Maze exploration and learning in *C. elegans*

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The soil dwelling nematode, *Caenorhabditis (C.) elegans*, is a popular model system for studying behavioral plasticity. Noticeably absent from the *C. elegans* literature, however, are studies evaluating worm behavior in mazes. Here, we report the use of microfluidic mazes to investigate exploration and learning behaviors in wild-type *C. elegans*, as well as in the dopamine-poor mutant, *cat-2*. The key research findings include: (1) *C. elegans* worms are motivated to explore complex spatial environments with or without the presence of food/reward, (2) wild-type worms exhibit a greater tendency to explore relative to mutant worms, (3) both wild-type and mutant worms can learn to make unconditioned responses to food/reward, and (4) wild-type worms are significantly more likely to learn to make conditioned responses linking reward to location than mutant worms. These results introduce microfluidic mazes as a valuable new tool for biological behavioral analysis.

Introduction

Despite having a simple nervous system comprised of only 302 neurons, the soil dwelling nematode, *Caenorhabditis (C.) elegans*, exhibits complex behavioral modalities, including habituation and sensitization,[1,2] and conditioning in response to stimulus.[3–9] This, combined with the extensive information available regarding the anatomy, development, and genetics of *C. elegans*, makes it an attractive model for analysis of behavioral plasticity, defined as the relationship between neurochemistry and neurobiology and behavior. A common tool used to evaluate behavioral plasticity in vertebrate animals is the maze.[10,11] As there is little evidence that invertebrate animals such as *C. elegans* can sense spatial cues, they have not historically been evaluated in mazes.† Here, we report the use of mazes, formed from simple microfluidic platforms, to evaluate *C. elegans* behavior. Microfluidic mazes have been used previously to demonstrate the ability of organic[12] and non-organic[13,14] systems to solve for shortest paths, but to our knowledge, have never before been used for behavioural analysis.

C. elegans adults are ~1 mm long and ~100 μm wide, which is a perfect match for the dimensions of microfluidics. Curiously, despite the similarity in dimensions, microfluidics has been relatively under-used for applications involving worms, with only a few reports regarding analysis of olfaction[15] and miniaturized imaging.[16] In the work presented here, *groups of worms* were evaluated for their *tendency to explore* and *individual worms* for their *capacity for associative learning*. In the former set of experiments, worms were challenged by mazes with and without the presence of food to evaluate their tendencies to search and explore novel environments. In the latter set of experiments, worms were evaluated as to whether they could form an association between food reward and spatial location. In both kinds of experiments, the role of the neurotransmitter, dopamine, was inferred by comparing the behavior of wild-type animals with that of the dopamine-poor mutant, *cat-2*. This work, which introduces microfluidic mazes as a valuable new tool for behavioral analysis, demonstrates the amazing ability that *C. elegans* has to explore its environment and learn to associate its spatial location with the presence of food.

Methods

C. elegans culture

Wild-type (N2) and *cat-2* mutant (e1112) *C. elegans* strains were obtained from the Caenorhabditis Genetics Center at the University of Minnesota (St. Paul), and cultivated as described in Brenner.[17] Briefly, worms were cultivated at 20 °C on nematode growth medium (NGM) agar seeded with *Escherichia coli* OP50 (food). Prior to seeding, bacteria were incubated overnight at 37 °C. All experiments used synchronized well-fed young adults.

Maze fabrication and use

Microfluidic mazes were fabricated in poly(dimethyl siloxane) (PDMS, or silicone rubber) using the rapid prototyping technique.[18] Briefly, SU-8-2100 photoresist (Microchem, Newton, CA), was spin-coated onto silicon wafers (900 RPM, 30 s) and patterned by photolithography. Positive-relief SU-8 features had widths and heights of 1 mm and 150 μm,

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† The most "maze-like" assay that we are aware of for *C. elegans* is the use of lines of unattractive odorants on an open culture plate to evaluate chemotaxis (ref. 9).

respectively. After patterning, masters were silanized by exposure to tridecafluoro-1,1,2,2-tetrahydrooctyl trichlorosilane vapor (Sigma Chemical) and positioned in Petri dishes. Sylgard 184 PDMS (Dow Corning, Midland, Michigan) base and curing agent were mixed thoroughly (10 : 1 by mass), degassed under vacuum, and poured onto the master. The assembly was cured in an oven (70 °C, 4 h); after cooling, devices were gently peeled from the master and trimmed to size. Holes (~8 mm dia.) were punched to form vias for introduction of worms and *E. coli* OP50 into the maze.

Prior to each experiment, a maze was positioned on an agar plate to form an enclosed network of microchannels. It should be noted that channels were filled with air; this stands in contrast to conventional microfluidic experiments, which make use of channels filled with liquid. With care, PDMS mazes could be repeatedly peeled off and replaced onto an agar plate for reuse. Schematics depicting the different configurations are shown in Fig. 1. The six T-maze (Fig. 1a) and complex U-maze (Fig. 1b) were used to characterize the exploratory performance of groups of worms. The continuous T-maze (Fig. 1c) was used to test reward-directed associative learning in individuals.

Behavioral assay type I: group exploration

A general assay was developed to characterize the tendency of worms to explore mazes. A group of worms (~300–500) was collected from culture plates, washed (suspended in 5 mL S-basal medium and collected by gravity sedimentation), and then transferred to a microfluidic maze entry chamber. For rescue experiments, worms were exposed to exogenous dopamine in agar (~4 mM) prior to analysis. In these experiments, a 400 μL aliquot of a solution of dopamine hydrochloride (Sigma Chemical, Oakville, ON) in M9 buffer (100 mM) was added to a 5 cm dia. plate containing 10 mL of agar and bacteria, and allowed to dry at room temperature with lid removed (1 h). Groups of synchronized young adult animals of each genotype (wild-type and *cat-2*) were incubated on these plates at 20 °C for 4 h prior to testing.

Groups of worms were evaluated using the six T-maze and complex U-maze to investigate exploratory performance in the presence and absence of food. Note that each T-maze experiment made use of a single maze; having six mazes enabled several experiments to be conducted simultaneously. 15 min prior to each assay, 1 μL of a solution of NaN_3 (1 M in H_2O) was deposited onto the exposed agar in the exit chambers (*i.e.*, L and R in the six T-maze, and A–D in the complex U-maze). NaN_3 acts as an anesthetic to immobilize animals that reach these regions during the assay. Groups of 30–50 worms were washed twice and then transferred to a maze entry chamber. For all experiments, the worms were allowed to freely explore the maze, and the number of animals within a 0.4 cm radius of each outlet chamber was recorded as a function of time. For assays without food, the *exploratory performance* was defined as the pooled number of animals observed within 0.4 cm of all outlet chambers divided by the total number of animals. For assays with food, worm transfer was immediately preceded by deposition of 2.5 μL of bacterial suspension onto the agar in the center of one of the exit chambers.

Behavioral assay type II: associative learning

To test worms for the capacity to learn, animals were trained individually using the continuous T-maze assay (Fig. 1c). For these experiments, each worm (wild-type or *cat-2* mutant) completed 10 successive trials, divided into phase 1 (trials 1–5) and phase 2 (trials 6–10). In phase 1, an individual animal was picked from an agar growth plate (with bacteria) and transferred to a fresh 20 cm dia. agar plate (without bacteria) for testing. The animal was allowed to conduct two body bends to reduce the amount of bacteria remaining on its body.

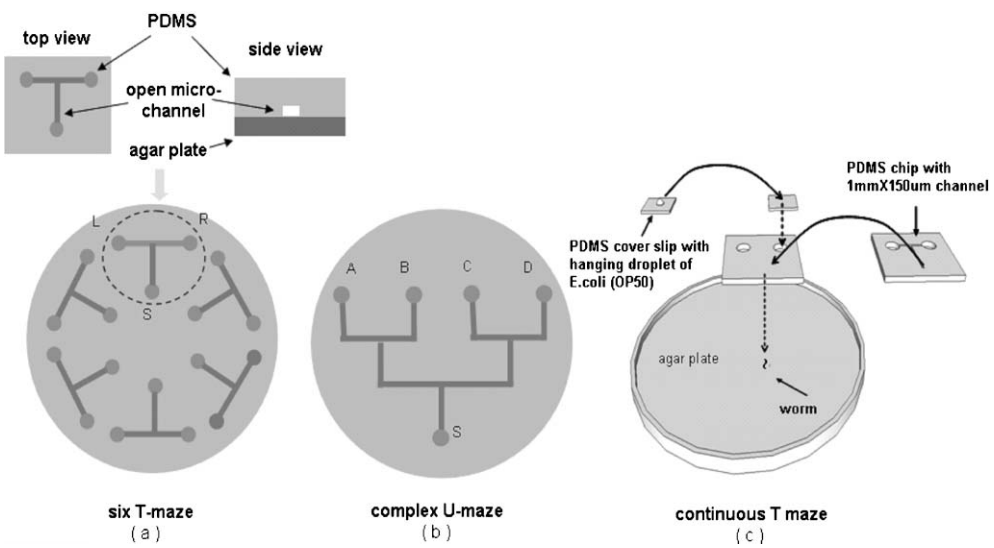

Fig. 1 Schematic showing the maze configurations used in this work. All open microchannels are 1 mm wide × 150 μm deep. (a) Six T-maze on agar plate. The distances from start point (S) to outlets (L–R) are equal (SL = SR = 2.0 cm; 1 cm per leg). (b) Complex U-maze. The distances from the start point (S) to the outlets (A–D) are equal (SA = SB = SC = SD = 2.0 cm). (c) Continuous T-maze assay platform. (a) and (b) were used for group exploration assays, while (c) was used for analysis of individual animals in associative learning assays.

Then, a PDMS T-maze was gently placed onto the animal (without touching the body), such that the animal was positioned in the start point of the maze. The worm was allowed to acclimate for 1 min, after which a PDMS coverslip with a hanging droplet of OP50 (4 μL) was placed on the top of the left or right outlet chamber, as depicted in Fig. 1c. Care was taken to prevent contact between the hanging droplet and any part of the maze or agar. Note that, for consistency, we use "reward" to describe the droplet of bacteria in this assay, even though the worms are never permitted to reach the food. In control experiments, an empty coverslip (without bacteria) was placed on the top of the right or left outlet chamber.

The worm was allowed to explore the T-maze, and its choice, *i.e.*, the first entry into the left or right arm of the maze, was recorded. The choice signaled the end of the first trial; the process was then repeated four more times (trials 2–5), by peeling the maze off the agar and replacing it such that the worm was again positioned in the start-point. In this manner, each successive trial covered fresh agar to prevent the possibility of the worm using a trail formed on a previous trial to navigate the maze. Following phase 1, the maze was either replaced with a fresh maze, or rinsed with water and dried twice, and then placed, peeled and replaced for five additional trials (phase 2, trials 6–10). In this phase, there was no coverslip or hanging droplet.

Each individual animal was required to complete ten trials in a row. Phase 1 investigated the unconditioned response of individuals seeking a reward, and phase 2 investigated a conditioned response of individuals associating a choice (left *vs.* right) with rewards experienced previously. The *degree of response* for each trial was defined as the number of individuals that made the "correct" choice divided by the total number of individuals tested (*i.e.*, 26 wild-type and 21 *cat-2* mutants). As a control, separate groups of wild-type and mutant worms (20 worms each) were required to perform trials 1–5 without reward.

Locomotory rate assay

Locomotory rates were measured by collecting five individuals from growth plates (with bacteria) washing the animals twice, and depositing them onto an assay plate (without bacteria). The remaining S buffer was absorbed with a Kimwipe, and the worms were allowed to acclimate for five min. The number of body bends in 20 s intervals was then recorded for each animal on the assay plate.

Data analysis

For group assays (*i.e.*, type I, above), exploratory performance with and without food was calculated for each population. Each experiment was repeated three times on three separate plate/maze assemblies (using a different group of 30–50 worms for each assay) to yield averages and variances. For individual behavior assays (*i.e.*, type II, above), the degree of response for each trial was calculated. These values were then pooled by experimental phase (trials 1–5, and 6–10, respectively) to yield averages and variances. Student's *t*-tests were used to evaluate statistical significances between each population (details in the text).

Results

C. elegans exhibits exploratory behavior in a maze environment

To investigate the exploratory behavior of *C. elegans* in a maze environment, groups of well-fed hermaphrodites that had been continuously cultured on *E. coli* OP50 were washed in S-medium and transferred to entry chambers in a six T-maze (Fig. 1a) positioned on a bacterium-free assay plate. Animals were allowed to move undisturbed for 1 h in the maze, during which time their movements were observed and recorded. Animals were observed to initially ($\sim$3–5 min) probe the local environment in the start-point (S) of the maze; this behavior was characterized by slow, random movements interspersed with long pauses. After this period, animals switched to long-range roaming, characterized by forward movement in the maze coupled with omega turns and pirouettes (a series of direction reversals and sharp turns[19]), such that most of them explored the entire maze (Fig. 2a). As shown in Fig. 2b, the number of worms observed to approach the outlet chambers increased with time, with no apparent preference for left or right turns.

When a drop of bacteria was placed in either of the outlet chambers, for the first few minutes, animals were observed to exhibit a similar searching behavior as in mazes without food. Upon switching to long-range roaming, however, they exhibited a lower frequency of pirouettes, and most migrated towards the food. In fact, as shown in Fig. 2c, at each time point, the fraction of animals reaching the reward chamber was significantly higher than that without reward ($P < 0.01$), indicating the innate ability of animals to explore and locate food resources in a novel environment. After demonstration of exploration in simple T-mazes, groups of animals were further characterized in complex U-mazes (Fig. 1b). As was the case for T-mazes, the worms were observed to explore, often wandering convoluted paths (Fig. 3a). The number of animals that approached each outlet when no reward was present was distributed randomly (Fig. 3b). When food was placed in one of the outlets, as shown in Fig. 3c, the majority of animals were attracted to that outlet.

Dopamine mediates *C. elegans* exploratory behavior

To investigate the effects of dopamine on exploration, we evaluated the behavior of the *cat-2* mutant strain, which is known to be deficient in dopamine, but not in other neurotransmitters.[20] In addition, the effects on worm behavior of pre-exposure to exogenous dopamine were evaluated. Wild-type and *cat-2* mutants were evaluated in T-mazes, and, as shown in the solid bars in Fig. 4a, wild-type worms were significantly more likely to explore the maze than *cat-2* animals at all times evaluated ($P < 0.05$). When worms were stimulated with exogenous dopamine, shown in the striped bars in Fig. 4a, animals of either phenotype were more likely than unstimulated animals to explore the maze. Similar trends were observed for maze exploration in the presence of food (data not shown). One possible explanation for this effect is simply a dopamine-mediated increase in locomotory rate. To test for this possibility, body bend rates of mutants and wild-type individuals on agar without food were measured, both with

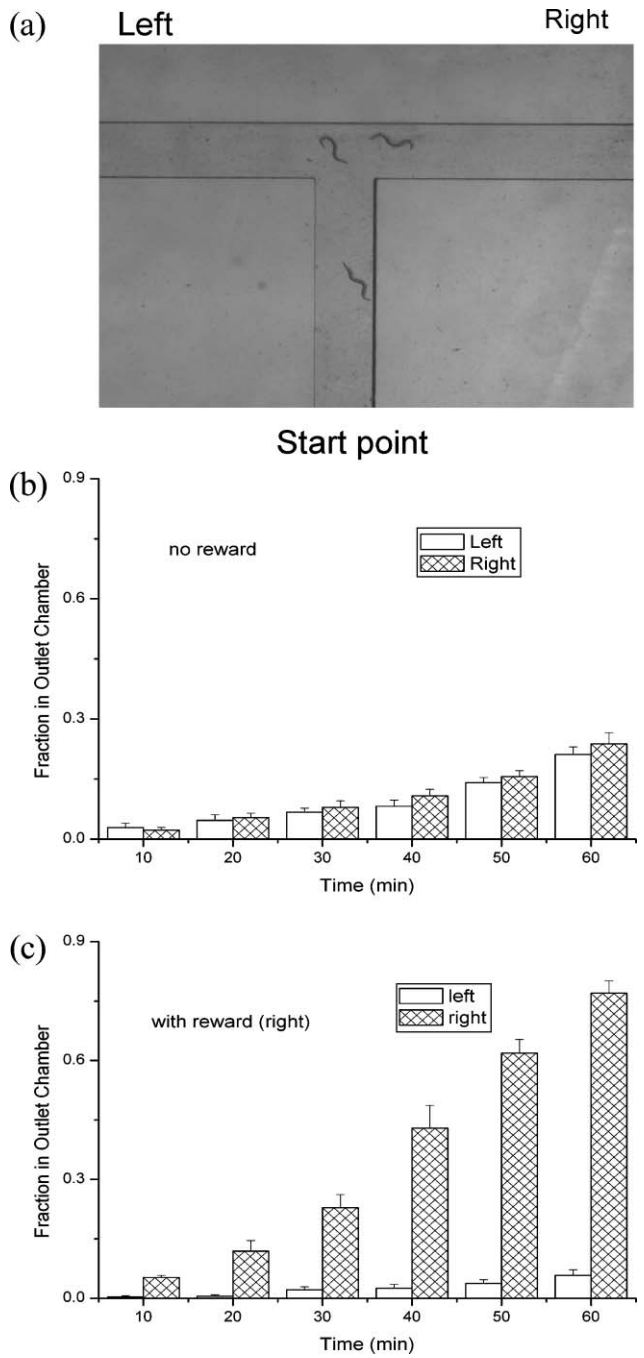

Fig. 2 T-maze exploration in the absence/presence of reward (*E. coli*). Well-fed young adult animals were evaluated for 1 h. (a) Photograph of animals navigating a maze. (b) Graph summarizing animal exploration in the absence of reward. (c) Graph summarizing exploration in the presence of reward; a droplet of *E. coli* was placed in the right chamber of the maze. Each experiment ($n = 6$) used 40–60 wild-type animals. Data are expressed as the fraction of animals that were within a 0.4 cm radius of each outlet relative to the total number of animals tested; error bars are 1σ.

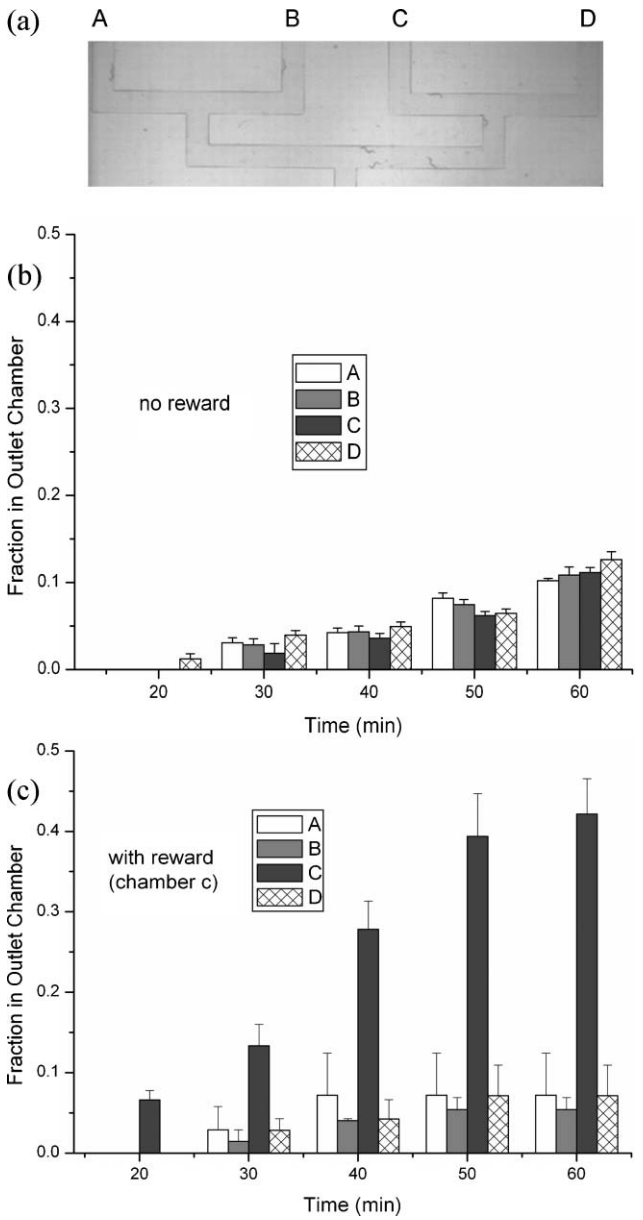

Fig. 3 Complex U-maze exploration in the absence/presence of food (*E. coli*). Well fed young adult animals were evaluated for 1 h. (a) Photograph of animals navigating a complex U-maze. (b) Graph summarizing exploration in the absence of reward. (c) Graph summarizing worm exploration in the presence of food; a droplet of *E. coli* was placed in chamber *C* of the maze. Each experiment ($n = 3$) used 40–60 worms. Error bars are 1σ.

and without pre-exposure to dopamine (Fig. 4b). Although slightly higher body bend rates were observed to correlate with exposure to dopamine and with wild-type worms, these differences were not statistically significant ($P > 0.05$).

Reward related associative learning

To investigate reward related associative learning, animals were evaluated individually in what we have called a continuous T-maze assay (Fig. 1c). As described in the Experimental procedures section, each assay required an individual worm to complete ten successive trials in a T-maze. In phase 1 (trials 1–5), a naive, wild-type animal was exposed to a maze containing a reward in the right outlet chamber. After the first (and each subsequent) trial, the maze was peeled off and replaced such that the worm was re-positioned in the inlet chamber. This measure insured that

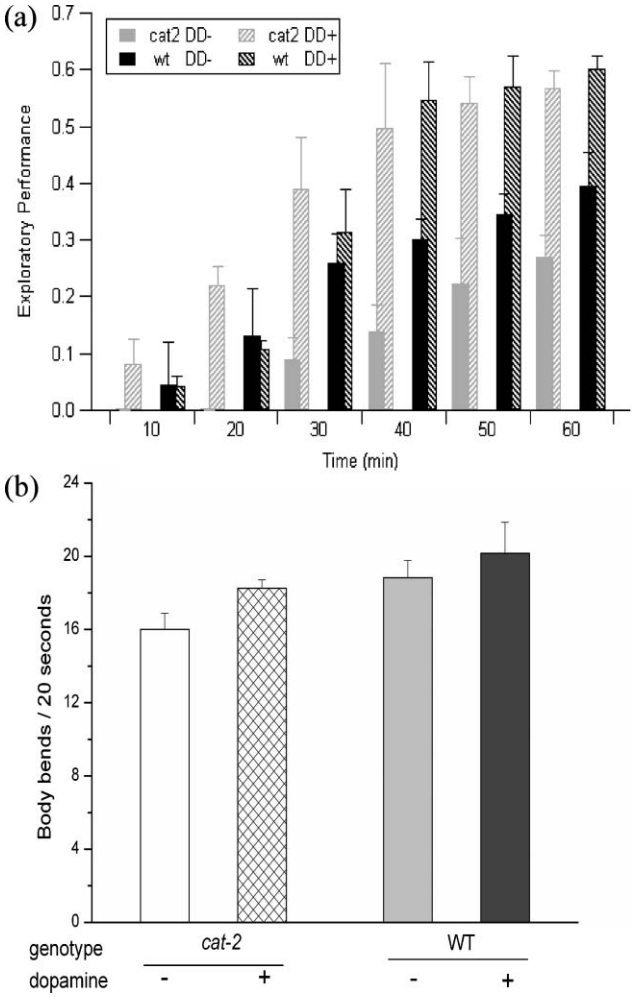

Fig. 4 Effects of dopamine on exploratory behavior. (a) Graph comparing exploratory performance without reward for wild-type (WT) and *cat-2* (dopamine-poor) mutants, with (DD+) or without (DD−) pre-exposure to exogenous dopamine ($n = 3$ for each of the four populations). (b) Graph comparing locomotory rates for wild-type and *cat-2* mutants, with and without pre-exposure to dopamine, on agar lacking bacteria. Error bars are 1σ.

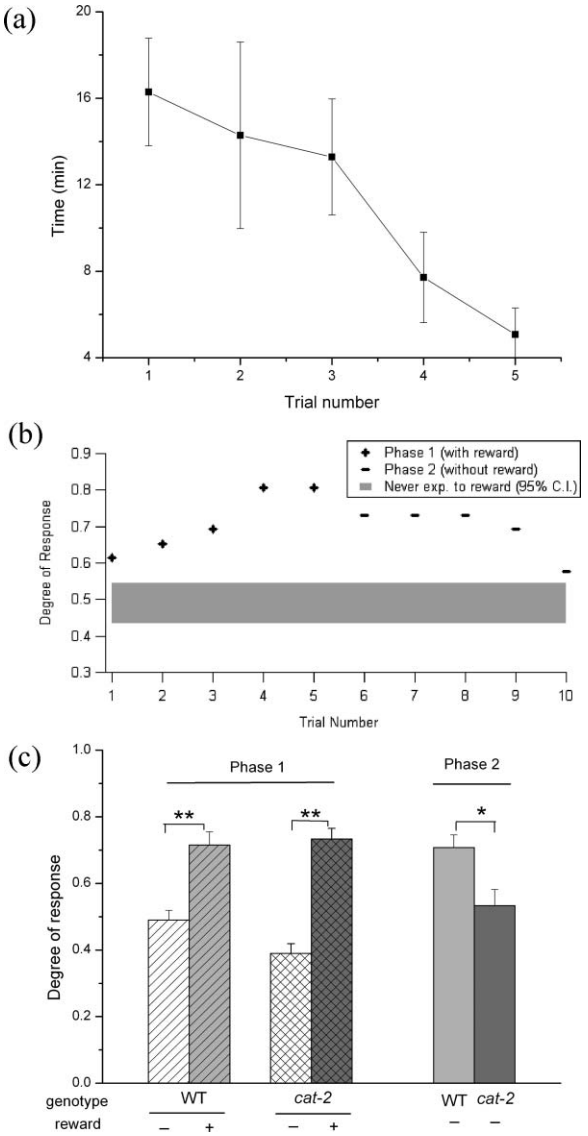

Fig. 5 Results of reward motivated learning in *C. elegans* individuals in a continuous T-maze assay. Individual worms were sequentially challenged by five trials with reward (phase 1) and five trials without reward (phase 2). (a) Graph summarizing the average time spent of wild-type worms in the maze for five sequential trials with reward; error bars are 1σ. (b) Graph depicting the fraction of 26 wild-type worms that selected the right outlet chamber of the maze with a food reward (+ marker) and without a reward (− marker). For comparison, the pooled results (shown as a 95% confidence interval) of 20 wild-type worms exposed to five trials in a maze without reward is also plotted. (c) Graph comparing pooled results of reward related associative learning assays in a continuous T-maze assay. In phase 1, both wild-type and *cat-2* individuals had a significant response to reward ($P < 0.01$, **). In phase 2, wild-type animals exhibited a higher tendency for conditioning relative to *cat-2* animals after the reward was removed ($P < 0.05$, *). Error bars are 1σ.

each trial covered a fresh surface to avoid the possibility of cues from previous trials affecting performance. As summarized in Fig. 5a, the average time required for each worm to reach the reward (for those that chose correctly) decreased steadily from more than 15 min in trial 1 to less than 5 min in trial 5. After completing trials 1–5, each worm was challenged with five additional trials without reward (*i.e.*, phase 2). Fig. 5b summarizes the results for 26 wild-type worms in phase 1 (+ marker) and phase 2 (− marker). As shown, in phase 1, worms were conditioned to associate reward with position, and in phase 2, the effect gradually extinguished as a function of trial number. For comparison, the pooled results (shown as a 95% confidence interval) for 20 additional individual worms challenged with five trials without reward is also plotted. A few experiments were also performed with reward placed on the left instead of the right, and similar trends were observed (data not shown).

To evaluate the extent of associative learning statistically, the results for trials 1–5 (phase 1) and 6–10 (phase 2) were pooled and compared. As shown in the first two bars in Fig. 5c, individual wild-type worms were significantly more likely to choose the right side of the maze (with reward) than the left ($P < 0.01$). To evaluate the effects of dopamine on this behavior, 21 *cat-2* mutants were tested using the same method, and the results are summarized in the second pair of bars in

Fig. 5c. These data suggest that *cat-2* mutants, like wild-type individuals, were also significantly biased towards choosing the reward ($P < 0.01$). However, the results for phase 2, summarized in the third pair of bars in Fig. 5c, indicate that *cat-2* mutants were significantly less likely to remain conditioned after training ($P < 0.05$). The trend for *cat-2* worm degree of response in phase 2 as a function of trial number (not shown) was similar to that of wild-type worms—the effect was gradually extinguished over the course of five trials.

Discussion

The *C. elegans*/maze data presented here have implications for two kinds of behaviors: (1) exploration (Fig. 2–4), and (2) associative learning (Fig. 5). As context for discussion of the former, we note that many animals use complex foraging strategies to find resources essential for their survival. These behaviors are widely conserved, even in very simple species; for example, *C. elegans*, uses area restrictive search to maximize the chance of encountering food.[21,22] The advantage of evaluating exploratory behavior in simple animal models is that the precise roles of each cell in the neural pathway can be determined.[23]

In the results presented here, we observed two distinct states of exploration: a local search state, and a long-range traveling state. While the former state was independent of the presence of reward, worms in the traveling state were observed to pirouette less frequently when one of the outlet chambers contained food. This observation suggests that the search behavior was biased by environmental cues (*i.e.*, olfactory sensing), which is consistent with recent work demonstrating that pirouettes are a mechanism used by *C. elegans* to move along chemical gradients in chemotaxis.[19,24,25]

In addition to characterizing exploratory tendencies in wild-type animals, we also evaluated the behavior of the dopamine-poor *cat-2* mutant. *Cat-2* animals were observed to exhibit reduced tendencies to explore; furthermore, exposure to exogenous dopamine promoted this behavior (as well as enhancing it in wild-type animals). As body bend rate was not significantly correlated to exposure to dopamine, this finding suggests that the decreased exploration observed in *cat-2* mutants may be a result of slow adaptation to new environments, possibly an effect of reduced neuronal dopamine. An alternate possibility is that dopamine and/or the genetic defect in *cat-2* modulates the frequency of direction reversal, which is known to correlate with worm foraging abilities in complex environments.[9] As dopamine is implicated in a broad range physiological functions, further tests are required to evaluate this phenomenon.

Turning from exploration to learning, we note that the use of geometric maze platforms to evaluate spatial conditioning in response to reward is an archetypal analysis tool, typically applied to advanced species such as mice and rats.[26,27] While nematodes have never before been used in mazes, *C. elegans* is known to be capable of other forms of conditioning, including association of food with a variety of olfactory cues,[4–6] as well as with specific temperature ranges.[7,8] Here, we have shown that *C. elegans* can be conditioned to associate a reward with a maze choice (*i.e.*, the right outlet chamber) in a continuous T-maze assay (*i.e.*, sequential, repetitive trials exposing a single worm to the same maze). Wild-type animals were able to make an unconditioned response to the reward (*i.e.*, right turn), and, after training, the animals displayed a conditional response, by continuing to turn right for five trials in the absence of reward (Fig. 5b). As shown, over the course of the five trials without reward, the conditioning was extinguished, and worms that were evaluated for further trials were indistinguishable from worms never exposed to reward (data not shown). Several measures were taken to eliminate confounding effects, such as using a fresh agar surface for each trial, and using new mazes or rinsing mazes between experimental phases; in addition, several controls were performed, including verification that worms had no preference for direction when never exposed to food, and were capable of being conditioned to choose either direction, depending on where the reward was placed. These results indicate that *C. elegans* might be able to associate spatial features of its environment with the presence of food reward.

This novel finding is remarkable, leading lead us to echo Rankin[28] in wondering "what can't a worm learn?" More importantly, the results pose a conundrum: it is unclear what mechanism the worms could be using to recognize and "remember" paths through a maze. Visual cues, which are typically associated with maze responses in mammals,[26,27] are unlikely, as worms lack eyes. Perhaps even more puzzling, worms typically position themselves in a side-dependent manner, which would presumably affect their ability to sense left and right. Simple mechanosensory feedback from muscles is not likely, as worms were observed to choose "left *vs.* right" through complex sequences of omega turns and/or pirouettes, rather than more straightforward 90° directional shifts. One possibility is the existence of a neural circuit that uses and stores sensory cues to modulate spatial orientation and learning. Our preliminary findings, discussed below, implicate dopamine in this putative circuit.

In mammals, the associative learning hypothesis posits that dopamine signaling is required for animals to learn to associate rewards with predictive cues. This hypothesis is supported by the observation that transient increases in neuronal dopamine concentration correlate with reward related learning,[29,30] as well as the observed role of dopamine in synaptic plasticity in models of learning at the molecular level.[29,31] In the present study, we investigated the effects of dopamine on learning by evaluating the behavior of the *cat-2* mutant in the continuous T-maze assay. The results in phase 1 (Fig. 5c) indicate that *cat-2* mutants are not impaired in choosing reward; however, the results for phase 2 indicate that mutants suffer from impaired conditioning after training. These results suggest a possible role for dopamine in a neural circuit involved in *C. elegans* learning; however, we note that many other factors in *cat-2* physiology could contribute to the observed behavioral difference. Clearly, more work is required to further investigate this phenomenon.

Conclusion

In conclusion, we present a new tool for analysis of behavior in *C. elegans*: microfluidic mazes. In this work, *C. elegans* worms

were found to explore mazes in a dopamine-dependent fashion, and were able to make conditioned responses linking rewards to maze/choices, which is evidence of associative learning. These results suggest future work in which the molecular/genetic mechanisms underlying multi-stimulus learning may be elucidated. More generally, this work serves to introduce microfluidics as a useful tool for behavioral analysis, and suggests the possibility of future lab-on-a-chip systems scaled for high-throughput behavioral analysis and/or integration with neurochemical assays.

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