Research

Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory

Yufeng Pan,^{1,2} Yanqiong Zhou,^{1,2} Chao Guo,^{1,2} Haiyun Gong,¹ Zhefeng Gong,^{1,3} and Li Liu^{1,3}

¹State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, Chaoyang District, Beijing 100101, People's Republic of China; ²Graduate University of Chinese Academy of Sciences, Beijing 100039, People's Republic of China

The central complex is a prominent structure in the *Drosophila* brain. Visual learning experiments in the flight simulator, with flies with genetically altered brains, revealed that two groups of horizontal neurons in one of its substructures, the fan-shaped body, were required for *Drosophila* visual pattern memory. However, little is known about the role of other components of the central complex for visual pattern memory. Here we show that a small set of neurons in the ellipsoid body, which is another substructure of the central complex and connected to the fan-shaped body, is also required for visual pattern memory. Localized expression of *rutabaga* adenylyl cyclase in either the fan-shaped body or the ellipsoid body is sufficient to rescue the memory defect of the *rut*²⁰⁸⁰ mutant. We then performed RNA interference of *rutabaga* in either structure and found that they both were required for visual pattern memory. Additionally, we tested the above rescued flies under several visual pattern parameters, such as size, contour orientation, and vertical compactness, and revealed differential roles of the fan-shaped body and the ellipsoid body for visual pattern memory. Our study defines a complex neural circuit in the central complex for *Drosophila* visual pattern memory.

The central complex is a prominent structure in the central brain of adult insects. Numerous studies have indicated that it is an important center for higher-order brain functions including locomotor control (Strauss and Heisenberg 1993; Martin et al. 1999; Strauss 2002; Poeck et al. 2008), processing of *E*-vector orientation information (Vitzthum et al. 2002; Heinze and Homberg 2007), courtship behavior (Popov et al. 2005; Sakai and Kitamoto 2006), olfactory long-term memory (Wu et al. 2007), visual pattern memory (Liu et al. 2006; Wang et al. 2008), and spatial orientation memory (Neuser et al. 2008).

In *Drosophila melanogaster*, the central complex consists of four interconnected substructures: the protocerebral bridge (pb), the fan-shaped body (fb), the ellipsoid body (eb), and the paired noduli (no) (Hanesch et al. 1989; Renn et al. 1999). The central complex has been found to be involved in multiple forms of learning and memory. Memory for two visual pattern parameters in tethered flight requires *rutabaga* (*rut*) function in two distinct large field neurons (F neurons) in the fan-shaped body (Liu et al. 2006). The central complex is not involved in short-term olfactory memory, but a subset of large field neurons in the ellipsoid body that are called “R neurons” (R2/R4m neurons) participate in the NMDA receptor-dependent long-term memory consolidation (Wu et al. 2007). Another group of R neurons (R3/R4d) are necessary for spatial orientation memory, which is similar to the working memory in vertebrates (Neuser et al. 2008).

Concerning *Drosophila* visual pattern memory in tethered flight, large field neurons in the first layer (F1 neurons) and fifth layer (F5 neurons) of the fan-shaped body are, respectively, responsible for *rut*-dependent memory for “contour orientation” and “elevation” (Liu et al. 2006), which are two out of five known learnable pattern parameters (Ernst and Heisenberg 1999; Tang

and Guo 2001). Meanwhile, protein kinase G (PKG) was found to be required in the ellipsoid body for visual pattern memory (Wang et al. 2008). Memory for “contour orientation” and “elevation” both needed PKG function in a subset of the ellipsoid body large field neurons. However, the functional relationship between the fan-shaped body and ellipsoid body in visual pattern memory is not well understood.

To address whether the fan-shaped body and ellipsoid body work together or function redundantly in *Drosophila* visual pattern memory, we performed *rut* rescue experiments by targeted expression of wild-type *rut* cDNA in *rut* mutant flies, and knocked down *rut* in the central complex by RNA interference. We found that both the fan-shaped body and ellipsoid body were involved in visual pattern memory. As for the four learnable pattern parameters: “contour orientation,” “elevation,” “size,” and “vertical compactness” (Ernst and Heisenberg 1999), F1 and F5 neurons were required for memory for “contour orientation” and “elevation,” respectively, while a subset of R neurons (R2/R4m) seemed to be involved in memory for all the parameters in a parameter-independent manner. Our results indicated that the fan-shaped body and ellipsoid body neurons might be both required, but in different ways, for *Drosophila* visual pattern memory.

Results

Overexpression of *rut* cDNA in either the fan-shaped body or ellipsoid body neurons was sufficient to restore *rut*²⁰⁸⁰ visual pattern memory defect

In *Drosophila*, visual pattern memory has been studied in detail in tethered flight (Fig. 1A; Wolf and Heisenberg 1991; Brembs and Heisenberg 2000). It was previously found that *rut*²⁰⁸⁰, a *P*-element insertion allele, was defective in visual pattern memory (Fig. 1C; Heisenberg et al. 2001; Liu et al. 2006). We successfully rescued visual pattern memory in the *rut*²⁰⁸⁰ mutant flies with restricted

³Corresponding authors.

E-mail liuli@sun5.ibp.ac.cn; fax 86-10-64853625.

E-mail zfgong@moon.ibp.ac.cn; fax 86-10-64853625.

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Figure 1. Visual pattern memory in the wild-type and rut^{2080} mutant flies in the flight simulator. (A) Schematic diagram of the flight simulator. (B) Performance of wild-type Canton S flies in an 18-min procedure ($n = 44$). Bars show performance indices (PIs) of nine consecutive 2-min periods including pretest (open bars, PI₁ and PI₂), training (dark gray bars, PI₃, PI₄, PI₅, and PI₆), and memory test (open bars, PI₇ and PI₈). PI₈ is used as an index of 2-min short-term memory throughout. T patterns were used as visual stimuli for pattern parameter "elevation." (C) Overexpression of rut cDNA targeted by $c205$ could restore the memory defect of rut^{2080} flies. Numbers below bars indicate numbers of flies. (***) $P < 0.001$ compared with the chance level zero (one-sample t -test); (a) $P < 0.01$ in comparison with zero (one-sample t -test) and $P > 0.05$ in comparison with wild type (two independent samples t -test). Error bars are SEMs throughout.

expression of rut cDNA using the UAS/GAL4 system (Brand and Perrimon 1993). Control flies carrying rut^{2080} and a copy of UAS- rut^{+} but no GAL4 driver ($rut^{2080}/y; UAS-rut^{+}/+$) were compared with those bearing GAL4 drivers. Introduction of UAS- rut^{+} did not increase the memory index of the rut^{2080} flies (Fig. 1C). According

to Liu et al. (2006), in a total of 27 driver lines expressing GAL4 in different neuropil regions, there were seven lines with an expression in the F5 neurons in which the memory for "elevation" was restored.

As we recently found that PKG was required in a subset of the ellipsoid body neurons (R2/R4m) for visual pattern memory (Wang et al. 2008), we asked whether Rut also was required there. Indeed, when using T patterns as conditioned stimuli, rut^{2080} flies expressing rut^{+} ectopically in the R2/R4m neurons (c819) (Fig. 2C) showed significantly higher memory levels than control flies ($t = 2.41$, $P < 0.05$; Fig. 2A). In contrast, expression of rut^{+} in the R3/R4d neurons (c232) (Fig. 2C) failed to restore the rut^{2080} memory defect ($t = 0.14$, $P = \text{NS}$ [not significant]; Fig. 2A). These results indicated that the R2/R4m neurons might also be required for visual pattern memory for "elevation," like the F5 neurons in the fan-shaped body. To confirm this, we targeted rut^{+} expression using two other GAL4 lines, c42 and c547, which also labeled the R2/R4m neurons (Fig. 2C). Consistent with the rescue results with c819, rut^{+} expression driven by c42 or c547 could successfully restore the rut^{2080} memory defect ($t = 4.13$, $P < 0.001$ and $t = 3.37$, $P < 0.01$, respectively; Fig. 2A). Taking these results together, we concluded that aside from the F5 neurons in the fan-shaped body, the R2/R4m neurons in the ellipsoid body could independently restore the rut^{2080} memory defect when using T patterns as conditioned stimuli.

Adult-restricted expression of Rut was sufficient to rescue rut^{2080} memory defect

To exclude potential developmental defects, we used tub -GAL80^{ts} to suppress GAL4 function in the R2/R4m neurons during development. Flies with induced expression of rut^{+} for 12 h at 30°C in the R2/R4m neurons ($rut^{2080}/y; tub$ -GAL80^{ts}/+; UAS- rut^{+} /c819) had significantly higher memory levels compared with those without induced expression of rut^{+} ($t = 2.65$, $P < 0.05$; Fig. 2B). Furthermore, these flies showed significantly better memory than those without GAL4 driver ($rut^{2080}/y; tub$ -GAL80^{ts}/+; UAS- rut^{+} /+) that had also been heat shocked for 12 h ($t = 2.97$, $P < 0.01$; Fig. 2B). These results demonstrated that acute Rut function in the adult R2/R4m neurons was sufficient for visual pattern memory.

RNAi knockdown of rut in either the fan-shaped body or ellipsoid body neurons abolished visual pattern memory

As indicated above, independent expression of rut^{+} in the F5 or R2/R4m neurons could both restore visual pattern memory for "elevation" of the rut^{2080} mutant. This suggests that Rut function in either of these two neuronal subtypes was sufficient for normal visual pattern memory. To test if they were functionally interchangeable, we performed RNA interference (RNAi) in either the fan-shaped body or ellipsoid body neurons. Flies with rut RNAi expression in the F5 neurons, labeled by c205, showed significantly impaired visual pattern memory compared with control flies ($t = 3.12$, $P < 0.01$ against the UAS- $rut^{RNAi}/+$ flies; $t = 2.79$, $P < 0.01$ against the c205/+ flies), so did those with rut RNAi driven by c819 ($t = 3.02$, $P < 0.01$ against the UAS- $rut^{RNAi}/+$ flies; $t = 2.72$, $P < 0.05$ against the c819/+ flies; Fig. 3A). Meanwhile, RNAi-mediated knockdown of rut driven by c232 did not affect visual pattern memory ($t = 0.58$, $P = \text{NS}$ compared with the UAS- $rut^{RNAi}/+$ flies; $t = 0.54$, $P = \text{NS}$ compared with the c232/+ flies; Fig. 3A). Hence, we concluded that the F5 (c205) and R2/R4m (c819) neurons were both necessary for visual pattern memory.

A critical role of rut mutant allele

The RNAi result is not consistent with the rescue experiments described above. If both neurons (F5 and R2/R4m) were necessary for the constitution of visual pattern memory for "elevation," then

Figure 2. Overexpression of *rut* cDNA in the R2/R4m neurons was sufficient to rescue *rut²⁰⁸⁰* visual pattern memory defect. (A) c819, c547, and c42 could fully restore visual pattern memory for “elevation” by driving *rut⁺* expression, but c232 could not. (B) Flies with genotype *rut²⁰⁸⁰/y;tub-GAL80^{ts}/+;UAS-rut⁺/c819* showed no memory if always kept at 19°C, but they showed significantly higher memory if transferred to 30°C 12 h before behavioral experiments. This acute rescue effect was not observed in the flies without GAL4 driver (*rut²⁰⁸⁰/y;tub-GAL80^{ts}/+;UAS-rut⁺/+*). (C) Expression patterns of c232, c819, c547, and c42 in the fan-shaped body and the ellipsoid body are shown by green fluorescent protein (GFP) (green). The synaptic neuropil is stained by nc82 antibody (magenta). c232 labels the R3/R4d neurons, while the other three drivers label the R2/R4m neurons. Only c42 has expression in the first layer of the fan-shaped body (F1 neurons). Numbers below bars indicate numbers of flies. (a) $P < 0.05$ compared with genotype *rut²⁰⁸⁰/y;UAS-rut⁺/+* and $P > 0.05$ compared with corresponding GAL4 controls. (b) $P > 0.05$ compared with *rut²⁰⁸⁰/y;UAS-rut⁺/+* flies. (*) $P < 0.05$; (**) $P < 0.01$. All comparisons were made using two independent samples *t*-test. Error bars indicate SEM. Scale bars are 20 μ m for both. The scale bars in the first column apply to all panels in the same row.

for only one of them the rescue should be expected to be unsuccessful. However, this was not the case: In both neurons the memory was successfully restored by expression of *rut⁺*. One possible explanation could be that there exists residual *rut* function in the fan-shaped body or ellipsoid body of *rut²⁰⁸⁰* flies. To compare the *rut* expression levels in the *rut²⁰⁸⁰* mutant and RNAi-expressing flies, we performed quantitative polymerase chain reaction (PCR) and found that the *rut* mRNA level in the *rut²⁰⁸⁰* flies was significantly lower than in the wild-type flies, but indeed significantly higher than in the *rut* RNAi flies (Fig. 3B).

The fact that *rut²⁰⁸⁰* is a hypomorph implies that the independent rescue in only the F5 or R2/R4m neurons might not be successful in a *rut* null mutant. Among the *rut* mutants, *rut¹* was generated by EMS (ethyl methane sulfonate) mutagenesis, which caused a point mutation in a conserved site and abolished Rut activity (Dudai and Zvi 1984; Livingstone et al. 1984; Han et al. 1992; Levin et al. 1992). Hemizygous male *rut¹* mutant flies with one copy of *UAS-rut⁺* showed a significant visual pattern memory defect, while heterozygous *rut¹* female flies with one copy of *UAS-*

rut⁺ showed normal visual pattern memory (Fig. 4A). Pan-neuronal expression of *rut* cDNA with *elav*-GAL4 rescued the *rut¹* memory defect ($t = 2.47$, $P < 0.05$), while targeted expression of *rut⁺* in neither the F5 neurons (c205: $t = 0.79$, $P = \text{NS}$; 104y: $t = 1.21$, $P = \text{NS}$) nor R2/R4m neurons (c819: $t = 1.15$, $P = \text{NS}$) could restore the memory defect (Fig. 4A). However, overexpression of *rut⁺* in both the F5 and R2/R4m neurons (104y + c819; Fig. 4B) significantly improved the visual pattern memory level ($t = 2.1$, $P < 0.05$; Fig. 4A). These results indicated that the combination of the F5 and R2/R4m neurons, but neither of them alone, were sufficient for visual pattern memory in the *rut¹* mutant.

Functional differentiation of the fan-shaped body and ellipsoid body neurons

Since the fan-shaped body and ellipsoid body neurons are both required for visual pattern memory, they may work together but function differentially. Liu et al. (2006) indicated that the F1 neurons were specific for memory for the parameter “contour orientation” and F5 for “elevation,” suggesting that the F neurons

Figure 3. Targeted RNA interference of *rut* could impair visual pattern memory. (A) RNAi silencing of *rut* in either the F5 (c205) or R2/R4m (c819) neurons abolished visual pattern memory for “elevation,” while knockdown of *rut* in the R3/R4d (c232) neurons had no effect on visual pattern memory. (B) *rut* mRNA level in the RNAi silencing flies was significantly lower than in the wild-type and *rut*²⁰⁸⁰ mutant flies ($n = 4$ for all). Numbers below bars indicate numbers of flies. (a) $P < 0.05$ compared with the UAS-*rut*^{RNAi}/+ flies or with corresponding GAL4 controls; (b) $P > 0.05$ compared with the UAS-*rut*^{RNAi}/+ or c232/+ flies (two independent samples *t*-test). (*) $P < 0.05$; (***) $P < 0.001$ (ANOVA). Error bars indicate SEM.

might participate in processing specific visual pattern information. So we turned to use other pattern parameters such as size and vertical compactness, which could be discriminated and memorized by the wild-type but not the *rut*²⁰⁸⁰ mutant flies (Fig. 5A). Overexpression of *rut*⁺ in the F5 neurons (c205) fully rescued visual pattern memory for “elevation” but none of the other three pattern parameters ($t = 3.07$, $P < 0.01$ for “elevation”; $t = 0.47$, $P = \text{NS}$ for “contour orientation”; $t = 0.46$, $P = \text{NS}$ for “size”; $t = 0.34$, $P = \text{NS}$ for “vertical compactness”), while overexpression of *rut*⁺ in the F1 neurons driven by NP6510 could only restore visual pattern memory for “contour orientation” ($t = 3.35$, $P < 0.01$ for “contour orientation”; $t = 0.09$, $P = \text{NS}$ for “elevation”; $t = 0.52$, $P = \text{NS}$ for “size”; $t = 0.55$, $P = \text{NS}$ for “vertical compactness”; Fig. 5B). However, overexpression of *rut*⁺ in the R2/R4m neurons driven by c819 or c42 could restore visual pattern memory for all four parameters (c819: $t = 3.59$, $P < 0.001$ for “elevation”; $t = 3.23$, $P < 0.01$ for “contour orientation”; $t = 3.36$, $P < 0.01$ for “size”; $t = 2.89$, $P < 0.01$ for “vertical compactness”; c42: $t = 2.54$, $P < 0.05$ for “elevation”; $t = 4.46$, $P < 0.001$ for “contour orientation”; $t = 2.33$, $P < 0.05$ for “size”; $t = 2.76$, $P < 0.01$ for “vertical compactness”; Fig. 5C). These results indicated that the F neurons (F1 and F5) might participate in visual pattern memory in a parameter-dependent manner, whereas the R2/R4m neurons might do so in a parameter-independent manner. Thus, the F and R neurons might function differentially for visual pattern memory.

Discussion

In this study, we reported that a subset of the ellipsoid body neurons were also necessary for Rut-dependent visual pattern memory, in addition to the previously described horizontal neurons in the

fan-shaped body (Liu et al. 2006). We demonstrated that these substructures of the central complex play different roles in visual pattern memory. Moreover, our experiments revealed that the choice of mutant allele was crucial when using the *rutabaga* rescue strategy.

Necessary and sufficient structures of the central complex for visual pattern memory

To localize the physical correlates of memory in the *Drosophila* central nervous system, one usually utilizes two distinct ways: (1) functional knockdown of “memory genes” or neural transmission in specific brain regions (Connolly et al. 1996; Dubnau et al. 2001; McGuire et al. 2001; Liu et al. 2006), and (2) functional rescue by targeted expression of a “memory gene” in the respective mutant (Zars et al. 2000a,b; Akalal et al. 2006; Liu et al. 2006; Thum et al. 2007). The former defines a structure necessary for memory formation, while the latter identifies a structure that is sufficient. *rutabaga* is such a gene that is involved in many forms of learning and memory in *Drosophila*. Functional rescue of the *rut*²⁰⁸⁰ mutant in olfactory aversive learning, olfactory reward learning, spatial learning, and visual pattern memory in tethered flight revealed distinct brain structures for memory formation: mushroom bodies for aversive olfactory learning (Zars et al. 2000a; Akalal et al. 2006), projection neurons or mushroom bodies for olfactory reward learning (Thum et al. 2007), median bundle for spatial learning (Zars et al. 2000b), and fan-shaped body for visual pattern memory (Liu et al. 2006). It should be noted that all these rescue experiments were done in the *rut*²⁰⁸⁰ mutant. The mutation is caused by a *P*-element insertion 155 bp upstream of the *rut* gene (Han et al. 1992), which leads to a reduced *rut* level (Fig. 3B). The results of

Figure 4. Rescue in the *rut*¹ mutant. (A) Targeted expression of *rut* cDNA in both the F5 and R2/R4m neurons restored *rut*¹ visual pattern memory defect. Overexpression of *rut*⁺ in the F5 (104y or c205) or R2/R4m neurons (c819) alone could not recover visual pattern memory for “elevation”; however, a combinatorial expression in these two neuronal subtypes led to successful rescue. (B) Combinatorial expression patterns of 104y and c819 in the F5 and R2/R4m neurons are shown by GFP (green). The synaptic neuropil is stained by nc82 antibody (magenta). 104y labels the F5 neurons and weakly the F1 neurons, and c819 labels restrictively the R2/R4m neurons. Numbers below bars indicate numbers of flies. (a) $P < 0.05$ and (b) $P > 0.05$ compared with *rut*¹/y;UAS-*rut*¹/+ flies. All comparisons were made using two independent samples *t*-test. Error bars indicate SEM. Scale bar, 50 μm . The scale bar applies to all panels.

our rescue experiments demonstrate that using a hypomorphic mutant allele is not optimal for determining the sufficiency of a brain region for a given task. For example, although restoring Rut function in the fan-shaped body rescued visual pattern memory successfully, this cannot exclude the involvement of other regions owing to the residual Rut activity in the *rut²⁰⁸⁰* flies. Therefore, it is crucial to perform rescue experiments in a null mutant. *rut¹* appears to be a suitable candidate, as the point mutation in the gene leads to a complete loss of Rut activity in both cultured cells (Levin et al. 1992) and head homogenate extracts (Dudai and Zvi 1984; Livingstone et al. 1984; Han et al. 1992). Overexpression of *rut¹* in either the F5 or R2/R4m neurons alone in the *rut¹* mutant failed to restore visual pattern memory, implying that neither the fan-shaped body nor ellipsoid body neurons were sufficient. However, a combination of these two regions did succeed in rescuing the *rut¹* memory defect. Taken together with our RNAi results that indicated necessary roles of both the F5 and R2/R4m neurons, it could be concluded that these fan-shaped body and ellipsoid body neurons seemed to be the sufficient brain regions where Rut functions, in the *rut¹* mutant, to form visual pattern memory.

We currently do not know the exact role of *rut* in the fan-shaped body and ellipsoid body; however, we can infer from previous studies on the larval neuromuscular junction (Zhong and Wu 1991; Cheung et al. 1999; Renger et al. 2000) that *rut* may mediate synaptic plasticity in these neurons. We assume that *rut*-dependent synaptic plasticity may be lost in the *rut¹* or RNAi silencing flies, but only compromised in the *rut²⁰⁸⁰* flies. Our results could be interpreted as that the loss of *rut*-dependent synaptic plasticity in either the fan-shaped body or ellipsoid body impaired visual pattern memory (Fig. 3A), but flies with a compromised fan-shaped body and a restored ellipsoid body, or a compromised ellipsoid body and a restored fan-shaped body, could form stable, wild-type memories (Figs. 1C and 2A). It seems that an operating range for the underlying neural circuit exists. Complete loss of *rut*-dependent synaptic plasticity in either the fan-shaped body or ellipsoid body moves the circuit out of the operating range, while restoring either of the compromised fan-shaped body or ellipsoid body can bring the circuit back into the operating range.

Functional differentiation of the fan-shaped body and ellipsoid body

It has been found in many insects including *Drosophila* that the central complex is involved in visual signal processing and motor control (Bausenwein et al. 1994; Martin et al. 1999; Strauss 2002; Vitzthum et al. 2002; Heinze and Homberg 2007; Ritzmann et al. 2008). However, the exact roles of the central complex substructures are not well understood. What we knew until now is that the F1 neurons are necessary for visual pattern memory for “contour orientation” and F5 neurons for “elevation,” which raises the possibility that visual signals are processed in the fan-shaped body and distinct F neurons are responsible for different visual pattern parameters. Recently, the R2/R4m neurons in the ellipsoid body were proved to be involved in ethanol sensitivity and tolerance (Urizar et al. 2007), and later in olfactory long-term memory consolidation (Wu et al. 2007). In our study, the R2/R4m neurons were found to be required for visual pattern memory for all tested parameters and thus may be parameter independent. However, the exact role of the R2/R4m neurons for visual pattern memory could not be determined yet.

Concluding remarks

Our studies indicated that Rut function in the central complex was crucial for *Drosophila* visual pattern memory; however, there

might be some other Rut-independent neurons that also contribute to the neural circuit. Future work should focus on loss-of-function studies by blocking neural signaling in targeted subsystems. Furthermore, a temporal dissection of memory acquisition and retrieval would help us to understand how the different neuropils are involved. As the F and R neurons are all large field neurons that connect to other brain regions or other parts of the central complex (Hanesch et al. 1989), it is also crucial to identify the upstream and downstream neurons. Although the picture is far from complete, it seems that the central complex might be the major center for visual pattern memory. Studying such adaptive

Figure 5. (Legend on next page)

behaviors from a single gene to multiple types of neurons within a circuit is a challenging but indispensable step to unravel the neural basis of complex behaviors.

Materials and Methods

Fly stocks

All flies were maintained at 25°C (except for flies carrying *tub-GAL80^{ts}*) on standard corn meal/molasses medium (Guo et al. 1996) in a 12-h light/12-h dark cycle at 60% humidity. The *rut²⁰⁸⁰;UAS-rut⁺* flies were previously described by Zars et al. (2000a,b). GAL4 lines c819, c232, c547, and c42 were kindly provided by U. Heberlein (University of California, San Francisco). The *UAS-rut^{RNAi}* flies were from VDRC (Vienna *Drosophila* RNAi Center), and the *rut¹* flies were from the Bloomington stock center. All the GAL4 lines were outcrossed with *w¹¹¹⁸* flies for six generations before use.

Visual pattern memory assays

Visual learning experiments were carried out in the *Drosophila* flight simulator (Fig. 1A). For apparatus, preparation, and handling of the flies, see the detailed description in Heisenberg and Wolf (1984). For the learning paradigm, procedure, and definition of performance index (PI), see Wolf and Heisenberg (1991) and Figure 1B. The reinforcer is an adjustable infrared laser (wavelength, 10.6 μ m; power, 300 mW) that is directed from above at the abdomen of the fly and controlled by the computer. The diameter of the laser spot is 0.4 mm, and the laser beam is pulsed with 50-msec duration and 50-msec interval.

Flies carrying *tub-GAL80^{ts}* were cultured at 19°C. The experimental groups were shifted for 12 h to 30°C prior to behavioral tests, while the control groups without heat shock treatment were raised at 19°C through development, then kept at 25°C for adaptation just before behavioral tests. All behavioral experiments were performed at 25°C.

For statistics, the two independent samples *t*-test was used to compare experimental groups with control ones, and the one-sample *t*-test was carried out for the wild-type and mutant flies.

Conditioned stimulus

Upright and inverted T-shaped patterns were used for parameter “elevation” if not specially mentioned. T patterns measure 36° vertically and 39° horizontally; the bars of the Ts are 12° wide. Four other patterns were used in this study (Fig. 5): (1) Parameter “elevation”: horizontal bars (40° × 10°) placed at 10° above and below the horizon; (2) parameter “contour orientation”: bars of two different orientations (−45° and 45°); (3) parameter “size”: 20° × 20° and 34° × 34° squares; (4) parameter “vertical compactness”: two horizontal bars (40° × 10°) with vertical distance of 20° and 30°. All bars and squares were in black.

Figure 5. The F and R neurons function differentially for visual pattern memory. (A) Wild-type flies could form memories for four visual pattern parameters: elevation, contour orientation, size, and vertical compactness, but the *rut²⁰⁸⁰* mutant flies with a copy of *UAS-rut⁺* (*rut²⁰⁸⁰/y;UAS-rut⁺/+*) did not. (B) Rescue in the fan-shaped body. Overexpression of *rut⁺* in the F5 neurons (c205) specifically rescued memory for “elevation” but not the other three pattern parameters, while overexpression of *rut⁺* targeted to the F1 neurons (NP6510) could only restore memory for “contour orientation” but none of the others. (C) Rescue in the ellipsoid body. Overexpression of *rut⁺* in the R2/R4m neurons (c819) could restore memory for all parameters to wild-type levels, and the rescue effect could be repeated by another GAL4 driver line c42, which also labels the R2/R4m neurons. Numbers below the bars indicate numbers of flies. (****) $P < 0.001$ (compared with zero, one sample *t*-test); (a) $P < 0.05$ compared with flies without GAL4 driver (*rut²⁰⁸⁰/y;UAS-rut⁺/+*) and $P > 0.05$ compared with the wild-type flies. (b) $P > 0.05$ compared with the *rut²⁰⁸⁰/y;UAS-rut⁺/+* flies. Comparisons were made between two groups using the same pattern parameter (two independent samples *t*-test). Error bars indicate SEM.

Flies’ pattern discrimination abilities were measured as previously described (Liu et al. 2006). In brief, the orientation distribution of an individual fly during pretest was analyzed by Fourier analysis. The discrimination value (*D*), which was used to evaluate a fly’s pattern discrimination ability, was calculated as the rate of the amplitude of the 180° component to the mean amplitude of the (near by) 120° and 72° components ($D = 2A_{180}/(A_{120} + A_{72})$ subscripts refer to Fourier components; *A* is amplitude). The chance value for *D* is 1. We evaluated the wild-type and *rut²⁰⁸⁰* flies’ ability to discriminate visual patterns and found that they both could discriminate all four pattern parameters used above (Table 1).

Quantitative PCR

Quantitative PCR (qPCR) was used in a standard way. In brief, 0.5 μ L of cDNA mixtures prepared from the heads of 3- to 4-d-old flies was used as template and tested on a Chromo 4 system (MJ Research/Bio-Rad). For each fly strain, cDNA was obtained from four independent RNA preparations for repeating and averaging. The relative differences in *rut* mRNA expression levels were quantified by comparing their levels with standard curves, which were constructed using the corresponding recombinant plasmids, and normalized to the level of *actin*. One-way analysis of variance (ANOVA) was used for the statistical analysis of relative mRNA levels. The primers used for qPCR were as follows:

qPCR-*actin* upper, 5′-CAGGCGGTGCTTCTCTCTA-3′;
qPCR-*actin* lower, 5′-AGCTGTAACCGCGCTCAGTA-3′;
qPCR-*rut* upper, 5′-TGTGATAAACGACGAGCTGA-3′;
qPCR-*rut* lower, 5′-AGCAACGAGATGTTCTCCAG-3′.

Immunohistochemistry

Dissection of adult brains was performed in cold PBS (phosphate buffer saline) to remove the cuticle and connective tissues. After a brief wash in PBS, samples were fixed in freshly prepared 4% paraformaldehyde in PBS for 1 h at room temperature and rinsed 3 × 15 min each in 0.5% PBT (PBS with 0.5% Triton X-100). Subsequently, samples were incubated with primary antibodies in 10% PNT (10% normal goat serum in PBT) overnight at 4°C, washed 3 × 15 min each with PBT, and then incubated in PNT containing secondary antibody. After three 15-min washes in PBT, brains were mounted in Vectashield (Vector Laboratories) and viewed with a Leica TCS SP5 confocal microscope. Raw confocal image sequences were imported into ImageJ software to reconstruct 3D images. Modifications were made using Adobe Photoshop (Adobe Systems) to obtain optimal visual effects. Care was taken to ensure that brightness and contrast alterations were performed on the entire panel without losing any detail. Monoclonal mouse anti-nc82 (kindly provided by E. Buchner, University

Table 1. Visual pattern discrimination of the wild-type and *rut²⁰⁸⁰* flies

Visual stimuli	Discrimination value (<i>D</i>)	
	wtcs	<i>rut²⁰⁸⁰/y; UAS-rut⁺/+</i>
Four identical T patterns	1.05 ± 0.19 (<i>n</i> = 20)	N.D.
Elevation (T and inverted T)	2.43 ± 0.46 (<i>n</i> = 44)	2.60 ± 0.42 (<i>n</i> = 45)
Elevation (horizontal bars)	3.58 ± 0.86 (<i>n</i> = 24)	2.44 ± 0.41 (<i>n</i> = 22)
Contour orientation	3.18 ± 0.57 (<i>n</i> = 29)	2.32 ± 0.42 (<i>n</i> = 28)
Size	2.39 ± 0.41 (<i>n</i> = 28)	3.59 ± 0.68 (<i>n</i> = 28)
Vertical compactness	2.55 ± 0.62 (<i>n</i> = 20)	3.12 ± 0.77 (<i>n</i> = 19)

For all the four tested visual pattern parameters, discrimination values (*D*) of wild-type and *rut²⁰⁸⁰* flies were not significantly different ($P > 0.05$ for each pattern parameter, two independent samples *t*-test), although both significantly higher than the chance value ($D = 1$) ($P < 0.05$ for all comparisons, one sample *t*-test). As a control, in the experiment of wild-type flies with four identical T patterns, the discrimination values are not different from the chance value.

of Wuerzburg, Germany) primary antibody was used at a dilution of 1:20. TRITC-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) was used at a 1:200 dilution.

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References

- Akalal, D.B., Wilson, C.F., Zong, L., Tanaka, N.K., Ito, K., and Davis, R.L. 2006. Roles for *Drosophila* mushroom body neurons in olfactory learning and memory. *Learn. Mem.* **13**: 659–668.
- Bausenwein, B., Muller, N.R., and Heisenberg, M. 1994. Behavior-dependent activity labeling in the central complex of *Drosophila* during controlled visual stimulation. *J. Comp. Neurol.* **340**: 255–268.
- Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**: 401–415.
- Brembs, B. and Heisenberg, M. 2000. The operant and the classical in conditioned orientation of *Drosophila melanogaster* at the flight simulator. *Learn. Mem.* **7**: 104–115.
- Cheung, U.S., Shayan, A.J., Boulianne, G.L., and Atwood, H.L. 1999. *Drosophila* larval neuromuscular junction's responses to reduction of cAMP in the nervous system. *J. Neurobiol.* **40**: 1–13.
- Connolly, J.B., Roberts, I.J., Armstrong, J.D., Kaiser, K., Forte, M., Tully, T., and O'Kane, C.J. 1996. Associative learning disrupted by impaired Gs signaling in *Drosophila* mushroom bodies. *Science* **274**: 2104–2107.
- Dubnau, J., Grady, L., Kitamoto, T., and Tully, T. 2001. Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. *Nature* **411**: 476–480.
- Dudai, Y. and Zvi, S. 1984. Adenylate cyclase in the *Drosophila* memory mutant *rutabaga* displays an altered Ca^{2+} sensitivity. *Neurosci. Lett.* **47**: 119–124.
- Ernst, R. and Heisenberg, M. 1999. The memory template in *Drosophila* pattern vision at the flight simulator. *Vision Res.* **39**: 3920–3933.
- Guo, A., Li, L., Xia, S.Z., Feng, C.H., Wolf, R., and Heisenberg, M. 1996. Conditioned visual flight orientation in *Drosophila*: Dependence on age, practice, and diet. *Learn. Mem.* **3**: 49–59.
- Han, P.L., Levin, L.R., Reed, R.R., and Davis, R.L. 1992. Preferential expression of the *Drosophila rutabaga* gene in mushroom bodies, neural centers for learning in insects. *Neuron* **9**: 619–627.
- Hanesch, U., Fischbach, K.F., and Heisenberg, M. 1989. Neuronal architecture of the central complex in *Drosophila melanogaster*. *Cell Tissue Res.* **257**: 343–366.
- Heinze, S. and Homberg, U. 2007. Maplike representation of celestial E-vector orientations in the brain of an insect. *Science* **315**: 995–997.
- Heisenberg, M. and Wolf, R. 1984. Vision in *Drosophila*: Genetics of microbehavior. In *Studies of brain function* (ed. V. Braitenberg), Vol. 12. Springer, Berlin, Germany.
- Heisenberg, M., Wolf, R., and Brembs, B. 2001. Flexibility in a single behavioral variable of *Drosophila*. *Learn. Mem.* **8**: 1–10.
- Levin, L.R., Han, P.L., Hwang, P.M., Feinstein, P.G., Davis, R.L., and Reed, R.R. 1992. The *Drosophila* learning and memory gene *rutabaga* encodes a Ca^{2+} /calmodulin-responsive adenylyl cyclase. *Cell* **68**: 479–489.
- Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., Heisenberg, M., and Liu, L. 2006. Distinct memory traces for two visual features in the *Drosophila* brain. *Nature* **439**: 551–556.
- Livingstone, M.S., Sziber, P.P., and Quinn, W.G. 1984. Loss of calcium/calmodulin responsiveness in adenylyl cyclase of *rutabaga*, a *Drosophila* learning mutant. *Cell* **37**: 205–215.
- Martin, J.R., Raabe, T., and Heisenberg, M. 1999. Central complex substructures are required for the maintenance of locomotor activity in *Drosophila melanogaster*. *J. Comp. Physiol.* **185**: 277–288.
- McGuire, S.E., Le, P.T., and Davis, R.L. 2001. The role of *Drosophila* mushroom body signaling in olfactory memory. *Science* **293**: 1330–1333.
- Neuser, K., Triphan, T., Mronz, M., Poeck, B., and Strauss, R. 2008. Analysis of a spatial orientation memory in *Drosophila*. *Nature* **453**: 1244–1247.
- Poeck, B., Triphan, T., Neuser, K., and Strauss, R. 2008. Locomotor control by the central complex in *Drosophila* an analysis of the *tay bridge* mutant. *Dev. Neurobiol.* **68**: 1046–1058.
- Popov, A.V., Peresleni, A.I., Ozerskii, P.V., Shchekanov, E.E., and Savvateeva-Popova, E.V. 2005. The role of the flabellar and ellipsoid bodies of the central complex of the brain of *Drosophila melanogaster* in the control of courtship behavior and communicative sound production in males. *Neurosci. Behav. Physiol.* **35**: 741–750.
- Renger, J.J., Ueda, A., Atwood, H.L., Govind, C.K., and Wu, C.F. 2000. Role of cAMP cascade in synaptic stability and plasticity: Ultrastructural and physiological analyses of individual synaptic boutons in *Drosophila* memory mutants. *J. Neurosci.* **20**: 3980–3992.
- Renn, S.C., Armstrong, J.D., Yang, M., Wang, Z., An, X., Kaiser, K., and Taghert, P.H. 1999. Genetic analysis of the *Drosophila* ellipsoid body neuropil: Organization and development of the central complex. *J. Neurobiol.* **41**: 189–207.
- Ritzmann, R.E., Ridgel, A.L., and Pollack, A.J. 2008. Multi-unit recording of antennal mechanosensitive units in the central complex of the cockroach, *Blaberus discoidalis*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **194**: 341–360.
- Sakai, T. and Kitamoto, T. 2006. Differential roles of two major brain structures, mushroom bodies and central complex, for *Drosophila* male courtship behavior. *J. Neurobiol.* **66**: 821–834.
- Strauss, R. 2002. The central complex and the genetic dissection of locomotor behaviour. *Curr. Opin. Neurobiol.* **12**: 633–638.
- Strauss, R. and Heisenberg, M. 1993. A higher control center of locomotor behavior in the *Drosophila* brain. *J. Neurosci.* **13**: 1852–1861.
- Tang, S. and Guo, A. 2001. Choice behavior of *Drosophila* facing contradictory visual cues. *Science* **294**: 1543–1547.
- Thum, A.S., Jenett, A., Ito, K., Heisenberg, M., and Tanimoto, H. 2007. Multiple memory traces for olfactory reward learning in *Drosophila*. *J. Neurosci.* **27**: 11132–11138.
- Urizar, N.L., Yang, Z., Edenberg, H.J., and Davis, R.L. 2007. *Drosophila* homer is required in a small set of neurons including the ellipsoid body for normal ethanol sensitivity and tolerance. *J. Neurosci.* **27**: 4541–4551.
- Vitzthum, H., Muller, M., and Homberg, U. 2002. Neurons of the central complex of the locust *Schistocerca gregaria* are sensitive to polarized light. *J. Neurosci.* **22**: 1114–1125.
- Wang, Z., Pan, Y., Li, W., Jiang, H., Chatzimanolis, L., Chang, J., Gong, Z., and Liu, L. 2008. Visual pattern memory requires foraging function in the central complex of *Drosophila*. *Learn. Mem.* **15**: 133–142.
- Wolf, R. and Heisenberg, M. 1991. Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *J. Comp. Physiol.* **169**: 699–705.
- Wu, C.L., Xia, S., Fu, T.F., Wang, H., Chen, Y.H., Leong, D., Chiang, A.S., and Tully, T. 2007. Specific requirement of NMDA receptors for long-term memory consolidation in *Drosophila* ellipsoid body. *Nat. Neurosci.* **10**: 1578–1586.
- Zars, T., Fischer, M., Schulz, R., and Heisenberg, M. 2000a. Localization of a short-term memory in *Drosophila*. *Science* **288**: 672–675.
- Zars, T., Wolf, R., Davis, R., and Heisenberg, M. 2000b. Tissue-specific expression of a type I adenylyl cyclase rescues the *rutabaga* mutant memory defect: In search of the engram. *Learn. Mem.* **7**: 18–31.
- Zhong, Y. and Wu, C.F. 1991. Altered synaptic plasticity in *Drosophila* memory mutants with a defective cyclic AMP cascade. *Science* **251**: 198–201.

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Yufeng Pan, Yanqiong Zhou, Chao Guo, et al.

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