tests were used to identify statistically significant differences in survival between treatments.

## Results

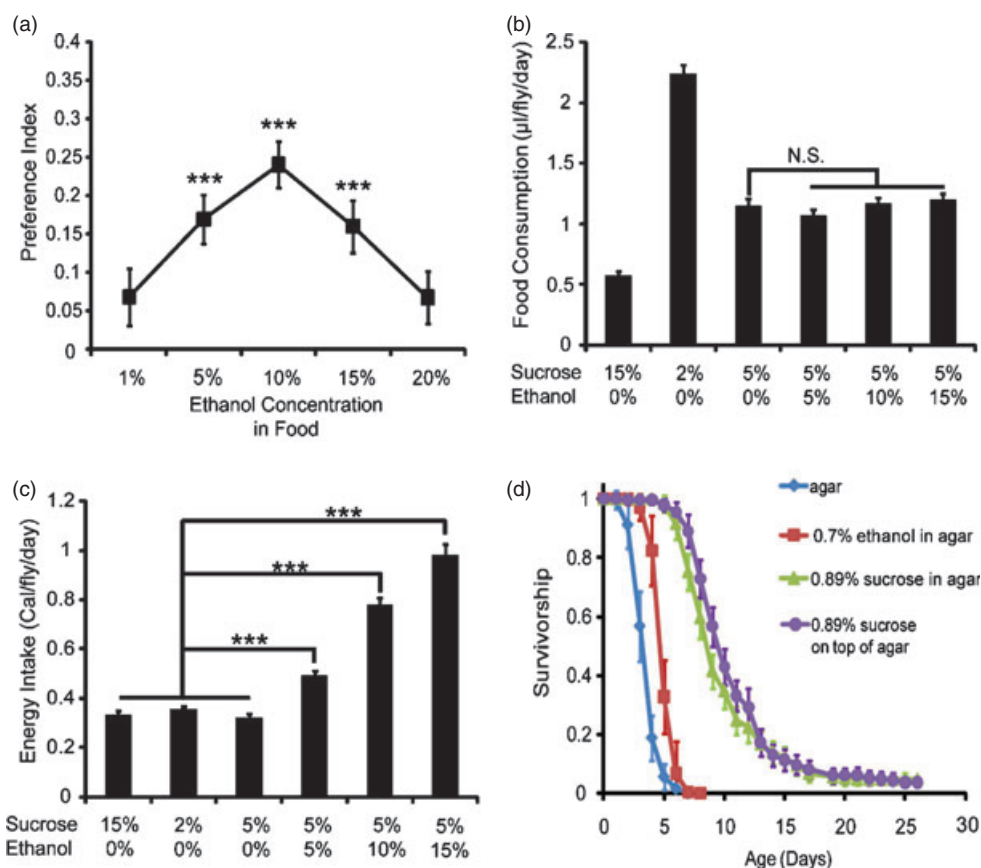
### Wild-type *Drosophila* prefer food-containing ethanol

To measure ethanol preference in *Drosophila*, we modified the Capillary Feeder (CAFE) assay (Devineni & Heberlein 2009; Ja *et al.* 2007). The CAFE assays are conceptually similar to the two-bottle choice assays commonly used to assess ethanol preference in rodents (Belknap *et al.* 1993; Cicero 1980). In our CAFE assay, single males were placed in a small humidified chamber and allowed to feed from two microcapillary tubes containing liquid food (5% yeast extract and 5% sucrose) or liquid food plus ethanol for 4 days (Fig. S1). The preference index was calculated as previously described to measure the daily surplus of food-containing ethanol consumed (Devineni & Heberlein 2009). Initially, we examined the preference index for food containing different concentrations of ethanol during a 4-day period. In this experiment, wild-type CS male flies significantly preferred 5%, 10% and 15% ethanol (Fig. 1a; for 5% ethanol,  $t_{(30)} = 5.193$ ,  $P < 0.0001$ ,  $n = 30$  flies; for 10% ethanol,  $t_{(31)} = 5.756$ ,  $P < 0.0001$ ,  $n = 31$  flies; for 15% ethanol,  $t_{(28)} = 5.537$ ,  $P < 0.0001$ ,  $n = 28$  flies), but did not display a preference for 1% and 20% ethanol (Fig. 1a; for 1% ethanol,  $t_{(33)} = 1.997$ ,  $P = 0.0545$ ,  $n = 33$  flies; for 20% ethanol,  $t_{(34)} = 1.762$ ,  $P = 0.0873$ ,  $n = 34$  flies). Ethanol preference was stable over the 4 days examined (data not shown). The preferred range of ethanol concentrations is similar to that found in C57BL, the most widely used mouse strain in alcohol self-administration assay (Belknap *et al.* 1993), but appears significantly lower than that found for  $w^-$  Berlin strains of *D. melanogaster* (Devineni & Heberlein 2009).

For a useful model of ethanol self-administration, the drug should be consumed for its pharmacological effects, rather than for calories, taste or smell (Cicero 1980). Interestingly, flies can associate the nutritional strength of food with an odor to form a robust memory, which may reinforce the preference in CAFE assay (Burke & Waddell 2011; Fujita & Tanimura 2011). Yet, a previous study suggested that ethanol preference was unlikely to be a byproduct of caloric attraction (Devineni & Heberlein 2009). To further confirm this result, we compared the daily food consumption and energy intake of CS males for which we varied either the sucrose content or the ethanol content in the food.

When the concentration of sugar is varied in the CAFE assay, the flies will consume differing amounts of food, so they balance their daily caloric intake to approximately 0.33 calorie/fly/day (Fig. 1c;  $F_{5,113} = 132.111$ ,  $P < 0.0001$ , each  $n = 19$ –20 flies; no significance for 5% sucrose group vs. 2% sucrose or 15% sucrose group). However, when the ethanol concentration within the food is varied, the flies consume the same total volume as the comparable non-ethanol containing food (Fig. 1b;  $F_{5,113} = 88.551$ ,  $P < 0.0001$ ,  $n = 19$ –20 flies; no significance between the four groups in which sucrose concentration is 5%), and do not balance food intake to compensate for any changes in caloric content added by the ethanol (Fig. 1c;  $F_{5,113} = 132.111$ ,  $P < 0.001$ , each  $n = 19$ –20 flies;  $P < 0.001$  for the 5% ethanol group vs. the other 3 non-ethanol groups,  $P < 0.001$  for 10% ethanol group vs. other three non-ethanol groups,  $P < 0.0001$  for 15% ethanol group vs. other three non-ethanol groups). To confirm that *Drosophila* can utilize ethanol for metabolic energy, we examined the survivorship of CS and  $w^{1118}$  wild-type strains on agar alone, agar mixed with 0.7% ethanol or agar mixed with 0.89% sucrose. Interestingly, ethanol prolongs survival without other energy source (Fig. S2;  $P < 0.05$ , log-rank test,  $n = 10$  groups). However, CS can survive much longer on the same amount of sugar calories than on ethanol calories (Fig. 1d;  $P < 0.001$ , log-rank test,  $n = 10$  groups). In these experiments the ethanol was mixed into the food, which substantially reduces the evaporation (Gibson *et al.* 1981). Moreover, the ethanol vial was exchanged after 4 days, allowing for the flies access to ethanol as a food source during this experiment. Hence, ethanol contains significantly less nutrition than sucrose. Since *Drosophila* do not alter the total food consumed based on ethanol content, the few calories received from ethanol are likely ignored and an energy bias is not a source of innate ethanol preference in the CAFE assay.

A second explanation for ethanol preference is a sensory bias; the food-containing ethanol may either smell or taste better than the normal food. To  $w^-$  flies in a Berlin background, high ethanol concentrations suppressed the PER to sucrose, indicating a gustatory aversion to ethanol. However, the PER response was not suppressed much by low ethanol concentrations (Devineni & Heberlein 2009). We also found that food containing 5–15% ethanol did not inhibit PER significantly, relative to food without ethanol, while low ethanol concentrations are at best neutral (Fig. S3a;  $F_{3,52} = 1.948$ ,  $P > 0.05$ ,  $n = 13$ –15 groups). Previously, the  $w^-$  Berlin flies were found to have an olfactory bias toward ethanol odor, but they preferred ethanol even when olfactory ability was removed, indicating that olfaction was not necessary for ethanol preference (Devineni & Heberlein 2009). Interestingly, in an olfactory trap assay CS flies in a  $w^-$  background were attracted more by yeast than by yeast plus 25% ethanol odorants (Kim & Smith 2001), which suggests that these flies have an olfactory bias for non-ethanol food over ethanol-containing food. To further explore possible roles for olfactory plasticity in ethanol preference, we examined  $w^+$ ; *orco*<sup>2</sup> and  $w^+$ ; *lush*<sup>1</sup> mutants in the CAFE assay. The  $w^+$ ; *orco*<sup>2</sup> mutants are broadly anosmic, display increased stress resistance and altered metabolism, and are long-lived (Larsson *et al.* 2004; Libert *et al.* 2007). The  $w^+$ ;



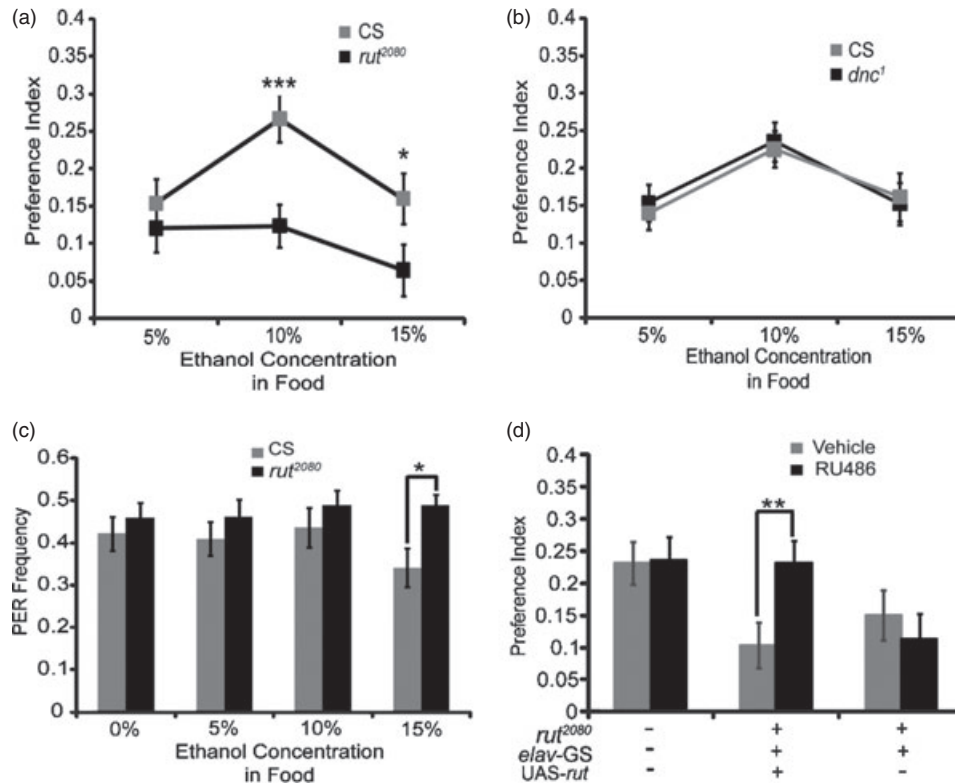
**Figure 1: The preference for ethanol-containing food is independent of nutrient value.** (a) Canton-S flies were analyzed in the CAFE assay for a preference for liquid food-containing ethanol at the indicated percentages. CS flies display a significant preference for food containing 5%, 10% and 15% ethanol. (b) The daily food consumption of CS flies over a 4-day period was determined for the indicated liquid foods that also contained 5% yeast extract. In this experiment, the flies were allowed access to only one kind of food if without ethanol in food, or were tested in the standard two choices CAFE assay. Although CS modulates the amount of food consumed per day based on the sucrose content, they did not alter food consumption based on ethanol concentration. (c) The caloric value consumed by each fly daily for each food substrate was determined. In this assay, CS flies balanced the energy intake from sugar, but failed to make a similar adjustment to calories provided by ethanol, because the daily energy intake from each kind of ethanol-containing food was more than these three kinds of non-ethanol food. (d) The ability of CS flies to utilize ethanol as an energy source was examined through a survivorship assay. CS flies fed with 0.7% ethanol survive significantly longer than completely starved flies, but significantly shorter than flies fed with 0.89% sucrose. Since 0.7% ethanol contains similar caloric content as 0.89% sucrose, the shorter survival time on ethanol as a food source compared to sucrose suggests that *Drosophila* cannot use a majority of the calories present in ethanol for sustenance. Data are means  $\pm$  SEM. N.S., no significance. \*\*\* $P < 0.001$ .

*orco*<sup>2</sup> flies did not display any significant defects in ethanol preference (Fig. S3b; for 5% ethanol,  $t_{(44)} = 0.212$ ,  $P > 0.05$ ,  $n = 23$  each; for 10% ethanol,  $t_{(74)} = 0.959$ ,  $P > 0.05$ ,  $n = 38$  each; for 15% ethanol,  $t_{(48)} = 0.542$ ,  $P > 0.05$ ,  $n = 25$  each). The *lush* gene encodes an odorant binding protein required for detecting a pheromone, 11-*cis* vaccenyl acetate (Laughlin *et al.* 2008). The *lush*<sup>1</sup> mutants are more attracted to high ethanol concentrations in olfactory trap assays than wild-type flies (Kim *et al.* 1998). However, the *lush*<sup>1</sup> mutants did not show a preference phenotype within our CAFE assay (Fig. S3c; for 5% ethanol,  $t_{(36)} = 0.421$ ,  $P > 0.05$ ,  $n = 19$  each; for 10% ethanol,  $t_{(56)} = 1.303$ ,  $P > 0.05$ ,  $n = 29$  each; for 15% ethanol,  $t_{(38)} = 0.328$ ,  $P > 0.05$ ,  $n = 20$  each). The

absence of a phenotype in the anosmic *w*<sup>+</sup>; *orco*<sup>2</sup> and the ethanol preferring *lush*<sup>1</sup> mutants support an absence of a significant role for an olfactory bias in the ethanol self-administration (Devineni & Heberlein 2009).

### **The rutabaga type I adenylyl cyclase in mushroom body is essential for the expression of an ethanol preference**

The *rutabaga* (*rut*) gene encodes a type I calcium-activated adenylyl cyclase and is essential for both associative learning and for normal responses to the sedative effects of ethanol vapor (Levin *et al.* 1992; Moore *et al.* 1998; Tempel *et al.* 1983). We therefore examined *rut*<sup>2080</sup> reduction-of-function



**Figure 2: The *rutabaga* adenylyl cyclase is acutely required for an ethanol preference.** (a) *rut*<sup>2080</sup> mutants and control CS flies were examined in the CAFE assay. The *rut*<sup>2080</sup> mutants display a significantly reduced preference for 10% and 15% ethanol compared to wild-type CS. (b) Mutants in the *dunce* phosphodiesterase were also examined in the CAFE assay. The *dnc*<sup>1</sup> loss-of-function mutants exhibited a normal preference for 5%, 10% and 15% ethanol. (c) In the PER assay, *rut*<sup>2080</sup> did not show a significant difference with CS, to liquid food without or with ethanol, except for 15% ethanol, indicating that the *rut*<sup>2080</sup> reduced preference is not due to altered gustatory responses to ethanol. (d) The reduced ethanol preference of *rut*<sup>2080</sup> can be rescued by the induced expression of a *rut* cDNA in the nervous system using the *elav*-GeneSwitch driver. In this experiment, *rutabaga* expression was induced by RU486, resulting in significantly greater preference for 10% ethanol than that within the same genotype but vehicle-fed flies, and also more than the genotype *rut*<sup>2080</sup>; +; *elav*-GS/+ induced by RU486. The same treatment did not influence the ethanol preference in CS flies and the negative control flies, *rut*<sup>2080</sup>; +; *elav*-GS/+. Data are means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

mutants in our CAFE assay. The *rut*<sup>2080</sup> mutants displayed a normal preference to 5% ethanol; however, the preference for 10% and 15% ethanol was significantly less than that of CS flies (Fig. 2a; for 5% ethanol,  $t_{(46)} = 0.951$ ,  $P > 0.05$ ,  $n = 24$  each; for 10% ethanol,  $t_{(69)} = 3.545$ ,  $P = 0.0007$ ,  $n = 35$  each; for 15% ethanol,  $t_{(52)} = 2.329$ ,  $P = 0.025$ ,  $n = 27$  each). These data suggest a role for cAMP signaling in the expression of an ethanol preference. The *rut*<sup>2080</sup> mutants also displayed decreased food consumption in the CAFE assay, raising the possibility that decreased total food consumption may shape the expressed ethanol preference (Fig. S4a; for 5% ethanol,  $t_{(46)} = 2.989$ ,  $P = 0.0045$ ,  $n = 24$  each; for 10% ethanol,  $t_{(69)} = 2.722$ ,  $P = 0.0082$ ,  $n = 35$ –36 each; for 15% ethanol,  $t_{(52)} = 2.550$ ,  $P = 0.014$ ,  $n = 27$  each).

The *dunce* gene (*dnc*) encodes a cAMP phosphodiesterase that is required during negatively reinforced associative conditioning, but is not required in a positively reinforced olfactory-learning assay (Tempel *et al.* 1983; Tully & Quinn

1985). The *dnc* gene product is also the primary and perhaps only phosphodiesterase active in adult mushroom body neurons (Gervasi *et al.* 2010). Interestingly, the *dnc*<sup>1</sup> mutants displayed a normal preference for 5%, 10% and 15% ethanol (Fig. 2b; for 5% ethanol,  $t_{(57)} = 0.422$ ,  $P > 0.05$ ,  $n = 29$  each; for 10% ethanol,  $t_{(58)} = 0.450$ ,  $P > 0.05$ ,  $n = 30$  each; for 15% ethanol,  $t_{(38)} = 0.105$ ,  $P > 0.05$ ,  $n = 20$  each). These differences between *dnc*<sup>1</sup> and *rut*<sup>2080</sup> in the CAFE assay also mirrors that of an ethanol sedation assay, where the *rut*<sup>2080</sup> mutants are hypersensitive, and the *dnc*<sup>1</sup> mutants lack an obvious phenotype (Moore *et al.* 1998).

To eliminate trivial causes for the *rut*<sup>2080</sup> ethanol preference phenotype, we examined the gustatory response of this mutant to ethanol-containing food. The PER elicited by 5% or 10% ethanol-containing food by *rut*<sup>2080</sup> mutants was not significantly different from the wild-type controls (Fig. 2c; for 5% ethanol,  $t_{(18)} = 0.924$ ,  $P > 0.05$ ,  $n = 9$ –11 groups; for 10% ethanol,  $t_{(17)} = 0.907$ ,  $P > 0.05$ ,  $n = 8$ –11 groups). In contrast, the PER index of *rut*<sup>2080</sup> flies to food-containing

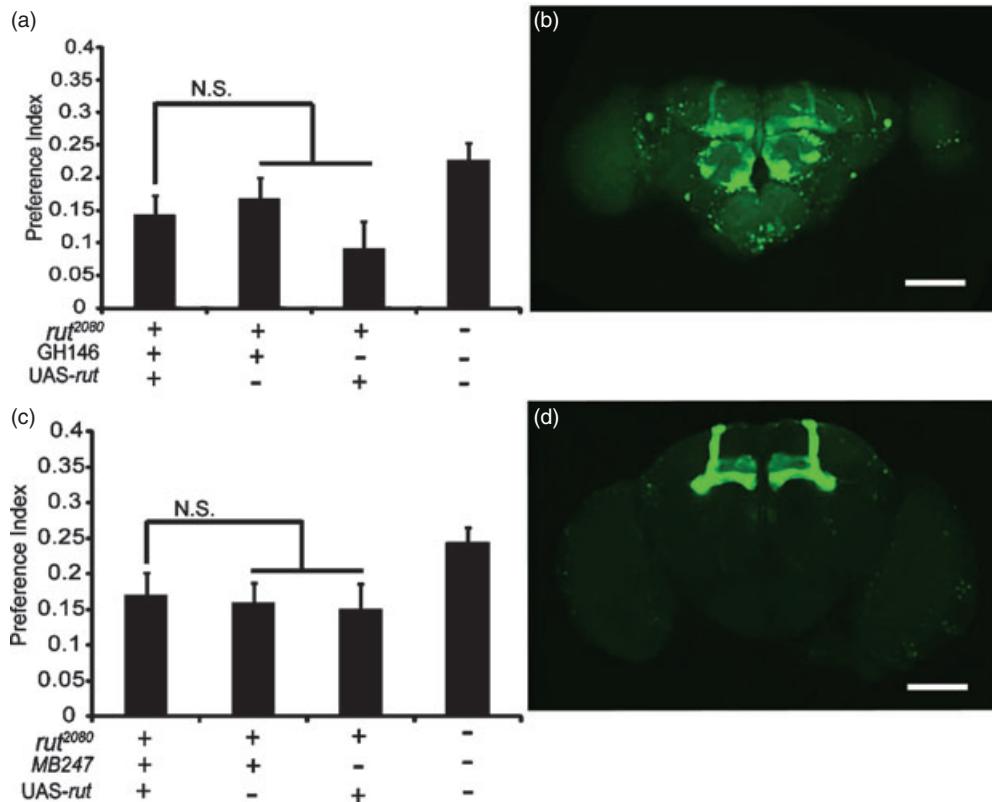
15% ethanol was significantly greater than that of CS flies (Fig. 2c;  $t_{(17)} = 2.64$ ,  $P = 0.017$ ,  $n = 9-10$  groups), which suggested that 15% ethanol was less aversive to *rut*<sup>2080</sup> than to CS controls. The expectation from the reduced aversive gustatory response of *rut*<sup>2080</sup> flies to 15% ethanol-containing food is that they would drink more ethanol than CS. Thus, the reduced ethanol preference of the *rut*<sup>2080</sup> mutants is unlikely to be due to reduced aversive taste of ethanol. Furthermore, it was previously reported that: (1) the olfactory response of *rut*<sup>2080</sup> flies was not different than that of wild-type flies and (2) the absorption and metabolism of ethanol is not altered between *rut*<sup>2080</sup> and wild-type flies (Moore et al. 1998; Tully & Quinn 1985).

Loss of *rut* activity may lead to developmental defects in neural function (Zhong & Wu 1993). To examine the possibility of defects in developmental underlying the *rut* function in ethanol self-administration we determined whether the deficit in ethanol preference of *rut*<sup>2080</sup> flies could be rescued by *rut* expression post-developmentally. This was accomplished using the RU486-inducible *elav*-GeneSwitch driver (Osterwalder et al. 2001; Roman 2004). One- to two-day-old CS, *rut*<sup>2080</sup>; +; *elav*-GeneSwitch/+ and *rut*<sup>2080</sup>; UAS-*rut*/+; *elav*-GeneSwitch/+ flies were fed with either RU486 or vehicle for 24 h, followed by a second 24-h period of habituation to the CAFE chamber. The RU486 did not alter the ethanol preference behavior of CS and *rut*<sup>2080</sup>; +; *elav*-GeneSwitch/+ flies (Fig. 2d). However, the RU486-induced expression of the *rut* cDNA within the nervous tissue completely rescued the *rut*<sup>2080</sup> ethanol preference phenotype (Fig. 2d;  $t_{(71)} = 3.08$ ,  $P = 0.003$  for induced *rut*<sup>2080</sup>; UAS-*rut*/+; GS/+ vs. un-induced *rut*<sup>2080</sup>; UAS-*rut*/+; GS/+ planned comparison,  $n = 36-37$  each). Also, this post-developmental expression of the *rut* cDNA did not significantly increase the total food consumption phenotype (Fig. S4b; planned comparison for *rut*<sup>2080</sup>; UAS-*rut*/+; GS/+ with un-induced *rut*<sup>2080</sup>; UAS-*rut*/+; GS/+,  $t_{(71)} = 0.500$ ,  $P > 0.05$ ,  $n = 36-37$  flies), genetically distinguishing these two phenotypes. Moreover, the OK107 or *c772* driven *rutabaga* expression rescued the abnormal ethanol preference in *rut*<sup>2080</sup> (see below), but did not rescue the total food consumption phenotype (Fig. S4c;  $F_{3,150} = 9.891$ ,  $P < 0.0001$ , in *post hoc* comparisons,  $P > 0.05$  for *rut*<sup>2080</sup>; *c772*/+; UAS-*rut*/+ vs. *rut*<sup>2080</sup>; *c772*/+,  $n = 37-40$  each; Fig. S4d;  $F_{3,140} = 17.650$ ,  $P < 0.0001$ ; in *post hoc* comparisons,  $P > 0.05$  for *rut*<sup>2080</sup>; UAS-*rut*/+; OK107/+ vs. *rut*<sup>2080</sup>; OK107/+,  $n = 27$  for the *rut*<sup>2080</sup>; OK107/+ group and  $n = 39$  for the *rut*<sup>2080</sup>; UAS-*rut*/+; OK107/+ group). These data suggested that total food consumption is not measurably affecting the preference for ethanol in our CAFE assay. Thus, the ethanol preference phenotype is due to the acute lack of *rut* expression within the nervous system, and not due to the absence of *rut* during development.

The identification of the neural foci for the *rut*-dependent function in ethanol self-administration will elucidate neurons that are required for this behavior and may also provide insight into the underlying behavioral mechanisms. The *rut* gene is broadly expressed in the adult nervous system, and is enriched within the mushroom body neurons of the central brain (Levin et al. 1992). These mushroom body neurons are critical sites for both positively reinforced

and negatively reinforced olfactory learning and memory formation (reviewed by Zars 2011). The decreased ethanol self-administration of *rut*<sup>2080</sup> mutants could be due to the role of this gene in forming positively reinforced associations. Ethanol can act as both a long-term positive reinforcer and shorter term negative reinforcer in an olfactory-learning paradigm in *Drosophila* (Kaun et al. 2011), but it is not known whether *rut* has a role in acquiring these memories. The activity of *rut* is however required for wild-type levels of sugar-reinforced olfactory memories in *Drosophila* (Schwaerzel et al. 2003; Tempel et al. 1983). We next considered the possibility that the *rut*-dependent component of the ethanol preference behavior in the CAFE assay is due to similar appetitive olfactory learning. The sugar-reinforced olfactory memory deficit of *rut*<sup>2080</sup> can be fully rescued through the expression of a wild-type *rut* cDNA within the antennal lobe projection neurons defined by the GH146-Gal4 driver, or the mushroom body neurons defined by the MB247-Gal4 driver (Thum et al. 2007). The expression of *rut* driven by either GH146 or MB247 failed to rescue the *rut*<sup>2080</sup> ethanol preference phenotype (Fig. 3;  $F_{3,123} = 2.800$ ,  $P = 0.042$ , no significance for *rut*<sup>2080</sup>; GH146/+; UAS-*rut*/+ vs. *rut*<sup>2080</sup>; GH146/+ or *rut*<sup>2080</sup>; UAS-*rut*/+,  $n = 31-32$  each; Fig. 3b;  $F_{3,190} = 2.047$ ,  $P = 0.109$ ,  $n = 48-50$  each). This result genetically separates the role of *rut* in sugar-reinforced olfactory memory formation from ethanol self-administration.

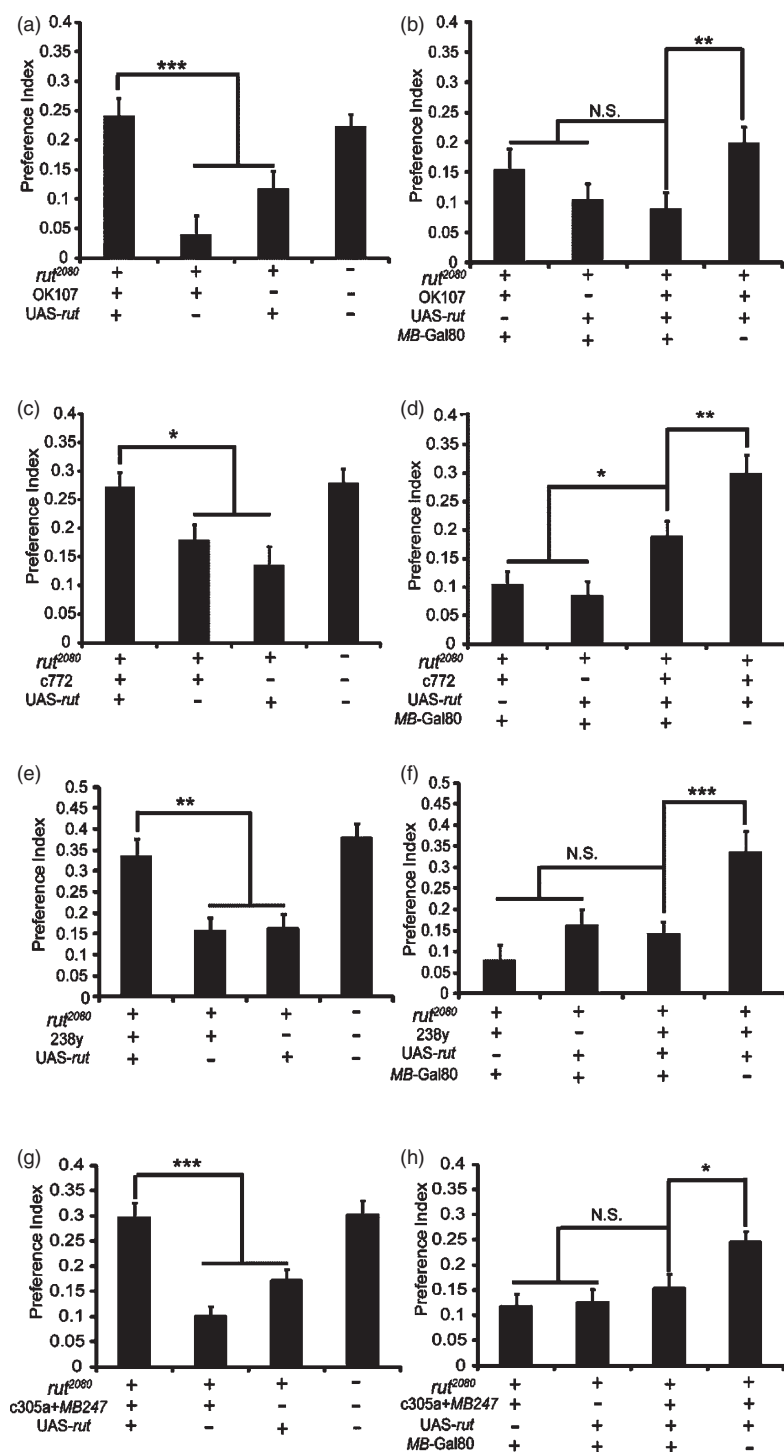
In contrast, the expression of *rut* driven by either the OK107, *c772*, 238y or *c305a*+MB247 Gal4 mushroom body drivers rescued the ethanol preference phenotype of *rut*<sup>2080</sup> (In Fig. 4a,  $F_{3,140} = 10.398$ ,  $P < 0.0001$ , in *post hoc* comparisons,  $P < 0.001$  for both *rut*<sup>2080</sup>; UAS-*rut*/+; OK107/+ vs. *rut*<sup>2080</sup>; OK107/+ or *rut*<sup>2080</sup>; UAS-*rut*/+, in *rut*<sup>2080</sup>; OK107/+ group,  $n = 27$  flies and each  $n = 39$  in other groups. In Fig. 4c,  $F_{3,150} = 6.638$ ,  $P = 0.0003$ , in *post hoc* comparisons,  $P = 0.023$  for *rut*<sup>2080</sup>; *c772*/+; UAS-*rut*/+ vs. *rut*<sup>2080</sup>; *c772*/+ and  $P = 0.0005$  for *rut*<sup>2080</sup>; *c772*/+; UAS-*rut*/+ vs. *rut*<sup>2080</sup>; UAS-*rut*/+,  $n = 37-40$  each. In Fig. 4e,  $F_{3,115} = 9.623$ ,  $P < 0.0001$ , in *post hoc* comparisons,  $P < 0.01$  for both *rut*<sup>2080</sup>; UAS-*rut*/238y vs. *rut*<sup>2080</sup>; 238y/+ or *rut*<sup>2080</sup>; UAS-*rut*/+,  $n = 29-30$  each. In Fig. 4g,  $F_{3,175} = 14.238$ ,  $P < 0.001$ , in *post hoc* comparisons,  $P < 0.001$  for *rut*<sup>2080</sup>; *c305a*/+; UAS-*rut*/MB247 vs. *rut*<sup>2080</sup>; *c305a*/+; UAS-*rut*/MB247 or *rut*<sup>2080</sup>; UAS-*rut*/+,  $n = 44-45$  each). These four Gal4 lines drive expression within the whole mushroom bodies, and also provide weak expression in antennal lobe, optic lobe, pars intercerebralis, antennal nerve, tritocerebrum and subesophageal ganglion (Fig. 5) (Aso et al. 2009). The *rut* foci for ethanol preference were further narrowed down by inhibiting Gal4 with the MB-Gal80 transgene (Krashes et al. 2007). In the MB-Gal80 transposon, Gal80 is driven by the *dme2* mushroom body enhancer, which is as also used in the MB247-Gal4 driver (Zars et al. 2000a). However, this enhancer drives the Gal80 expression in the whole mushroom body, rather than in the  $\alpha/\beta$  and  $\gamma$  lobes in MB247-Gal4 (Krashes et al. 2007). In the presence of MB-Gal80, the GFP expression in mushroom bodies driven by the OK107, *c772*, 238y Gal4 lines or the *c305a* + MB247 combination was significantly reduced or eliminated (Fig. 5). Moreover, the four Gal4 drivers were no longer capable of rescuing the *rut*<sup>2080</sup> ethanol preference phenotype in the presence



**Figure 3: The ethanol preference phenotype of *rut*<sup>2080</sup> mutants is separable from simple appetitive olfactory learning.** Both GH146 and MB247 rescue the olfactory appetitive-learning defect in *rut*<sup>2080</sup>. However, they cannot rescue ethanol preference defect in *rut*<sup>2080</sup>. (a) The expression of *rut* driven by the projection neuron driver GH146-Gal4 failed to rescue the *rut*<sup>2080</sup> ethanol preference phenotype, since the ethanol preference of the genotype *rut*<sup>2080</sup>; GH146/+; UAS-*rut*/+ was not significantly different from both negative controls. (b) GH146-Gal4 drives GFP expression in antennal lobe, olfactory projection neurons and partial mushroom body. (c) Likewise, the expression of *rut* driven by the MB247-Mushroom body Gal4 line also failed to rescue the *rut*<sup>2080</sup> ethanol preference phenotype, since the preference index of the genotype *rut*<sup>2080</sup>; UAS-*rut*/MB247 was not significantly different from both negative controls. (d) MB247-Gal4 drives GFP expression in  $\alpha/\beta$  and  $\gamma$  lobe. Data are mean  $\pm$  SEM. N.S., no significance.

of MB-Gal80 (In Fig. 4b,  $F_{3,114} = 3.011$ ,  $P = 0.033$ , no significance for *rut*<sup>2080</sup>; MB-Gal80/+; UAS-*rut*/+; OK107/+ vs. *rut*<sup>2080</sup>; MB-Gal80/+; OK107/+ or *rut*<sup>2080</sup>; MB-Gal80/+; UAS-*rut*/+, however,  $P = 0.0074$  for *rut*<sup>2080</sup>; MB-Gal80/+; UAS-*rut*/+; OK107/+ vs. *rut*<sup>2080</sup>; UAS-*rut*/+; OK107/+). In Fig. 4d,  $F_{3,110} = 13.898$ ,  $P < 0.0001$ , in *post hoc* comparisons,  $P = 0.018$  for *rut*<sup>2080</sup>; MB-Gal80/c772; UAS-*rut*/+ vs. *rut*<sup>2080</sup>; MB-Gal80/c772, however,  $P = 0.0013$  for *rut*<sup>2080</sup>; MB-Gal80/c772; UAS-*rut*/+ vs. *rut*<sup>2080</sup>; c772/+; UAS-*rut*/+,  $n = 28-29$  each. In Fig. 4f,  $F_{3,80} = 7.73$ ,  $P = 0.0001$ , no significance for *rut*<sup>2080</sup>; MB-Gal80/+; UAS-*rut*/238y vs. *rut*<sup>2080</sup>; MB-Gal80/+; 238y/+ or *rut*<sup>2080</sup>; MB-Gal80/+; UAS-*rut*/+, however,  $P = 0.0008$  for *rut*<sup>2080</sup>; MB-Gal80/+; UAS-*rut*/238y vs. *rut*<sup>2080</sup>; UAS-*rut*/238y,  $n = 20-22$  each. In Fig. 4h,  $F_{3,165} = 4.938$ ,  $P = 0.0026$ , no significance for *rut*<sup>2080</sup>; MB-Gal80/c305a; UAS-*rut*/MB247 vs. *rut*<sup>2080</sup>; MB-Gal80/c305a; MB247/+ or *rut*<sup>2080</sup>; MB-Gal80/+; UAS-*rut*/+, however,  $P = 0.0209$  for *rut*<sup>2080</sup>; MB-Gal80/c305a; UAS-*rut*/MB247 vs. *rut*<sup>2080</sup>; c305a/+; UAS-*rut*/MB247,  $n = 42-43$  each). The expression of *rut* in the  $\alpha/\beta$ ,  $\alpha'/\beta'$  and  $\gamma$  lobe

neurons is capable of fully rescuing the *rut*<sup>2080</sup> ethanol self-administration phenotype, whereas *rut* expression in the  $\alpha/\beta$  and  $\gamma$  lobe neurons driven by MB247 alone is insufficient for rescue. This difference suggests that the expression of *rutabaga* in the  $\alpha'/\beta'$  neurons may be sufficient to rescue the *rut*<sup>2080</sup> ethanol self-administration phenotype. We examined the isolated c305a  $\alpha'/\beta'$  Gal4 driver for an ability to rescue the *rut*<sup>2080</sup> self-administration phenotype. The resulting phenotype lies between the CS positive control and the *rut*<sup>2080</sup>; c305/+ genotype control, and is not significantly different from either (Fig. S5;  $F_{3,147} = 5.767$ ,  $P = 0.0009$ . In *post hoc* comparisons,  $P = 0.487$  for *rut*<sup>2080</sup>; c305a/+; UAS-*rut*/+ vs. CS,  $P = 0.137$  for *rut*<sup>2080</sup>; c305a/+; UAS-*rut*/+ vs. *rut*<sup>2080</sup>; c305a/+ and  $P = 0.002$  for *rut*<sup>2080</sup>; c305a/+; UAS-*rut*/+ vs. *rut*<sup>2080</sup>; UAS-*rut*/+,  $n = 37-39$  each). Hence, it remains possible that *rut* expression within the  $\alpha'/\beta'$  lobe neurons is critical for the expression of a normal ethanol self-administration. Together, these data indicate that *rut* expression in the mushroom body is essential for ethanol preference in *Drosophila*.



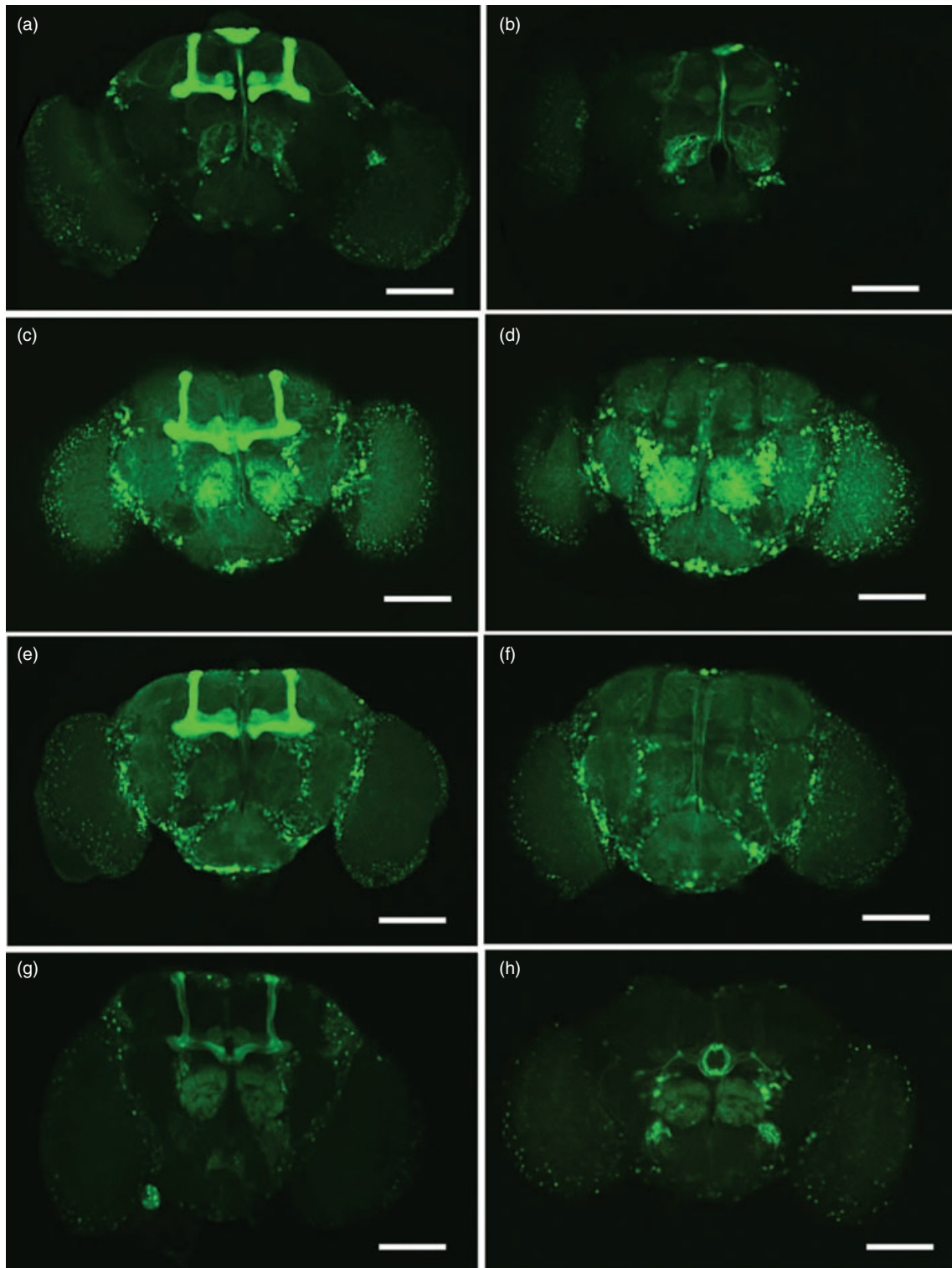
**Figure 4: The *rutabaga* requirement for normal ethanol preference maps to the mushroom body neurons.** The indicated genotypes were examined in the CAFE assay for an ethanol preference. (a) In the presence of both the UAS-*rut* and the OK107 mushroom body Gal4 driver, the *rut<sup>2080</sup>* ethanol preference phenotype is not significantly different than CS, and is significantly higher than the control *rut<sup>2080</sup>* genotypes. (b) The MB-Gal80 transgene inhibits the expression of Gal4 in specifically mushroom body neurons (Krashes *et al.* 2007). In the presence of MB-Gal80, UAS-*rut* and OK107 did not display a significantly ethanol preference in comparison to the *rut<sup>2080</sup>*; UAS-*rut*/+; OK107/+ genotype, and also no significance in comparison to *rut<sup>2080</sup>*; MB-Gal80/+; OK107/+ and *rut<sup>2080</sup>*; UAS-*rut*/MB-Gal80 genotype. (c) Similarly, the *rut<sup>2080</sup>* ethanol preference phenotype is only rescued in the presence of both the UAS-*rut* and c772 mushroom body Gal4 driver. (d) The MB-Gal80 transgene similarly reduced the ethanol preference of *rut<sup>2080</sup>* flies carrying the UAS-*rut* and c772 transgenes. However, even in the presence of MB-Gal80, UAS-*rut* and c772 Gal4 displayed a significantly increased ethanol preference, compared with the two negative control. (e) In the presence of both the UAS-*rut* and the 238y Gal4 driver, the *rut<sup>2080</sup>* ethanol preference phenotype is significantly higher than the control *rut<sup>2080</sup>* genotypes. In all genotypes, the *ry<sup>506</sup>* allele was also present. (f) In the presence of MB-Gal80, UAS-*rut* and 238y-Gal4 display a significantly rescued ethanol preference in comparison to the *rut<sup>2080</sup>*; UAS-*rut*/+; 238y/+ genotype, and also no significance in comparison to *rut<sup>2080</sup>*; MB-Gal80/+; 238y/+ and *rut<sup>2080</sup>*; UAS-*rut*/MB-Gal80 genotype. In all genotypes, a *ry<sup>506</sup>* allele was taken in third chromosome. (g) In the presence of the UAS-*rut* and the combination of c305a + MB247 Gal4 drivers, the *rut<sup>2080</sup>* ethanol preference phenotype is significantly higher than the control *rut<sup>2080</sup>* genotypes. (h) The MB-Gal80 transgene similarly reduced the ethanol preference of *rut<sup>2080</sup>* flies carrying the UAS-*rut* and c305a+MB247 transgenes, and also displayed no significance in comparison to *rut<sup>2080</sup>*; MB-Gal80/c305a; MB247/+ and *rut<sup>2080</sup>*; UAS-*rut*/MB-Gal80 genotype. Data are means  $\pm$  SEM. N.S., no significance. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

**Discussion**

Why alcohol is consumed habitually and compulsively remains a fundamental unanswered question in addiction research. The use of diverse model systems and approaches appears necessary to unravel this question. We have used

the CAFE self-administration assay to address the behavioral mechanism, molecular mechanism and neural circuitry of ethanol-feeding preference in *D. melanogaster*. In our assay, wild-type CS flies exhibit a significant preference for ethanol concentrations from 5% to 15%. This preference does not depend on the recognition of extra calories gained from





**Figure 5: MBGal80 inhibits Gal4 activity within all the mushroom body neurons.** GFP was driven by four Gal4 drivers: (a) OK107; (c) c772; (e) 238y; (g) c305a+*MB247*, and detected by immunohistochemistry. The genotypes in panels (b), (d), (f) and (h) contain the MB-Gal80 transgene which effectively removes the expression of GFP from the mushroom bodies. Scale bar, 100  $\mu$ m.

the ethanol, nor does it depend on olfactory or gustatory biases for ethanol. The preference does, however, require the activity of the *rut* type I  $\text{Ca}^{2+}$ -stimulated adenylyl cyclase within the mushroom body neurons, indicating a role for cAMP signaling in ethanol consumption behavior. This result is very significant as *rut* activity within the mushroom bodies is also involved in both reward and punishment learning. Since ethanol can act as a positive unconditioned stimulus in olfactory learning, and mushroom bodies are required to learn the association of odorants with alcohol (Kaun *et al.* 2011), there is a strong likelihood that the ethanol preference found in our assay is due to the rewarding properties of this drug.

The *rut*<sup>2080</sup> mutants are defective in ethanol preference. This mutant phenotype was rescued acutely through the induced expression of a wild-type *rut* cDNA within the nervous system, indicating a physiological need for this cyclase for ethanol self-administration behavior. Knockout mutants of the mouse calcium-activated type VIII adenylyl cyclase (AC8), but not knockouts of the type I adenylyl cyclase, also display reduced ethanol self-administration (Maas *et al.* 2005). In mouse cortical membrane preparations, ethanol does not increase  $\text{Ca}^{2+}$ -stimulated adenylyl cyclase activity, but in the AC8 knockout mutants there is a reduction of ethanol-induced PKA phosphorylation events (Maas *et al.* 2005). Moreover, the reduction of G(s) $\alpha$  activity within mice results in reduced adenylyl cyclase activity and ethanol self-administration (Wand *et al.* 2001). A knockout mutation of protein kinase A regulatory subunit II $\beta$  leads to a decrease in basal cAMP-stimulated PKA activity and an increase in ethanol self-administration, indicating additional complexities in cAMP pathway modulation of ethanol self-administration (Thiele *et al.* 2000). These findings suggest a phylogenetically conserved role for cAMP production in modulating ethanol consumption, however in vertebrates the role for this pathway during development and the critical neural foci are currently unknown.

The spatial rescue of the *rut* self-administration phenotype suggests that cAMP signaling within the mushroom bodies is critical for this behavior. Recent imaging data has shown that both dopamine and octopamine induce *rut*-dependent increases in cAMP and PKA activation within mushroom body neurons (Gervasi *et al.* 2010; Tomchik & Davis 2009). Interestingly, the *dnc*<sup>1</sup> phosphodiesterase mutants exhibited a normal ethanol preference even though the cAMP concentration within the heads is almost twofold higher than in wild-type CS (Byers *et al.* 1981), and the forskolin-induced PKA activity within mushroom body axons is increased by more than 20% (Gervasi *et al.* 2010). Within the mushroom bodies of *dnc*<sup>1</sup> mutants, the spatial dynamics of PKA activation induced by dopamine but not octopamine are altered. In wild-type flies, the application of dopamine leads to significant PKA activation in the vertical  $\alpha$  lobe, but not the horizontal  $\beta$  and  $\gamma$  lobes. In the *dnc*<sup>1</sup> mutants, this PKA restriction is removed and PKA is significantly increased within both horizontal lobes (Gervasi *et al.* 2010). The compartmentalization of PKA activation by octopamine is unchanged in the *dnc*<sup>1</sup> mutants (Gervasi *et al.* 2010). The absence of an ethanol self-administration phenotype in the *dnc*<sup>1</sup> mutants suggests that the compartmentalization

of dopamine-induced PKA activity does not have a major role in this behavior. Octopamine may be responsible for *rut* activation during ethanol feeding, but additional neuromodulators are also possible (Feany & Quinn 1995).

The expression of *rut* driven by either *MB247* or *GH146 Gal4* cannot rescue the defective ethanol preference. This result differentiates self-administration from sugar-reinforced olfactory appetitive learning (Thum *et al.* 2007). Yet, ethanol as a rewarding unconditioned stimulus likely requires different neural populations for reinforcement. In a recent adaptation of the olfactory-learning paradigm using ethanol vapor as the unconditioned stimulus, learning ethanol reward required synaptic release from the  $\gamma$  lobe neurons during training, the  $\alpha'/\beta'$  lobes during consolidation, and the  $\alpha/\beta$  lobe neurons during retrieval (Kaun *et al.* 2011). In negatively reinforced olfactory memory, *rut* activity is required in different subsets of neurons for short-term and long-term memory formation, indicating both spatially and temporally distinct roles for *rut* within mushroom bodies (Blum *et al.* 2009). Thus, for the acquisition, consolidation and retrieval of an appetitive memory formed during the CAFE assay, *rut* activity in multiple lobes of the mushroom bodies may be required.

If the *rut*-dependent ethanol preference found in the CAFE assay originates from a positively reinforced behavior, the actions or stimuli that may be specifically conditioned are currently unknown. Devineni and Heberlein (2009) showed that flies drink ethanol-containing food in shorter duration bouts than non-ethanol food and that flies visit ethanol-containing food more frequently. Since the ethanol preference is expressed by repeatedly going back to the ethanol-containing capillary rather than continuously feeding, and the capillaries used in the CAFE assay are identical, a conditioning of feeding behavior *per se* would not produce the ethanol preference. In our experiments, we exchanged the locations and capillary tubes of regular food and ethanol-containing food daily to prevent a long-term place preference memory. The expression of *rut* in mushroom body neurons is not required in independent spatial or visual operant-learning paradigms (Liu *et al.* 2006; Zars *et al.* 2000b). In another negatively reinforced place preference paradigm, the mushroom bodies have no appreciable role (Ofstad *et al.* 2011). Nevertheless, we cannot currently exclude the possibility that a shorter term place preference for the ethanol-containing capillary may have taken place during each 24-h period and this spatial memory is dependent on *rut* activity within the mushroom bodies. Alternatively, appetitive associations of ethanol reinforcement to specific ethanol sensory cues such as the taste of ethanol may be responsible for this *rut*-dependent component of the ethanol preference behavior. The brief and frequent drinking bout structure may be due to the complex negative- and positive-reinforcing properties of ethanol (Kaun *et al.* 2011); after consuming alcohol the drinking behavior is temporally inhibited by the early and temporarily aversive properties of this drug, but subsequently, the positive reinforcement brings the flies back to drink more.

The *rut*-dependent preference for ethanol-containing food may also result from a more general role for mushroom body plasticity in modulating motivation. Intrinsic mushroom

body neurons are also critical for ethanol-induced locomotion hyperactivity (King *et al.* 2011), suggesting these neurons are important for the stimulating effects of ethanol (Wolf *et al.* 2002). The knockdown of the *tequila* neurotrypsin-like gene with the inducible mushroom body Gene-Switch driver displayed a reduced response to sugar after starvation (Colomb *et al.* 2009). If this requirement for *teq* in a motivated response to starvation lies within the mushroom body neurons, a similar role for a *rut*-dependent cAMP signaling within mushroom bodies for a motivated response toward alcohol is also plausible.

In conclusion, our data support previous findings that *Drosophila* displays an innate preference for ethanol-containing food (Devineni & Heberlein 2009; Ja *et al.* 2007). This preference is largely independent of an innate olfactory or gustatory bias for ethanol, and is not due to the nutrition in the ethanol-containing food. We further found that the *rut* type I adenylyl cyclase is required acutely and broadly within the mushroom bodies for the expression of an ethanol preference. Hence, cAMP signaling within these neurons is likely required for continued ethanol self-administration. We propose that a major component of the preference for ethanol-containing food comes from the hedonic reward of feeding behavior by ethanol and that this is regulated by a *rut*-induced cAMP signaling cascade in mushroom body neurons.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1:** Schematic of two-choice CAFE assay (not to scale). A single male fly was housed in inner vial, with water in outer vial, to keep a high humidity inside. Two kinds of liquid food were provided to the fly through two capillaries separately. One contains 5% sucrose and 5% yeast extract, which is represented by green color. The other contains 5% sucrose, 5% yeast extract and ethanol in a certain concentration, which is represented by red color. The two capillaries were replaced every 24 h with their locations were exchanged.

**Figure S2:** Ethanol is not an efficient energy source for *Drosophila*. *w*<sup>1118</sup> mutants in a CS genetic background did not survive as long on agar as flies fed with 1% ethanol. Consistent with the data for wild-type CS, the ethanol fed flies did not survive for very long. Hence, ethanol can be used as a food substrate by these flies, but not efficiently. Each data point is mean ± SEM.

**Figure S3:** Ethanol preference in the CAFE assay does not rely on gustatory or olfactory attraction. (a) The PER index of CS flies was not different between liquid food without ethanol and liquid food with 5%, 10% or 15% ethanol, which suggested that ethanol was not an appetitive gustatory cue. (b) The *orco*<sup>2</sup> mutant ethanol preference to 5%, 10% or 15% ethanol is not significantly different from the ethanol preferences of CS. (c) The ethanol preferences of *lush*<sup>1</sup> to 5%, 10% or 15% ethanol are not significantly different from the preferences of CS. Panels (b) and (c) suggest that ethanol preference on *Drosophila* is not due to olfactory attraction of ethanol. Data are mean ± SEM.

**Figure S4:** The decreased ethanol preference in *rut*<sup>2080</sup> is not due to decreased food consumption. (a) In the CAFE assay, *rut*<sup>2080</sup> consumed significantly less food than CS at each ethanol concentration. (b) This defect in food consumption was not increased significantly by the post-developmental expression of a wild-type *rut* cDNA in the nervous system with the *elav*-GeneSwitch driver. However, the same treatment (RU486 feeding) induced a higher ethanol preference than the vehicle-feeding group (Fig. 2). (c and d) The defect in food consumption was not rescued by the *rutabaga* expression driven by the OK107 or c772 Gal4 driver. However, this defect of ethanol preference in *rut*<sup>2080</sup> was rescued by OK107 or c772 driven *rutabaga* expression in mushroom body (Fig. 4). Panels (b), (c) and (d) indicated that the *rut*<sup>2080</sup> ethanol preference phenotype is independent of the total food consumption phenotype. Data are mean ± SEM. N.S., no significance. \**P* < 0.05 and \*\**P* < 0.01. Because the negative control *rut*<sup>2080</sup>; +; 238y and *rut*<sup>2080</sup>; c305a/+; MB247/+ genotype displayed no difference with CS in food consumption (data not shown), these results cannot indicate whether the two phenotypes are independent each other in *rut*<sup>2080</sup> or not.

**Figure S5:** The expression of *rutabaga* in the  $\alpha'/\beta'$  lobe neurons alone is not sufficient for a full rescue of the *rut*<sup>2080</sup> ethanol preference phenotype. (a) The expression of UAS-*rut* driven by the c305a  $\alpha'/\beta'$  Gal4 drive was not sufficient to fully rescue the *rut*<sup>2080</sup> ethanol preference phenotype. The *rut*<sup>2080</sup>; c305a/+; UAS-*rut* ethanol preference phenotype was not significantly different than CS, and was significantly

higher than one control, *rut*<sup>2080</sup>; UAS-*rut*/+ genotype. However, because it was not significantly different than the *rut*<sup>2080</sup>; c305a/+ genotype control, it is still a question whether the *rutabaga* expression in  $\alpha'/\beta'$  lobe is required for ethanol preference or not. Data are mean  $\pm$  SEM. N.S., no significance. (b) c305a Gal4 drives the GFP expression in the  $\alpha'/\beta'$  lobe mushroom body neurons. Scale bar: 100  $\mu$ m.

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