Social feeding in *Caenorhabditis elegans* is induced by neurons that detect aversive stimuli

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Natural *Caenorhabditis elegans* isolates exhibit either social or solitary feeding on bacteria. We show here that social feeding is induced by nociceptive neurons that detect adverse or stressful conditions. Ablation of the nociceptive neurons ASH and ADL transforms social animals into solitary feeders. Social feeding is probably due to the sensation of noxious chemicals by ASH and ADL neurons; it requires the genes *ocr-2* and *osm-9*, which encode TRP-related transduction channels, and *odr-4* and *odr-8*, which are required to localize sensory chemoreceptors to cilia. Other sensory neurons may suppress social feeding, as social feeding in *ocr-2* and *odr-4* mutants is restored by mutations in *osm-3*, a gene required for the development of 26 ciliated sensory neurons. Our data suggest a model for regulation of social feeding by opposing sensory inputs: aversive inputs to nociceptive neurons promote social feeding, whereas antagonistic inputs from neurons that express *osm-3* inhibit aggregation.

In many animal species individuals live in groups for part or most of their lives[1,2]. Environmental signals such as predators, food availability and season can regulate aggregation behaviour[1,2]. In turn, aggregation can profoundly influence population dynamics, community structure and biodiversity[3,4]. Although our understanding of the ecological and evolutionary significance of aggregation has advanced considerably, the neural mechanisms underlying this form of behaviour have rarely been explored.

Many nematode species can aggregate, both in culture and in the wild[5,6]. Natural isolates of the soil nematode *C. elegans* feed on bacteria either alone or in groups[7,8]. Solitary feeders such as the standard N2 strain reduce locomotory activity and disperse on encountering bacterial food. By contrast, social feeders such as strain CB4856 continue moving rapidly on food and aggregate together. Social feeders also accumulate on the border of a bacterial lawn[8]. These behavioural differences are associated with natural variation at a single amino acid residue of NPR-1, a putative neuropeptide receptor related to the neuropeptide Y (NPY) receptor family[8]. Solitary strains bear valine at residue 215 of the *npr-1* gene, whereas social strains bear phenylalanine. Animals lacking *npr-1* strongly aggregate, indicating that *npr-1* represses this behaviour. Because the laboratory standard N2 strain is solitary, little is known about the sensory signals or neural mechanisms that promote social behaviour in *C. elegans*. Thus, *npr-1* mutants provide an opportunity to investigate this behaviour.

Stimulation of aggregation behaviour

To ascertain conditions required for aggregation of *npr-1* animals, we varied cultivation conditions and population density. Social feeders aggregated within 20 min of being placed on a fresh food source and remained in groups, whereas animals from the solitary strain N2 did not aggregate, or showed weak transient aggregation and then dispersed (Fig. 1a). Increasing population density, which favours encounters with other individuals, stimulated aggregation in *npr-1* strains (Fig. 1a).

Well-fed animals from both social and solitary strains disperse when food is absent. To investigate whether social animals would aggregate in the absence of food if they were maintained at a high population density, we trapped animals using a chemical or physical fence (see Methods). In the absence of food neither well-fed *npr-1(ad609)* (where *ad609* is a loss-of-function allele) mutants nor well-fed wild social animals showed a significant tendency to aggregate at any density (Fig. 1b).

One possible mediator of aggregation is a pheromone that induces dauer larva formation. Production of dauer pheromone requires the *daf-22* gene[9]. Social feeding by *npr-1* animals was not suppressed by a mutation in *daf-22*, or by mutations in other dauer pathway genes that affect a transforming growth factor (TGF)-β signalling cascade[10] (*daf-3*,*-5*,*-7*,*-8*) or an insulin receptor signalling pathway (*daf-2*, *daf-16*)[9] (data not shown). Thus the aggregation signal is unlikely to correspond to the dauer pheromone.

Mutants in *daf-1*, *daf-7*, *daf-8* and *daf-14* aggregate and border on food, although less strongly than *npr-1* mutant animals[11]. This observation prompted a further investigation of the dauer pathway's relationship to *npr-1*. The aggregation and bordering behaviour of these *daf* mutants is completely suppressed by mutations in *daf-3* and *daf-5* (ref. 11), whereas *daf-3* and *daf-5* mutations did not inhibit aggregation and bordering of *npr-1* mutant animals (data not shown). To investigate whether the *daf* genes and *npr-1* regulate social feeding by a common pathway, we compared social feeding in adult *npr-1* and *daf-7* mutant animals with the behaviour of adult *daf-7*; *npr-1* double mutant animals. *daf-7* encodes a TGF-β homologue that is expressed in the ASI neurons in well-fed animals, but is transcriptionally repressed at high nematode population density by dauer pheromone[10,12]. Social behaviour was enhanced in the *daf-7*; *npr-1* double mutants (Fig. 1c–f): the average number of animals in a group is greatly increased in *daf-7*; *npr-1(null)* strains compared with *npr-1(null)* or mutant *daf-7* strains alone. This result suggests that *daf-7* and *npr-1* act in parallel pathways to repress social feeding. For example, *daf-7* may mediate some of the effect of density on aggregation.

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Mutations in OSM-9 and OCR-2 disrupt social feeding

The observation that aggregation behaviour is modulated by food and population density initiated a search for sensory mechanisms that might mediate these effects. *Caenorhabditis elegans* responds to mechanosensory stimuli, chemical attractants and repellants, and temperature gradients[13,14]. We asked whether mutations that disrupt one or more of these behaviours prevent *npr-1* mutant animals from aggregating. Mutants with defective responses to water-soluble attractants (*che-13*, *osm-1*, *osm-3*), attractive odorants (*che-13*, *osm-1*, *odr-1*, *odr-2*, *odr-3*, *odr-7*), aversive chemicals (*che-13*, *osm-1*, *osm-3*, *odr-3*), gentle touch on the body (*mec-3*, *mec-4*), touch to the head (*che-13*, *osm-1*), mechanical properties of bacteria (*cat-4*), or temperature (*ttx-3*) were still able to aggregate efficiently (Fig. 2a, and data not shown). Bordering behaviour on a bacterial lawn was also unaffected by these mutations (Fig. 2b, and data not shown).

Mutations in the *osm-9* and *ocr-2* genes abolished aggregation of *npr-1* mutant animals, and substantially reduced accumulation at the border of a bacterial lawn (Fig. 2c, d). *osm-9* and *ocr-2* encode predicted TRPV cation channel subunits that are thought to form a sensory transduction channel in several *C. elegans* chemosensory neurons[15,16]. *osm-9* and *ocr-2* are required for some forms of olfactory chemoattraction as well as avoidance of hyperosmotic stimuli, nose touch, and certain volatile aversive stimuli. The closest vertebrate relatives of OSM-9 and OCR-2 are also sensory channels: the vanilloid (capsaicin) receptor TRPV1 (VR1) that is implicated in sensing thermal pain, and the osmo-mechanosensitive channel TRPV4 (refs 17, 18).

The *odr-4* and *odr-8* genes are required for some olfactory responses, and act to localize a subset of olfactory receptors to olfactory cilia[19]. *odr-4* encodes a transmembrane protein, whereas the molecular identity of *odr-8* is unknown. Mutations in both *odr-4* and *odr-8* strongly disrupted the ability of *npr-1* mutant animals to aggregate and to border on food (Fig. 2c, d).

Mutations in *ocr-2*, *osm-9*, *odr-4* and *odr-8* could disrupt aggregation of social *npr-1* animals by reducing production of the stimuli that promote aggregation, or by reducing the detection of these stimuli. To distinguish between these two possibilities we investigated whether individual *npr-1* animals defective in *ocr-2*, *osm-9*, *odr-4* or *odr-8* function would join groups of social *npr-1* animals. All four double mutants, like the solitary wild strain N2, failed to join groups of *npr-1* social animals (Fig. 2e). These results suggest that the double mutants cannot sense signals that promote aggregation. Bordering behaviour of the double-mutant strains was also unaffected by the presence of social *npr-1* animals (Fig. 2f).

Animals from wild solitary strains such as N2 respond to food by strongly reducing their locomotory activity. This slowing response is mediated by a dopamine-containing neural circuit that senses a mechanical attribute of bacteria, and is enhanced by food-deprivation through a mechanism that involves serotonin[20]. *npr-1* mutants and wild social strains do not show this slowing response, even after food deprivation (Fig. 3)[8]; however, double mutants between *npr-1* and *ocr-2* or *osm-9*, displayed a slowing response to food similar to that of N2 solitary animals (Fig. 3). *ocr-2*; *npr-1* and *osm-9*; *npr-1* animals also displayed the enhanced slowing response to food after food deprivation. These results suggest that *npr-1* is not

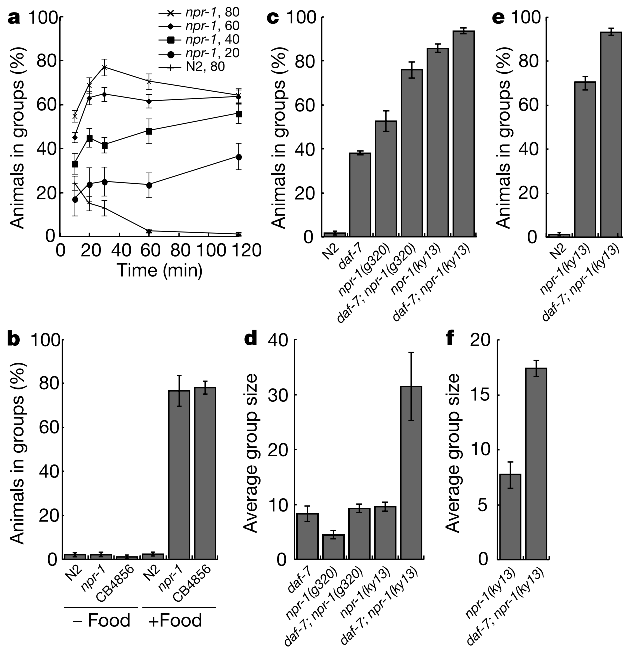

Figure 1 Aggregation of *npr-1* mutant animals requires food and is enhanced by increased population density and *daf-7* TGF-β mutations. **a**, Time courses for aggregation of 20, 40, 60 or 80 *npr-1(ad609)* animals, or of 80 N2 animals, as indicated. Populations of 20, 40 and 60 N2 animals show less aggregation than 80 N2 animals (data not shown). **b**, Well-fed social feeders do not aggregate without food. Population density in these assays was about 26 animals per cm^2. The *npr-1* allele is *ad609*; CB4856 is a wild social strain bearing the *npr-1 215F* allele. **c**, Aggregation behaviour with 80 adult animals per assay. *ky13* is a predicted null allele of *npr-1*; *g320* is the natural *npr-1 215F* allele. The *daf-7(e1372)* allele is a point mutation that acts like a strong loss-of-function allele[10]. *daf-7(e1372)* enhances the aggregation of both *npr-1(ky13)* ($P < 0.02$) and *npr-1(g320)* animals ($P < 0.01$). **d**, Average number of animals in a group for the assays in **c**. Only animals in groups were scored to get the average group size. *daf-7(e1372)*; *npr-1(ky13)* animals form significantly larger groups than *npr-1(ky13)* animals ($P < 0.05$). **e**, **f**, Per cent aggregation and average group size in assays involving a population of 40 adult animals. *daf-7(e1372)*; *npr-1(ky13)* animals aggregate more strongly and form larger groups than *npr-1(ky13)* animals ($P < 0.001$ for both behaviours). In all panels $n \geq 5$ assays.

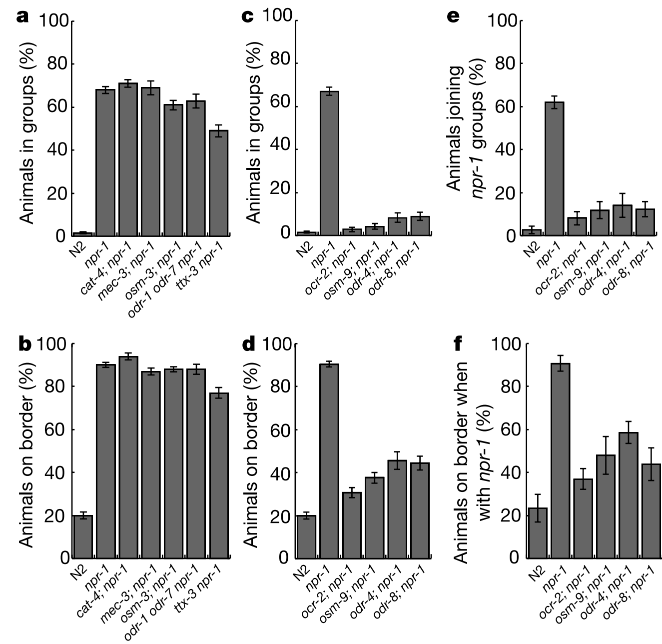

Figure 2 Social feeding of *npr-1* is lost in *ocr-2*, *osm-9*, *odr-4* or *odr-8* mutant backgrounds. **a**, **b**, *cat-4(e1141)*, *mec-3(e1338)*, *osm-3(p802)*, *odr-1(n1936) odr-7(ky4)* and *ttx-3(ks5)* mutations do not disrupt aggregation or bordering of *npr-1(ad609)* animals. The *odr-1 odr-7 npr-1* and *ttx-3 npr-1* animals also bear the *lon-2(e678) X* mutation. **c**, **d**, Mutations in *ocr-2*, *osm-9*, *odr-4* and *odr-8* strongly reduce aggregation and bordering of *npr-1* animals ($P < 0.001$). **e**, **f**, Individual animals of each genotype were tested for their ability to join groups of marked *npr-1* animals, or to border in the presence of *npr-1* animals (see Supplementary Information). For **a**–**d**, $n \geq 9$ population assays; for **e**, **f**, $n \geq 30$ individual animals, each tested 2–3 times.

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essential for a food-induced slowing response. Instead, loss of *npr-1* may activate locomotion in a way that bypasses or suppresses its downregulation by food.

Various slow-moving *npr-1* double-mutant strains showed strong aggregation and bordering behaviours, including *mec-3(e1338)*; *npr-1(ad609)*, *unc-25(e156)*; *npr-1(ad609)*, *unc-30(e191)*; *npr-1(ad609)* and *unc-36(e251)*; *npr-1(ad609)*. Thus high locomotory activity is not necessary for social feeding behaviour.

OCR-2 and ODR-4 are required in nociceptive neurons

The expression patterns of the sensory transduction genes provided a set of candidate neurons for social feeding behaviour. OCR-2 and OSM-9 are co-expressed in six pairs of sensory neurons: ADF, ADL, ASH, AWA, PHA and PHB. To identify the cells in which OCR-2 activity is required for social behaviour, we expressed an *ocr-2* minigene in subsets of these six neurons using neuron-specific promoters. Expression of OCR-2 in either ASH or ADL neurons was able to restore social behaviour to *ocr-2*; *npr-1* animals; expression in the other types of neurons did not alter the solitary behaviour of *ocr-2*; *npr-1* animals (Fig. 4a).

The *odr-4* gene is expressed in 12 pairs of neurons, including ASH and ADL[19]. To investigate where *odr-4* activity is required for social behaviour, we expressed functional green fluorescent protein (GFP)-tagged ODR-4 in subsets of these 12 neurons in *odr-4*; *npr-1* animals, and assayed aggregation and bordering behaviour. Expression of *odr-4* in ADL, but not other neurons, restored both aggregation and bordering behaviours of *odr-4*; *npr-1* animals (Fig. 4b). This analysis of *ocr-2* and *odr-4* implicates the ASH and ADL neurons in social feeding behaviour.

In the *C. elegans* olfactory neuron AWA, the OCR-2/OSM-9 channel is thought to be gated by a G-protein-coupled cascade activated by odorant binding to olfactory receptors such as ODR-10 (refs 15, 21, 22). Similar pathways may operate in the ASH and ADL neurons, which also express predicted chemosensory receptors[23]. ODR-4 acts to localize a subset of chemosensory receptors, including ODR-10, to the cilia of olfactory neurons[19]. The requirement of *odr-4* in ADL neurons for social feeding suggests that chemosensory receptors act in ADL to support this behaviour.

Ablation of ASH and ADL abolishes social feeding behaviour

To investigate whether the ASH and ADL neurons are required for social feeding, we ablated these neurons using a laser microbeam. The aggregation behaviour of ablated animals expressing a GFP marker was examined after mixing them with unmarked *npr-1* mutant animals. Ablation of both ASH neurons, or of both ADL neurons, did not disrupt social behaviour. However, simultaneous ablation of all ASH and ADL neurons strongly disrupted the ability of *npr-1* animals to aggregate (Fig. 4c). These data suggest that input from either the ASH neurons or the ADL neurons is required for social feeding behaviour.

The ASH and ADL neurons are implicated in aversive responses to noxious stimuli. ASH neurons are important for avoidance of touch to the head, hyperosmotic solutions, extracts of dead worms, high concentrations of benzaldehyde, and toxic heavy metal ions such as Cu^{2+} and Cd^{2+} (refs 23–26). The ADL neurons are important for long-range avoidance of 1-octanol and contribute with ASH to short-range avoidance of 1-octanol[24,27]. The well-documented roles for ASH and ADL in mediating responses to aversive stimuli suggests that such stimuli have a function in social behaviour: ASH and ADL may activate social feeding behaviour in response to repulsive cues. The most probable source of the aversive cues in standard culture conditions is bacteria.

To investigate whether bacterial signals induce social feeding, we assayed *npr-1* mutant animals on a lawn of *Escherichia coli* that had been killed using streptomycin. *npr-1* mutant animals became solitary feeders under these conditions (data not shown). Notably, *npr-1* mutant animals feeding on bacteria killed by streptomycin became social feeders when they were exposed to the odour of live bacteria placed on the lid of the assay plate. N2 animals continued to feed alone under these conditions. These results suggest that a bacterial odour induces social feeding behaviour.

Nociceptive neurons are antagonized by other neurons

Ablation of the ASH and ADL pairs of nociceptive neurons strongly disrupted social feeding of *npr-1* animals; however, a mutation in *osm-3* kinesin that truncates the sensory cilia of ASH and ADL[28] had no effect on social behaviour (Figs 2a, b and 5a, b). *osm-3* affects development of the distal cilia in 26 *C. elegans* sensory neurons that are open to the external environment[29], including ASH and ADL. As *osm-3* acts in other neurons apart from ASH and ADL, we reasoned that high levels of social behaviour in *osm-3*; *npr-1* animals could reflect a balanced removal of positively and negatively acting signals that regulate aggregation (Fig. 5c). In this model disruption of ASH and ADL neurons only removes stimuli that activate social behaviour, whereas *osm-3* mutations remove both the ADL/ASH positive signals and opposing negative ones (Fig. 5c). This hypothesis predicts that the triple mutants *odr-4*; *osm-3*; *npr-1* and *osm-3 ocr-2*; *npr-1* should aggregate indistinguishably from *osm-3*; *npr-1*

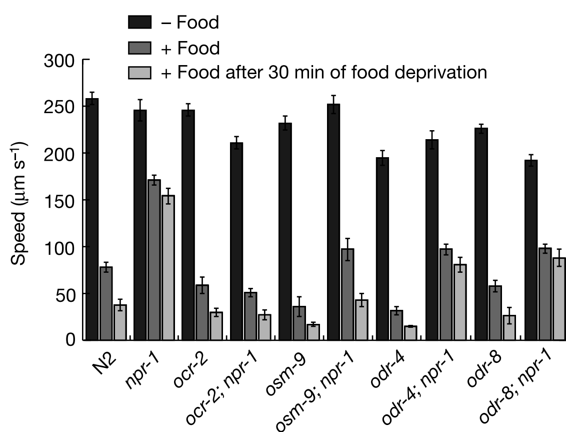

Figure 3 Mutations in *ocr-2*, *osm-9*, *odr-4* and *odr-8* restore the ability of *npr-1* mutant animals to slow down upon encountering food. $n \geq 25$ animals each recorded for 4 min.

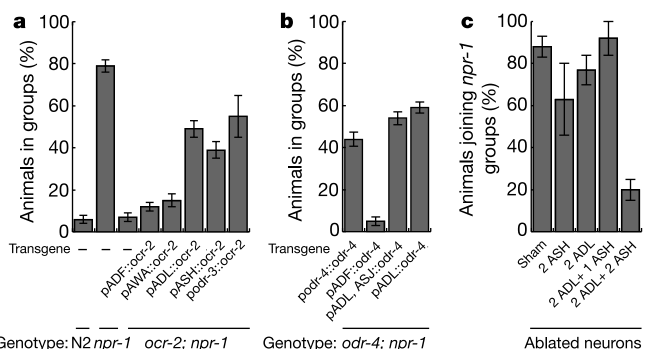

Figure 4 The nociceptive neurons ASH and ADL are required for social feeding behaviour. **a**, Expression of *ocr-2* in the ASH or ADL neurons restores aggregation behaviour to *ocr-2*; *npr-1* mutant animals ($P < 0.003$ compared to *ocr-2*; *npr-1*), whereas expression in the ADF or AWA neurons does not. *odr-3* is expressed in ASH, AWA, AWB, AWC and ADF neurons. **b**, *odr-4* expression in ADL restores social behaviour to *odr-4*; *npr-1* mutant animals ($P < 0.001$ compared to *odr-4*; *npr-1*), whereas expression in ADF does not. Expression of *odr-4* in AWA, AWB, ASI and ASK neurons also fails to restore aggregation behaviour to *odr-4*; *npr-1* animals (data not shown). **c**, Ablation of both pairs of ADL and ASH neurons suppresses aggregation ($P < 0.001$ compared to sham). Ablation of the ASH neuron pair, the ADL neuron pair, or the ASH neuron pair and 1 ADL neuron, does not suppress aggregation.

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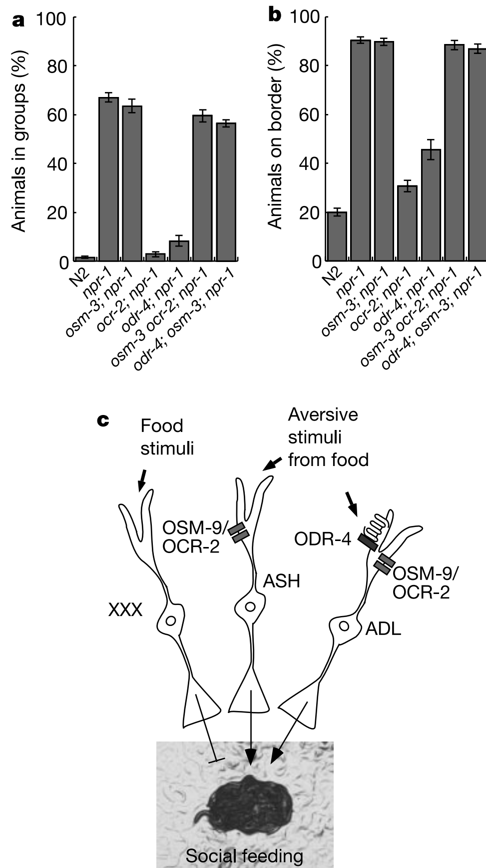

Figure 5 Disruption of *osm-3* kinesin restores social feeding to mutants defective in nociceptive neuron function. **a**, **b**, Aggregation (**a**) and bordering (**b**) behaviours were scored in animals of the indicated genotype. *osm-3 ocr-2*; *npr-1* and *odr-4*; *osm-3*; *npr-1* animals aggregate and border strongly to similar levels as *npr-1* and *osm-3*; *npr-1* animals ($P > 0.05$). $n \geq 10$ assays. **c**, A model for social feeding in *C. elegans*. The ASH and ADL nociceptive neurons are proposed to respond to aversive stimuli from food to promote social feeding. This function requires the putative OCR-2/OSM-9 ion channel. The ODR-4 protein may act in ADL to localize seven transmembrane domain chemoreceptors that respond to noxious stimuli. In the absence of ASH and ADL activity, an unidentified neuron (XXX) represses social feeding, perhaps in response to a different set of food stimuli. The photograph shows social feeding of a group of >30 *npr-1* mutant animals on a lawn of *E. coli*.

animals. This prediction is borne out: the triple mutant strains aggregated and bordered to the same level as *npr-1* and *osm-3*; *npr-1* mutants (Fig. 5a, b). Moreover, both *osm-3* and *osm-3*; *ocr-2* animals were solitary feeders if they expressed *npr-1* bearing valine at residue 215 (data not shown), indicating that the suppression of social feeding by the *npr-1* gene does not require *osm-3*.

These data suggest that social feeding behaviour in *C. elegans* is regulated by antagonistic signals from sensory neurons. Signalling from the ASH and ADL nociceptive neurons promotes social feeding behaviour, whereas signalling from another, as yet unidentified, neuron that expresses *osm-3* suppresses social behaviour by an *npr-1*-independent pathway.

Discussion

Repulsive or stressful environmental conditions may represent important inducers of aggregation behaviour in *C. elegans*. Signalling from either the ASH or the ADL nociceptive neurons is required for social feeding by *npr-1* mutants, suggesting that a repulsive signal stimulates social feeding and related behaviours. However, ASH and ADL neurons are not always essential for social behaviour: a mutation in *osm-3* restores social behaviour to *ocr-2*; *npr-1* and *odr-4*; *npr-1* mutants. We suggest that ASH and ADL transmit information about aversive stimuli in the environment to a circuit that is responsible for aggregation, rapid locomotion, and food bordering behaviour. This circuit may also respond to an antagonistic set of neurons that express *osm-3* and inhibit social feeding (Fig. 5c).

Aggregation can be regulated by two different sensory pathways that respond to environmental stress: the *daf-7*/TGF-β dauer pathway[11] and the ASH/ADL nociceptive pathway. Similar to aggregation, dauer larva development represents an adaptive response to adverse conditions that is under complex sensory regulation by pheromones, food, and temperature. As double mutants between *daf-7* and *npr-1(null)* mutants show enhanced aggregation compared with either mutant alone, it is likely that these two genes use different pathways to inhibit aggregation behaviour.

Food, food acquisition and population density are important regulators of aggregation in a variety of species[1,2]. Aggregation in nematodes is also regulated by food: well-fed animals from wild social *C. elegans* strains as well as from *npr-1* mutant strains do not aggregate when food is absent. Our results suggest that a nociceptive ASH/ADL stimulus is produced in standard culture conditions, which indicates that it is made at least in part by *E. coli*. It is surprising to think that food would be a source of aversive stimuli, but both *E. coli* and many soil bacteria that form part of the *C. elegans* diet in the wild can kill *C. elegans* under certain conditions[30–32].

In mammals, NPY has sedative effects and decreases sensitivity to nociceptive stimuli[33]; these effects are reminiscent of our observation that *npr-1* activity suppresses a behaviour induced by noxious stimuli. It will be interesting to study the role of aversive pathways in regulating social behaviour in mammals and other animals. As social feeding is initiated by aversive stimuli, we propose that *C. elegans* aggregation supplies a defence to the animal. Possible advantages to group feeding would include the secretion of enzymes that inactivate bacterial toxins, or the stimulation of dauer formation and dispersal in the next generation. The data in this paper and in the accompanying paper[34] suggest that the regulation of social feeding behaviour in *C. elegans* is complex, involving several layers of positive and negative inputs. Such complexity may have evolved as a result of the tension between cooperation and competition that underlies social behaviour, and may be important to ensure that social behaviour is induced only when it offers a selective advantage. □

Methods

Strains, genetics and germline transformation

We grow nematodes at 20 °C under standard conditions[35]. Double mutants between *npr-1 X* and other loci were made by replacing marked balancer chromosomes with chromosomes carrying the mutation of interest. Mutations used have been described previously[36]. Strains used or generated in this work are listed in Supplementary Information. Germline transformation was carried out as described[37]. The *lin-15* clone pJMZ (50 ng μl^{-1})[38] was used as a co-injection marker. Strains injected were either AX215 *odr-4(n2144) III*; *npr-1(ad609) lin-15(n765*ts*) X* or CX4827 *ocr-2(ak47) IV*; *npr-1(ad609) lin-15(n765*ts*) X*. Test DNA was injected at 50 ng μl^{-1}. At least four transgenic lines were examined for each tested clone.

Behavioural assays

Aggregation and bordering were quantified as described previously, except that 80 animals were used on bacterial lawns of 2-cm diameter[8]. Speed of locomotion was quantified with the DIAS software program[8]. For more detailed information on behavioural assays, see Supplementary Information. The statistical significance of population and single animal behavioural assays was determined using the two-tailed *t*-test and the Mann–Whitney rank sum test, respectively. Error bars represent the s.e.m.

Molecular biology

Details of plasmid construction are available on request. Promoters for *odr-4* constructs were: *odr-10* (AWA); *str-1* (AWB); *odr-3* (AWC, AWB, AWA, ASH, ADF); pT08G3 (ADF); *sra-6* (ASH; ASI); *srg-8* (ASK); *sre-1* (ADL, ASJ); pF47C12 (ADL); *str-3* (ASI). *sre-1::odr-4–gfp* and F47C12::*odr-4–gfp*, but not other plasmids, restored social feeding to *odr-4*; *npr-1* animals. Expression patterns were verified by examining GFP fluorescence. Most functions of ASH are *odr-4*-independent, even those that are mediated by G-protein pathways (C.I.B., unpublished data). Promoters for *ocr-2* constructs were: pT08G3 (ADF);

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odr-10 (AWA); *sra-6* (ASH; ASI); pF47C12 (ADL); *odr-3* (AWC, AWB, AWA, ASH, ADF). Laser ablation was used to confirm that rescue of *ocr-2*; *npr-1* social feeding was due to transgene expression in the expected cells. Laser ablation of ADL eliminated rescue by the pF47C12::*ocr-2* transgene. Laser ablation of ASH eliminated rescue by the *sra-6::ocr-2* transgene. To aid in the identification of ADL or ASH and to confirm cell death, *ocr-2* trangenic strains also contained either F47C12::GFP (ADL) or *sra-6*::GFP (ASH) markers. Promoters used were described previously[19,21–23,27,39].

Laser ablation

Neurons were identified for ablation using DIC optics by a combination of positional and morphological cues[40,41]. Neurons were killed during the L1 or L2 stages using a laser microbeam[41], and operated animals were assayed within 48 h of the L4 to adult moult. Operated animals were *mIs10 V*; *npr-1(ad609)* X, which expressed GFP in muscle cells under the *myo-2* promoter. To test aggregation, they were mixed with *npr-1* control animals that did not express GFP.

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