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Article

Behavioral Motifs and Neural Pathways Coordinating O_2 Responses and Aggregation in *C. elegans*

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Summary

Background: Simple stimuli can evoke complex behavioral responses coordinated by multiple neural circuits. O_2 is an important environmental variable for most animals. The nematode *C. elegans* avoids high O_2, and O_2 levels regulate its foraging and aggregation.
Results: Here, we dissect aggregation and responses to O_2 gradients into behavioral motifs and show how O_2 responses can promote aggregation. To remain in a group, *C. elegans* continually modify their movement. Animals whose heads emerge from a group will reverse or turn, thereby returning to the group. Re-entry inhibits further reversal, aiding retention in the group. If an animal's tail exits a group during a reversal, it switches to forward movement, returning to the group. Aggregating *C. elegans* locally deplete O_2. The rise in O_2 levels experienced by animals leaving a group induces both reversal and turning. Conversely, the fall in O_2 encountered when entering a clump suppresses reversal, turning, and high locomotory activity. The soluble guanylate cyclases GCY-35 and GCY-36, which are expressed in head and tail neurons, promote reversal and turning when O_2 rises. Avoidance of high O_2 is also promoted by the TRP-related channel subunits OCR-2 and OSM-9, and the transmembrane protein ODR-4, acting in the nociceptive neurons ASH and ADL. Both O_2 responsiveness and aggregation can be modified by starvation, but this is regulated by natural variation in the *npr-1* neuropeptide receptor.
Conclusions: Our work provides insights into how a complex behavior emerges from simpler behavioral motifs coordinated by a distributed circuit.

Introduction

Most organisms, including humans, are highly sensitive to changes in ambient O_2. To monitor O_2 levels, animals have evolved sensors that are exposed either to their body fluids or to the outside milieu [1]. In response to changes in pO_2, these sensors can induce multiple changes in behavior and physiology. For example, in mammals hypoxia induces hyperventilation, increased heart output, systemic arterial vasodilation, pulmonary vasoconstriction, and activation of glucose uptake by muscle and fat cells [2]. Several cells modulating these O_2 responses have been described. For example, neurosecretory cells in the carotid bodies activate respiratory centers when O_2 levels fall, and endothelial cells and smooth muscle mediate arterial vasodilation in response to hypoxia. O_2-sensing mechanisms probably operate in most mammalian cells, and a variety of ionic conductances that are acutely modulated by O_2 tension have been identified by electrophysiological methods [1]. However, in many cases the nature of the sensor molecules, the pathways through which they mediate their effects, and the mechanisms by which these pathways are modulated are unclear.

The free-living nematode *Caenorhabditis elegans* can grow in the laboratory at O_2 tensions that range from 100% to less than 1% [3]. In the wild, this animal thrives in rich decaying organic matter. For example, *C. elegans* can grow to large numbers in mushroom beds [4, 5] and has repeatedly been isolated from compost heaps [6]. Because O_2 has a low diffusion coefficient in water and is rapidly consumed by microbes, O_2 tensions can vary from 21% to close to 0% across a few millimeters in such habitats [7–9]. *C. elegans* exhibit behavioral preferences for ambient-O_2 levels. Cultivated under standard laboratory conditions, it prefers 5%–11% O_2, but this response can be modified by environment and experience [10, 11]. Acute changes in pO_2 can induce *C. elegans* to modify multiple aspects of their movement, including locomotory speed, turning, and rate of reversal [11].

C. elegans foraging varies across different wild isolates. Some strains disperse on a bacterial food lawn and feed as individuals. Other strains accumulate where bacteria are most abundant and aggregate into groups [12, 13]. Aggregation and preference for thick bacteria are strongly induced in 21% O_2 but are progressively inhibited at lower O_2 levels [10]. *C. elegans* may thus use O_2 gradients to locate bacteria if O_2 levels are high. Aggregation, often a prelude to burrowing, may be part of a mechanism to avoid the high ambient O_2 associated with the soil surface.

Much of the natural variation in foraging can be accounted for by two wild alleles of the neuropeptide receptor *npr-1* (*n*euro*p*eptide receptor *r*esemblance). These alleles, called *npr-1 215F* and *npr-1 215V* (they bear phenylalanine and valine, respectively, at residue 215 of the receptor) reconfigure the sensory landscape of the animal [10, 11, 13, 14]. *C. elegans* that bear the *npr-1 215V* allele respond to food by dampening O_2-sensing circuits and strongly reducing movement. In contrast, isolates that bear the *npr-1 215F* allele, such as the Hawaiian strain CB4856, maintain high locomotor activity on food if ambient O_2 is close to 21%, but reduce movement when O_2 levels drop closer to their preferred levels [11].

Genetic studies have uncovered a network of neural pathways that promote aggregation and accumulation where bacteria are most abundant [11, 13, 15–17]. One of these pathways involves cGMP signaling in the AQR, PQR, and URX sensory neurons that reside or

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have sensory endings in the animal's body fluid [17]. These neurons express the atypical soluble guanylate cyclases GCY-35 and GCY-36, which are required for aggregation [16]. The heme prosthetic group of GCY-35 can bind O_2, suggesting that this soluble guanylate cyclase can act as an O_2 sensor [10]. Consistent with this, loss of *gcy-35* disrupts aerotaxis [10], and coexpression of GCY-35 and GCY-36 confers O_2 responsiveness to olfactory neurons in vivo [11]. However, GCY-35 and GCY-36 are unlikely to be the only O_2 sensors that act acutely to regulate *C. elegans* O_2 responses: Double mutants lacking both these molecules still respond to O_2 changes [11].

Here, we dissect the constellation of behavioral motifs used by *C. elegans* to aggregate and relate them to those used by the nematode to avoid high ambient O_2. We show that *C. elegans* create steep O_2 gradients as they aggregate. We examine how the network of signaling pathways and sensory neurons that regulate aggregation and bordering modulate movement in response to O_2 changes. Our data allow us to build a model for how animals aggregate and accumulate at preferred O_2 environments.

Results

C. elegans Responses to Starvation Are Polymorphic

When collected from the wild, *C. elegans* are usually either starved or in the dauer state [6]. This prompted us to examine whether responses to starvation can vary across different wild isolates of this species. We trapped well-fed animals from two strains, the Hawaiian CB4856 and the Bristol N2 strains, without food and monitored them as they starved. Animals from both strains initially dispersed (Figure 1A). However, after 5 hr without food, the Hawaiian animals began to aggregate. After 7 hr without food, more than 60% of CB4856 individuals had aggregated, usually into a single active group (Figure 1A). By contrast, animals from the N2 strain aggregated only weakly under these conditions. The Hawaiian animals often burrowed into the agar substrate after they aggregated; in contrast, the dispersed N2 animals rarely burrowed (data not shown). Wild strains of *C. elegans* thus can vary not only in their responses to food [12, 13], but also in their responses to starvation.

Aggregation of starved animals was reminiscent of *C. elegans* aggregation on bacterial food, an aggregation that is regulated by the NPR-1 neuropeptide receptor [13]. *C. elegans* strains bearing the *npr-1 215F* allele, such as the CB4856 Hawaiian isolate, or lacking *npr-1* activity aggregate strongly on food. In contrast, animals bearing *npr-1 215V*, such as the N2 Bristol strain, disperse on food. We examined whether the *npr-1* receptor could influence responses to starvation. Consistent with this possibility, N2 (Bristol) animals defective in *npr-1* began to aggregate after 4 hr of starvation, and almost all joined groups after 7 hr of food deprivation. These data suggest that natural variation in NPR-1 alters behavioral responses of both feeding and starved animals.

As well as regulating aggregation, the NPR-1 neuropeptide receptor modulates the locomotory response of *C. elegans* to bacterial food. Animals lacking *npr-1* or having the *npr-1 215F* allele are "roamers" and maintain high locomotory activity on food when O_2 levels are close to 21% [11, 13]. In contrast, animals bearing *npr-1 215V* are "dwellers" and slow down when they encounter food even if ambient O_2 is 21% [11, 13, 18]. We wondered whether variation at *npr-1* would modulate the exploratory behavior of starving animals. To test this, we

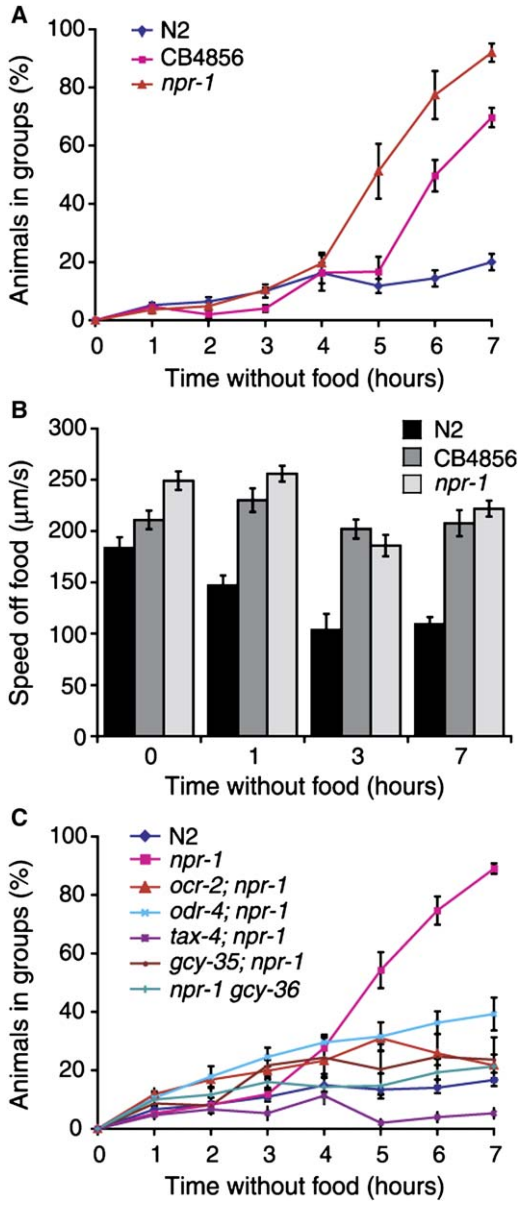

Figure 1. Starvation Induces Aggregation Behavior

(A) Well-fed N2 (Bristol), CB4856 (Hawaii), and *npr-1* mutant animals disperse when removed from food. However, as starvation ensues, CB4856 and *npr-1* mutants, but not N2 animals, reinitiate aggregation.

(B) N2 (Bristol) animals, which encode *npr-1 215V*, reduce locomotory activity as they starve; in contrast, CB4856 (Hawaii), which encode *npr-1 215F*, and *npr-1* mutants continue roaming even after 7 hr of starvation.

(C) Similar neural pathways regulate aggregation of feeding and starved animals.

The soluble guanylate cyclases GCY-35 and GCY-36, the cGMP-gated cation-channel subunit TAX-4, the TRPV-related channel OCR-2, and the novel transmembrane protein ODR-4 are required for starved animals to aggregate.

Error bars indicate SEM.

compared locomotory activity after different periods of food deprivation of N2 animals (*npr-1 215V*), of CB4856 animals (*npr-1 215F*), and of *npr-1(ad609)* loss-of-function mutants in an N2 genetic background. Animals bearing *npr-1 215F* or deficient in *npr-1* maintained high roaming even after 7 hr of starvation (Figure 1B). In contrast, N2 animals bearing *npr-1 215V* reduced exploratory activity as they starved. Thus the "roamer" and "dweller" behavioral polymorphism found in different wild isolates of *C. elegans* is exhibited not only in response to food, but also in response to starvation. These data suggest that natural variation at *npr-1* can alter multiple features of the *C. elegans* lifestyle in different contexts.

Similar Neural Pathways Control Aggregation of Starved and Feeding Animals

Opposite contexts—the presence of food and its prolonged absence—could promote *C. elegans* aggregation. The phenotypic similarity of these responses prompted us to examine whether they are orchestrated by similar neural pathways.

Two pathways that induce feeding animals to aggregate act in the ASH and ADL nociceptive neurons and require the TRPV-related ion-channel subunit OCR-2 [15]. The pathway in the ADL neurons also requires the novel single-pass transmembrane protein ODR-4 [15], which localizes chemosensory receptors to sensory cilia [19]. A further pathway acts in the body-cavity neurons AQR, PQR, and URX and is mediated by the soluble guanylate cyclases GCY-35/GCY-36 and the cGMP-gated ion channel TAX-4 [16, 17]. All these pathways also promote starvation-induced aggregation: *ocr-2; npr-1*, *odr-4; npr-1*, *tax-4; npr-1*, *gcy-35; npr-1*, and *npr-1 gcy-36* double mutants all failed to aggregate in response to starvation (Figure 1C). Thus, the same neural pathways promote aggregation of both feeding and starved animals.

Behavioral Subcomponents of Aggregation

To investigate the behavioral mechanisms by which *C. elegans* aggregate, we recorded freely moving animals from wild aggregating strains and *npr-1* mutant lines as they formed groups. Aggregation of both feeding and starved animals followed a stereotyped pattern (Movie 1 in the Supplemental Data available online; data not shown). A group began to form when two individuals, usually moving in different directions, encountered one another along their body lengths. Both animals suppressed forward movement, reversed, and started to move backward and forward while remaining close together. Further animals joined the group in similar fashion, reducing locomotory speed and initiating forward and backward movements to stay together (Movie 1).

To quantify these responses, we analyzed the behavior of feeding *npr-1* animals joining or residing in clumps of 6–16 animals. Several behavioral subcomponents contributed to aggregation: (1) isolated *npr-1* animals moved rapidly [13], facilitating encounters with other animals. (2) On joining a group, an animal reduced its speed of movement (Movie 1). (3) When the head and anterior part of an animal's body emerged from a group, the animal usually initiated a reversal that returned the animal to the group (Figures 2A, 2C, and 2D). (4) Animals that did not reverse upon emerging from a group often initiated a turn that led into the group (Figure 2D). Failure to initiate one of these movements was the predominant way animals left a group, usually in a straight run of forward movement (Figure 2C). (5) In the case of reversals, after the head of the animal had returned back into the group, animals quickly suppressed further backward movement. Most reversals were thus short—less that one-sixth of a body length (Figure 2E). Short reversals usually prevented animals from reversing out of the clump. (6) However, if the tail and posterior part of an animal emerged from a group, animals almost invariably switched to forward movement (Figures 2B and 2F). The animal thus re-entered the group rather than leaving it. This observation raises the possibility that *C. elegans* uses posteriorly located sensors to switch its direction of movement when its tail exits a group. Thus *C. elegans* aggregation results from a series of different behavioral motifs acting together in space and time. We next sought to dissect the neural pathways mediating each of these behavioral motifs.

Regulation of Starvation-Induced Aggregation by Ambient O_2

The observation that starved animals can aggregate on a simple agar substrate suggested that aggregating animals themselves, without the need of bacteria, generate the sensory signals for aggregation. Involvement of the soluble guanylate cyclases GCY-35 and GCY-36, which confer O_2 responsiveness [10, 11], implicated a role for O_2 in this behavior.

Previous work has shown that lowering ambient O_2 from 21% to 7% rapidly causes animals feeding in groups to disperse and to feed in isolation [10]. We therefore asked whether starved *npr-1* mutants could aggregate de novo in environments containing 5% O_2. Consistent with a role for O_2 responses in aggregation, *npr-1* animals could only aggregate to intermediate levels under these conditions compared to animals in an atmosphere containing 21% O_2 (Figure 3A). Thus, aggregation of starved animals is stimulated by high ambient O_2, although other stimuli probably also play a role.

C. elegans Locally Reduce Ambient O_2 as They Aggregate

We have previously shown that feeding *C. elegans* bearing the *npr-1 215F* allele, or defective in *npr-1* activity, progressively reduce movement as O_2 levels drop below 21% [11]. This modulation is dependent on the GCY-35 and GCY-36 soluble guanylate cyclases. If *C. elegans* reduce O_2 as they aggregate, this would locally suppress locomotory activity and promote group formation. To investigate this possibility, we measured O_2 levels in and around clumps of starved *npr-1* animals. Because starved animals can aggregate when food is absent, we could measure the effect of *C. elegans* on local O_2 concentration without the confounding presence of bacteria. To measure pO_2, we used a purpose-made microsensor with an active tip diameter of 20 μm (see Experimental Procedures). For comparison, adult *C. elegans* are ~1300 μm long and have a diameter of ~80 μm. We placed the sensor tip outside clumps and at various positions inside clumps (Figure 3B). O_2 tension 0.5 mm

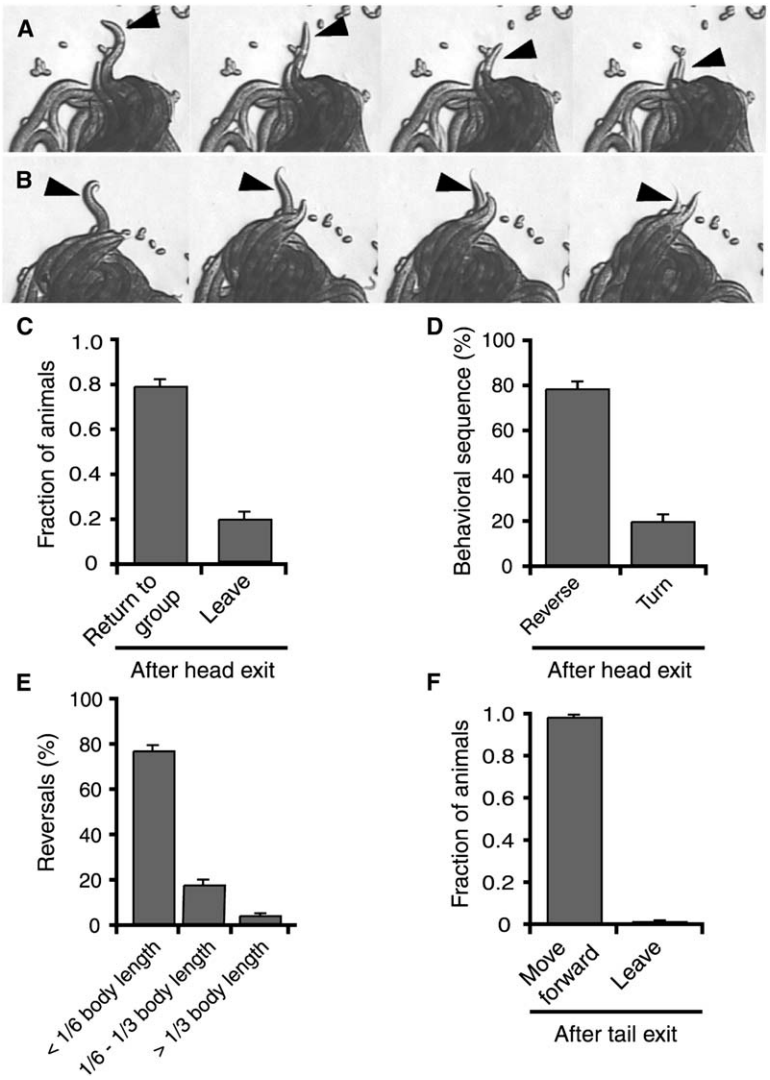

Figure 2. Behavioral Subcomponents Promoting Aggregation

(A and B) Exit of the head and anterior part of an animal from a clump usually results in a reversal that returns the animal to the clump (A). Emergence of the tail of the animal from a clump during backward movement almost invariably ends in a switch to forward movement before the animal leaves the group (B). Arrowheads indicate the head (A) and the tail (B) of an animal as it returns into the clump. Photographs are of starved, aggregated animals.

(C) The fraction of animals that return to the group or leave after their heads exits a clump. Data for this and subsequent panels in this figure are from groups of adult *npr-1(ad609)* animals aggregating on food. n = 10 clumps, each recorded for >5 min.

(D) Animals that leave the group head-first return to the group by using either a reversal or, less frequently, a turn.

(E) The size distribution of reversals that return animals to a group after head exit.

(F) The fraction of animals that switch to forward movement or reverse out of the group when their tails emerge from a clump.

Error bars indicate SEM.

away from the outside edge of clumps of 40–50 starved animals was 20.5% ± 0.11 (n = 5 clumps), close to atmospheric levels. In contrast, the average pO_2 just inside a clump was 13.7% ± 1.0 (n = 5). O_2 levels in the middle of clumps were lower, averaging 6.4% ± 0.7 (n = 5). Thus, as *C. elegans* aggregate, they locally deplete O_2 and create steep gradients that can range from 20.5% to 6.4% O_2 over 1 mm—the length of adult *C. elegans*.

Starved *npr-1* Animals Respond to a Drop in O_2 by Reducing Locomotory Activity

If food is absent, well-fed animals do not strongly suppress movement when ambient-O_2 levels drop [11]. We wondered whether starvation might stimulate aggregation by enhancing the slowing response to a drop in pO_2. To test this hypothesis, we measured the rate of movement of N2, CB4856, and *npr-1* animals at 21% O_2 and after a switch to 16% O_2 following various periods of food deprivation. Consistent with our hypothesis, starved CB4856 and *npr-1* animals reduced their movement significantly more than well-fed animals when O_2 levels fell (Figures 3C–3E). Thus, feeding state dynamically regulates both aggregation and the slowing response to a drop in ambient O_2. Bacterial food promotes these responses, the absence of food leads to their suppression in well-fed animals, and starvation reverses this suppression (Figure 3G). Interestingly, whereas 3 hr of food deprivation was sufficient to induce the slowing response to an O_2 drop, 5 hr of food withdrawal was required before these animals began to aggregate. Starvation may therefore modulate other pathways that regulate aggregation.

Modulation of *C. elegans* speed by O_2 when food is present requires the soluble guanylate cyclases GCY-35 and GCY-36 [11]. We asked whether these genes also couple the locomotory activity of starved animals to O_2 levels. Consistent with this possibility, starved *npr-1 gcy-36* and *gcy-35; npr-1* animals failed to strongly inhibit movement when O_2 levels were reduced from 21% to 16% (Figure 2F and data not shown). Interestingly, however, after 3 hr of starvation, *npr-1 gcy-36* mutant animals also failed to maintain the high locomotor activity typical of starved *npr-1* and CB4856 (Hawaii) animals when O_2 levels are at 21%. This suggests that GCY-36 upregulates locomotory activity of starved animals when O_2 levels are high (see discussion).

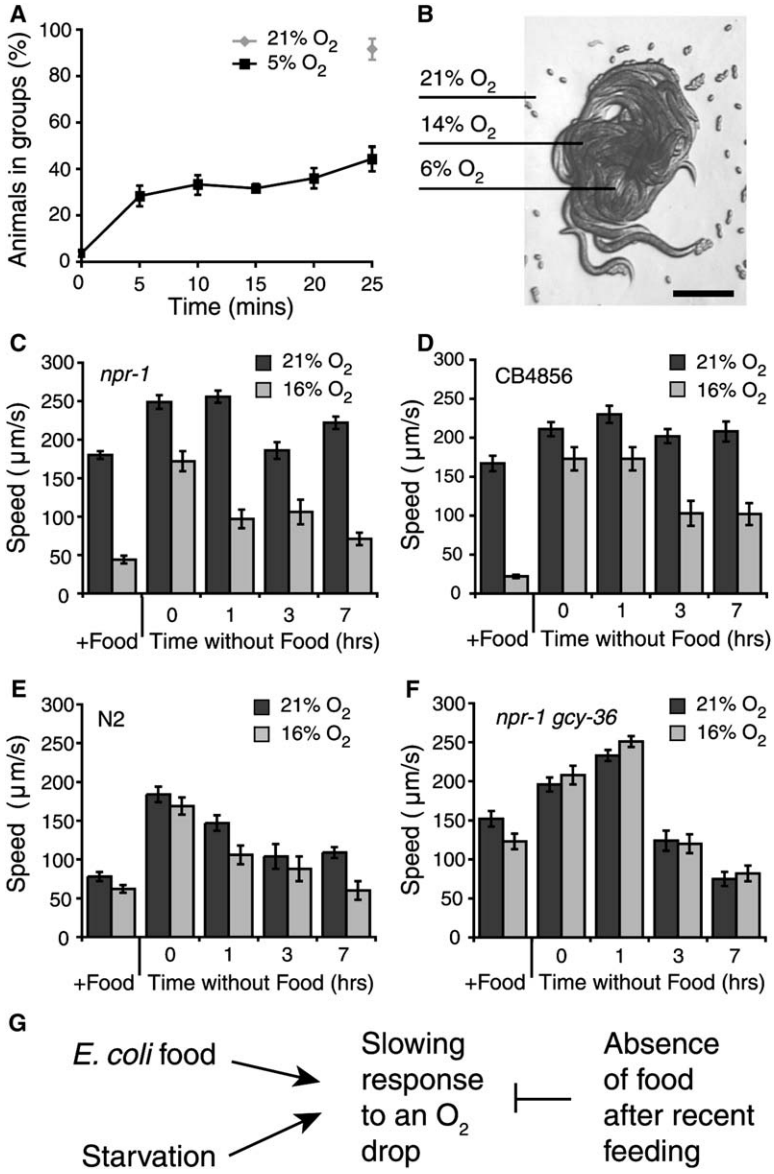

Figure 3. Regulation of Aggregation and Locomotory Activity by O_2 in Starved Animals

(A) Starved *C. elegans* aggregate more strongly at high ambient O_2.

(B) Aggregated animals locally reduce O_2 levels. Lines indicate O_2 levels in different areas relative to the clump. The scale bar represents 0.5 mm.

(C–E) Starvation modulates locomotory responses to changes in pO_2 in an *npr-1*-dependent manner. If they have recently been on food, CB4856 (Hawaii) and *npr-1* mutant animals continue roaming when ambient-O_2 levels drop; however, as these animals starve, they respond to drops in ambient O_2 by suppressing locomotory activity. N2 animals reduce locomotory activity as they starve, but only respond weakly to changes in pO_2.

(F) The slowing response of starved *npr-1* animals to a drop in O_2 requires GCY-36. GCY-36 is also required to maintain the high locomotory activity of starved *npr-1* animals at 21% O_2 (compare [C] and [F] after 3 and 7 hr of starvation, $p < 0.001$).

(G) A simple model for how food and food deprivation regulate the slowing response of CB4856 and *npr-1* animals to a drop in pO_2.

Error bars indicate SEM.

Regulation of Reversal and Turning Rates by a Rise in Ambient O_2

Another behavioral subcomponent of aggregation involves the initiation of a reversal when the head of an animal emerges from a group. Previously, we showed that feeding *C. elegans* transiently elevate the probability of a reversal when they experience a rise in ambient O_2 from 11% to 21% [11]. Because O_2 tension is significantly lower inside than outside a clump, animals exiting a group should experience a steep rise in O_2. This rise would be expected to prompt a reversal, returning the animal to the group and promoting aggregation. This scenario predicts that some of the neural circuitry promoting aggregation could promote the reversal response to a rise in O_2 levels.

To investigate this further, we first characterized in detail how *C. elegans* responds to rises and falls in ambient O_2 in the absence of food. We changed O_2 levels between 21% and 12% by rapidly pumping defined O_2:N_2 mixtures into the behavioral arena while capturing video footage of the animals' responses. To relate behavioral responses to changes in O_2, we simultaneously measured O_2 levels in the arena once every second. During upsteps, animals experienced a rise from 12% to 20% O_2 in the first 15 s after the gas switch and then a further rise to 21% in the subsequent 15 s (Figure 4A). Conversely, during downsteps, O_2 fell from 21% to 13% in the first 15 s after the gas switch and then stabilized at 12% by 30 s after the switch. To score behavioral responses, we counted reversals and omega turns executed in 15 s time intervals during and after the change in O_2 tension (Figures 4B and 4C). In an omega turn, an animal executes a deep bend that results in the animal changing its direction of movement [20, 21]. During O_2 upsteps, *npr-1* animals exhibited a rise in the probability of both reversals and omega turns (Figures 4B and 4C). This rise was most striking in the 30 s during which O_2 levels were increasing. Interestingly, however, rates of reversals and omega turns remained elevated for more than 1 min after O_2 levels had stabilized, suggesting

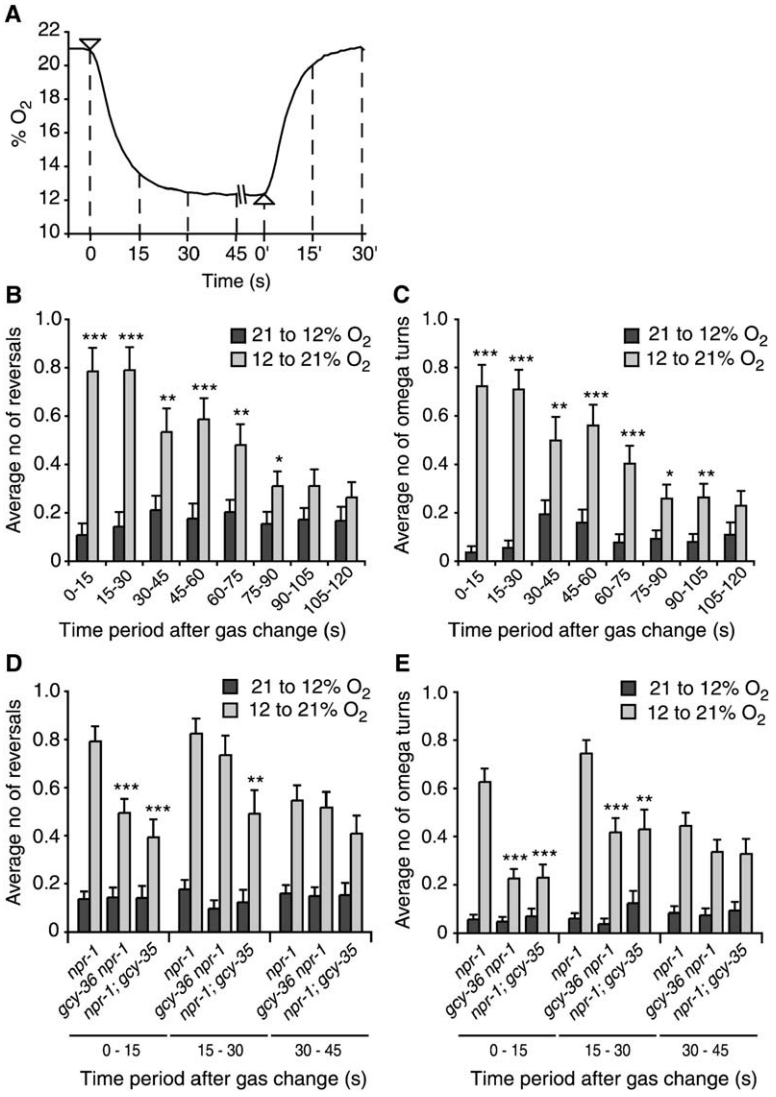

Figure 4. Regulation of Reversals and Turns by Changes in Ambient O_2

(A) Time course of O_2 changes used in our assays. Arrowheads indicate the time point at which a gas switch was initiated. Dashed lines demarcate 15 s intervals after switches.
(B and C) Raising ambient O_2 from 12% to 21% induces reversals (B) and omega turns (C) in *npr-1* animals. The increase in reversals and turns perdures after O_2 levels have stabilized at 21%. Asterisks indicate p values for comparisons between responses to shifts from 21% to 12% and 12% to 21% O_2.
(D and E) Loss-of-function mutations in *gcy-35* or *gcy-36* reduce the ability of *C. elegans* to respond to a rise in O_2 by increasing reversal frequency (D) and omega turns (E). Asterisks indicate p values for comparisons of equivalent responses of *npr-1* and *gcy; npr-1* double-mutant animals.
Data in this figure and in Figures 5 and 6 refer to average number of maneuvers per animal. Error bars indicate SEM.

maintenance of neuronal signaling either in sensory neurons or downstream interneurons during this period.

We next examined how loss-of-function mutations in *gcy-35* or *gcy-36* altered these responses to O_2 steps. Loss of either gene significantly attenuated the increase in reversals and omega turns observed in *npr-1* animals after an increase from 12% to 21% O_2 (Figures 4D and 4E). Thus, the soluble guanylate cyclases GCY-35 and GCY-36 regulate not only *C. elegans* speed of movement but also reversals and omega turns in response to changes in pO_2. However, whereas the increase in turning rate elicited by an O_2 rise was transient, and persisted for only 1 min after O_2 levels stabilized, the change in locomotory speed is persistent, lasting many minutes [11]. Perhaps distinct neural circuits mediate these different behavioral outputs.

ODR-4 and the TRPV-Related Channels OCR-2 and OSM-9 Promote O_2 Avoidance

The increase, stimulated by a rise in O_2, in the frequency of reversals and omega turns was only partly disrupted by mutations in *gcy-35* and *gcy-36*, suggesting that *C. elegans* also uses other O_2-sensing pathways to regulate these responses. Consistent with this, *gcy-35; npr-1 gcy-36* triple mutants are still able to avoid high O_2 in aerotaxis assays, although less effectively than *npr-1* animals [11]. This prompted us to examine whether other neural pathways that promote aggregation also modulate O_2 responses.

As mentioned previously, the TRP channel-related subunits *ocr-2* and *osm-9* promote aggregation. We asked whether loss of either of these genes disrupted aerotaxis. Because NPR-1 inhibits O_2 responses in some conditions, we performed our assays in animals lacking this receptor. Interestingly, when assayed in an O_2 gradient, both *ocr-2; npr-1* and *osm-9; npr-1* mutants showed defects in avoidance of high ambient O_2 (Figure 5A). Thus the OCR-2 and OSM-9 receptor subunits promote O_2 avoidance.

The ODR-4 protein acts in the same neural pathway as OCR-2/OSM-9 to induce aggregation behavior. We therefore asked whether *odr-4; npr-1* mutants also displayed aerotaxis defects. Like *ocr-2* and *osm-9* mutants, animals lacking *odr-4* showed reduced avoidance of

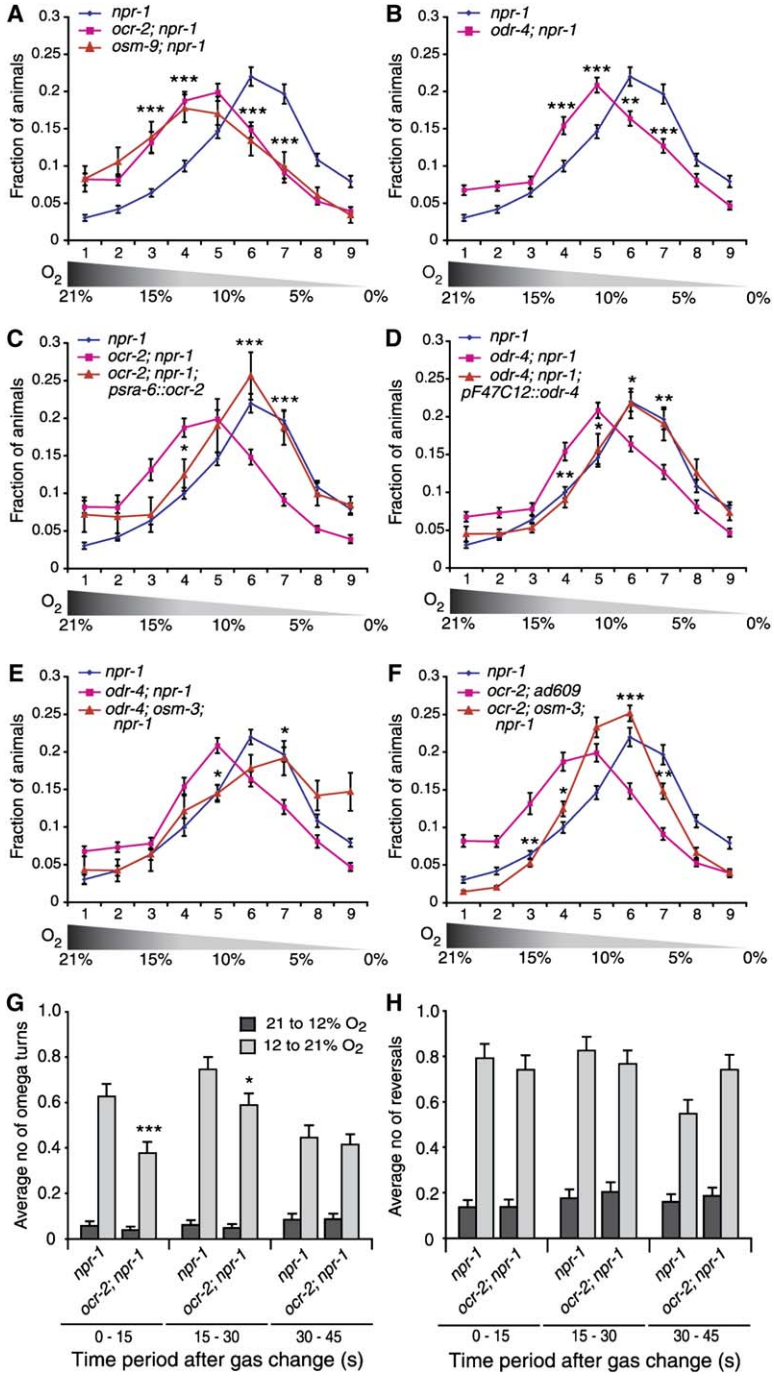

Figure 5. OCR-2 and ODR-4 Act in Nociceptive Neurons to Mediate Avoidance of High Ambient O_2

(A) *ocr-2; npr-1* and *osm-9; npr-1* animals are defective in avoidance of high ambient O_2. Asterisks indicate p values for comparisons between *npr-1* and double mutants in bins 3 to 7.

(B) Loss of the single-pass transmembrane protein ODR-4, which is required to localize chemosensory GPCRs to sensory cilia, also disrupts hyperoxia avoidance. Asterisks indicate p values for comparisons between *npr-1* and *odr-4; npr-1* in bins 3 to 7.

(C) OCR-2 expression in the ASH nociceptive neurons, from the *sra-6* promoter, is sufficient to restore strong avoidance of high ambient O_2 to *ocr-2* mutants. Asterisks indicate p values for comparisons between *ocr-2; npr-1* and *ocr-2; npr-1 dbEx sra-6::ocr-2* in bins 3 to 7.

(D) ODR-4 expression in the ADL nociceptive neurons, from the F47C12.6 promoter, restores strong avoidance of high ambient O_2 to *odr-4* mutants. Asterisks indicate p values for comparisons between *odr-4; npr-1* and *odr-4; npr-1 dbEx F47C12::odr-4* in bins 3 to 7.

(E and F) Loss of *osm-3* kinesin restores avoidance of high ambient O_2 to *odr-4* (E) and *ocr-2* (F) mutants. Asterisks indicate p values for comparisons between double and triple mutants.

(G and H) A loss-of-function mutation in *ocr-2* reduces the ability of *C. elegans* to respond to a rise in O_2 by increasing omega turns (G) but does not significantly reduce induction of reversals (H). Asterisks indicate p values for comparisons of equivalent responses of *npr-1* and and *ocr-2; npr-1* animals.

Error bars indicate SEM.

high ambient O_2 (Figure 5B). Together, these data suggest that a pathway mediated by ODR-4, OCR-2, and OSM-9 promotes O_2 avoidance.

OCR-2/OSM-9 and ODR-4 Act in Nociceptive Neurons to Mediate Avoidance of High O_2

OCR-2 acts in the nociceptive neurons ASH and ADL to promote aggregation, whereas ODR-4 acts in ADL [15]. Both ASH and ADL can respond to a variety of noxious cues and direct an escape response by initiating a reversal that is usually followed by an omega turn (reviewed in [22]). We therefore asked whether OCR-2 and ODR-4 act in nociceptive neurons to promote avoidance of high O_2. Consistent with this hypothesis, expression of OCR-2 in ASH (Figure 5C) and expression of ODR-4 in ADL (Figure 5D) restored strong avoidance of high ambient O_2 to *ocr-2; npr-1* and *odr-4; npr-1* mutants, respectively. Thus the nociceptive neurons ASH and ADL promote avoidance of high ambient O_2 and may be O_2-sensing neurons.

Loss of OSM-3 Kinesin Restores Avoidance of High Ambient O_2 to *ocr-2* and *odr-4* Mutants

Neither OCR-2 nor ODR-4 is absolutely required for *C. elegans* to aggregate. Animals lacking either of these genes can be restored to strong aggregation behavior

by loss-of-function mutations in the kinesin *osm-3* [15]. *osm-3* is expressed in chemosensory neurons whose ciliated endings communicate with the outside milieu [23]. Mutations in *osm-3* cause truncation of the distal parts of these cilia and consequent loss of sensory function [24]. Because OCR-2 and ODR-4 promoted O_2 responses, we speculated that rescue of aggregation behavior in *ocr-2* and *odr-4* mutants by *osm-3* could reflect restored avoidance of high ambient O_2. To investigate this possibility, we compared the aerotaxis of *ocr-2; npr-1* and *odr-4; npr-1* animals with *ocr-2 osm-3; npr-1* and *odr-4; osm-3; npr-1* mutants. Consistent with our hypothesis, mutating *osm-3* kinesin restored strong avoidance of high ambient O_2 to both *ocr-2* and *odr-4* mutants (Figures 5E and 5F). Thus *ocr-2* and *odr-4* are not essential for efficient avoidance of high ambient O_2, perhaps because other pathways can compensate for their absence.

To examine the behavioral mechanisms by which OCR-2 promotes avoidance of high pO_2, we compared the responses of *npr-1* and *ocr-2; npr-1* animals to O_2 downsteps and upsteps. Loss of *ocr-2* reduced the number of omega turns executed during the first 30 s of a switch from 12% to 21% O_2 (Figure 5G), although it did not significantly alter reversal in response to the O_2 upstep under our assay conditions (Figure 5H). Because omega turns serve to reorient *C. elegans*, this defect could explain reduced avoidance of high O_2 by these animals.

C. elegans Suppress Reversals and Turns When O_2 Levels Fall from 21% to 12%

In our studies of aggregation behavior, we noticed that the reversals initiated when the head of an animal leaves a group are typically short (<1/6 body length, Figure 2E) because they are usually suppressed when the head reverses back into the group. In contrast, many of the reversals induced by a 12% to 21% rise in O_2 (Figure 4) were much longer, often of more than one body length (data not shown). These observations raised the possibility that *C. elegans* can suppress reversals if O_2 falls toward preferred levels, as occurs when an animal rejoins a group.

To explore this, we compared the behavior of animals that experienced a rise from 12% to 21% O_2 and were then retained at 21% O_2 to those of animals that experienced the same 12% to 21% O_2 upstep but were then immediately returned to 12% O_2 (Figure 6A). As expected, the upstep from 12% to 21% O_2 induced reversal and turning behavior, both in N2 and *npr-1* animals (Figures 6B and 6C). Interestingly, however, whereas animals retained at 21% O_2 continued to reverse and turn for more than 1 min after O_2 had stabilized, animals returned to 12% O_2 immediately suppressed both reversal and turning when O_2 levels began to fall. Thus *C. elegans* suppress reversals and turns when O_2 levels fall toward preferred levels.

To explore the genetic basis for this suppression, we compared the responses of *npr-1* animals to those of *ocr-2; npr-1* and *gcy-35; npr-1* mutants in the same behavioral paradigm. Interestingly, both *ocr-2; npr-1* and *gcy-35; npr-1* animals retained the ability to suppress reversals and turns in response to a downstep from 21% to 12% O_2 (Figures 6B and 6C). These data suggest

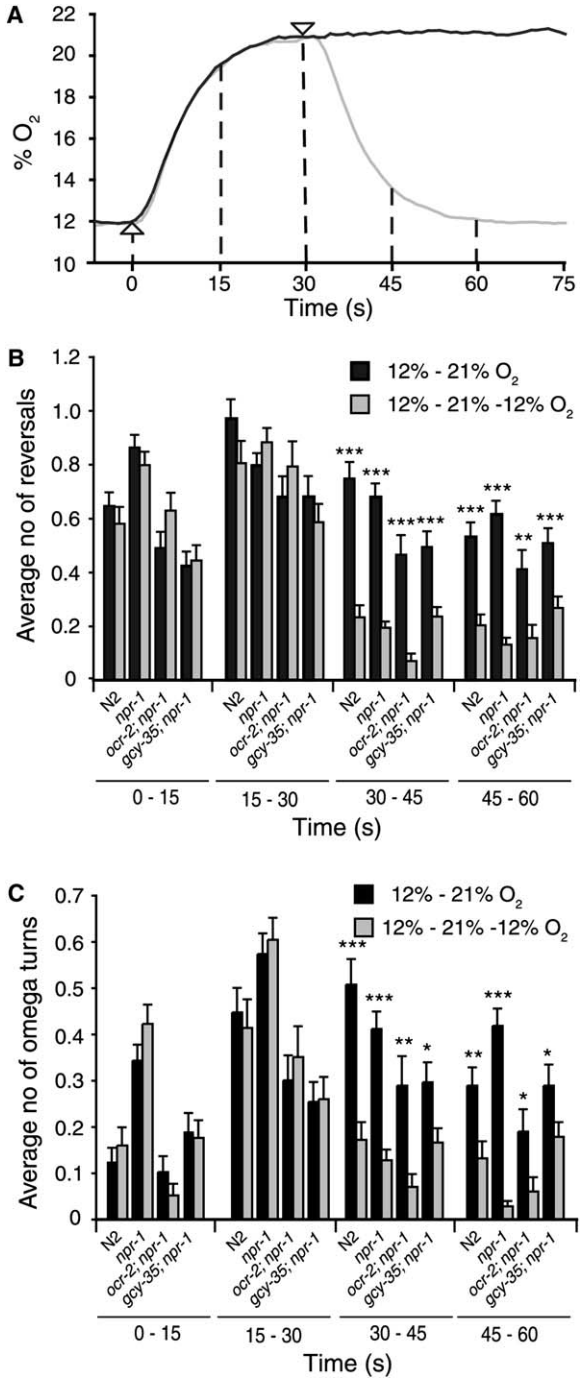

Figure 6. *C. elegans* Suppress Reversal and Turning Behavior When Ambient O_2 Falls toward Preferred Levels

(A) Time course of O_2 changes used in our assays. Arrowheads indicate the time point at which a gas switch was initiated. The dark line indicates O_2 changes from 12% to 21%. The light line indicates O_2 changes from 12% to 21% to 12%. Dashed lines demarcate 15 s intervals after switches.

(B and C) Comparison of the frequency of reversals (B) and omega turns (C) induced by the O_2 changes outlined in (A). Reversals and omega turns are suppressed in all tested genotypes when O_2 levels return to 12% after a brief rise to 21%. Asterisks indicate p values for comparisons of responses to a 12% to 21% O_2 step and a 12% to 21% to 12% O_2 step at similar time intervals.

Error bars indicate SEM.

the existence of other pathways that act to suppress turning and reversal when ambient-O_2 levels fall.

Discussion

A Distributed Circuit for O_2 Sensing in *C. elegans*

Multiple sensory neurons coordinate *C. elegans* responses to ambient-O_2 changes (Figures 7A and 7B). A distributed circuit involving several O_2 sensors may enable the animal to respond reliably to small changes in O_2. O_2 sensors in different locations may also allow the nematode to monitor internal as well as external O_2 levels. To navigate O_2 gradients, *C. elegans* respond to both rises and falls in O_2 levels. When O_2 rises above preferred levels, *C. elegans* reverse, turn, and can increase locomotory activity. Conversely, when O_2 levels fall toward preferred levels, the animal suppresses reversals and turns and can reduce locomotory activity. The GCY-35 and GCY-36 soluble guanylate cyclases that are coexpressed in the AQR, PQR, and URX body-cavity neurons couple both speed of movement and turning rates to changes in ambient O_2.

The TRPV-related channel subunits OCR-2 and OSM-9 and the transmembrane protein ODR-4 also promote avoidance of high ambient O_2. Transgenic studies suggest that OCR-2 and ODR-4 act in nociceptive neurons to promote avoidance of high O_2: expression of OCR-2 in ASH and of ODR-4 in ADL restores strong O_2 avoidance. Although we have not directly shown that OCR-2 or OSM-9 transduces an O_2-sensing pathway, this is a possibility. TRPC channels, which are related to TRPV channels, have previously been implicated as redox sensors in endothelial cells [25]. It will be interesting to explore whether TRPV channels can transduce O_2 signals in vertebrates.

A Model for Aggregation and Accumulation at Preferred O_2 Tensions

C. elegans aggregation can be dissected into a constellation of behavioral motifs (Figure 7C). Upon joining a group, *C. elegans* reduce locomotory activity. When its head emerges from a group an animal reverses, but this reversal is suppressed when the head re-enters the group. Because aggregated *C. elegans* locally deplete O_2, animals leaving or joining a group experience a rise and fall in O_2 levels, respectively. The behavioral responses to these O_2 changes would be expected to promote aggregation.

In our experiments, we examined how *C. elegans* respond to temporal changes in O_2, with little or no spatial component to the O_2 gradient that animals experienced. However, aggregating animals experience a spatial O_2 gradient as they enter or leave a clump. Our studies of aggregation raise the possibility that *C. elegans* can respond to spatial O_2 gradients by making head-to-tail comparisons of O_2 concentration. When the tail of an individual exits a group during a reversal, the animal almost invariably shifts to forward movement. If this is a response to aversive high O_2 outside the clump, it suggests that animals have O_2 sensors located in the tail. A combination of head and tail sensors might enable animals to compare O_2 levels across their body length, inducing backing if pO_2 is higher anterior and forward movement if pO_2 is higher posterior, as in fact occurs during aggregation. The location of the cell bodies of GCY-35- and GCY-36-expressing neurons is consistent with this hypothesis (Figure 7A). AQR, PQR, and URX lie at opposite ends of the animal: URX and AQR in the head and PQR in the tail. The ALN, PLN, and SDQL neurons that express GCY-35 also lie posterior, whereas SDQR, another GCY-35-expressing neuron, lies anterior (Figure 7A). GCY-36, one of the presumed O_2 sensors, is

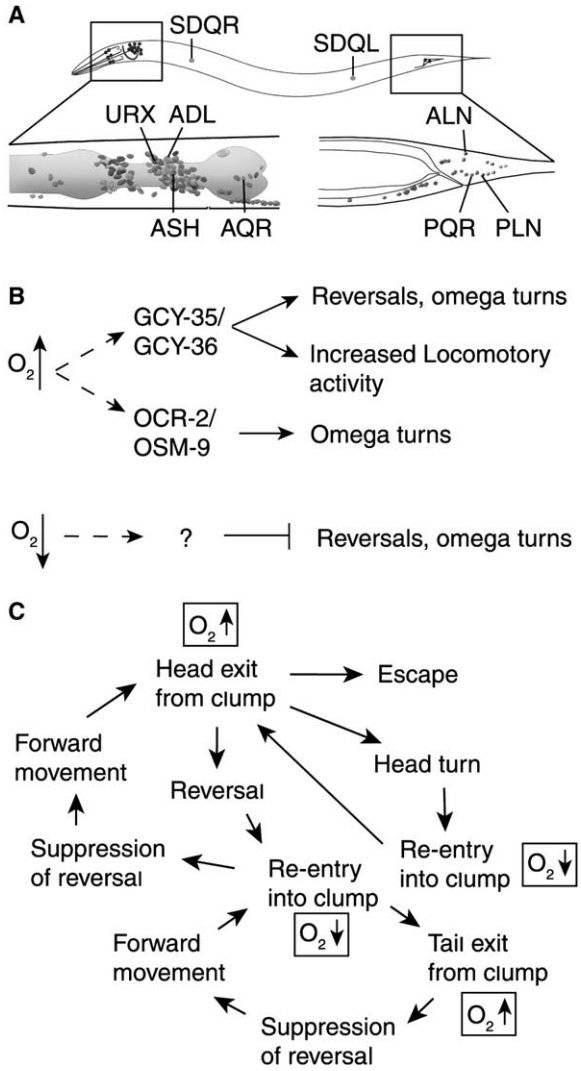

Figure 7. Model for Genes and Neurons Regulating *C. elegans* Aggregation and O_2 Responses

(A) Anatomical location of identified neurons implicated in aggregation and O_2 sensing. The ASH and ADL neurons express the TRPV-related channels OCR-2 and OSM-9. GCY-36 is expressed in AQR, PQR, and URX, whereas GCY-35 is expressed in AQR, PQR, URX, ALN, PLN, SDQL, and SDQR. Putative O_2-sensing neurons lie both at the anterior and posterior of the animal and may allow a comparison of O_2 concentrations at the two ends of the animal.

(B) Pathways promoting avoidance of high ambient O_2. When O_2 levels rise above 12%, regulated GCY-35 and GCY-36 activity can induce reversals, omega turns, and high locomotory activity. The OCR-2 pathway promotes omega turns when O_2 levels rise.

(C) Schema for behavioral subcomponents promoting aggregation and accumulation at preferred ambient-O_2 levels. Not indicated is the reduction in locomotory activity exhibited by *npr-1* and CB4856 animals when they encounter preferred low-O_2 environments.

Error bars indicate SEM.

appropriately targeted subcellularly for localized sensing. Functional GFP-tagged GCY-36 is enriched at the ciliated endings of AQR and PQR [11]. The AQR cilium projects anterior and lies in the head between body-wall muscle and the terminal bulb of the pharynx. The cilium of PQR projects posterior into the tail spike [26]. The synaptic outputs of AQR, PQR, and URX deduced from electron micrographs are consistent with these neurons controlling direction of movement [26]. All these neurons make synaptic connections with command interneurons that control forward versus backward movement. Although this model for head-to-tail O_2 comparison is attractive, we have not provided direct evidence for it.

Regulation of Aggregation and O_2 Responses by Context and Experience

Both food and prolonged food deprivation can stimulate aggregation. Food and starvation also regulate *C. elegans* locomotory response to O_2 changes: CB4856 (Hawaii) and *npr-1* animals strongly reduce locomotory activity when O_2 levels fall if they are feeding or are starved (this work; [11]). Interestingly, however, loss of *gcy-36* has different effects on locomotory activity in the two contexts (Figure 3; Table S1). *npr-1 gcy-36* animals that are feeding maintain high locomotory activity at both 21% and 16% O_2. The simple inference from this loss-of-function phenotype is that GCY-36 becomes active when O_2 levels fall and induces slowing (Figure 3, [11]). In contrast, however, starved *npr-1 gcy-36* animals exhibit low locomotory activity at both 21% and 16% O_2, typical of *npr-1* animals at 16% O_2 but much lower than *npr-1* animals at 21% O_2 (Figures 3C and 3F , 7 hr time point). This second set of data suggests that GCY-36 is activated by high O_2 levels and induces high locomotory activity. These contradictory inferences appear to reflect plasticity in the O_2-sensing circuits regulating locomotory activity. In contrast to normoxia-cultivated *npr-1 gcy-36* animals (Figure 3, [11]), *npr-1 gcy-36* animals raised in 11% or 1% O_2 exhibit low locomotory activity on food at both 21% and 16% O_2, lower than *npr-1* controls at 21% O_2 (A.P. and M.d.B., unpublished data). Together, these genetic data are more consistent with a model in which GCY-35/GCY-36 are activated when they bind O_2, much like how classical soluble guanylate cyclases are activated by binding nitric oxide. To reflect this altered interpretation, we have modified our model ([11], Figure 7B), mindful of the need of biochemical experiments to test it further. The plastic nature of neural networks may frequently make it complicated to infer the wild-type function of a gene from its loss-of-function phenotype.

Ecologically, enhancement of the slowing response to reduced O_2 tension by starvation could aid animals in finding O_2-consuming microbial food. It could also promote long-term survival by helping to ensure that animals avoid the surface where dessication may be a threat. Changes in nutritional state regulate aggregation behavior in other nematodes apart from *C. elegans*. For example, in the plant parasitic nematode *Tylenchorhynchus martini*, rapid host-plant growth favors aggregation, whereas slow growth inhibits group formation [27–29]. In contrast, in the mushroom-eating nematode *Ditylenchus myceliophagous*, swarming occurs after food is exhausted [30].

After experiencing a rapid rise in O_2 from 12% to 21%, *C. elegans* engage in a prolonged bout of reversals and turns that lasts more than 1 min after O_2 levels have stabilized at 21%. This behavior is reminiscent of an area-restricted search by which animals locally seek a preferred environment that they have recently left before moving away (reviewed in [22]). It will be interesting to explore the neural circuitry properties that encode the delay between stabilization of O_2 levels and resumption of persistent forward movement.

How is sensory information integrated across a distributed O_2-sensing circuit? For control of forward versus backward movement, integration of information from different sensory neurons may ultimately occur in the command interneurons, which receive direct and indirect synaptic input from many of the sensory neurons outlined in Figure 7A. However, the architecture of the circuit—how broadly information is distributed across different neurons and whether context and experience modify single or multiple elements in it—remains unclear. Combined genetic and neural imaging studies should make it possible to uncover this logic.

Experimental Procedures

Strains

Except where indicated, nematodes were grown under standard conditions on OP50 *E. coli* [31]. Double-mutant strains were created by following visible phenotypes, or by using balancer chromosomes [15–17]. Strains made or used in this study are listed in Supplemental Data.

Behavioral Assays

To measure aggregation in the absence of food, we trapped 50 animals in a 1.7-cm-diameter arena by a copper washer [15] on an unseeded agar plate, and we counted animals in groups at hourly intervals. Speed of animal movement under different O_2 atmospheres was quantified with DIAS software [11, 13, 16]. Defined O_2::N_2 gas mixtures were obtained from BOC. Aerotaxis assays were carried out as described previously [10]. Behavioral data represent means of at least four, and usually six or more, assays carried out over several days. Statistical significance was determined with the two-tailed t test. In all figures, error bars represent the standard error of the mean (SEM).

To monitor O_2 levels, we used a fiber-optic microsensor (Loligo). In experiments measuring turning rates (Figures 4 and 6), we studied animals that had been food-deprived for 1 hr and modified our previous assay [11] to increase the rate of change of O_2 concentration. We used six evenly spaced luers inserted into the lid of the 3.5 cm petri-dish arena, each connected to gas-filled syringes. Three luers connected to syringes containing the same gas mix were active at any one time. Gas flow rate in each syringe was 20 ml/minute, at atmospheric pressure, giving a total flow rate into the arena of 1 ml/second.

Molecular Biology and Transgenic Studies

Expression constructs were made with Gateway (Invitrogen) [17, 32] and have been published previously [15, 17]. Details of plasmid construction are available upon request. The following promoters were used (with expression pattern in parentheses): *sra-6* (ASH, ASI) and F47C12. 6 (ADL) [33]. Transgenic strains used *lin-15* (pJMZ, 30 ng μl^{-1}) or *unc-129::gfp* as coinjection markers following standard methods [34]. Several transgenic lines were examined for each experiment; figures show data from one representative line.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, one table, and one movie are available with this article online at: http://www.current-biology.com/cgi/content/full/16/7/649/DC1/.

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