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Supplemental information

Caenorhabditis elegans learning in a structured maze is a multisensory behavior

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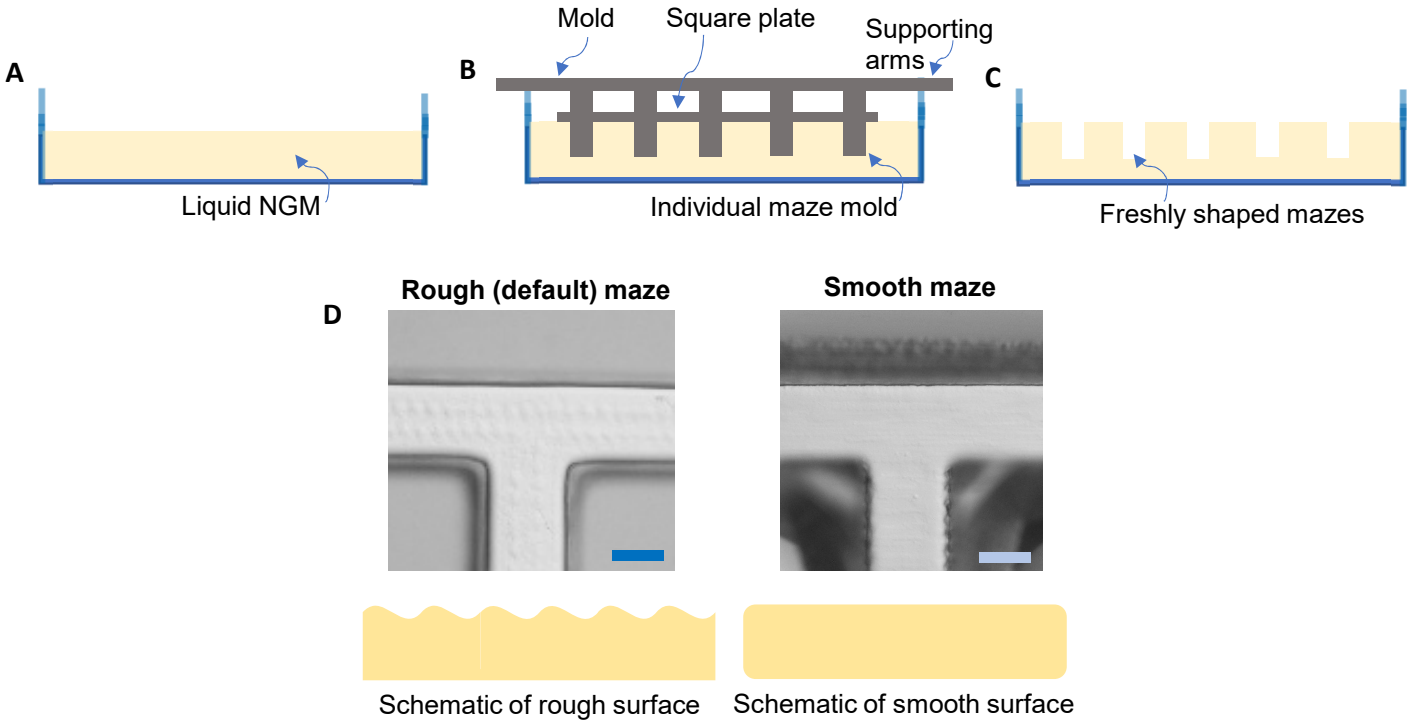
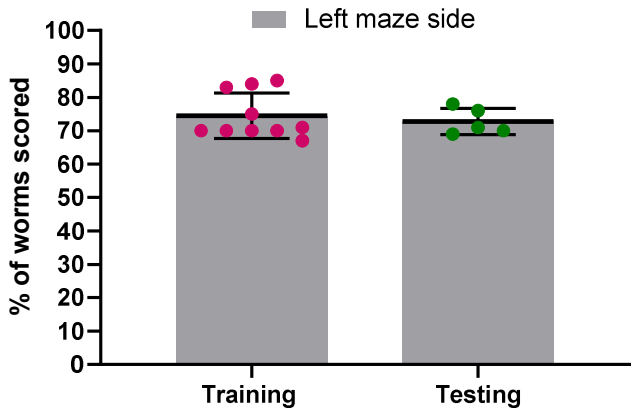
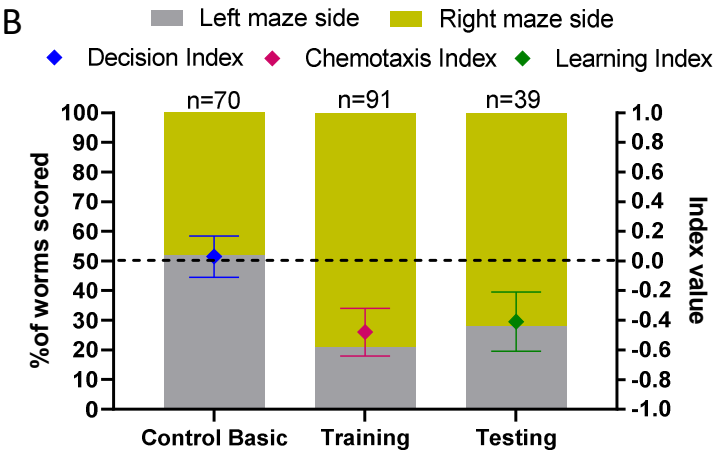


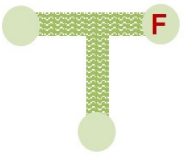
Figure S1: Maze fabrication process, related to Fig. 1. (A): Liquid NGM is poured in a 60mm plastic petri dish, up to 2/3 of the dish depth; **(B):** The mold is immersed, with the supporting arms firmly standing on the plate rim, and the square-shaped plate prevents liquid agar from forming a meniscus around each individual maze mold; (**C**): Once NGM cools down and solidifies, the mold is pulled out and mazes are shaped, made entirely out of NGM; (**D**): Left: Top view image and side view schematic of the surface of a rough (default) maze (i.e. Fig. 1C) used in experiments of Figs. 1-7; Right: Top view image and side view schematic of a smooth maze (used in experiments of Fig. 2C). Scale bars: 1mm.

A



B

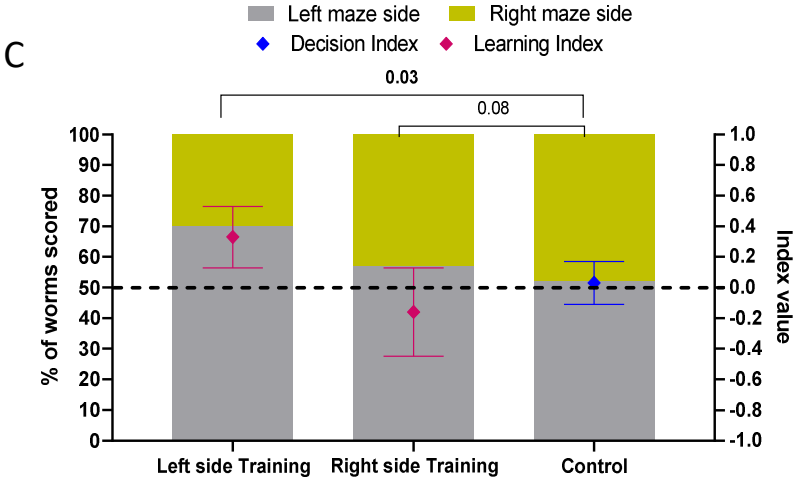




Food on the right side of the maze

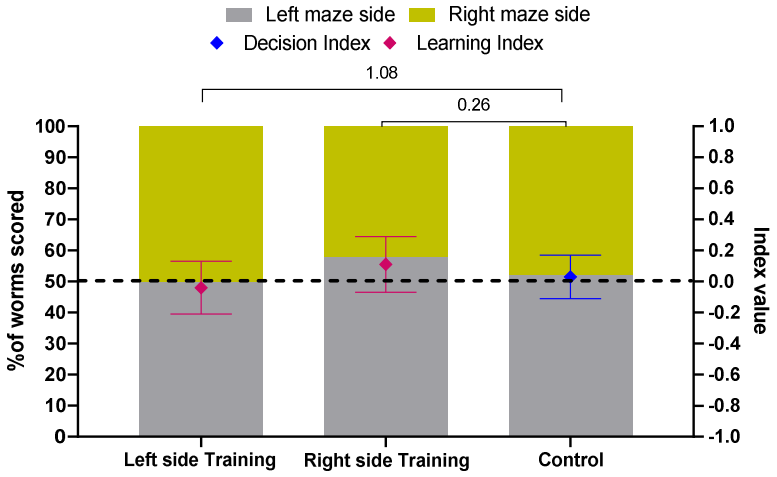
comparisons	Food Right
Train to Control$_{Basic}$	**<0.001**
Train to Train$_{Basic}$	0.13
Test to Control$_{Basic}$	**0.001**
Test to Test$_{Basic}$	0.76

C



Training, n=50	L: 70%	R: 30%
Testing, n=42 (Training maze/Testing maze)	(first bar) L/L: 70% L/R: 30%	(second bar) R/L: 57% R/R: 43%

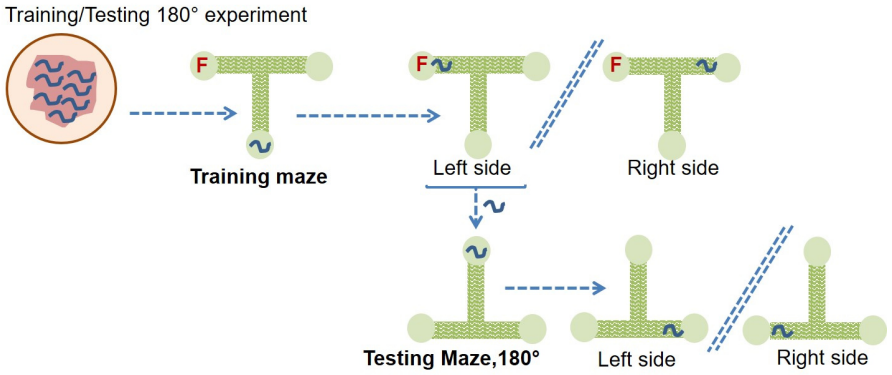
D

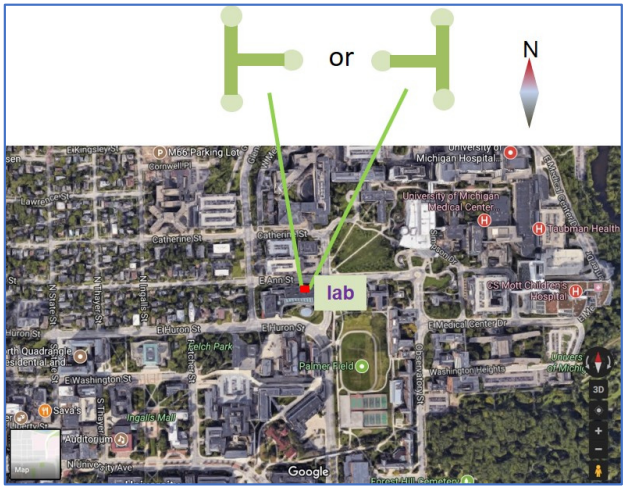


Training, n=153	L: 52%	R: 48%
Testing, n=133 (1st maze/2nd maze)	(first bar) L/L: 50% L/R: 50%	(second bar) R/L: 58% R/R: 42%

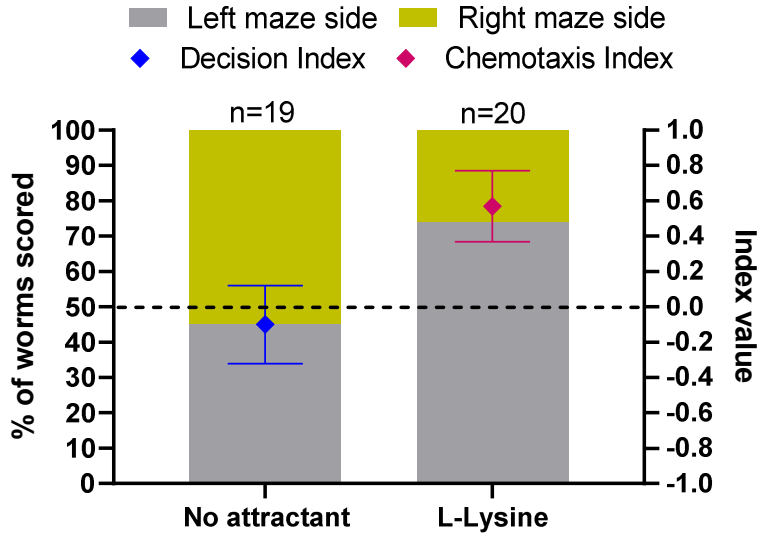
Figure S2: Range of % scored in Training and Testing, side of food location in Training, and absence of learning when food is not located, related to Fig. 1. (**A**): Graph showing the range of % scored during Training and Testing experiments, performed with adult Day 1 fully fed N2 *C. elegans* nematodes, experimental steps as illustrated in Fig. 2B. Each dot represents one Training or Testing experiment, black horizontal line depicts the mean, error bars indicate standard deviation; Training experiments included are shown in Figs 1, 2A, 3A (all 3 Training experiments), 3B, 3C (both Training experiments), 7A (Day 1 adult fully-fed), S3B, n_{total}=824; Testing experiments included are shown in Figs 1, 2A, 3B (1st Testing), 7A (Day 1 adults fully fed), S3A, n_{total}=255. (**B**): Side of food location in the Training maze. Graph shows % of worms that were scored in Basic Control and Food-on-the-Right Training/Testing experiments (left y axis), with the respective indices (right y axis, Training: Chemotaxis Index, Testing maze: Learning Index); experimental steps as shown in Fig. 1, with the exception of food placed on the right end of the Training maze (see schematic). Bar diagram shows the % of worms scored; dashed line illustrates the 50% and index=0 level. Indices error bars represent the standard deviation of the index; above bars is indicated the number of nematodes scored (n). Table shows *p*-values (binomial distribution probability) for indicated comparisons. (**C**) No learning is observed in *C. elegans* that do not locate the food during Training. Graph shows % of worms scored in the Testing maze, with respect to their performance during Training (food placed on the left side of the Training maze). Worms that reached the right (non-food-containing) side of the Training maze make an unbiased decision in the Testing maze (second bar), in contrast with the ones that reached the left (food-containing) side in the Training maze (first bar). (**D**) No learning is observed in *C. elegans* that have experienced empty mazes. Graph shows % of worms scored in the Testing maze, after experiencing an empty Training maze (no food present), with respect to their performance during Training. After traversing an empty Training maze, the decision of all worms in the Testing maze is unbiased. (**C**), (**D**): Bar diagrams show the % of worms scored; dashed line illustrates the 50% and index=0 level, *p*-values (binomial distribution probability) are shown above bars for comparison; indices error bars represent the standard deviation of the index; tables show the % of worms that reached a L or R maze arm in each case and the number of worms scored (n).

A



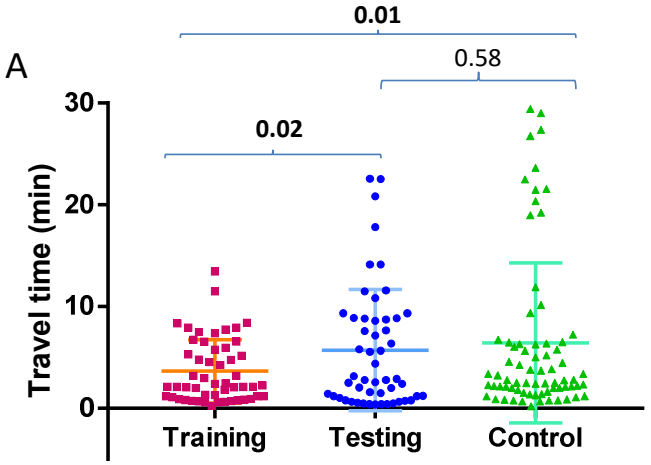


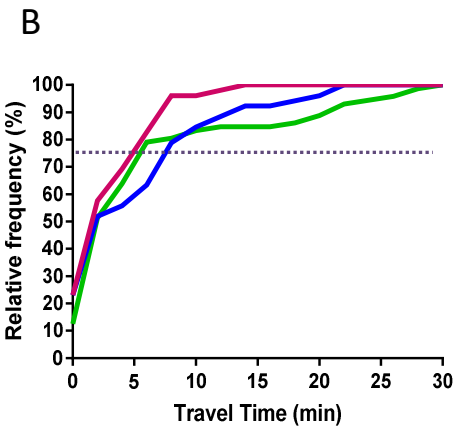
B



	Left	Right
L-Lysine	74%	26%
No attractant	45%	55%

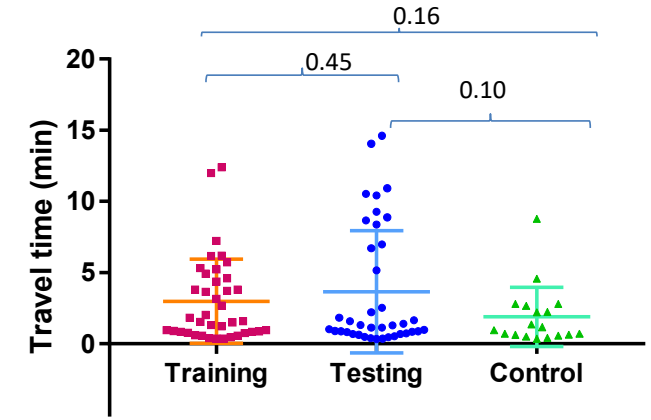
Figure S3: Cardinal directions, and L-lysine trial, related to Fig. 2 and Fig. 7. (**A**): The effect of cardinal directions on *C. elegans* maze learning. Left panel: Schematic of the Training/Testing180° experiment on N2 Day 1 adult *C. elegans* (Fig. 2). Right panel: Schematic of the maze orientation before rotation (Training), with respect to lab location (Medical Campus, University of Michigan, Ann Arbor) and to cardinal directions. Both configurations where used. Map snapshot captured from Google Maps, with modifications. (**B**): *C. elegans* N2 Day 1 adult performance in a T-maze, when instead of *E. coli* OP50 there is 1.5μL of L-lysine 3M placed on the left end of the maze. To compare, results of a parallel control experiment (no attractant used-empty maze) are also shown. n=total number of worms scored; dashed line illustrates the 50% level. The use of L-lysine gives similar results to the use of *E. coli* OP50.

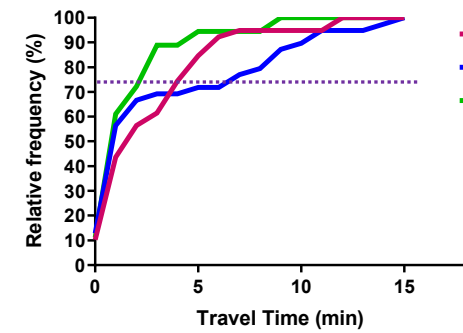




C

	Training	Testing	Control
Total number of values	52	52	72
Number of excluded values	0	0	0
Number of binned values	52	52	72
Minimum	0.238	0.376	0.194
25% Percentile	1.109	1.068	1.842
Median	2.193	2.834	2.774
75% Percentile	5.898	8.823	6.439
Maximum	13.454	22.554	29.412
Mean	3.636	5.719	6.431
Std. Deviation	3.116	5.951	7.848
Std. Error of Mean	0.432	0.825	0.924





	Training	Testing	Control
Total number of values	39	39	18
Number of excluded values	0	0	0
Number of binned values	39	39	18
Minimum	0.357	0.353	0.368
25% Percentile	0.840	0.733	0.619
Median	1.598	1.309	1.074
75% Percentile	4.613	6.989	2.713
Maximum	12.407	14.609	8.790
Mean	2.984	3.655	1.901
Std. Deviation	2.953	4.283	2.070
Std. Error of Mean	0.473	0.685	0.488

Figure S4. Analysis of the time needed for N2 Day 1 adult worms to traverse a T-maze, related to Fig. 2 and Fig. 5. Top row: data for standard (rough) T-mazes, bottom row: data for smooth T-mazes. (**A**): Scatter plot of the time needed for the worms to run a Training, a Testing or an empty maze (Control). On each plot the *p*-values of Student's two-tailed t-test, unpaired for comparisons with Control, paired for Training/Testing comparison, are provided. Symbols (squares, circles, triangles) indicate individual data points (worms), horizontal lines indicate the mean, vertical lines are SEM error bars. (**B**): Cumulative % probability of the time needed for the worms to run a Training (magenta), a Testing (blue) or an empty maze (Control-green); Plots reveal differences in the frequency distribution of time spent in the maze, dashed lines indicate the 75% percentile. (**C**): Descriptive statistics of the frequency distribution analysis. Data analyzed are from the Basic Training/Testing experiment and Basic Control experiment (Top C, Fig. 1) and from the Smooth Training/Testing experiment and Basic Control experiment (Bottom C, Figs 1,2).

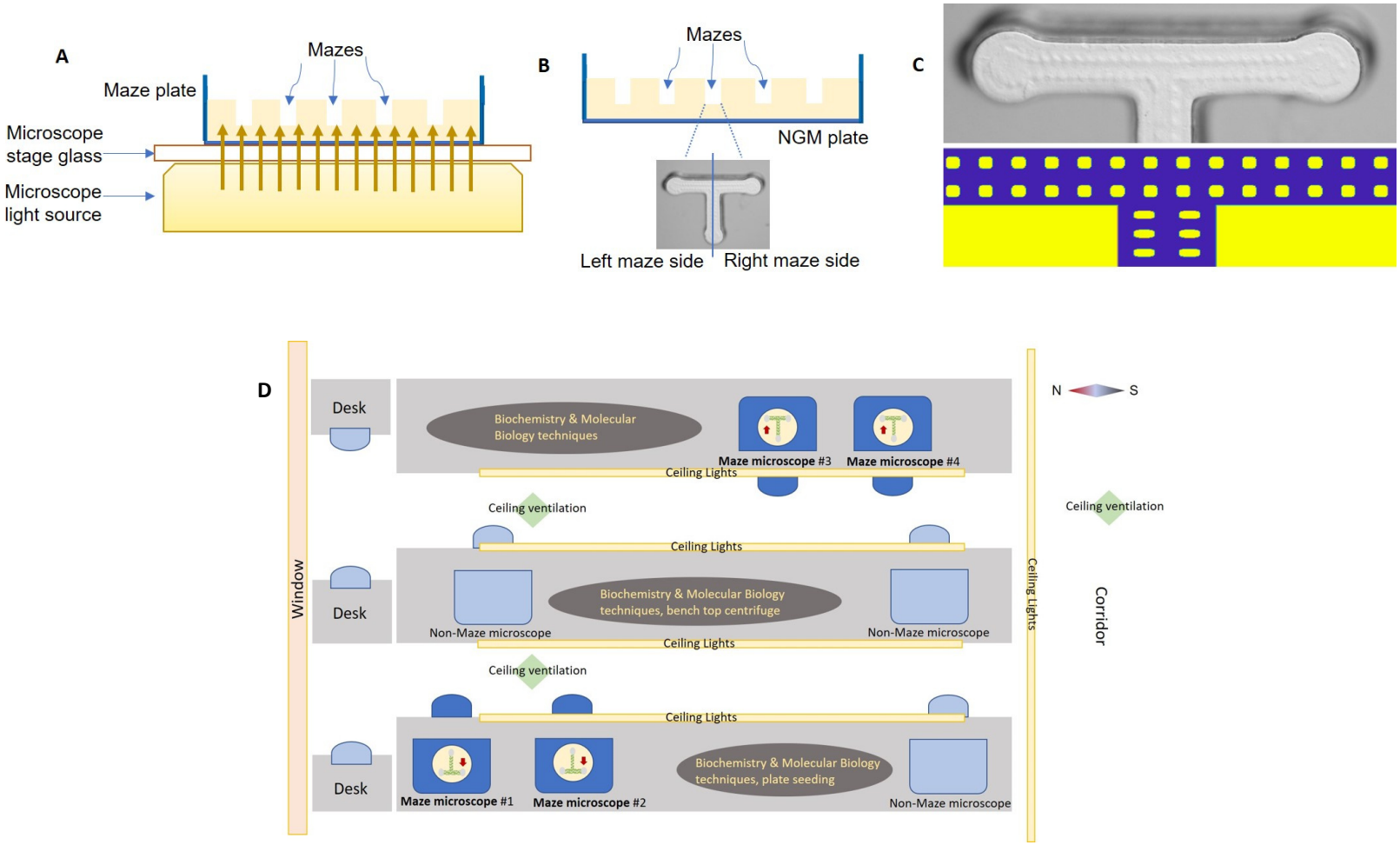
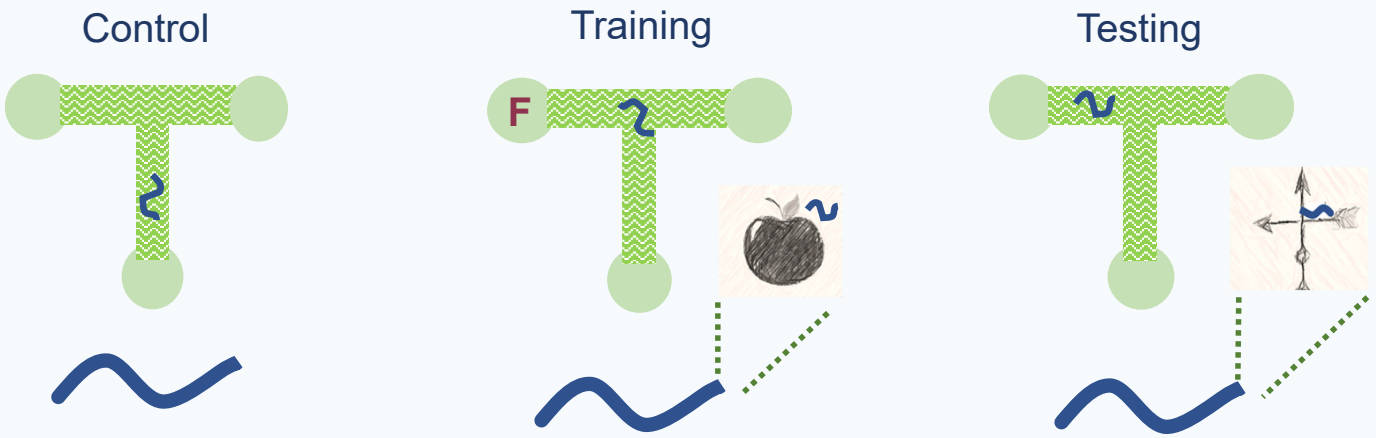

A
Mazes
Maze plate
Microscope
stage glass
Microscope
light source
B
Mazes
NGM plate
Left maze side
Right maze side
C
D
Desk
Biochemistry & Molecular
Biology techniques
Maze microscope #3
Maze microscope #4
Ceiling Lights
Ceiling ventilation
Window
Desk
Biochemistry & Molecular Biology
techniques, bench top centrifuge
Non-Maze microscope
Non-Maze microscope
Ceiling Lights
Ceiling ventilation
Desk
Maze microscope #1
Maze microscope #2
Biochemistry & Molecular Biology
techniques, plate seeding
Non-Maze microscope
Ceiling Lights
N
S
Ceiling ventilation
Corridor

Figure S5. Maze chemical consistency and surface texture, and out of the maze cues, related to Figs. 1 to 7. (**A**): Light coming from the microscope illumination source reaches uniformly all individual mazes in the plate. (**B**): Due to the fabrication process (see Fig S1) all mazes in the same plate (and all plates of the same batch) have the same chemical consistency across all maze arms. Note that maze plates used on the same experimental day, namely Training and Testing mazes used on the same experimental day, are made of the same NGM batch. (**C**): On the floor surface of rough mazes the microfeatures (micro-indentations, est. ~20microns, i.e. ~1/4 of *C. elegans* body thickness) have the same pattern across the two maze sides. Any variations that occur randomly, because of occasional discrepancies during the fabrication of the 3D-printed maze mold, are not consistent nor always present on the same maze side (bottom panel credit: Bennet Sakelaris). (**D**): Schematic of the maze setup locations in the lab. Maze experiments were run at four different microscopes, shown in dark blue. The maze orientation at each setup is indicated, along with a red arrow pointing at the left maze side in each case. Microscopes not used for maze experiments are shown in light blue, and other bench uses are explained in grey ellipses, to indicate space use and traffic. Lab window (orange rectangle, left), ceiling ventilation locations (green rectangles) and ceiling lights (yellow rectangles) are shown to note potential sources of air flow or light. Maze orientation in all four working stations is not consistent regarding air or light flow on one specific maze side.

Suggested model of *C. elegans* learning in a T-maze

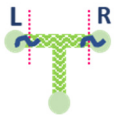
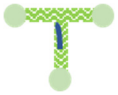


	Food location 	Learning (formation & expression) 
Chemosensation	◎	Food location needed
Mechanosensation	◎	◎
Proprioception	-	◎
Maze structural features	-	◎
Body orientation	-	-

◎: modality/feature involved, **-** : not involved

Figure S6: Suggested model of *C. elegans* learning strategy in T-mazes, related to Fig. 1, Fig. 4, Fig. 5, Fig. 6. In an empty maze, naïve *C. elegans* (control experiment) display an explorative behavior that results in unbiased behavioral outcome regarding which side of the maze the nematodes will eventually reach (Fig. 1, S2B). In a maze that contains food on one end (Training maze), naïve *C. elegans*' behavior is strongly biased, and the majority of nematodes reach the food-containing arm (Fig. 1, S2B). Successful food location requires the contribution of chemical and tactile input (Fig. 4A, 4B, 2C). In the current assay it is not possible to distinguish between food detection and subsequent food location. *C. elegans* experience in the Training maze leads to learning. When successfully trained worms are placed in a second, similar, empty maze (Testing maze), they express a biased behavior, and the majority of them reach the same maze side with the one that contained food previously. Learning requires the contribution of tactile and proprioceptive cues (Fig. 4, 2C). Input on *C. elegans* body actions is collected through proprioception (Fig. 4C), and information on worm's body interaction with mazes' structural features is acquired through touch sensation (Fig. 5). Animals' body ventral/dorsal orientation is not imperative for learning in the maze environment (Fig. 6). The current assay cannot distinguish between learning formation and learning expression (retrieval) processes. We suggest that *C. elegans* learn to associate food with a combination of proprioceptive cues about their body actions (interoceptive cues), and mechanosensory cues about their body's interaction with the structural features of the maze (exteroceptive cues). Therefore, we suggest that *C. elegans* follow a kind of response learning strategy to achieve learning in the maze environment.

Supplementary Table S1. Categorization of *C. elegans'* behavioral outcome in mazes (related to Figures 1, 2, 3, 4, 5, 7).

Decision	Interpretation	Visualization
L/R maze side (scored animals)	Worm reached either the left-side end or the right-side end of the maze (first decision recorded)[#]	
Escaped[*]	Worm escaped the maze, usually by climbing out of it	
Inconclusive[*]	Worm wandered around, without reaching a maze arm after 30 min	
Immobile[*]	Worm immobile for at least 5min while inside the maze, at any stage of the process	
Lost[*]	Worm burrowed in agar, or was lost during transfer from one maze to the next	

[#]: Left maze side: the arm of the maze that lies on the left side of the maze, with regard to the starting point; Right maze side: the arm of the maze that lies on the right side of the maze, with regard to the starting point. A nematode is considered to have made a left-side or a right-side decision when they reach the end of the respective arm (>half its body passed into the circular auxiliary area, as shown in Visualization column, see also Fig. 1);
[*]: censored worms, i.e. not included in indices or percentages calculations.

Supplementary Table S2 (related to Figures 1, 2, 3, 4, 5, 7): Raw data, including number of worms categorized as immobile, inconclusive, lost, or escaped (i.e., censored) for all experiments of Figs 1-7. Categories are defined as described in Table S1. Note: Animals that reached the left (food-containing) arm in Training equal the ones that are processed (N) in Testing. In the Rotated Testing maze experiment this is not the case, because a number of worms were lost during transfer from the Training to the Testing.

	Total (N)			Scored (n)			Left Maze Arm			Immobile			Inconclusive			Lost			Escape			Total censored		
	TRAIN	TEST	CTRL	TRAIN	TEST	CTRL	TRAIN	TEST	CTRL	TRAIN	TEST	CTRL	TRAIN	TEST	CTRL	TRAIN	TEST	CTRL	TRAIN	TEST	CTRL	TRAIN	TEST	CTRL
N2, Day 1 adults, Figs.1-4	116	90	73	106	70	70	90	51	36	1	4	0	1	3	0	2	1	0	6	12	19	10	20	19
Rotated Testing maze, Fig. 2A	116	66	73	96	56	70	83	34	36	5	6	0	4	2	0	1	3	0	19	10	19	29	21	19
Angled maze, Fig. 2B	100	62	84	88	45	75	62	33	29	2	6	0	1	0	2	0	0	0	9	7	7	12	13	9
Smooth maze, Fig. 2C	135	68	50	100	40	45	68	22	24	3	8	0	8	0	0	0	7	0	19	7	0	30	22	0
Seeded intermediate, 10min interval, Fig. 3A	100	52	73	74	45	70	52	20	36	0	0	0	6	5	0	2	1	0	18	1	19	26	7	19
Unseeded intermediate, 10min interval, Fig. 3A	102	51	73	74	44	70	51	19	36	0	0	0	13	7	0	2	0	0	13	0	19	28	7	19
Seeded intermediate, 5min interval, Fig. 3A	81	47	73	71	38	70	47	20	36	0	0	0	7	4	0	0	0	0	31	5	19	38	9	19
Training/Seq. Testing, Fig. 3B	114	72 31	73	87	44 21	70	72	31 10	36	0	8 2	0	6	0 1	0	0	2 2	0	13	15 4	19	19	25 9	19
Training-Stay5min-Testing, Fig. 3C	160	88	73	119	48	70	88	34	36	6	9	0	6	1	0	1	0	0	25	9	19	38	19	19
Training-Stay10min-Testing, Fig. 3C	55	36	73	45	20	70	36	11	36	0	1	0	0	3	0	5	12	0	5	16	19	10	32	19

odr-3 (n2150), Fig. 4A	115	52	40	95	40	33	52	18	16	0	3	3	0	2	0	1	0	0	6	11	8	7	16	11
odr-10 (ky32), Fig. 4A	92	44	33	85	39	24	44	20	12	0	1	3	0	0	1	0	0	0	7	4	5	7	5	9
mec-4 (e1611), Fig. 4B	117	55	74	96	37	63	55	18	27	1	4	1	2	0	0	0	3	0	6	8	11	9	15	12
mec-10 (e1515), Fig. 4B	99	55	44	88	40	36	55	22	20	0	3	0	3	1	2	0	6	0	8	4	1	11	14	3
trp-4 (ok1605), Fig. 4C	71	51	44	68	44	43	51	17	21	0	2	0	0	0	0	0	1	0	2	4	1	2	7	1
trp-1;trp-2 (sy690 sy691), Fig. 4C	95	65	45	84	42	41	65	22	21	0	2	0	3	11	1	1	5	0	4	5	3	8	23	4
crh-1 (tz2), Fig. 4D	141	65	44	105	44	35	65	19	17	4	9	2	8	1	1	0	5	0	16	7	6	28	22	9
dop-3 (vs106), Fig. 4D	130	57	39	109	40	32	57	15	15	4	6	2	5	1	1	0	2	0	12	3	4	21	12	7
pmyo3-mcherry, Fig. 6	97	63	27	80	37	25	63	27	13	7	11	0	7	3	0	3	3	1	8	6	1	25	23	2
N2, Day 1,fully fed, Fig. 7	166	72	73	102	50	70	72	38	36	0	4	0	16	10	0	4	0	0	44	8	19	64	22	19
N2, Day 5,fully fed, Fig. 7	85	51	73	76	41	70	51	21	36	0	0	0	1	3	0	0	0	0	4	5	19	5	8	19
N2, Day 1, starved, Fig. 7	105	52	73	72	50	70	52	35	36	0	0	0	7	1	0	2	0	0	24	1	19	33	2	19
N2, Day 5, starved, Fig. 7	77	48	73	67	43	70	48	30	36	0	1	0	5	1	0	0	1	0	6	1	19	11	3	19

Transparent Methods

Maze mold design, 3D-printing, and maze fabrication

Computer-aided design software SolidWorks, Dassault Systèmes, France, was used to design all maze molds, and Form1+ and Form2 (FormLabs, USA) 3D-printers were used to print them (Fig. 1), except from the smooth mazes molds (Supplementary Figure S1), which were printed in a ProJet 3500 HD Max 3D-printer. To generate the mazes, liquid NGM (2% agar) was poured in a 60mm petri dish up to 2/3 of the dish depth, and the mold was placed in, with the supporting arms firmly standing on the plate rim (Supplementary Figure S1). Once the agar based NGM solidified, the mold was pulled out and the mazes were imprinted in the NMG plate (Fig. 1, Supplementary Figure S1). The edges of the smooth mazes were trimmed to become leveled with the agar surface using a pair of tweezers. All the other mazes were built using a no-trim design, as explained in Fig. 1A and Supplementary Figure S1. Maze plates were usually prepared the day before the experiment, and in no case were they used >2 days post fabrication. These precautions were necessary to achieve a consistently moist surface. Because of the fabrication process, mazes were made of the exact same material used to culture worms in the lab (NGM) thus minimizing stress due to environment change and unfriendly material.

Experimental process

We used *C. elegans* N2 adult Day 1 hermaphrodites, grown in 20°C and well fed, unless stated otherwise. Each maze was used only once, and only one worm was placed in each maze for each trial. Each maze plate contained nine mazes, as a result of the maze mold design (see Fig. 1A). Maze plates were used either as Training or as Testing plates. In Training plates, mazes were loaded with food -and used- in triplets (by row or by column), to avoid food diffusion to neighboring mazes. Each Training maze plate was used within 1 hour from the first loading or was discarded. To load a Training maze, the maze plate was tilted so that the target maze arm was lower than the rest of the maze, and 1.2 microliters of liquid *E. coli* OP50 were carefully pipetted onto the floor of the circular auxiliary area (Fig. 1B, Supplementary Figure S2B schematic). For the aging

experiments (Fig. 7), OP50 was mixed in 1:1 ratio with 3M L-lysine, a well-known attractant (Bargmann, 2006), to a final volume of 1.2 microliters, to enhance the mixture's attractiveness to older *C. elegans*. Next, the maze plate remained tilted for about 10 min, until the OP50 solution was absorbed. Food was placed on the left side of the maze, unless stated otherwise (Supplementary Figure S2B).

Using a platinum wire worm picker, no food attached, a worm crawling outside the bacterial lawn was picked from the culture plate. These precautions were necessary to minimize the transfer of food along with the nematode. Next, the worm was placed on the floor of the starting area of the food-containing Training maze (bottom auxiliary area, Fig. 1B). Finally, a thin agar pad, made by gently pressing a droplet of NGM between two glass slides, was quickly placed on the open maze, to cover it and prevent the worm's escape. It is noted that the agar cover does not result in air-tight sealing of the maze.

Each nematode was recorded until it reached one of the maze arms (first decision recorded) or until 30 minutes had passed without reaching either end of the maze (Supplementary Table S1). The time limit of 30 min was selected based on the finding that the vast majority of nematodes reaches a maze arm in less than 30min (Supplementary Figure S4). If the worm reached the maze side with no food, it was not processed any further (except for experiments in Supplementary Fig. S2C,D). If the worm reached the maze side that contained food, it was allowed to remain in the area for ~3min. After 3 min, or as soon as the worm departed from the food-containing area, whichever happened first, the nematode was picked up using the picker. If the assay was concluded, the worm was discarded after it reached one of the maze ends. The same process was followed for worms tested in empty mazes (control experiments for all cases, and Fig. S2), with the omission of food placement.

If a testing trial was to follow, then immediately after removal from the Training maze, the worm was placed into a new, empty maze (Testing maze), in a different plate, made from the same NGM batch. The maze was covered using a thin agar pad and the worm was once again recorded until it reached one of the maze arms (first decision recorded) or until 30 minutes had passed without reaching either end of the maze (Supplementary Table S1).

The same process was followed when testing mutant strains (Fig. 4) and the Pmyo-3::mcherry::unc-54 strain (Fig. 6).

For the Training-Interval-Testing experiment (Fig. 3A), after the Training session, the worm in study was placed in an intermediate NGM 60mm plate, which was either unseeded or seeded with OP50. The worm remained there for 10 or 5min. Next, it was picked with a worm picker and placed in the Testing maze. If the intermediate plate was seeded, then the worm was picked when crawling outside the bacterial lawn, or if that was not possible within the interval time, then after the worm was picked it was lightly touched on a clean spot of the plate, to discard food stuck on its cuticle. The remaining steps were conducted as described above.

For the Training-Sequential Testing experiment (Fig. 3B), nematodes that reached the food-containing side of the first Testing maze were allowed to remain in the area for ~3 min. Next, or as soon as they departed, whichever happened first, they were transferred into a new, empty, second Testing maze. The remaining steps were conducted as described above.

To run the Training-Stay-Testing experiment (Fig. 3C), after they reached the food-containing area of the Training maze, nematodes were allowed to stay in the maze for ~8 or ~13min (an additional 5 or 10 minutes to the 3 min allowed in the food-containing area in other experiments). Then, they were transferred to the Testing maze and following steps were conducted as described above.

For the starvation experiments (Kaeberlein et al., 2006) (Fig. 7), nematodes were transferred to an unseeded 60mm NGM plate 24hours before the experiment took place, namely before they reached the age of interest.

Recording *C. elegans* in mazes

C. elegans' actions were recorded with a DP22 camera, mounted on a SZ61 dissection microscope, using CellSens Software (all by Olympus, Japan). For experiments with strain AVG09 Pmyo-3::mcherry::unc-54, a SZX12 Olympus fluorescent microscope was used. Videos were recorded with a time lapse, with a 1.2sec interval, or using the “movie”

module. A worm was considered to have made a left-side or a right-side decision when it reached the end of the respective functional maze arm (see also Fig. 1, and visualization in Supplementary Table S1). Nematodes that were inconclusive, immobile or lost, as well as worms that escaped, were censored (not scored) and not included in the indices and percentages calculations (Supplementary Table S1). To watch *C. elegans* traverse a maze, see Supplementary Videos.

Decision, Chemotaxis and Learning Indices

To quantify *C. elegans*' behavioral outcome in each experiment, we introduce the Decision Index (DI). Thus, for each maze experiment, the DI is

$$(DI) = (n_L - n_R)/(n_L + n_R)$$

where $n_L =$ worms that reached the left side of the maze, $n_R =$ worms that reached the right side of the maze. Note that $n_L + n_R = n$=scored worms= $N - n_{censored}$, where $N =$ total number of worms processed, and $n_{censored}$ =worms that were censored, namely worms categorized as inconclusive, immobile, lost, or escaped (Supplementary Table S1). All raw data for each experiment are reported in Supplementary Table S2, and in addition *n* for each experiment is also reported in the respective figures. The DI for the control experiment is $(DI)_{Control}$, for the Training Maze experiments is $(DI)_{Train}$, and for the Testing Maze experiments is $(DI)_{Test}$.

The Chemotaxis Index (CI), which refers to *C. elegans* performance in the Training Maze (in the presence of food on one maze end) is calculated as

$$(CI) = (DI)_{Train} - (DI)_{Control}$$

The Learning Index (LI), which refers to *C. elegans* performance in the Testing Maze is calculated as

$$(LI) = (DI)_{Test} - (DI)_{Control}$$

The LI and CI for each strain or experimental group, as well as the DI, are reported in the respective figures or/and in the text (see Results).

Note that we calculate all percentages and indices over the number of worms that reached a maze arm, because we calculate a conditional probability: the probability of a worm reaching the same maze arm, conditioned that it has reached an arm in the first maze.

C. elegans Strains

The strains used were N2 Bristol (wild type), YT17 *crh-1(tz2)* III, LX703 *dop-3(vs106)* X, CB1611 *mec-4(e1611)* X, CB1515 *mec-10(e1515)* X, CX2205 *odr-3(n2150)* V, CX32 *odr-10(ky32)* X. Mutant trains were provided by the CGC (Caenorhabditis Genetics Center). Strain VC1141 *trp-4(ok1605*) was acquired from CGC (Caenorhabditis Genetics Center, provided by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, which is part of the international *C. elegans* Gene Knockout Consortium) and was outcrossed 6 times before use (new strain EG001). Strain *trp-1(sy690) trp-2(sy691)* was a kind gift from Kyuhyung Kim Lab. Strain AVG09 Pmyo-3::mcherry::unc-54 + ppezo-1::gcamp6s::unc-54 was a kind gift from Andres Vidal-Gadea Lab.

Statistical Analyses

Each experiment was performed over a period of 2-4 weeks. Key experiments were replicated by different lab members, in two different lab locations and four different working stations. Comparisons between experiments, presented in Figures 1-7, were made by applying the binomial distribution probability test in MATLAB R2016b (Mathworks, USA) using the binomial cumulative distribution function of the Statistics and Machine Learning Toolbox. Details on the analysis and statistics, and the code used are provided in the Supplementary Information. In all cases, comparisons were considered statistically significant when p-value<0.05.

As described above, each worm was tested only once, and only one single worm was placed in the maze for each trial. Therefore, each worm corresponds to one single experiment. The percentages and indices reported are the percentages and indices that illustrate the behavior of the entire population in study (e.g., 85% of the worms that were

scored reached the left arm of the maze, with an index value equal to 0.68). In each figure we report the standard deviation of the preference indices (index error bars), along with the total number of worms that were scored (n) and the *p*-value of the binomial distribution test. For more details, see also Supplementary Information. All raw data are reported in Supplementary Table S2.

Comparisons presented in Supplementary Figure S4 were made by applying Student's two-tailed t-test, unpaired or paired, as explained in captions of Supplementary Figure S4, using GraphPad Prism 8 (GraphPad, USA). In all cases, comparisons were considered statistically significant when *p*-value<0.05.

On the standard deviation of the binomial probability and the preference indices

The binomial distribution, used to model our experimental results, has both a mean and a variance. The standard deviation of the binomial distribution is calculated using the formula

$$m = n * p$$

where m=actual number of worms that reached the left (food-containing) maze arm, and n=number of worms that were scored, and p=% of worms that reached the left (food-containing) maze arm, and then we define

$$m' = p$$

This leads to defining the variance as

$$\sigma^2 = \frac{p(1-p)}{n}$$

And therefore, standard deviation (STD) is defined as

$$\sigma = \sqrt{\frac{p(1-p)}{n}}$$

We report the standard deviation of the calculated index as follows:

For the DI of each experiment, it is (see Methods)

$$(DI) = (n_L - n_R)/(n_L + n_R)$$

where $n_L =$ worms that reached the left side of the maze, $n_R =$ worms that reached the right side of the maze, and n_L+$n_R = \mathrm{n} = \text{scored worms}$.

Therefore, it is

$$DI = \frac{2n_L - n}{n} = 2\,\frac{n_L}{n} - 1$$

Therefore, the mean is $\mu = 2p_{ctrl} - 1$, and the STD is $\sigma_{DI} = 2\sqrt{\frac{p_{ctrl}(1-p_{ctrl})}{n}} = 2\sigma$

Consequently, for the CI of each experiment, it is

$$(CI) = (DI)_{Train} - (DI)_{Ctrl} = 2\left(\frac{n_{LTrain}}{n_{Train}} - \frac{n_{Lctrl}}{n_{ctrl}}\right) = 2(p_{Train} - p_{ctrl})$$

Therefore, the mean is $\mu_{CI} = 2(p_{Train} - p_{ctrl})$

And the STD of the CI is $\sigma_{CI} = 2\sqrt{\frac{p_{ctrl}(1-p_{ctrl})}{n_{ctrl}} + \frac{p_{Train}(1-p_{Train})}{n_{Train}}}$

Similarly, the STD of the LI is $\sigma_{LI} = 2\sqrt{\frac{p_{ctrl}(1-p_{ctrl})}{n_{ctrl}} + \frac{p_{Test}(1-p_{Test})}{n_{Test}}}$

The index STD is illustrated in Figs 1-7 by index error bars, in the same color with the index symbol.

On the binomial distribution probability test in MATLAB using the binocdf function

In MATLAB, the binomial cumulative distribution function binocdf*(x,n,p*) computes a binomial cumulative distribution function at each of the values in x using the corresponding number of trials in *n* and the probability of success for each trial in *p*, where

x: the number of worms that reached the target location (i.e., left maze side),

n: the number of worms scored, and

p: the number of worms that reached the target location over the number of worms scored, in the reference (e.g. control) experiment.

The binomial cumulative distribution function binocdf*(x,n,p*) allows the user to obtain the probability of observing less than or equal to *x* successes in *n* trials, with the probability *p* of success on a single trial. The function 1-binocdf*(x-1,n,p*) allows the user to obtain the probability of observing greater than or equal to *x* successes in *n* trials, with the probability *p* of success on a single trial. These two functions were used to calculate the *p*-value under the null hypothesis in each comparison.

We provide below the MatLab code used, for reference.

```
n=32; %number of scored worms
% treatment experiment
n1=15; % actual number of worms who reached target location (e.g. left arm)
n1control=0.52*n; % expected number of worms that would have reached the
target location if the control ratio applied
t1=min([n-n1,n1]); % lower critical threshold for H0 (exclusive)
t2=max([n-n1,n1]); % upper critical threshold for H0 (exclusive)
pvalue = 1-binocdf(t2-1,n,n1control/n)+binocdf(t1,n,n1control/n)
```

References

Bargmann, C. I. 2006. Chemosensation in C. elegans. *WormBook*, 1-29.

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