Supplemental Data

Sensory Regulation of *C. elegans*

Male Mate-Searching Behavior

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Supplemental Results

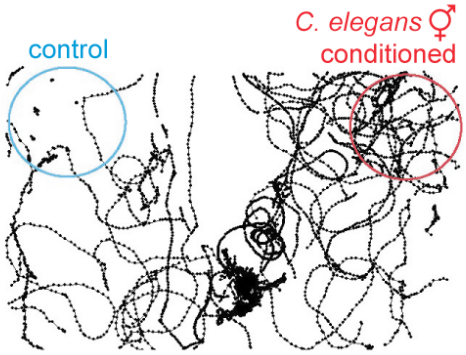
Hermaphrodite secreted accumulation cues do not block male exploratory behaviour.

To assess the behavioural response of males to hermaphrodite secretions, we developed the *accumulation assay*, a modification of the Simon and Sternberg assay [1]. In this assay a small population of males is given the choice, in a plate where food is present everywhere, between a spot of agar soaked with M9 buffer pre-incubated with hermaphrodites and a control spot of agar soaked with M9. We observed an accumulation of males at the conditioned spot (supplemental Fig.1A) indicating that males can sense hermaphrodite-secreted chemical cues released into the environment. During a 20 minutes observation period, males repeatedly visited the conditioned spot more than the control spot and once a male entered the conditioned spot, it spent on average 148 sec ±14 sec compared with 47 sec ±7 sec when it entered the control spot (supplemental Fig.1B).

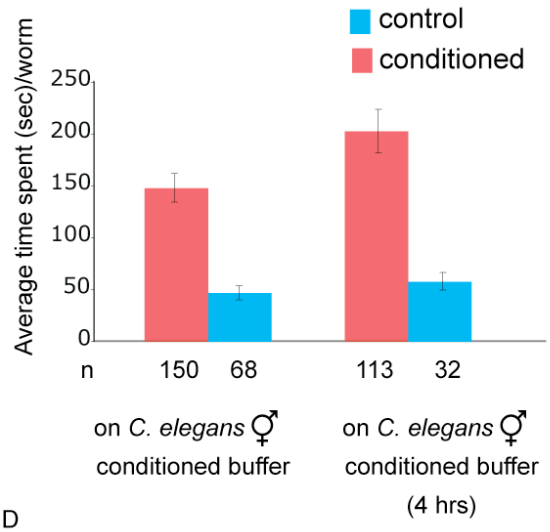
To assess the contribution of this behavioural response to the suppression of male exploratory behaviour in the leaving assay, we placed each individual male in a spot of food soaked either with a drop of M9 as control or soaked with a

drop of hermaphrodite-conditioned M9. In this experiment, males left both spots of food at the same rate, with C_r= 0 (supplemental Fig.1C). In a parallel experiment, spots of agar that had been soaked with the same hermaphrodite-conditioned M9 could, after 4 hours (a time at which almost half of the population of males had left in the leaving assay) still elicit a behavioural response in a new population of males (202 ±21 sec spent on conditioned spot versus 58 ±8 sec in control spot) (supplemental Fig.1B), indicating that the hermaphrodite cues are not labile and are still present at that time. From this we conclude that the behavioural change induced by hermaphrodite-secreted cues is not sufficient to account for the inhibition of male exploratory behaviour.

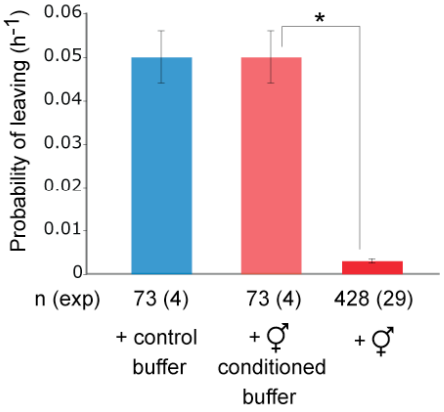
A


control
C. elegans ♂
conditioned

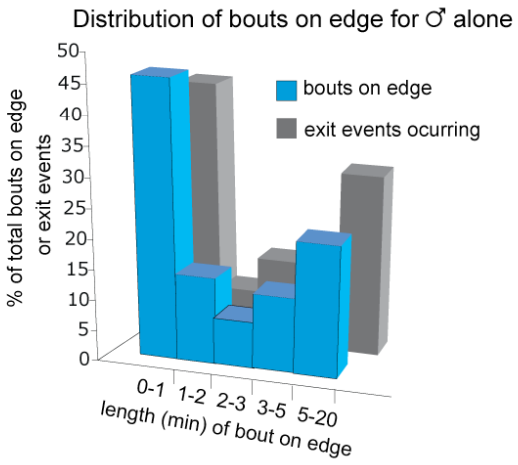
B


control
conditioned
Average time spent (sec)/worm
250
200
150
100
50
0
n
150
68
113
32
on C. elegans ♂ conditioned buffer
on C. elegans ♂ conditioned buffer (4 hrs)

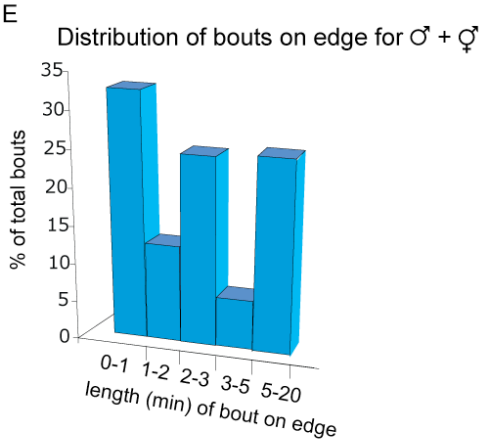
C


Probability of leaving (h-1)
0.06
0.05
0.04
0.03
0.02
0.01
0
*
n (exp)
73 (4)
73 (4)
428 (29)
+ control buffer
+ ♂ conditioned buffer
+ ♂

D


Distribution of bouts on edge for ♂ alone
% of total bouts on edge or exit events
50
45
40
35
30
25
20
15
10
5
0
bouts on edge
exit events ocurring
0-1
1-2
2-3
3-5
5-20
length (min) of bout on edge

E


Distribution of bouts on edge for ♂ + ♀
% of total bouts
35
30
25
20
15
10
5
0
0-1
1-2
2-3
3-5
5-20
length (min) of bout on edge

F

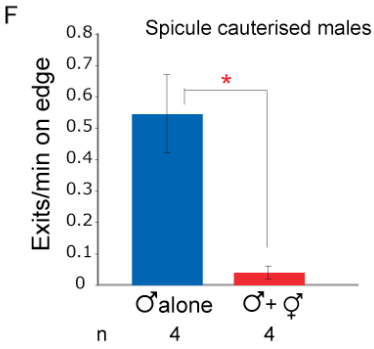

Spicule cauterised males
Exits/min on edge
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
♂alone
♂+ ♀
n
4
4

Figure S1. Hermaphrodite secreted accumulation cues do not inhibit mate searching behaviour.

(A) Males accumulate at a spot of agar conditioned with *C. elegans* hermaphrodite secretions. Video tracking of a population of 12 males in response to a control spot of agar soaked with buffer (M9) and one soaked with buffer conditioned with *C. elegans* hermaphrodites. 3 cm plates with food were used and the spots were placed 1.5 cm away from the males and from each other. Locomotion was recorded for 20 min and analysed with DIAS software.

(B) *C. elegans* hermaphrodites secrete cues that attract males and are long lasting. Average time that a male spent every time it encountered a region pre-treated with hermaphrodite-conditioned buffer or control M9 buffer on a food seeded agar plate. Buffer (M9) was conditioned for 4 hrs with *C. elegans* hermaphrodites and a drop allowed to soak into the agar. Males response was assayed either within 5 minutes after placing the media onto the plate or 4 hours later. n= number of events, note the higher frequency at which a male enters a spot conditioned with *C. elegans* hermaphrodites compared with a control spot. Mann-Whitney test was used to compare time spent in conditioned versus control spots; * indicates $p<0.01$. Error bars indicate SEM.

(C) Hermaphrodite secreted accumulation cues do not retain males. Probability of leaving (P_L) for males in the presence of buffer, buffer conditioned with *C. elegans* hermaphrodites or intact *C. elegans* hermaphrodites. The presence of buffer alone slowed down the rate at which males left, possibly due to salt

attraction. * indicates $p< 0.001$ when P_L values are compared to each-other. Error bars indicate SEM.

(D) For males alone, most exit events are produced during bouts on the food edge that are less than 1 minute in length. Distribution of the total bouts on the food edge according to their duration (in minutes) and distribution of the total exit events according to the length of the bout on the food edge during which they occurred. n=4 worms in 1h 30 min observation periods.

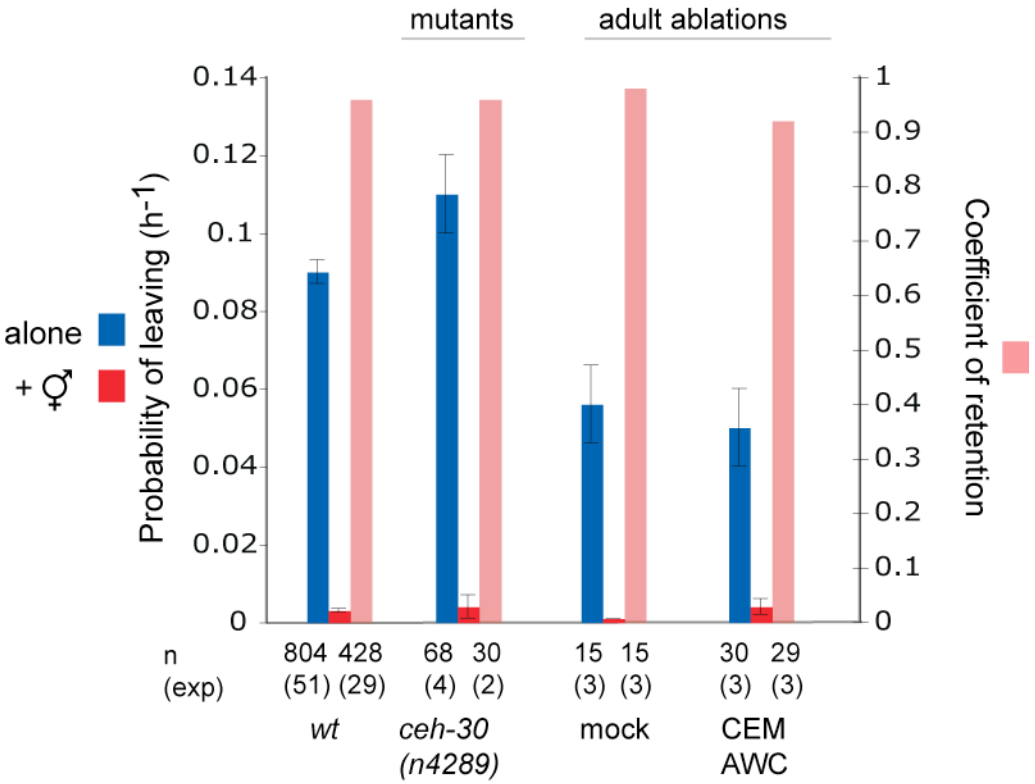
(E) For males with hermaphrodites, most bouts on the food edge are 1 minute or more than 5 minutes in length. Distribution of the total bouts on the food edge according to their duration (in minutes). n=4 worms in 1h 30 min observation periods. Only 2 exit events were observed in these observations.

(F) Exit events at the food edge are suppressed in males without copulatory organs. Exit events were scored in males with cauterized spicules alone and in the presence of hermaphrodites. For each condition, four males were analysed during observation periods of 1 hour and 30 minutes. * indicates $p< 0.003$ compared to each-other (t-test). Error bars indicate SEM.

Hermaphrodite pheromones contribute minimally to retention of males with high exploratory behaviour.

Two olfactory amphid neurons AWA and AWC and the male-specific CEM neurons constitute the circuit for male chemotaxis towards secreted hermaphrodite pheromones [2]. This circuit is impaired in *osm-9* and *ocr-2;ocr-1* double mutants but not in *ocr-2* single mutants [2]. All these genes encode subunits of a TRPV channel widely expressed in the sensory nervous system [3] and required for food attraction in hermaphrodites (supplemental Fig.2B). To assess the contribution of these pheromone-sensing neurons to the inhibition of male exploratory behaviour by hermaphrodites we disrupted their function by laser ablation and genetic mutations. Ablation of the CEM and AWC neurons in adult males caused no defects in exploratory behaviour or in retention (supplemental Fig.2A). In contrast, *osm-9(ky10)* and *ocr-2(ak47);ocr-1(ok132)* double mutants displayed a higher rate of exploratory behaviour and a small but significant loss of retention (Cr=0.83 and Cr=0.91 respectively) (supplemental Fig.2B). These results suggest that secreted hermaphrodite pheromones may contribute minimally to retention in males with broader sensory defects.

A


mutants
adult ablations
alone
+ ♀
Probability of leaving (h-1)
Coefficient of retention
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
n
(exp)
804 428
(51) (29)
68 30
(4) (2)
15 15
(3) (3)
30 29
(3) (3)
wt
ceh-30
(n4289)
mock
CEM
AWC

B

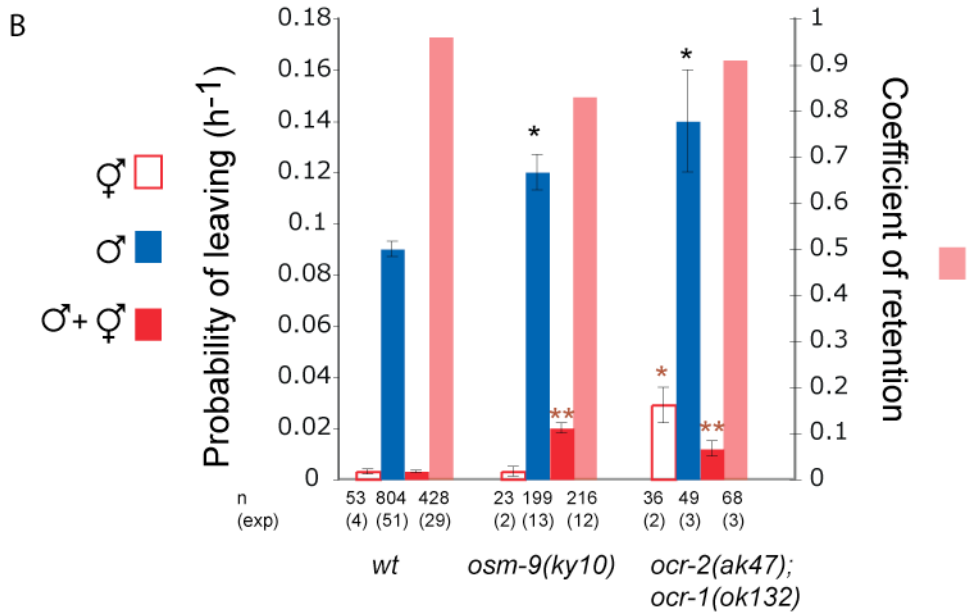

♀
♂
♂+ ♀
Probability of leaving (h-1)
Coefficient of retention
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
n
(exp)
53 804 428
(4) (51) (29)
23 199 216
(2) (13) (12)
36 49 68
(2) (3) (3)
wt
osm-9(ky10)
ocr-2(ak47);
ocr-1(ok132)

Figure S2. Hermaphrodite pheromones contribute minimally to retention of males with high exploratory behaviour.

(A) Loss of CEM neurons during embryogenesis or during adulthood does not affect male exploratory behaviour or retention. P_L and C_r values alone and in the presence of hermaphrodites for *wild type*, *ceh-30(n4289)* mutant males (which lack CEM neurons), mock ablated males and males in which 4 to 6 out of 4 CEM and 2 AWC neurons have been ablated. Ablation of AWC and CEM neurons was performed in young adults in a *bxIs14*(P(*pkd-2)::gfp)*; *oyIs44*(P*(odr-1)::rfp)* background to visualize the target neurons. Mock animals underwent the same manipulations as ablated animals with the exception of being shot with the laser microbeam. These manipulations slightly reduced the rate of *leaving* alone compared to non-manipulated males. Error bars indicate SEM.

(B) TRPV channels modulate male exploratory behaviour and retention. Bars show the P_L and C_r values of hermaphrodites alone, males alone and males with hermaphrodites for wt and mutants in TRPV channels. Strains used: *wt*, *osm-9(ky10)* and *ocr-2(ak47);ocr-1(ok132)*; * indicates $p< 0.001$ when P_L values are compared to wt males alone; * indicates $p< 0.001$ when P_L values are compared to wt hermaphrodites; ** indicates $p< 0.001$ when P_L values are compared to wt males with hermaphrodites. Error bars indicate SEM.

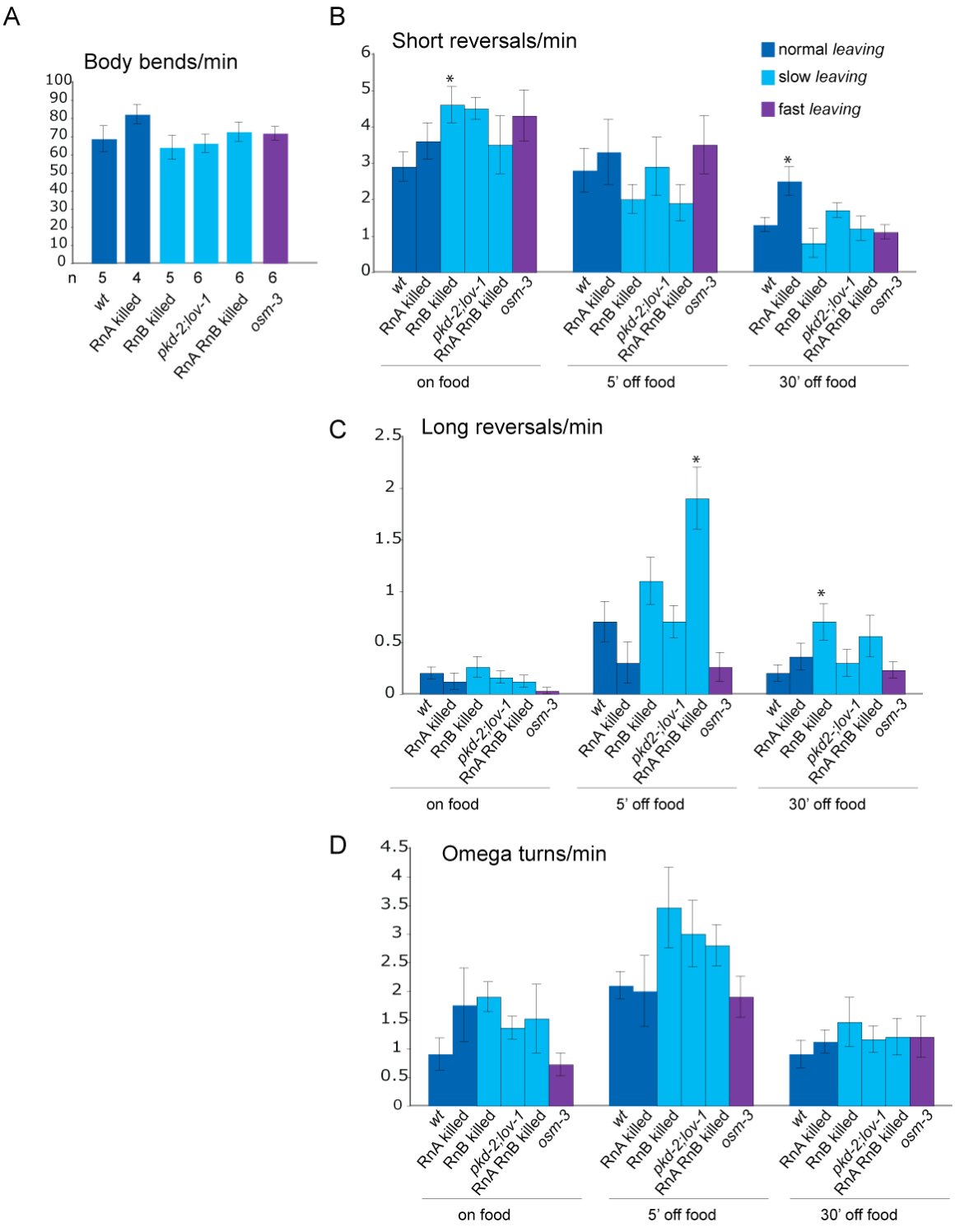

A
Body bends/min
100
90
80
70
60
50
40
30
20
10
0
n 5 4 5 6 6 6
wt
RnA killed
RnB killed
pkd-2;lov-1
RnA RnB killed
osm-3
B
Short reversals/min
normal leaving
slow leaving
fast leaving
6
5
4
3
2
1
0
*
*
wt
RnA killed
RnB killed
pkd-2;lov-1
RnA RnB killed
osm-3
on food
5' off food
30' off food
C
Long reversals/min
2.5
2
1.5
1
0.5
0
*
*
wt
RnA killed
RnB killed
pkd2-;lov-1
RnA RnB killed
osm-3
on food
5' off food
30' off food
D
Omega turns/min
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
wt
RnA killed
RnB killed
pkd-2;lov-1
RnA RnB killed
osm-3
on food
5' off food
30' off food

Figure S3. Locomotion analysis of males with defects in exploratory behaviour

(A) Frequency of body bends is similar in males with normal, reduced and increased rate of leaving behaviour. Bar graph plots the average number of body bends/minute for *wild type, bxEx137* (RnA killed)*, bxEx136* (RnB killed)*, pkd-2*(sy606);*lov-1*(sy582), *bxEx136;bxEx137* (RnA and RnB killed) and *osm-3(p802)* males on food during 2 minutes assays. n= number of assayed animals; t-test was used for statistical analysis. Error bars indicate SEM.

(B-C-D) Males with reduced leaving behaviour show an increase in omega turns or long reversals immediately off food. Frequency of short reversals (B), long reversals (C) and omega turns (D) measured during 5 minutes on food, immediately off food and after 30 minutes off food. Same genotypes as in A, number of worms assayed was 7 for *wild type,* 5 for RnA killed and 6 for the rest. Short reversals were measured as lasting two or fewer head swings and long reversals as lasting 3 head swings or more. * indicates $p < 0.03$ when compared to wild type; t-test. Error bars indicate SEM.

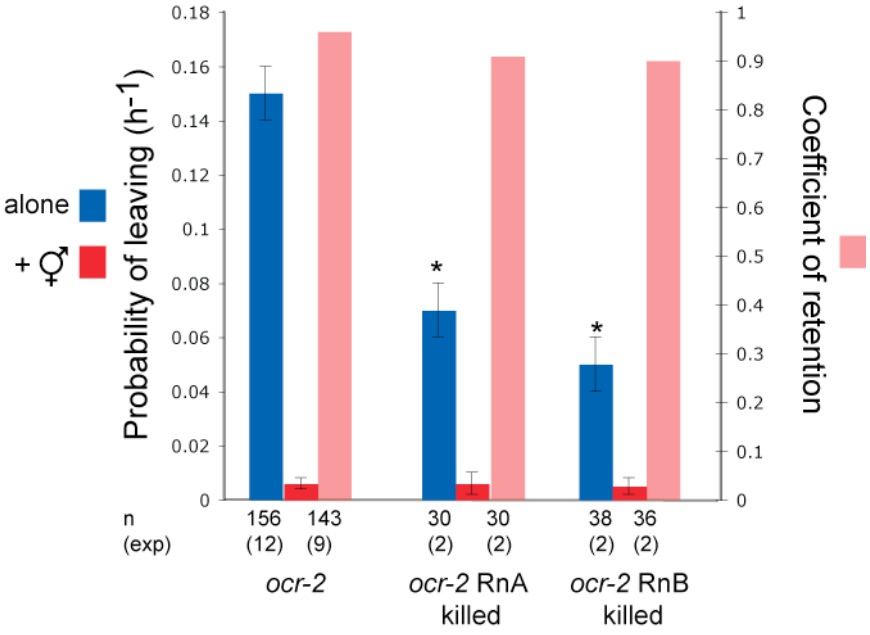


Figure S4. Amphid and ray neurons regulate male exploratory behaviour antagonistically.

Bars show the P_L and C_r values of males alone and with hermaphrodites for *ocr-2* mutants with and without RnA and RnB neurons. Strains used: *ocr-2(ak47);bxIs14(*P*(pkd-2)::gfp); bxEx136*[P*(pkd-2)::ICE*+P*(unc-122)::gfp*] for RnB genetic ablations and *ocr-2(ak47);bxEx138*[P*(trp-4)::*ICE+P(*elt-2)::gfp*] for RnA genetic ablations. * indicates $p < 0.001$ when compared to *ocr-2* mutants. Error bars indicate SEM.

Table 1. Death efficiency in caspase-expressing lines

	Number of RnA neurons alive (out of 18 total)	
	bxEx137 (reconstituted caspase)	bxEx138 *(trp-4::ICE)*
male 1	3	6
male 2	2	7
male 3	4	5
male 4	5	4
male 5	2	9
male 6	3	6
male 7	0	8
male 8	3	3

Table 2. Mating assays in caspase-expressing lines

Genotype	Response % (n=number of males)	Defective Turning % (n=number of males)
wt	90 (10)	8 (18)
bxEx136 (noRnB)	6 * (46)	n.d.
pkd-2(sy606);lov-1(sy582)	50*(20)	n.d.
bxEx137 (noRnA)	n.d.	38* (11)
cat-2 (e1112)	n.d.	36 *(14)

Table S1. Death efficiency in caspase-expressing lines.

Caspase trans-genes were observed in the *bxIs19* background which labels RnA neurons. The number of green cells was counted in 8 worms of each line.

Table S2. Mating assays in caspase-expressing lines.

Assays for response to hermaphrodite contact were performed in males with defects in RnB neurons (*bxEx136* and *pkd-2*(*sy606); lov-1(sy582)).* In each assay, one or two males were placed with 40 hermaphrodites in a 0.5 cm patch of food for 3 minutes. A male was scored as positive for response if its tail was placed ventral down on the hermaphrodite body for 20 consecutive seconds. Assays were done on different days.

Turning assays were performed on males with defects in RnA neurons (*bxEx137* and *cat-2(e1112)*). In each assay, one male was placed with 20 paralysed hermaphrodites in a 0.5 cm patch of food for 5 minutes. The percentage of

defective turns were scored for each male and averaged for the genotype. Assays were performed through different days.

(* indicates $p<0.01$ compared to wild type; Mann Whitney test)

Supplemental Experimental Procedures

Leaving assay: Male exploratory behaviour is measured with the leaving assay, which exploits the male's tendency to leave a food source in the absence of mating partners as previously described in [4]. A population of between 10 and 20 males was assayed by placing each male individually in a Petri plate (9 cm diameter and 10 ml agar) in the centre of a 20 μl (10 mm diameter) patch of food (*E. coli* OP50 seeded the night before). To assay retention, each individual test male was placed together with 2 paralysed *unc-51(e369)* hermaphrodites or males. Plates were kept at 20^{o}C during the 24 hrs assay and at 4 time points the proportion of males that had left the food was scored. A male was considered a leaver and left censored if at the scoring time point it had reached a 3 cm distance from the food patch. To assay retention with fixed worms, adult males and hermaphrodites were fixed in freshly made 4% PFA in PBS at 4^{o}C overnight. Fixed worms were then rinsed twice in PBS before being placed in the patch of food for the retention assay. To block contact between tester male and fixed worms, after being placed on the patch of food, a layer of 5% agarose (Sigma) was applied on top of the fixed worms with a tungsten worm pick. This resulted in a layer of agarose 0.5 mm thin on either side of the worm, a few microns thin on top of the worm and nothing underneath, between the worm and the food.

To determine the proportion of time a male spends on the food edge, in contact with the hermaphrodite and the number of exit events, leaving assays were recorded for 1hr 30 min with a video camera attached to a dissecting scope (at x10). The number of exit events was scored blindly. An exit event is scored when the whole body of the male is outside of the patch of food. Mann-Whitney test was used for statistical analysis.

To make the graphs that illustrate the behaviour of the male in relation to the food edge and the centre of the plate/hermaphrodite in the leaving assay, 60 min digital movies were analyzed with Image J software. Using a tracking plugging, the distance between two objects was calculated over time. The male was marked as object 1 and a region in the centre of the plate (empty for a male alone or containing both hermaphrodites) was marked as object 2 (immobile). The size of the diameter of the food patch was used to scale pixels into mm.

Calculation of leaving rates and statistical analysis: As previously demonstrated in [4] male leaving behaviour was modelled with the exponential function: $N(t)/N(0)=exp(-\lambda t)$. $N(0)$ is equal to the number of worms at time zero, $N(t)$ is the number of worms at time t (in hours). λ is the P_L value or probability of leaving, per worm, per hour. P_L values for each genotype and condition were calculated using R (http://www.R-project.org) to fit the censored data with an exponential parametric survival model, using maximum likelihood. The hazard values obtained were reported as the P_L values. To estimate the P_L values, S.E.M. and the 95% confidence intervals, worms from each experimental

treatment were then pooled across replicas and contrasted against controls using maximum likelihood. Contrasts were performed as follows: for male exploratory behaviour, P_L values for mutant males alone were contrasted against P_L value for wild type males alone; because retention can be partial or total, two types of contrasts were made, P_L values for wild type or mutant males with hermaphrodites were contrasted against P_L values for the same males alone to identify any level of retention; to identify any loss from maximum level of retention, P_L values for mutant males with hermaphrodites were contrasted against P_L values for wt males with hermaphrodites.

Laser ablations: The standard protocol was used [5]. Animals were mounted on a glass slide on a 5% agar pad with 10mM NaAzide as anaesthetic. L1 stage animals were used for the ablation of ray, post-cloacal sensilla and EF/DX inter-neuron precursor cells, L2 animals were used for ablation of hook precursor cells. All these cells were identified by morphology and position with Normanski optics. Young adults were used for CEM, AWC, SPD, SPV and SPC neurons ablation and for spicule tips cauterisation. Neurons were identified using reporter constructs: *P(pkd-2)::gfp* for CEMs, *P(odr-1)::rfp* for AWC, *P(gpa-1)::gfp* for SPD, SPV and SPC. NaAzide causes the spicules to protract allowing the cauterisation of the protruding tips. All ablated animals were allowed to recover for at least 40 hrs or until adulthood before assaying. Animals were assayed on two consecutive days first for retention and then for exploratory behaviour. After the

assay, loss of the targeted sensilla or neuron was confirmed under the microscope and animals were discarded if the target neurons were still alive.

Strains: Nematodes were maintained as described in Brenner [6]. Bristol (N2)-derived strain *him-5(e1490),* which generates a high incidence of males [7], was used as reference wild type strain. All *C. elegans* strains used in this paper, except *lin-39(n1760), lin-7(e1413), mec-4(n1611)* and *ocr-2(ak47);ocr-1(ok132)*, were made in the *him-5(e1490)* background. These include *unc-51(e369), ceh-30(e4289), bxIs14(P(pkd-2)::gfp*), *oyIs44(P(odr-1)::rfp*), *mab-3(mu15), lin-32(e1926), syIs33(P(gpa-1)::gfp), osm-3(p802), pkd-2(sy606); lov-1(sy582), tax-2(p691)*, *osm-9(ky10)*, *ocr-2(ak47), zdIs31(P(dat-1)::gfp); akEx387(P(dat-1)::ICE*, *P(dat-1)::gfp*) and *cat-2(e1112)*. *C. briggsae* AF16 and *C. briggsae mip-6(sy5019)* were also used.

Caspases strains and DNA constructs:

RnB neurons were killed by expressing the human ICE caspase [8] under the *pkd-2* promoter [9], P*(pkd-2)::ICE*. RnB death was monitored in the *bxIs14(P(pkd-2)::gfp*) reporter background and only males in which GFP had disappeared from all ray neurons were assayed. RnA neurons were killed using the reconstituted caspase approach [10]. To drive caspase expression in RnA neurons, we used the *grd-13* promoter, expressed in the ray precursor cells (the seam cells) [11] and the *trp-4* promoter [12] which we see drives expression in RnA and possibly HOA neurons in the tail. We also made a strain where RnA

neurons were killed by expression of P*(trp-4)::ICE*. RnA death efficiency for both strains was assessed in the *bxIs19(P(trp-4)::gfp)* reporter background and it was found to be not fully penetrant (Supl table 1 and 2). Because the *bxIs19* reporter line displayed defects in exploratory behaviour, leaving assays of RnA killed males were performed in a reporter-absent wild type background and therefore the number of RnA cells surviving could not be assessed in assayed animals.

The transgenic strain *bxEx136* was built in the background of the *bxIs14(P(pkd-2)::gfp*) reporter line [13] by co-injecting the PCR fusion construct P*(pkd-2)::ICE* (10 ng/μl) together with the marker P*(unc-122)::gfp* (40 ng/μl). The P*(pkd-2)::ICE* fusion was generated by amplifying the 1.8 kb region upstream of the *pkd-2* gene and the ICE coding region out of the P*(dat-1)::ICE* plasmid (pTH5, [8]). The *P(trp-4)::gfp* plasmid EM#316 was generated by inserting the 8kb region upstream of the *trp-4* gene (from 25692 to 33658 of Y71A12B) into the pPD95.75 vector. The line *bxIs19* was generated by co-injecting EM#316 (25 ng/μl) with the marker *P*(*ttx-3)::gfp* (50 ng/μl) and integrating the array by γ irradiation as described in [14]. The *P(trp-4)::caspase3-NZ* plasmid EM#317 was generated by removing the *gfp* coding region from EM#316 and introducing the *caspase3-NZ* coding region from the pTU813 plasmid (BamHI-EcoRI region) [10]. The *P(grd-13)::CZ-caspase3* plasmid EM#318 was generated by inserting the 712 nt region upstream of the *grd-13* gene (from 3911 to 4623 of W05E7) between the HindIII-BamHI sites of the *CZ-caspase3* plasmid pTU#814 [10]. The *bxEx137* line was generated by co-injecting EM#317 and EM#318 (25 ng/μl each) with the marker *elt-2::gfp* (50 ng/μl) and RnA neuronal death was monitored by crossing *bxEx137*

into *bxIs19*. The *P(trp-4)::ICE* construct EM#319 was generated by removing the *gfp* coding sequence from EM#316 and replacing it by the *ICE* coding region from the P(*dat-1)::ICE* plasmid (SalI-ApaI region in both plasmids). *bxEx138* transgenic worms were generated by co-injecting EM#319 at 20 ng/μl with the marker *elt-2::gfp* (50 ng/μl) into *bxIs19*.

Response to hermaphrodite secreted cues: hermaphrodite secreted cues were prepared by incubating hermaphrodites in M9 buffer at a concentration of 1 hermaphrodite per 1 μl of M9 for up to 4 hrs. To determine a male's behavioural response to hermaphrodite secretions we used the *accumulation assay*. Two μl of M9 and two μl of M9 incubated with hermaphrodites were placed 1.5 cm apart from each other in a 3.5 cm plate (seeded the night before with OP50). A population of typically 12 males was placed 1.5 cm away from each spot immediately after the spots were dry (circa 5 min) and for 20 minutes, their locomotion was recorded (1 frame/second) with a digital camera attached to a dissecting microscope. The spots of conditioned and control M9 were visible by the rim left on the food loan after drying. Each time a male entered one of the spots, the time spent within the rim was counted. Typically, a male stayed for a length of time within either spot before leaving and exploring other areas of the plate away from the spot. A male may come back to the spot or not. Each entrance to the spot was considered an independent event if after leaving, the worm had moved at least 0.5 cm away from the spot. The number of events for each spot was also counted. Mann-Whitney test was used for statistical analysis

to compare the average time for each event. Diagrams representing video tracks were obtained with DIAS ver.3.1 software (Soltech).

To assess the ability of the hermaphrodite-conditioned medium to retain males in the retention assay, 1 μl of either control or conditioned M9 was soaked on the patch of food. The patch of 20 μl of food occupied an area of 10 mm diameter in the agar plate and 1 μl of M9 occupied an area of 4 mm diameter within the patch of food. Males were assayed after the medium was soaked into the agar/food (circa 5-10 minutes).

Locomotion assays

Single worms assays were performed on 5 cm plates with food. Body bends were scored by eye during 2 minutes observation periods under a dissecting microscope. Subsequently, reversals and omega turns for that same worm were scored during 5 minutes. The worm was then transferred to a plate without food and reversals and omega turns were scored for 5 minutes one minute after transfer and 30 minutes later. Worms of one same genotype were scored in two different days and a wild type control was assayed in parallel. Short reversals were measured as lasting two or fewer head swings and long reversals as lasting 3 head swings or more. t- test was used for statistical analysis

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