LETTERS

A hub-and-spoke circuit drives pheromone attraction and social behaviour in *C. elegans*

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Innate social behaviours emerge from neuronal circuits that interpret sensory information on the basis of an individual's own genotype, sex and experience. The regulated aggregation behaviour of the nematode *Caenorhabditis elegans*, a simple animal with only 302 neurons, is an attractive system to analyse these circuits. Wild social strains of *C. elegans* aggregate in the presence of specific sensory cues, but solitary strains do not[1–4]. Here we identify the RMG inter/motor neuron as the hub of a regulated circuit that controls aggregation and related behaviours. RMG is the central site of action of the neuropeptide receptor gene *npr-1*, which distinguishes solitary strains (high *npr-1* activity) from wild social strains (low *npr-1* activity); high RMG activity is essential for all aspects of social behaviour. Anatomical gap junctions connect RMG to several classes of sensory neurons known to promote aggregation, and to ASK sensory neurons, which are implicated in male attraction to hermaphrodite pheromones[5]. We find that ASK neurons respond directly to pheromones, and that high RMG activity enhances ASK responses in social strains, causing hermaphrodite attraction to pheromones at concentrations that repel solitary hermaphrodites. The coordination of social behaviours by RMG suggests an anatomical hub-and-spoke model for sensory integration in aggregation, and points to functions for related circuit motifs in the *C. elegans* wiring diagram.

Many naturally isolated social strains of *C. elegans* aggregate into feeding groups with dozens of animals, although other strains, including the laboratory strain N2, are solitary[1,2]. Aggregating strains show several behavioural changes compared to solitary feeders: they accumulate on the border of a lawn of bacterial food (bordering) and move rapidly on food. Aggregation, bordering and rapid movement are coordinately controlled by the neuropeptide Y receptor homologue NPR-1 (ref. 2). Solitary strains have a high-activity form of NPR-1 (215-valine), whereas aggregating strains have a low-activity form of NPR-1 (215-phenylalanine); *npr-1* null mutants also aggregate[2,6]. Neuropeptide control of aggregation provides an analogy with mammalian social behaviour, which is regulated by the neuropeptides oxytocin and vasopressin[7]. In addition to genetic regulation by *npr-1*, aggregation is sensitive to environmental signals. It is stimulated by URX sensory neurons that detect environmental oxygen[4], and ASH and ADL sensory neurons that sense noxious stimuli[3]. Attraction to low-oxygen environments promotes accumulation at the lawn border and feeding in groups, which have low oxygen levels compared to the open lawn[4,8]. Population density, food availability[3] and environmental stressors[9] also modulate aggregation. The site of integration of these diverse cues is unknown.

How NPR-1 acts to regulate behaviour is not well understood. A previous report using a genomic *npr-1* fragment identified the oxygen-sensing URX neuron as a site of *npr-1* action, but behavioural rescue was incomplete, with rescue of aggregation, partial rescue of bordering, and no rescue of rapid movement[10]. To identify other neurons in which NPR-1 promotes solitary behaviour, we first established that a full-length *npr-1* complementary DNA expressed from the endogenous *npr-1* promoter rescued solitary behaviour in the strong loss-of-function mutant *npr-1*(*ad609lf*), then refined the essential site of expression using other characterized promoters (Fig. 1a, b). Because promoter expression patterns in *C. elegans* can vary between transgenes, we used a bicistronic messenger RNA to express both *npr-1* and green fluorescent protein (GFP), and identified GFP-positive neurons in each rescued line with solitary behaviour (Supplementary Table 1 and Methods). Only promoters driving expression in the inter/motor neuron RMG showed robust rescue of aggregation, bordering, and locomotion speed (Fig. 1b and Supplementary Fig. 1).

We next addressed whether RMG expression of *npr-1* is sufficient to suppress aggregation. No RMG-specific promoter is known, so an intersectional strategy was developed to drive *npr-1* expression only in cells that express both *flp-21* and *ncs-1*, using Cre-mediated recombination between *loxP* sites that flanked transcriptional stop sequences. When *ncs-1::nCre* and *flp-21::LoxStopLox::GFP* strains were crossed together, the intersection between *ncs-1* and *flp-21* allowed strong and consistent GFP expression only in RMG and M2 pharyngeal neurons (Fig. 2a). We next inserted the *npr-1* cDNA into the *flp-21::LoxStopLox* plasmid (Fig. 2a); in *npr-1*(*lf*) animals expressing both *flp-21*::*LoxStopLox*::*npr-1* and *ncs-1*::*nCre*, aggregation, bordering and high speed on food were strongly suppressed (Fig. 2b). M2 is synaptically isolated from neurons implicated in these behaviours, so we conclude that RMG expression of *npr-1* can block aggregation and related behaviours.

Mammalian neuropeptide Y receptors generally inhibit neurotransmitter release[11,12]. To determine whether NPR-1 suppresses aggregation by inhibiting or by activating RMG, we killed RMG in wild-type and *npr-1*(*lf*) animals using a laser microbeam, anticipating an effect on the genotype(s) in which RMG is normally active. Killing RMG in *npr-1*(*lf*) eliminated aggregation, bordering and rapid movement (Fig. 2c, d), whereas killing RMG in solitary wild-type animals had no effect (Fig. 2d). These results show that RMG neurons stimulate aggregation-related behaviours in *npr-1* mutants, and indicate that NPR-1 inhibits RMG activity in solitary strains.

Inspection of the *C. elegans* wiring diagram[13] revealed that RMG is the hub of a gap-junction network connecting seven classes of neurons, including the oxygen-sensitive URX neurons and the nociceptive ASH and ADL neurons previously implicated in aggregation behaviour[3,10] (Fig. 3a). RMG-ablated *npr-1* animals were normal in their avoidance of high osmolarity, a behaviour mediated by ASH[14] (Supplementary Fig. 2). Therefore RMG is not essential for all functions of associated sensory neurons, but selectively required for aggregation and related behaviours.

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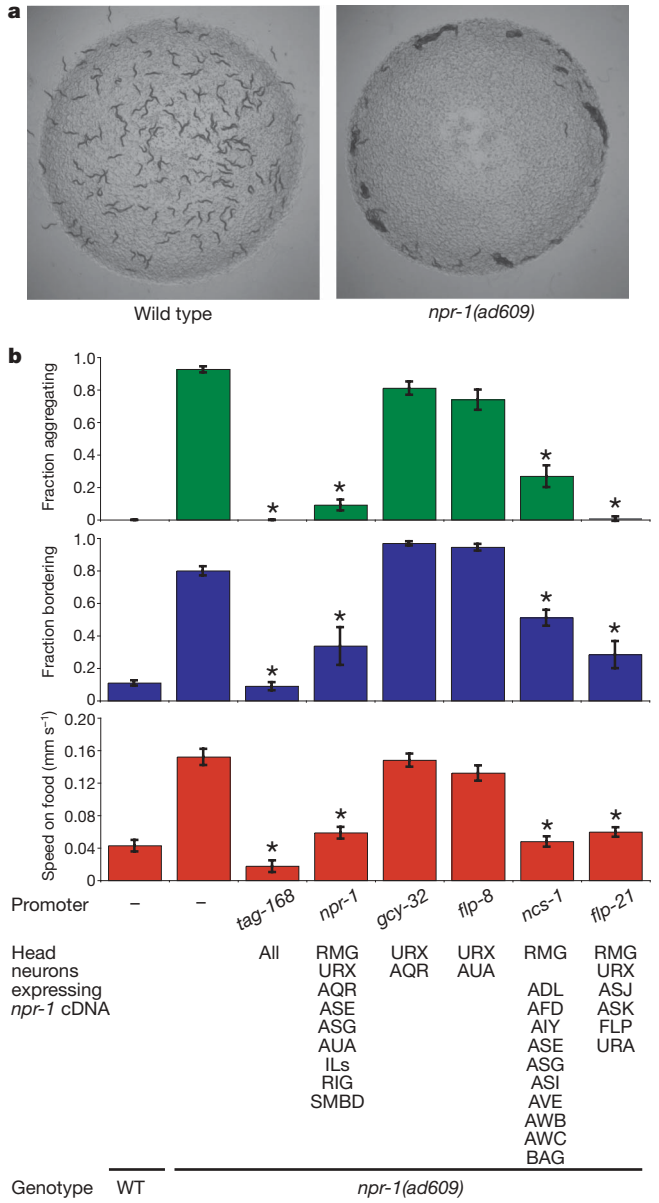

Figure 1 | Selective expression of NPR-1 suppresses aggregation and related behaviours in *npr-1* mutants. **a**, Solitary behaviour of 150 wild type N2 animals (left) and aggregation behaviour of 150 *npr-1*(*ad609*) animals (right). Magnification × 2. **b**, Behavioural phenotypes of *npr-1*(*ad609*) animals expressing an *npr-1* cDNA under a pan-neuronal promoter (*tag-168*), its endogenous promoter (*npr-1*), URX promoters (*gcy-32* and *flp-8*) and RMG promoters (*ncs-1* and *flp-21*). For all figures, full promoter expression patterns are in Supplementary Table 1. Error bars indicate s.d. Asterisk, different from *npr-1*(*ad609*) ($P < 0.01$, Bonferroni test).

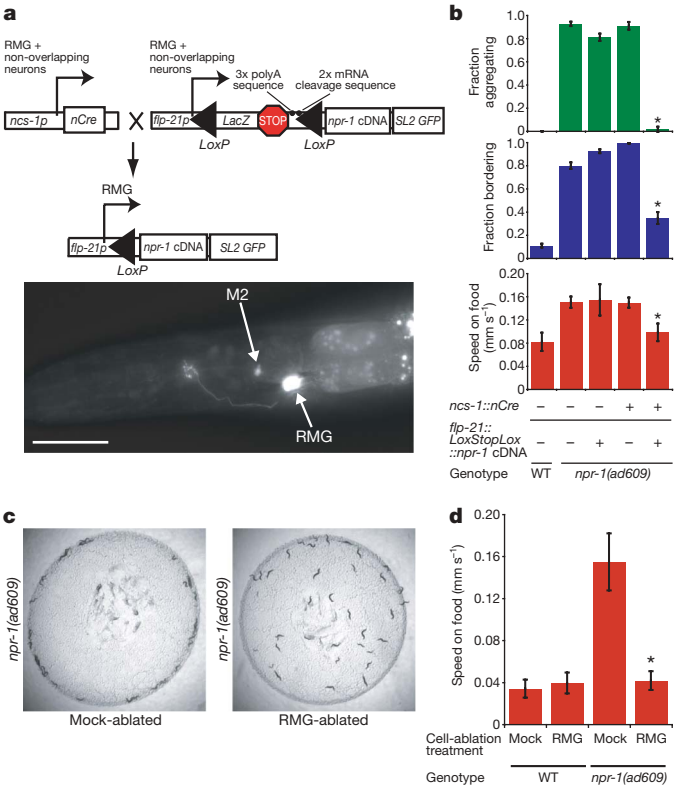

Figure 2 | Inhibition of RMG by NPR-1 suppresses social behaviour. **a**, Top, intersectional Cre/*Lox* strategy to express *npr-1* specifically in RMG. Bottom, L4 larva expressing *ncs-1::nCre* and *flp-21::LoxStopLox::GFP*. Scale bar, 30 μm. **b**, Aggregation and related behaviours of *npr-1*(*ad609*) animals carrying *ncs-1::nCre* and/or *flp-21::LoxStopLox::npr-1* transgenes. Asterisk, different from *npr-1*(*ad609*) ($P < 0.01$, Student's *t*-test). WT, wild type. **c**, Mock-ablated or RMG-ablated *npr-1*(*ad609*) animals (mock-ablated: 97.1% bordering, 40% aggregating; RMG-ablated: 17% bordering, 0% aggregating; $\chi^2 = 43.05$, $P < 0.001$). Magnification × 1.6. **d**, Locomotion speed of wild-type and *npr-1*(*ad609*) animals, mock-ablated or RMG-ablated. Asterisk, different from mock-ablated *npr-1*(*ad609*) ($P < 0.01$, Student's *t*-test). Error bars indicate s.d.

Among the other neurons anatomically coupled to RMG, the ASK neurons were of particular interest. ASK is one of several neurons that integrate pheromone and food signals to regulate *C. elegans* development[15], and it has recently been implicated in male attraction to hermaphrodite pheromones[5]. The role of ASK was probed using a *tax-4* mutation that affects sensory transduction: *tax-4* encodes a cyclic GMP-gated transduction channel expressed in ASK and other sensory neurons, but not in RMG[16]. *tax-4;npr-1*(*lf*) double mutants are strongly suppressed for aggregation and related behaviours[10], and rescue of these behaviours requires *tax-4* expression in URX and an unknown sensory neuron[10]. We addressed whether ASK might be the unknown neuron. Indeed, the simultaneous expression of *tax-4* in URX and ASK resulted in near-complete rescue of aggregation and related behaviours in *tax-4;npr-1*(*lf*) double mutants (Fig. 3b). Rescue was also observed after expression of *tax-4* in URX and ASJ neurons, which synapse onto ASK (Fig. 3b). Thus ASK and ASJ promote aggregation-related behaviours.

The connectivity of RMG suggests two models of behavioural output: RMG could integrate sensory input through gap junctions and stimulate aggregation using its own chemical synapses; or RMG could modify the output of associated sensory neurons, which all have chemical synapses. RMG is presynaptic to head muscles and interneurons that control forward and backward locomotion[13] (Fig. 3a). To determine whether the synaptic output of RMG promotes aggregation, we used the Cre/*Lox* system to express the light chain of tetanus toxin (TeTx) in RMG of *npr-1*(*lf*) mutants. TeTx inhibits synaptic transmission by cleaving the synaptic vesicle protein synaptobrevin[17]. Aggregation and related behaviours were partially suppressed by TeTx expression in RMG (Fig. 3c). Coexpression of TeTx with a cleavage-resistant *C. elegans* synaptobrevin (Q68V) mutant[17] significantly suppressed the RMG::TeTx effect, confirming that TeTx acts via synaptobrevin cleavage (Supplementary Fig. 3). Aggregation was also suppressed by the expression of TeTx in ASK and ASJ, further implicating these neurons in aggregation behaviours, and TeTx expression in both RMG and ASK+ASJ neurons had additive effects (Fig. 3c). Silencing or killing URX neurons suppresses aggregation[10,18], but TeTx expression in URX neurons had little effect unless the other neurons were silenced (Fig. 3c). These results indicate that synaptic outputs for aggregation are distributed, with contributions from both RMG and ASK+ASJ neurons.

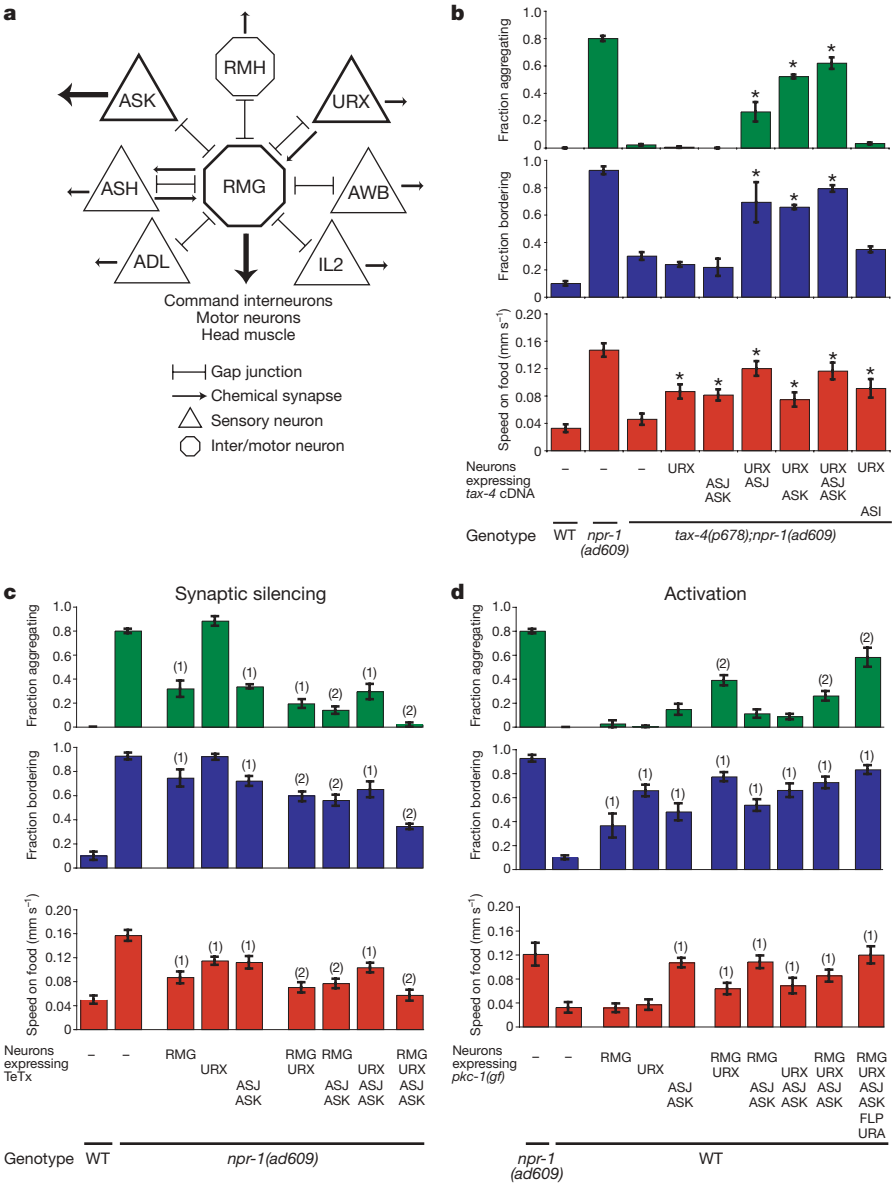

Figure 3 | ASK and ASJ sensory neurons promote aggregation. **a**, Circuit diagram of neurons with gap junctions to RMG. RMG may also form gap junctions with RMF[13]. **b**, Rescue of aggregation and related behaviours in *tax-4*(*p678*);*npr-1*(*ad609*) animals expressing a *tax-4* cDNA. Asterisk, different from *tax-4*;*npr-1*. **c**, Aggregation and related behaviours of *npr-1*(*ad069*) animals expressing TeTx. Statistics: (1), different from *npr-1*(*ad609*); (2), different from *npr-1*(*ad609*) and overlapping single-transgene strains. **d**, Aggregation and related behaviours of wild-type animals expressing gain-of-function protein kinase C (*pkc-1*(*gf*)). Statistics: (1), different from wild type; (2), different from wild type and overlapping single-transgene strains. In **b**–**d**, $P < 0.01$, Bonferroni test. Error bars indicate s.d.

We next determined whether activation of RMG or coupled neurons in solitary wild-type animals might induce aggregation. Neurons were activated by expressing a constitutively active protein kinase C homologue of *C. elegans* (*pkc-1*(*gf*)) that promotes synaptic transmission[19] and neuropeptide release[20], and may have further excitatory properties[21]. Expression of *pkc-1*(*gf*) in most RMG-coupled neurons elicited aggregation, bordering and high speed in solitary strains, a near-complete transformation of their behaviour (Fig. 3d). Expression in subsets of neurons had partial effects, suggesting contributions from RMG, URX, ASK+ASJ and possibly other cells (Fig. 3d). Like the behaviour of *npr-1*(*lf*) strains, *pkc-1*(*gf*)-induced behaviours were suppressed by killing RMG (Supplementary Fig. 3b). Thus simultaneous activation of RMG and sensory neurons by *pkc-1*(*gf*) can drive aggregation and related behaviours.

The dual involvement of ASK in aggregation and male attraction to hermaphrodites[5] prompted an examination of pheromone responses in aggregating strains. A class of *C. elegans* pheromones termed ascarosides is attractive to males, but repulsive to solitary hermaphrodites, suggesting a role in sex-specific attraction for mating (Supplementary Fig. 4a)[5,22]. Ascarosides are constitutively secreted by *C. elegans*, providing a plausible aggregation signal[23,24]. Solitary wild-type hermaphrodites were repelled by ascarosides; in contrast, *npr-1*(*lf*) hermaphrodites were attracted to low levels of ascarosides, with responses resembling those of males (Fig. 4a, b). Expression of *npr-1* in RMG restored pheromone avoidance to *npr-1*(*lf*) hermaphrodites, linking this behaviour to the RMG circuit (Fig. 4b).

Attraction to pheromones was absent in *tax-4*;*npr-1*(*lf*) double mutants, suggesting that *tax-4*-expressing sensory neurons detect ascarosides (Fig. 4c). Rescue of *tax-4* in ASK neurons restored ascaroside attraction to *tax-4*;*npr-1*(*lf*) strains, providing evidence that ASK is a relevant pheromone sensor (Fig. 4c). In a systematic analysis of the three pheromone components, a combination of C3 ascaroside with C6 or C9 drove effective attraction via ASK (Supplementary Fig. 4b, c). Developmental effects of ascarosides are greatest in the

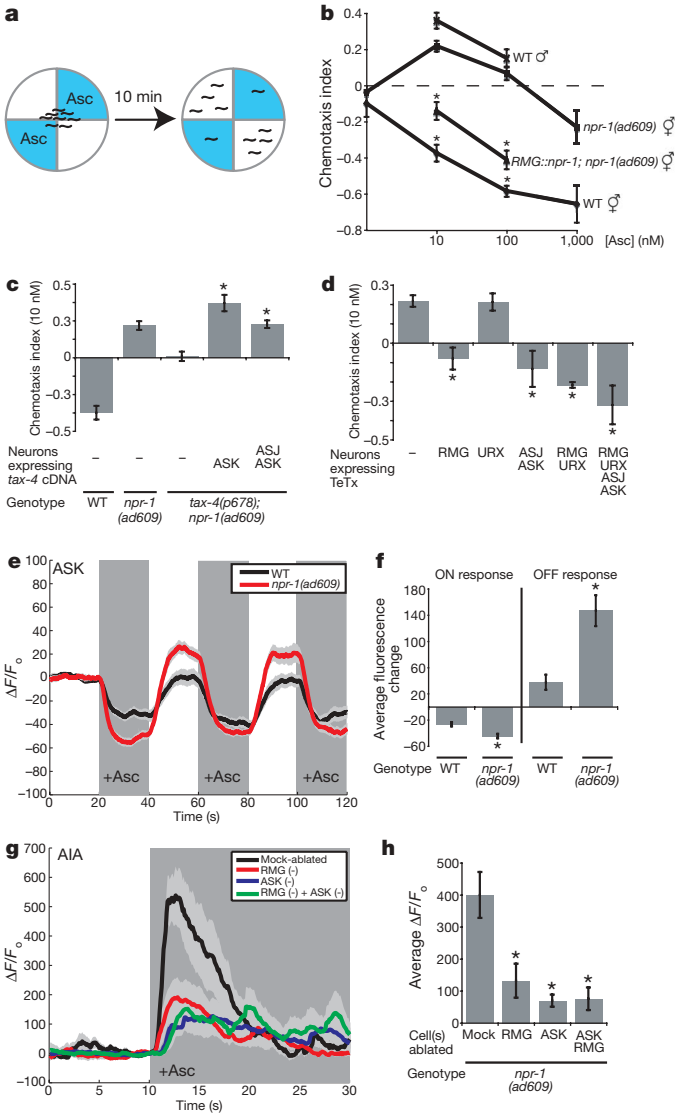

Figure 4 | Behavioural and neuronal responses to pheromones. a, Diagram of pheromone chemotaxis assay. Asc, three equimolar ascarosides (C3, C6 and C9). **b**, Ascaroside chemotaxis. Asterisk, different from *npr-1*(*ad609*). **c**, ASK expression of *tax-4* restores pheromone attraction to *tax-4;npr-1*. Asterisk, different from *tax-4;npr-1*. **d**, TeTx expression in RMG or ASJ and ASK eliminates pheromone attraction in *npr-1*(*ad609*). Asterisk, different from *npr-1*(*ad609*). In **b**–**d**, $P < 0.01$, Bonferroni test. **e**, Ascaroside (100 nM) decreases G-CaMP calcium signals in ASK ($n = 17$ animals each). **f**, Average ASK fluorescence change to first ascaroside addition (ON) and removal (OFF). Asterisks, different from wild type ($P < 0.01$, *t*-test). **g**, Ascaroside (1 μM) induces G-CaMP calcium signals in AIA interneurons of *npr-1*(*ad609*); for ablations, $n \geq 10$ animals; mock-ablated, $n = 16$. In **e** and **g**, dark shading indicates presence of ascarosides, light shading indicates s.e.m. **h**, Average AIA fluorescence change in the 5 s after ascaroside addition. Asterisk, different from mock-ablated ($P < 0.01$, Bonferroni test). In **b**–**d**, **f** and **h**, error bars indicate s.e.m.

same combinations, and require higher pheromone concentrations than attraction, suggesting that attraction occurs at physiological pheromone levels[24]. Inhibiting synaptic transmission from ASK+ASJ or RMG neurons eliminated ascaroside attraction (Fig. 4d). The correlation between cells required for pheromone attraction and aggregation supports the hypothesis that these behaviours are functionally related.

Sensory properties of ASK were examined directly by monitoring sensory-evoked calcium transients with the genetically encoded calcium indicator G-CaMP[25]. In both wild-type and *npr-1*(*lf*) animals, ASK responded to ascaroside cocktails (100 pM–1 μM) with a rapid diminution of fluorescence suggesting decreased calcium levels; fluorescence recovered after ascaroside removal (Fig. 4e and Supplementary Fig. 4d). The rapid response in ASK neurons supports their identification as ascaroside-sensing neurons; the calcium decrease suggests that ASK uses a hyperpolarizing mode of sensory transduction[26,27]. At attractive nanomolar ascaroside concentrations, ASK calcium responses were reliably greater in *npr-1* animals than in wild type, with a greater calcium decrease after ascaroside addition and a greater rebound after ascaroside removal (Fig. 4e, f).

To determine whether this apparent change in ASK activity could propagate across synapses, ascaroside responses were monitored in a synaptic target of ASK, the AIA interneuron. Ascaroside cocktails elicited increased G-CaMP fluorescence in AIA, suggesting depolarization (Fig. 4g); the average magnitude of this signal was significantly greater in *npr-1*(*lf*) than in wild-type animals (Supplementary Fig. 4e). The AIA response was diminished in *npr-1* animals whose ASK neurons were killed with a laser, suggesting that ASK sensory input is an important source of ascaroside signals to AIA (Fig. 4g, h). The inversion of calcium signals (decrease in ASK, increase in AIA) suggests that ASK makes inhibitory synapses onto AIA. Ascaroside-induced AIA calcium signals were also diminished when the RMG neurons were killed (Fig. 4h). An RMG–ASK double ablation resembled an ASK ablation alone, indicating that RMG and ASK affect AIA through a common process (Fig. 4g, h). The imaging results indicate that ASK senses ascarosides (along with other neurons), that the ASK response is propagated to downstream neurons, that RMG enhances ASK signalling, and that high *npr-1* activity diminishes it.

These results provide insight into behavioural mechanisms of aggregation, the anatomical circuit underlying the behaviour, and the regulatory role of *npr-1*. Solitary animals ignore oxygen in the presence of food, and are repelled by ascarosides produced by other animals. In social *npr-1*(*lf*) animals, oxygen-sensing URX neurons promote accumulation at the lawn border, and ascaroside-sensing ASK neurons promote attraction to other animals (or neutralize repulsion). The altered pheromone response in *npr-1* hermaphrodites demonstrates that aggregation involves directed responses to other nematodes, not just a shared preference for low-oxygen environments. The analysis of RMG suggests a hub-and-spoke model for aggregation behaviour, in which distributed sensory inputs are coordinated through gap junctions with the RMG hub to produce distinct, distributed synaptic outputs. Mechanistically, calcium imaging suggests that RMG amplifies weak sensory signals in ASK to stimulate pheromone attraction. For example, the RMG circuit could depolarize ASK to increase tonic transmitter release at rest, and thereby increase the signal ASK sends when hyperpolarized by ascarosides.

In solitary strains, the neuropeptide receptor NPR-1 inhibits RMG function. In one model, NPR-1 might act by closing RMG gap junctions, gating access of sensory neurons to a shared circuit, but sparing their individual synaptic outputs. This instructive model for gap junction regulation is analogous to the dopaminergic regulation of gap junctions in the mammalian retina, in which gap junctions link rod and cone visual pathways to increase light sensitivity at night; during the day, dopamine inhibits gap junctions to increase spectral and spatial resolution[28]. Alternatively, NPR-1 could alter RMG excitability, and gap junctions could passively propagate this information from RMG to the sensory neurons to change their properties. In both models, *npr-1* shifts the properties of an entire anatomical circuit by modulating a single neuron. As RMG gap junctions are at present defined purely by anatomical criteria, further experiments are needed to determine whether RMG propagates electrical signals, calcium, cAMP, or other information.

Within the *C. elegans* wiring diagram, gap junction distributions are highly skewed. Most neurons have only a few gap junctions, but 17 classes of neurons are gap junction hubs that link seven or more neuronal classes[29]. We suggest that this circuit motif performs a characteristic computation wherever it appears.

METHODS SUMMARY

Aggregation and bordering behaviours were measured essentially as described[2]; values report the average fraction of three or more behavioural assays of 150 animals each. Average locomotion speed was calculated by tracking 20 animals for 10 min with an automated tracking system[30].

For RMG-selective expression of transgenes, a *LoxP*-flanked *LacZ* sequence containing a transcriptional stop, three repeated poly(A) sequences, and two repeated mRNA cleavage sequences, was inserted upstream of *npr-1::SL2::GFP* under the control of the *flp-21* promoter (*flp-21::LoxStopLox::cDNA[GFP*, *npr-1*, *TeTx*, or *pkc-1*(*gf*)*]*). Transgenic animals containing this plasmid were crossed with animals expressing *nCre* under the *ncs-1* promoter (*ncs-1::nCre*). Strong and consistent expression was observed in RMG and M2 neurons; ADL, ASJ and ASK were seen weakly and inconsistently.

For ascaroside chemotaxis assays, washed animals were placed in the centre of a four-quadrant plate with ascarosides in alternating quadrants, and scored after 10 min. A chemotaxis index was calculated as (the number of animals on pheromone quadrants − the number of animals on buffer quadrants)/(total number of animals). In the cartoon in Fig. 4a, the chemotaxis index = −0.6. In Fig. 4 a cocktail of three ascarosides was used; individual ascarosides and other combinations are shown in Supplementary Fig. 4.

Calcium imaging of the AIA and ASK neurons was performed in a custom-fabricated microfluidic device, essentially as described[26]. For ASK imaging, the transgene *kyEx2866* was used, with *GCaMP2.2b* (gift from L. Looger) expressed under the *sra-9* promoter. For AIA imaging, the transgene *kyEx2916* was used, with *GCaMP2.2b* expressed under the T01A4.2 promoter. ASK fluorescence was recorded in the neuronal cell body, and AIA fluorescence was measured in the dorsal AIA process in the nerve ring.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 26 October 2008; accepted 11 February 2009.
Published online 6 April 2009.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank L. Looger for *GCaMP2.2b*, M. Nonet for cleavage-resistant synaptobrevin, and J. Ragains for synthesizing ascarosides. This work was funded by the Howard Hughes Medical Institute, the Harold and Leila Y Mathers Charitable Foundation, the Jensam Foundation, and National Institute of Health grants GM07739 (E.Z.M. and E.H.F.), CA24487 (J.C.) and GM077943 (R.A.B.). C.I.B. is an Investigator of the Howard Hughes Medical Institute.

Author Contributions E.Z.M. performed experiments; N.P., E.H.F., S.C., R.A.B. and J.C. developed experimental methods and reagents; E.Z.M. and C.I.B. designed and interpreted experiments and wrote the paper.

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METHODS

Strains were grown and maintained under standard conditions[31]. Standard methods were used for molecular biology. A complete strain list and information on clones and transgenes is included in Supplementary Information.

Behavioural analysis. Aggregation and bordering behaviours were on NGM plates seeded with 200 µl OP50 for 48 h and 150 adult animals per assay[2]. Behaviours were scored 2 h after picking the animals to the assay plates. An animal was scored as aggregating if it touched two or more other animals, and bordering if it was less than 2 mm from the edge of the bacterial lawn. To determine the rate of locomotion on food, 10-cm NGM agar plates were seeded with a thin layer of OP50 bacteria and allowed to grow overnight at 25 °C. Filter paper soaked in 20 mM copper chloride was placed around the perimeter of the plate to prevent animals from escaping from view. Twenty animals were then picked to the centre of the plate, allowed to rest for 1 h, and recorded for 10 min. Average speed was calculated from speed values determined every ten seconds by an automated worm tracker (http://wormsense.stanford.edu/tracker)[30].

A previous report indicated that social behaviour could be partially suppressed by expressing *npr-1* in URX neurons under *gcy-32* or *flp-8* promoters[10]. We did not observe strong suppression using any of the three tested URX-expressing promoters: *gcy-32*, *flp-8* or *gcy-35*. One possible explanation for this incongruity is that the published rescue uses an intron-containing genomic fragment of *npr-1*, whereas we used an *npr-1* cDNA. Some introns in *C. elegans* act as tissue-specific or nonspecific transcriptional enhancers[32]. Therefore the *npr-1* genomic constructs may have directed some expression in RMG; furthermore, some fragments of the *flp-8* promoter, which was more potent in the published report, are occasionally expressed in RMG[33]. An alterative explanation is that the published genomic clone may result in higher *npr-1* expression in URX than the cDNA clone. Social behaviour can be partially suppressed by silencing or killing URX[10,34] and the reported partial suppression is consistent with silenced URX neurons. Whether the higher or lower levels of *npr-1* are more physiological is unclear, but it is a concern that transgenes can be toxic when expressed at high levels, even when they drive 'inert' molecules such as GFP. In any case, the effects of RMG *npr-1* expression described here are much stronger than the reported effects of URX *npr-1* expression.

Chemotaxis to pheromone was assayed in 10-cm Petri plates divided into four quadrants[35] with ascarosides mixed into the agar in alternating quadrants. For each assay, 200 worms were picked to a seeded plate for 2–3 h. The worms were then washed three times with chemotaxis buffer[36], and placed in the centre of the assay plate.

Osmotic avoidance was measured using the dry drop test[37]. Eight to ten animals per condition were placed on individual NGM plates without food. After 10 min, a small drop of 1 M glycerol, dispensed from a micropipette, was placed in the path of a forward-moving animal, so that the drop soaked into the agar as the animal reached the drop. A reversal away from the glycerol was scored as an avoidance event. Each animal was tested 8–10 times, from which a fractional response was calculated. The avoidance index is the average fractional response for all animals tested in a given condition.

Laser ablation. For behavioural assays, the RMG inter/motor neurons were targeted for laser killing using a transgenic strain expressing GFP in RMG (*ncs-1::GFP*) and a MicroPoint laser system. Cells were ablated at the L1 stage as described[38], and behaviour was assayed approximately 72 h later. Individual ablated animals were examined for the absence of RMG fluorescence to confirm cell death. For calcium imaging experiments, neurons were identified using Nomarski optics on the basis of their position and morphology, then killed with a laser. Adult animals were visually scored for aggregation-related behaviours to confirm the death of RMG.

Calcium imaging. Calcium imaging of the AIA and ASK neurons was performed in a custom-fabricated polydimethylsiloxane chamber that physically immobilized animals with their nose in a stream of fluid[26]. Ascaroside stimuli were presented by switching the fluid streams that bathed the nose. G-CaMP fluorescence was recorded by a Nikon CoolSnap camera, regions of interest were tracked, and fluorescence intensity analysed using Matlab scripts.

The near-ultraviolet-light wavelengths used to excite the G-CaMP fluorophore elicited fluorescence changes in ASK; therefore, as described for other light-sensitive neurons[39], the ultraviolet-response was desensitized by a 2–3 min light pre-exposure before the beginning of all experiments monitoring ASK and AIA activity. Previous studies are consistent with a possible light-sensitive function of ASK[40], and with a demonstrated light-sensitive activity of the synaptically connected ASJ neurons[40]. All imaging experiments used a 1:1:1 ratio of three ascarosides (Supplementary Fig. 4a), each at the same concentration (100 pM–1 µM). Behavioural responses to pheromones were examined in strains used for imaging, and resembled those of the parental *npr-1* strains.

To quantify ASK responses, the average response in the first 20 s after ascaroside addition in each recording was subtracted from the average of 10 s before ascaroside addition (ON response), and the average response in the first 20 s after ascaroside removal was subtracted from the average of 10 s before ascaroside removal (OFF response) (Fig. 4f). To quantify AIA responses, the average change in fluorescence during the first 5 s after presentation of the ascaroside stimulus was calculated for each recording (Fig. 4h).

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