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

Shiyu Xu¹, Tammy Chan^{2,3}, Vrantant Shah¹, Shixing Zhang¹, Scott D. Pletcher^{2,3}, and Gregg Roman^{1,4,*}

¹Department of Biology and Biochemistry, University of Houston, 4800 Calhoun St. Houston, TX 77204-5001

²Program in Developmental Biology, Baylor College of Medicine, Houston, TX 77030

³University of Michigan Geriatrics Center, Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI 49108

⁴Biology of Behavior Institute, University of Houston, Houston TX, 77204

Abstract

Alcohol activates reward systems through an unknown mechanism, in some cases leading to alcohol abuse and dependence. Herein, we utilized a two-choice Capillary Feeding assay to address the neural and molecular basis for ethanol self-administration in *Drosophila melanogaster*. Wild-type *Drosophila* demonstrates 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 *Drosophila melanogaster*.

Keywords

Drosophila; Ethanol Self-Administration; Learning; cAMP; Mushroom Body

Introduction

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.

*Corresponding author: Gregg Roman, Dept. Biology and Biochemistry, University of Houston, 4800 Calhoun St., Houston, TX 77204-5001, gwroman@uh.edu.

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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

1. 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 background for a minimum of six generations prior to behavioral analysis. All the Gal4 lines were out-crossed into *w¹¹¹⁸* (CS10) background for at least seven generations. The *orco²* mutation is a loss-of-function disruption of the *orco* locus (Larsson *et al.*, 2004). The *lush¹* mutation has a deletion of *lush* coding sequences (Laughlin *et al.*, 2008). The *rut²⁰⁸⁰* mutation is severe hypomorphic allele caused by a P{1ArB} insertion within *rutabaga* (Levin *et al.*, 1992). The UAS-*rutabaga* line is capable of rescuing *rut²⁰⁸⁰* 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 α , β and γ lobes of mushroom body, and the *c305a* Gal4 transgene drives expression in α' / β' 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 *rut²⁰⁸⁰*, the experimental genotype was generated by crossing

virgin females of *rut*²⁰⁸⁰, UAS-*rut*/*CyO* (or *rut*²⁰⁸⁰; +; UAS-*rut*) with males of the indicated Gal4 lines. All Gal4 insertions were autosomal. F₁ male progeny were selected for analysis.

2. 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, 3.5 cm long) was plugged by a rubber stopper (standard 0#) with two holes inserted by two 200- μ 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 μ l micropipettes (VWR, West Chester, PA) filled with liquid food by capillary action were inserted through the 200- μ l pipette tips. A mineral oil (CAS: 8042-47-5; Sigma-Aldrich, St. Louis, MO) overlay was applied in micropipette to minimize evaporation.

Liquid food was prepared with the designated amount of sucrose (CAS: 57-50-1; Sigma-Aldrich, St. Louis, MO), yeast extract (Bacto yeast extract; BD Diagnostic Systems, Franklin Lakes, NJ), 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 anaesthetized by CO₂, 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 four 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.

3. 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 to 20 hours to increase their gustatory sensitivity. A vial containing the flies was placed on ice for 2 minutes to briefly immobilize the flies. The flies were then transferred to a Petri dish. Small drops, each approximately 0.7 μ l of Loctite 404 (Henckel CO., Lewisville, TX), 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 to 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, then at 5% sucrose + 5% yeast + 5% (or 10%, or

15%) ethanol. PER index was defined as the ratio between the flies showing proboscis extension and total flies tested.

4. 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, St. Louis, MO) dissolved in 80% ethanol was diluted to 500 μ 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.

5. 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-hour period and allowed to mate for 2–3 days. After this time, females were collected under light CO₂ anesthesia and housed at 25 flies per group, and transferred to fresh food every other day. At ten 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 hours during the period of highest mortality until 100% mortality was reached.

6. Immunohistochemistry

The methods for immunohistochemical detection of 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, St. Louis, MO) in PBS for 20 minutes at room temperature and washed with PBS containing 0.4% Triton X-100 (PBT) (CAS: 9002-93-1; Sigma-Aldrich, St. Louis, MO). The fixed brains were subsequently rinsed with PBT for 20 minutes three times. After being blocked with PBT containing 5% normal goat serum (Sigma-Aldrich, St. Louis, MO) for 30 minutes at room temperature, the brains were incubated with the mouse polyclonal antibody against green fluorescent protein (GFP) (1:200; Roche, Germany) in PBT at 4 °C for 48 hrs. The brains were then washed with PBT for 20 minutes three times. The prepared labeled brains were next incubated with Alexa Fluor®488 conjugated goat anti-mouse (1:500; Invitrogen, U.S.A) in blocking solution for 48 hr at 4 °C. Finally, the brains were rinsed with PBT (3×20 minutes) and mounted in mounting medium (Vectashield; Vector Laboratories, U.S.A). The prepared brains were imaged with confocal microscopy on a Fluoview FV1000 microscope (Olympus, Japan).

7. Statistical analysis

All statistical analysis was performed using Statview software (SAS Institute, Inc., USA). In the CAFE assay, energy intake, food consumption or preference index was analyzed using Student's *t*-test, or one-way 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), 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, 5% sucrose) or liquid food plus ethanol for four days (Sup. Fig. 1). 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 four day period. In this experiment, wild-type Canton-S (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; 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 four 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 *Drosophila 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 $n=18-20$ 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; $F_{5,113}=88.551$, $P<0.0001$, $n=18-20$ flies; no significance between the 4 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; $F_{5,113}=132.111$, $P<0.0001$, each $n=18-20$ 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 3 non-ethanol groups, $P<0.001$ for 15% ethanol group vs. other 3 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 (Sup. Fig. 2; $P<0.05$, log-rank test, $n=10$ groups). However, Canton-S 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, 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 proboscis extension reflex (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% to 15% ethanol did not inhibit PER significantly, relative to food without ethanol, while low ethanol concentrations are at best neutral (Sup. Fig. 3a; $F_{3,52}=1.948$, $P>0.05$, $n=12-14$ groups). Previously, the w^- Berlin flies were found to have an olfactory bias towards 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 Canton-S 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*² and w^+ ; *lush*¹ mutants in the CAFE assay. The w^+ ; *orco*² 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^+ ; *orco*² flies did not display any significant defects in ethanol preference (Sup. Fig. 3b; 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*¹ mutants are more attracted to high ethanol concentrations in olfactory trap assays than wild-type flies (Kim *et al.*, 1998). However, the *lush*¹ mutants did not demonstrate a preference phenotype within our CAFE assay (Sup. Fig. 3c; 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^+ ; *orco*² and the ethanol preferring *lush*¹ 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*²⁰⁸⁰ reduction-of-function mutants in our CAFE assay. The *rut*²⁰⁸⁰ mutants displayed a normal preference to 5% ethanol; however, the preference for 10% and 15% ethanol was significantly less than that of Canton-S flies (Fig. 2a; for 5% ethanol, $t_{(46)}=0.951$, $P>0.05$, $n=24$ each; for 10% ethanol, $t_{(69)}=3.545$, $P=0.0007$, $n=36$ 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*²⁰⁸⁰ mutants also displayed decreased food consumption in the CAFE assay, raising the possibility that decreased total food consumption may shape the expressed ethanol preference (Sup. Fig. 4a; for 5% ethanol, $t_{(46)}=2.989$, $P=0.0045$, $n=24$ flies; for 10% ethanol, $t_{(69)}=2.722$, $P=0.0082$, $n=35-36$ flies; for 15% ethanol, $t_{(52)}=2.550$, $P=0.014$, $n=27$ flies).

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^l* mutants displayed a normal preference for 5%, 10% and 15% ethanol (Fig. 2b; for 5% ethanol, $t_{(57)}=0.422$, $P>0.05$, $n=29$ flies; for 10% ethanol, $t_{(58)}=0.450$, $P>0.05$, $n=30$ flies; for 15% ethanol, $t_{(38)}=0.105$, $P>0.05$, $n=20$ flies). These differences between *dnc^l* and *rut²⁰⁸⁰* in the CAFE assay also mirrors that of an ethanol sedation assay, where the *rut²⁰⁸⁰* mutants are hypersensitive, and the *dnc^l* mutants lack an obvious phenotype (Moore *et al.*, 1998).

To eliminate trivial causes for the *rut²⁰⁸⁰* 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 *rut²⁰⁸⁰* 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 *rut²⁰⁸⁰* flies to food containing 15% ethanol was significantly greater than that of Canton-S flies (Fig. 2c; $t_{(17)}=2.64$, $P=0.017$, $n=9-10$ groups), which suggested that 15% ethanol was less aversive to *rut²⁰⁸⁰* than to Canton-S controls. The expectation from the reduced aversive gustatory response of *rut²⁰⁸⁰* flies to 15% ethanol-containing food is that they would drink more ethanol than Canton-S. Thus, the reduced ethanol preference of the *rut²⁰⁸⁰* mutants is unlikely to be due to reduced aversive taste of ethanol. Furthermore, it was previously reported that: 1) the olfactory response of *rut²⁰⁸⁰* flies was not different than that of wild-type flies and 2) the absorption and metabolism of ethanol is not altered between *rut²⁰⁸⁰* 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 *rut²⁰⁸⁰* 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 Canton-S, *rut²⁰⁸⁰;+; elav*-GeneSwitch/+ and *rut²⁰⁸⁰; UAS-rut/+; elav*-GeneSwitch/+ flies were fed 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 Canton-S and *rut²⁰⁸⁰;+; elav*-GeneSwitch/+ flies (Fig. 2d). However, the RU486-induced expression of the *rut* cDNA within the nervous tissue completely rescued the *rut²⁰⁸⁰* ethanol preference phenotype (Fig. 2d; $t_{(71)}=3.08$, $P=0.003$ for induced *rut²⁰⁸⁰;UAS-rut/+;GS/+* vs. un-induced *rut²⁰⁸⁰;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 (Sup. Fig. 4b; planned comparison for *rut²⁰⁸⁰;UAS-rut/+;GS/+* with un-induced *rut²⁰⁸⁰;UAS-rut/+;GS/+*, $t_{(71)}=0.500$, $P>0.05$, $n=35$ flies), genetically distinguishing these two phenotypes. Moreover, the OK107 or *c772* driven *rutabaga* expression rescued the abnormal ethanol preference in *rut²⁰⁸⁰* (see below), but did not rescue the total food consumption phenotype (Sup. Fig. 4c; $F_{(3,150)}=9.891$, $P<0.0001$, in *post hoc* comparisons, $P>0.05$ for *rut²⁰⁸⁰;c772/+;UAS-rut/+* vs. *rut²⁰⁸⁰;c772/+*, $n=37-39$ each; Sup. Fig. 4d; $F_{(3,140)}=17.650$, $P<0.0001$; in *post hoc* comparisons, $P>0.05$ for *rut²⁰⁸⁰;;UAS-rut/+;OK107/+* vs. *rut²⁰⁸⁰;;OK107/+*, $n=27$ for the *rut²⁰⁸⁰;;OK107/+* group and $n=39$ for the *rut²⁰⁸⁰;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*²⁰⁸⁰ 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*²⁰⁸⁰ 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*²⁰⁸⁰ ethanol preference phenotype (Fig. 3; $F_{(3,123)}=2.800$, $P=0.042$, no significance for *rut*²⁰⁸⁰;GH146/+;UAS-*rut*/+ vs. *rut*²⁰⁸⁰;GH146/+ or *rut*²⁰⁸⁰;UAS-*rut*/+, n=30–32 each; Fig. 3b; $F_{(3,190)}=2.047$, $P=0.109$ n=43–46 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*²⁰⁸⁰ (In Fig. 4a, $F_{(3,140)}=10.398$, $P<0.0001$, in *post hoc* comparisons, $P<0.001$ for both *rut*²⁰⁸⁰;UAS-*rut*/+;OK107/+ vs. *rut*²⁰⁸⁰;OK107/+ or *rut*²⁰⁸⁰;UAS-*rut*/+, in *rut*²⁰⁸⁰;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*²⁰⁸⁰;c772/+;UAS-*rut*/+ vs. *rut*²⁰⁸⁰;c772/+, and $P=0.0005$ for *rut*²⁰⁸⁰;c772/+;UAS-*rut*/+ vs. *rut*²⁰⁸⁰;UAS-*rut*/+, n=38–40 each. In Fig. 4e, $F_{(3,115)}=9.623$, $P<0.0001$, in *post hoc* comparisons, $P<0.01$ for both *rut*²⁰⁸⁰;UAS-*rut*/238y vs. *rut*²⁰⁸⁰;238y/+ or *rut*²⁰⁸⁰;UAS-*rut*/+, n=28–30 each. In Fig. 4g, $F_{(3,175)}=14.238$, $P<0.001$, in *post hoc* comparisons, $P<0.001$ for *rut*²⁰⁸⁰;c305a/+;UAS-*rut*/MB247 vs. *rut*²⁰⁸⁰;c305a/+;UAS-*rut*/MB247 or *rut*²⁰⁸⁰;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 *dmef2* 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 α/β and γ 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*²⁰⁸⁰ ethanol preference phenotype in the presence of MB-Gal80 (In Fig. 4b, $F_{(3,114)}=3.011$, $P=0.033$, no significance for *rut*²⁰⁸⁰;MB-Gal80/+;UAS-*rut*/+;OK107/+ vs. *rut*²⁰⁸⁰;MB-Gal80/+;OK107/+ or *rut*²⁰⁸⁰;MB-Gal80/+;UAS-*rut*/+, however, $P=0.0074$ for *rut*²⁰⁸⁰;MB-Gal80/+;UAS-*rut*/+;OK107/+ vs. *rut*²⁰⁸⁰;UAS-*rut*/+;OK107/+. In Fig. 4d, $F_{(3,110)}=13.898$, $P<0.0001$, in *post hoc* comparisons, $P=0.018$ for *rut*²⁰⁸⁰;MB-Gal80/c772;UAS-*rut*/+ vs. *rut*²⁰⁸⁰;MB-Gal80/c772, however, $P=0.0013$ for *rut*²⁰⁸⁰;MB-Gal80/c772;UAS-*rut*/+ vs. *rut*²⁰⁸⁰;c772/+;UAS-*rut*/+, n=28–30 each. In Fig. 4f, $F_{(3,80)}=7.73$, $P=0.0001$, no significance for *rut*²⁰⁸⁰;MB-Gal80/+;UAS-*rut*/238y vs. *rut*²⁰⁸⁰;MB-Gal80/+;238y/+ or *rut*²⁰⁸⁰;MB-Gal80/+;UAS-*rut*/+, however, $P=0.0008$ for *rut*²⁰⁸⁰;MB-Gal80/+;UAS-*rut*/238y vs. *rut*²⁰⁸⁰;UAS-*rut*/238y, n=20–22 each. In Fig. 4h, $F_{(3,165)}=4.938$, $P=0.0026$, no significance for *rut*²⁰⁸⁰;MB-Gal80/c305a;UAS-*rut*/MB247 vs. *rut*²⁰⁸⁰;MB-Gal80/c305a;MB247/+ or *rut*²⁰⁸⁰;MB-

Gal80/+;UAS-*rut*+, however, $P=0.0209$ for *rut*²⁰⁸⁰, *MB-Gal80/c305a;UAS-*rut*/MB247* vs. *rut*²⁰⁸⁰;c305a/+;UAS-*rut*/MB247, $n=41-42$ each). The expression of *rut* in the α/β , α'/β' and γ lobe neurons is capable of fully rescuing the *rut*²⁰⁸⁰ ethanol self-administration phenotype, whereas *rut* expression in the α/β and γ lobe neurons driven by *MB247* alone is insufficient for rescue. This difference suggests that the expression of *rutabaga* in the α'/β' neurons may be sufficient to rescue the *rut*²⁰⁸⁰ ethanol self-administration phenotype. We examined the isolated c305a α'/β' Gal4 driver for an ability to rescue the *rut*²⁰⁸⁰ self-administration phenotype. The resulting phenotype lies between the Canton-S positive control and the *rut*²⁰⁸⁰;c305/+ genotype control, and is not significantly different from either (Sup. Fig. 5; $F_{(3,147)}=5.767$, $P=0.0009$. In *post hoc* comparisons, $P=0.487$ for *rut*²⁰⁸⁰;c305a/+;UAS-*rut*+ vs. CS, $P=0.137$ for *rut*²⁰⁸⁰;c305a/+;UAS-*rut*+ vs. *rut*²⁰⁸⁰;c305a/+, and $P=0.002$ for *rut*²⁰⁸⁰;c305a/+;UAS-*rut*+ vs. *rut*²⁰⁸⁰; UAS-*rut*+. $n=36-38$ each). Hence, it remains possible that *rut* expression within the α'/β' 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*.

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 *Drosophila melanogaster*. In our assay, wild-type Canton-S 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 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 Ca²⁺-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*²⁰⁸⁰ 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. Knock-out mutants of the mouse calcium-activated type VIII adenylyl cyclase (AC8), but not knock-outs 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 Ca²⁺-stimulated adenylyl cyclase activity, but in the AC8 knock-out mutants there is a reduction of ethanol-induced PKA phosphorylation events (Maas *et al.*, 2005). Moreover, the reduction of G(s) α activity within mice results in reduced adenylyl cyclase activity and ethanol self-administration (Wand *et al.*, 2001). A knock-out mutation of Protein kinase A Regulatory subunit II β 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

demonstrated 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 *dnc¹* phosphodiesterase mutants exhibited a normal ethanol preference even though the cAMP concentration within the heads is almost two-fold higher than in wild-type Canton-S (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 *dnc¹* mutants, the spatial dynamics of PKA activation induced by dopamine but not octopamine is altered. In wild-type flies, the application of dopamine leads to significant PKA activation in the vertical α lobe, but not the horizontal β and γ lobes. In the *dnc¹* 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 *dnc¹* mutants (Gervasi *et al.*, 2010). The absence of an ethanol self-administration phenotype in the *dnc¹* 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 γ lobe neurons during training, the α'/β' lobes during consolidation, and the α/β 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 knock-down 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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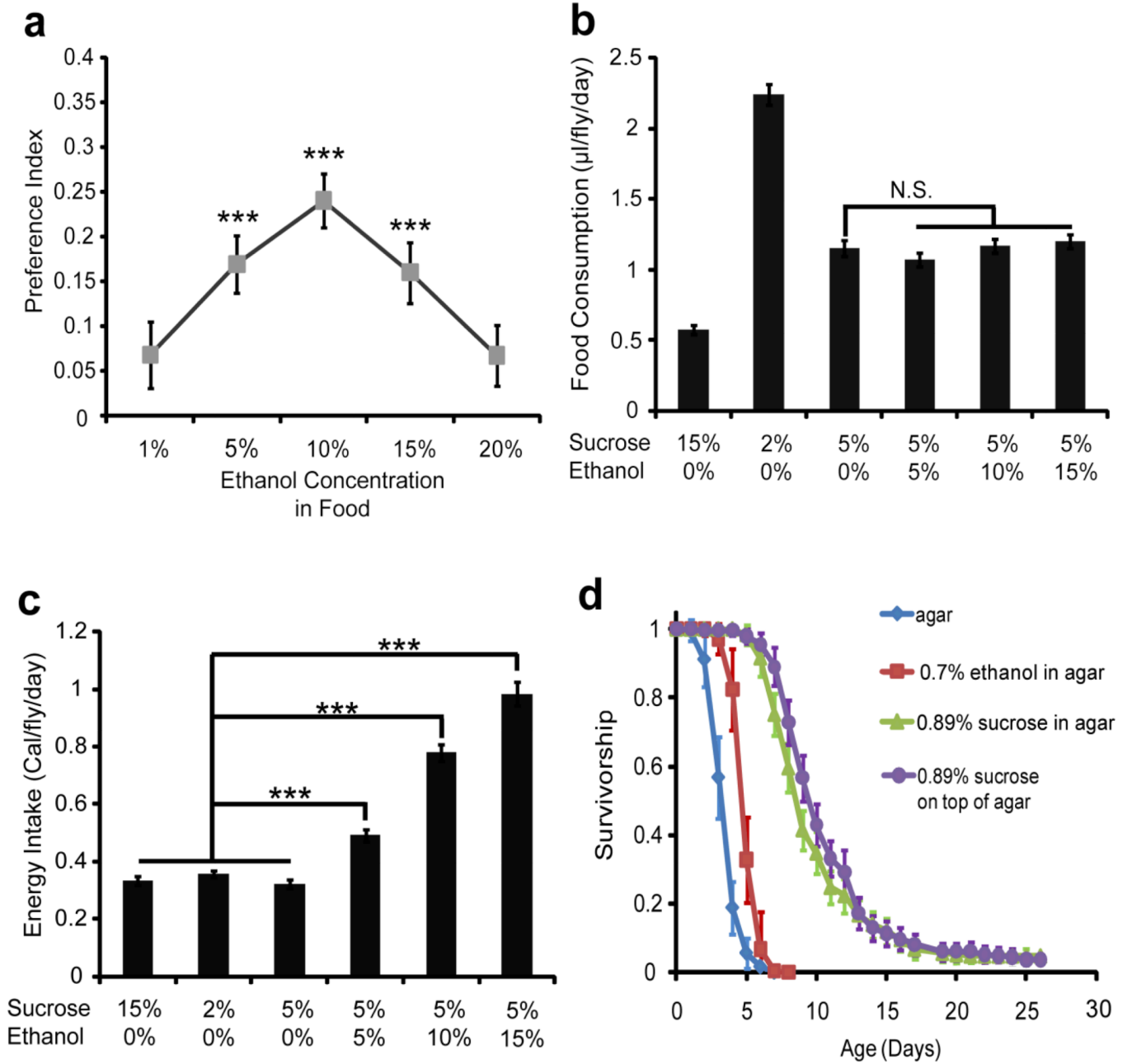


Figure 1. The preference for ethanol containing food is independent of nutrient value

(a) Canton-S (CS) 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 four 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 of 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 S.E.M. "N.S." means no significance. *** $P < 0.001$.

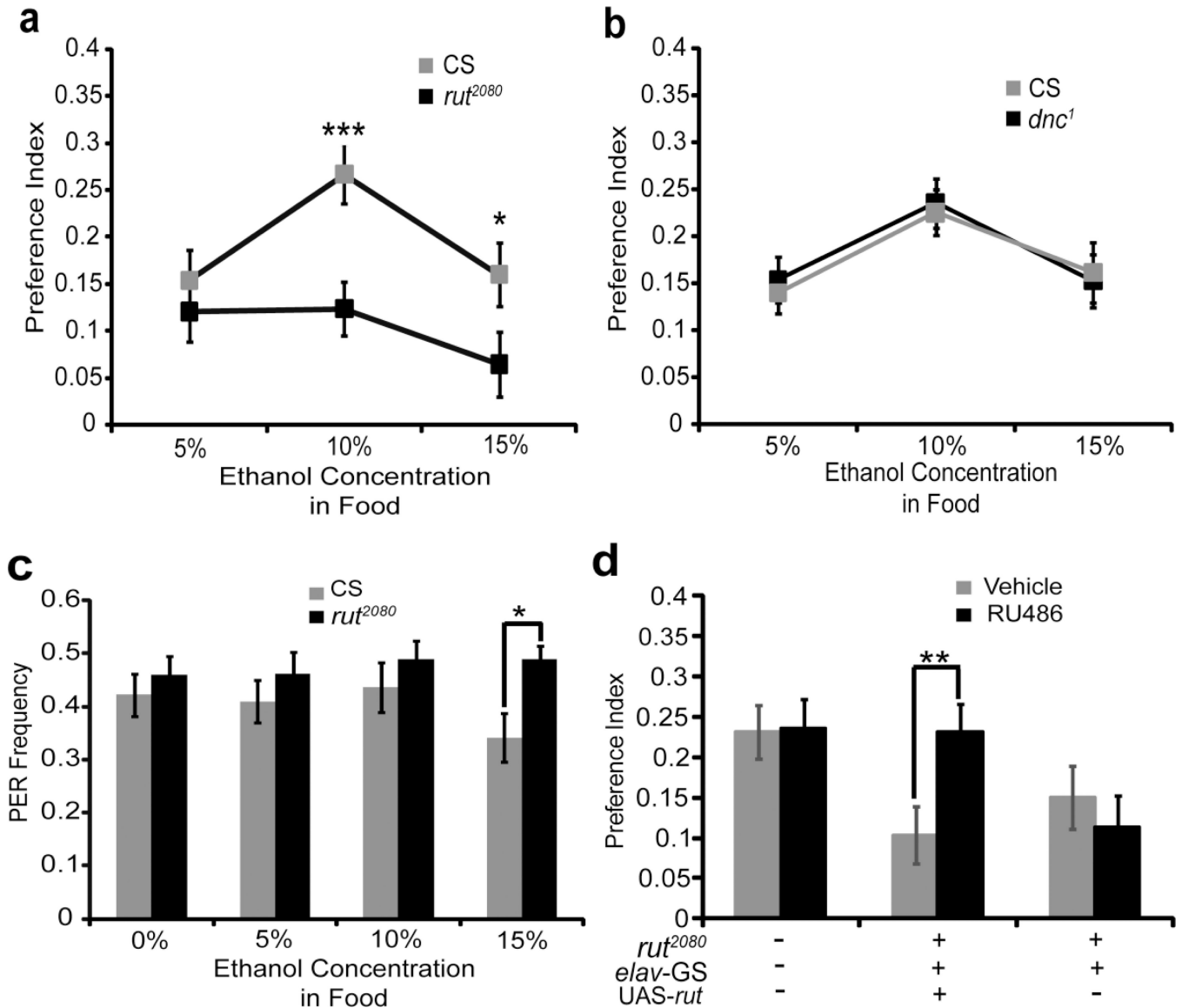


Figure 2. The *rutabaga* adenylyl cyclase is acutely required for an ethanol preference
 (a) *rut*²⁰⁸⁰ mutants and control CS flies were examined in the CAFE assay. The *rut*²⁰⁸⁰ mutants display a significantly reduced preference for 10% and 15% ethanol compared to wild-type CS. (b) Mutants in the *dunce* phosphodiesterase were also examined in the CAFE assay. The *dnc*¹ loss-of-function mutants exhibited a normal preference for 5%, 10% and 15% ethanol. (c) In the PER assay, *rut*²⁰⁸⁰ did not show a significant difference with CS, to liquid food without or with ethanol, except for 15% ethanol, indicating that the *rut*²⁰⁸⁰ reduced preference is not due to altered gustatory responses to ethanol. (d) The reduced ethanol preference of *rut*²⁰⁸⁰ 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*²⁰⁸⁰,+;*elav*-GS/+ induced by RU486. The same treatment did not influence the ethanol preference in CS flies and the negative control flies, *rut*²⁰⁸⁰,+;*elav*-GS/+. Data are means ± S.E.M. **P*<0.05, ***P*<0.01, and ****P*<0.001.

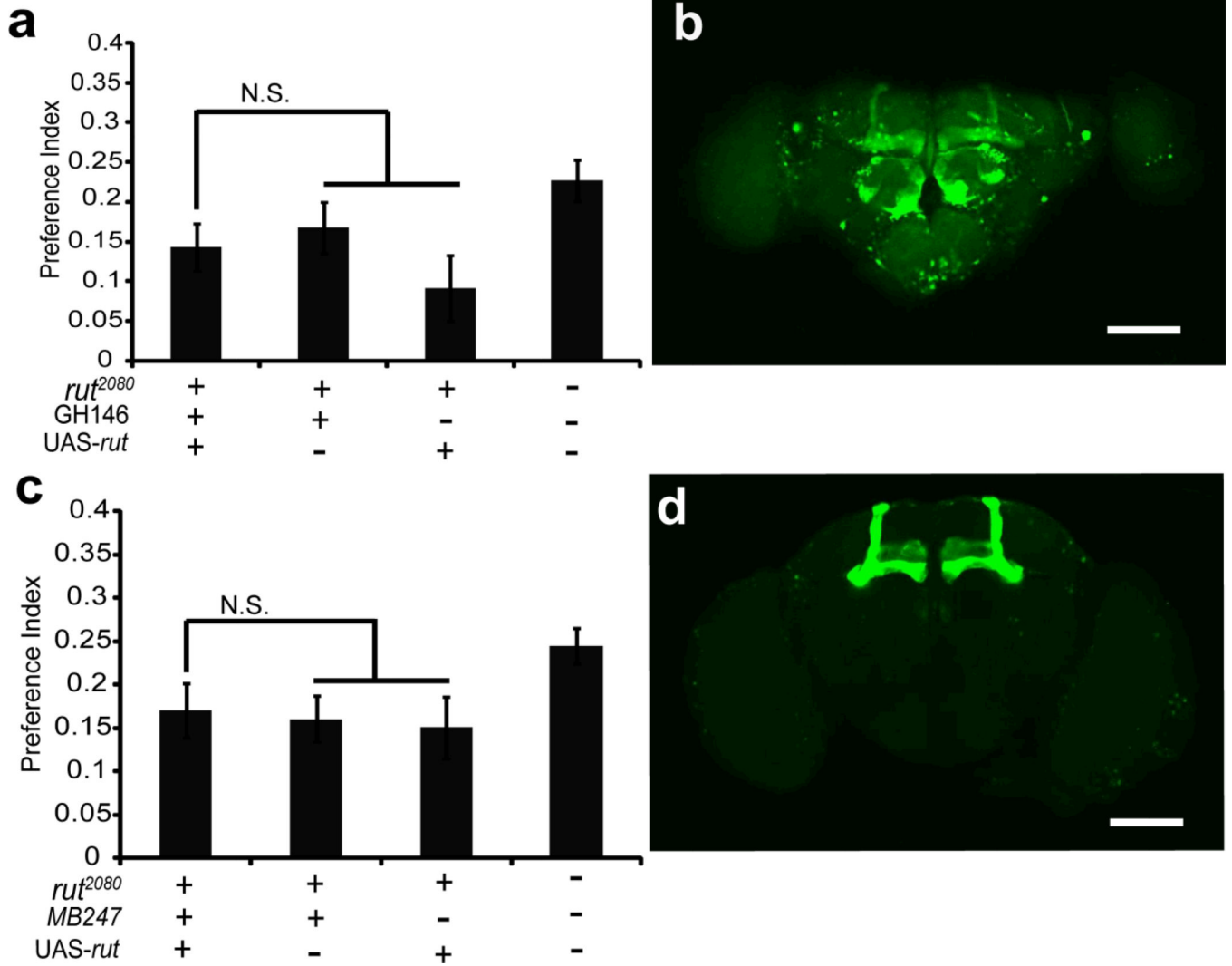


Figure 3. The ethanol preference phenotype of *rut²⁰⁸⁰* mutants is separable from simple appetitive olfactory learning
 Both GH146 and *MB247* rescue the olfactory appetitive learning defect in *rut²⁰⁸⁰*. However, they cannot rescue ethanol preference defect in *rut²⁰⁸⁰*. (a) The expression of *rut* driven by the projection neuron driver GH146-Gal4 failed to rescue the *rut²⁰⁸⁰* ethanol preference phenotype, since the ethanol preference of the genotype *rut²⁰⁸⁰; 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 *rut²⁰⁸⁰* ethanol preference phenotype, since the preference index of the genotype *rut²⁰⁸⁰; UAS-*rut*/MB247* was not significantly different from both negative controls. (d) *MB247*-Gal4 drives GFP expression in α/β and γ lobe. Data are mean \pm S.E.M. "N.S." means no significance.

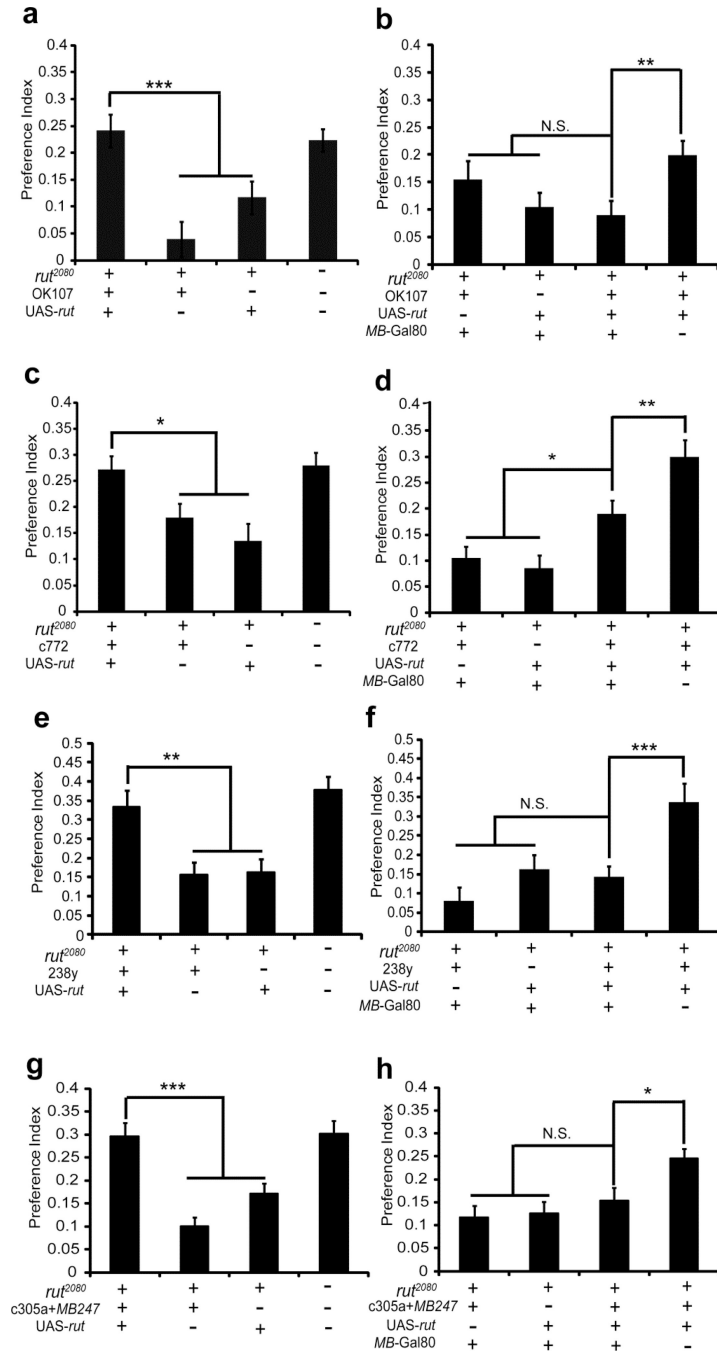


Figure 4. The *rutabaga* requirement for normal ethanol preference maps to the mushroom body neurons

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*²⁰⁸⁰ ethanol preference phenotype is not significantly different than CS, and is significantly higher than the control *rut*²⁰⁸⁰ 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 rescued ethanol preference in comparison to the *rut*²⁰⁸⁰; UAS-*rut*/+; OK107/+ genotype, and also no significance in comparison to *rut*²⁰⁸⁰; *MB-Gal80*/+; OK107/+ and *rut*²⁰⁸⁰; UAS-*rut*/*MB-*

Gal80 genotype. (c) Similarly, the *rut*²⁰⁸⁰ 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*²⁰⁸⁰ 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*²⁰⁸⁰ ethanol preference phenotype is significantly higher than the control *rut*²⁰⁸⁰ genotypes. In all genotypes, the *ry*⁵⁰⁶ 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*²⁰⁸⁰, UAS-*rut*/+; 238y/+ genotype, and also no significance in comparison to *rut*²⁰⁸⁰, *MB*-Gal80/+;238y/+ and *rut*²⁰⁸⁰, UAS-*rut*/*MB*-Gal80 genotype. In all genotypes, a *ry*⁵⁰⁶ allele was taken in third chromosome. (g) In the presence of the UAS-*rut* and the combination of *c305a* + *MB247* Gal4 drivers, the *rut*²⁰⁸⁰ ethanol preference phenotype is significantly higher than the control *rut*²⁰⁸⁰ genotypes. (h) The *MB*-Gal80 transgene similarly reduced the ethanol preference of *rut*²⁰⁸⁰ flies carrying the UAS-*rut* and *c305a*+*MB247* transgenes, and also displayed no significance in comparison to *rut*²⁰⁸⁰, *MB*-Gal80/*c305a*; *MB247*/+ and *rut*²⁰⁸⁰, UAS-*rut*/*MB*-Gal80 genotype. Data are means ± S.E.M. “N.S.” means no significance. **P*<0.05, ***P*<0.01 and ****P*<0.001.

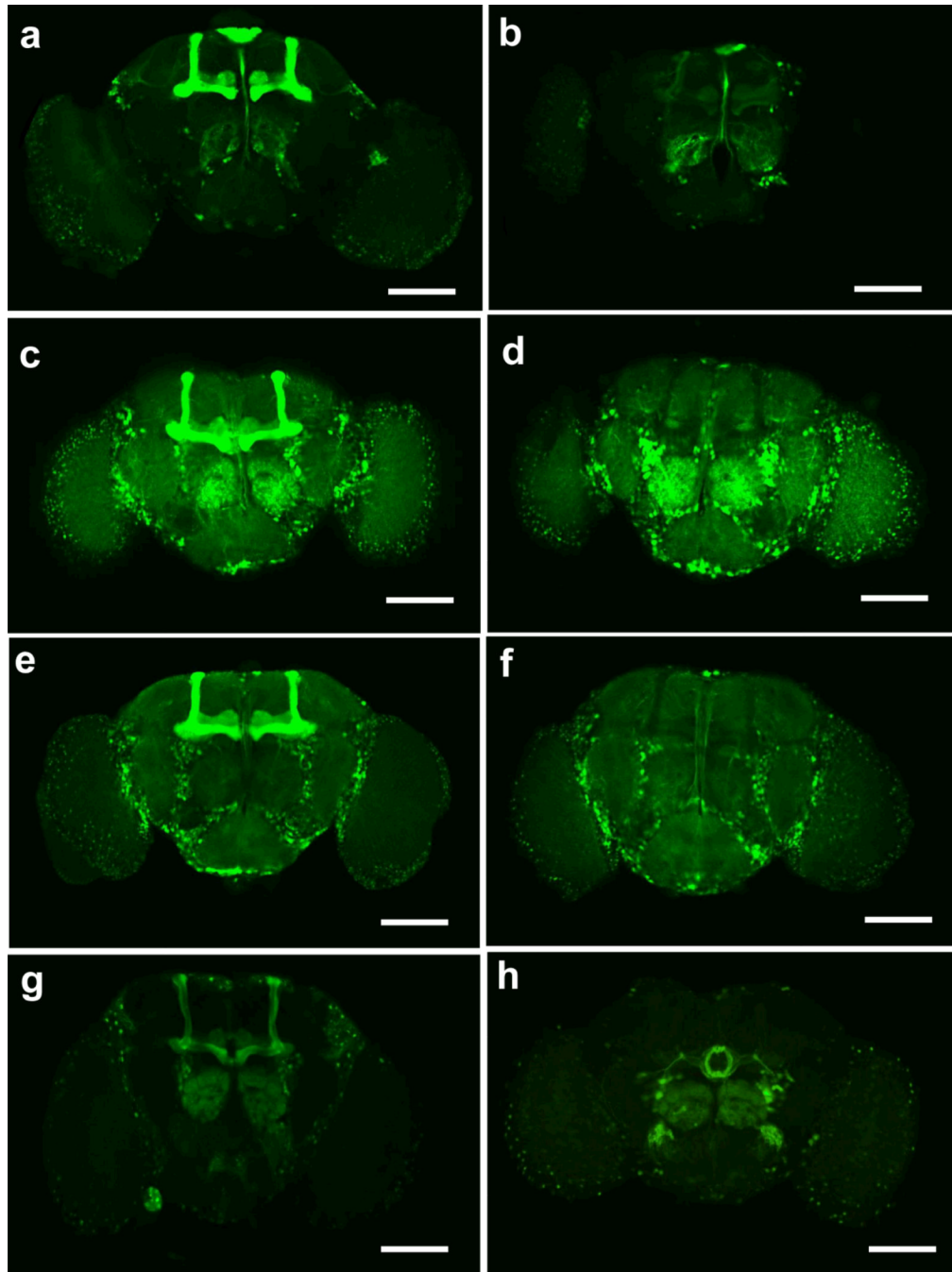


Figure 5. MBGal80 inhibits Gal4 activity within all the mushroom body neurons
 GFP was driven by four Gal4 drivers (a, OK107; c, *c772*; e, *238y*; g, *c305a+MB247*), and detected by immunohistochemistry. The genotypes in panels b, d, f and h contain the MB-Gal80 transgene which effectively removes the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