Hyperoxia Avoidance and Aggregation Behavior in *C. elegans*

Annelie Persson

UNIVERSITY OF GOTHENBURG

Department of Cell and Molecular Biology
University of Gothenburg, Sweden
2010

ISBN 978-91-628-8181-8

Department of Cell and Molecular Biology
University of Gothenburg, Sweden

Printed by Chalmers Reproservice
Göteborg, Sweden

ABSTRACT

Living in the soil, *C. elegans* can move in three dimensions in search for food. To navigate, it partly uses oxygen levels as a description of its habitat. Oxygen tension may indicate presence of microbial food and location with respect to the surface, where oxygen is 21%. The N2 groups of *C. elegans* strains differ in their oxygen responses from other strains of this species collected in the wild [1-4]. This difference is due to a polymorphism in the NPY receptor homologe, NPR-1. The result is two distinct feeding strategies; solitary feeding and feeding in groups (aggregation) [1]. NPR-1 antagonizes hyperoxia avoidance on food [4] and N2-like strains, carrying a gain of function mutation in the receptor, feed alone and do not respond strongly to changes in ambient oxygen. In contrast, strains carrying the ancestral form of the receptor, NPR-1 215F, exhibit robust hyperoxia avoidance. These animals aggregate on food, at least in part because animals create a low oxygen environment as they form groups [1, 4].

In paper I we examined how hyperoxia avoidance can trigger aggregation. We showed that when animals encounter a rise in oxygen they initiate a reversal and turn. We showed that similar behaviors direct the animal to stay in an aggregate, and that aggregated animals create a sharp oxygen gradient. We further showed that soluble guanylate cyclases, expressed in the body cavity neurons, and TRPV channels expressed in the nociceptive neurons ASH and ADL, regulate these behaviors.

In paper II ---text removed from public version ---

In paper III we showed that a polymorphic locus, encoding the neuroglobin *glb-5*, regulates hyperoxia avoidance. The ancestral allele, *glb-5(Haw)*, acts in the body cavity neurons and tunes the dynamic range of these neurons to a narrow range close 21% oxygen. ---text removed from public version ---

The data presented in this thesis thus provide novel insights into oxygen sensing in a metazoan, and highlight how oxygen responses promote aggregation behavior of a nematode.

PAPERS DISCUSSED

This thesis is based on the following papers, which will be referred to by their Roman numerals within the text.

Paper I
Behavioral motifs and neural pathways coordinating O_2 responses and aggregation in *C. elegans.*
Rogers C[1], Persson A[1], Cheung B[1], de Bono M.
Curr Biol. 2006 Apr 4;16(7):649-59.

Paper II
---text removed from public version ---
Persson A, Wolfram V, Couto A, Tremain N, de Bono M.
Manuscript

Paper III
Natural variation in a neural globin tunes oxygen sensing in wild *Caenorhabditis elegans*.
Persson A[1], Gross E[1], Laurent P, Busch KE, Bretes H, de Bono M.
Nature. 2009 Apr 23;458(7241):1030-3.

Paper IV
---text removed from public version ---.
Persson A, de bono M.
Manuscript

[1]These authors contributed equally to this paper

BACKGROUND

C. elegans

In the wild

Caenorhabditis elegans is a ~ 1.3 mm long, free-living, soil nematode with a diameter of ~ 80 um. It habitats nutrient rich organic materials and eats anything that fits into its mouth. It has almost never been found outside anthropogenic territory and their natural habitat is therefore largely unknown [5].

When conditions are good, the population size grows quickly. A single hermaphrodite lays about 300 eggs that develop through four larval stages into adults. The fast growing population quickly runs out of resources and extinction and de novo colonization follows [6]. When an environment becomes crowded and food limiting the developing larvae can enter a stress resistant stage, dauer larvae, which can survive for long periods of time. It is usually in this state *C. elegans* is found when isolated from the wild [7]. Dauer larvae can hitch-hike with carriers such as snail and spread to new areas [5].

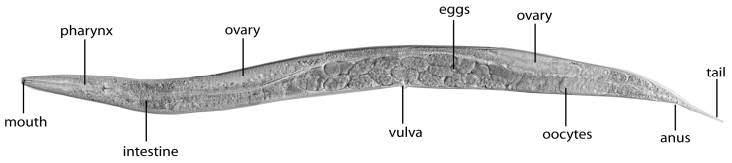

Fig. 1 A *C. elegans* hermaphrodite. Picture taken by C. Mörk

C. elegans is composed of only 959[1] somatic cells. Despite this simplicity it contains most major differentiated tissue types. The body plan is easily visualized under the microscope due to the transparency of the animal (fig. 1). It includes a feeding organ, the pharynx, that connects to the intestine running along the body length. Other organs lie outside this central tube. The reproductive system is easily seen in fig. 1 as it occupies a large portion of the body. A hermaphrodite has two gonad arms and eggs are fertilized and develop internally for several hours before laid. The pseudocoelom, also referred to as the body fluid, surrounds the inner tube of pharynx, intestine and separates them from the gonads as well as the outer tube made up of muscles, neurons, excretory system, hypoderm and cuticle.

In the lab

C. elegans fills the need of a very simple genetic model for addressing biological problems of multicellular organisms. It is well suited for forward genetic studies since it has short generation time (3.5 days at 20°C) and can easily be grown in large quantities. Techniques to mutate and to identify unknown mutations in the genome are well established, and mapping is facilitated by the presence of both hermaphrodites (XX) and males (X0).
It was for its potential using forward genetics, that *C. elegans* was picked by Brenner as a model in the 1960s. *C. elegans* also offers opportunities for reverse genetic approaches. It is a fast system for generating transgenes or transient knockouts using RNAi. It is also particularly suited for large scale studies, since it can be cultivated as a microbe. To give an idea of its relevance as a genetic model, about 30% of its genes have a human orthologue [8]. Gene homology with humans can approach 80% [9]. Its evolutionary relationship to humans, and other model organisms [10] is illustrated in fig 2.

[1] *1031 cells in males*

Divergence among wild *C. elegans* strains and *Caernorhabditis* species
In paper III we looked at the distribution of a polymorphic locus among different wild *C. elegans* isolates. Using close relative species we also established its ancestral heritage. Below is a short description of the genetic diversity among strains used in this study. It is however important to note that the divergence time in the nematode phyla must be taken with caution, and it is given here only for estimation of the evolutionary distance between strains.

C. elegans belongs to the huge nematode phylum, which is highly diverse and spread to incredibly different ecological niches. Its closest identified relatives are *Caenorhabditis briggsae*, *Caenorhabditis remanei* and *Caenorhabditis brenneri*. Of these, *C.briggsae* and *C.remanei* are most closely related with *C. elegans* an outgroup [11]. *C. elegans* and *C. briggsae* are estimated to have diverged 100 million yrs ago [12], comparable to the time elapsed since human and mice last shared a common ancestor. In such interval nucleotides at neutral sites have been exchanged almost to saturation [13]. A more distant group of relatives are briefly studied in paper III and is represented by PS1010, RGD1 and RGD2. It can be mentioned that PS1010 and *C.briggsae* are slightly more diverged than mice compared to zebra fish [11].

The relative large distance between *C. elegans* and its closest identified relatives is in contrast with the quite low genetic diversity among *C. elegans* strains captured in the wild. The reference wild-type strain, N2 was isolated from a mushroom compost near Bristol and the different strains discussed in paper III were sampled from various location world wide. Interestingly, there is no correlation found between genetic and geographic distances between different *C. elegans* isolates [7, 14], probably reflecting high migration rates. The actual divergence among these strains is low (for example 20 fold lower than what is found in *Drosophila* [6]). However, the Hawaiian isolate CB4856 and JU258 from Madeira are exceptions and show a higher polymorphism. 70% of the SNPs in CB4856 are not found in other strains [15]. The divergence time between for example N2 and CB4856 is estimated to correspond to several million years of evolutionary drift [16], equivalent to a polymorphism on average every 840 bp [17, 18].

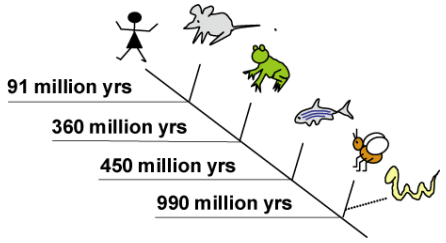

Fig 2) *C. elegans* in the tree of life.
Due to small sampling of the taxa and to rapid evolution for nematodes it is difficult to assign their exact branching point in the tree of life. It is a debate whether nematodes belong to the clade of molting animals, Ecdysozoa, as shown here, or if diverging before the Ecdysozoa branch [19].

Sensing the environment

C. elegans responds to chemical, thermal, osmotic and mechanical stimuli. It also orients itself in electric fields and avoids UV light [20-26]. *C. elegans* possess a few sensilla with sensory neurons arranged in groups [27-29]. These include two large amphid sensilla situated in the tip of the nose, laterally. Through openings in the cuticle, generated by glial cells (socket and sheath cells), a subset of the amphid neurons are directly exposed to the external milieu. These include sensory neurons of importance for this work: ---text removed from public version ---.
In the tail region there are two structures that are analogous to but simpler than amphids, called the phasmids. The amphids and phasmids are the main chemoreceptive sites of *C. elegans*. An additional class of chemoreceptor, the IL2 neurons, extend dendrites to the inner labial ring around the mouth where their cilia are also exposed to the outside milieu.

In addition to these sense organs there are several classes of neurons that serve as sensory receptors, but are not in sensilla. Of particular importance for this thesis are AQR, PQR and URX neurons that are exposed to the body fluid.

In total there are 32 presumed chemosensory neurons that read the external milieu. Nevertheless, the *C. elegans* relies heavily on olfactory and gustatory signals for navigation and the relative low number of chemosensory neurons is compensated by the predicted high number of functional chemoreceptors (potentially more than 500) [26]. This result in multifunctional neurons and several different receptors can be expressed in the same neuron. The dominant type of chemoreceptors in *C. elegans* are G-protein coupled receptors distantly related to rhodopsin-related GPCRs [26]. Downstream of these GPCRs there are primarily two types of signal transductions, based either on cGMP-gated channels or on TRPV channels [26]. Receptor expression determines whether a stimulus is repellent or attractive when a neuron is associated with a certain response [30, 31].

Neuronal control of movement

C. elegans navigation is based on forward and backward sinusoidal movements, interrupted by turns. A change in direction is achieved either by a reversal (a short backward movement, followed by forward locomotion in a new direction) or by a turn. Turning is established by changing the magnitude of head swings in dorsal or ventral direction, swinging stronger to one side or the other. A weak curve is made by many swings biased to one side. A greater change in direction can be achieved by a larger head swing. *C. elegans* can even turn 180° in this way, making a deep turn called an omega turn (when the head touches the tail its body resembles the Greek letter Ω). This extreme turn is usually preceded by a reversal, but can also occur in isolation [32-34]. Turning is more frequent when animals are moving away from an attractant compared to when moving towards it, allowing the animal with time to end up at the attractant. The turns are described to be random in direction [35], but it has also been suggested that for some sensations a spatial gradient over the body is sensed and determines in which direction the animal turns [36].

The *C. elegans* nervous system is composed of 302 neurons (for comparison, the mammalian cerebellum has more than 10^{10} neurons [37]). The major area of neuropil is a nerve ring in the head and two nerve cords running along the body on the dorsal and ventral sides. Many sensory neurons and interneurons have their cell bodies close to the nerve ring and receive and make synaptic connections with each other in the ring. As this is a likely place for integrating and processing information, it is sometimes referred to as *C. elegans'* brain. The neuronal circuits coupling sensation to locomotory behavior are largely unknown, even though the connectivity of the entire nervous system has been described [38]. Knowledge of the anatomical structure is an important platform, guiding experiments to reveal complete circuits. Studies to identify responsible neurons have largely been based on laser ablations and genetic manipulations. In the past, studies of sensory and motor neuron classes have dominated [22, 26, 39-44], but the field is shifting towards trying to understand the more complex roles of interneurons [45-47]. Recently developed techniques for calcium imaging will be important for determining complete circuits, linking stimuli to a behavioral response. The aim of this thesis is not focused on determining, or particularly discussing neuronal circuits. The next section is only brief, aiming to exemplify how studied genes and neurons could exert effect on behavior.

C. elegans swims on its side and alternating ventral and dorsal muscle contractions result in a sinusoidal movement. The motor circuit underlying this movement has been identified (illustrated in fig. 3) [22], although it is still poorly understood. Muscle contraction is controlled by DA, DB, DD motor neurons on the dorsal side and by VA, VB, and VD on the ventral side. Interneurons AVA, AVD, and AVE together with motor neuron DA and VA initiate backward movement. Interneurons

PVC and AVB together with motor neuron VB and DB control forward locomotion. The VD and DD motor neurons get input from the other motor neurons, inhibiting muscle contraction on the contra-lateral side. This helps ensure alternating dorsal and ventral contraction. Sensory neurons relevant for this thesis connect to interneurons in the locomotory circuit. Direct connections are shown in figure 3. This figure also points out direct connections to the inter/motor neuron RMG. This neuron has been suggested to function as a hub in the circuitry regulating aggregation behavior [48]. RMG connects to the interneurons AVB, AVA, AVD and AVE and also to head muscles.
Besides indicating potential pathways regulating forward and backward locomotion, the connectivity patterns also support a function in regulation of turns. ---text removed from public version --- RIA that connects to the motor neuron SMD, controlling the steep amplitude of omega [34]. RIA also connects to RIV motor neuron that sets ventral bias of turns [34].

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Note that the connectivity patterns mentioned above are just examples of possible signal transductions and theoretically multiple circuits, involving different layers of interneurons, could be used to achieve locomotory responses.

Aggregation behavior

There are two distinct types of feeding behavior described in *C. elegans* strains. Aggregating strains feed together in clumps and accumulate where food is most abundant. Under laboratory conditions food is thickest at the edge of the food lawn and animals accumulate here; this is referred to as bordering. Non-aggregating strains, typified by the laboratory wild-type strain N2 and its relatives, feed in isolation and disperse evenly over a bacterial lawn (fig. 4).

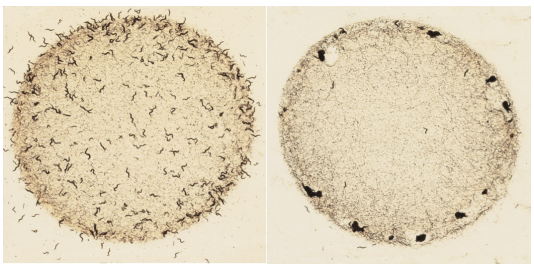

Fig. 4. Solitary and aggregating strains as they feed on bacterial lawns. Aggregation is defined to involve at least 3 animals, feeding together, but a clump can contain several hundred individuals [1]. When animals are put on fresh food, they aggregate within 20 minutes [51]. The clumps are dynamic and animals leave and enter the clump over time. More than half of the animals of the aggregating strain RC301 are in clumps under laboratory conditions. This can be compared to the non-aggregating strain N2, where less than 2% of the animals clump [1]. In addition, more than 95% of the RC301 animals show bordering, compared to about 15% for N2. Picture taken by M. de Bono.

Genetic components and neuronal pathways regulating aggregation

A key determinant between the two feeding strategies in *C. elegans* is variation in the neuropeptide Y receptor homolog, NPR-1 [1]. Aggregating strains carry the ancestral form of the receptor [52], whereas solitary strains carry a gain of function mutation (Phe-215 →Val-215) in the third intracellular loop of the receptor. For many seven transmembrane receptors, this region is important for G-protein coupling and the aromatic phenylalanine residue is conserved among receptors of the NPY family [1]. Overexpressing the recessive, ancestral *npr-1 215F* allele, results in solitary feeding, and if the locus is mutated animals aggregate. The NPR-1 receptor thus appears to inhibit aggregation behavior. The gain of function mutation, carried by the reference wild-type strain N2, enhances this inhibition and triggers solitary feeding.

C. elegans' genome does not encode any NPY peptides, but does encode several FMRF-amide related peptides. Of these, FLP-21 was shown to activate both NPR-1 215V and NPR-1 215F, and FLP-18 was shown to activate NPR-1 215V [52]. Overexpressing *flp-21* turns aggregating animals into solitary feeders, whereas if *flp-21* is mutated aggregation is enhanced [52]. The NPY receptor family are typical G-protein coupled receptors and they activate heterotrimeric G-proteins, modulating activity of enzymes, ion channels, and second messenger pathways [53, 54]. Mammalian NPY inhibit neurotransmitter release [55, 56], but the consequences of NPR-1 activation is unknown.

npr-1 is primarily expressed in neuronal cells, but expression is also seen in a muscle in the terminal bulb of the pharynx, in the excretory duct cell and in the excretory canal [49]. The expression pattern highlighted candidate neurons involved in control of aggregation behavior. A transgenic rescue experiment, suppressing aggregation in *npr-1* mutants by neuron-specific expression of the gene, suggested the body cavity neurons AQR, PQR, URX and the interneuron AUA (which is a synaptic target to URX) play a role. The rescue observed in this experiment was incomplete and in a recent study the inter/motor neuron RMG was pointed out as a central site of action for NPR-1 [48]. The body cavity neurons are exposed to the body fluid and are proposed to monitor the internal milieu. Based on an experiment using an activated K^+ channel, that inhibits neuronal activity, these neurons appear to promote aggregation when active. Hence NPR-1 is likely to inhibit signalling from these neurons. By contrast, cyclic GMP-gated cation channels, encoded by *tax-2* (beta subunit) and *tax-4* (alpha subunit) promote aggregation. Based on rescue experiments with the *tax-4* gene, this occurs in one or more of the body cavity neurons and also in one or more of ASG, ASI, ASJ, ASK, AWB and AWC [49].

To search for additional signalling pathways and neurons involved in aggregation behavior, a candidate gene approach was performed. This involved asking if mutations in the selected genes disrupted aggregation [51]. This approach highlighted several additional molecules that in turn have led to identification of further neurons controlling aggregation [1]. Aggregation is promoted by *odr-4*, which encodes a transmembrane protein needed for correct localization of some olfactory receptors [57]. ODR-4 was shown to act in ADL to promote aggregation. In addition, *odr-8* was shown to stimulate aggregation. *odr-8* has similar function as *odr-4*, but its molecular identity is unknown. *ocr-2* and *osm-9*, encoding TRPV-related transduction channels, were also shown to promote aggregation. The related mammalian capsaicin receptor, TRPV1, responds to the hot substance in chilli peppers and to high temperature. *ocr-2* and *osm-9* are expressed in many amphid sensory neurons and rescue experiments indicated that *ocr-2* acts in ASH and ADL to regulate aggregation. ASH and ADL are known to be involved in aversive responses to various noxious stimuli. ASH neurons are important for responses to hyperosmotic solutions, high concentrations of benzaldehyde, toxic heavy metal ions, extracts of dead worms and head touch [58-61]. ADL and ASH are involved in avoidance of 1-octanol [30, 59]. A role for these neurons in aggregation suggests that the behavior might be a protective response to aversive

environmental cues. When ASH and ADL are ablated together, but not separately, the result is solitary feeding. Signalling from either neuron is thus needed to promote aggregation. An antagonizing signal seems to come from other ciliated neurons. *osm-3* is needed for the development of 26 ciliated sensory neurons, including ASH and ADL [62, 63]. However, mutations in *osm-3* do not repress aggregation. This leads to the conclusion that other ciliated neurons could act to inhibit aggregation. In agreement with this, mutations in *osm-3* restore aggregation in *ocr-2* and *odr-4* mutants.

A third approach, to identify components contributing to aggregation behavior, involved forward genetics: a mutagenesis screen seeking mutants that disrupt aggregation. This approach identified the soluble guanylate cyclases *gcy-35* and *gcy-36* as important for aggregation [2]. Mammalian soluble guanylate cyclases are activated by gaseous ligands [64, 65], principally nitric oxide, which binds to the haeme prostetic group. A constitutively active mammalian version could partly, albeit weakly, replace *gcy-35* and *gcy-36* showing that they act as soluble guanylate cyclases [2]. In mammals, these molecules are known to function as alpha/beta homo- or hetero-dimers [65]. GCY-35 was shown to act as an alpha subunit (has an active aspartate) and GCY-36 as a beta subunit (has an active asparagine). Actually all seven soluble guanylate cyclases encoded by the *C. elegans* genome resembles more the beta subunit in mammalians [66], but only GCY-36 has the conserved asparagine. The observation that two soluble guanylate cyclases are involved in regulating aggregation behavior initiated experiments testing if any other of the sGCs encoded by the *C. elegans* genome is also involved. Double mutants with *npr-1* suggested that in addition to *gcy-35* and *gcy-36*, *gcy-32* and *gcy-34* also play a role in aggregation. Like *gcy-35* and *gcy-36*, *gcy-32* and *gcy-34* are selectively expressed in the body cavity neurons AQR, PQR and URX. Mutations in *gcy-32* and *gcy-34* did affect aggregation of *npr-1* animals, but only when both genes were simultaneously disrupted. These two sGCs show high sequence similarity, raising the possibility that they can compensate for each other [3].

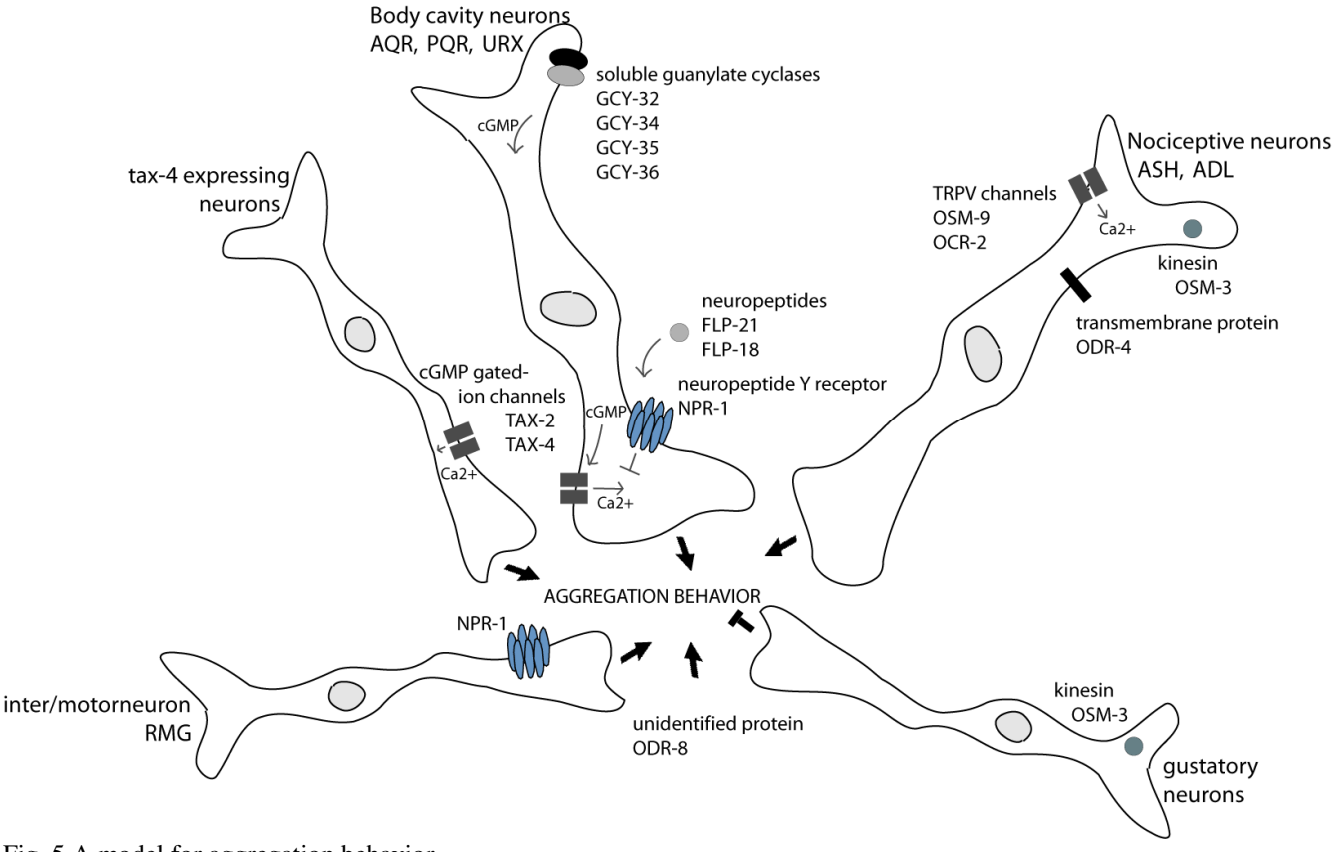

Fig. 5 A model for aggregation behavior

The soluble guanylate cyclases *gcy-32*, *gcy-34*, *gcy-35* and *gcy-36* are all expressed in the body cavity neurons [2, 4, 67]. In addition, *gcy-35* neuronal expression is also seen in SDQ, BDU (interneurons), AVM, PLM (mechanosensory neuron) and ALN and PLN (putative sensory neurons) [2, 4]. Rescue experiments point out URX and potentially also the other body cavity neurons as the location where *gcy-35* and *gcy-36* act to promote aggregation [2]. URX is not a ciliated neuron, but in AQR and PQR *gcy-36* where enriched in sensory cilia, suggesting that it might function as sensors. *C. elegans* lacks a nitric oxide synthase, suggesting another activator than seen in mammalians. In a study by Gray et al it was shown that at least GCY-35 binds oxygen in vitro [4]. In addition a transgene experiment show that when co-expressed with *gcy-36* it can also function as oxygen sensor in vivo [3]. The other sGCs of importance for aggregation, as well as *tax-2* and *tax-4*, also appear to mediate oxygen responses [3, 4].
C. elegans and bacteria consume oxygen faster than it diffuses, hence locally lowering oxygen levels. Aggregation and bordering is therefore linked to areas with low oxygen. The oxygen level at the border of the lawn is 12.8% and in middle it is 17.1% [4]. In the middle of a worm aggregate of 50 animals oxygen level is 6% (paper I). Importantly, both aggregation and bordering are disrupted when oxygen levels are lowered [4]. Together these data point at a close relationship between oxygen and aggregation behavior.

Aerotaxis

Living in decaying organic matter such as fruit, *C. elegans* can move in three dimensions in search for food. Its ecological niche is likely to experience a wide span of oxygen levels [68], although *in situ* studies of oxygen levels in areas inhabited by natural *C. elegans* populations have yet to be carried out. *C. elegans* appears to use oxygen levels to help navigate its environment. Oxygen levels could be interpreted as distance below ground or indicate presence of food (oxygen consuming microbes). Besides being a guide, describing the surroundings, oxygen is crucial for survival. *C. elegans* does not have a respiratory system and relies on diffusion to maintain aerobic respiration [69]. Under laboratory conditions it can grow in oxygen tensions varying between 100% and 1% [70], and can maintain the same metabolic rate (as measured by CO_2 production) until oxygen levels fall below 4% [70]. At 1% oxygen *C. elegans* reduce their metabolic rate by 50% [70]. Since the metabolic rate is not much affected as long as oxygen levels are above 4%, but damaging ROS [71] increases at higher levels, it makes sense that *C. elegans* prefers the lower range of oxygen. However when oxygen levels reaches below 1% the hypoxia response pathway [72-74] is induced and oxygen levels below 0.1% are lethal [75].
Under standard laboratory conditions *C. elegans* has a preference of oxygen concentrations between 5% and 12%, avoiding both higher and lower concentrations [4]. Hypoxia and hyperoxia avoidance are mediated by distinct pathways and the following sections focus only on hyperoxia avoidance and its relation to aggregation behavior.

Aerotaxis and Aggregation

It was first shown by Gray et al and Cheung et al that molecules in the body cavity neurons regulate oxygen responses. TAX-2/TAX-4 cGMP-gated channels were shown to mediate hyperoxia avoidance [4] and the sGCs, GCY-32, GCY-34, GCY-35 and GCY-36 were shown to all play roles in hyperoxia avoidance under different contexts [3, 4, 76]. In *npr-1* mutants, *gcy-32* and *gcy-34* appear to be particularly important after precultivation in hypoxia [3]. *gcy-35* and *gcy-36* regulate hyperoxia avoidance regardless of previous cultivation [3, 4]. Besides acting in the body cavity neurons, *gcy-35* also mediates hyperoxia avoidance in one or more of SDQ, ALN and PLN [76]. The two SDQ neurons are similar in linage morphology and connectivity as AQR and PQR and are therefore interesting candidates.

NPR-1 regulates hyperoxia avoidance on food

The major difference between aggregators (*npr-1 215F* animals) and non-aggregators (*npr-1 215V* animals) is that hyperoxia avoidance is blunted in animals carrying *npr-1 215V* if food is present [4]. In contrast *npr-1 215F* animals show strong hyperoxia avoidance regardless of the presence or absence of food.

npr-1 215V animals dramatically slow locomotion when encountering food (N2 does a 2-fold reduction) [1]. Animals bearing *npr-1 215F* in N2 background, or carry a loss of function allele of *npr-1*, instead keep high locomotory rate on food if oxygen levels are high. These strains integrate oxygen signals in the decision and they progressively slow on food with decreasing oxygen levels and increased food concentrations [3]. In *npr-1 215V* animals, high locomotory activity, mediated partly by sGCs in the body cavity neurons, is strongly antagonized by NPR-1. A transgene, expressing *npr-1 215V* only in the body cavity neurons, resembles *gcy-36* mutants [3]. Interestingly, these animals maintain a higher locomotory rate at 21% compared to *npr-1 215V* animals, suggesting that NPR-1 acts in additional neurons to regulate locomotory rate [3]. A recent paper point out RMG as this key neuron, important for *npr-1* regulation of locomotory activity [48].

In proposed models [3, 76], food leads to activation of NPR-1. If the *npr-1* gene would be the sole regulator of food dependence, then a background defective for *npr-1* would not show changed aerotaxis behavior with changed food status. However, if AQR, PQR and URX are ablated in *npr-1* mutants the animals regain food regulation and a similar observation is made if deleting *ocr-2*, encoding a TRPV channel [76]. It seems that the primary role for NPR-1 V in changing aerotaxis on food is by repressing the output from the oxygen sensing neurons [3]. This would uncover the food sensitivity of other circuits. The ADF neurons produce serotonin that mediate food regulated hyperoxia avoidance. Chang et al. demonstrated that this is the key determinant in the food regulation seen in *npr-1 215V* animals [76]. Chang et al also showed that *daf-7* mutants have higher levels of serotonin in ADF and that this promote hyperoxia avoidance in an *npr-1 215V* background [76]. Actually *daf-7* mutants exhibit hyperoxia avoidance both on and off food, thus resembling *npr-1 215F* animals. DAF-7 is therefore similar to NPR-1 215V in blunting hyperoxia avoidance on food. Interestingly, along these lines, *daf-7* mutants also aggregate [77]. *daf-7* encodes a TGF beta protein that affects dauer formation. *npr-1 215V* animals also show aggregation if other genes in this signalling pathway are mutated. Mutants defective in *daf-1*, *daf-7*, *daf-8* and *daf-14* aggregate and border on food and this aggregation is suppressed by *daf-3* and *daf-5* [77]. The aggregation associated with this pathway is less pronounced than *npr-1* regulated aggregation. *daf-7, npr-1* double mutants have greatly enhanced aggregation, suggesting parallel pathways [51].

AIM

This thesis aims to dissect, in behavioral and molecular terms, how *C. elegans* avoid high ambient oxygen and aggregate. To achieve this goal I combine classic forward genetics, and study of mutants with defective behavior. A particular strategy was to use wild strains of *C. elegans* as a source of genetic and behavioral variation from the standard laboratory reference strain N2.

SUMMARY OF RESULTS

The starting point for this study was the hypothesis that hyperoxia avoidance and aggregation is related and mediated by molecules expressed in the body cavity neurons [2-4]. We therefore asked if other pathways, previously shown to promote aggregation, could modulate oxygen responses.
In paper I we focused on the nociceptive neurons ASH and ADL [51] ---text removed from public version --- In addition, we analysed in behavioral detail how oxygen responses actually can trigger aggregation, paper I.
We next identified two new genes functioning in the oxygen avoidance circuit, ---text removed from public version --- *glb-5* was identified by studying variation in hyperoxia avoidance across different wild strains, ---text removed from public version ---.

Paper I

In this paper we asked if the nociceptive neurons ASH and ADL can modulate oxygen responses. In aggregation, signalling from these neurons involve the TRPV channel subunits OCR-2 and OSM-9 and the transmembrane protein ODR-4 [51]. We used an aerotaxis chamber (developed by Gray et al [4]), in which animals can choose their oxygen environment, ranging from 0% to 21% oxygen. Mutants, or rescued animals, were allowed to distribute themselves in the oxygen gradient in the aerotaxis chamber. From these experiments we concluded that *ocr-2*, *osm-9* and *odr-4* are all needed for hyperoxia avoidance. *ocr-2* is needed in ASH and *odr-4* in ADL. In addition, we showed that loss of *osm-3* restored hyperoxia avoidance in both *odr-4* and *ocr-2* mutants, in a similar way as it restores aggregation [51].

Aggregation had previously only been described on food, in well fed animals. In this paper we showed that prolonged starvation also lead to aggregation. This indicates that aggregating animals themselves, without the need of bacteria, generate the sensory signals triggering aggregation. Starvation induced aggregation involve similar neuronal pathways as aggregation in well fed animals. *gcy-35*, *gcy-36*, *tax-4*, *ocr-2* and *odr-4* are all required for aggregation off food. In addition *gcy-36* is required to keep high locomotory rate at 21% oxygen under these conditions.

We dissected the complex aggregation behavior into simpler sub-behaviors and investigated how changes in oxygen levels can trigger these behavioral motifs.
A clump is formed as animals encounter each other, slow their locomotory rate, suppress forward movement and start to move backward and forward to remain together. Regardless if the head or tail leaves the clump, the animal changes direction in order to stay in the group.
To analyze these behavioral motifs, we developed a new assay where the animals encounter a step oxygen gradient, similar to that encountered by animals entering or leaving clumps. This assay allows rapid changes of oxygen levels (changing from 12% to 20% in 15 sec). We showed that *gcy-35* and *gcy-36* mediate reversals and omega turns and that *ocr-2* mediate omega turns in response to a rise in oxygen. We also showed that if re-exposed to low oxygen after experiencing a rise in oxygen (relevant for re-entry the clump), turning is immediately suppressed. This occurred independently of *gcy-35* and *ocr-2*.

Paper II

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Paper III

We had observed that *npr-1 215F* associated phenotypes were influenced by genetic background. In this paper we compared 20 wild strains, bearing the *npr-1 215F* allele, to *npr-1 215F* animals with a N2 background. Animals from the later strain progressively decrease locomotory rate with decreasing oxygen levels, but the wild strains switched from roaming to dwelling just below 21% oxygen. We decided to map the trait of wild aggregating strains using single nucleotide polymorphisms. For this, we made recombinant inbred lines between CB4856 (wild strain carrying *npr-1 215F*) and AX613 (*npr-1 215F* in N2 background).The behavioral difference observed was caused by a duplication/insertion in the recessive *glb-5(N2)* allele. RT-PCR experiments indicated that this duplication might interfere with correct splicing.

By examining orthologues in other *Caenorhabditis* species we concluded the dominant *glb-5(Haw)* allele to be ancestral. To establish the prevalence of the two *glb-5* alleles in natural populations we examined 98 wild strains collected from all over the world. Only 7 of these carried the *glb-5(N2)* allele. The *glb-5* locus seems to be coinherited with the *npr-1* locus although situated on different chromosomes. *glb-5(N2); npr-1 215V*, and the *glb-5(Haw);npr-1 215F* allele combinations seem to dominate. Only one exception to this was found; the Wisconsin isolate *glb-5(Haw)*; *npr-1 215V*.

glb-5 encodes a hexacoordinated globin and absorbance spectra of purified protein confirmed oxygen binding. We determined *glb-5* expression by fusing DNA, encoding mCherry fluorescent protein, just upstream of *glb-5* genomic fragment. Expression was seen in URXL/R AQR/PQR BAGL/R, the pharynx, the intestine, coelomocytes, the uv2 vulval cells and the excretory cell. Partial expression, using selective promoters, determined that *glb-5* acts in AQR, PQR and URX to tune oxygen responses. Studies of animals carrying the *glb-5(Haw)* allele in different mutant backgrounds also revealed that *gcy-35* and *gcy-36* are required for its affect on behavior.

Calcium imaging, using the ratiometric Ca^{2+} sensor cameleon, in AQR, PQR and URX revealed that the oxygen response is graded in these neurons, and that both rises and falls in oxygen trigger Ca^{2+} changes. Higher oxygen elicited higher Ca^{2+} plateaus that persisted as long as the stimulus was kept. Quantification of Ca^{2+} responses in PQR showed that mutations in *gcy-35* or *tax-4* disrupted Ca^{2+} responses. Comparison between *npr-1* animals and *glb-5(Haw); npr-1* animals revealed that the later responded significantly more strongly to small oxygen changes close to 21% oxygen, consistent with the behavioral data.

Paper IV

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DISCUSSION

Importance of *npr-1* and *glb-5* polymorphisms in a natural habitat

Strong hyperoxia avoidance triggers aggregation and seems to be the behavior of most *C. elegans* wild strains. Solitary strains are underrepresented among wild isolates, but have been collected from several locations that are geographically distant [78]. Unexpectedly, a recent study questions them as independent wild isolates, and suggests that solitary feeding has evolved in the laboratory [79].
In *Drosophila,* a similar difference in feeding behavior is observed. Rovers keep high locomotory activity on food whereas sitters feed in a smaller area. Rovers, being reminiscent of aggregating *C. elegans* strains, outcompete sitters under crowded conditions. Sitters on the other hand have better success under lower population densities [80]. Besides roaming on food, if oxygen levels are high, aggregating *C. elegans* compete for food in aggregates. This raise the questions why aggregating strains seem to dominate in the wild? Aggregations is only triggered at high oxygen levels [4] and the underlying hyperoxia avoidance might therefore be the selected behavior. The importance of aggregation in the wild is unknown, but animals in an aggregate burrow much more strongly into surrounding materials [78]. This might be advantageous by further facilitating escape of high oxygen levels, for example at desiccating surfaces.

The very strong hyperoxia avoidance associated with the *npr-1(lf)* allele, generated in the lab, is likely disadvantageous in the wild since preventing access to nutrient rich areas closer to surface. In paper I and paper II we studied animals that are mutant for *npr-1* in a non-aggregating background (N2). These animals are enhanced aggregators and they show stronger hyperoxia avoidance compared to wild aggregating strains. Wild aggregators seem to have a higher tolerance, keeping the hyperoxia avoidance triggering aggregation, but allowing more flexibility in the feeding. ---text removed from public version --- In paper III we map this locus to a neuroglobin, *glb-5*, shown to act in the body cavity neurons. ---text removed from public version ---

C. elegans is very vulnerable to desiccation. Sharp avoidance of 21% oxygen present at the soil surface, allowing animals to dwell at nutrient rich areas below, would therefore be advantageous. Aggregating strains carry the ancestral form of *glb-5*, *glb-5(Haw)*. This allele enables a strong reduction of locomotory rate if oxygen drops below 21% (paper III). An independent study describes that *glb-5(Haw)* animals also initiate omega turns when oxygen levels approach 21% [79]. ---text removed from public version ---As a result, strains carrying *glb-5(Haw)* exhibit sharp avoidance of oxygen tensions present at the desiccating surface.

GLB-5 and NPR-1 both antagonize hyperoxia avoidance in the same neuronal circuit. The coinheritance of the two loci (paper III), although on different chromosomes, suggests that the combinations *glb-5(Haw); npr-1 215F* and *glb-5(N2); npr-1 215V* are particularly advantageous. Overlapping functions of *npr-1 215V* and *glb-5* genes could have permitted duplication/insertion and reduced gene function of *glb-5(N2)* in the *npr-1 215V* background. Both *npr-1 215V* and *glb-5(N2)* seem to have evolved recently (paper III) and in a common background [79]. McGrath et al. suggested that the isolates bearing *npr-1 215V* and *glb-5(N2)* are re-isolates of laboratory N2 strains. This paper also suggests that the two N2 alleles have evolved under laboratory conditions [79]. Such scenario would be interesting since laboratory conditions increase exposure to 21% oxygen. The gain of function mutation in NPR-1 215V decrease hyperoxia avoidance and fits the N2 strain to the high oxygen present in the laboratory. Fitness to cultivation condition is not the only selection force in the laboratory. Experimentalists controlling transfer to the next generation also influence the outcome. It is likely that solitary feeding N2-like animals are favoured, over aggregating and burrowing animals,

when individuals are chosen for maintaining stocks [79]. Adaptations to laboratory conditions have previously been described for example in mice and yeast [81]. In a natural habitat the unduplicated *glb-5* allele and the recessive *npr-1 215F* allele dominate; most wild *C. elegans* strains, and strains of the related *Caernorhabditis* species *brenneri*, *briggsae* and *remanei* carry these alleles. This gene combination allows animals to feed at repelling oxygen levels, but robustly avoid 21% oxygen, providing a successful foraging strategy for these *Caernorhabditis* species in their natural habitat. Since *glb-5(Haw)* antagonizes hyperoxia avoidance below 21%, it might also interfere with aggregation. In fact *glb-5(Haw)* was shown to inhibit aggregation in *npr-1(lf)* animals [79]. It is therefore likely that it also down regulate aggregation in *npr-1 215F* animals. This raises the question if aggregation in the wild only occurs close to surface as a way to burrow below ground again?

Modelling in the lab

Aggregation behavior and hyperoxia avoidance have been modelled in the laboratory with use of different assays, permitting study of the oxygen sensing circuit. The primary assays used are the aerotaxis chamber, the locomotory rate assay, and the turning assay.

In the aerotaxis chamber, animals are allowed to distribute themselves in an oxygen gradient [4]. The gradient used is quite shallow and the preferred oxygen levels (6-12% oxygen) cover an area of 4 x 15 body lengths. This contrasts the gradient observed in a clump of aggregating animals. In a clump, levels can change from 6% to 21% over a body length (paper I). The aerotaxis chamber is thus useful for dissecting the oxygen sensing circuit, but the measure of hyperoxia avoidance can not be used to predict aggregation behavior. This is further discussed in the next section.
In the locomotory rate assay and in the turning assay, animals are instead recorded while moving in a chamber with defined oxygen levels. The gas in the chamber can be changed within a few seconds. If switching to a repelling oxygen level, animals initiate turning and reversals to avoid the stimuli. This exposure is more comparable to the oxygen changes that animals experience while aggregating. In the locomotory assay, the rate of movement is measured using DIAS software [1-3], starting 2 minutes after the gas switch. At this time point the gas level is stabilized and turning have ceased. High locomotory activity can be used as a read out of oxygen avoidance, but again, this assay is to crude to predict aggregation.

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Proposed model

Oxygen assays have revealed that both aggregating and solitary strains seem to share the same toolbox for exhibiting hyperoxia avoidance; a distributed neuronal network involving many players. The difference between the two feeders results from differences in total contribution from individual neurons.

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The body cavity neurons AQR, PQR and URX have a central role in hyperoxia avoidance and aggregation [2, 3, 49]. Cheung et al established the key role for these neurons in regulation of locomotory rate in *npr-1* mutants in response to oxygen changes. Behavioral data pointed out the sGCs (GCY-32, GCY-34, GCY-35 and GCY-36) and the TAX-2/TAX-4 Ca^{2+} channels as important [2, 3]. This was confirmed in paper III, where we showed that sGCs acts in these neurons to promote high locomotory rate in oxygen avoidance. Neuronal imaging also indicated that Ca^{2+} levels increase in the

body cavity neurons, when oxygen levels rise, and that this requires *gcy-35* and *tax-4* (paper III). Such Ca^{2+}peaks, triggered by oxygen upsteps, were also reported by Zimmer et al. in a study of the URX neuron [83]. In the model in fig. 6, the body cavity neurons are illustrated as a circle. cGMP signalling mediated by sGCs opens TAX-2/TAX-4 Ca^{2+} channels and evokes neuronal signalling. This promotes hyperoxia avoidance and aggregation. The polymorphic *glb-5* antagonizes cGMP signalling in these neurons on food (paper III). The precise mechanism by which GLB-5 exerts its affect is unclear. ---text removed from public version ---
NPR-1 has also been suggested to antagonize hyperoxia avoidance, acting in the body cavity neurons. Coates and de Bono showed that NPR-1 antagonize aggregation and bordering acting in these neurons [49]. A recent study questions this result and instead point out the RMG motor/interneuron as the main centre for NPR-1 determination of aggregation behavior [48], as well as for the high locomotory activity of *npr-1* mutants.

The model that emerges when looking at data of locomotory activity is supported by results from the aerotaxis chamber. *tax-2, tax-4, gcy-32, gcy-34, gcy-35* and *gcy-36* all promote hyperoxia avoidance in different genetic backgrounds, under different contexts [2-4]. Besides acting in the body cavity neurons, *gcy-35* also seem to regulate aerotaxis, acting more broadly in the circuit [4].

Using the aerotaxis chamber we showed in paper I that molecules in the nociceptive neurons also mediate avoidance of high oxygen. TRPV related channel subunits OCR-2 and OSM-9 acts in ASH and the transmembrane protein ODR-4, acts in ADL to promote hyperoxia avoidance in an *npr-1(lf)* background. Loss of *osm-3* restored hyperoxia avoidance in both *odr-4* and *ocr-2* mutants (paper I). These data were later strengthened by Chang et al. [76]. He observed that *osm-9* is needed in ASH for hyperoxia avoidance in *npr-1 215V* animals. In addition, Chang reported recue of hyperoxia avoidance if *osm-9* was expressed in ADF [76].

In paper I we analyzed turning and reversals triggered by high oxygen levels in *npr-1* mutants. We showed that *gcy-35* and *gcy-36* mediate reversals and omega turns and that *ocr-2* mediate omega turns in this context. ---text removed from public version ---

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ACKNOWLEDGEMENT

My mentor Mario de Bono
Thank you for giving me the opportunity to work with you. You have been hugely supportive, patiently discussing science, guiding me and helping me out in the lab. I owe my deepest gratitude to you for all the time you invested.

Peter Carlsson, thanks for all your support, for effort you put on administration work and for being the best coach.

Marika Hellqvist-Greberg, I'm grateful for the time we worked together and for the continuous financial support.

Brit Corneliussen, I had the best of times working in your team, learning the advantages of using *C. elegans* as a model. I feel very fortunate to have you as a friend.

I also want to show my gratitude to my colleagues working with *C. elegans*. Thanks for interesting and fun discussions. Africa, Andrew, Benny, Beate, Birgitta, Candida, Catarina, Claes, Einav, Emanuel, Fabien, Fausto, Fredrik, Gino, Helen, Hugo, Luis, Manish, Maria, Marc, Merav and Verena. Thanks for sharing ideas, worms and laughter.

I would also like to thank all helpful and generous people at MRC Laboratory of Molecular Biology, Cambridge and at Department of Cell and Molecular Biology, University of Gothenburg. Special thanks to Gunnar, Jeanette, Julie, Lars, Marie, Magnus, Marc, Per and Peter L.

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