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Multiple Memory Traces for Olfactory Reward Learning in *Drosophila*

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Physical traces underlying simple memories can be confined to a single group of cells in the brain. In the fly *Drosophila melanogaster*, the Kenyon cells of the mushroom bodies house traces for both appetitive and aversive odor memories. The adenylate cyclase protein, Rutabaga, has been shown to mediate both traces. Here, we show that, for appetitive learning, another group of cells can additionally accommodate a Rutabaga-dependent memory trace. Localized expression of *rutabaga* in either projection neurons, the first-order olfactory interneurons, or in Kenyon cells, the second-order interneurons, is sufficient for rescuing the mutant defect in appetitive short-term memory. Thus, appetitive learning may induce multiple memory traces in the first- and second-order olfactory interneurons using the same plasticity mechanism. In contrast, aversive odor memory of *rutabaga* is rescued selectively in the Kenyon cells, but not in the projection neurons. This difference in the organization of memory traces is consistent with the internal representation of reward and punishment.

Key words: **learning; memory; insect; memory trace; olfaction; adenylate cyclase; *Drosophila*; short-term memory**

Introduction

For associative memories, the underlying traces can be localized to single groups of neurons or synapses in the corresponding neural circuits, although the study cases for memory circuits at the cellular level are limited (Kim and Thompson, 1997; Roberts and Glanzman, 2003; Gerber et al., 2004). To map them, it was asked whether the neuronal plasticity in specific subsets of neurons is necessary and/or sufficient for mediating the conditioned behavior (Gerber et al., 2004; Thompson, 2005). In particular, if learning generates multiple redundant memory traces, multiple sets of "sufficient" neurons should be identified, because one memory trace would compensate the loss of others (Gerber et al., 2004).

Concerning the short-term odor memory of *Drosophila*, the intrinsic neurons of the mushroom body (MB), the Kenyon cells (KCs), are supposed to be a converging site of olfactory information and electric shock punishment or sugar reward (Schwaerzel et al., 2003; Gerber et al., 2004) (see Fig. 1*A*). The primary olfactory information is represented in the antennal lobe (AL), the first olfactory brain center (Laurent, 2002; Hallem and Carlson, 2004; Komiyama and Luo, 2006). From there, the projection neurons (PNs) via the inner antennocerebral tract (iACT) transmit the processed olfactory information to the secondary centers, the MB and lateral horn (LH) (Stocker, 2001) (Fig. 1*A*). Because the retrieval, but not acquisition, of short-term olfactory memories requires the outputs of subsets of KCs (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002), the underlying traces should be formed upstream in the olfactory pathway, arguably at the output synapses of KCs (Gerber et al., 2004).

In the honeybee, in addition to the MB, the AL has been shown to contribute to olfactory reward memories (Erber et al., 1980; Hammer and Menzel, 1998; Faber et al., 1999; Farooqui et al., 2003; Peele et al., 2006). Similarly, appetitive conditioning in *Manduca* has been shown to change odor-evoked activity patterns of PNs (Daly et al., 2004). In *Drosophila*, aversive conditioning recruits more PNs to respond to the trained odor (Yu et al., 2004). In addition, the prolonged protein kinase A activation or degradation of the RNA-induced silencing complex pathway component in AL may regulate long-term memories (Müller, 2000; Ashraf et al., 2006). Thus, PNs might harbor a memory trace in addition to that in KCs.

To address the possibility of multiple traces in PNs and KCs for the short-term olfactory memory, we decided to map sufficient cell groups for rescuing the memory of *rutabaga* (*rut*) mutant with targeted expression of wild-type *rut* cDNA (Zars et al., 2000). The *rut* gene encodes a type I calcium/calmodulin-activated adenylate cyclase (AC) (Han et al., 1992; Levin et al., 1992) onto which the internally processed conditioned stimulus (CS; odor) and unconditioned stimulus (electroshock or sugar) are supposed to converge (Abrams et al., 1998). In both olfactory shock learning and sugar reward learning, short-term memory of the *rut* mutant is reduced (Tempel et al., 1983; Tully and Quinn,

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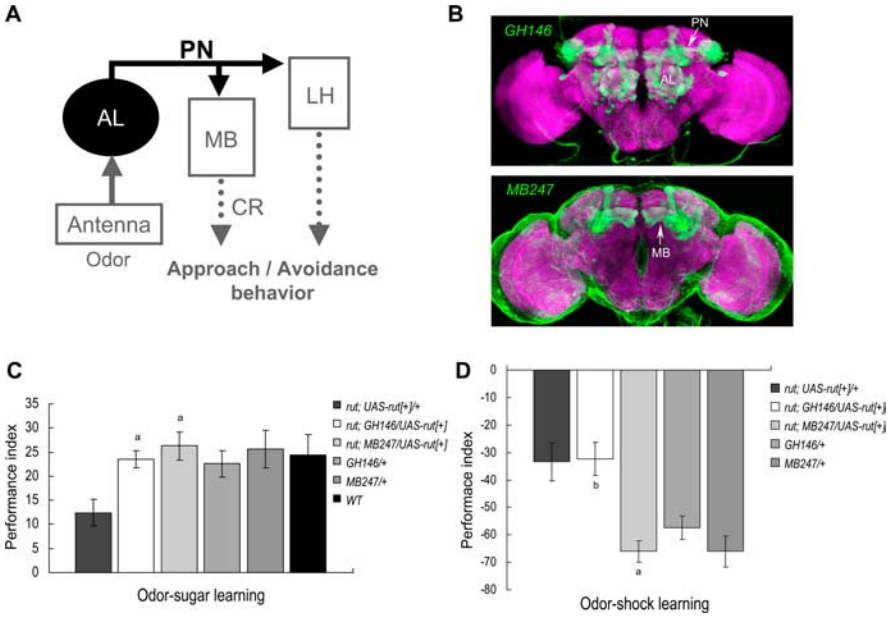

Figure 1. ***A***, Simplified diagram of *Drosophila* olfactory circuits. The odor signals received in the antennae are first conveyed to the AL. The PNs transmit the processed olfactory information to the MB and LH. The neurons conveying the conditioned response (CR) or innate odor response are unknown (dotted line). ***B***, The GAL4 expression patterns of *GH146* (top) and *MB247* (bottom) in the brains is monitored by *UAS-mCD8::GFP* (green) with counterstaining by presynaptic protein, Brp (magenta). *GH146* has strong expression in the PNs innervating the AL, the calyx of MB, and the LH. In addition, it labels the MB extrinsic neurons. *MB247* has predominant expression in MB Kenyon cells and surface glial cells. ***C***, The rut^{+} expression was targeted to the PNs or KCs with *GH146* or *MB247*, respectively. *GH146*, like *MB247*, fully rescues the immediate odor memory of the *rut* mutant in appetitive learning ($n = 8–12$). ***D***, The same rut^{+} expression in the PNs does not rescue the mutant aversive memory if tested immediately after training, whereas the one in KCs does ($n = 5–8$). $^{a}p < 0.05$ in comparison with rut^{2080}*/Y; UAS-rut*$^{+}$*/+* and $p > 0.05$ in comparison with the group with respective *GAL4/+*; $^{b}p > 0.05$ in comparison with rut^{2080}*/Y; UAS-rut*$^{+}$*/+* and $p < 0.05$ in comparison with the group with respective *GAL4/+*. Error bars indicate SEM.

1985). Wild-type *rut* expression localized to the KCs rescues the aversive and appetitive olfactory memory of the mutant to the wild-type level (Zars et al., 2000; Schwaerzel et al., 2003), suggesting that these KCs can serve as a shared sufficient site for two differently reinforced memory traces. Taking the advantage of this experimental system, we addressed the ability of PNs to form the *rut*-dependent trace for rewarded and punished olfactory memories at different time points (up to 3 h).

Materials and Methods

Flies. For all behavioral experiments, F1 progeny of the following crosses were measured: females of rut^{2080}*; UAS-rut (III)* or wild-type Canton-S were mated to males of *w; MB247 (III)*, *w; GH146 (II)*, *w; NP225 (II)*, *w; MB247 (III)*, *Tub-GAL80*^{ts} *(III)*, *w; GH146; Tub-GAL80*^{ts} *(III)*, or w^{1118} (Zars et al., 2000; Heimbeck et al., 2001; McGuire et al., 2003). *MB247*, *GH146*, and *NP225* are the GAL4 driver lines directing the expression in the KCs (*MB247*) and PNs (the other two). McGuire et al. (2003) reported that transgenic Rutabaga protein is detected specifically in the targeted tissue. Mixtures of male and female progeny were measured in olfactory conditioning or relevant preference/avoidance of odors, electroshock, and sugar. Flies with balancers were eliminated from calculation and genders were separated afterward. For immunohistochemistry, brains from the progeny of the cross between *UAS-mCD8::GFP* and *GH146*, *MB247*, or *NP225* were prepared. All flies were raised and starved at 25°C, unless stated otherwise.

Behavioral analyses. Olfactory conditioning reinforced either by electroshock punishment or sugar reward was as described previously (Tully and Quinn, 1985; Schwaerzel et al., 2003). For reward learning, flies were starved for 16–18 h before measurement. The flies in the experiments using *Tub-GAL80*^{ts} (see Fig. 4*A*, *B*) were starved for 14 h at 30°C (see Fig. 4*A*) and 38–40 h at 18°C (see Fig. 4*B*) until the measurements. We noticed that the memory defect of rut^{2080}*; UAS-rut (III)* in sugar-reward learning tended to be weaker if the starvation was longer than indicated above (see Fig. 3*A*, compare mutant memory performance, better in 3 h than 1 h retention).

Differential conditioning with two odors (CS^{+} and CS^{-}) was used. Flies were trained by receiving the CS^{+} for 1 min in the presence of 12 pulses of electric shocks (90 V direct current) or, for appetitive learning, dried filter paper having absorbed 2 M sucrose solution (Tully and Quinn, 1985; Schwaerzel et al., 2003). Subsequently, they received CS^{-} for 1 min, but without electroshock or sugar. In sugar-reward learning, the training consists of two sets of differential conditioning by transferring the flies between two respective training tubes with or without sugar-containing paper. For 1 or 3 h memory retention, the trained flies were kept in empty vials. To observe the conditioned response, the trained flies were allowed to choose between CS^{+} and CS^{-} for 2 min in the T maze. A learning index was then calculated by taking the mean preference of two groups, which had been trained reciprocally in terms of the two odors used as CS^{+} and CS^{-} (Tully and Quinn, 1985; Schwaerzel et al., 2003).

Statistics. Only the data from male progeny were taken in this study because the male progeny of the crosses was either hemizygous for rut^{2080} or wild-type, whereas females were heterozygous. The significance level of statistical tests was set to 0.05. All groups tested in associative learning did not violate the assumption of the normal distribution and the homogeneity of variance. Therefore, mean learning indices were compared with one-way ANOVA followed by the planned pairwise multiple comparisons (Fisher's least significant difference). For the *rut* rescue experiments, the mean learning index of the experimental group in which rut^{+} was expressed in the mutant background were compared with those of the two different control groups: the flies without a driver (rut^{2080}*/Y; UAS-rut*$^{+}$*/+*) and heterozygotes for respective GAL4 drivers (*GAL4/+*). The data distribution of the groups in Table 1 was significantly different from the normal distribution. Therefore, the medians of all groups in Table 1 were compared with nonparametric methods (Kruskal–Wallis test). The sample size of each group is reported in the figure legends.

Immunohistochemistry. Whole-mount immunofluorescent preparation of the brain was described in the previous study (Thum et al., 2006). Microdissection was performed in Ringer's solution to remove cuticle and connective tissues. The brains were fixed with 4% formaldehyde in PBS containing 0.3% Triton X-100 (PBT) and then rinsed with PBT three times. Samples were blocked with 3% normal goat serum, and subsequently incubated with the primary antibodies in the blocking solution at 4°C overnight. Mouse monoclonal anti-Bruchpilot (Brp; nc82) for labeling the neuropile (Wagh et al., 2006) and rabbit polyclonal anti-green fluorescent protein (GFP; 1:1,000; A6455; Invitrogen, Eugene, OR) were used as primary antibodies. After washing the brains with PBT, samples were incubated with secondary antibodies in the blocking solution at 4°C overnight. Alexa Fluor 488- or Cy3-conjugated goat anti-rabbit or anti-mouse, respectively, was used to detect the primary antibody. After rinsing the samples with PBT, the brains were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Confocal detection and organization of raw image data followed the standard procedure (Thum et al., 2006).

Results

Rescuing *rutabaga*-dependent memory deficit with localized expression in PNs or KCs

To determine the role of PNs compared with that of KCs, we first expressed wild-type *rutabaga* (*rut*) cDNA in the PNs using the GAL4/upstream activating sequence (UAS) system in the *rut* mu-

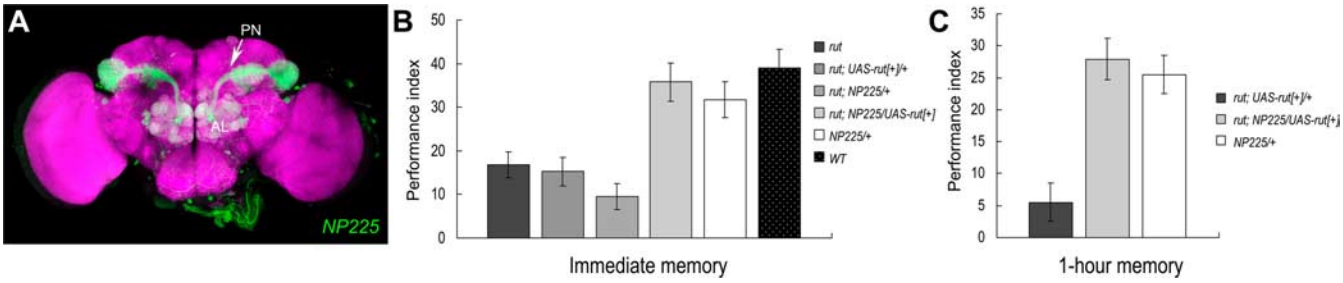

Figure 2. ***A***, GAL4-expressing cells in *NP225* monitored by *UAS-mCD8::GFP* (green). Brp protein is visualized as a marker of the neuropile using the monoclonal antibody nc82 (magenta). *NP225* has more specific GAL4 expression in the PNs. ***B***, The rut^+ expression is targeted to the PNs with *NP225*. As in *GH146*, *NP225* fully rescues the short-term odor memory of the *rut* mutant in appetitive learning ($n = 14–23$). ***C***, The rut^+ expression with *NP225* also repairs the *rut* memory defect at 1 h retention ($n = 9–12$). Error bars indicate SEM.

tant background (Zars et al., 2000). We chose the driver line *GH146*, which predominantly labels, among other cells, 83 PNs innervating 34 of 43 glomeruli in the AL (Fig. 1*B*) (Heimbeck et al., 2001). The driver line *MB247* was, in parallel, used to drive *rut* expression in KCs (Fig. 1*B*). We first tested short-term reward learning with the previously used combination of odors (3-octanol and 4-methyl cyclohexanol) (Tempel et al., 1983), which are most commonly used for *Drosophila* olfactory learning.

Mutant *rut* flies expressing rut^+ in the PNs learned significantly better than the control mutant flies without a GAL4 driver ($p = 0.019$) (Fig. 1*C*). As reported previously, the rut^+ expression in the KCs using the driver line *MB247* also restored the *rut* learning deficit. The performance of rescued flies was indistinguishable from those of wild-type and heterozygotes for respective GAL4 drivers ($p > 0.05$) (Fig. 1*C*), indicating that the localized transgenic expression driven by *GH146* or *MB247* is fully sufficient to rescue the memory defect in the mutant. These results imply that at least two memory traces for odor–sugar association may exist in *Drosophila*: one in the first- and another in the second-order interneurons of the olfactory system (i.e., PNs and KCs).

If electric shock was used as a reinforcer (unconditioned stimulus), the expression of rut^+ in the PNs failed to rescue the learning deficit of the control mutant flies without a GAL4 driver ($p > 0.05$) (Fig. 1*D*). Because heterozygotes for *GH146* do not show obvious deficit in punished memory, these results indicate that the effect of *rut* expression with *GH146* is specific for reward conditioning. In contrast, the *rut* expression in the KCs did rescue the odor memory in electroshock learning ($p = 6.5 \times 10^{-4}$) (Fig. 1*C*), as reported previously (Zars et al., 2000; Schwaerzel et al., 2003). Together, the PNs and MBs may independently establish the Rut-dependent olfactory memory traces, if reinforced by the sugar reward, but not if reinforced by electroshock punishment. Thus, the differentiation of electroshock punishment and sugar reward should underlie the selective contribution of PNs.

Rescue of *rutabaga* memory with another PN-driver line

As is the case for many GAL4 drivers, *GH146* has additional GAL4 expression in addition to that in the PNs, including neurons projecting to the MB (Heimbeck et al., 2001) (Fig. 1*B*), although these are not KCs (H. Tanimoto and K. Ito, unpublished observation). Therefore, we directed *rut* expression to the PNs using the independent GAL4 driver *NP225*. This strain predominantly labels 75 PNs projecting to 35 glomeruli with few neurons in other parts of the brain potentially overlapping with the cells in *GH146* (Tanaka et al., 2004) (Fig. 2*A*). Consistent with the results with *GH146*, rut^+ expression targeted by *NP225* in the *rut* mutant background was sufficient to restore the mutant memory to the wild-type level (Fig. 2*B*). The memory of the flies with the targeted expression was significantly better than the control mutant flies without a GAL4 driver and/or *UAS-*rut^+ ($p < 0.05$) (Fig. 2*B*). Moreover, *NP225* was also sufficient to repair 1 h memory of the mutant to the level of the GAL4 control ($p = 2.8 \times 10^{-5}$) (Fig. 2*C*). Thus, it is likely that PNs are a sufficient site of the *rut*-dependent memory trace.

Memory retention in flies with localized rut^+ expression

In the honeybee, intervention in the antennal lobe and the MB affects conditioned behavior with different dynamics (Erber et al., 1980). Similarly in *Drosophila*, the decay of glomerular recruitment of PNs is much shorter than the detectable conditioned behavior (Yu et al., 2004). This raises the possibility that the memory traces in PNs and MB might be differentiated in their temporal stability. We therefore measured 1 and 3 h memories, because the consolidated component of olfactory reward memory develops within 3 h (Tempel et al., 1983). Intriguingly, we did not find temporally distinct rescue effects for *GH146* and *MB247* ($p > 0.05$) (Fig. 3*A*). rut^+ expression targeted to both cell types was fully sufficient to rescue the mutant memory to the control level at both of the tested retention intervals ($p = 1.8 \times 10^{-9}$ and 4.0×10^{-4} for 1 and 3 h retention, respectively) (Fig. 3*A*). These results, together with immediate memory, suggest that the *rut*-dependent trace in PNs is as stable as the one in MB for up to 3 h.

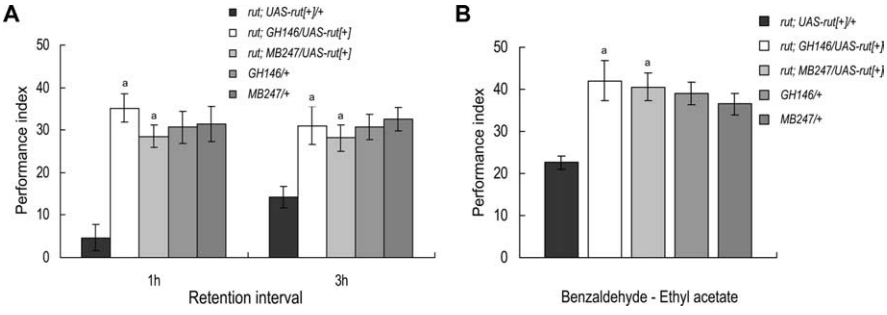

Figure 3. ***A***, The appetitive memory retention of the *rut* mutant flies with rut^+ expression by *GH146* or *MB247* at 1 (left) or 3 h (right) after training. The expression in the PNs, as in KCs, fully restores memory at both tested retention intervals ($n = 10–14$). ***B***, The rut^+ expression in the PNs, as in KCs, is sufficient to rescue immediate memory with another odor combination (benzaldehyde and ethylacetate; $n = 7–15$). $^a p < 0.05$ compared with rut^{2080}/Y; *UAS-*$rut^+/+$ and $p > 0.05$ compared with the group with respective *GAL4/+*. Error bars indicate SEM.

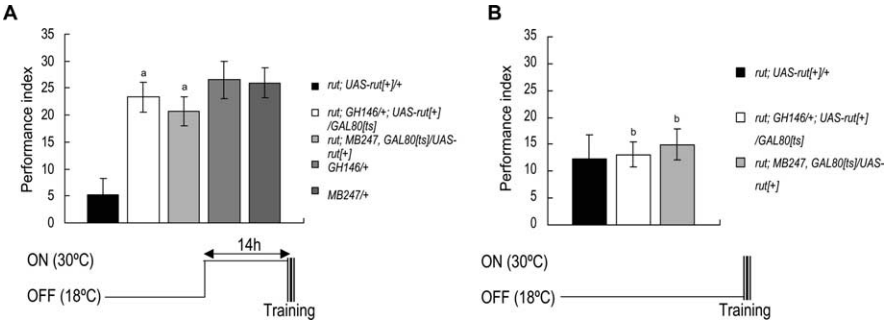

Figure 4. ***A***, ***B***, Rescuing the immediate appetitive memory deficit of *rut* with (***A***) or without (***B***) adult-induced rut^{+} expression either by *GH146* or *MB247*. All flies in the experiments are raised at 18°C, where $GAL80^{ts}$ is supposed to suppress GAL4 transactivation. Before the measurement, the flies are starved at 30°C (***A***) or 18°C (***B***). The temperature shift can derepress the GAL4-dependent *rut* expression (McGuire et al., 2003). ***A***, Adult-induced rut^{+} expression in either the PNs or KCs is sufficient to rescue the short-term appetitive memory of the *rut* mutant ($n = 12$–13). ***B***, Induction of rut^{+} expression is necessary to rescue the short-term appetitive memory of the *rut* mutant ($n = 8$–12). $^{a}p < 0.05$ compared with rut^{2080}/Y; *UAS-rut*$^{+}$/+ and $p > 0.05$ compared with the group with respective *GAL4*/+; $^{b}p > 0.05$ compared with rut^{2080}/Y; *UAS-rut*$^{+}$/+. Error bars indicate SEM.

Odor specificity of memory traces

We next asked whether PNs can form reward memories not only with the standard pair of odors, but also with other ones. *rut* expression in KCs has been shown to rescue memories of different odor combinations (Zars et al., 2000; McGuire et al., 2003; Schwaerzel et al., 2003; Akalal et al., 2006). We therefore used benzaldehyde and ethyl acetate as conditioned stimuli in short-term reward learning, and examined whether the *rut* mutant memory defect for these odors also can be rescued with wild-type *rut* expression in the PNs. Benzaldehyde has been reported to be unique in memory formation for *Drosophila*, because it is perceived not only through the antennae (Keene et al., 2004). If benzaldehyde uses PNs and/or KCs differently from other odors, the degree of the rescue could be different. Contrary to this assumption, *rut* mutant flies with transgenic expression by *GH146* or *MB247* showed benzaldehyde/ethyl acetate memories significantly better than those of the mutant without transgenic expression ($p = 1.6 \times 10^{-5}$) (Fig. 2*B*). The rescued memories were similar to the control heterozygotes for the respective driver lines ($p > 0.05$) (Fig. 2*B*). Thus, as with KCs, the *rut*-dependent memory traces in PNs do not appear to be restricted for a particular odor combination.

Rescuing *rutabaga*-dependent memory deficit with adult-induced expression in PNs or KCs

The results above suggest that PNs as well as KCs require Rut-AC during adulthood for neuronal plasticity. Alternatively, Rut-AC might be specifically involved during development in setting up the circuitry required later in the adult for memory formation. Regarding the memory trace of aversive odor conditioning in the MB, previous experiments have already shown the adult requirement and sufficiency of Rut-AC in KCs, despite the reported additional role of Rut in MB development (McGuire et al., 2003; Mao et al., 2004).

To investigate the temporal requirement for *rut*-dependent memory formation in sugar-reward conditioning, rut^{+} expression was induced specifically in the adult, either with *GH146* or *MB247* in the *rut* mutant background using the temperature-sensitive GAL4 repressor $GAL80^{ts}$ (McGuire et al., 2003). By rearing the flies at 18°C, GAL4-dependent expression throughout development had been blocked and was induced in adulthood by shifting the temperature to 30°C. We found that, for both PNs and KCs, 14 h of induction just before training could rescue the short-term memory in appetitive learning ($p = 4.7 \times 10^{-5}$ and 3.9×10^{-4} for rescue with *GH146* and *MB247*, respectively) (Fig. 4*A*). If the flies were kept constantly at 18°C throughout the experiments, thereby suppressing GAL4-dependent *rut* expression, neither of the GAL4 drivers could rescue the phenotype ($p > 0.05$) (Fig. 4*B*), confirming the integrity of $GAL80^{ts}$. Moreover, this indicates that the rescue is attributable to the transactivation of *UAS-rut*$^{+}$, but not mere GAL4 insertion effects. Hence, similar to its action in odor–shock learning, Rut-AC driven by *GH146* and *MB247* in the adult is sufficient for restoring the memories in sugar-reward conditioning. This also suggests that transgenic supply of rut^{+} cDNA during development is dispensable for adult short-term memory. These results are in line with the hypothesis that Rut-AC acutely acts in neuronal plasticity, possibly as a coincidence detector.

To exclude the possibility that GAL4-dependent *rut* expression in the PNs changes sensory perception, we measured the behavioral responses to the odors, sugar, and electroshock, which we used for training and testing. Reactivity to these sensory stimuli of all the genotypes was not significantly different ($p > 0.05$) (Tables 1, 2). Thus, as in the case of the MB, memory rescue by rut^{+} expression is probably attributable to restoring the neuronal plasticity in these cells, rather than to enhancing sensory perception.

Table 1. Response to sucrose solution

Genotype	Sugar reactivity	Water reactivity
rut/Y; rut[+]/+	95.0/4.6	1.7/4.6
GH146/+	90.8/10.0	0.8/3.3
MB247/+	90.0/15.4	0.0/0.0
NP225/+	96.7/9.2	0.0/0.0
rut/Y; GH146/+; rut[+]/+	93.3/11.3	1.7/6.3
rut/Y; MB247/rut[+]	92.5/19.2	2.5/3.3
rut/Y; NP225/+; rut[+]/+	96.7/9.2	0.0/2.5
GH146/+; Gal80[ts]/+	98.3/1.7	0.0/7.1
MB247 Gal80[ts]/+	85.0/14.2	0.0/0.0
rut/Y; GH146/+; Gal80[ts]/rut[+]	90.0/14.6	0.0/2.5
rut/Y; MB247 Gal80[ts]/rut[+]	96.7/2.5	0.0/0.0

Sugar and water sensitivities of experimental and control animals. No significant differences ($p > 0.005$) in either assay are detected between experimental and control flies. Because data points are not normally distributed, the median and interquartile range of 10 experiments are shown in percent.

Discussion

Multiple memory traces and underlying potential mechanisms

Using appetitive olfactory learning in *Drosophila*, we showed that the PNs as well as KCs can accommodate a Rutabaga-dependent memory trace (Figs. 1*C*, 2*B*,*C*, 3, 4). In contrast, aversive odor memory of *rutabaga* can be rescued in the KCs, but not in the PNs (Fig. 1*D*). Thus, appetitive, but not aversive, olfactory learning may induce two memory traces: one in the first- and another in the second-order olfactory interneurons (i.e., PNs and KCs).

The search for an "engram" in the brain has been a focus in neuroscience with a history of more than 70 years (Lashley, 1929). Since then, behavioral plasticity has been measured as a signature of memory. To our knowledge, it has never been shown at the cellular level that different types of neurons independently

Table 2. Sensory acuity

Genotype	Odorant avoidance		Shock avoidance
	3-OCT (1:10; 4 mm)	4-MCH (1:10; 5 mm)	
rut/Y; rut[+]/+	38.8/4.7	27.8/5.9	63.8/5.3
GH146/+	43.8/4.7	33.7/5.5	68.9/6.5
MB247/+	41.5/6.5	30.9/5.6	64.6/11.1
NP225/+	42.5/6.2	29.5/5.2	N.D.
rut/Y; GH146/+; rut[+]/+	34.3/5.2	34.9/4.3	64.6/8.5
rut/Y; MB247/rut[+]	38.3/4.6	39.5/4.3	75.1/5.1
rut/Y; NP225/+; rut[+]/+	48.8/8.2	25.9/4.2	N.D.
GH146/+; Gal80[ts]/+	44.4/6.5	33.2/5.4	N.D.
MB247 Gal80[ts]/+	31.5/6.6	30.2/7.3	N.D.
rut/4; GH146/+; rut[+]/+	40.6/4.0	28.9/5.6	N.D.
rut/Y; MB247 Gal80[ts]/rut[+]	31.5/6.6	31.7/5.5	N.D.

Olfactory and electric shock sensitivities of experimental and control animals. Odors are diluted 10 times (1:10) in paraffin oil; 4-methyl cyclohexanol (4-MCH) is applied in an odor cup of 5 mm diameter and 3-octanol (3-OCT) in an odor cup of 4 mm diameter. No significant differences ($p > 0.005$) in any of the assays are detected between experimental and control flies. For each experiment, mean and SEM of 7–10 experiments are shown in percent. N.D., Not determined.

provide memory traces for the same behavioral task. In the honeybee, the independent application of octopamine to the AL or the MB as a substitute of positive reinforcement induced olfactory memory, implying multiple traces formed at different sites in the brain (Hammer and Menzel, 1998). Because the kinetics of memory acquisition was different when octopamine was applied to the AL and the MB, these two traces do not appear to be fully redundant.

In this study, we mapped equivalent traces to two sequential interneurons in the olfactory pathway, the PNs and KCs. Because both of the traces are mediated by the same molecule, the type I adenylate cyclase, these could undergo the same type of plasticity. Our findings therefore suggest that multiple cellular memory traces can be localized within a common neuronal circuit. At the same time, the multiple traces raise the possibility that AC activity in one of these cell types might be dispensable. This could be tested by examining the appetitive memories by cell-type specific knock-down of *rut* mRNA or impairment of Rut-AC regulation.

It would also be important to identify the synaptic terminals of PNs undergoing Rut-dependent associative plasticity. The PNs projecting via the iACT synapse onto three different brain structures: the AL, the calyx of the MB, and the lateral horn. Because calcium imaging techniques allow to compare spatially separated synaptic outputs of the same neurons in vivo (Fiala et al., 2002; Yu et al., 2005, 2006), optophysiological measurement of the PNs during odor–sugar conditioning might be able to identify the corresponding terminals. The strengthened synaptic outputs after conditioning might be qualitatively distinct from those elicited by the perception of the higher concentration of odors, because the corresponding behavioral outputs are different. In any case, the Rut-dependent memory trace in the PNs should drive conditioned responses via the KCs, because blocking subsets of KCs (α/β and γ neurons) at the moment of test abolished the retrieval of appetitive memory (Schwaerzel et al. 2003). Although the memories supported by the PNs and MB in the present study were equivalent in terms of the level and temporal stability of the learning index, other experimental conditions might well qualitatively differentiate these two traces. For example, the difference might become obvious if odors are difficult to discriminate because of the different odor representations in PNs and KCs (Perez-Orive et al., 2002) or if tested for long-term memory (e.g., Müller, 2000).

Odor-evoked activity of the PNs is altered after associative aversive training by an effect called "synaptic recruitment" (Yu et al. 2004). More glomeruli in the AL responded to the conditioned odor specifically after associative (odor–shock) training (Yu et al. 2004). Yet, the synaptic recruitment and the Rut-dependent trace in the PNs in this study might have different behavioral correlates. The Rut-dependent trace lasted for 3 h after training (Fig. 3*A*), whereas the synaptic recruitment was short-lived, decaying within 7 min (Yu et al., 2004). In addition, transgenic expression of *rut* cDNA in the PNs did not support the memory rescue if the odor was paired with electric shocks (for discussion, see Heisenberg and Gerber, 2007).

Consolidation of the Rut-dependent trace in PNs

The mechanisms to maintain olfactory memories for several hours are, so far, little understood (Keene and Waddell, 2007). Whereas Rut-AC is supposed to mediate memory formation in certain types of KCs (α/β and γ neurons) and the PNs, the stabilization of both appetitive and aversive odor memories (e.g., 3 h memory) requires additional neurons specifically after memory acquisition. One of these is the dorsal paired medial (DPM) neuron that innervates the entire MB lobes (Keene et al. 2004, 2006). The requirement of the DPM neurons has been reported to be limited to the first hour of the 3 h retention period (Keene et al. 2004). Intriguingly, DPM neurons project exclusively to MB lobes and possibly function only in a subsystem (α'/β' lobes) of the MB (Keene et al., 2006). Because we showed here that the Rut-dependent trace in the PNs can also support 3 h appetitive memory (Fig. 3*A*), the question arises how the memory trace in the PNs is related to the activity of DPM neurons and α'/β' neurons during consolidation of 3 h olfactory memory.

There are at least two possible interpretations. One is that DPM neurons would be temporally gating KCs to respond to or to transmit the associative plasticity formed in the PNs and KCs. In fact, the calcium response to aversively conditioned odors in the KCs is transiently increased, and this increase depends on Amnesiac protein expressed in the DPM neurons (Yu et al., 2006). Another possibility is that Rut-independent plasticity in KCs that can initially be induced by the trace in PNs might be responsible to drive 3 h memory. This plasticity in KCs could be maintained by the activity of DPM neurons during the retention period.

Yet, additional experiments regarding 3 h memory would be necessary for an integrated network model accommodating distinct roles of PNs, KCs, and DPM neurons. As suggested by Krashes et al. (2007), blocking PN output during the retention period would address whether memory stabilization by DPM neurons requires the sustained activity of PNs.

Reinforcement systems for the memory trace in PNs

The Rut-dependent memory trace with *GH146* was specific to sugar-reward learning; there was no rescue on shock learning. This differential effect could have its underpinnings in the neuronal wiring of the octopaminergic and dopaminergic neurons, which respectively mediate the appetitive and aversive reinforcers in insect brains (Schwaerzel et al., 2003; Unoki et al., 2005; Schroll et al., 2006). Octopamine immunoreactivity has been found in the main synaptic terminal areas of the PNs: antennal lobes, MB calyx, and lateral horn in *Drosophila* (Sinakevitch and Strausfeld, 2006). A single reward-representing neuron in the honeybee, VUMmx1, also terminates in these neuropiles (Hammer, 1993). Thus, the Rut-dependent memory trace in the *Drosophila* PNs and MBs could directly be induced by octopaminergic processes.

In contrast, projections of dopaminergic neurons to the antennal lobe are, if at all, very faint, and are minimal in the calyx (data not shown). Instead, they strongly innervate the peduncu-lus and all lobes of the MB where KCs have presynaptic terminals (Riemensperger et al., 2005). Thus, the behavioral specificity of the memory trace in the PNs has its anatomical parallel in the target specificity of the internal reinforcement systems. Additional behavioral and physiological analyses of modulatory neurons could clarify the circuit differentiation of appetitive and aversive memory traces.

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