ARTICLES

Distinct memory traces for two visual features in the *Drosophila* brain

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The fly *Drosophila melanogaster* can discriminate and remember visual landmarks. It analyses selected parts of its visual environment according to a small number of pattern parameters such as size, colour or contour orientation, and stores particular parameter values. Like humans, flies recognize patterns independently of the retinal position during acquisition of the pattern (translation invariance). Here we show that the central-most part of the fly brain, the fan-shaped body, contains parts of a network mediating visual pattern recognition. We have identified short-term memory traces of two pattern parameters—elevation in the panorama and contour orientation. These can be localized to two groups of neurons extending branches as parallel, horizontal strata in the fan-shaped body. The central location of this memory store is well suited to mediate translational invariance.

Drosophila tethered to a torque meter, with its head (and hence its eyes) fixed in space, can control its orientation with respect to the artificial scenery in a flight simulator[1]. In this set up, the fly is conditioned to avoid certain flight directions relative to virtual landmarks (Fig. 1) and recognizes these visual patterns for up to at least 48 h (ref. 2). Visual pattern recognition in *Drosophila* has been studied in some detail[3–7]. Flies store values of at least five pattern parameters: size, colour, elevation in the panorama, vertical compactness, and contour orientation. Moreover, they memorize spatial relations between parameter values. The neuronal substrate underlying visual pattern recognition is little understood in any organism.

In *Drosophila*, memory traces can be localized to groups of neurons in the brain[8]. Using the enhancer GAL4/UAS expression system[9,10], short-term memory traces of aversive and appetitive olfactory conditioning have been assigned to output synapses of subsets of intrinsic neurons of the mushroom bodies (MBs). The Rutabaga protein—a type 1 adenylyl cyclase that is regulated by Ca^{2+}/Calmodulin and G protein, and is considered a putative convergence site of the unconditioned and conditioned stimulus in olfactory associative learning[11–14]—selectively restores olfactory learning if expressed in these cells in an otherwise *rutabaga* (*rut*)-mutant animal[15,16]. Moreover, expressing a mutated constitutively activating $G\alpha_s$ protein ($G\alpha_s$*) in the MBs interferes with olfactory learning[17]. Blocking the output from these neurons during memory retrieval has the same effect, while blocking it during acquisition has no effect[16,18,19]. Interestingly, memory traces for other learning tasks seem to reside in other parts of the brain: for remembering its location in a dark space, the fly seems to rely on a *rut*-dependent memory trace in neurons of the median bundle and/or the ventral ganglion[20].

In the present study, we localize short-term memory traces for visual pattern recognition to the fan-shaped body (FB), the largest component of the central complex (CX; also called the central body in other species). The CX is a hallmark of the arthropod brain. It has been characterized functionally as a pre-motor centre with prominent, but not exclusive, visual input (for a review see refs 21, 22). In the locust, large-field neurons sensitive to the *e*-vector orientation of polarized light have been described in the CX[23]. Because of its repetitive structure and the precisely ordered overlay of fibre projections from the two hemispheres in the FB, neighbourhood relations of visual space might still be partially preserved at this level (retinotopy)[24]. Using the genetic approach[15,16,20], we now show that a small group of characteristic stratified neurons in the FB house a memory trace for the pattern parameter 'elevation', and a different set of neurons forming a parallel stratum contain a memory trace for 'contour orientation'.

Central complex defects impair visual pattern memory

Of ten mutants with structural abnormalities in the CX, all were impaired in visual pattern recognition[25,26]. They were able to fly straight and to avoid heat, yet they failed to remember the patterns (Fig. 2a). Did they really lack the memory or had they lost their ability to discriminate between patterns? Fortunately, individual flies often display spontaneous preferences for one of the patterns (see Fig. 2c and the Supplementary Information for the evaluation procedure (Table S1)). In three lines, these preferences were consistent enough to reveal intact pattern discrimination, suggesting that aberrant circuitry of the central complex can affect visual learning independent of visual pattern discrimination.

As the developmental and structural defects in these mutants are not well characterized, we used the GAL4/UAS system to acutely interfere with CX function. We chose a GAL4 driver line (c205–GAL4) with expression in parts of the CX (expression pattern shown in Fig. 3c) and, as the effector, the gene for tetanus toxin light chain (CntE)[27]. CntE blocks neurons by cleaving neuronal Synaptobrevin, a protein controlling transmitter release[28]. For temporal control, we added the temperature-sensitive GAL4-specific silencer GAL80 under the control of a *tubulin* promoter (*tub*–$GAL80^{ts}$)[10]. Flies (UAS–CntE/+; *tub*–$GAL80^{ts}$/c205–GAL4) were raised at 19 °C, and were transferred for 14 h to the restrictive temperature (30 °C) just before the behavioural experiment to induce GAL4-driven toxin expression. Flies kept at the low temperature showed normal

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memory scores, while after inactivation of GAL80^{ts} no pattern memory was observed (Fig. 2b). Again, flight control and heat avoidance were normal, and Fourier analysis confirmed that flies at the high temperature had retained their ability to tell the patterns apart (Supplementary Table S1). As with the structural mutants, interrupting the circuitry of the CX by tetanus toxin expression seemed to specifically interfere with visual pattern memory. The use of *tub*–GAL80^{ts}, in addition, excluded the possibility that toxin expression in unknown tissues during development might cause the memory impairment in the adult. These results do not, as yet, address the question of memory localization.

The *rutabaga* gene is required for pattern memory

Visual pattern memory in the flight simulator requires an intact *rut* gene (Fig. 2c, left)[29]. Mutant *rut* flies (*rut*^{2080}) showed normal visual flight control, heat avoidance and pattern discrimination (Fig. 2d). To confirm that the defect was indeed due to the mutation in the *rut* gene rather than an unidentified second-site mutation, we rescued it by the expression of the wild-type *rut* cDNA (UAS–*rut*$^{+}$) using the

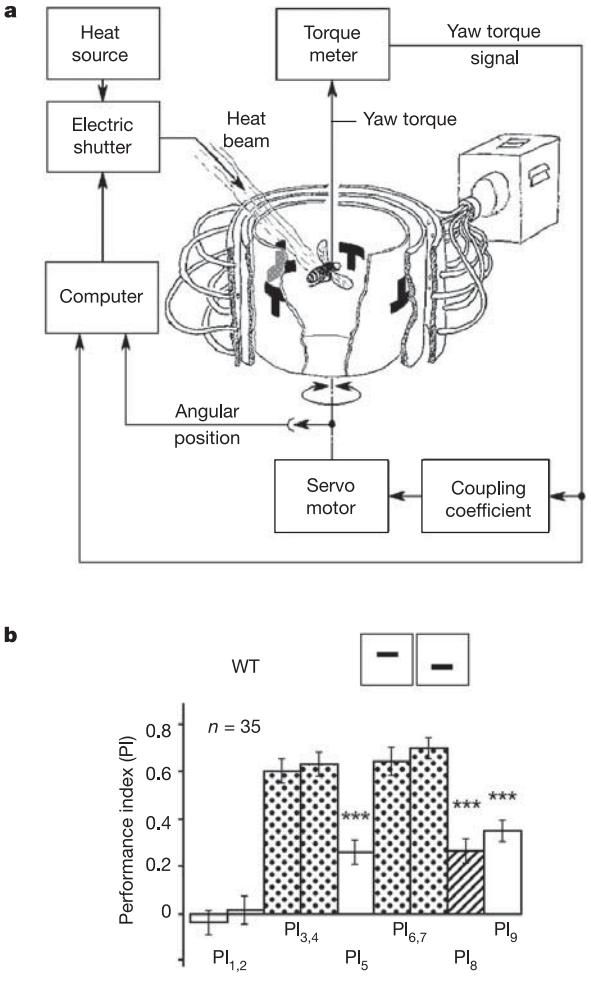

Figure 1 | Flight simulator for measuring visual pattern recognition. **a**, Experimental setup. The fly is attached to a torque meter and its yaw torque controls the angular velocity of the panorama surrounding it. It can fly straight and chooses its flight direction relative to the visual patterns in the panorama. With a beam of infrared light, the fly can be conditioned to avoid certain flight directions (see the Methods for more details). **b**, Course of experiment. Bars show performance indices (PI) of successive 2-min intervals of pretest (PI_1, PI_2), training (dotted bars; PI_3, PI_4, PI_6, PI_7) and memory test (PI_5, PI_8, PI_9) (see the Methods for experimental details and definition of PI). The following figures all show PI_8 (hatched bar) exclusively. Error bars are s.e.m. throughout. WT, wild-type *Berlin* strain.

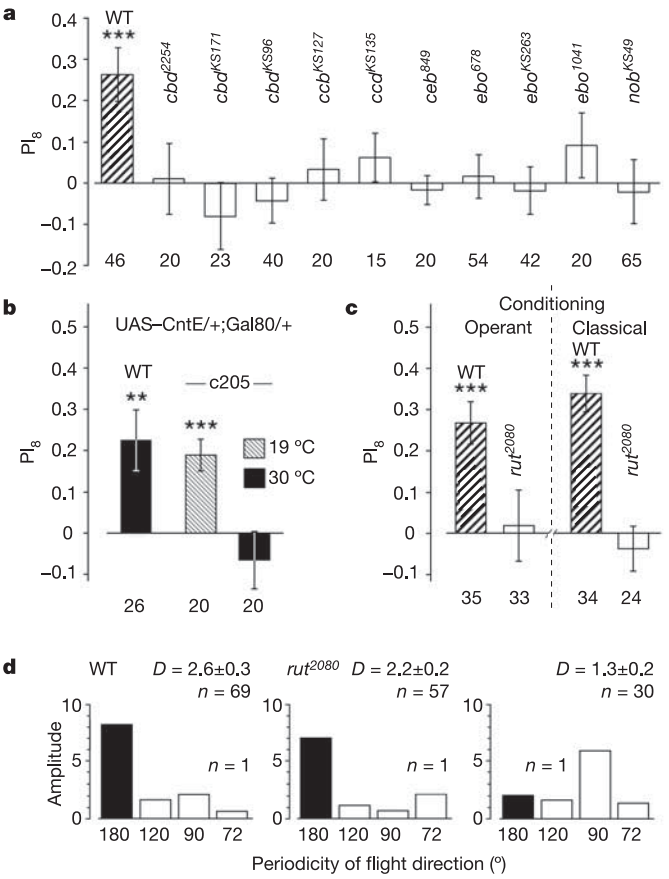

Figure 2 | Visual pattern memory is impaired in central complex mutants. Memory (PI_8) for the pattern parameter 'elevation' was tested using either horizontal bars at different elevations in the panorama or upright and inverted Ts. (The fly can conditionally distinguish the latter only by the different elevations of their centres of gravity[6].) **a**, Visual pattern memory in wild-type (WT) *Berlin* flies (hatched bar) and ten CX mutants in six independent genes. Mutants are in the genetic background of WT *Berlin*. Experimental details can be found in the Methods, Supplementary Information and Fig. 1. None of the mutant memory scores are significantly different from zero. **b**, Expression of tetanus toxin light chain (CntE) in the driver line c205–GAL4 labelling parts of the CX interferes with visual pattern memory. Owing to the *tub*–GAL80^{ts} element, a conditional silencer of GAL4, CntE is expressed only in the adult during a 14 h incubation at 30 °C (the restrictive temperature for *tub*–GAL80^{ts}) before the experiment. GAL4 lines are derived from wild-type Canton-S. As WT control, therefore, Canton-S was chosen. **c**, Visual pattern memory is impaired in the mutant *rut*^{2080}. Memory scores after operant (left) and classical (pavlovian) conditioning (right). Note that during classical conditioning, flies are not in command of panorama motion and are exposed to heat for precisely 50% of the time (PI = 0). Panorama motion alternates between smooth clockwise and anticlockwise rotation, each for 12 s at an angular velocity of 30° sec^{-1}. Memory test is the same as in the operant experiment. **d**, Pattern discrimination in the flight simulator. If flies show spontaneous individual pattern preferences, orientation histograms (not shown) have a 180° periodicity owing to identical patterns in opposing quadrants. Left and middle panels show principal Fourier coefficients during the 4-min pre-test period for single WT (left) and *rut* (middle) flies. The 180° Fourier components (black bars) have the largest amplitude. The same evaluation of a 4-min pre-test with four identical patterns (upright Ts) is also shown (right panel). The 180° component is at background level, and the high 90° component indicates equal choice for all four quadrants. Discrimination values (D) are calculated as $D = 2A_{180}/(A_{120} + A_{72})$ (subscripts refer to Fourier components; A = amplitude). Numbers for D above Fourier spectra are averages ± s.e.m. for n flies (for further details, see the Supplementary Information). Numbers below bars in **a–c** indicate numbers of flies. Error bars are s.e.m.

pan-neuronally expressing driver line *elav*–GAL4. Indeed, flies of the genotype rut^{2080}/Y;*elav*–GAL4/UAS–rut^{+} had normal memory (Supplementary Table S2A).

Visual pattern memory in the flight simulator has been shown to depend upon at least two kinds of behavioural plasticity. For one, an associative classical (pavlovian) memory trace is formed linking a particular set of values of pattern parameters to heat. Second, the fly's control of the panorama operantly facilitates the formation of this memory trace[30]. Either of the two processes might depend upon the Rut cyclase.

To address this issue, we tested *rut* mutant flies in a purely classical variant of the learning paradigm. During training, panorama motion was uncoupled from the fly's yaw torque and the panorama was slowly rotated around the fly. Heat was made contingent with the appearance of the 'punished' pattern in the frontal quadrant of the fly's visual field. All other parameters were kept as described (see the Methods). For testing memory, panorama motion was coupled again to yaw torque and the fly's pattern preference was recorded as usual. Even in the absence of operant facilitation, visual pattern memory required the intact *rut* gene (Fig. 2c, right). Therefore, the *rut*-dependent memory trace investigated here represents the association of a property of a visual pattern with the reinforcer.

Local rescue of visual pattern memory in the *rut* mutant

As a first step in localizing the memory trace, we asked in which neurons of the *rut* mutant expression of the wild-type *rut* gene would be sufficient to restore learning. To this end, a total of 27 driver lines expressing GAL4 in different neuropil regions of the brain were used to drive the UAS–rut^{+} effector gene in the *rut* mutant background. The parameter 'elevation' was measured. With seven of the driver lines, pattern memory was restored (104y, 121y, 154y, 210y, c5, c205 and c271; Fig. 3 and Supplementary Fig. S2; Supplementary Table S2B, C).

Comparison of the expression patterns of the 27 lines allowed us to narrow down the putative site of the memory trace to a small group of neurons in the brain. The seven rescuing lines all showed transgene expression in a stratum in the upper part of the FB. In three of them staining is rather selective. It comprises, in addition to the FB, only a layer in the medulla, several cell clusters in the suboesophageal ganglion and a few other scattered neurons.

Evidently, rut^{+} expression in the MBs is neither necessary (104y, c5, c205, 154y) nor sufficient for rescue (Supplementary Table S2B). This result is in line with the earlier observation that elimination of more than 90% of the MBs by hydroxyurea treatment of first-instar larvae[31] has no deleterious effect on visual pattern memory[32]. We ablated the MBs in one group of rescue flies (rut^{2080}/Y;UAS–rut^{+}/+;c271/+). They showed full visual pattern memory (data not shown).

Although GAL4 expression in the optic lobes is prominent in all seven rescuing lines, it occurs in distinctly different layers that do not overlap (Supplementary Fig. S2). For instance, in 104y expression is restricted to layer 2, whereas in 210y it is found only in the serpentine layer (layer 7)[33]. A similar situation is found for the suboesophageal ganglion, although there the staining patterns are more difficult to evaluate. Finally, expression in the ellipsoid body is again not necessary (104y, c5, c205, 154y) or sufficient (c232, 78y, 7y, and so on) for rescue. Thus, the expression patterns favour the conclusion that the neurons of the upper stratum of the FB (Supplementary Fig. S2) might be the site of the memory trace for the parameter 'elevation' in visual pattern memory.

Neurons in this stratum, labelled in all seven rescuing lines, have a very characteristic shape (Supplementary Fig. S3). Their cell bodies are located just lateral to the calyces. Their neurites run slightly upward in an antero–medial direction, forming an upward-directed tufted arborization just behind the α/α'-lobe of the MB. From there, the fibre turns sharply down and backward towards the midline just in front of the FB. Finally, it turns horizontally backward, spreading as a sharp stratum through all of the FB across the midline. These

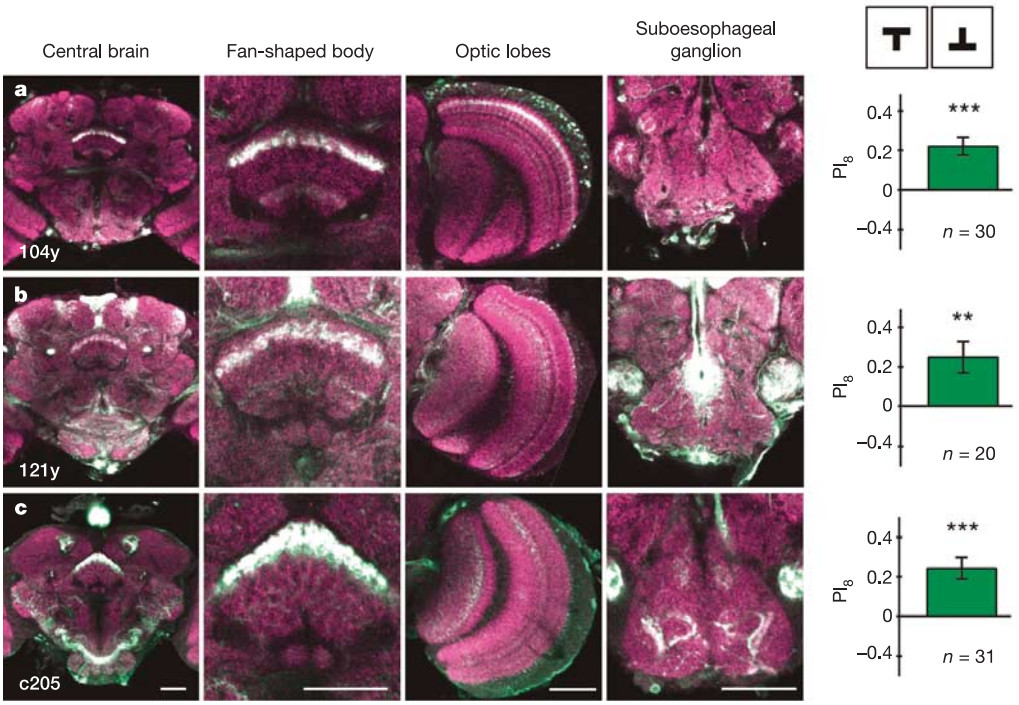

Figure 3 | Expression patterns of three driver lines rescuing pattern memory. GAL4-driven green fluorescent protein (GFP) expression in three of the seven 'rescuing' driver lines. Flies mutant for *rut* show normal visual pattern memory (PI_8, green bars on the right), (**a**), if 104y–GAL4, (**b**)121y–GAL4y or (**c**) c205–GAL4 drive rut^{+} cDNA. Frontal virtual sections of whole-mount preparations stained with anti-nc82 antibody (staining the synaptic neuropil, magenta) and anti-GFP antibody (GAL4 expression, green; overlay white). Scale bars 50 μm. All 'rescuing' driver lines (see Supplementary Fig. S2) show labelling in a dorsal horizontal layer (layer 5) of the FB. Error bars are s.e.m.

neurons have been described before in Golgi preparations[34]. They belong to a larger group of tangential FB neurons called F neurons[34]. Besides the stratum in FB, most of them have an arborization in a particular part of the unstructured neuropil. We tentatively classify the layer stained in 104y, and the other six rescuing lines, as layer 5 (from bottom upward), and hence provisionally call the neurons F5, although, without further markers, it is difficult to reliably number the layers. In summary, expression of Rut cyclase in F5 neurons rescued the *rut*-dependent memory defect for pattern elevation, whereas no rescue effect was observed in any of 20 strains without expression of Rut cyclase in F5 neurons (though Rut cyclase was expressed in other regions of the brain). Hence, a *rut*-dependent memory trace for pattern elevation may reside in F5 neurons.

This finding does not exclude the possibility that memory is redundant, and that other *rut*-dependent memory traces for pattern elevation might be found elsewhere. Therefore, we asked whether plasticity in the F5 neurons is necessary for visual pattern memory. As mentioned above, the Rut cyclase is regulated by G protein[11–14], and olfactory learning/memory can be blocked by a constitutively active form of the $G\alpha_s$ protein subunit ($G\alpha_s$*)[17]. We expressed the $G\alpha_s$* mutant protein in the FB using the driver line c205, and tested the flies for their memory of 'elevation'. Memory was fully suppressed (Fig. 4a). As in olfactory learning[17], overexpression of the wild-type protein did not interfere with learning (Fig. 4a). These results support the hypothesis that continuous upregulation of Rut cyclase in the F5-neurons interferes with visual short-term memory, implying that F5 neurons are the only site of a *rut*-dependent memory trace for pattern elevation.

Pattern specificity of the memory trace

The patterns used in the experiments so far exclusively addressed the parameter 'elevation' (upright and inverted Ts or horizontal bars at different elevations). We wondered whether the mutant defect in *rut* and the Rut rescue in the F5 neurons affected only this parameter, or whether it applied to other pattern parameters as well. We therefore extended the study to two further parameters: 'size' and 'contour orientation'. Three driver lines—c205, NP6510 and NP2320—were chosen showing different expression patterns in the FB (Fig. 5a–c). In the line NP6510, as in c205, a group of F neurons is marked. They are putatively classified as F1, as their horizontal stratum lies near the lower margin of the FB. Their cell bodies form a cluster in the dorsofrontal cellular cortex above the antennal lobes. Like the F5 neurons, they have large arborizations in the dorsal unstructured neuropil. The line NP2320 expresses the driver in columnar neurons running perpendicular to the strata of F neurons, with their cell bodies scattered singly or in small groups between the calyces. As they seem to have no arborizations outside the FB, they are tentatively classified as pontine neurons[34].

First, we showed that pattern memory required the *rut* gene for each of the three parameters (data not shown). Next, we studied the Rut rescue flies (for example, rut^{2080}/Y;c205/UAS–rut^{+}). In the line c205, memory was restored only for 'elevation', not for 'size' or 'contour orientation' (Fig. 5a). Correspondingly, the memory impairment by expression of dominant-negative $G\alpha_s$* in this driver line should be specific for 'elevation', as is indeed the case (Fig. 4b). With the driver line NP6510, memory was not restored for either 'elevation' or for 'size,' but memory was restored for 'contour orientation'. The third driver line, NP2320, labelling columnar neurons of the FB, did not restore the memory for any of the three

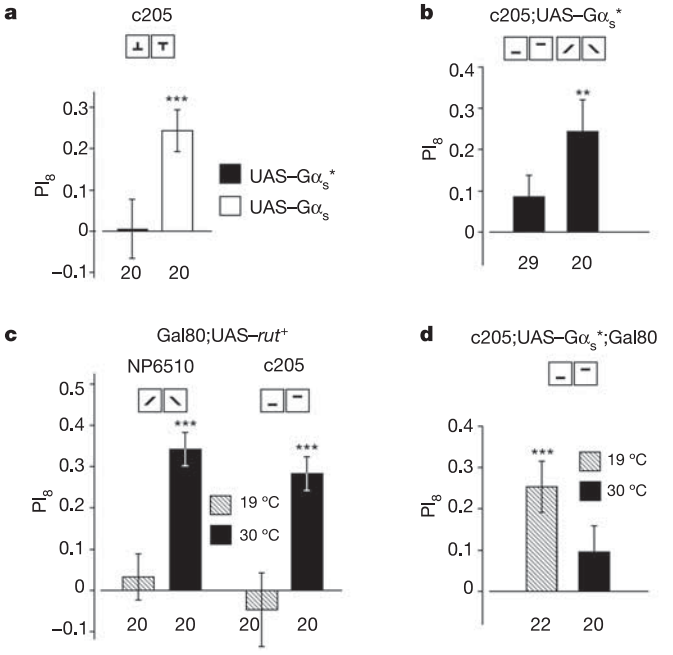

Figure 4 | Rescue and suppression of pattern memory occur in the adult. **a**, Expression of a constitutively active $G\alpha_s$* protein subunit in the FB (driver line c205–GAL4) abolishes pattern memory. Overexpression of wild-type $G\alpha_s$ protein has no effect. **b**, $G\alpha_s$* driven by c205–GAL4 disrupts memory for 'elevation' but leaves memory for 'contour orientation' intact. **c**, Temporal specificity of pattern memory. Expression of rut^{+} cDNA in a mutant *rut* background is blocked during development using *tub*–$GAL80^{ts}$ (light hatched bars). Only after inactivation of $GAL80^{ts}$ at 30 °C in the adult, Rut expression is induced and pattern learning/memory restored (black bars). **d**, $G\alpha_s$* protein interferes with pattern memory if expression is delayed until adulthood. Temperature control as in panel **c**. Numbers below the bars indicate numbers of flies. Error bars are s.e.m.

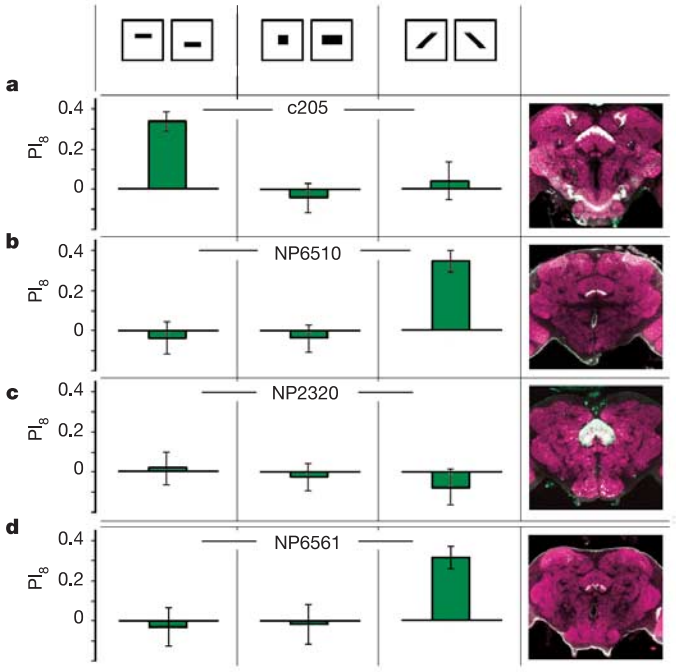

Figure 5 | Memory traces for pattern parameters are spatially separated. **a**–**c**, Three GAL4 lines—c205 (**a**), NP6510 (**b**), NP2320 (**c**)—driving rut^{+} cDNA in a mutant *rut* background, were tested for 3-min memory using patterns specifically testing for the single parameters 'elevation', 'size' or 'contour orientation'. Expression patterns in the FB, using GFP as effector, are shown on the right (staining as in Fig. 3). Note that in NP2320 (**c**), only columnar elements (presumably 'pontine neurons')[34] are stained. **d**, Driver line NP6561 has a similar expression pattern as NP6510. In flies of the genotype rut^{2080}/Y;NP6561/UAS–rut^{+}, again only the memory for pattern parameter 'contour orientation' is rescued. Number of flies: $n = 20$ in all experiments. Error bars are s.e.m.

pattern parameters (Fig. 5c). Among the 27 GAL4 lines above (Supplementary Table S1) we found a second with a very similar expression pattern as NP6510 (NP6561). The P-element insertions in the two lines are only 124 nucleotides apart from each other. Like NP6510, NP6561 restores the memory for 'contour orientation' but not for 'size' or 'elevation' (Fig. 5d). These results strongly suggest that memory traces for distinct visual pattern parameters are located in different parts of the FB, and that, in addition to the memory trace in F5 neurons, a memory trace for the parameter 'contour orientation' is located in F1 neurons.

Developmental versus adult restoration

A pertinent question in rescue experiments is whether the rescue is due to the provision of an acute function in the adult or to the avoidance of a developmental defect. Therefore, the *tub*–GAL80^{ts} transposon was added to the system. We chose the driver lines c205 and NP6510. Groups of adult males (for example, *rut*^{2080}/Y;+/*tub*–GAL80^{ts};NP6510/UAS–*rut*$^{+}$), raised at 19 °C, were kept as adults for 14 h at 19 °C or 30 °C. Afterwards, pattern memory for the corresponding pattern parameter was tested. In both cases, flies that had been kept at 30 °C showed normal memory, indicating that Rut cyclase induced just a few hours before the experiment had restored an immediate neuronal function rather than preventing a developmental defect (Fig. 4c). This conclusion was further supported by the finding that Gα_s* expression in the adult (using *tub*–GAL80^{ts}) was sufficient to disrupt memory (Fig. 4d).

Conclusions

Several conclusions can be drawn from the above results. Memory traces in *Drosophila* are associated with specific neuronal structures: odour memories with the MBs (reviewed in ref. 8), visual memories with the CX, and place memory (tentatively) with the median bundle[20]. Memory traces are not stored in a common all-purpose memory centre. Even within the visual domain, memories for distinct pattern parameters are localized within distinct structures: a *rut*-dependent short-term memory trace for the pattern parameter 'elevation' to F5 neurons, and a corresponding memory trace for 'contour orientation' to F1 neurons. Moreover, if the constitutively activating Gα_s* protein indeed interferes with the regulation of Rut cyclase, it follows that the brain contains no other redundant *rut*-dependent memory traces for these pattern parameters. The Rut-mediated plasticity is necessary and sufficient, at least in F5 neurons. As in the earlier examples, the memory traces are confined to relatively small numbers of neurons. At least in flies, and probably in insects in general, memory traces appear to be part of the circuitry serving the respective behaviour.

Our study provides a first glimpse of the circuitry within a neural system for visual pattern recognition. Though the picture is far from complete, it invites (and may guide) speculation. The FB is a fibre matrix of layers, sectors and shells[34]. The F1- and F5-neurons form two sharp parallel horizontal strata in this matrix. If the width of the FB represents the azimuth of visual space as recently proposed[24], the horizontal strata of the F neurons would be well suited to mediate translation invariance (see ref. 7 and the Supplementary Information). In any case, it is satisfying to find a translation invariant memory trace in the CX where visual information from both brain hemispheres converges[35,22]. These first components of the circuitry may encourage modelling efforts for pattern recognition in small visual systems[7] (see the Supplementary Information for a discussion of the pattern recognition mechanism).

METHODS

The apparatus for studying visual learning is shown in Fig. 1a (see also refs 36, 37). The tethered fly, suspended at a torque meter, is flying stationarily in the centre of an arena that is illuminated from behind and carries two upright and two inverted T-shaped patterns in alternating sequence on its wall. The arena is rotated such that its angular velocity is proportional to, but directed against, the fly's yaw torque; that is, the fly's yaw torque determines the angular velocity of the arena instead of the fly's own body. This arrangement allows the tethered fly to stabilize and choose its flight orientation with respect to the arena by adjusting its yaw torque. A computer continuously registers yaw torque and angular position of the arena. For visual pattern learning, a beam of infrared light is directed at the fly as an instantaneous source of heat. The arena is virtually divided into four quadrants with the patterns at their respective centres (Fig. 1a; Supplementary Fig. S1). During training, the computer switches the heat beam on whenever the fly is heading towards a quadrant with, for example, an upright T, and switches it off when the fly is oriented towards one of the other two quadrants. Hence, half of all possible orientations in the arena are paired with heat, the other with ambient temperature. During tests, heat is permanently switched off. (In some experiments, a circular LED display is used instead of the mechanically rotating arena.)

Angular position is recorded every 50 ms and orientation preferences are calculated for nine consecutive 2-min periods (performance index (PI) 1–9). Pattern A is paired with ambient temperature during training and pattern B with heat. The two patterns alternate as A and B from fly to fly. If t_A is the time the fly spends heading towards the quadrants of pattern A, and t_B the time heading towards pattern B quadrants, the performance index is calculated as $PI = (t_A - t_B)/(t_A + t_B)$. Supplementary Fig. S1 gives a further explanation. Technical details regarding the flight simulator and data processing routines can be found in refs 36, 37. Error bars in the figures are s.e.m.; asterisks indicate levels of significance against zero (***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s., $P > 0.05$ (one-sample *t*-test; two-sided *P*-value)).

Details about the visual figures, fly maintenance, mutant lines, histological procedures and image processing are available in the Supplementary Information.

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