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

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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* adenyl cyclase in either the fan-shaped body or the ellipsoid body is sufficient to rescue the memory defect of the *rut*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.
expression of rut cDNA in the UAS/GAL4 system (Brand and Perrimon 1993). Control flies carrying rut2080 and a copy of UAS-rut" but no GAL4 driver (rut2080/+;UAS-rut"+/>+) were compared with those bearing GAL4 drivers. Introduction of UAS-rut" did not increase the memory index of the rut2080 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, rut2080 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 rut2080 memory defect (t = 0.14, P = 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 rut2080 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 rut2080 memory defect when using T patterns as conditioned stimuli.

Adult-restricted expression of Rut was sufficient to rescue rut2080 memory defect

To exclude potential developmental defects, we used tub-GAL80ts 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 (rut2080/y; tub-GAL80ts+/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 (rut2080/y; tub-GAL80ts+/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 rut2080 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 = NS compared with the UAS-rut"RNAi/+ flies; t = 0.54, P = 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
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-rut1 showed a significant visual pattern memory defect, while heterozygous rut1 female flies with one copy of UAS-rut1 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 rut1 in neither the F5 neurons (c205: t = 0.79, P = NS) nor R2/R4m neurons (c819: t = 1.21, P = NS) nor R2/R4m neurons could restore the memory defect (Fig. 4A). However, overexpression of rut1 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...
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<sup>2080</sup> mutant flies (Fig. 5A).

Overexpression of rut<sup>+</sup> 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 = NS for “contour orientation”; t = 0.46, P = NS for “size”; t = 0.34, P = NS for “vertical compactness”), while overexpression of rut<sup>+</sup> 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 = NS for “elevation”; t = 0.52, P = NS for “size”; t = 0.55, P = NS for “vertical compactness”; Fig. 5B). However, overexpression of rut<sup>+</sup> 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<sup>2080</sup> 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<sup>2080</sup> 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
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 rut<sup>2080</sup> flies. Therefore, it is crucial to perform rescue experiments in a null mutant. rut<sup>1</sup> 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 rut<sup>+</sup> in either the F5 or R2/R4m neurons alone in the rut<sup>1</sup> 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 rut<sup>1</sup> 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 rut<sup>1</sup> 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 rut<sup>1</sup> or RNAi silencing flies, but only compromised in the rut<sup>2080</sup> 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
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) 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 tub-GAL80 flies were from VDRC (Vienna Drosophila RNAi Center), and the rut+ 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

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

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

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 significantly higher than the chance value (D = 1) (P < 0.05 for all comparisons, one sample t-test).
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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