Visual Place Learning in *Drosophila melanogaster*

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Abstract

The ability of insects to learn and navigate to specific locations in the environment has fascinated naturalists for decades. While the impressive navigation abilities of ants, bees, wasps, and other insects clearly demonstrate that insects are capable of visual place learning^{1–4}, little is known about the underlying neural circuits that mediate these behaviors. *Drosophila melanogaster* is a powerful model organism for dissecting the neural circuitry underlying complex behaviors, from sensory perception to learning and memory. Flies can identify and remember visual features such as size, color, and contour orientation^{5, 6}. However, the extent to which they use vision to recall specific locations remains unclear. Here we describe a visual place-learning platform and demonstrate that *Drosophila* are capable of forming and retaining visual place memories to guide selective navigation. By targeted genetic silencing of small subsets of cells in the *Drosophila* brain we show that neurons in the ellipsoid body, but not in the mushroom bodies, are necessary for visual place learning. Together, these studies reveal distinct neuroanatomical substrates for spatial versus non-spatial learning, and substantiate *Drosophila* as a powerful model for the study of spatial memories.

Vision provides the richest source of information about the external world, and most seeing organisms devote enormous neural resources to visual processing. In addition to visual reflexes, many animals use visual features to recall specific routes and locations, such as the placement of a nest or food source. When leaving the nest, bees perform structured “orientation flights” to learn visual landmarks. If subsequently displaced from their outbound flight, bees take direct paths back to their nest using these learned visual cues⁷.

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Author Contributions

All authors designed the study and wrote the manuscript. TAO carried out the experiments and data analysis.

Competing Financial Interests

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How do insects, with relatively compact nervous systems, perform these navigational feats? In mammals, the identification of place, grid, and head direction cells suggests the existence of a “cognitive map”⁸. Unfortunately, little is known about the cellular basis of invertebrate visual place learning. In order to identify the neurons and dissect the circuits that underlie navigation, we studied place learning in a genetically tractable model organism, *Drosophila melanogaster*.

To explicitly test for visual place learning in *Drosophila*, we developed a thermal-visual arena inspired by the Morris Water Maze⁹ and the Heat Maze of Mizunami et al.^{1, 2} (Fig. 1a). In the *Drosophila* place learning assay, flies must find a hidden “safe” target (i.e. a cool tile) in an otherwise unappealing warm environment (36° C) (Fig. 1b). Notably, there are no local cues that identify the cool tile. Rather, the only available spatial cues are provided by the surrounding electronic panorama that displays a pattern of evenly spaced bars in three orientations (Fig. 1a and c). To assay spatial navigation and visual place memory, fifteen adult flies are introduced in the arena and confined to the array surface by placing a glass disk on top of a 3mm high aluminum ring. During the first 5 minute trial, nearly all (94% of flies) eventually succeed in locating the cool target (data not shown). In subsequent trials, the cool tile and corresponding visual panorama are rapidly shifted to a new location (90° clockwise or 90° counterclockwise chosen at random). Importantly, the target and visual panorama are *coupled* so that although the absolute position of the cool tile changes, its location relative to the visual panorama remains constant (Fig. 1c). Our results (Fig. 2a, red trace and Supplementary movie 1) demonstrate that over the course of 10 training trials flies improve dramatically in the time they require to locate the cool tile. This improvement is accomplished by taking a shorter (Fig. 2b), more direct route to the target (Fig. 2c), without noteworthy changes in the mean walking velocity (Fig. 2d). To ensure that social interactions between flies were not influencing place learning (e.g. flies following each other to the safe spot), we also trained single flies and found that flies tested individually show equivalent place learning (Supplementary Fig. 1). As would be predicted for *bona-fide* visual place learning, the improvement in place memory is critically dependent on the visual panorama. Flies tested in the dark show no improvement in the time, path length, or directness of their routes to the target (Fig. 2, black trace).

To verify that flies are using the spatially distinct features of the visual panorama to direct navigation, we also tested flies using an *uncoupled* condition whereby the cool tile was still randomly relocated for each trial, but now the display remained stationary throughout. With this training regime the visual panorama provides no consistent location cues, but idiothetic and weaker spatial cues such as distance and local orientation of the arena wall are still available to the flies. Our results (Fig. 2, grey trace) demonstrate that flies trained with the uncoupled visual panorama exhibit little improvement in the time to find the cool tile, and no improvement in the directness of their approaches. Thus, spatially relevant visual cues are required for flies to learn the location of the target.

As a further test of visual place memory, flies were challenged immediately after training with a probe trial (trial #11) where the visual landscape is relocated as usual, but no cool tile is provided (i.e. can the flies be fooled into going to the non-existent safe spot?). We hypothesized that if the flies learned to locate the cool tile by using the peripheral visual

landmarks, then they should bias their searches to the area of the arena where the visual landscape indicates the cool tile should have been, even when the target is absent. Indeed, flies preferentially search in the arena quadrant where they have been trained to locate the now “imaginary” cool tile (Fig. 3, Supplementary Fig. 2 and 3a, Supplementary movie 2). Whereas, if flies were trained in the dark or with an uncoupled visual landscape, conditions that contain no specific information about the location of the cool tile, the flies instead search the arena uniformly during the probe trial (Fig. 3c, Supplementary Fig. 2 and 3a, Supplementary movie 3). Together, these results demonstrate that fruit flies can learn spatial locations based on distal visual cues and use this memory to guide navigation. Interestingly, by varying the time between the end of a single round of training (10 trials) and testing during a probe trial, we could also show that flies retain these visual place memories for at least 2 hours (Fig. 3d).

Where are spatial memories processed (or stored) in the *Drosophila* brain? We reasoned that specific regions of the fly brain would function as the neuroanatomic substrate for visual place learning and therefore set out to engineer and test animals where different brain areas were selectively inactivated using the GAL4/UAS expression system. In essence, we conditionally silenced small subsets of neurons in adult flies by targeting expression of the inward rectifying potassium channel Kir2.1¹⁰; to limit potential side-effects of Kir2.1 expression during development, we used a temperature-sensitive GAL80^{ts} which blocks Kir2.1 expression when flies are reared at 18°C but allows expression when flies are shifted to 30°C prior to testing¹¹. GAL4 driver lines were selected for expression in two areas: the mushroom bodies (Fig 4a–c) and the central complex (Fig 4d–f). The mushroom bodies have been the subject of extensive studies of learning and memory in *Drosophila*¹², and have been shown to be essential for associative olfactory conditioning¹³, but not for some other forms of learning like tactile, motor, and non-visually guided place learning^{14–16}. The central complex is hypothesized to be a site of orientation behavior, multisensory integration and other “high order” processes^{17, 18}. In some social insects the mushroom bodies have been implicated in visual place learning^{19, 20}, and in the cockroach bilateral surgical lesions to these structures abolish spatial learning¹. However, we see no evidence for involvement of the mushroom bodies (mb) in our assay. In fact, silencing mb intrinsic neurons using the GAL4 drivers R9A11, R10B08, R67B04 (Fig. 4a–c, g and Supplementary Fig. 3b), OK107, or even chemically ablating the mb (hydroxyurea¹³; Supplementary Fig. 4) had no significant effect on the performance of flies in visual place learning. The differing requirement for the mb between *Drosophila* and other species may be explained by the observations that mb inputs in *Drosophila* are predominantly olfactory^{1, 21}. In sharp contrast, silencing subsets of neurons with projections to the central complex ellipsoid body (eb, lines R15B07 and R28D01) dramatically impaired visual place learning (Fig. 4d–h and Supplementary Fig. 3b). Notably, silencing a different subset of ring neurons with line R38H02 leaves visual place learning intact (Fig. 4f–h). Thus, specific circuits within the eb (but not the entire structure) are necessary for visual place learning.

To confirm that silencing the eb neurons in lines R15B07 and R28D01 produces a specific impairment in visual place memory, we tested these flies in a series of behavioral paradigms and showed they display normal locomotor, optomotor, thermosensory and visual pattern

discrimination behaviors (Fig. 4i–l, Supplementary Fig. 5). In addition, we reasoned that if these flies have a general defect in memory (or in processing thermally-driven learned behaviors), then they should exhibit impairment in multiple types of learning (or in using thermal signals to drive learning and memory). Thus, we developed a novel olfactory conditioning paradigm using temperature (rather than electric shock²²) as the unconditioned stimulus (Fig. 4m, Supplementary Fig. 6). As expected, silencing the mushroom bodies leads to a total loss of odor learning (Fig. 4m). In contrast, silencing subsets of neurons in the ellipsoid body (eb) has no effect on olfactory learning, yet ablates visual place learning. Taken together, these results demonstrate that subsets of cells in the eb are specifically required for visual place learning and substantiate distinct neuroanatomical substrates for visually guided spatial (place) versus non-spatial (olfactory) learning in *Drosophila*.

Mammals likely use place, grid, and head direction cells to solve and perform navigational tasks⁸. The tight correlation between place cell activity and an animal's position in space has established the hippocampus as the substrate for a cognitive map. This map is likely informed by head directions cells (indicating an animal's orientation) and grid cells which tile the surrounding environment and could support path integration. While it is not known if there are direct correlates to these cells in flies, invertebrates are capable of solving similarly challenging navigational feats and do so using significantly smaller brains. Indeed, flies are able to use idiothetic cues, and path integration, to aid navigation^{15, 17, 23, 24}. Now, our studies demonstrate that *Drosophila* can learn and recall spatial locations in a complex visual arena, and do so with remarkable efficacy.

Here, we also show that subsets of neurons in the fly brain (ring neurons of the ellipsoid body) are critical for visual place learning, likely by implementing, storing or reading spatial information. Strikingly, flies in which we silenced eb neurons exhibit a basic “circling” search routine (Supplementary Fig. 2d) that is reminiscent of the behavior displayed by rats with hippocampal lesions²⁵. Imaging of neuronal activity in the fly brain while the animal is executing a navigation task should help further define the role of the central complex, and eb neurons in particular, in spatial memory (for example in a head-fixed preparation with a virtual reality arena^{26, 27}). Ultimately, elucidating the cellular basis for place learning in the fruit fly will help uncover fundamental principles in the organization and implementation of spatial memories in general.

Note added in proof: While our paper was under review another study reported the use of a heat maze to study spatial search strategies in *Drosophila*³⁰.

Methods Summary

To control the thermal landscape, we developed an array of sixty-four 1 inch-square individually addressable thermoelectric modules arranged in an 8×8 grid (Fig. 1b). This array forms the floor of our test arena and is covered with black masking tape. To confine flies to this surface, a 3mm high, 8 inch diameter heated aluminum ring was placed around the outer perimeter of the arena and covered with a glass disk coated with a slippery surface. Visual cues were provided by an LED display positioned around the outer perimeter of the arena²⁸ (Fig. 1a). The experimental protocol included 10 training trials (5 minutes each)

followed by a probe trial (trial 11) where the visual display was relocated in the absence of a cool spot. The navigational behavior of the flies during a session was tracked off-line using Ctrax²⁹. At the end of the experiment, flies were tested in the same arena for thermal preference and optomotor behavior to confirm normal thermal and visual responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *Drosophila* trained in a thermal-visual arena exhibit place learning

(a) Illustration of the arena. The floor is composed of 64 thermoelectric modules, the panorama is provided by a 24×192 LED display, and flies are recorded using a CMOS camera under infrared (IR) illumination. (b) Thermal imaging view of the arena's floor showing the uniformly warm surface with a single cool tile; also shown is the heated ring barrier. Bottom panel: temperature readings across the arena (black line). (c) Trajectories of 4 representative flies from trials #1,2 and 10 are shown below a diagrammatic representation of the visual panorama denoting the location of the cool tile in the previous trial (dashed square), and the current location of the cool tile (blue square). In this *coupled* condition the position of the cool tile relative to the visual panorama remains constant even as its absolute position changes between trials.

Figure 2. Flies use visual cues to improve in place learning task

Flies were trained with a *coupled* visual panorama (red, $n=33$ experiments, 495 flies), with an *uncoupled* (gray, $n=21$ experiments, 315 flies) or *dark* (black $n=23$ experiments, 345 flies) visual surround. (a) When trained in the coupled condition (red) flies reduce the time to find the cool tile by nearly half, whereas flies trained with an uncoupled panorama (gray) or in the dark (black) show little or no improvement in time to locate the target. The improvement exhibited with the coupled visual panorama is due to flies taking (b) shorter, (c) more direct paths to the target (d) rather than simply increasing walking velocity. See Methods for details of calculations. Values are mean $\pm$ SEM.

Figure 3. Following training flies exhibit a persistent search bias in the absence of the cool tile and retain this memory for several hours

Flies are tested in a probe trial (#11) where the visual display is relocated but no cool tile is present. **(a)** Trajectories from four representative flies, each plotted for 60 s after leaving their starting quadrant. Flies start in the top-left quadrant (Q1, Start); the dashed square denotes the “expected” location of the cool tile (Q2, Target). **(b)** Flies preferentially search in the quadrant where they have been trained to find the cool spot (Q2), even when the cool spot is absent; values are mean $\pm$ SEM, $n=33$ experiments, 495 flies. **(c)** Probe learning index is significantly greater than zero (indicating learning) when flies are trained with a coupled visual panorama (red, $p<0.0001$, $n=33$), but not when trained with an uncoupled (grey, $p=0.28$, $n=21$) or dark (black, $p=0.39$, $n=23$) visual panorama. **(d)** To test place memory retention, flies were tested in a probe trial at several time intervals following training ($n=5$). Flies retain visual place memories for at least 2 hours after training. Box plots indicate the median value (solid black line), 25 and 75 percentiles (box), and the data range (dashed whiskers). For details of calculations and additional statistics, see Methods.

Figure 4. Subsets of ellipsoid-body ring neurons are required for place learning

(a–c) GAL4 driver lines targeting subsets of cells in the mushroom body or (d–f) the ellipsoid body; shown are the expression patterns for each driver using a GFP reporter. (g) White boxes denote spatial learning prior to Kir2.1 induction; grey boxes indicate performance following Kir2.1 expression. Silencing ellipsoid body neurons projecting to R1 and R4 (R15B07) or R1 (R28D01) severely impairs place learning ($p < 0.001$, red lines) while silencing mushroom body neurons (a–c), or a separate subset of eb neurons projecting to R4 alone (R38H02), leaves place learning intact. Box plots are as described in Fig 3, n 8

experiments. **(h)** Schematic representation of ellipsoid body ring neuron anatomy. **(i–m)** Flies with impaired place learning (expressing Kir2.1) show normal **(i)** walking velocity, **(j)** heat aversion, **(k)** optomotor response, **(l)** visual pattern discrimination during tethered flight, and **(m)** olfactory learning. For i–k (n = 8 experiments), values are mean $\pm$ SEM. For l (n = 6 flies) & m (n=8 experiments), values are mean $\pm$ SD. See Methods for details of calculations and additional statistical analysis.