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Identical Shapes Results in a Failed Memory Formation of Location of Dark Surroundings in Planarians

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Planarians, the first kind of animal to have evolved a brain structure[1] yet has not evolved visual sense, was demonstrated to have a capability of spatial learning in the last several decades[2], but what does the navigation of planarians depends on is still unknown. Here, we provide an objective, strictly variable-controlled planarian training method using 3D printing techniques[3] fabricated mazes. Then we use modifications of the mazes to first demonstrate a learning paradigm that worms can memorize the location of a darkened surrounding through training. However, a memory formation failure was found that in the situation of providing identical shapes in a maze, planarians cannot memorize the location of the darkened surrounding. Thus, this result shows the planarians associated darkness with the crude shape of the objects they have crawled on, which is a kind of spatial learning. This finding provides not only a key insight into spatial learning information that planarians are processing but also an interpretation of the origin of memory formation where higher grades of memory formation might originate from.

Planarians are free-living flatworms to have first evolved a centralized brain[1] with synaptic structure[4], their primitive eyes allow them to feel and avoid the light, which is an innate behavior[5]. However, their eyes can only sense the existence of light instead of imaging[6]. Planarians are able to handle simple associative learning like associating light with electric shock[7]. For freely living in the natural environment, spatial learning abilities are also needed to handle tasks like foraging, homing and predator avoidance more efficiently[8], suggesting that they might have already evolved spatial learning.

Spatial learning is known to be a process that animals use landmarks (stimuli that are relatively close to the goal but are not themselves the goal) as cues to find the objective beacon(stimuli that are directly navigated to)[9]. The spatial learning memory in human is a map full of visual cues. When using mazes to train animals like honey bees[10] and rats[11] to learn spatial tasks, visual cues are provided as landmarks to them, and they performed excellent spatial learning for remembering these landmarks to navigate to their beacons. When vision is deprived, for instance, spatial learning of rats in water maze are somehow impaired [12]. Thus, visual cues are mostly needed for spatial learning of higher animals, but when it comes to the first animal that has evolved the central nervous system, their eyes were not yet able to image. If they have spatial learning memory, the map in their mind must be different from higher animals.

The intriguing area about the worms' spatial learning was first investigated by behavioral scientists in the 1950s-1960s, by training worms to go through a T-maze[13] or a triangle maze[2] lack of water to return to watery places, which made the worms show a preference to the direction of the watery places, even the worms regenerated from a trained worm's tail showed a preference[14]. However, these experiments were criticized for poor variable control and reproducibility[15], nor could they demonstrate the real information that worms are truly processing. Recent research with large samples revealed that the worms can form familiarity with the environment to start feeding but still unable to illustrate what the familiarity is [16]. Thus, the question, what might these planarians have learned in spatial learning tasks is still waiting to be resolved.

The fabrication of mazes by 3D printing can also be an advantage for further investigation of this question. In order to make the experiment easy to reproduce, this training and testing procedure is easy to handle and strictly variable controlled. Our experiment here first establishes a maze learning paradigm that worms can memorize the location of a darkened surrounding through training, then uses this paradigm to illustrate a learning failure that the worms cannot memorize two identical shapes with one strongly lighted and one darkened in two different directions. This fact not only shows the worms are associating shapes of objects with light intensity, but also demonstrated a property of memory formation when memory was firstly starting to emerge.

We totally designed 8 E-mazes to train and test the worms, as shown in Fig.1a. A dark chamber (white translucent areas in Fig.1a) can be attached to or detached from the mazes. Worms are put in the start point in the thick arm once a day to form a training effect on the association of darkness with the location of the surrounding for consecutive 6 days. On day 7, worms are tested to make choices on either of the arms. When worms choose the correct arm (the dark chamber arm), it is recorded as a correct response; when choosing the incorrect arm, it is recorded as an incorrect response. Light is used to propel the worms to move, and its direction is opposite the route to the dark chamber so that light will not provide worms with any guidance to the dark chamber for the worms are photophobic. Worms are tested for consecutive

8-10 times based on their fatigue state. The rate of correct response for each worm is counted. The relation between light intensity and location is shown in Fig.1b.

We firstly find that worms in grooved F-maze showed a significantly higher correct response than control worms (Fig.2a). During each training and testing procedure, the worms sometimes put their head into the grooves, and this somehow influenced their heading direction. Therefore, we speculate that the grooves might provide guidance for the worms. Then we checked whether worms could learn in the flat F-maze. Surprisingly, worms in flat F-maze showed learning as well (Fig.2a). We calculated the difference between the grooved and flat groups, found there is no significant difference in the direction preference between the worms trained in grooved F-maze with arrow “<” shape pattern grooves and the worms trained in flat F-maze without the pattern grooves (Fig.2c). The result shows that grooves cannot provide guidance for the worms.

Although the designed grooves cannot provide guidance for the worms, the worm can still learn to find the place in the testing procedure that used to be the dark chamber during the training period. We suppose that it is the shapes of the of the objects the worms crawled on that provide guidance for the worms. We checked whether grooves would provide guidance for worms in the E-maze, the result shows it cannot (Fig.2b). We calculated the difference between the grooved and flat groups, found there is no significant difference of the direction preference between the worms trained in grooved EC-maze with arrow “<” shape pattern grooves and the worms trained in flat and EC-maze (Fig.2c).

For there is no significant difference between the grooved and flat mazes, we merge the groups of grooved and flat mazes together and will not mention this henceforth. As shown in Fig.2d, the worms in the F-maze and ECP-maze showed great significance in learning compared with control groups, which means they did memorize the dark chamber's location. The preference to the wrong direction of the control group is caused by the gradient of the light direction, which the trained worms can overcome this preference.

As shown in Fig.2e, trained worms in EC-maze and EP-maze did not show significance in learning compared with control groups. Compared with the differently-shaped-ending maze (DM), worms trained in the identically-shaped-ending maze (IM) did not learn significantly, as shown in Fig.2f, which means worms trained in the IM did not memorize the dark chamber's location, but worms trained in the DM did. This can further support that worms may get confused and unable to form a memory of the preferred darkened location, and demonstrate that worms did not leave chemical trails to help them find the preferred darkened location.

The toughest challenge in the former experiments decades ago is the variable control. There are too many variables that can influence a worm's behavior, including light intensity[17], water temperature[18], water existence[13], time of day[18], time of year[19], chemical components of water[19], chemical components of food[20], worm's appetite level[16], slime trails [21], worm fatigue state[22], magnetic fields[23], training conditions and manipulation of the experimenter[19]. All of the irrelevant variables above were obstacles to former studies and made the experiments unable to be reproduced, which caused the whole line of research to have become abandoned[24]. Therefore, the crucial thing of the worm's behavioral research is to control the variables strictly.

Based on former studies, neither the lack of water nor the food lures are seemingly easy to be controlled, for there is no criterion for the degree of deprivation of water and components of food for the planarians. Our experiment using light was designed to decrease the number of the variables needed to be controlled, which only uses light to train the worms to learn the shapes of the mazes. Other variables, including water components, temperature, time of day for training, appetite level, geomagnetic fields, are all easy to control. Although we did not remove slime trails, the result clearly shows that worms did not use chemical cues to navigate to a formerly recognized dark place, for the worms cannot memorize darkened surroundings facing identical shapes (Fig.2e). The light is easy to control either in training and preserving the worms using fixed LED light tubes. The woolen brush very softly handling the worms in the mazes, avoiding rapid water current caused by transfer pipe that might hurt the worms.

The criterion we designed for the worms' correct response is touching the red line shown in Fig.1b. Some worms might be persistently crawling in a circle between the two red lines and refuse to make a choice in a long time, which might cause the worms to get in a fatigued state and cannot insist on taking a total of ten tests, so we counted the worms that continuously took 8-10 tests. Criterions judging the correct response of a worm can be further developed. We did not control the initial orientation of the worms when testing for the following reasons: 1. Correcting the direction accurately requires much manual effort, which might disturb and hurt the worms 2. Worms confront multiple direction choices no matter which orientation it is headed, and it may also turn back on the route to the dark chamber.

The fabrication of mazes using 3D printing techniques is a great advantage in this research. Designing such a number of mazes of different shapes requests a much higher cost than 3D printing techniques[3]. The toxicity released by the stereolithography (STL) printing materials (photosensitive resin in this experiment)[25] is fatal to worms, which causes the worms to die and disintegrate in less than 20 hours, even in the mazes handled by ultraviolet to have reduced toxicity[25]. Although the fused deposition modeling (FDM) materials like PLA is non-toxic, it is of lower printing precision, and its water leaking problem may cause worms to escape from the maze while training. We found that a biocompatible encapsulation material called Parylene[26] to totally block the toxicity from the STL printed parts, using which can make worms safely live in the maze without health problems for over 2 weeks. With the assistance of the 3D printing techniques, further investigation of the worm’s memory of shapes in spatial learning will be much more convenient.

Although this is a spatial learning task, it can still be categorized into classical conditioning[27]: the dark is the unconditioned stimulus (US), the shape of the apparatuses are the conditioned stimulus (CS), worms are anticipated to go to the designed place darkened in the training session (unconditioned response, UR) and lightened in the testing session (conditioned response, CR). Therefore, this experiment can also be analyzed as a classical conditioning paradigm.

The mazes with or without identical shapes illustrate that the worms can learn crude shapes to navigate the environment, for they cannot distinguish between identical shapes. Here, we propose some possible mechanisms of the worms' spatial learning. As shown in the former studies, the worms can learn to have a left or right direction preference based on their body axis[13]. We speculate that there are three factors that play a crucial role in this memory code formation of beacon's location: the shapes of the beacon, the light intensity of the beacon, and the worm's left-right direction based on its body axis and landmarks. If here we use A and B to represent two different shapes of the beacon, I and D to represent the lightness and darkness, L and R to represent left and right directions, the worms are able to form memory like AIL&BDR, AIR&BDL, with all three factors different but unable to form memory like AIL&ADR, BIR&BDL, with one factor identical, it is also easy to demonstrate that the worms cannot learn in the situation of AIL&BIR, AIL&BDL. This process is illustrated in Fig.3. Hence, we might have an insight on the origin of memory that it might not be able to distinguish different objectives with one identical feature. Our experiment shows that the planarians recognize the beacons by their shapes, we deduce that they recognize landmarks by their shapes as well. If this is the case, the identification of landmarks does not seem to be influenced by the identical shapes provided since the F-maze is totally symmetrical except its beacon (in F-maze worms can be trained to learn the location of dark surroundings) However, we still lack information on how does the worms utilize landmarks.

For higher animals like rats, they can be trained to use different strategies to solve spatial learning tasks. For instance, they can use “response strategy” (using the turning response reinforced during training) rather than “place strategy” (using the location of the place) [28]. For honeybees, they are even able to category similar images with the same features to earn a reward[29]. Nonetheless, our finding showed that when tracing back to the origin of learning, animals cannot form this kind of associative memory. We might even believe that the ability to distinguish between two similar items with identical features in a certain memory was evolved later before memorizing items with totally different features. There is still a long way to go to unravel the mystery of the origin of memory, and research with planarians might shed light on this question.

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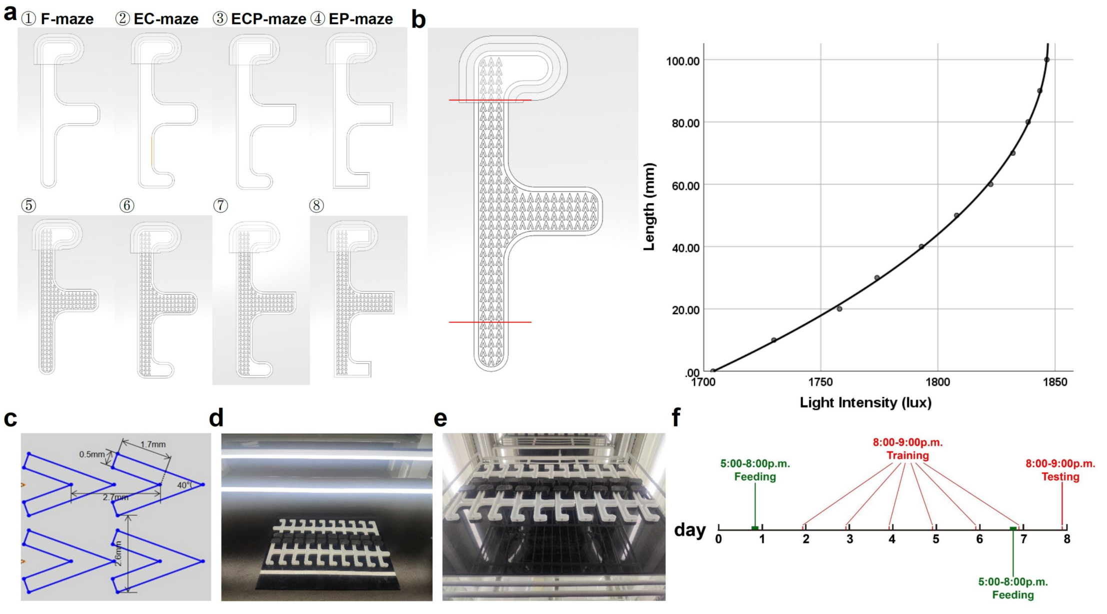

Figure 1 | Maze Parameters and Light Conditions.

a. Top view of the mazes. ①F-maze ②E-maze with curving end walls, EC-maze ③E-maze with perpendicular end walls, EP-maze ④E-maze one side with curving end walls and the other side with perpendicular end walls, ⑤-⑧ are modifications of ①-④ adding arrays of

‘>’ (arrow) pattern grooves to the ground and walls

b. The relation between light intensity and location in the maze. The y axis refers to the location of the maze corresponding to the maze in the picture shown on the left. Cubic curve estimation, R^2=0.9993. The variation of the light intensity in the total 10 mazes in transverse location is ±50Lux, the variation of the light intensity in one maze in transverse location can be ignored. Worms’ touching the red line shown in the maze is the criterion to record either correct (the upper red line) or incorrect response (the lower red line).

c. Top view of the arrow grooves. Some parameters are shown. The depth of the grooves is 0.5mm.

d. The training or testing (dark chamber taken off) procedure. Two LED light tubes are above the mazes, and mazes are put on a black acrylic board.

e. Daily preservation of the training worms. Worms are put in a light incubator in daily light and dark cycles.

f. Flow chart of the experiment process.

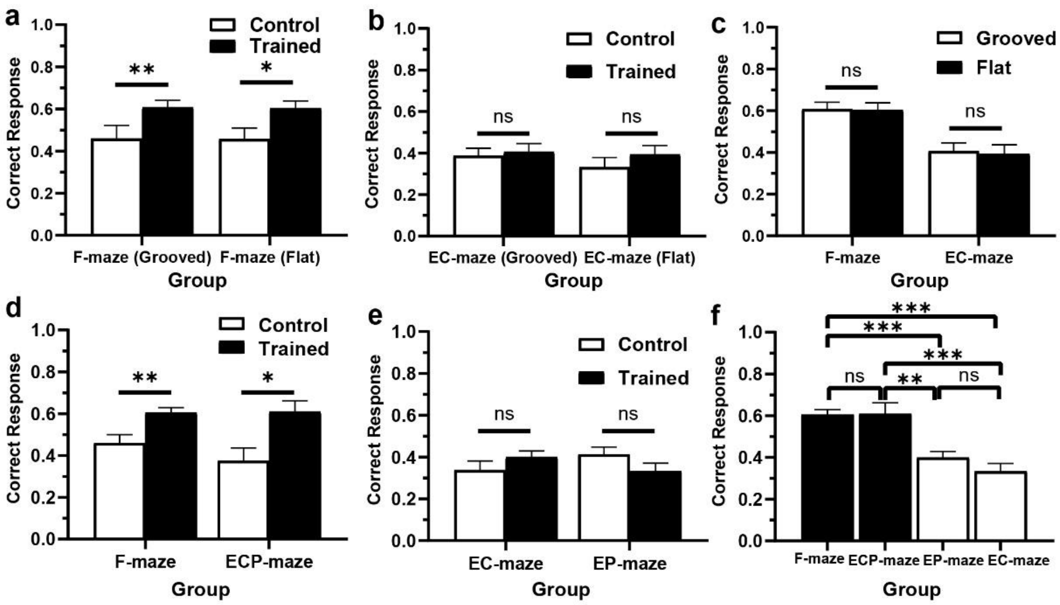

Figure 2 | Worms can learn in DM but not in IM.

a． Worms trained in grooved (n=19) and flat (n=17) F-maze showed higher correct response than control worms in grooved (n=13) and flat (n=16) F-maze.

b． Trained worms in grooved (n=17), flat (n=17) EC-maze did not show significance in learning compared with control worms in grooved (n=15), flat (n=14) EC-maze.

c． There is no statistical significance between trained worms in the grooved (n=19) and flat (n=17) F-maze or grooved (n=17) and flat (n=17) EC-maze of the correct response.

d． Trained worms in F-maze (n=36) and ECP-maze (n=10) showed great significance in learning compared with control groups in F-maze (n=28) and ECP-maze (n=10).

e． Trained worms in EC-maze (n=34) and EP-maze (n=10) did not show significance in learning compared with control groups in EC-maze (n=30) and EP-maze (n=10).

f. A comparison of the result between the 4 groups of trained worms whether or not with two identical shapes of beacons. F-maze (n=36), ECP-maze (n=10), EC-maze (n=34), EP-maze (n=10)

Values are means ± S.E.M. Two-tailed Mann-Whitney U test is used in this data analysis. ***P < 0.001; **P < 0.01; *P < 0.05. ns denotes P > 0.1.

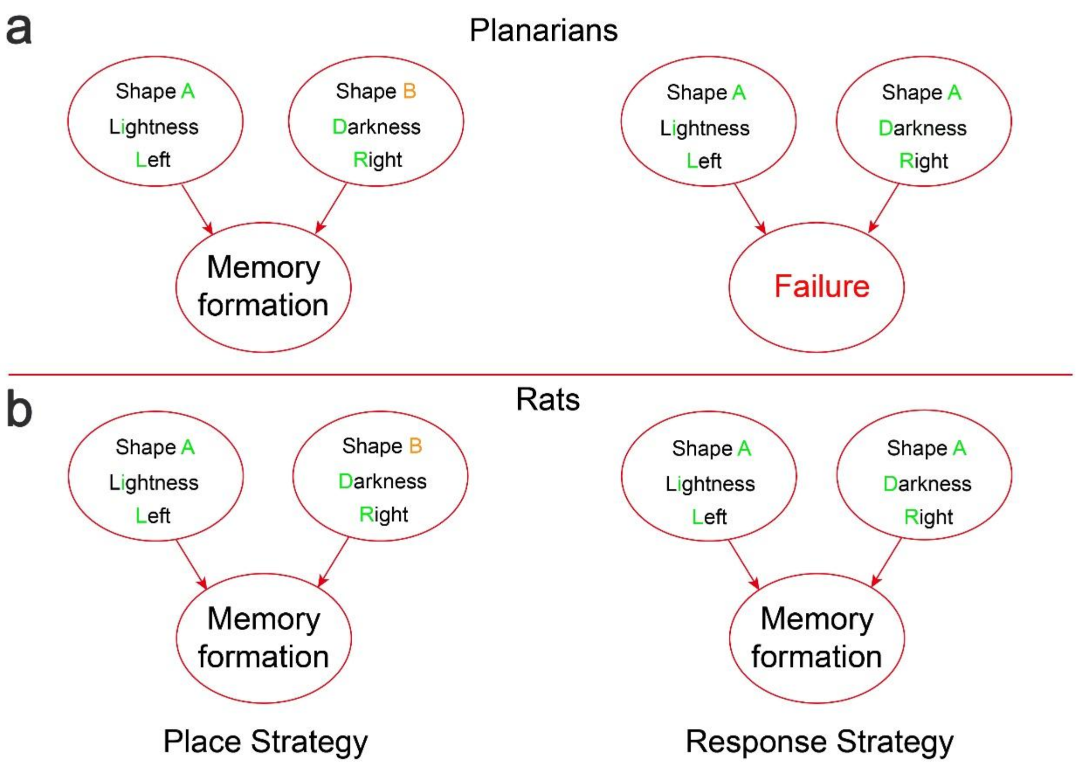

Figure 3 ｜ Model of the Planarians' memory formation.

a. When facing two different shapes, planarians can memorize the direction of the two places differently shaped. However, when facing identical shapes, planarians cannot memorize the direction of the two places identically shaped.

b. Rats can choose different strategies to either choose to memorize the property of the place or the direction of the place. Thus, it can form a correct memory.

Method

Worm maintenance

All planarians used in the study were *Dugesia japonica*. Planarian colonies were stored in rectangular plastic containers, filled with 1 × Montjuic water[30] (1.6 mM NaCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl, 1.2 mM $NaHCO_3$ in distilled water. Adjusted pH to ~7.5 with 1 M HCl) at constant water temperature of 20 ± 0.5 ℃ . The Environment for the worms is completely dark. Worms are fed with raw chicken liver 3 times a week and changed water the following day.

Experimental apparatuses

3D printed E-mazes (derived from the classical T-maze) are used for the whole training and testing process. The material used for 3D printing is photosensitive resin and PLA (Polylactic acid), the main body of the mazes is printed in white using photosensitive resin, and the dark chamber is printed in black using PLA (The dark chamber can be detached from the main body of T-maze). The main body of the mazes is coated with Parylene film (thickness of 12 μ m) to block the toxicity from the resin. There are a total of 8 kinds of apparatuses with different modifications of the E-maze (①F-maze ②E-maze with curving end walls, EC-maze ③E-maze with perpendicular end walls, EP-maze ④E-maze one side with curving end walls and the other side with perpendicular end walls, ECP-maze, ⑤-⑧ are modifications of ①-④ adding arrays of ‘>’(arrow) pattern grooves to the ground and walls), all the mazes are of similar parameters like total length, width, and height. The precision of the printing is 0.1mm. The configuration and some parameters of both mazes and arrow grooves are shown in Fig.1a, b, and c. Blueprints showing details and parameters are shown in the SI. The light while training is provided by two paralleled LED lighting tubes. The distance between the tubes is 155 mm, the height of the tubes is 301.9mm. A light incubator with constant temperature is used for daily training and reinforcement of the worms' spatial memory, shown in Fig.1d. While training and testing, the mazes are placed on a flat rectangular black acrylic board.

Daily preservation of the training worms

Experimental worms were each placed in their individual maze. Each maze and worm inside are numbered. Worms were fed to satiety the day (day0) before the first training day (day1). All of the worms in their own mazes undergo a 12-hour light (800-900 Lux) and 12-hour dark cycle for 6 days in the incubator to make worms preserve the association of darkness with the dark chamber under constant water temperature of 20 ± 0.5℃ (Fig.1 d). The first day is counted when each of the naive worms is put in their own maze for a training procedure (Shown in the next section). No food was provided in the apparatuses. The worms are taken out at 8:00 p.m. of each day for training. Worms are fed on day 6 from 5:00 p.m. to 8:00 p.m. in a 12-well-plate. Each numbered worm is in a single well. The procedure is shown in Fig.1d. Worms that are of bad health or undergo self-fission are excluded from the experiment.

Training procedure

Training procedure happens at 8:00 p.m. of each day except the day that performs the test, both maze and stored Montjuic water is taken out from the incubator (to control water temperature). Each worm in its maze is carefully taken out by a smooth brush to a single well of a 12-well plate and kept in the dark. Each apparatus is immersed in and washed with Montjuic water. The slime is not removed to protect the Parylene membrane. Strong light is provided by two parallel LED lighting tubes, which is used to propel the worms to find the dark chamber to rest, but the light direction is opposite the route to the dark chamber so that light will not provide worms with any guidance to the dark chamber for the worms are photophobic. The relation between light intensity and location is shown in Fig.1b. The verification procedure starts to count when each of the worms is put in their starting point (1cm to the base wall of the T-maze) quickly of each of their own apparatus. The geographic orientation of the mazes kept the same in the training procedure and testing procedure to exclude the influence of the geomagnetic field. The training procedure ends at 60 min, before which most of the worms stop moving, probably due to fatigue. This generates a training effect for the testing procedure. The control group is kept in the dark in single wells of 12-well plates in the same incubator, and their feeding synchronizes with the training worms. The procedure is shown in Fig.2c.

The test procedure for direction preference

On day 7, 8:00 p.m., each worm is taken out to the 12-well plate as mentioned in the training procedure. Each worm was tested in its training maze. The control worms were tested in corresponding mazes which tested trained worms. The light is provided identically to the training procedure. The starting point is the same. The dark chamber is taken off while testing. In each trial of a single worm, when the worm reached either left (correct) or right(incorrect) arm and touches the red line shown in Fig1.b (The red line goes through the center of the semicircle, for the worms' eyespot will receive stronger light stimulus once the worms start to turn which might cause worms to withdraw, if the maze is an F-maze, the red line is the same distance to its middle axis as shown in Fig1.b), a correct or incorrect result is recorded. Then the worms are taken out to a 12-well plate and kept in the dark to wait for mazes to be washed as described above and refilled with 1× Montjuic water. We totally perform the trial for one worm for consecutive 10 times or until the worm shows extreme fatigue and does not move. One experimenter simultaneously handles 10 worms in one trial. Worms are not repeatedly used in this experiment for both trained and control groups. The slime is not removed to protect the Parylene membrane. The procedure can be described in Fig.2c with the dark chamber taken off.

Method References

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Author contributions.

Kaiyuan Huang: idea, experiment design, pre-experiment, main experiment, worm maintenance, data analysis, article writing, correspondence

Yufei Liu: pre-experiment, main experiment, worm maintenance

Yixi Duan: pre-experiment, main experiment, worm maintenance

Kehan Chen: drawing blueprints of 3D-printed parts

Ziyun Xiao: pre-experiment, worm maintenance

Peiao Zhang: pre-experiment, worm maintenance

Bangqi Zhu: pre-experiment, worm maintenance

Yunhao Shi: worm maintenance

Zhengxin Ying: experiment guidance, article revising

Baoqing wang: experiment guidance, article revising

Competing interests.

The authors declare that they have no competing interests

Supplementary information line

We would like to provide 9 STL format 3D printing blueprints: 8 mazes and a dark chamber. These files contain the exact parameters of the mazes, which can not be inserted in PDF and do not fit your SI format. The documents' sizes are less than 30MB. We wish to be allowed to upload these files.

Materials & Correspondence.

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Additional Information

Supplementary Information is available for this paper.

Figures

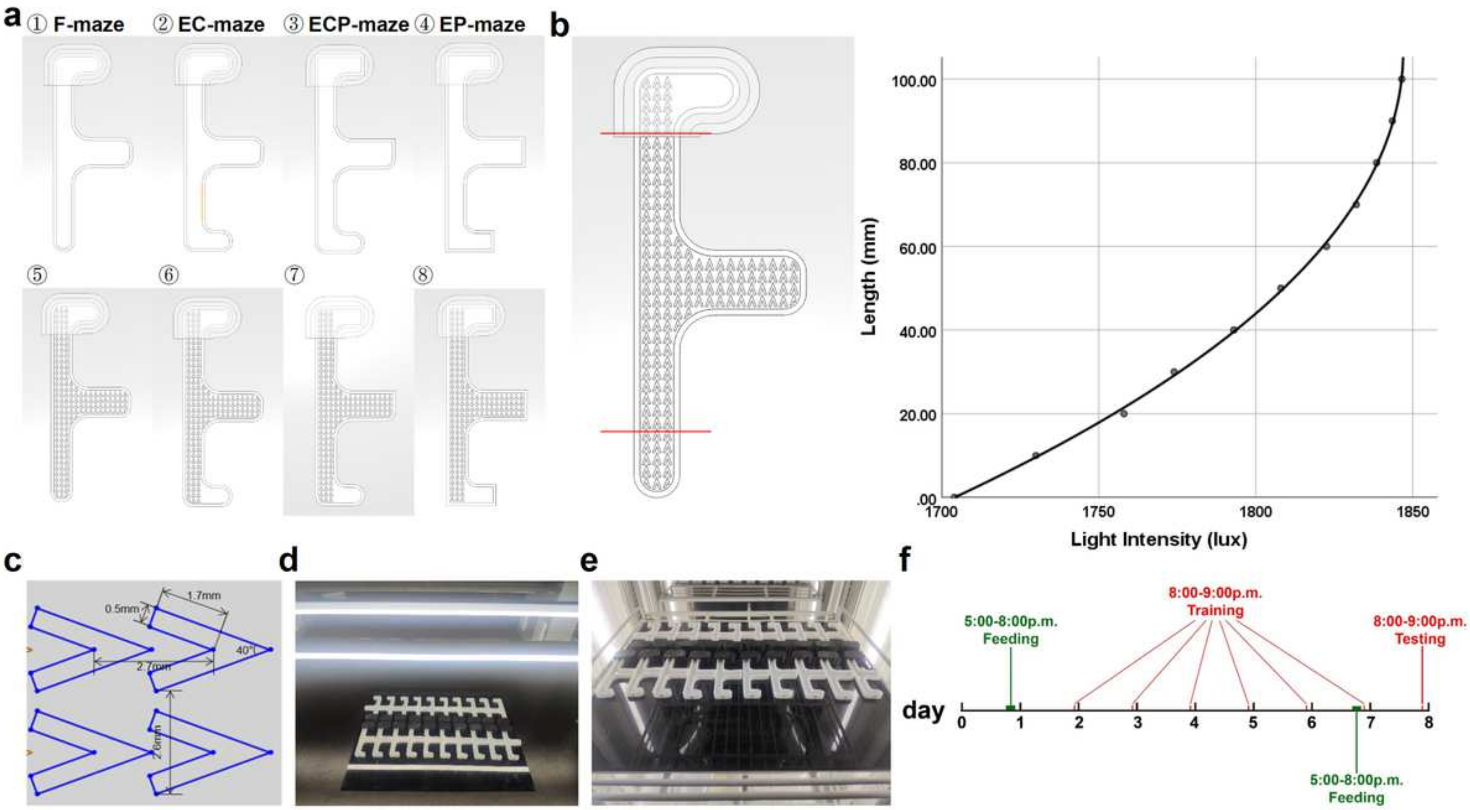

Figure 1

Maze Parameters and Light Conditions. a. Top view of the mazes. ①F-maze ②E-maze with curving end walls, EC-maze ③E-maze with perpendicular end walls, EP-maze ④E-maze one side with curving end walls and the other side with perpendicular end walls, ⑤-⑧ are modifications of ①-④ adding arrays of '>'(arrow) pattern grooves to the ground and walls b. The relation between light intensity and location in the maze. The y axis refers to the location of the maze corresponding to the maze in the picture shown on the left. Cubic curve estimation, R2=0.9993. The variation of the light intensity in the total 10 mazes in transverse location is ± 50Lux, the variation of the light intensity in one maze in transverse location can be ignored. Worms' touching the red line shown in the maze is the criterion to record either correct (the upper red line) or incorrect response (the lower red line). c. Top view of the arrow grooves. Some parameters are shown. The depth of the grooves is 0.5mm. d. The training or testing (dark chamber taken off) procedure. Two LED light tubes are above the mazes, and mazes are put on a black acrylic board. e. Daily preservation of the training worms. Worms are put in a light incubator in daily light and dark cycles. f. Flow chart of the experiment process.

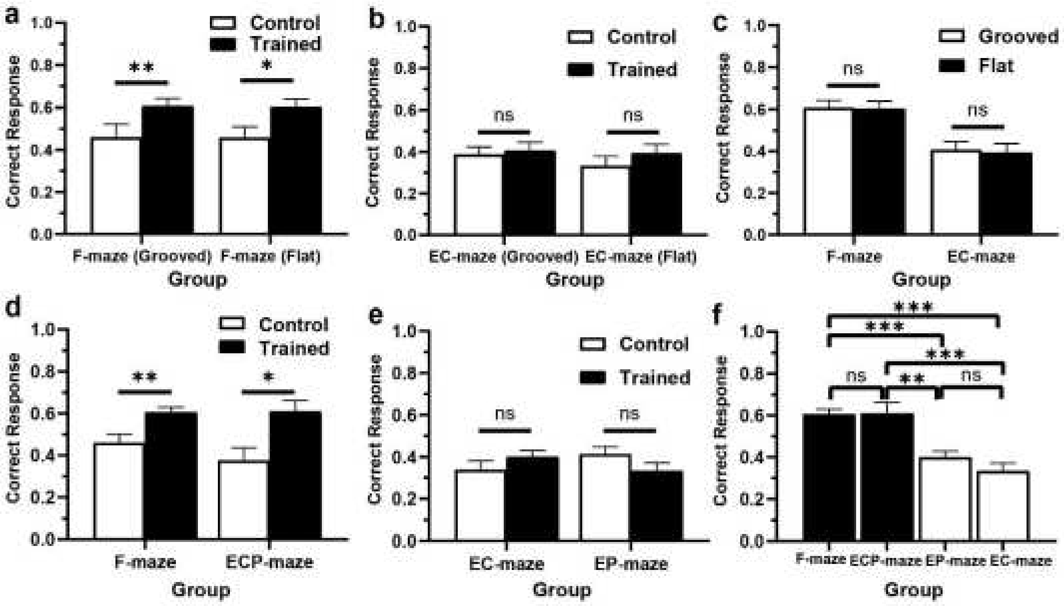

Figure 2

Worms can learn in DM but not in IM. a Worms trained in grooved (n=19) and flat (n=17) F-maze showed higher correct response than control worms in grooved (n=13) and flat (n=16) F-maze. b Trained worms in grooved (n=17), flat (n=17) EC-maze did not show significance in learning compared with control worms in grooved (n=15), flat (n=14) EC-maze. c There is no statistical significance between trained worms in the grooved (n=19) and flat (n=17) F-maze or grooved (n=17) and flat (n=17) EC-maze of the correct response. d Trained worms in F-maze (n=36) and ECP-maze (n=10) showed great significance in learning compared with control groups in F-maze (n=28) and ECP-maze (n=10). e Trained worms in EC-maze (n=34) and EP-maze (n=10) did not show significance in learning compared with control groups in EC-maze (n=30) and EP-maze (n=10). f A comparison of the result between the 4 groups of trained worms whether or not with two identical shapes of beacons. F-maze (n=36), ECP-maze (n=10), EC-maze (n=34), EP-maze (n=10) Values are means ± S.E.M. Two-tailed Mann-Whitney U test is used in this data analysis. ***P < 0.001; **P < 0.01; *P < 0.05. ns denotes P > 0.1.

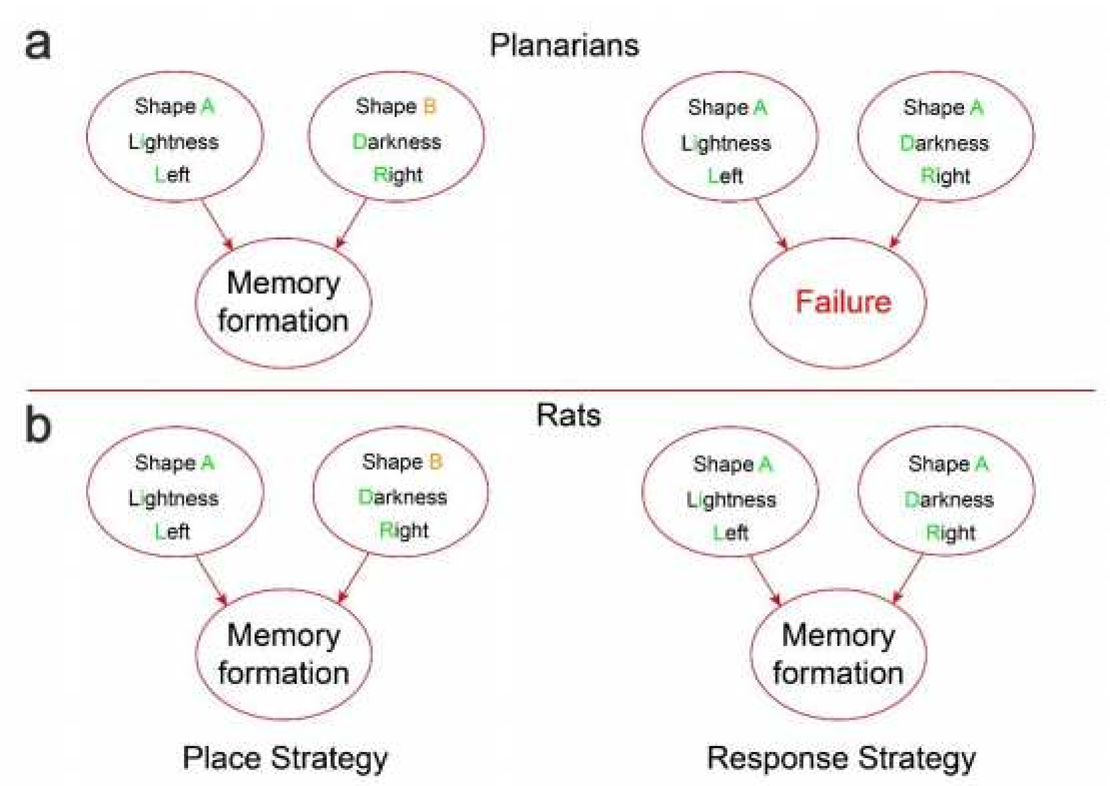

Figure 3

Model of the Planarians' memory formation. a. When facing two different shapes, planarians can memorize the direction of the two places differently shaped. However, when facing identical shapes, planarians cannot memorize the direction of the two places identically shaped. b. Rats can choose different strategies to either choose to memorize the property of the place or the direction of the place. Thus, it can form a correct memory.