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Chemosensory receptors in Caenorhabditis elegans

by

Emily Troemel

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

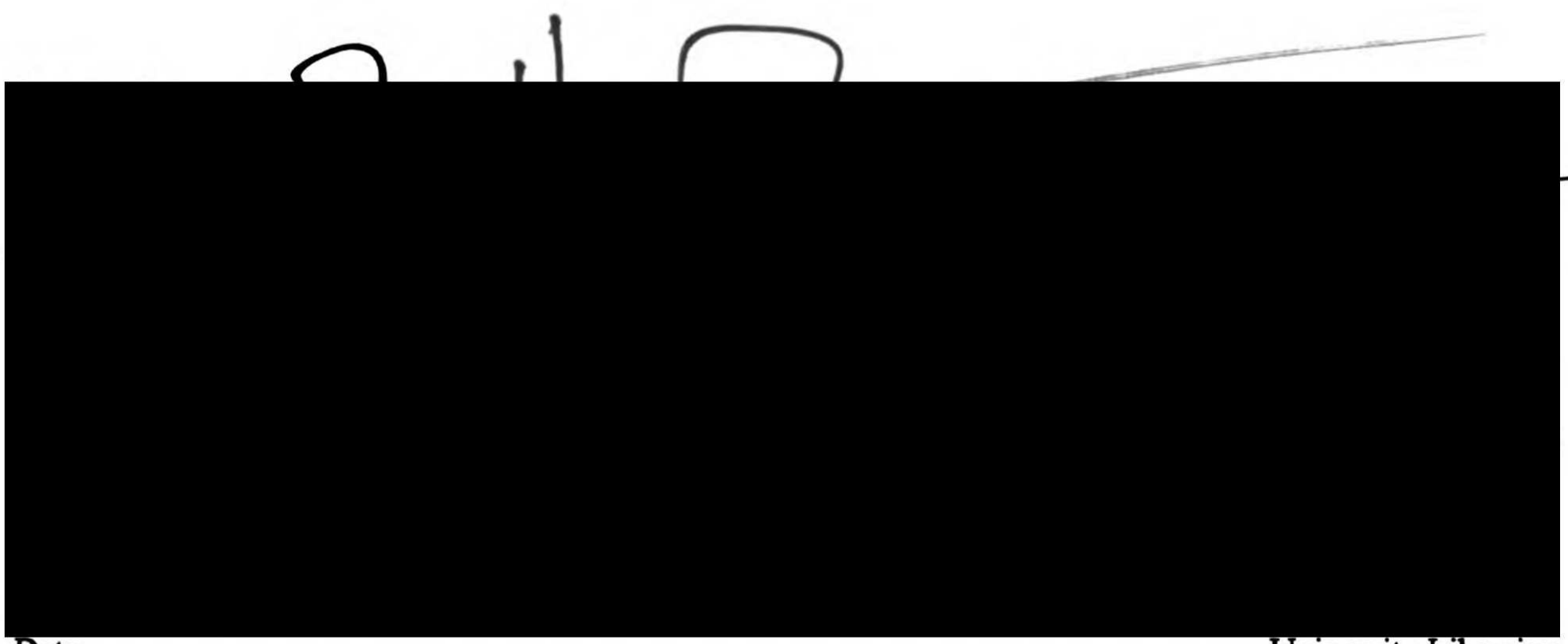
Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO



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This thesis is dedicated to my parents.

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Advisor Statement

The work in Chapter 2a was previously published in Cell, Vol. 83, No. 2, pp. 207-218, 1995. Aside from Emily Troemel, authors were Joseph Chou, Noelle Dwyer, Heather Colbert and myself. This publication was a group effort and Emily Troemel was involved in all aspects and stages of the work described. The first four authors made similar contributions to this work.

Chapter 3 was published as Cell, Vol. 91, No. 2, pp. 161-9. Bruce Kimmel performed the laser ablations and assays in Figure 1C, D and I performed laser ablations of the AWB neurons for Figure 3D. All other experiments were performed by Emily Troemel.

Chapter 4 is a manuscript in preparation. Alex Santoso integrated the *str-2::GFP* fusion and Noelle L'Etoile examined *str-2::GFP* expression in *odr-1* and *daf-11* adults. Alvaro Sagasti analyzed *str-2::GFP* expression in axon guidance mutants, performed the screen for mutants that express *str-2::GFP* in both AWC cells and did the majority of the epistasis analysis. All other experiments were performed by Emily Troemel.

Chapter 5 is work done in collaboration with Erin Peckol. Erin performed analysis on *srd-1* and *str-3*, while Emily Troemel studied *str-2*.

Cori Bargmann

Cori Bargmann, Ph. D.

Associate Professor

HHMI and UCSF, Department of Anatomy

Chemosensory receptors in *Caenorhabditis elegans*

Emily R. Troemel

Abstract

The small nematode *Caenorhabditis elegans* detects and responds to hundreds of chemicals using a small number of chemosensory cells. These cells are found in bilaterally symmetric pairs in the head, with each pair detecting a distinct subset of chemicals. We have used the nematode as a model system to investigate chemosensory signalling and regulation of chemosensory receptor expression.

Large multigene families of candidate chemosensory receptors were identified through expression studies and information from the *C. elegans* genome sequencing project. Over 1000 predicted G protein-coupled receptors exist in the genome, and about half of those examined so far are expressed in chemosensory neurons. If those genes are representative, *C. elegans* may have about 500 different functional kinds of chemosensory receptors.

Misexpression experiments with the *odr-10* receptor provided insights into chemosensory signalling and information coding in *C. elegans*. The *odr-10* receptor is normally expressed in the AWA neurons, where it mediates attraction to the odorant diacetyl. When *odr-10* is expressed in the AWB neurons instead of the AWA neurons, it elicits a repulsive response to diacetyl. These experiments demonstrate that ODR-10 expression can confer

diacetyl sensitivity onto AWB. Furthermore, they indicate that sensory neurons can define behavioral responses to odorants.

Studies with the *str-2* candidate odorant receptor uncovered a novel mechanism for specifying alternative cell fates. The two bilaterally symmetric AWC neurons can be distinguished from one another by *str-2* expression: half the animals in a population express *str-2* in the left AWC and the other half in the right AWC. Ablation and mutant analysis suggests that the two neurons communicate through their axons to specify *str-2* expression in one of them via Ca^{++} and cGMP signalling. Thus, activity appears to regulate the specification of olfactory neurons.

Chemosensory receptor expression can be altered by dauer pheromone and entry into the dauer larva stage. These changes in expression can be regulated by the *daf* genes, which control entry into the dauer phase. Alterations in receptor expression may be a strategy for olfactory plasticity that allows *C. elegans* to respond appropriately to its changing environment.

Cori Bargmann

Cori Bargmann
Graduate Advisor
Associate Professor
HHMI and UCSF, Dept of Anatomy

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Chapter 1

Introduction

Chemosensory signalling in *Caenorhabditis elegans*

(Bioessays 1999, in press.)

Summary

The nematode *Caenorhabditis elegans* can sense and respond to hundreds of different chemicals with a simple nervous system, making it an excellent model for studies of chemosensation. The chemosensory neurons that mediate responses to different chemicals have been identified through laser ablation studies, providing a cellular context for chemosensory signalling. Genetic and molecular analyses indicate that chemosensation in nematodes involves G protein signalling pathways, as it does in vertebrates, but the receptors and G proteins involved belong to nematode-specific gene families. About 500 different chemosensory receptors are probably used to detect the large spectrum of chemicals to which *C. elegans* responds, and one of these receptors has been matched with its odorant ligand. *C. elegans* olfactory responses are also subject to regulation based on experience, allowing the nematode to respond to a complex and changing chemical environment.

I. Introduction

How do animals sense and respond to their environment? For the small nematode *C. elegans*, most information about the environment comes through detection of chemicals, or chemosensation. *C. elegans* lives in the soil, where it must compete with other species for food while avoiding predators, parasites and toxic compounds. Chemosensation, and specifically olfaction (detection of volatile chemicals), allows the worm to thrive by guiding it to food, warning it of harmful chemicals, and alerting it to high population density. *C. elegans* has an impressive chemosensory repertoire, considering the size of the its nervous system; *C. elegans* detects hundreds of

chemicals using only 20-30 chemosensory neurons. By comparison, *Drosophila* detects a similar number of chemicals with 1000 neurons, and mice detect thousands of chemicals with 10^7 neurons. *C. elegans* also has surprising complexity in its chemosensory responses; it can discriminate among multiple chemicals, respond to them in different ways, and alter its responses based on experience.

This review will outline the chemosensory neurons and behavior of *C. elegans*, but will focus on the genes it uses for signalling. These genes have been identified through genetic screens as well as the genome sequencing project. They share a considerable number of similarities with the genes involved in vertebrate chemosensation. In addition, experience-dependent effects on chemosensory responses will also be summarized.

II. *C. elegans* chemosensation

A. Repertoire of *C. elegans* chemosensory responses.

C. elegans normally lives in the air/water interface of the soil, where it can detect both volatile and aqueous chemicals. Sensation of aqueous chemicals, analogous to the sense of taste, was first described by Ward and Dusenbery (Ward, 1973; Dusenbery, 1974). *C. elegans* chemotaxes to water-soluble attractants such as the ions Na^+ and Cl^-, cyclic nucleotides, amino acids, biotin and basic pH (Ward, 1973; Dusenbery, 1974), and avoids water-soluble repellents like Cu^{++}, acid, SDS and high osmotic strength (Dusenbery, 1974; Dusenbery, 1975; Culotti and Russell, 1978). *C. elegans* has an acute sense of smell as well, with the ability to detect at least 60 volatile chemicals, or

odorants, at a wide range of concentrations (Bargmann et al., 1993). As with aqueous chemicals, certain odorants are attractive to *C. elegans* while others are repulsive. In general, short chain ketones and alcohols are attractive, while longer chain ketones and alcohols tend to be repellent. Some odorants are attractive at low concentrations but repellent at high concentrations. *C. elegans* is also capable of discriminating among attractive chemicals; in the presence of a saturating concentration of one attractant, an animal will still chemotax up a gradient of another (Ward, 1973; Bargmann et al., 1993).

Chemotaxis assays have been used to identify about 100 aqueous and volatile chemicals detected by *C. elegans*, although this number is likely to be a small fraction of the total. Only about 300 compounds have been tested in chemotaxis assays, and certain ones have been identified as attractants only in assay conditions designed to detect weaker responses (Wicks et al., 1999). In addition, uncharacterized chemical cues are known to elicit other kinds of behavioral and developmental responses (see below). Therefore, the total *C. elegans* chemosensory repertoire is likely to include far more than the characterized 100 chemicals.

Chemosensation has been implicated in the regulation of larval development (Golden and Riddle, 1984), egg-laying (Sawin, 1996), detection of hermaphrodites by males (Sulston, J., personal communication), and various steps in the male-mating pathway (Liu and Sternberg, 1995). The chemical structures of the pheromones that elicit these responses are unknown, but the most well-characterized response is dauer larval development. Dauer pheromone is secreted constitutively by *C. elegans* and is used as a measure of population density. When pheromone is detected at a high enough

concentration, *C. elegans* larvae will develop as dauer larvae, which are resistant to harsh environmental conditions.

B. Laser ablation studies identify chemosensory neurons.

The nematode has a compact, stereotyped nervous system consisting of 302 neurons (White et al., 1986). The function of specific cells in this network can be studied by eliminating them through laser killing. This approach has led to the identification of sensory neurons required for detection of both aqueous and volatile chemicals, as well as dauer pheromone (Bargmann et al., 1990; Bargmann and Horvitz, 1991; Bargmann and Horvitz, 1991; Bargmann et al., 1993; Kaplan and Horvitz, 1993; Liu and Sternberg, 1995). The 11 pairs of chemosensory neurons with known functions are found in a pair of bilaterally symmetric sensory organs in the head called the amphids, which can be considered to be the nose of the worm. The cell bodies of the amphid neurons send dendrites to the tip of the nose where they terminate in specialized sensory cilia. The cilia protrude through a pore exposed to the environment to allow detection of chemicals. A number of other neurons in the head and tail are predicted to be chemosensory because they also have exposed cilia, but their function is unknown.

The neurons that primarily detect aqueous chemicals have morphologies distinct from those that detect volatile chemicals (Fig. 1-1) (Ward et al., 1975; Ware et al., 1975). The eight pairs of neurons known to sense aqueous chemicals have simple single or double ciliary endings in the amphid opening with direct contact to the environment. The ASE pair, along with some input from the ASI, ADF, ASG, and ASK neurons, senses aqueous

attractants (Bargmann and Horvitz, 1991). ASI, ADF and ASG neurons are also implicated in dauer pheromone detection, suggesting that these neurons are able to send at least two different outputs (Bargmann and Horvitz, 1991). ASJ neurons also sense pheromone (Bargmann and Horvitz, 1991; Schackwitz et al., 1996). Unlike the others in this group, the ASH and ADL neurons are not restricted to sensing aqueous chemicals; the ASH neurons detect high osmolarity, certain volatile repellents, and nose touch (Kaplan and Horvitz, 1993; Troemel et al., 1995), while the ADL neurons detect aqueous and volatile repellents (Bargmann et al., 1990; Troemel et al., 1995).

Most volatile molecules are sensed by three pairs of neurons, called AWA, AWB and AWC. These neurons have elaborate ciliary endings enclosed in a sheath cell near the amphid opening. They have wing-like, flattened ciliary endings with a large surface area, presumably to allow optimal detection of odorants. The AWA and AWC neurons detect attractive odorants, while the AWB neurons detect repulsive odorants (Bargmann et al., 1993; Troemel et al., 1997). In addition to these 11 pairs of chemosensory neurons, the amphid contains a thermosensory pair of neurons, called AFD (Mori and Ohshima, 1995), which have elaborate, brush-like endings enclosed in the same sheath cell as AWA, AWB and AWC.

C. Chemosensory mutants from genetic screens.

Genetic screens have uncovered many genes important for chemosensation. These screens have employed a number of different chemosensory assays (Ward, 1973; Dusenbery et al., 1975; Culotti and Russell, 1978; Bargmann et al., 1990; Bargmann et al., 1993) Twenty-five genes required for development of

sensory cilia or amphid openings are known from mutants defective for chemotaxis to aqueous chemicals (*tax* and *che* mutants (Dusenbery et al., 1975; Lewis and Hodgkin, 1977)), avoidance of high osmolarity (*osm* mutants (Culotti and Russell, 1978)), pheromone-induced dauer formation (*daf* mutants (Albert et al., 1981; Riddle et al., 1981)) or from direct visual screening for structural defects (*dyf* mutants (Starich et al., 1995)). Genes encoding chemosensory signalling components have also been identified in these screens. Subsequent screens have found genes required specifically for chemotaxis to volatile chemicals (*odr* mutants (Bargmann et al., 1993; Sengupta et al., 1996; Dwyer et al., 1998)). Most *odr* genes analyzed so far are involved in olfactory neuron differentiation or olfactory signalling.

III. Chemosensory receptors

A. The diacetyl receptor, ODR-10, is a G protein-coupled receptor.

The first step in chemosensation is the binding of a chemical ligand to its receptor. Behavioral studies demonstrated that *C. elegans* can discriminate among odorants sensed by the same neuron, suggesting that multiple receptors may be expressed in the same neuron. For example, diacetyl and pyrazine are both attractants sensed by the AWA neurons. However, an animal in the presence of a high uniform concentration of pyrazine is still able to respond to a gradient of diacetyl, suggesting that there are separate receptors for diacetyl and pyrazine. Therefore, in order to identify a diacetyl receptor, a screen was performed to isolate mutants that failed to chemotax toward diacetyl, but still retained the ability to chemotax toward pyrazine. This approach identified the gene *odr-10*, which encodes a receptor for

diacetyl (Sengupta et al., 1996). *odr-10* mutants are defective for chemotaxis to low concentrations of diacetyl, but not other odorants. The ODR-10 protein is a predicted G protein-coupled receptor with seven transmembrane domains that localizes to the cilia of the AWA neurons, where diacetyl detection is believed to occur. Reintroducing ODR-10 into an AWA-defective mutant is sufficient to restore responses to diacetyl, but not pyrazine. Furthermore, ODR-10 is sufficient to confer diacetyl sensitivity upon both heterologous cells, such as HEK293 cells (Zhang et al., 1997), as well as other *C. elegans* chemosensory neurons, such as the AWB neurons (Troemel et al., 1997) (see Sect. IVD).

Behavioral and heterologous expression experiments indicate that ODR-10 is quite specific for diacetyl. ODR-10 is known to be required only for chemotaxis to low concentrations of diacetyl, although behavioral experiments suggest it may also weakly detect structurally similar odorants (Chou et al., 1996; Troemel et al., 1997). Heterologous expression of ODR-10 in HEK293 cells confers responsiveness only to diacetyl and two structurally similar compounds (Zhang et al., 1997). Intriguingly, this degree of specificity is comparable to that found recently for rodent olfactory receptors in overexpression and heterologous expression studies (Krautwurst et al., 1998; Zhao et al., 1998). In these experiments, receptors showed a robust response only to one odorant, and a much weaker response to structurally similar compounds. Ligand specificity of mouse receptors studied by *in vivo* imaging also indicates that receptors respond to just a small number of odorants, especially at low concentrations (Malnic et al., 1999). Although only one *C. elegans* receptor has been matched with a ligand, these results suggest that

both rodents and nematodes may have receptors with relatively narrow odorant specificity.

B. Four large families of genes encode candidate chemosensory receptors.

The identification of ODR-10 as a receptor for diacetyl provides an example of the molecular nature and specificity of one odorant receptor. But what about other receptors? Fortunately, the *C. elegans* genome sequencing project, which has recently completed the sequence of the entire genome, has provided a large number of candidate chemosensory receptors (*C. elegans* sequencing consortium, 1998). These genes all code for G protein-coupled seven transmembrane domain receptors, and are highly divergent in sequence. These receptors fall into four large families based on sequence (Fig. 1-2), the largest of which is composed of genes similar to *odr-10*. There are about 700 putative G protein-coupled receptor genes with sequence identity to *odr-10* that fall into four subfamilies; *str*, *stl*, *stx* and *srd*. These genes are fairly divergent in sequence; among members of each of these subfamilies there is approximately 10-30% sequence similarity, with even less similarity between subfamilies. The *odr-10* receptor is a member of the *str* subfamily, which contains about 300 genes. In addition to the *odr-10*-like receptors, three other families of candidate chemosensory receptors, called *sra*, *srg* and *sro*, have been identified (Troemel et al., 1995; *C. elegans* sequencing consortium, 1998). The *sra* family is further subdivided into the *sra*, *srb* and *sre* subfamilies, each of which have about 40 members. The *srg* family has around 40 receptors, while the *sro* family has about 80 receptors that are related to the opsin subfamily of seven-transmembrane receptor genes.

To test if these predicted G protein-coupled receptors might encode chemosensory receptors, their expression patterns were studied using promoter fusions to the green fluorescent protein (GFP). Of 20 genes examined from the *sra*, *srg*, and *sro* families, 10 showed expression in chemosensory neurons, including ASK, ASI, ASH, and ADL (Troemel et al., 1995). These genes may therefore encode receptors for aqueous attractants, volatile or aqueous repellents, or dauer pheromone. Interestingly, certain receptors are expressed in a male-specific manner and therefore might be receptors for mating signals from hermaphrodites. Expression patterns of 21 *str* genes have also been examined with promoter-GFP fusions and over half are expressed in chemosensory neurons (see Chapter 2b). These genes are expressed in both aqueous and olfactory neurons, and their protein products appear to localize to the ciliary endings of these neurons, consistent with a role in detecting chemicals.

How many chemosensory receptors does *C. elegans* have? The *odr-10*-like, *sra*, *srg*, and *sro* families contain a total of 1000 genes, and the expression patterns of 65 of these have been examined with promoter-GFP fusions (Zhang, M., Grantner, S. and Bargmann, C. I., personal communication; Chapter 2b; Troemel et al., 1995). Over half of these 65 genes are expressed in chemosensory neurons, with the rest expressed in non-sensory neurons, non-neuronal cells, or not expressed at all. Surprisingly, about 30% of the *C. elegans* receptor genes appear to be pseudogenes, meaning they contain stop codons due to base changes or frameshifts (Robertson, 1998). However, most of the pseudogenes examined so far appear not to be transcribed, based on promoter-GFP studies. If the genes examined so far are representative, *C. elegans* may have about 500 different kinds of functional chemoreceptors. *C.*

elegans therefore appears to dedicate not only a major portion of nervous system to chemosensation, but also a large fraction (about 4%) of its protein repertoire (Robertson, 1998).

GFP fusion genes showed that multiple receptors can be expressed in the same pair of neurons. With a total of 20-30 chemosensory neurons and perhaps 500 chemosensory receptors, each pair of chemosensory neurons may therefore express 15-25 receptors. There seems to be little correlation between receptor expression and gene family; receptors from the same family can be expressed in different neurons, and receptors from different families can be expressed in the same neuron. For example, the ASK neurons are known to express at least six different receptors from three different families.

Many of the *odr-10*-like, *sra*, *sro*, and *srg* genes are found in clusters in the genome, which can contain up to 10-15 related genes (*C. elegans* sequencing consortium, 1998). Clustering may have resulted from repeated duplication of genes due to unequal cross-over events, and this organization may continue to contribute to receptor evolution. A number of *C. elegans* receptors appear to have already diverged significantly from their orthologs in the related nematode, *C. briggsae* (Robertson, 1998). Comparison of a *C. briggsae* cluster of 11 candidate receptor genes with their *C. elegans* orthologs revealed a lower level of conservation than is seen for other genes compared between *C. briggsae* and *C. elegans*. In addition, this cluster exhibited distinct duplication and pseudogene events in each organism, as well as movement of genes to new genomic locations.

Based on data from ODR-10, a strong case can be made that *C. elegans* detects odorants with G protein-coupled receptors. However, candidate chemosensory receptors of another class have also been identified. *C. elegans* has 26 receptor guanylyl cyclases (*C. elegans* sequencing consortium, 1998), a number of which are expressed in chemosensory neurons and localize to the cilia (Yu et al., 1997). Although some of these receptor guanylyl cyclases are likely to be involved in signal transduction (Birnby, D. and Thomas, J., personal communication; L'Etoile, N. and Bargmann, C.I., personal communication), others may be chemoreceptors. A receptor guanylyl cyclase has been implicated in vertebrate olfaction as well (Juilfs et al., 1997).

C. Comparison of *C. elegans* and vertebrate receptors

Like *C. elegans,* vertebrates have multiple families of candidate chemosensory receptors. The ORs, the V1Rs and the V2Rs are all families of predicted G protein-coupled receptors expressed in vertebrate chemosensory neurons. Unlike *C. elegans* receptors, these receptors have high sequence similarity within a family, although the only similarity between families is that they all encode putative G protein-coupled receptors. The OR family is the largest, composed of genes that belong to the opsin subfamily of seven-transmembrane receptors (Buck and Axel, 1991). The ORs are expressed in the main olfactory epithelium, and rodent ORs have been shown to function in odorant detection (see Sect. IIIA). The V1Rs and the V2Rs are predicted G protein-coupled receptors expressed in the vomeronasal organ (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997), which has been implicated in detecting chemical cues that regulate social and sexual responses. The V1Rs have no sequence similarity

to other G protein-coupled receptors, while the V2Rs are similar in sequence to the metabotropic glutamate receptor and the extracellular calcium-sensing receptor. The V1Rs are expressed more highly in the apical region of the vomeronasal organ, while the V2Rs are enriched in the basal region. Thus, different families of vertebrate chemosensory receptors have characteristic regions of expression, in contrast to *C. elegans*, where several kinds of receptors are present in one cell.

There are a few other notable differences between the *C. elegans* and vertebrate chemosensory systems. Most vertebrate receptor genes lack introns (Buck and Axel, 1991), while *C. elegans* receptor genes have multiple introns. A number of experiments indicate that vertebrates express only one olfactory receptor per neuron (Buck and Axel, 1991; Chess et al., 1994; Malnic et al., 1999), while *C. elegans* expresses multiple receptors per neuron. Interestingly, vertebrate receptors appear to play a role in neuronal wiring. All the olfactory neurons expressing a particular receptor project to the same post-synaptic target, and this target can be altered by changing receptor expression (Mombaerts et al., 1996; Wang et al., 1998). *C. elegans* receptors have not been found to play a significant role in neuronal wiring (Troemel et al., 1997).

Despite the vast difference in their complexity, mammals and *C. elegans* appear to have similar numbers of chemosensory receptors. For example, the three identified rat receptor families contain a total of about 1200 genes, while the four *C. elegans* receptor families contain around 1000 genes. As in *C. elegans*, many of the mammalian genes appear to be pseudogenes. The exact number of functional receptors is not known for any animal, but estimates

suggest that rats have 1000 kinds of functional receptors, which is not far from the estimate of 500 receptors for *C. elegans*.

Chemosensory receptors appear to have arisen independently multiple times in evolution, since there is no sequence similarity among the ORs, the V1Rs, the V2Rs, or the four families of *C. elegans* receptors. However, all of these genes are found in clusters in the genome, suggesting a common mechanism for their creation. Perhaps founder chemosensory receptors arose from G protein-coupled receptor genes in genomic areas especially prone to duplication and mutation (*C. elegans* sequencing consortium, 1998). As these genes progressively duplicated and diversified, they gave rise to a large number of different chemosensory receptors that provide the chemosensory system with its large detection capability.

D. *odr-4* and localization of chemosensory receptors

Chemosensory receptors are localized to the ciliary endings of neurons, presumably to facilitate detection of chemicals in the environment. Expression studies using GFP-tagged receptors indicate that most of the ODR-10 protein, as well as other candidate chemoreceptor proteins, are present in the cilia (Sengupta et al., 1996; Dwyer et al., 1998). How are these receptors properly processed and transported to the cilia? Two mutants, *odr-4* and *odr-8*, are defective in localization of a subset of receptors, including ODR-10 (Dwyer et al., 1998). *odr-4* and *odr-8* mutants are also defective in a subset of olfactory responses, including response to diacetyl, suggesting that their olfactory defects are due to mislocalization of a subset of receptors. Interestingly, *odr-4* does not appear to be required for cilia localization of

other olfactory signalling components, such as G proteins and channels. *odr-4* codes for a novel protein with a putative C terminal transmembrane domain that localizes to intracellular membranes in the cell body, the dendrite, but not the cilia. Therefore, *odr-4* may be involved in folding, sorting, or active targeting of receptors to the cilia. *odr-8* mutants have the same defects as *odr-4* mutants, but the molecular identity of *odr-8* is not yet known.

The requirement for special cofactors to process and transport G protein-coupled receptors appears to be a common theme. For example, *Drosophila* photoreceptors have a special cyclophilin, called Nina A, which is required for localization of a subset of opsin proteins (Colley et al., 1991; Stamnes et al., 1991). The RAMP proteins are important for transport and ligand-specificity of the human calcitonin-receptor-like receptor (McLatchie et al., 1998). There are hints that vertebrate odorant receptors require special cofactors for processing as well. Efficient expression of vertebrate odorant receptors in heterologous expression systems is difficult to achieve (Krautwurst et al., 1998), which may be explained by the lack of essential cofactors such as ODR-4 in cultured cells.

IV. Chemosensory signalling downstream of receptors.

A. G protein alpha subunits are involved in aqueous, volatile and pheromone chemosensation.

Seven-transmembrane domain receptors like ODR-10 are predicted to interact with G proteins. One important olfactory G protein is encoded by *odr-3*

(Roayaie et al., 1998). ODR-3 is a G_i-like alpha subunit required for robust responses to odorants sensed by the AWA and AWC neurons, but not required for responses to most aqueous attractants. In addition to its role in AWA and AWC-mediated responses, *odr-3* is also essential for responses to ASH-sensed stimuli, such as high osmolarity and nose touch. The ODR-3 protein is expressed in and localizes to the cilia of AWA, AWC and ASH, where it would be poised to receive signals from chemosensory receptors. This expression pattern suggests that ODR-3 is directly involved in the transduction of chemical stimuli. Proper levels of *odr-3* appear to be critical for chemosensory signalling: overexpression of *odr-3* interferes with chemotaxis, to an even greater extent than the *odr-3* null phenotype. *odr-3* null mutants may have residual responses because AWC has other G protein subunits that play a role in the absence of *odr-3*. Perhaps overexpression of *odr-3* leads to a more pronounced olfactory defect because it titrates out factors required for signalling of these other G proteins.

Other G alpha subunits are known to play a role in chemosensation. *C. elegans* has 20 G alpha subunits in total, 15 of which, including *odr-3*, *gpa-2* and *gpa-3*, are expressed in chemosensory neurons (Jansen et al., 1999). *gpa-2* and *gpa-3* are G protein alpha subunits of a novel G_i-like class that regulate entry into the dauer stage (Zwaal et al., 1997). Knock-outs of *gpa-2* or *gpa-3* reduce the sensitivity to dauer pheromone, while constitutively active forms of these genes are dauer-constitutive. *gpa-2* appears also to play a role in AWC-mediated olfaction: an *odr-3 gpa-2* double mutant is more defective for response to one AWC-sensed odorant than an *odr-3* single mutant. The 12 other G protein alpha subunits with expression in chemosensory neurons are also involved in chemosensation (Jansen et al., 1999). Gain of function

mutations in a number of these G proteins interfere with chemosensation of water-soluble attractants, repellents or pheromone. However, the loss of function phenotypes of these genes are subtle compared to *odr-3*, suggesting that they may encode modulatory or redundant G proteins. One especially interesting G protein is encoded by the *gpa-5* gene. A *gpa-5* knock-out can suppress one odorant defect of an *odr-3* knock-out, implying that *gpa-5* may be a negative regulator of olfaction.

B. Two distinct channels are involved in chemosensation.

A cyclic nucleotide-gated channel encoded by the *tax-2* and *tax-4* genes is required for chemotaxis to many chemicals, both volatile and aqueous (Coburn and Bargmann, 1996; Komatsu et al., 1996). Mutations in these genes cause virtually identical phenotypes; they are defective in response to water-soluble attractants, AWC-sensed odorants, certain volatile repellents and temperature. These genes are also involved in dauer formation. *tax-2* and *tax-4* are expressed in the neurons required for these responses, namely, ASE, AWC, AWB, AFD and ASI. Functional expression of TAX-2 and TAX-4 in HEK293 cells has shown they form a channel that is gated by cGMP and permeable to Na^+ and Ca^{++} (Komatsu et al., 1999).

The *tax-2/4* genes are not required for function of other chemosensory neurons, such as AWA and ASH, nor are they expressed in these cells. A strong candidate for a channel involved in transducing signals in these cells is encoded by the *osm-9* gene. OSM-9 has similarity to the *Drosophila* phototransduction channel, TRP, as well the vertebrate capsaicin receptor, VR1 (Caterina et al., 1997; Colbert et al., 1997). *osm-9* mutants are defective in

response to odorants sensed by AWA as well as osmotic, volatile and tactile stimuli sensed by ASH. *osm-9* is expressed in all amphid neurons except AWB and AFD, as well as in non-amphid sensory neurons and non-neuronal cells, but does not appear to be required for primary transduction in these cells. One likely role for *osm-9* in chemosensory cells other than AWA and ASH is a modulatory one; *osm-9* mutants are defective in adaptation to certain AWC-sensed odorants (see Sect. V), suggesting that *osm-9* regulates the responsiveness of AWC toward certain odorants.

C. Comparison of G proteins and channels in *C. elegans* and vertebrates.

Both vertebrates and *C. elegans* express multiple G protein alpha subunits in chemosensory neurons, and have one G alpha known to play a critical role in olfaction. The G_s-like protein G_{olf} is expressed in the vertebrate olfactory epithelium and is required for normal electrophysiological responses to most odorants (Jones and Reed, 1989; Belluscio et al., 1998). However, G_{olf} is not expressed in the vomeronasal organ, which expresses two other G alpha subunits, G_{i2} and G_o (Halpern et al., 1995; Berghard and Buck, 1996). G_{i2} is expressed at high levels in the apical region coinicident the with V1R receptors, while G_o is expressed more highly in the basal region along with the V2R receptors. Therefore, the ORs in the main olfactory epithelium appear to signal through G_{olf}, while the V1Rs and the V2Rs in the vomeronasal organ probably use G_{i2} and G_o, respectively.

A number of parallels exist between channel use in *C. elegans* and vertebrate chemosensation. First, a cyclic nucleotide-gated channel (CNG) is crucial for transducing olfactory signals in both systems. In mouse, a CNG is required

for electrical response to all odorants detected by the main olfactory epithelium (Brunet et al., 1996). However, unlike TAX-2/4, the mouse channel is gated by cAMP, not cGMP. As in *C. elegans*, the mouse CNG is not the only channel involved in transducing chemosensory signals. It is not expressed in the vomeronasal organ and electrophysiological studies have indicated that cyclic nucleotides are not required for responses in these neurons (Liman and Corey, 1996). This raises the possibility that a channel similar to OSM-9 is functioning in this chemosensory system and that vertebrates, like *C. elegans*, use two kinds of chemosensory transduction channels. Another intriguing parallel between *C. elegans* and vertebrates is the conservation of channels used for nociception. In the ASH neurons, OSM-9 is required for sensation of repulsive stimuli, while its vertebrate homolog, VR1, is required for detection of painful stimuli, like capsaicin and noxious heat (Caterina et al., 1997).

D. Olfactory neurons specify behavioral responses.

Once an odorant is detected, the animal must match the odor to the appropriate behavioral or developmental response. This problem is complicated, because each neuron expresses many receptors, including receptors from different gene families. One possibility is that there are different types of receptors, with some, like ODR-10, biased toward attraction and others biased toward repulsion. Alternatively the neuron could interpret information from all receptors as equivalent. This question was addressed by misexpressing the diacetyl receptor, ODR-10, in another olfactory neuron. Normally, ODR-10 is expressed in the AWA neurons and animals are attracted to diacetyl. However, when ODR-10 is misexpressed in the

repellent-sensing AWB neurons, animals are repelled by diacetyl (Troemel et al., 1997). This result suggests that ODR-10 can confer diacetyl sensitivity upon another neuron. In addition, it shows that the behavioral response directed by ODR-10 is defined by the cell that expresses it. If this phenomenon proves to be generally true, it suggests that receptors simply detect chemicals while the cells in which they are expressed direct behavioral responses.

AWA and AWB require different transduction molecules for their normal activity. Both neurons use the G protein ODR-3, but AWA uses the OSM-9 channel and AWB the TAX-2/4 channel. When ODR-10 was introduced into the AWB neurons, it required ODR-3 and TAX-2 for signalling, but not OSM-9. This suggests that while ODR-10 coupled to the same G protein in AWB that it uses in AWA, this led to activation of a different channel.

V. Olfactory discrimination and adaptation

The olfactory environment encountered by *C. elegans* in lab-controlled chemotaxis assays is an extremely simplified version of the complex chemical milieu that it encounters in the wild. In order to maneuver through this environment, *C. elegans* must be able to determine which cues to pay attention to and which ones to ignore. For example, some odorants are thought to be attractive to the worm because they are indicative of a food source. However, if such an odorant is ubiquitous in a certain environment when food is not, it no longer provides information about food, and responses to that odorant should be down-regulated. Indeed, *C. elegans* does attenuate its responses to odorants, or adapts, in an odorant-specific manner (Colbert and Bargmann, 1995).

The odorants benzaldehyde and butanone are both attractive odorants sensed by the AWC neurons. After prolonged exposure to high concentrations of benzaldehyde, chemotaxis toward that odorant is reduced, while butanone responses are unaffected. Some aspects of adaptation in AWC appear to involve influx of extracellular Ca^{++} and two mutants that affect AWC adaptation, *adp-1* and *osm-9*, have been identified. *adp-1* mutants have reduced adaptation (i.e. stronger response after prolonged exposure) to benzaldehyde and butanone, while *osm-9* mutants have reduced adaptation to benzaldehyde and isoamyl alcohol. *adp-1* appears to affect the same responses that are sensitive to Ca^{++} influx, although the identity of the *adp-1* gene remains unknown. *osm-9*, as discussed above, encodes a channel with similarity to VR1 and TRP and appears to have a modulatory, rather than an essential function in AWC.

If chemotaxis toward attractive chemicals is used as a strategy for finding food, it would make sense to modulate those responses depending on the feeding state of an animal. Indeed, worms exposed to an odorant while starved adapt more completely to that odorant than they adapt when exposed to the odor in a well-fed state (Colbert and Bargmann, 1997). Thus, an odorant is paired with a negative stimulus, i.e. the absence of food, to make the odorant less attractive. Starvation also increases the nematode's ability to discriminate among different odorants, which may allow the nematode to respond more precisely to its environment. The end result of these starvation-induced changes is to enable the nematode to seek out novel environments that may have more plentiful food. Interestingly, treating with exogenous serotonin can substitute for food in both its effect on adaptation as well as on

discrimination, suggesting that serotonin may signal a well-fed state. Serotonin has also been shown to substitute for food in the regulation of other *C. elegans* behaviors (Horvitz et al., 1982).

VI. Summary and future directions

Nematodes and vertebrates use a large number of G protein-coupled receptors from multiple families to detect chemical cues (Fig. 1-3). These receptors signal through different G proteins in different cells, with ODR-3 in worms and G_{olf} in vertebrates known to play critical roles. Signalling in both systems culminates in the activation of a cyclic nucleotide-gated channel in certain cells, but not others. While certain details differ, like the type of receptor and the use of cGMP vs. cAMP, the similarities in the logic used in such divergent animals are quite striking.

Many of the detection components of *C. elegans* chemosensory neurons have now been identified, but the details of how they signal have yet to be deciphered. For example, a simple model for diacetyl detection in the AWA neurons is that diacetyl is detected by the ODR-10 receptor, which then signals via the ODR-3 G protein to a channel containing OSM-9. However, it is not clear how ODR-3 signalling leads to OSM-9 activation, whether OSM-9 functions on its own or with other channel subunits, or if detection of odorants leads to depolarization or hyperpolarization. These questions could be addressed through heterologous expression studies, or perhaps *in vivo* in *C. elegans*. Advances have been made in characterizing the electrophysiological properties of *C. elegans* chemosensory neurons

(Goodman et al., 1998), but it has not yet been possible to record responses to specific chemicals.

An immense number of chemosensory receptors and G proteins are used by this simple organism to detect its chemical environment. Why so many? The chemical sense is a crucial mode of information gathering for the worm, and since *C. elegans* lacks a complex brain, it makes sense to do much processing and regulation at the periphery. In the wild, *C. elegans* confronts chemical cues emanating from a wide variety of sources and needs to respond appropriately to those cues; perhaps the use of a large number of receptors and G proteins allows for flexibility in response to the environment in the context of a single neuron.

The large number of chemosensory receptors, the high percentage of pseudogenes, as well as the significant divergence from *C. briggsae* orthologs indicates that *C. elegans* chemosensory receptors are rapidly evolving. What allows such diversification? One possibility is that there is partial functional redundancy among receptors, which would allow them to evolve more rapidly. However, the *odr-10* mutant phenotype suggests there may not be much redundancy for detection of individual odors at low concentrations. In addition, analysis of duplicated receptors indicates that there is often strong selection for functionality in both (Robertson, 1998). Perhaps it is more relevant to consider that the pressures on *C. elegans* in the wild are not to detect every possible odor, but just the informative ones. Pseudogenes might therefore arise in receptors that detect unimportant odors, or odors that are always co-emitted from sources with odors detected by other receptors. It would be interesting to examine the receptor repertoire of *C. elegans* strains

that live in various environments to determine whether different receptor genes have been inactivated and duplicated. We are looking at a snapshot in evolution, and the pressures on an individual receptor and therefore its importance are likely everchanging. At any given time there will probably be some receptors that are functionally redundant, others that play a unique role in chemosensation, and only a subset of these that serve a critical purpose.

Our understanding of how *C. elegans* interprets chemosensory information is at its early stages. Mutational and misexpression analysis with the ODR-10 diacetyl receptor suggests that a given odorant is detected by an individual receptor, which is expressed in a pair of neurons to drive a single output. However, for odorant specificity, receptor expression and neuronal output, these studies examine the simplest case scenario. Behavioral experiments with higher odorant concentrations indicate that ODR-10 can detect odorants structurally similar to diacetyl, and that diacetyl is detected by other receptors (Chou et al., 1996; Sengupta et al., 1996). These results are reminiscent of mouse *in vivo* studies, in which a single odorant was detected by multiple receptors, and an individual receptor could detect multiple structurally similar odorants, especially at higher concentrations (Malnic et al., 1999). Such a "combinatorial coding" scheme could theoretically provide more information about odor quality than a one receptor/one odorant scheme. Whether *C. elegans* employs a similar strategy requires examining more receptor/odorant relationships. However, candidate receptors have been found that are expressed in multiple neurons (Troemel et al., 1995), which could functionally be similar to an individual odor activating multiple receptors. Regardless of how individual odors are detected, *C. elegans* likely

uses combinatorial coding to interpret natural stimuli, which are usually mixtures of compounds.

The events that occur downstream of the sensory neurons are largely mysterious. Reconstruction of electron micrographs has revealed the likely connectivities among chemosensory and interneurons, but the functional relevance of those connections has yet to be resolved. For example, AWA and AWB appear to have many similar downstream connections, yet direct opposite behaviors. Do their non-overlapping connections lead to different outputs or do they send different signals to the same interneurons? A further level of complexity in chemosensory coding arises with neurons that are capable of directing multiple outputs. For example, the ASI neurons can direct chemoattraction or dauer development, depending on the chemical detected. It will be interesting to elucidate the signalling and regulation of separate pathways in the same neuron, which then lead to distinct outputs. Sorting out the relationship among receptors and chemicals, how multiple inputs are processed and regulated within sensory and interneurons will provide much interesting work for the future.

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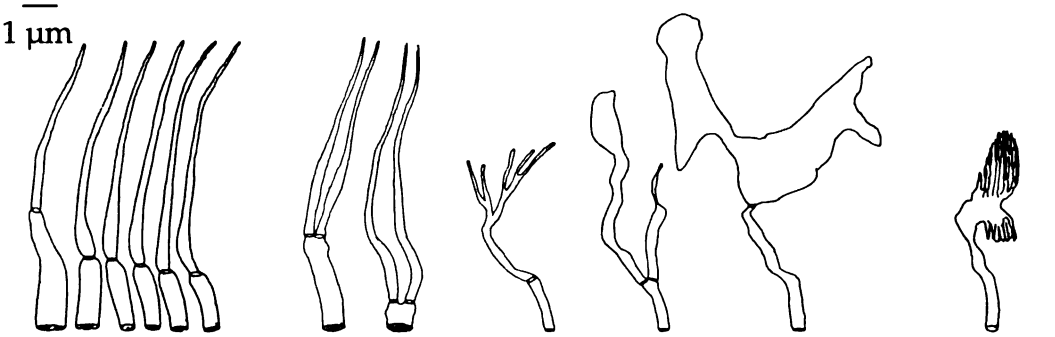
Fig. 1-1. *C. elegans* chemosensory and thermosensory neuron structure and function

A. Morphology of chemosensory and thermosensory neuron endings



B. Chemicals detected by simple ciliated neurons

Cell	Aqueous attractants and pheromone
ASE	Na^+, Cl^-, cAMP, biotin, lysine
ADF	Pheromone; (minor) Na^+, Cl^-, biotin, cAMP
ASI, ASG	Pheromone; (minor) Na^+, Cl^-, biotin, cAMP, lysine
ASK	Lysine
ASJ	Pheromone
Cell	**Aqueous and volatile repellents**
ADL	Octanol
ASH	High osmolarity, benzaldehyde (high conc.), octanol

C. Chemicals detected by wing-like ciliated neurons

Cell	Volatile attractants
AWA	Diacetyl, pyrazine, 2,4,5 trimethyl thiazole
AWC	Benzaldehyde (low conc.), isoamyl alcohol, butanone, 2,4,5 trimethyl thiazole
Cell	**Volatile repellents**
AWB	2-nonanone

Figure 1-2

Candidate chemosensensory receptor families

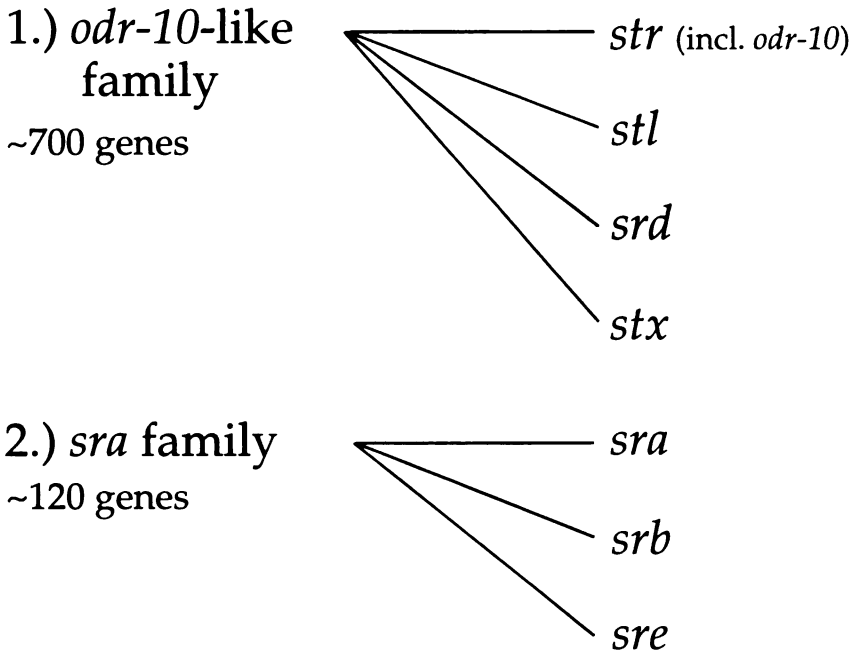
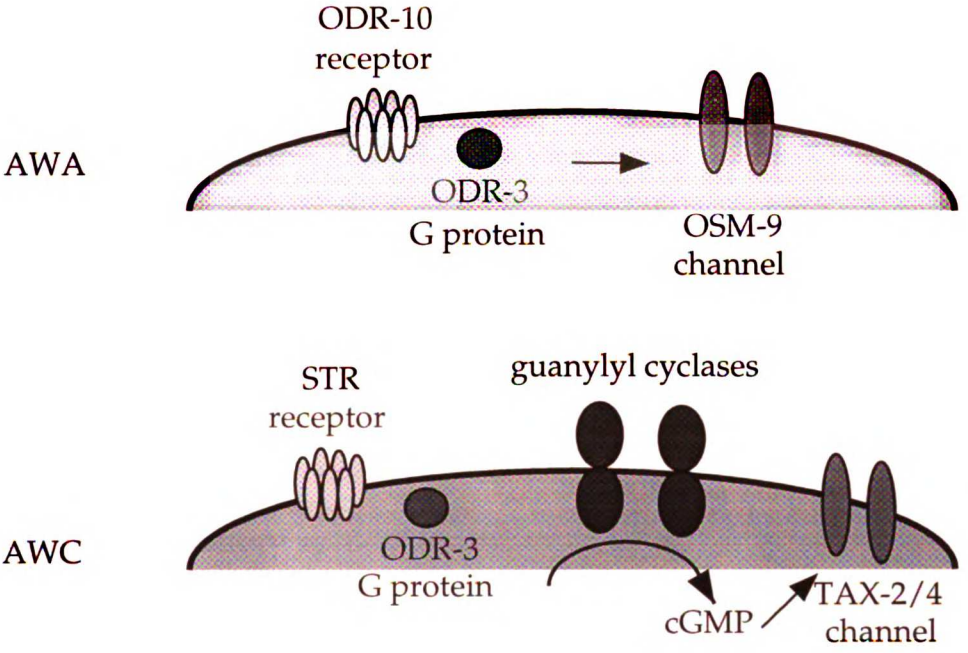


Figure 1-3. Models for *C. elegans* and vertebrate olfactory signalling.

A. *C. elegans* olfactory signalling



B. Vertebrate olfactory signalling

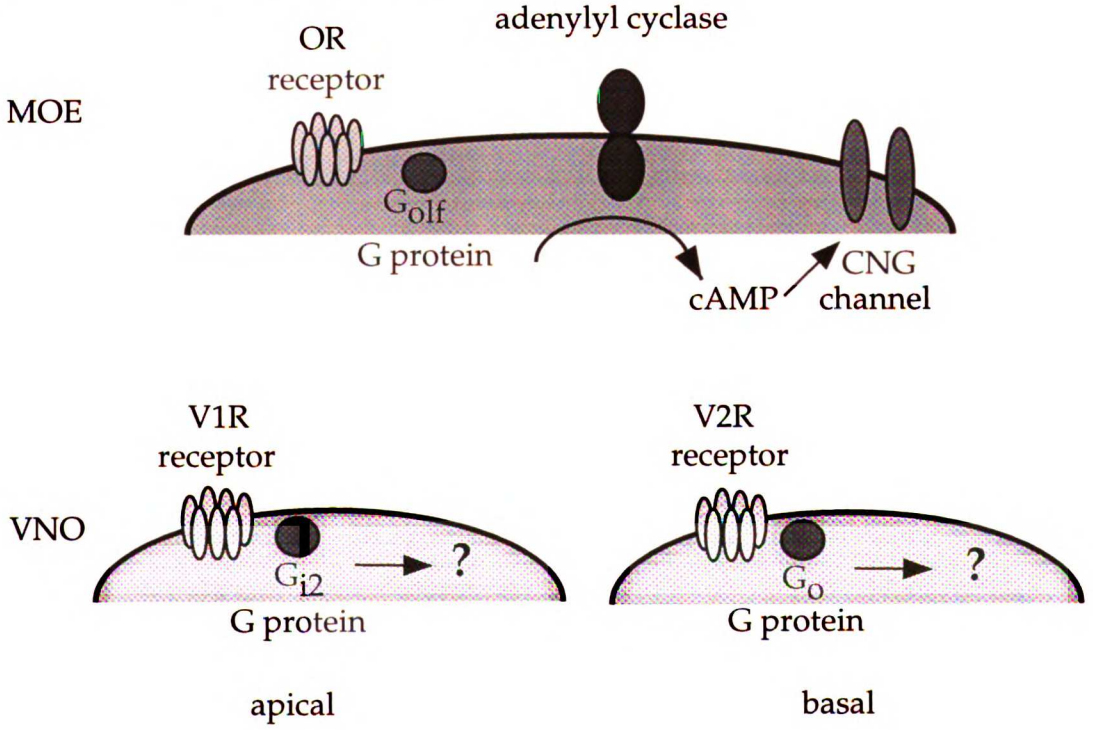


Figure 1-3

Models for olfactory signalling in *C. elegans* and vertebrates.

A. *C. elegans*:

In the AWA neurons, when receptors like ODR-10 bind odorant, they signal through a G protein containing the alpha subunit ODR-3, which regulates a channel containing the TRP-like OSM-9 protein. In AWC, when G protein-coupled receptors bind odorant, they activate G proteins, including ODR-3, which regulate activation of guanylyl cyclases (or phosphodiesterases), which generate cGMP to gate the TAX-2/4 cyclic nucleotide-gated channel.

B. Vertebrates:

In the main olfactory epithelium (MOE), ORs signal through a G protein containing G_{olf}, leading to activation of the AC_{III} adenylyl cyclase, which generates cAMP to gate the cyclic nucleotide-gated channel (CNG). In the apical region of the vomeronasal organ (VNO), V1Rs signal through a G protein containing the G_{i2} alpha subunit, while in the basal region, V2Rs signal through a G protein containing the G_o alpha subunit. The channels activated in these neurons are not yet known.

Chapter 2a

Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*.

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Divergent Seven Transmembrane Receptors Are Candidate Chemosensory Receptors in C. elegans

Emily R. Troemel,* Joseph H. Chou,* Noelle D. Dwyer,* Heather A. Colbert,* and Cornelia I. Bargmann
Howard Hughes Medical Institute
Programs in Developmental Biology, Neuroscience, and Genetics
Department of Anatomy
University of California, San Francisco
San Francisco, California 94143-0452

Summary

Using their senses of taste and smell, animals recognize a wide variety of chemicals. The nematode C. elegans has only fourteen types of chemosensory neurons, but it responds to dozens of chemicals, because each chemosensory neuron detects several stimuli. Here we describe over 40 highly divergent members of the G protein-coupled receptor family that could contribute to this functional diversity. Most of these candidate receptor genes are in clusters of two to nine similar genes. Eleven of fourteen tested genes appear to be expressed in small subsets of chemosensory neurons. A single type of chemosensory neuron can potentially express at least four different receptor genes. Some of these genes might encode receptors for water-soluble attractants, repellents, and pheromones.

Introduction

The olfactory and gustatory systems detect a variety of structurally unrelated molecules, from ions to complex organic compounds. These heterogeneous chemical signals are recognized by specialized sensory receptors and sensory neurons. Unlike the visual system, which detects many wavelengths of light but uses only a few types of receptor neurons, the olfactory system contains many types of sensory neurons that each detect particular chemical cues.

To analyze the mechanisms of sensory recognition and discrimination by the nervous system, we are studying chemosensation in the nematode Caenorhabditis elegans. C. elegans can detect touch, temperature, and light, but its responses to chemicals are the most diverse responses in its behavioral repertoire. C. elegans eats bacteria; chemicals produced by bacteria stimulate chemotaxis, egg laying, feeding, and defecation (Ward, 1973; Dusenbery, 1974; Horvitz et al., 1982; Avery and Horvitz, 1990; Thomas, 1990; Bargmann et al., 1993), while toxic or aversive compounds are avoided (Culotti and Russell, 1978). Pheromones contribute to mating between males and hermaphrodites (Liu and Sternberg, 1995). A pheromone also controls the development of an alternative larval stage called a dauer larva (Golden and Riddle, 1984).

The neurons involved in these chemosensory responses can be precisely defined within the nervous system of C. elegans. An adult hermaphrodite has exactly 302 neurons, whose positions, morphology, and synaptic connections are reproducible from animal to animal (White et al., 1986). Among these neurons are 32 neurons that appear to be chemosensory, since they have ciliated endings that are exposed to the environment through specialized sensory structures (Ward et al., 1975; Ware et al., 1975). These neurons can be divided into fourteen types, where one neuron type typically consists of two bilaterally symmetric neurons. For ten types of neurons, chemosensory function has been directly demonstrated by observing behavioral deficits after laser killing of defined cell types. For example, the two ASE chemosensory neurons respond to water-soluble attractants including salts, cAMP, and biotin; the two AWC olfactory neurons respond to volatile aldehydes, ketones, alcohols, and thiazoles; and the two ASH neurons respond to both chemical and mechanical stimuli (Bargmann and Horvitz, 1991a; Bargmann et al., 1993; Kaplan and Horvitz, 1993). The neurons that sense attractants (seven types) and repellents (two types) do not overlap, and they synapse onto distinct synaptic targets that mediate chemotaxis and avoidance behaviors (White et al., 1986).

Interestingly, different kinds of sensory information can be sorted out within a single type of sensory neuron. For example, three pairs of neurons regulate both chemotaxis and dauer larva formation, while two pairs of neurons regulate both chemotaxis and egg laying (Bargmann and Horvitz, 1991b; E. Sawin and H. R. Horvitz, personal communication). Thus, two distinct responses can be generated by a single sensory cell type. In addition, animals can adapt independently to two different chemicals that are detected by the same chemosensory neuron (Colbert and Bargmann, 1995), and the response to one chemical detected by a chemosensory neuron can be saturated without blocking the response to a second chemical detected by that neuron (Ward, 1973; Bargmann et al., 1993).

How can a small number of chemosensory neurons generate responses to a much larger number of chemicals? One possibility is that each chemosensory neuron possesses multiple receptor proteins or binding sites for different compounds. In that case, some aspects of discrimination between sensory stimuli could occur within a single sensory neuron. Alternatively, each sensory neuron might express only one type of receptor that binds to many chemicals; in this case, downstream integration of information from several types of sensory neurons could be used to generate diverse responses. Unfortunately, it has not been possible to examine chemosensory receptor expression directly. A family of G protein–coupled vertebrate olfactory receptors has been identified (Buck and Axel, 1991), but homologs of these receptors have not been identified in C. elegans. G protein–mediated second messengers have been implicated in insect chemoreception (Breer et al., 1990), but the receptors that mediate chemosensation in invertebrates are unknown.

*These authors made similar contributions to this work.

We describe here a family of seven transmembrane receptor genes whose products might mediate chemosensation in C. elegans. These genes were sequenced by the C. elegans genome sequencing consortium, which has sequenced about 15% of the genome (Sulston et al., 1992; J. Sulston, A. Coulson, R. Waterston, et al., personal communication). The genes are highly divergent from known genes and from one another. However, they have secondary structures and key residues that define them as members of the G protein–coupled receptor superfamily. The receptor genes are clustered in the genome, with up to nine genes present in a single cluster. Most of these genes are expressed in sensory neurons, and multiple receptors can be expressed by a single sensory neuron.

Results

A Large Family of Potential Seven Transmembrane Receptors

In C. elegans, genes with related functions are often found clustered in the genome in operons: a primary transcript encoding several genes is cleaved to produce multiple mature mRNAs (Zorio et al., 1994). Therefore, we examined regions around potential olfactory signaling molecules in the sequenced DNA of C. elegans for genes that might be chemosensory receptors. Immediately adjacent to a transmembrane guanylyl cyclase on chromosome II, we found nine novel genes that were related to one another. Although these genes were not homologous to any known genes, they encoded proteins with multiple predicted transmembrane domains, as would be expected of receptors. These sequences were used to search databases for related genes, which were then used in further sequence searches (Altschul et al., 1990). From this analysis, we identified 41 potential C. elegans receptor genes that fell into six families based on sequence similarity with one another. The gene families were named *sra*, *srb*, *srg*, *srd*, *sre*, and *sro* (for serpentine receptor classes a, b, g, d, e, and o). The chromosomal locations of these genes are shown on Figure 1A; their sequences are presented in Figure 2.

The *sra*, *srb*, *srg*, *srd*, and *sre* genes were not significantly similar to any known gene in homology searches. However, when their sequences were manually aligned with consensus sequences for seven transmembrane receptors, they were found to contain features that are characteristic of that family. Each gene displayed approximately seven hydrophobic peaks that could be transmembrane domains (Figure 1B) (Kyte and Doolittle, 1982) as well as some key residues that are usually present in G protein–coupled receptors (Probst et al., 1992). The regions of conservation in each subfamily were most pronounced in predicted transmembrane domains 3 and 7 and in linker regions between transmembrane domains. These regions tend to be most conserved among related seven transmembrane receptors, supporting the hypothesis that the *sra*, *srb*, *srg*, *srd*, *sre*, and *sro* genes encode G protein–coupled receptors from this superfamily (Probst et al., 1992).

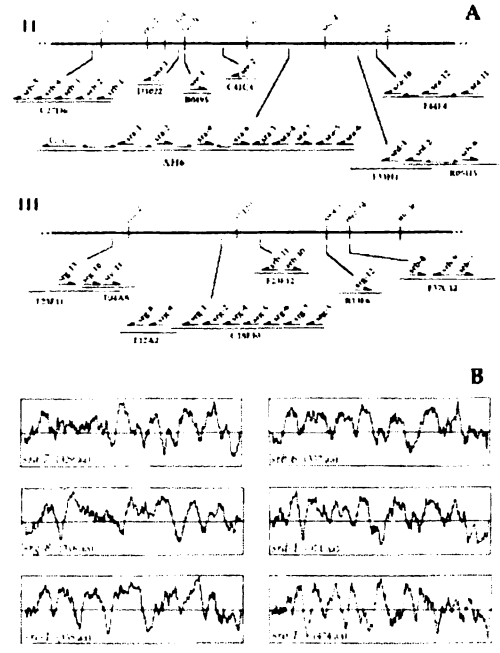


Figure 1. Genomic Organization and Structure of Predicted Receptor Genes

(A) Genomic organization of predicted receptor genes (not to scale). Approximately two thirds of chromosome III and one third of chromosome II were available through GenBank when these genes were discovered. At the top is shown the approximate genetic map position of each set of genes. In insets are shown the cosmids from which each gene was derived. Predicted receptor genes are indicated by the thicker lines; interspersed genes that did not belong in these receptor families are indicated by the thinner lines. In the *sra-1–sra-9* cluster, the interspersed genes were AH6.5, a zinc finger–containing protein, AH6.15, a transposon, and AH6.13, a fragment of an *sra*-like gene. G. C. is the guanylyl cyclase AH6.1. In the *sra-10–sra-12* cluster, the interspersed genes were F44F4.6, a β-1,6-N-acetylglucosaminyltransferase, and F44F4.8, a transposase. In the *srd-1–srd-2–srb-6* cluster, the interspersed genes were R05H5.2, a phosphatase, and R05H5.7, a novel protein.

(B) Hydrophobicity plots of representative genes. Six of the genes whose expression patterns are presented in Figures 3–6 are shown. Hydrophobic peaks predicted by Kyte–Doolittle analysis (Kyte and Doolittle, 1982) appear above the center line in each graph. Similar plots were obtained for all family members shown in Figure 2.

The three largest families of genes were the *sra*, *srb*, and *srg* genes. The *sra* family included the nine genes that initiated the search, *sra-1* through *sra-9*, which shared about 35% amino acid identity overall, and three other genes, *sra-10* through *sra-12*, which were about 20%–25% identical with *sra-1* through *sra-9*. The eleven *srb* genes were distantly related to the *sra* genes (about 10%–15% amino acid identity) but significantly more closely related to one another (about 30% identity). The thirteen *srg* genes were essentially unrelated to the *sra* and *srb* genes by sequence, but between 10%–30% identical to one another. The *srg* genes were independently predicted to be G protein–coupled receptors by E. Sonnhammer of the Sanger Center (personal communication).

Fewer members of the *srd*, *sre*, and *sro* families of genes were detected. *srd-1* and *srd-2* were 48% identical to one another at the amino acid level. *sre-1* and *sre-2* were 28% identical to one another at the amino acid level, and marginally similar to members of the *srb* gene family. While the *sra–sre* genes were unrelated in sequence to known sensory receptors, the single *sro-1* gene displayed distant similarity to opsin genes (Figure 2). *sro-1* is highly diverged from known opsins; it lacks the lysine that forms a Schiff base with retinal, so its sequence similarity with opsins might not reflect functional similarity (Thomas and Stryer, 1982).

Most of the *sra*, *srb*, *srg*, and *srd* genes were found in clusters of two to nine related genes (see Figure 1A). Contrary to the initial rationale of the search, the nine original *sra* genes were not organized into a single operon with the guanylyl cyclase gene or each other. Although they were all found within a 30 kb region, different members were transcribed from different DNA strands and presumably had different promoters (see Figure 1A). The three additional *sra* genes *sra-10*, *sra-11*, and *sra-12* were also closely linked to one another, but they were far from *sra-1–sra-9* on chromosome II. In both cases, the *sra* genes were not strictly clustered: unrelated genes were interspersed among the *sra* genes. By contrast, many of the *srb*, *srg*, and *srd* clusters were uninterrupted and might encode polycistronic transcripts, since the genes were transcribed in the same orientation within 1 kb of each other (see Figure 1A) (Zorio et al., 1994). The five genes *srb-1–srb-5* and the nine genes *srg-1–srg-9* might each arise from a single transcript. With the exception of the *srd-1–srd-2–srb-6* cluster, closely linked genes fell within one sequence family (e.g., all *sra* or all *srb* genes), and with the further exception of two *srb* clusters (*srb-7–srb-9* and *srb-10–srb-11*), the linked genes were always the most closely related genes within a family.

The candidate vertebrate olfactory receptors are encoded by genes that are similar enough to cross-hybridize with one another at high stringency (Buck and Axel, 1991), but their level of sequence similarity is higher than that of the families of genes described here. To determine whether many other genes might belong to the *sra*, *srb*, and *srg* gene families, we used the coding regions of *sra-6*, *sra-7*, *srb-1*, *srb-8*, *srb-10*, and *srg-8* to probe genomic Southern blots of C. elegans DNA at high (65°C) or reduced (55°C) stringency. Each gene appeared to detect only its own sequence at both high and lowered stringency (data not shown).

Expression of Seven Transmembrane Receptors in Sensory Neurons

The large number of related sequences in the *sra*, *srb*, and *srg* gene families, their relatively small size (311–371 amino acids), and their clustering in the genome (Ben-Arie et al., 1994) were reminiscent of vertebrate olfactory receptors. To ask whether these genes might be expressed by chemosensory neurons, upstream regions of 22 genes were fused to the reporter gene *GFP* (green fluorescent protein) and introduced into the germline of C. elegans to produce transgenic animals (see Experimental Procedures) (Chalfie et al., 1994). Interestingly, many of these reporter gene constructs yielded highly specific expression patterns (see Figures 3–6; Table 1). By aligning *GFP* fluorescence with differential interference Nomarski images, expression could be localized to single cell types (Figure 4).

Of thirteen genes whose expression was observed in hermaphrodites, seven were expressed only in small subsets of chemosensory neurons. Fusion genes derived from the two linked *srg* genes *srg-2* and *srg-8* and the two linked *sra* genes *sra-7* and *sra-9* were expressed exclusively in the two ASK sensory neurons (Figure 3A). The ASK neurons, which are implicated in chemotaxis to the amino acid lysine and in sensory regulation of egg laying, are easily recognized by their positions in a trio of cell bodies at the dorsal midline (Figure 4A).

Three other fusion genes were also localized strictly to sensory neurons. *srd-1::GFP* was expressed in the sensory neuron ASI, which detects water-soluble attractants and the dauer pheromone that regulates nematode development (Figures 3B and 4C). *sre-1::GFP* was expressed in the ADL neuron, which is required for the response to some repellents (Figures 3C and 4B; Table 2; B. E. Kimmel, C. I. B., and J. H. Thomas, unpublished data). In addition, a lower level of *sre-1::GFP* expression was detected in the sensory neuron ASJ, which is implicated in pheromone detection. *srg-13::GFP* was expressed in the PHA neurons in the tail (Figure 5D). The function of these neurons is unknown, but their morphology is characteristic of chemosensory neurons.

In addition to the seven genes that were expressed exclusively in chemosensory neurons, three additional fusion genes were expressed predominantly in chemosensory neurons. *srb-6::GFP* was expressed in five types of sensory neurons, three in the head and two in the tail (see Table 1; Figures 5A and 5B). In addition to this neuronal expression, a low level of expression of the reporter gene was observed in the egg laying structures in the mid-body region. *sra-6::GFP* also showed both sensory and nonsensory expression. The ASH and ASI sensory neurons expressed the fusion gene (Figures 5C and 5E), as did the PVQ interneurons (Figure 5E). *sro-1::GFP* was expressed mainly in the ADL sensory neurons (see Figure 3C), but lower expression was observed in the SIA neurons, which have unknown functions.

Three gene fusions were expressed predominantly outside the chemosensory system (see Table 1; data not shown). The linked genes *sra-10* and *sra-11* were expressed in some sensory neurons, interneurons, and pharyngeal neurons and muscle. *srg-12*, the most divergent *srg* gene, was expressed in the excretory cell and the gut.

Expression of the gene fusions was examined in animals of all developmental stages. In all cases, *GFP* expression was observed in animals from the first larval stage through the adult, though some variability was apparent (for example, *srg-8* was consistently expressed more strongly in young larvae than in adults). A total of 22 predicted genes were tested by this approach (see Experimental Proce-

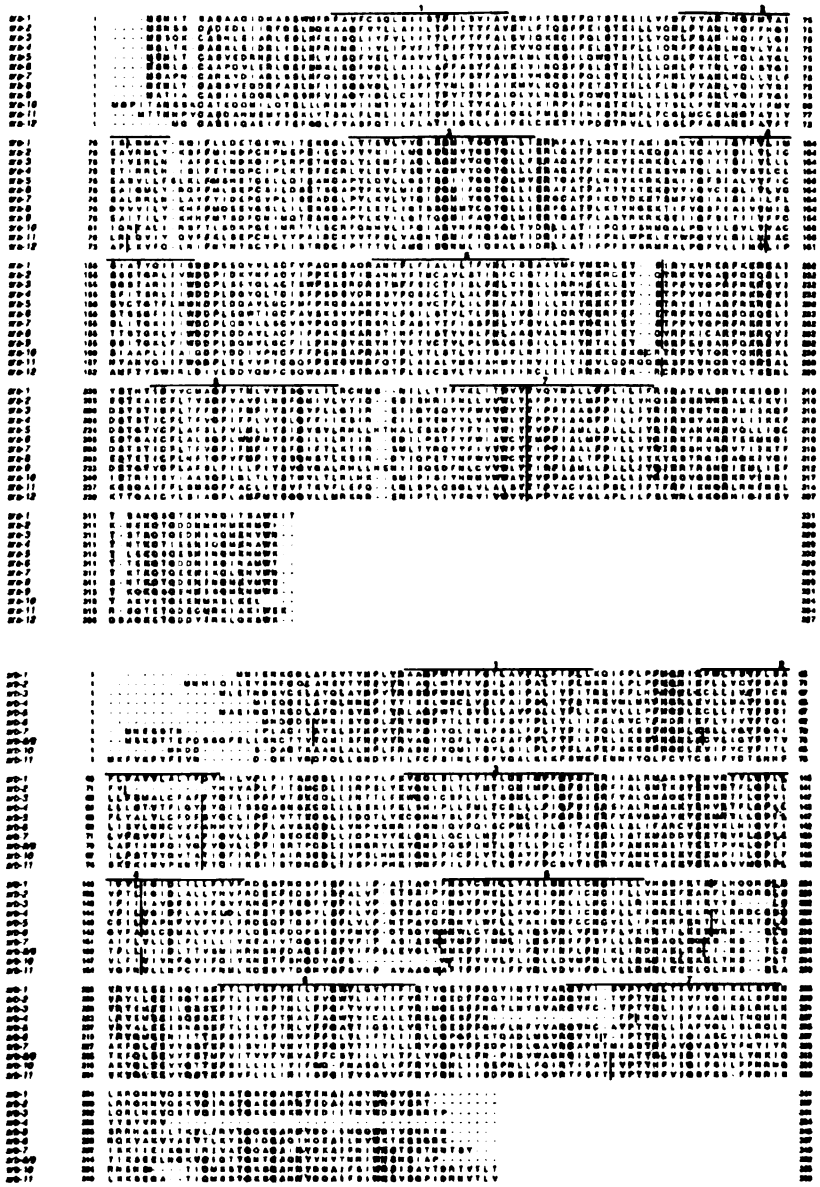


Figure 2. Sequence Alignments of Predicted Seven Transmembrane Receptors

Unless otherwise noted, all genes were as predicted by the C. elegans sequencing consortium. Residues conserved in ≥50% of the clones are shaded in grey. The approximate locations of predicted transmembrane domains 1 through 7 are noted. Exon/intron boundaries are denoted by slash marks.

The *sra* family: *sra-5* contained a single frameshift, which was introduced at the position marked with a number symbol in its sequence to align it with the other genes (see Experimental Procedures). *sra-11* and *sra-12* were modified from the previously predicted genes (see Experimental Procedures).

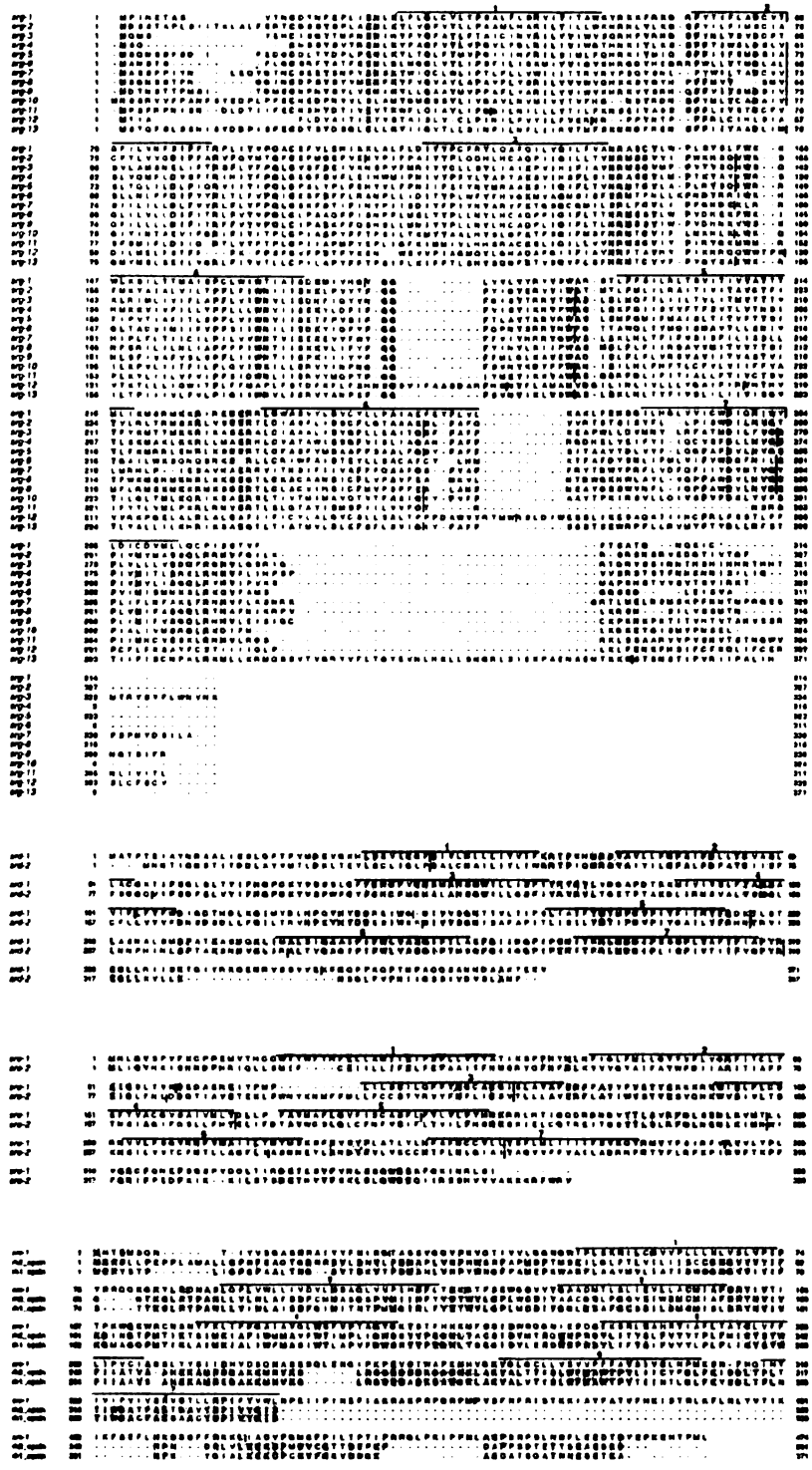
The *srb* family *srb-1*, *srb-2*, *srb-3*, *srb-4*, and *srb-5* were all different from the previously predicted genes. *srb-8* and *srb-9* were also different from the previously predicted genes; these two genes are identical at the amino acid level. *srb-4*, *srb-7*, and *srb-10* could only be aligned with these sequences by introducing frameshifts, denoted by a number symbol.

The *srg* family: *srg-1*, *srg-2*, *srg-3*, and *srg-9* were all modified from previously predicted genes. *srg-4*, *srg-5*, *srg-6*, and *srg-7* were identified on the basis of searches of genomic regions in the C18F10 cosmid.

The *srd* family: *srd-2* was modified from the previously predicted gene.

The *sre* family: *sre-2* was modified from the previously predicted gene.

sro-1: alignment of *sro-1* with the rh2 ocellar opsin from Drosophila pseudoobscura (Carulli and Hartl, 1992) and the rh1 photoreceptor opsin from Calliphora vicina (Huber et al., 1990). The lysine that forms a Schiff base with retinal is denoted with an asterisk.



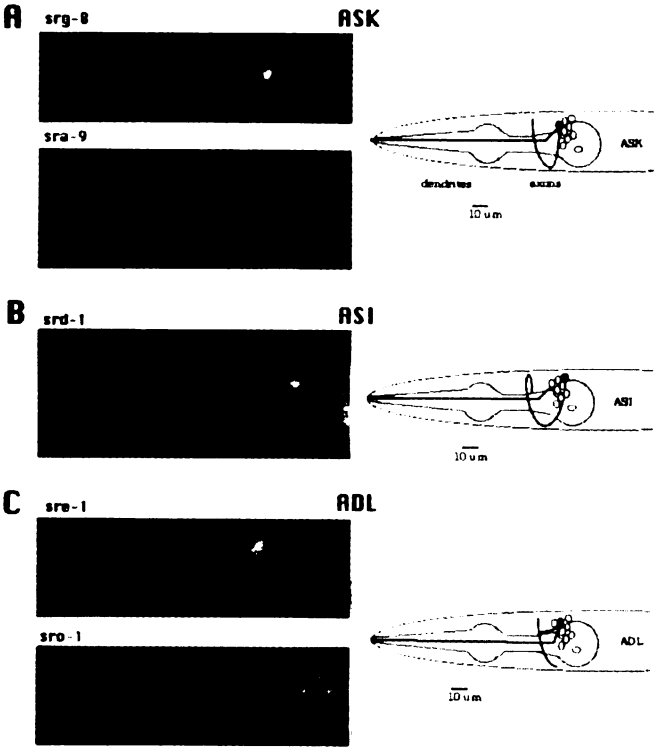


Figure 3. Expression of Reporter Gene Constructs in the ASK, ADL, and ASI Neurons

Fusions of the upstream regions of various genes were made to *GFP* and visualized in transgenic animals.

(A) Expression of *srg-8::GFP* and *sra-9::GFP* in ASK. Note the staining of axons and dendrites of the ASK neurons in the transgenic animals. Staining with *sra-7::GFP* and *srg-2::GFP* constructs was similar, but weaker.

(B) Expression of *srd-1::GFP* in the ASI neurons.

(C) Expression of *sre-1::GFP* and *sro-1::GFP* in the ADL neurons. Faint ASJ staining is also visible at lower right in *sre-1::GFP*. At right, morphology of the ASK, ASI, and ADL sensory neurons. Note the different axon morphologies, which were used to confirm the cell identifications made on the basis of position. The positions of the pharynx and other chemosensory neurons of the head are included for reference. Anterior is at left and dorsal up in all cases.

Table 1. Summary of *GFP* Expression Data

Gene	Cell	Function
Expression in chemosensory neurons		
sra-7, sra-9, src-2, src-8	ASK	Lysine chemotaxis; egg laying
srb-6, sre-1, sro-1	ADL	Octanol avoidance; water-soluble avoidance
srd-1, sra-6 (faint)	ASI	Dauer pheromone; Na^+, Cl^-, cAMP, biotin, lysine chemotaxis
srb-6 (faint), *srd-1* (males only)	ADF	Dauer pheromone; Na^+, Cl^-, cAMP, biotin chemotaxis
sra-6, srb-6	ASH	Osmotic avoidance, nose touch avoidance, volatile avoidance
sre-1 (faint)	ASJ	Dauer pheromone (recovery)
srg-13, srb-6	PHA	Unknown, chemosensory
srb-6	PHB	Unknown, chemosensory
sra-1 and *sra-6* (males only)	SPD/SPV	Sex pheromones/mating
srd-1 (males only)	R8/R9?	Sex pheromones/mating
Expression in other cells		
sra-6	PVQ	Interneuron (chemosensory)
sra-11	AIY	Interneuron (chemosensory)
	AVB	Interneuron, locomotion
		One pharyngeal neuron
sra-10	URX	Sensory neuron
	ALA	Interneuron
		Additional interneurons, pharyngeal neurons, and muscle
srb-6		Vulval region
srg-12		Gut, excretory cell
sro-1	SIA	Neuron, unknown function

The cellular pattern of expression of each gene is given, along with the predicted function of those cells.
References are as follows: ASK, Bargmann and Horvitz, 1991a; E. Sawin and H. R. Horvitz, personal communication. ADL, Table 2; J. H. Thomas, personal communication. ASI and ADF, Bargmann and Horvitz, 1991a, 1991b. ASH, Bargmann et al., 1990; Kaplan and Horvitz, 1993; Table 2. ASJ, Bargmann and Horvitz, 1991b. SPD, SPV, and ray neurons, Liu and Sternberg, 1995.

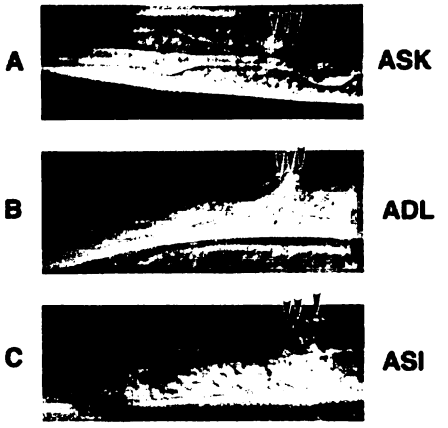


Figure 4. Alignment of Fluorescence and Nomarski Images

(A), *srg-8::GFP* staining ASK, (B), *sre-1::GFP* staining ADL, (C), *srd-1::GFP* staining ASI. In all cases, the positions of the three dorsal neurons ASK, ADL, and ASI are noted on the Nomarski image with arrowheads (compare relative positions of these three neurons in Figure 3). Slides of fluorescence and Nomarski images of the same animal were aligned with Adobe Photoshop.

Table 2. ADL and ASH Function in Avoidance of Volatile Repellents

Animals	Time to Reversal (seconds) Median	Mean	SEM	Number of Assays (number of animals)
Octanol avoidance				
Intact animals	4	6.07	0.56	60 (12)
ASH killed	19.5	14.03	0.88	66 (10)
ADL killed	8	10.47	0.93	47 (11)
AWB killed	5	6.75	1.0	20 (4)
Benzaldehyde avoidance				
Intact animals	4	4.97	0.63	33 (8)
ASH killed	20	15.45	1.23	22 (5)
ADL killed	3	4.96	0.90	28 (7)
AWB killed	5	6.3	1.13	16 (4)

Median and mean time to reversal for intact, ASH-killed, ADL-killed, and AWB-killed animals in the presence of the repellents 1-octanol and benzaldehyde are given. If animals did not reverse within 20 s, their time to reversal was scored as 20 s. Avoidance of octanol was significantly impaired in both ADL and ASH-killed animals (Mann–Whitney rank sum test, $p < 0.001$), but not in AWB-killed animals, while avoidance of benzaldehyde was impaired only in ASH-killed animals ($p < 0.001$).

dures), but reporter gene expression was only observed in 14. This success rate is typical for promoter fusions with C. elegans genes (Lynch et al., 1995). The absence of expression in some cases might occur because the fusions lacked control sequences within the body of the gene, because of errors in predicting gene structure from genomic sequence, or because these genes are actually not expressed under our culture conditions (e.g., they might be expressed in alternative larval stages, or they might be pseudogenes).

Some Receptor Genes Are Expressed in Sex-Specific Patterns

During mating, the C. elegans male exhibits a stereotyped series of behaviors whose progress is regulated by sensory feedback (Hodgkin, 1983; Liu and Sternberg, 1995). The mating structures in the male tail contain 75 male-specific neurons, out of a total of 79 extra neurons in the male adult (Sulston et al., 1980). Cell ablation experiments have revealed functions for many male-specific neurons in different steps of male mating (Liu and Sternberg, 1995). Over 20 of the male-specific neurons have exposed sensory endings and therefore are candidate chemoreceptor neurons that might detect pheromones during mating (Sulston et al., 1980). To investigate whether some of the *sra–sro* genes might function as mating pheromone receptors, each gene fusion was examined in adult male animals.

Most of the gene fusions had identical patterns of expression in males and hermaphrodites, but three genes showed interesting patterns of male-specific expression. The most striking difference was observed with the fusion gene *sra-1::GFP*, for which no staining was observed in hermaphrodites. In males, sensory neurons associated with the spicules stained brightly with this fusion gene (Figures 6B and 6C). The spicules are spikelike mating structures that probe the ventral surface of the hermaphrodite during mating. They contain the putative chemosensory neurons SPD and SPV, which coordinate sperm release into the vulva and have been proposed to sense vulval pheromones (Liu and Sternberg, 1995). The neuroanatomy of the male tail is not as well described as the neuroanatomy of the hermaphrodite, but the cells that stained with *sra-1::GFP* were probably the spicule neurons SPD and SPV. One of these neurons was unambiguously a spicule-associated sensory neuron, since its dendrite invaded the spicule shaft (Figure 6B).

The gene *sra-6::GFP* was also expressed in one neuron pair associated with the spicules (Figure 6D). In addition, *sra-6::GFP* males showed staining in the PVQ interneurons and the ASH and ASI head chemosensory neurons, as did hermaphrodites that contained the *sra-6::GFP* transgene.

srd-1::GFP also had a different staining pattern in males and hermaphrodites, but it was found in a sex-specific pattern in nonsex-specific neurons. Both males and hermaphrodites expressed this gene fusion in the ASI sensory neurons; in addition, in males the fusion was expressed in the ADF sensory neurons (Figure 6E). While the ADF neurons are present in both sexes, the promoter fusion reveals a potential sex-specific regulation of gene expression in these neurons. The ADF and ASI neurons detect pheromones in hermaphrodites (Bargmann and Horvitz, 1991b); these cells might participate in sex-specific pheromone detection in males. *srd-1::GFP* was also expressed in some male-specific neurons in the tail (Figure 6F). Their morphology and position did not permit unambiguous identification of the neurons, but they might be chemosensory neurons associated with the sensory rays.

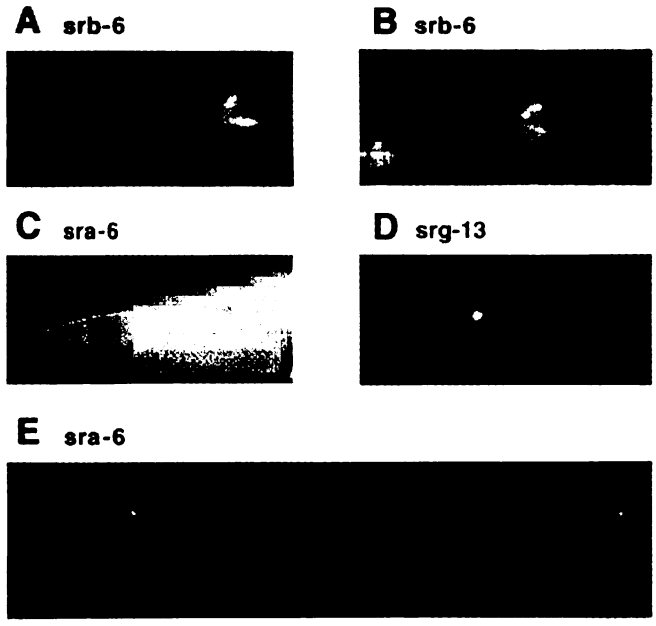


Figure 5. Expression of Additional Reporter Gene Constructs in Sensory and Nonsensory Cell Types

(A and B) Expression of *srb-6* in the ASH and ADL (A) and PHA and PHB (B) sensory neurons. ADF expression was weaker and is not obvious in this plane of focus. ASH and ADF morphologies are similar to that of ASK (Figure 3); PHA and PHB are bipolar sensory neurons in the tail with posterior dendrites and anterior axons. Gut autofluorescence is visible at the anterior edge of this photograph.

(C) Expression of *sra-6* in the ASH sensory neurons. Faint expression in the ASI neuron is visible just dorsal to the ASH neuron.

(D) Expression of *srg-13::GFP* in the PHA sensory neurons of the tail.

(E) Expression of *sra-6* in the ASH sensory neurons (anterior) and in the PVQ interneurons (posterior). Each PVQ neuron sends a single axon to the head. Anterior is at left and dorsal up in all cases.

Discussion

Novel Receptor-like Proteins from C. elegans

The *sra*, *srb*, *srg*, *srd*, *sre*, and *sro* genes have many properties reminiscent of the candidate olfactory receptor genes of vertebrates. First, they encode seven transmembrane receptors that could potentially be coupled to G proteins. The preponderance of evidence from both invertebrate and vertebrate systems supports the notion that such receptors are utilized in chemosensation (Breer et al., 1990; Buck and Axel, 1991; Boekhoff et al., 1994). Recent data implicate G protein–coupled receptors in C. elegans chemosensation as well. Animals mutant for the G proteins *gpa-2* and *gpa-3* are defective in pheromone detection (R. Zwaal and R. Plasterk, personal communication), and animals triply mutant for the G proteins *gpa-1*, *gpa-2*, and *gpa-3* are defective in chemotaxis to water-soluble attractants (E. R. T. and C. I. B., unpublished data). *gpa-1*, *gpa-2*, and *gpa-3* are expressed in sensory neurons, including those that express the *sra–sro* genes (J. Mendel and P. Sternberg, personal communication).

Second, at least eleven of these genes appear to be expressed in small numbers of chemosensory neurons. Eight genes were expressed only in sensory neurons, and six were expressed only in a single type of sensory neuron. Our results are based on expression of reporter gene constructs and therefore may not fully reflect the endogenous expression patterns of the receptor genes. Nonetheless, the highly reproducible patterns of sensory-specific expression are likely to reflect at least some aspects of the regulation of these genes in their natural context.

Third, a substantial number of these genes are present in the genome. Hermaphrodites have 14 classes of chemosensory neurons, and males probably have more; at a minimum, one receptor gene per type of chemosensory neuron would be expected to exist. Only a subset of these genes has been examined, but expression is already suggested for eight types of hermaphrodite chemosensory neurons and three types of male-specific chemosensory neurons.

Most of these genes are found in small clusters, like the vertebrate olfactory receptors, which are found in clusters of 10–100 genes (Ben-Arie et al., 1994). The largest *sra* and *srg* clusters are particularly striking, with nine genes each included within a region of about 30 kb. Unlike the immunoglobulin or T cell receptor genes, the vertebrate olfactory gene clusters do not seem to be rearranged or precisely coexpressed, so the reason for this clustering is unknown. Perhaps this arrangement helps coordinate receptor expression so that only one or a small number of receptors are expressed per sensory neuron (Chess et al., 1994). Although the C. elegans genes are clustered, all genes within a cluster did not share identical regulatory elements; for example, three different expression patterns were observed with fusion genes to four *sra* genes from one cluster.

Several other functions could be proposed for these receptor-like proteins. Some might be neurotransmitter receptors, but relatively few synaptic connections are made onto chemosensory neurons, so they are not expected to express many neurotransmitter receptors (White et al., 1986). Alternatively, they might be receptor molecules used for axon guidance or for the selection of synaptic targets by the chemosensory neurons. Each chemosensory neuron synapses onto several classes of target neurons, and no two types of neurons share the identical

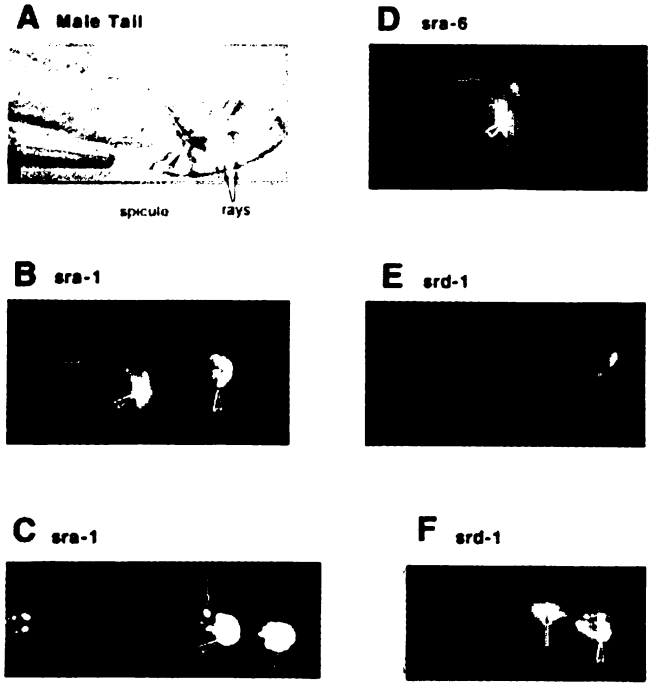


Figure 6. Male-Specific Expression of Fusion Genes

(A) Nomarski image of the male tail, with spicules and rays (compare hermaphrodite tail morphology in Figure 5). The male spicules and posterior male tail structures have an intense yellow autofluorescence (arrowheads here and in remainder of figure). Rays are located around the circumference of the male tail; only two are indicated.

(B) Expression of *sra-1::GFP* in the male tail. The *sra-1::GFP* fluorescence in SPD is green (arrow), while spicule and other autofluorescence is yellow (arrowheads). The dendrite of the spicule neuron SPD can be seen invading the shaft of the spicule.

(C) Axons of the SPD spicule neurons in the ventral nerve cord, as visualized in *sra-1::GFP* animals. An SPD cell body is denoted by an arrow.

(D) Expression of *sra-6::GFP*. One pair of male spicule neurons (arrow) and the PVQ neurons (unmarked; these neurons also stain in hermaphrodites) are visible.

(E and F) Expression of *srd-1::GFP* in males (compare Figure 3C). Two pairs of neurons, the ASI and ADF neurons, are visible in the head (e). Two additional neurons in the tail are visible, one denoted with an arrow in (F); they are bipolar neurons with one apparent sensory dendrite and one axon. These cells may be the R8 or R9 ray neurons.

downstream targets (White et al., 1986). The mechanism by which these connections are made is unknown, but it might involve specific receptors like those described here. Since there are multiple gene families, these models are not mutually exclusive; it is possible that some of the genes described here encode sensory receptors, and some of the genes have developmental or synaptic functions.

The *sra-sro* Receptors Might Sense Attractants, Repellents, or Pheromones

Since each of the sensory neurons appears to sense multiple chemicals, it is not obvious which chemical might be detected by a particular receptor gene. However, various fusion genes were expressed in chemosensory neurons that sense attractants, repellents, and pheromones.

A role in pheromone detection is suggested for the three genes for which sex-specific expression patterns were observed. Males demonstrate multiple responses to hermaphrodites that might involve chemosensation, including long-range chemotaxis to hermaphrodite pheromones, short-range behavioral changes in response to proximal hermaphrodites, and responses to vulval cues during mating (Liu and Sternberg, 1995; J. Hodgkin, E. Jorgensen, and J. H. Thomas, personal communication). Specific neurons mediate each successive step in male mating, suggesting that several sensory cues are detected during this process. Two fusion genes (to *sra-1* and *sra-6*) were expressed in sensory neurons that recognize the vulva during mating, suggesting that a mating pheromone might be detected by these receptors. Interestingly, there are indications that some of the vertebrate olfactory receptor genes are expressed in sperm (Vanderhaeghen et al., 1993). In addition, the *srd-1::GFP* fusion gene was expressed in neurons implicated in both pheromone detection and chemotaxis, suggesting that these receptors might direct male responses to hermaphrodite pheromones.

All of the neurons that express the *sra–sro* genes are thought to detect water-soluble compounds, although the ASH and ADL neurons can also detect some volatile compounds. At this point it is unclear whether the receptors used for volatile chemotaxis in C. elegans will belong to this gene family. The C. elegans genes have no primary sequence similarity to the candidate vertebrate olfactory receptors (Buck and Axel, 1991), nor are they similar to a novel family of genes from the rat vomeronasal organ that might encode vertebrate pheromone receptors (Dulac and Axel, 1995 [this issue of *Cell*]). Water-soluble molecules might be recognized by a different type of receptor than volatile molecules; alternatively, the chemosensory systems of nematodes and mammals may have evolved independent, though related, receptor systems. One gene, *sro-1*, was distantly related to opsin genes, but on the basis of its sequence it is unlikely to be covalently linked to a retinal chromophore (Thomas and Stryer, 1982). One possibility is that this receptor might transiently interact with a hydrophobic retinal-like chemical and act as a chemoreceptor rather than a photoreceptor.

Despite the sensory enrichment of expression observed, some of these genes were expressed predominantly in

nonsensory cell types. These genes might encode receptors for other molecules used in communication between cells. For example, the receptors for attractive amino acids in chemosensory neurons might be similar to receptors for peptide or amino acid neurotransmitters in other cells.

C. elegans Sensory Neurons Are Likely to Express Multiple Receptors

The *GFP* expression patterns predict that several receptor genes, from different gene families, will be expressed in one chemosensory cell type. Four different fusion genes were expressed in the two ASK neurons, three genes in the ADL neurons, and two genes each in the ASI neurons, the ASH neurons, and the PHA neurons. Since only fourteen genes have been examined, it is likely that the total number of genes expressed per neuron will increase.

Even considering possible problems with expression of fusion genes, it is likely that more than one of these receptor genes is expressed per sensory neuron. Over 40 candidate receptor genes were found in the sequenced DNA of C. elegans, which encompasses about 15% of the genome. Even if only half of these genes are sensory receptors, the approximate number of receptors expected in the genome would be about 100, suggesting that many receptors could be found in one cell type.

These results contrast with observations in the vertebrate olfactory epithelium, where single neurons express a very small number of receptor genes, probably one per cell (Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994). Mammalian neurons expressing a single receptor gene appear to project to common targets in the olfactory bulb, where sensory information is integrated (Ressler et al., 1994; Vasser et al., 1994). By contrast, in C. elegans much of this integration may occur within the peripheral sensory neurons. The neurons detect multiple chemically dissimilar compounds; we propose that this occurs because each neuron expresses multiple receptors with different specificities. This prediction is also consistent with the behavioral data that indicate that responses to different attractants sensed by one neuron saturate and adapt independently (Ward, 1973; Colbert and Bargmann, 1995).

In addition, the possibility that multiple cells might express one receptor gene could explain other features of the C. elegans sensory system. In cell ablation studies, it was often found that a particular molecule was sensed, not by one cell type, but by a small group of neurons (Bargmann and Horvitz, 1991a, 1991b). The expression of *sra-6*, *srb-6*, and *sre-1* in two, five, and two sensory cell types, respectively, suggest that this cellular redundancy might derive from less specific expression of some receptor genes.

One open question raised by these results is the extent to which multiple receptors expressed on a single cell type contribute to perceptual discrimination, as opposed to recognition of various compounds. Behavioral discrimination in C. elegans was demonstrated by distinct patterns of cross-saturation and adaptation between different compounds sensed by one neuron. However, cross-saturation and adaptation experiments in humans suggest that different bitter compounds are recognized by different receptors, even though the perception of all bitter compounds is the same to a human subject (McBurney et al., 1972; Lawless, 1987). Thus, the existence of several independently adapting receptors might not generate a true discrimination on the part of the animal. However, discrimination is strongly indicated in cases where one neuron mediates qualitatively different responses to different chemical stimuli. For example, the ADF and ASI sensory neurons affect both development, probably by detecting a pheromone, and chemotaxis, probably by detecting chemoattractants (Bargmann and Horvitz, 1991a, 1991b). It should be possible to ask whether this functional discrimination between chemicals arises at the level of receptors, signal transduction mechanisms, or assemblies of activated neuronal types.

Experimental Procedures

Sequence Analysis

The sequence of the cosmid AH6, which contains homology to a predicated guanylyl cyclase, was obtained from the Sanger Center Network site maintained by the C. elegans sequencing consortium (Sulston et al., 1992). AH6 was first analyzed by use of the BLASTX program, which translates sequences in all six frames before searching protein databases. All BLASTP (protein sequence) and BLASTX (DNA sequence) searches were performed with the NCBI (National Center for Biotechnology Information) BLAST network service to search databases including GenBank, SwissProt, PIR, and the Brookhaven Protein Data Bank (Altschul et al., 1990). BLASTX searches revealed at least ten different regions of AH6 that detected the previously sequenced C. elegans gene F44F4.5 (now *sra-10*). These regions were subsequently found to correspond to ten genes predicted by the C. elegans sequencing consortium, *sra-1*–*sra-9* and AH6.13, which appears to be a gene fragment. Each gene contained multiple potential transmembrane domains predicted by hydropathy analysis using the Kyte–Doolittle algorithm in Geneworks (Kyte and Doolittle, 1982).

The sequences of other C. elegans cosmids and coding regions predicted by the sequencing consortium were obtained through GenBank. BLASTP searches with *sra-1*–*sra-10* revealed more distant similarities to genes in the *srb* family. Continued searches with *sra* and *srb* genes led to the identification of the *srg*, *srd*, *sre*, and *sro* genes. In general, even weak sequence similarities were pursued if several members of a gene family recognized a new gene, and if that new gene encoded multiple predicted transmembrane domains.

Sequence alignments were produced by using the CLUSTAL W program (Thompson et al., 1994). In the initial alignments of the receptor clusters, many genes appeared to be gene fragments rather than full-length coding regions, on the basis of a smaller number of transmembrane domains. Unlike the vertebrate olfactory and vomeronasal receptors, all of the genes contain predicted introns. Analysis of the genomic organization of the receptors revealed that genes within one receptor family often shared conserved splice junctions (see, for example, *sra-2*–*sra-9*, all of which are generated from three similar exons [Figure 2]). Where the C. elegans sequencing project had predicted gene fragments rather than full-length gene products, we searched the genomic DNA for possible splice sites that corresponded precisely to those splice sites that were conserved in other family members. In several cases, changing the predicted genes to incorporate these conserved splice sites generated the full-length gene products shown in Figure 2.

In addition, for all genes with incomplete sequences, genomic DNA was examined for alternative exons or splice patterns. Genomic DNA was used to search the GenBank databases by using the BLASTX program as described above. This analysis revealed that many cosmids contained additional regions of homology to the receptor gene families. This information was used to predict full-length coding regions for those genes in which frameshifts or poor splice junctions were included (see below and Figure 2).

Genes that correspond exactly to genes predicted by the C. elegans sequencing project were as follows (the names for these genes given

by the sequencing project are shown in parentheses): *sra-1* (AH6.4), *sra-2* (AH6.6), *sra-3* (AH6.7), *sra-4* (AH6.8), *sra-6* (AH6.10), *sra-7* (AH6.11), *sra-8* (AH6.12), *sra-9* (AH6.14), *sra-10* (F44F4.5), *srb-6* (R05H5.6), *srb-11* (F23F12.10), *srg-8* (T12A2.9), *srg-10* (T04A8.1), *srg-12* (R13F6.3), *srg-13* (T23F11.5), *srd-1* (F33H1.5), *sre-1* (B0495.1), and *sro-1* (D1022.6).

Genes that differ in their splicing pattern from previously predicted genes include *srb-8* (modified from F37C12.6), *srb-9* (F37C12.8), *srg-1* (C18F10.4), *srg-2* (C18F10.5), *srg-3* (C18F10.6), *srg-9* (T12A2.10), *srd-2* (R05H5.1), and *sre-2* (C41C4.2). Similar analysis of a single predicted gene, C27D6.2, revealed five internal regions that could each encode a full-length receptor protein (renamed *srb-1–srb-5*). Analysis of the predicted gene F44F4.7 on the advice of S. Jones from the Sanger Center (personal communication) revealed that it most likely consisted of a false fusion of two genes, which were renamed *sra-11* and *sra-12*.

A few of the genes could only be aligned well to the other sequences if unfavorable splices or frameshifts were incorporated into their sequences. These genes might be pseudogenes or they might have sequencing errors; their alignments are shown in Figure 2 with incorporated frameshifts denoted by number symbols. They include *sra-5* (AH6.9, one frameshift), *srb-4* (one frameshift), *srb-7* (F37C12.5, one unfavorable splice, one frameshift), *srb-10* (F23F12.5, four frameshifts), and *srg-11* (T04A8.2, one unfavorable splice). BLASTP searches also revealed several predicted gene fragments that could not be aligned with complete coding regions of these genes; these genes have not been included here.

If a putative receptor gene was found near a region of 2 kb or more with no predicted coding regions, those regions were scanned for additional receptor genes with the BLASTX program. From this analysis, we found four additional genes in the C18F10 cluster (*srg-4*, *srg-5*, *srg-6*, and *srg-7*). Genes were predicted on the basis of a combination of amino acid similarity to other family members and conserved splice junctions.

Expression Constructs

Promoter fusions for the genes *sra-7* and *sra-11* were generated by using standard molecular biology methods to subclone these genes from cosmid clones (Sambrook et al., 1989). Four kilobases upstream of *sra-7* were included in a fusion to the reporter gene *GFP* (Chalfie et al., 1994); the fusion site was a HindIII site 31 amino acids into the predicted coding region of *sra-7*. Six kilobases upstream of *sra-11* and approximately two thirds of its coding region were included in an XhoI–XbaI fragment for a translational fusion to *GFP*.

All other promoter fusions were generated by using the polymerase chain reaction (PCR) to join *GFP* to the first 7–20 predicted amino acids of a given gene product. These fusions included 3–4 kb upstream of the predicted translational start site (2 kb of upstream region were used for *srg-8*). The downstream PCR primer was engineered to end in a BamHI site and the upstream primer in an SphI or PstI site, and the fragment was inserted into the *C. elegans GFP* expression vectors TU#61 and TU#62 (Chalfie et al., 1994). Promoters were amplified from purified cosmid DNA or *C. elegans* genomic DNA and checked for predicted restriction sites, and junctions were confirmed by DNA sequencing.

Most primers were amplified with Taq polymerase by PCR in 50 mM KCl, 10 mM Tris (pH 8.0), 2.5 mM $MgCl_2$, 200 μM each dNTP, and 50 ng of total genomic DNA or 10 ng of cosmid DNA. Cycling conditions were 94°C for 30 s, 55°C for 60 s, 72°C for 3–4 minutes for 30 cycles on an MJ Research thermal cycler. For some primer sets, this protocol did not give good amplification; in these cases, the amplifications were conducted with the Expand long template PCR kit (Boehringer) and conditions recommended by the manufacturer.

Microinjection and Analysis of Transgenic Animals

Expression constructs (10–50 ng/μl) were injected into the germline of *lin-15(n765ts)* animals together with the *lin-15* plasmid pJM23 (30–50 ng/μl), which was used as a coinjection marker to facilitate identification of transgenic animals (Mello et al., 1991; Huang et al., 1994). *lin-15* mutants have a multivulval phenotype; after injection, transgenic F1 animals were recognized by their normal vulval morphology at 20°C. These animals were used to establish transmitting lines of transgenic animals that expressed both plasmids from unstable arrays. Typically, 40%–90% of the F2 or F3 animals in these lines would be rescued for the *lin-15* phenotype and therefore presumed to be transgenic. At least four independent lines were established for each expression construct, and *GFP*-expressing cells were identified in at least ten independent animals (in most cases, 20–30 animals were examined for each fusion gene). For the genes *sra-6*, *sra-7*, *sra-9*, *sra-10*, *sra-11*, *srb-6*, *srg-12*, *srg-13*, *srd-1*, *sre-1*, and *sro-1*, all examined lines showed comparable expression patterns. Promoter fusions to *srg-2* and *srg-8* did not give reproducible expression in all lines; we observed staining in only 3 of 6 tested lines for both of these constructs. For both *srg-2* and *srg-8*, all of those lines showed identical expression patterns (i.e., only ASK expressed the fusion gene). Promoter fusions to the genes *sra-2*, *sra-3*, *sra-8*, *srb-1*, *srb-8*, *srb-9*, *srb-10*, and *srb-11* showed no detectable *GFP* expression in at least four independently derived lines. *sra-1* was expressed only in males (see below).

Cell identifications were made by comparing the fluorescence image with Nomarski images of the same animal. Particular neurons were identified by using a combination of their position and their morphology, as previously described (Bargmann and Horvitz, 1991a). In all cases, cells were observed in well-fed animals that had been grown on standard nematode culture plates under sparse conditions (Brenner, 1974).

To identify staining cells in males, wild-type males were crossed to adult transgenic hermaphrodites and the resulting cross-progeny viewed by fluorescence optics. Only a fraction of the male cross-progeny carried the transgenic array, but since *lin-15* is on the X chromosome, all males that did not carry the array were multivulval at high temperature.

Behavioral Assays

Avoidance assays for volatile odorants were conducted by presenting concentrated odorant in front of the animal's nose in a 20 μl microcapillary pipette, or dipping an eyebrow hair into the odorant and presenting it in front of the animal's nose. Animals were scored for the time until their next reversal. Typically, wild-type animals reversed less than once per minute in the absence of odorant, while they reversed in approximately 4 s when presented with octanol or benzaldehyde. Wild-type and laser-operated animals were presented with odorants for four to ten trials per animal, with at least 5 min of rest between trials, and scored for reversal responses. If animals did not reverse within 20 s, the odorant was removed and the animal scored as negative. Results were compared by use of a Mann-Whitney rank sum test in the Statview II program. Cell ablations were conducted with a nitrogen-pumped dye laser and standard methods (Avery and Horvitz, 1987).

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Chapter 2b

Expression analysis of the *odr-10*-like *str* genes.

Rationale

C. elegans detects volatile attractants with the AWA and AWC olfactory neurons (Bargmann et al., 1993). Because chemotaxis to volatile attractants is an easily assayed chemosensory behavior in *C. elegans* and many mutants defective in this behavior have been isolated and characterized, I was interested in identifying receptors expressed in the AWA and AWC neurons. Identification of such receptors could facilitate studies of olfactory signalling, discrimination and adaptation. Eleven candidate chemosensory receptors from the *sr* families (*sra, srb, srg, srd, sre* and *sro*) were identified through expression studies with promoter-GFP fusions (see Chapter 2a). These genes are expressed in neurons that detect pheromone, water-soluble attractants, water-soluble and volatile repellents. However, none were found in the AWA and AWC neurons.

One reasonable hypothesis is that AWA and AWC olfactory receptors might be similar in sequence to the only known olfactory receptor, *odr-10* (Sengupta et al., 1996). I therefore screened through expression of the *str* genes, which are similar in sequence to *odr-10*. Twenty-one *str* genes were analyzed using promoter-GFP fusions to examine expression. Eleven *str* genes were analyzed using an RT-PCR approach that was specifically designed to identify AWA receptors, based on previous work that indicated *odr-10* expression is regulated by the AWA-specific *odr-7* transcription factor. By both promoter-GFP fusions and RT-PCR analysis, *odr-10* transcription is markedly reduced in *odr-7* mutants (Sengupta et al., 1996). If other AWA receptors are regulated similarly by *odr-7*, their RNA levels should be reduced in *odr-7* mutants as

well. Thus, I compared RT-PCR products of candidate receptors from *odr-7* and wild-type RNA.

Results

Promoter-GFP studies analysis of *str* gene expression

Promoter-GFP fusions were used to examine expression of 21 *str* genes, as well as one gene from the related *srd* family (Table 2-1). In general a fusion gene included 4 kb upstream of the gene's predicted start site and the first few amino acids of its coding region fused to GFP. Expression patterns for these genes fall into four general categories:

1) Predominantly chemosensory neuron expression:	8 genes
2) Chemosensory and interneuron expression	5 genes
3) Interneuron or other cell expression	5 genes
4) No expression	4 genes

Over half of the *str* genes appeared to be expressed only in chemosensory neurons or in chemosensory and interneurons. These chemosensory neurons include the simple ciliated ASI, ASK, and ADL neurons and the wing-like ciliated AWB and AWC olfactory neurons. A cluster of four genes from the C42D4 cosmid were expressed in AWB, three of which also showed expression in interneurons. The promoter of the C42D4.5 gene (renamed *str-1*) drove high levels of expression in the AWB neurons and was used for misexpression experiments (see Chapter 3). C50C10.7 (renamed *str-2*) was expressed strongly in the AWC neurons and faintly in the ASI neurons.

Further analysis of *str-2* expression is described in Chapters 4 and 5. The predicted start site of R07B5.1 is 4.7 kb away from the *str-2* start site in the opposite orientation, and showed no expression in larvae or adults. However, it was expressed in about 10 cells in embryos, in a pattern similar to *str-2* embryonic expression.

A subset of *str* genes examined with promoter-GFP fusions were also examined with C terminal-GFP fusions to assess receptor subcellular localization (Table 2-1). In general, these epitope-tagged GFP constructs contained 4 kb of upstream region and the entire coding region with GFP fused to the C terminus. Two genes, *str-1* and *str-2*, yielded expression with these fusions: *str-1* localizes to the AWB cilia and *str-2* localizes to the AWC cilia.

Some *str* genes were expressed only in interneurons or in non-neuronal tissues. Because my objective in these studies was to identify olfactory receptors, these genes were not pursued further.

RT-PCR analysis of *str* genes

RT-PCR analysis of *str* genes was used to more rapidly screen for candidate AWA receptors. Piali Sengupta had demonstrated that *odr-10* expression is lower in *odr-7* mutants by Southern blotting of *odr-10* product amplified by RT-PCR. I developed conditions under which this difference could reproducibly be detected by RT-PCR alone. The amount of *odr-10* RT-PCR product after 45, 50 and 55 cycles of amplification from wild-type RNA was consistently similar to the amount generated by five more amplification

cycles from *odr-7* RNA (Figure 2-1). Assuming approximately two-fold amplification each cycle, *odr-10* expression is roughly 30-fold lower in *odr-7* mutants than wild-type animals.

RT-PCR analysis was performed on 11 *str* genes to compare their expression in *odr-7* mutant and wild-type animals (Table 2-2). No genes other than *odr-10* showed reduced expression in *odr-7* RNA compared to wild-type RNA. Eight of 11 genes showed RT-PCR product in both *odr-7* and wild-type at equal levels, and these products were of the expected size based on primer design and predicted splice sites. R11.2 gave a product of the expected size only from *odr-7* RNA, and none from wild-type RNA.

Methods

str gene names

The choice of which *str* genes to examine was determined in part by those with highest sequence similarity to *odr-10* and in part by which genes were sequenced first by the *C. elegans* sequencing project. Most genes were analyzed before being annotated by the Genome Sequencing Consortium, and so received provisional names for the analysis that were different from names assigned in Genbank. Genes that were not annotated by the sequencing project are named by the cosmid and a question mark.

Promoter-GFP analysis

In general, promoter-GFP fusions were made by amplifying about 4 kb of sequence upstream of the candidate receptor, plus the first few amino acids, and fusing the coding region in frame to GFP. For F10D2.4 and R07B5.1, such fusions did not show expression, and GFP fusions were made with 4 kb upstream sequence and no coding region. These fusions yielded the expression patterns listed in Table 1. For *str-1*, and *str-2*, GFP fusions with or without coding region showed similar expression patterns. The primers used to amplify upstream regions are named with a 5' and 3' suffix following the old name. For example, primers used to amplify the upstream region of T22H6.3 are T22.1-5', which anneals 4 kb upstream of the predicted start, and T22.1-3', which anneals in the first part of the coding region (see below).

T22.1-5'---> -4 kb- <--- T22.1-3'

ATG T22.1 coding

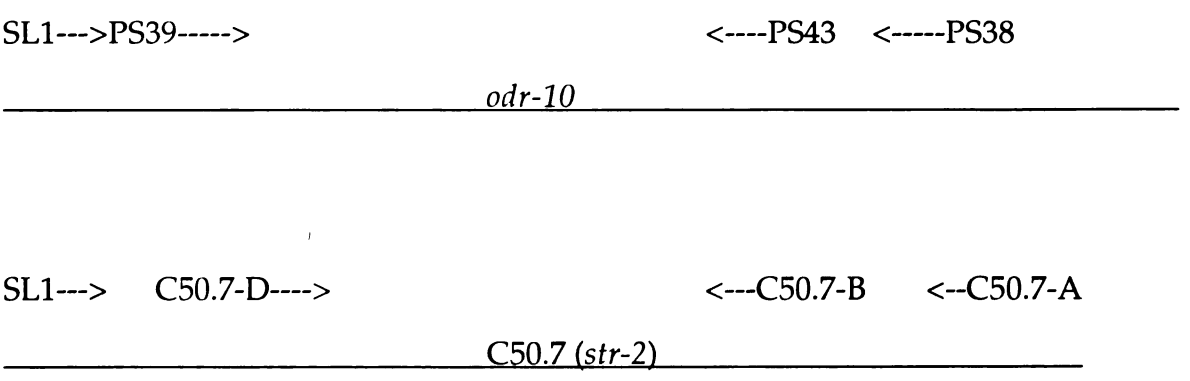
GFP transgenes were co-injected with the *lin-15* rescuing plasmid pJM23 into *lin-15* mutants. Transgenic animals were identified by the non-Muv phenotype and F3 animals were examined for expression. At least three transgenic lines were examined for genes in category #1. Cell identification was performed by comparing the fluorescence image with Nomarski images of the same animal.

RT-PCR analysis

Total RNA was prepared from mixed stage wild-type N2 and *odr-7(ky4)* mutant animals by Trizol extraction (GIBCO). First strand cDNA was reverse transcribed from N2 or *odr-7(ky4)* RNA using primers specific for *str* genes

(PS38 for *odr-10*, *str*-A for *str* genes). This cDNA was used for 30 cycles of PCR amplification with SL1 and the same primers used for reverse transcription. Analysis of each gene was performed in a separate tube, because artefactual PCR products were occasionally generated when reactions were performed in the same tube. One microliter (µl) of each reaction was used as a template in a new 25µl reaction with nested primers (PS39 + PS43 for *odr-10*, str-B + str-D for *str* genes). Following 10, 20 or 30 cycles, half of the reaction was removed for viewing on an agarose gel stained with ethidium bromide.

Diagram of primers used for *odr-10* and *str-2*:



Most genes were only analyzed once, and results should be considered preliminary.

Discussion

Twelve of 21 *str* genes examined by GFP fusions were expressed in chemosensory neurons and could encode chemosensory receptors. The *str* family contains about 300 genes, so if this proportion holds, there could be around 150 functional chemosensory receptors in the *str* family. The fraction

of *str* genes with chemosensory expression is similar to the *sr* genes, as described in Chapter 2a. In addition to the *sr* and *str* genes, two other families of predicted GPCRs with distant similarity to *odr-10*, called the *stl* and *stx* families, encode genes predominantly expressed in chemosensory neurons (M. Zhang, personal communication). These combined gene families include about 1000 predicted genes; based on these GFP experiments, *C. elegans* may have around 500 different functional kinds of chemosensory receptors.

The expression patterns seen for *str* genes are similar to *sr* genes, with several genes from each family expressed in the ADL, ASK and ASI neurons. A few *str* genes were also found in the AWB and AWC wing cells, which have not yet been found to express *sr* genes. So far, the only candidate receptors expressed in the wing cells of adult animals are from the *str* family, namely *odr-10*, *str-2* and the C42D4 genes (includes *str-1*). However, many *sr*, *str*, *stl* and *stx* genes (>900) remain to be examined, so this correlation may not be absolute. Other than wing cell expression, there appears to be little correlation between sequence similarity and expression pattern. For example, the closest relative to the AWA-specific *odr-10* is F10.1, which is expressed in the ASK neurons. Conversely, receptors from the *sre* and *sra* families that have no sequence similarity to each other or to F10.1 are also found in the ASK neurons.

Among the *sr* and *str* genes, there has been a preponderance of candidate receptors identified in the ASI (5 genes) and ASK (6 genes) neurons. Laser ablation and genetic studies indicate that ASI has an important role in detection of dauer pheromone (Bargmann and Horvitz, 1991). Dauer pheromone is a stimulus of unknown structure and may comprise multiple

chemicals, which are detected by multiple receptors. Even if dauer pheromone is only composed of one chemical, it is a crucial environmental signal for *C. elegans*, so expression of multiple receptors in ASI might be needed to ensure accurate detection. Ablation studies have suggested that ASI and ASK only make a minor contribution to chemoattraction (Bargmann and Horvitz, 1991). However, these experiments were optimized to identify cells that make a significant contribution to chemotaxis toward a small number of attractants and therefore provide just a small glimpse of the relative importance of different chemosensory neurons. Perhaps ASI and ASK detect a wide range of chemicals at low sensitivity and make a general contribution to chemoattraction. Alternatively, ASI and ASK may be more important in detecting chemicals that were not tested, or they might direct behavioral responses that were not tested. ASK has been implicated in a bacteria-induced egg-laying response, which likely involves detection of multiple chemicals (Sawin, 1996).

Many predicted GPCRs are found in genomic clusters, perhaps as a result of unequal cross-over events that led to local gene duplications. It was initially speculated that this organization could provide a method for co-regulating receptor expression in certain cell types, but this may not be the case. While some genes in a cluster are expressed in overlapping cells, such as the four C42D4 genes expressed in the AWB neurons, other genes are not. An incomplete correlation with clusterhood was also seen for the *sra* genes found in clusters: genes from the same cluster can be expressed in either overlapping or non-overlapping sets of cells.

Alternative splicing has been proposed as a possible mechanism to generate receptor diversity and RT-PCR analysis did yield multiple bands for a few genes, but further analysis is required to determine if these are authentic transcripts and are translated.

The initial motivation for this work was to identify AWA and AWC receptors, but only one such receptor was found. However, a side benefit has been the identification of cell-specific promoters that are useful experimental tools. For example, *str* and *sr* GFP strains that label individual cells have been used for analysis of axon guidance mutants. In addition, the promoters of these genes have been used for misexpression studies.

Although RT-PCR analysis did not identify AWA receptors, it supports the use of promoter-GFP fusions as a method to assess gene transcription. Three genes that showed expression with GFP fusions were analyzed by RT-PCR, and all three gave a product. In addition, the percentage of genes that failed to show a promoter-GFP expression pattern (18%, n=22) was the same as the percentage that did not yield RT-PCR product (18%, n=11). The genes that yielded an RT-PCR product are good candidates to analyze with promoter-GFP fusions.

str gene expression analysis provides more information about the distribution of chemosensory receptors in *C. elegans*, but we are far from a complete understanding of the *C. elegans* receptor repertoire. Because its entire genome sequence is known, *C. elegans* provides a unique opportunity to analyze the expression and possibly the function of every chemosensory receptor in a given animal. More than 900 predicted GPCRs remain to be

examined and some of these are quite likely to be AWA and AWC receptors. Continued brute-force promoter-GFP analysis of these genes would probably be successful in identifying AWA and AWC receptors, but may not be the most efficient approach. Microchip array technology might be a more rapid method for analysis of these receptors (Schena et al., 1996). This technique allows for massively parallel comparisons of gene expression in different backgrounds. DNA from all the predicted GPCRs could be spotted on a microchip, cDNA probes made from RNA of different strains and hybridized to the chip to assess expression of GPCRs. For example, probes made from *odr-7* could be compared to wild-type probes to identify AWA receptors, as in RT-PCR analysis. Not only could this method yield receptors expressed in specific cells, but it would allow for comparison of receptor expression under different environmental conditions to ask how receptor expression is regulated. Preliminary experiments of this kind were performed by a rotation student, Levente Egry, and myself, in collaboration with John Wang and Stuart Kim. Two post-doctoral fellows in the lab, Rob Rutherford and Paul Wes, have plans for continued development of these experiments

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Table 2-1. Expression of *str* gene GFP fusions

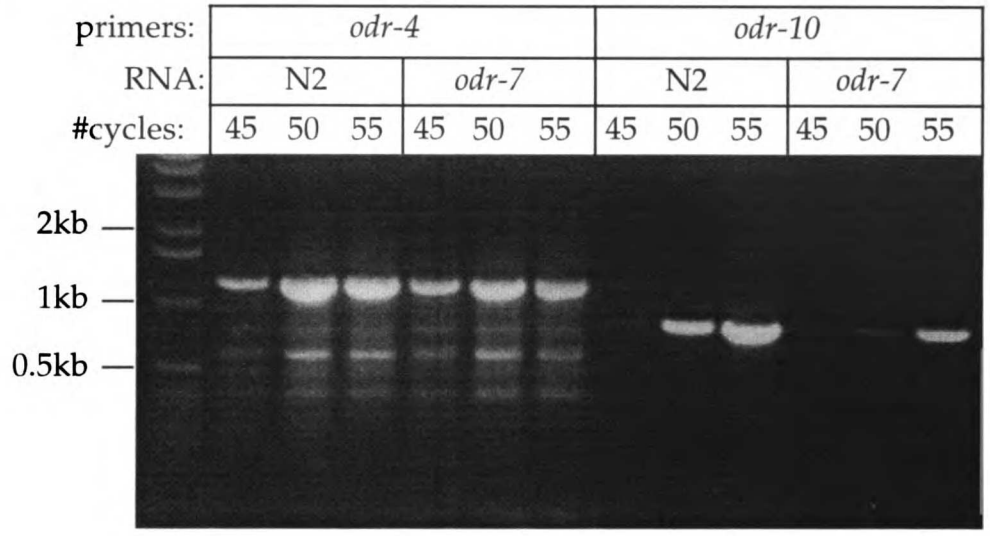
Old name	Annotated name	Expression	Expression category	C term tag?
C14.1	C14H10.?	none	4	no expn
C31.1	C31E10.1	AIA or AVL	3	
C42.1	C42D4.5 *(str-1)*	AWB(faint AIN)	1	AWB cilia
C42.2	C42D4.4	AWB, SIA, AIN	2	
C42.3	C42D4.12	none	4	
C42.4	C42D4.10	AWB, AIN	2	
C42.5	C42D4.9	AWB, SIA, AIN	2	
C50.6	C50C10.6	none	4	no expn
C50.7	C50C10.7 *(str-2)*	AWC(faint ASI)	1	AWC cilia
C50F4.1	C50F4.?	ASI, CEP antx RVG, SAB or RIF	2	
F07.1	F07C3.8-like	rectal gland cells	3	
F10.1	F10D2.4	ASK	1	pharynx
F17.12*	F17A2.12	ADL	1	
F40.1	F40F9.4	pharynx	3	
F57.1	F57A8.3	AVJ/AIN	3	
F58.7	F58G4.7	interneurons?	3	
M7.1	M7.13 *(str-3)*	ASI	1	
R07.1	R07B5.1	emb. neurons (*str*-2-like emb. expn)	2	
T01.1^	T01E8.?	ASK	1	
T18.1	T18H9.4	ASI	1	
T22.1	T22H6.3	ADL	1	no expn
T22.2	T22H6.4	none	4	no expn

Expression categories:
1) predominantly sensory neurons
2) sensory and interneurons
3) interneuron or other cells
4) no expression

**srd* family
^*str/srd* family

See Material and Methods for details on gene names.

A.



B.

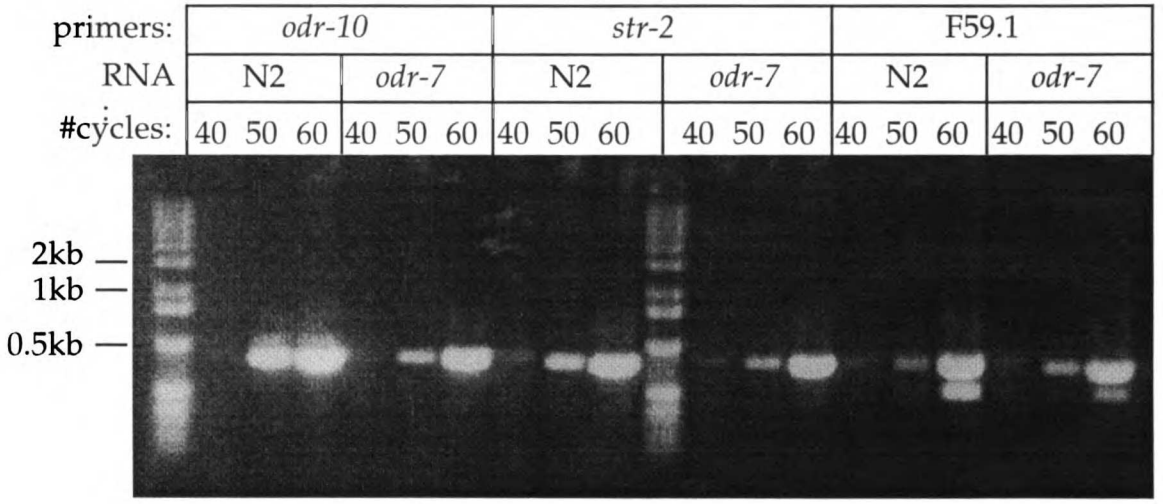


Figure 2-1. RT-PCR analysis of *odr-10* and other *str* genes.

Table 2-2. *str* genes analyzed by RT-PCR

Old name	Genefinder name	RT-PCR product of expected size?
C07.4	C07G3.3	yes, plus 2 smaller bands
C50.7	C50C10.7	yes
F10.1	F10D2.4	yes
F59.1	F59B1.?	yes, plus 1 smaller band
K05.1	K05D4.2	no
R07.1	R07B5.1	yes
R11.1	R11D1.6	yes
R11.2	R11D1.5	yes (only in *odr-7*)
T09.1	T09F5.3	yes
T09.2	T09F5.4	yes
W06.1	W06H8.?	no

Chapter 3

Reprogramming chemotaxis responses: sensory neurons define olfactory preferences in *C. elegans*.

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Reprogramming Chemotaxis Responses: Sensory Neurons Define Olfactory Preferences in C. elegans

Emily R. Troemel, Bruce E. Kimmel,* and Cornelia I. Bargmann
Howard Hughes Medical Institute
Programs in Developmental Biology,
Neuroscience, and Genetics
Department of Anatomy
The University of California
San Francisco, California 94143-0452

Summary

Different olfactory cues elicit distinct behaviors such as attraction, avoidance, feeding, or mating. In the nematode C. elegans, these cues are sensed by a small number of olfactory neurons, each of which expresses several different odorant receptors. The type of behavioral response elicited by an odorant could be specified by the olfactory receptor or by the olfactory neuron in which the receptor is activated. The attractive odorant diacetyl is detected by the receptor protein ODR-10, which is normally expressed in the AWA olfactory neurons. The repulsive odorant 2-nonanone is detected by the AWB olfactory neurons. Transgenic animals that express ODR-10 in AWB rather than AWA avoid diacetyl, while maintaining qualitatively normal responses to other attractive and repulsive odorants. Animals that express ODR-10 simultaneously in AWA and AWB have a defective response to diacetyl, possibly because of conflicting olfactory inputs. Thus, an animal's preference for an odor is defined by the sensory neurons that express a given odorant receptor molecule.

Introduction

Different olfactory cues can elicit distinct behavioral responses in an animal, allowing it to respond appropriately to its environment. To accomplish this task, olfactory neurons detect these cues, discriminate among them, and relay this information to the rest of the nervous system. For many animals, olfactory stimuli from food, predators, and pheromones induce stereotyped behaviors, suggesting these responses are specified by an innate genetic or developmental program. How such a program generates distinct responses for different stimuli remains to be understood fully.

The molecular and neural pathways that define specific behaviors can be identified and manipulated in the nematode C. elegans. C. elegans depends largely on its olfactory or chemosensory sense to acquire information about its environment. It can detect hundreds of water-soluble and volatile molecules, which can evoke attraction, repulsion, feeding, egg-laying, mating, and developmental changes in the animal (Bargmann and Mori, 1997). A given molecule is associated with a characteristic response in animals raised under standard conditions: for example, many short-chain ketone, alcohol, and aldehyde odorants are attractive to essentially all animals, while other ketones and alcohols are repulsive (Bargmann et al., 1993).

C. elegans detects chemicals using a small number of chemosensory neurons whose morphology and synaptic connections are known through reconstruction of the entire nervous system from serial electron micrographs (White et al., 1986). Functions for many of these neurons have been determined by examining the behaviors of animals in which specific neurons are killed with a laser microbeam (Bargmann and Mori, 1997). Eleven pairs of chemosensory neurons are found within the bilaterally symmetric amphid chemosensory organs, including three pairs of neurons with branched, extended sensory cilia called AWA, AWB, and AWC (Ward et al., 1975; Ware et al., 1975). The AWA and AWC neurons mediate responses to attractive volatile odorants (Bargmann et al., 1993). A single olfactory neuron can mediate responses to several compounds; for example, the AWA neurons detect both diacetyl and pyrazine, which can be distinguished by the animal in behavioral assays. These results imply that single olfactory neurons express multiple receptors, each of which binds different odorants.

Odorant receptors in C. elegans and other animals are G protein–coupled seven transmembrane proteins. The *odr-10* gene encodes a receptor in this class that likely detects the volatile attractant diacetyl (Sengupta et al., 1996). *odr-10* mutants fail to chemotax to low concentrations of diacetyl, but have normal responses to all other odorants tested, including pyrazine. An ODR-10::GFP fusion protein localizes to the cilia of the AWA neuron, where diacetyl detection is thought to occur. Experiments with *odr-7*, a mutant defective for AWA responses, provide further evidence for the diacetyl specificity of ODR-10. The *odr-7* gene encodes a transcription factor required for the expression of ODR-10 and possibly other AWA receptors. Expression of *odr-10* under an *odr-7*-independent promoter is sufficient to restore the diacetyl response but not the pyrazine response of *odr-7* mutants. These genetic arguments that ODR-10 is a diacetyl receptor have been confirmed by biochemical experiments demonstrating that expression of ODR-10 in human 293 kidney cells confers diacetyl sensitivity on those cells (Y. Zhang et al., unpublished data).

odr-10 is a member of a large family of candidate chemosensory receptor genes. At least 200 *odr-10*-like seven transmembrane-domain (*str*) genes are present in the 80% of the C. elegans genome that has been sequenced (Sulston et al., 1992). These genes encode putative G protein–coupled receptors, many of which are expressed in chemosensory neurons (E. R. T. and C. I. B., unpublished data). There are at least five other families of seven transmembrane-domain receptors that are also predominantly expressed in chemosensory neurons (*sra*, *srb*, *srd*, *sre*, and *srg* genes, over 100

* Present address: Ernest Orlando Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, California 94720.

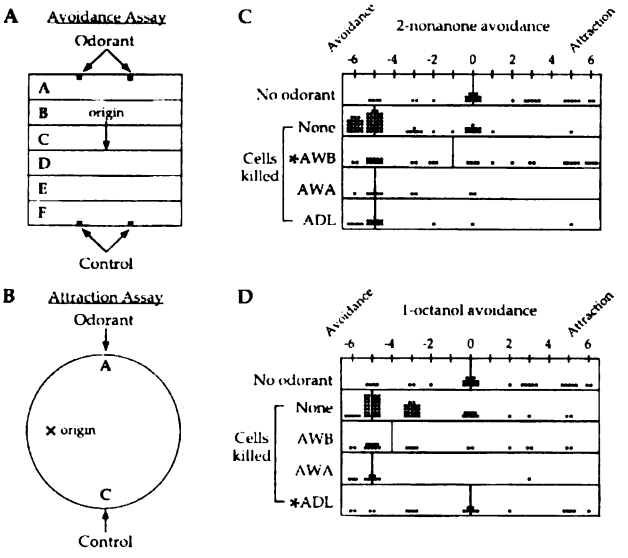

Figure 1. The AWB Olfactory Neurons Mediate Repulsion from 2-Nonanone

(A) Avoidance assays. For single animal assays, animals are placed in the center of the plate and observed to determine which sectors they enter during 1 hr (see Experimental Procedures). For population assays, azide is included to anaesthetize animals that enter sections A and F and animals are scored based on their final position after 1 hr. The scoring method for these square plate assays can detect either attraction (which generates a positive number) or repulsion (which generates a negative number). (B) Conventional chemotaxis assays are conducted on a round plate, which is less effective at scoring repulsion. (C) Avoidance of 2-nonanone (1:10 dilution) by intact and laser-operated animals in single animal assays. (D) Avoidance of 1-octanol (undiluted) by intact and laser-operated animals in single animal assays. For (C) and (D), each dot represents one assay, and the vertical line indicates the median response. Asterisks denote responses different from wild-type at $p < 0.01$. Intact animals were also scored in the absence of repellent.

members in total; Troemel et al., 1995, and our unpublished data). Although only ODR-10 has been shown to detect odorants, C. elegans may have a total of as many as 200–400 chemosensory receptor genes. Importantly, a single neuron can express multiple receptor genes, including receptors from different gene families. The expression and regulation of several different odorant receptor genes may allow each individual neuron to sense and discriminate among several odorants.

How is olfactory information encoded by the nervous system? In principle, all olfactory neurons might be equivalent, while olfactory receptors could be of different types. For example, attractant receptors could be different from repellent receptors, so that the response directed by a neuron depends on the type of receptor that is activated. In this model, olfactory information would be encoded by an odorant receptor and its effects on signal transduction within a neuron, and a neuron expressing receptors of both types could mediate both positive and negative responses. This sort of organization might be used in the lobster, where single olfactory neurons are depolarized by some odorants and hyperpolarized by others, suggesting that the cells express qualitatively distinct receptors (Michel et al., 1991). In an alternative model, each olfactory neuron might be dedicated to a characteristic response, so that any receptor it expresses drives the same behavior. In C. elegans, individual olfactory neurons have been shown to detect either attractants or repellents, but not both, suggesting that there are differences between these cell types (Bargmann and Mori, 1997). However, the interpretation of these cell ablation experiments is limited because they included only a small fraction of odorants; moreover, such loss-of-function experiments can only provide correlations. To determine whether the receptor or the neuron determines olfactory preference, we misexpressed *odr-10* in a cell that detects repellents rather than attractants. Our results indicate that ODR-10 generates a behavior that is specified by the sensory neuron in which it is expressed. Interestingly, the ODR-10 protein can couple to different signaling pathways in different sensory neurons.

Results

The AWB Sensory Neurons Detect the Repellent 2-Nonanone

The AWB neurons have branched, flattened cilia that are enclosed within a sheath cell, like the odorant-sensing AWA and AWC neurons (Ward et al., 1975; Ware et al., 1975). This morphology suggests that AWB neurons might recognize volatile molecules, like AWA and AWC, rather than water-soluble molecules, which are recognized by sensory neurons with more exposed single or double cilia. To analyze the function of the AWB neurons, we developed a new assay to measure long-range avoidance of volatile repellents (Figure 1A). The avoidance assay is similar to volatile attraction assays, but is conducted on a square plate with a repellent along one edge, while attraction assays are conducted on round plates with a point source of attractant (Figure 1B). Wild-type animals exhibit robust avoidance of repellents such as 1-octanol and 2-nonanone in the avoidance assay (Figures 1C, 1D, and 2C).

The contribution of the AWB neurons to avoidance was investigated by behavioral testing of animals in which the AWB neurons had been killed with a laser (Bargmann and Avery, 1995). AWB-ablated animals exhibited diminished avoidance of the volatile repellent 2-nonanone, but they had only minor defects in avoidance of 1-octanol (Figures 1C and 1D). Other forms of volatile avoidance rely on other neurons: killing the ADL neurons impaired long-range avoidance of 1-octanol (Figure 1D), and killing the ASH or ADL neurons impaired

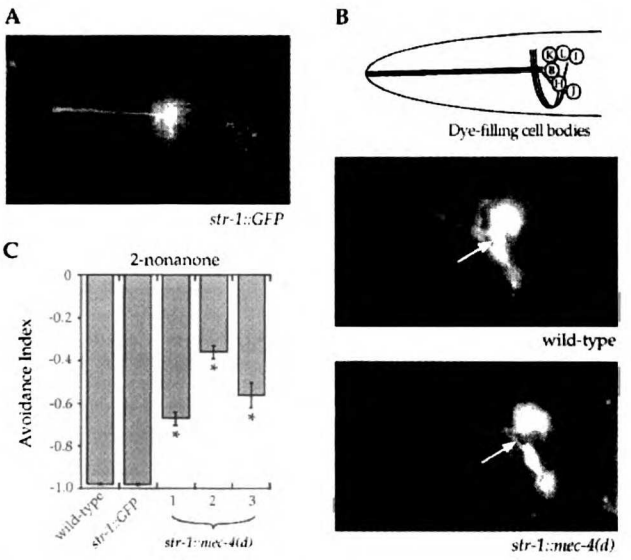

Figure 2. Regions Upstream of the *odr-10*-like Gene *str-1* Drive Expression in AWB

(A) Expression of the *str-1::GFP* reporter construct in AWB visualized by fluorescence microscopy. (B) Dye-filling with DiO of wild-type and *str-1::mec-4(d)* animals. K = ASK, L = ADL, I = ASI, B = AWB, H = ASH, J = ASJ. Arrows indicate the AWB cell body in the wild-type animal, and the expected position of the AWB cell body in an *str-1::mec-4(d)* animal. Anterior is at left and dorsal is up in (A) and (B). (C) Transgenic animals bearing the *str-1::mec-4(d)* transgene are defective in repulsion from 2-nonanone. Three transgenic *str-1::mec-4(d)* strains were compared, one with an integrated transgene (1), and two with extragenic arrays of the transgene (2 and 3). The percentage of animals that lacked DiO filling was 14% (1), 70% (2), 22% (3); in all strains, many of the remaining animals had only a single dye-filling AWB neuron. Asterisks denote responses that differ from wild-type at $p < 0.01$. Error bars equal the standard error of the mean (SEM). Each data point represents the average of at least eight independent assays.

short-range avoidance of 1-octanol (Troemel et al., 1995). Killing the ADL or AWA neurons did not affect 2-nonanone avoidance in this assay.

Since the AWB neurons appear to detect repulsive odorants, they should express odorant receptors and other signal transduction molecules. Indeed, in a survey of expression patterns of the *odr-10*-like *str* genes, we found that an *str-1::GFP* fusion gene was expressed at high levels in the two AWB neurons (Figure 2A). Expression from the *str-1* promoter in the AWB olfactory neurons suggests that *str-1* might encode a receptor for an AWB odorant. In addition, like the AWC olfactory neurons, the AWB neurons express the cyclic nucleotide–gated channel encoded by the *tax-2* and *tax-4* genes, which is predicted by genetic studies to transduce signals sent by G protein–coupled receptors such as the *str* genes (Coburn and Bargmann, 1996; Komatsu et al., 1996).

As noted above, avoidance of volatile repellents is distributed among several classes of neurons, and the contribution of each neuron appears to vary depending on the exact avoidance assay. Instead of performing the long-term tracking of single animals that is used to characterize laser-operated animals, we wished to examine the AWB contribution to avoidance in populations of animals. A population avoidance assay (Figure 1 and Experimental Procedures) was used to compare the behavior of wild-type animals and animals with compromised AWB function. In the latter animals, the AWB-specific *str-1* promoter was used to express the toxic gain-of-function degenerin gene *mec-4(d)* (*str-1::mec-4(d)* animals) (Driscoll and Chalfie, 1991; Maricq et al., 1995). AWB expression of *mec-4(d)* did not kill the AWB neurons efficiently, since their cell bodies could still be detected in the transgenic animals. However, the transgene resulted in a defect in the ability of the AWB sensory cilia to take up the lipophilic dye DiO (Herman and Hedgecock, 1990) (Figure 2B); in different transgenic strains, between 14% and 70% of animals lacked AWB dye-filling ($n > 35$ animals per strain). By contrast, no wild-type animals were defective in AWB dye-filling ($n = 70$). All other DiO-stained neurons retained normal dye-filling in *str-1::mec-4(d)* animals, confirming the cell type–specific effects of the transgene (Figure 2B).

In the population avoidance assay, which requires higher concentrations of 2-nonanone than single-animal assays to elicit robust avoidance, the transgenic *str-1::mec-4(d)* animals displayed diminished avoidance of 2-nonanone (Figure 2C). The *str-1::mec-4(d)* animals avoided 1-octanol normally and approached numerous volatile attractants normally (data not shown), indicating that AWB function is less important for these responses. The strength of the 2-nonanone defect in different strains correlated with the severity of the DiO-filling defect in the AWB neurons, but some nonanone avoidance persisted in all strains. It is unclear whether the residual 2-nonanone avoidance is due to residual AWB function or to the contribution of other sensory neurons to avoidance; because the population assay requires higher 2-nonanone concentrations than the single animal assay, it might involve more neurons. Regardless of whether other neurons sense 2-nonanone, these results provide evidence that the AWB neurons detect this repellent.

ODR-10 Expression in AWB Drives an Avoidance Response

odr-10 is normally expressed in the AWA olfactory neurons, but the expression of the *odr-10*-related gene *str-1* in AWB suggested that AWB might also support *odr-10* signaling. To determine whether ODR-10 could be expressed efficiently in AWB and localized to the AWB cilia, we used the *str-1* promoter to drive expression of

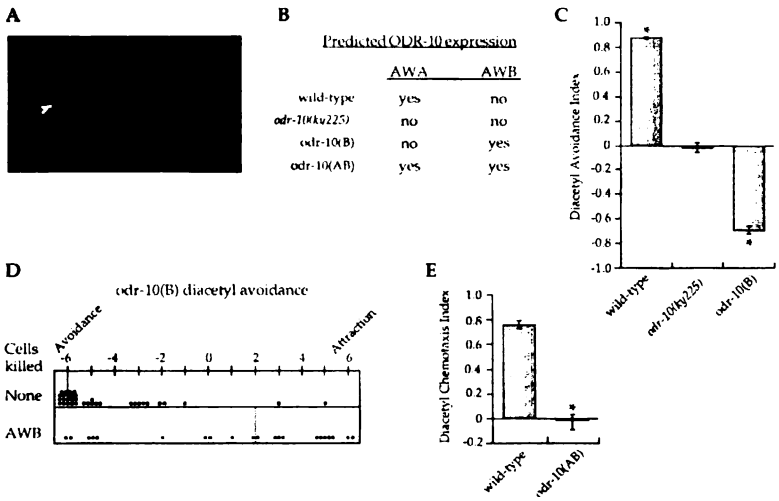

Figure 3. ODR-10 Expression in AWB Directs Repulsion from Diacetyl in an *odr-10* Null Background and Interferes with Attraction in a Wild-Type Background

(A) *str-1::odr-10::GFP* transgene expression in the AWB cilia. Identification of the AWB cilia was based on their characteristic bilobed structure, together with weak staining observed in the AWB cell body. Anterior is at left and dorsal is up. (B) Predicted ODR-10 expression in strains used for analysis. Expression in AWA indicates that animals are wild-type for *odr-10*, while expression in AWB indicates that the strain carries the *str-1::odr-10* transgene. (C) Diacetyl chemotaxis responses of wild-type, *odr-10(ky225)*, and odr-10(B) animals. Error bars equal the SEM. Asterisks denote responses that differ from *odr-10(ky225)* at $p < 0.01$. (D) Diacetyl responses of odr-10(B) intact and AWB ablated animals in single-animal avoidance assays. Data display is the same as in Figures 1C and 1D; differences are significant at $p < 0.01$. (E) Diacetyl chemotaxis responses of wild-type and odr-10(AB) animals. Asterisks denote responses that differ from wild-type at $p < 0.01$. Population assays in (C) were performed on square plates, which can measure either attraction or avoidance. Population assays in (E) were standard round-plate chemotaxis assays. Error bars equal the SEM. Each data point represents the average of at least 13 independent assays.

an *odr-10* cDNA fused to GFP at its carboxyl terminus. Animals with this transgene showed GFP expression predominantly in the AWB cilia (Figure 3A), confirming the expected cellular and subcellular distribution of the ODR-10 fusion protein.

To ask whether *odr-10* would function in AWB, we expressed an unmodified *odr-10* cDNA under control of the *str-1* promoter fragment. This transgene was introduced into *odr-10(ky225)* null mutants, to create a strain called odr-10(B) that expresses ODR-10 in AWB but not AWA (Figure 3B). *odr-10(ky225)* mutants are neither attracted nor repelled by low concentrations of diacetyl, and the *str-1::odr-10* transgene restored a diacetyl response to these nonresponsive animals (Figure 3C). Unlike wild-type animals, however, the odr-10(B) animals were repelled by diacetyl, indicating that misexpression of ODR-10 altered the behavior elicited by the odorant. Similar results were observed in eight independently derived odr-10(B) strains (data not shown; see Experimental Procedures).

Although *str-1::odr-10::GFP* and *str-1::GFP* fusions were expressed only in AWB, it was possible that a low level of *odr-10* expression in some other neuron was responsible for the diacetyl avoidance of the odr-10(B) strain. To ensure that repulsion from diacetyl in odr-10(B) animals was dependent on AWB, the AWB neurons were killed by laser ablation in the odr-10(B) strain, and the operated animals were tested in single-animal avoidance assays (Figure 3D). odr-10(B) animals in which AWB was killed no longer avoided diacetyl, indicating that diacetyl repulsion in this strain is probably due to ODR-10 signaling in AWB. After AWB killing, the response of these animals to diacetyl was not significantly different from the response of *odr-10(ky225)* animals (data not shown, $p > 0.5$).

Simultaneous Expression of *odr-10* in AWA and AWB Blocks Diacetyl Responses

Since wild-type animals expressing *odr-10* in AWA were attracted to diacetyl, and animals expressing *odr-10* only in AWB were repelled by diacetyl, the expression of the *str-1::odr-10* transgene in a wild-type strain might generate conflicting behavioral signals. The *str-1::odr-10* transgene was introduced into wild-type animals to generate the strain odr-10(AB), for ODR-10 in AWA and AWB (Figure 3B). These animals failed to respond to diacetyl (Figure 3E), suggesting that conflicting attractive and repulsive signals from AWA and AWB might be summed to generate a neutral response. Similar results were observed in eight independently derived transgenic strains that expressed ODR-10 in both AWA and AWB (data not shown). The severity of the diacetyl defect varied in magnitude among these strains, perhaps

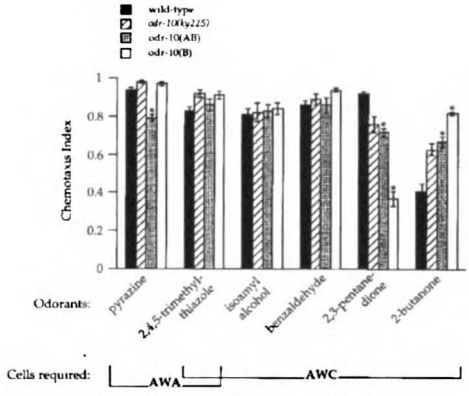

Figure 4. Odorant Responses of odr-10(AB) and odr-10(B) Strains

Responses of wild-type animals are indicated by closed bars, responses of *odr-10(ky225)* animals are indicated by hatched bars, responses of odr-10(AB) animals are indicated by gray bars, and responses of odr-10(B) animals are indicated by open bars. The cells required for responses to odorants are indicated below the odorant. Asterisks denote responses that differ from controls at $p < 0.01$. The control for odr-10(AB) is wild-type, and the control for odr-10(B) is *odr-10(ky225)*. Assays were standard population chemotaxis assays on round plates. Error bars indicate the SEM. Each data point represents the average of at least six independent assays. Dilutions of the odorants were as follows (in ethanol): 10 mg/ml pyrazine, 1:200 benzaldehyde, 1:100 isoamyl alcohol, 1:1000 2-butanone, 1:1000 2,3-pentanedione, 1:1000 2,4,5-trimethylthiazole.

because of differences in ODR-10 expression levels from different transgenes.

As a control for nonspecific promoter effects from *str-1*, the diacetyl responses of *str-1::mec-4(d)* and *str-1::GFP* strains in an *odr-10(+)* background were examined. Neither of these transgenes affected diacetyl responses, indicating that the promoter alone does not cause chemotaxis defects (data not shown). As a control for copy number of *odr-10*, we examined strains containing transgenic arrays of the wild-type *odr-10* gene under its endogenous promoter in both *odr-10(+)* and *odr-10(ky225)* backgrounds. These strains had normal attractive responses to diacetyl, indicating that high-copy expression of ODR-10 does not disrupt diacetyl responses or cause diacetyl repulsion (data not shown, and J. Chou and C. I. B., unpublished data).

To ask whether the effects of ODR-10 misexpression were specific to diacetyl, we examined the responses of the odr-10(B) and odr-10(AB) strains to pyrazine, which is sensed by AWA, and to 2,4,5-trimethylthiazole, which is sensed by AWA and AWC. Responses to these odorants were normal (Figure 4), except for a slight defect in the response of the odr-10(AB) strain to pyrazine, which was not present in other strains bearing the same transgene (data not shown). These results indicate that misexpression of *odr-10* did not interfere with all AWA functions.

odr-10(B) and odr-10(AB) animals responded normally to some volatile attractants sensed by AWC (Figure 4), such as isoamyl alcohol and benzaldehyde. However, the chemotaxis of the odr-10(B) strain to 2,3-pentanedione was diminished. 2,3-pentanedione is structurally similar to diacetyl (2,3-butanedione), but it is detected predominantly by the AWC neurons via an *odr-10*-independent pathway (Sengupta et al., 1996; P. Sengupta and C. I. B., unpublished data). The effect of the *str-1::odr-10* transgene suggests that ODR-10 may detect 2,3-pentanedione to some extent, even though this response is normally dominated by another receptor. The attractant 2-butanone is also sensed by AWC and is structurally similar to diacetyl; 2-butanone responses were somewhat enhanced in odr-10(B) and odr-10(AB) strains.

ODR-10 Requires Different Signaling Components in AWA and AWB

To define the signaling pathways used by ODR-10 in AWB, we examined avoidance responses in mutants defective in potential olfactory transduction molecules. *odr-3* encodes a putative G protein alpha subunit expressed in AWA, AWB, AWC, and other chemosensory neurons, and *odr-3* mutants are defective in chemotaxis to volatile attractants such as diacetyl (K. Roayaie, J. G. Crump, and C. I. B., unpublished data; Bargmann et al., 1993). *tax-2* and *tax-4* encode a putative cyclic nucleotide–gated channel that is expressed in AWB, AWC, and other neurons. TAX-2 and TAX-4 are hypothesized to be the transduction channel activated by olfactory receptors in AWC, but they are not required for AWA-mediated responses, including attraction to diacetyl, and do not appear to be expressed in AWA (Coburn and Bargmann, 1996; Komatsu et al., 1996). A candidate transduction channel for AWA is OSM-9, a novel protein with similarity to G protein–regulated channels that is required for AWA function and is expressed in AWA, AWC, and other sensory neurons, but does not appear to be expressed in AWB (Colbert et al., 1997). Candidate null alleles are available for each of these genes.

The effects of *odr-3*, *tax-2*, and *osm-9* mutations were analyzed in the odr-10(B) strain, whose diacetyl avoidance should be entirely dependent on AWB. odr-10(B); *odr-3* strains were defective in avoidance of diacetyl, suggesting that *odr-3* is required for ODR-10 signaling in AWB as it is in AWA (Figure 5A). However, odr-10(B); *osm-9* strains avoided diacetyl as effectively as odr-10(B) strains, demonstrating that OSM-9 is not required for ODR-10 signaling in AWB. odr-10(B); *tax-2* mutants did not avoid diacetyl, suggesting that ODR-10 signaling in AWB occurs through the TAX-2/TAX-4 channel (Figure 5A). Thus, the genetic requirements for diacetyl avoidance are different from those for AWA-mediated diacetyl chemotaxis, which depends on *odr-3* and *osm-9* but not *tax-2*.

odr-3, *tax-2*, and *osm-9* mutants were also tested using population avoidance assays for their avoidance of 2-nonanone, the normal AWB ligand (Figure 5B). *odr-3* and *tax-2* mutants displayed a partial impairment in 2-nonanone avoidance, but *osm-9* mutants were indistinguishable from wild-type animals (Figure 5B). The 2-nonanone defects could be caused by a lack of *odr-3*

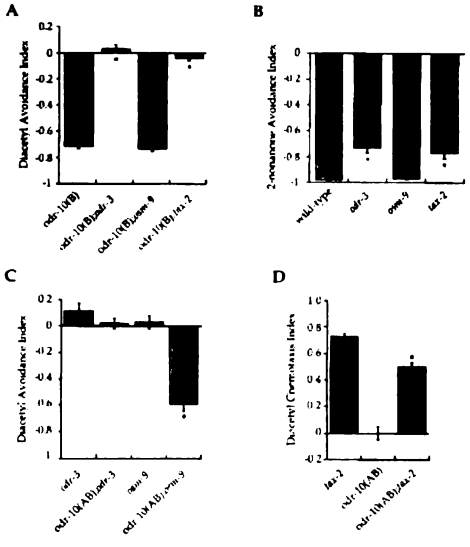

Figure 5. Diacetyl Avoidance Uses Some, but Not All, of the Signaling Components Required for Diacetyl Attraction

(A) Diacetyl responses of odr-10(B) animals in wild-type and mutant genetic backgrounds. (B) 2-nonanone responses of wild-type and mutant animals. (C) Diacetyl chemotaxis responses of odr-10(AB) animals in *odr-3* and *osm-9* mutant genetic backgrounds. (D) Diacetyl chemotaxis of odr-10(AB) animals in wild-type and *tax-2* mutant backgrounds. Asterisks denote responses that differ from controls at $p < 0.01$. For statistical comparisons, controls in (A) were odr-10(B) animals, controls in (B) were wild-type animals, and matched controls for odr-10(AB) transgenic animals in (C) and (D) were *odr-3*, *osm-9*, or *tax-2* mutants without the transgene. Population assays in (A), (B), and (C) were performed on square plates, which can measure either attraction or avoidance. Population assays in (D) were standard round-plate chemotaxis assays. Error bars equal the SEM. Each data point represents the average of at least 10 independent assays.

and *tax-2* function in the AWB neurons. Although these results are qualitatively similar to those observed with diacetyl avoidance, a strong second pathway for 2-nonanone avoidance persisted in each of these mutants. This second pathway could be due to other signaling molecules in AWB, since the *odr-3* and *tax-2* defects appeared to be milder than the defects in the *str-1::mec-4(d)* strain. If this were true, it would suggest that AWB has two signaling pathways, one used by ODR-10 to sense diacetyl that requires ODR-3 and TAX-2, and the other used to sense 2-nonanone that only partially requires ODR-3 and TAX-2. It also remains possible that other cells sense 2-nonanone at the high concentration used in the population assay.

Analysis of odr-10(AB) in *odr-3*, *tax-2* and *osm-9* mutant backgrounds also supported the conclusion that diacetyl avoidance and diacetyl attraction use different genetic pathways (Figures 5C and 5D). odr-10(AB); *odr-3* mutants had no diacetyl response, as expected if ODR-3 is required for both attraction to diacetyl in AWA and repulsion from diacetyl in AWB. odr-10(AB); *osm-9* mutants avoided diacetyl, consistent with the observation that *osm-9* is required for diacetyl attraction, but not diacetyl repulsion. Finally, diacetyl attraction was partially restored in an odr-10(AB); *tax-2* strain, consistent with a requirement for *tax-2* in diacetyl repulsion, but not diacetyl attraction. In summary, ODR-10 signaling in AWB requires the G protein ODR-3, as it does in AWA, but ODR-10 in AWB requires the TAX-2/TAX-4 channel, instead of the OSM-9 channel required in AWA.

Discussion

C. elegans Olfactory Neurons Have Preferred Behavioral Outputs

Individual olfactory neurons in C. elegans can detect several odorants, but there are no cases where one neuron detects both attractive and repulsive stimuli. One explanation for this bias is that receptor proteins intrinsically signal attraction or avoidance, and an individual neuron preferentially expresses receptors of only one class. An alternative explanation is that the receptors are versatile, and an odorant is attractive or repulsive based on the neuron in which its receptor is expressed and activated. Our results show that the diacetyl receptor ODR-10 can mediate distinct responses when expressed in different neurons, supporting the second model.

The AWB olfactory neurons mediate a long-range avoidance response to 2-nonanone; expression of ODR-10 in AWB generates a similar response to diacetyl, despite the fact that ODR-10 generates an attractive response in AWA. These results indicate that the olfactory neuron plays a major role in encoding information about chemotaxis responses. The mechanism by which AWA and AWB neurons direct different responses is unknown, but is likely to reside in the connections that they make with target neurons. AWA and AWB neurons share many of their presumed synaptic targets (White et al., 1986), but perhaps the precise combination of targets is crucial. Alternatively, AWA and AWB could differ in whether they are depolarized or hyperpolarized by odorant, or by the transmitter they release and its effect on target neurons.

These experiments have only been conducted with one receptor (ODR-10) and one olfactory cell type, the AWB neuron. They do not rule out the possibility that neurons, including AWA and AWB, are able to direct both an attractive and an avoidance response. It is possible that receptors encode information about olfactory preference in a cell type–specific manner: while ODR-10 directs attraction in AWA and repulsion in AWB, there may exist another class of receptors that can direct repulsion in AWA and attraction in AWB. Individual chemosensory neurons have been shown to be capable of directing more than one response: the ADF and ASI neurons direct chemotaxis responses to water-soluble attractants as well as developmental responses to the dauer pheromone, which controls progression through alternative larval stages (Bargmann and Horvitz, 1991a, 1991b). These neurons might use two classes of receptors to couple to chemotaxis responses or pheromone

responses. Distinct responses to sensory stimuli may also be generated by the ASH sensory neurons, which mediate avoidance of mechanical and chemical stimuli. *glr-1* codes for a glutamate receptor that is expressed in postsynaptic target cells of ASH, and *glr-1* mutants are defective for avoidance of mechanical, but not chemical stimuli (Kaplan and Horvitz, 1993; Hart et al., 1995; Maricq et al., 1995). The ASH neurons might use mechanoreceptors to couple to the *glr-1*-dependent synaptic pathway, while using chemoreceptors to couple to the *glr-1*-independent pathway.

Odorant Specificity and Signaling

Specificity of ODR-10

Previous studies demonstrated that ODR-10 was necessary for chemotaxis to diacetyl in wild-type animals and sufficient for chemotaxis to diacetyl in a mutant that lacks AWA responses (Sengupta et al., 1996). Expression of ODR-10 in AWB alters responses to diacetyl, but it also alters responses to two other odorants that are not strongly affected by loss of *odr-10* function. Both of these odorants, 2,3-pentanedione and 2-butanone, are structurally similar to diacetyl (2,3-butanedione).

Responses to the attractive odorant 2,3-pentanedione are reduced when ODR-10 is expressed in AWB, although they are less defective than diacetyl responses in the same odr-10(AB) strain. The reduced response might mean that ODR-10 can interact directly with 2,3-pentanedione, as it does with diacetyl, so that conflicting attraction and avoidance signals are generated in response to the odorant. In wild-type animals, 2,3-pentanedione is sensed mainly by the AWC neurons using an *odr-10*-independent receptor (Sengupta et al., 1996; P. Sengupta and C. I. B., unpublished data). ODR-10 may be part of a weaker pathway for sensing 2,3-pentanedione, and this response may be easier to detect in the AWB than the AWA neurons. Responses to the attractive odorant 2-butanone are paradoxically enhanced by *odr-10* expression in AWB, suggesting that there might be an antagonistic interaction between 2-butanone and ODR-10. It is also possible that ODR-10 does not bind 2,3-pentanedione or 2-butanone directly, but rather that complex interactions between sensory neurons can subtly alter the behavioral responses to some odorants.

The AWA, AWB, and AWC olfactory neurons, which are characterized by the complex morphology of their sensory cilia, are all utilized for directed movement in gradients of volatile odorants. However, the signaling molecules within these cells differ, and ODR-10 appears to be able to use different signaling pathways in different cell types. ODR-10 signaling in either AWA or AWB requires ODR-3, a putative G protein alpha subunit (K. Roayaie et al., unpublished data). Normal ODR-10 signaling in AWA requires OSM-9, a protein distantly related to the TRP phototransduction channel of Drosophila (Colbert et al., 1997), but not the cyclic nucleotide–gated channel proteins TAX-2 and TAX-4. By contrast, ODR-10 signaling in AWB requires TAX-2 but not OSM-9.

Interpretation of Olfactory Information by the Nervous System

Like mammals, C. elegans recognizes and discriminates among an enormous diversity of odorants using G protein–coupled receptors. The number of candidate chemosensory receptor genes is comparable between mammals and nematodes: perhaps 1000 genes in rodents and 200–400 genes in C. elegans. However, while the detection potential of the systems may be comparable, the smaller C. elegans nervous system has a more limited behavioral repertoire.

Our results suggest a model for the organization of olfactory information by the C. elegans nervous system. Individual C. elegans neurons express a spectrum of receptors that detect a characteristic set of odorants; each neuron also directs certain behavioral responses, which are presumably defined by the synapses it forms with other neurons. During normal development, we suggest that receptors that detect similar kinds of information are coexpressed in the same sensory neuron. For example, many structurally distinct odorants are produced by bacteria that serve as food for C. elegans (Zechman et al., 1986), and these odorants could have very different receptors, but all of those receptors might be expressed by the AWA and AWC neurons. This organization could allow the nematode to coordinate responses to its environment efficiently; for example, it could regulate responses from AWA and AWC depending on the availability of food (e.g., Colbert and Bargmann, 1997). The specificity of individual receptors can allow a high level of olfactory discrimination, and even specific olfactory adaptation, in which the attractiveness of an odorant can be altered without affecting responses to other odorants sensed by the same cell (Colbert and Bargmann, 1995). At the same time, the small nervous system constrains possible mechanisms of plasticity. Odorant-specific changes can be made by modifying signal transduction components, but modifying synapses will probably alter many odorant responses at once.

Our experiments indicate that if olfactory receptor expression is altered, the odorant detected by the receptor is interpreted in the context of the heterologous sensory neuron. In vertebrate olfactory neurons, altering the expression of olfactory receptors can lead to altered targeting of olfactory axons (Mombaerts et al., 1996). If this were true in C. elegans, altering the expression of receptors might change the behavioral responses generated by olfactory neurons. However, the AWA olfactory receptor ODR-10 can be expressed in AWB without altering the AWB behavioral output. Furthermore, ODR-10::GFP protein has only been observed in the AWA cilia, and not in the axons where target selection occurs (Sengupta et al., 1996). These results suggest that olfactory receptors are not major determinants of sensory axon targeting in C. elegans, though they are complicated by the fact that one neuron expresses many receptors, and ODR-10 was added to a complete endogenous repertoire of AWB receptors rather than replacing them.

Wild-type animals, odr-10(B) animals, and odr-10(AB) animals raised under identical conditions have different

responses to diacetyl, indicating that the intrinsic response to an odorant is defined not by experience but by the cells that express its receptor. A similar bias may underlie olfactory preferences in more complex animals. Many mammals display innate preferences for particular food sources and innate responses to pheromones the first time they are encountered (Halpern, 1987). Such responses can be directed by the vomeronasal olfactory organ, which evokes species-specific behavioral responses, or by the main olfactory organ. A developmental template for these intrinsic responses may be provided by the convergence of specific mammalian olfactory neurons onto invariant targets in the olfactory bulb (Mombaerts et al., 1996). We speculate that the initial projections from the olfactory bulb and the accessory olfactory bulb to brain centers that control feeding, mating, and other strongly determined behaviors may be similar from individual to individual as well, so that critical odorant responses are prewired to a behavioral map. The modification of olfactory preferences by experience may be superimposed on this initial hard-wired map of the olfactory system.

Experimental Procedures

Plasmid Construction

The predicted gene C42D4.5 is 21% identical to *odr-10* at the amino acid level, based on a splicing pattern altered from the Genefinder prediction, and has been named *str-1*. An *str-1::GFP* fusion gene was prepared by using the polymerase chain reaction (PCR) to amplify 4 kb of sequence upstream of the predicted *str-1* start site and the first 4 amino acids of the protein. A PstI site and a BamHI site engineered into the PCR primers were used to insert the amplified product into the GFP vector pPD95.75 (A. Fire et al., personal communication). The *str-1::odr-10::GFP* fusion gene was generated by PCR using the same 4 kb upstream region without any of the *str-1* coding region. A PstI site and a BamHI site engineered into the PCR primers were used to insert the amplified product into a vector containing an *odr-10* cDNA fused to GFP at its carboxyl terminus (Sengupta et al., 1996). The *str-1::odr-10* construct was made by replacing the *GFP* coding region in *str-1::GFP* with a KpnI/ApaI fragment containing the *odr-10* cDNA and *unc-54* 3′UTR. The *str-1::mec-4(d)* construct was made by replacing the GFP coding region in *str-1::GFP* with a KpnI/ApaI fragment containing the *mec-4(d)* allele (Driscoll and Chalfie, 1991; Maricq et al., 1995).

Transgenic Strains

Germline transformation was carried out as described (Mello et al., 1991). The *lin-15* clone pJM23 (50 μg/ml) (Huang et al., 1994) and *str-1::odr-10* (50 μg/ml) were injected into two *odr-10* mutant strains, *odr-10(ky225) lin-15(n765ts)* and *odr-10(ky32) lin-15(n765ts)*, and into *lin-15(n765)* animals. Transgenic animals were identified by rescue of the *lin-15(n765ts)* multivulval phenotype at 20°C. Many independent lines were characterized in each genetic background; eight in *odr-10(ky225) lin-15(n765ts)*, six in *odr-10(ky32) lin-15(n765ts)*, and eight in *lin-15(n765ts)*. Similar results were obtained in both *odr-10* mutant backgrounds. Transgenes were integrated into *odr-10(ky225) lin-15(n765ts)* and *lin-15(n765ts)* by gamma irradiation with 6000R delivered at 330R/min to generate the odr-10(B) and odr-10(AB) strains, respectively. The *str-1::odr-10* transgene integrated into chromosome V in the odr-10(B) strain, and into the X chromosome in the odr-10(AB) strain. An *str-1::GFP* transgene and an *str-1::mec-4(d)* transgene were also injected into *lin-15* animals with pJM23 and integrated. Four independent extragenic *str-1::mec-4(d)* lines were identified that had AWB dye-filling defects; the *str-1::mec-4(d)* transgene in strain 2 was integrated into chromosome V to generate strain 1 (Figure 2C). The *str-1::GFP* transgene integrated into the X chromosome, and is expressed strongly in AWB, with occasional weak staining in the interneurons SIA and AIN. Cell identification was based on the characteristic morphology and position of GFP-positive cell nuclei viewed by simultaneous fluorescence and Nomarski differential interference microscopy.

Chemotaxis Assays

Nematodes were grown at 20°C on E. coli strain HB101 under well-fed and uncrowded conditions (Brenner, 1974). Repulsion assays were performed on square plates containing 10 ml of 1.6% agar, 5 mM potassium phosphate, 1 mM calcium chloride, and 1 mM magnesium sulfate. These plates are divided into six equal sectors labeled A–F. For population assays, one microliter each of odorant and 1 M sodium azide were added in two spots in sector A, and 1 microliter each of control diluent (water or ethanol) and 1 M sodium azide were added in two spots in sector F. Adult animals were washed twice with S-Basal (Brenner, 1974) and once with water, placed in the center of the assay plate (between sectors C and D) and counted after 1 hr.

An avoidance index was calculated using the formula:

$$\text{Avoidance Index} = \frac{(A + B) - (E + F)}{N}$$

where A, B, E, and F are the number of animals in plate sectors A, B, E, and F respectively and N is the total number of animals in all six sectors of the plate.

Laser kills were performed on L1 animals as previously described (Avery and Horvitz, 1987; Bargmann and Avery, 1995). For single animal assays, square assay plates were prepared as for population assays except that just prior to the assay the plates were allowed to air dry for 1 hr with the lid of the plate removed. In addition, no sodium azide was used on these plates. Two spots with 1 μl each of 1-octanol, 2-nonanone, or diacetyl spotted onto sector A were used in single worm avoidance assays. 1-octanol was used undiluted, 2-nonanone was diluted 1:10, and diacetyl was diluted 1:1000 for these assays; diluent alone was spotted onto sector F. Laser-operated and control animals were transferred either in S-Basal or in halocarbon oil to the center of the plate and removed after 1 hr. No more than three repulsion assays were performed on any operated animal in one day. The order of repellent presentation was varied in an effort to minimize the possible effects of odorant interactions. All AWB ablations in the wild-type background were scored blind.

Upon completion of the assay, the path taken by the animal during the course of an assay was recorded by tracing the track on the agar. The nominal scores 3, 2, 1, −1, −2, −3 were assigned to sectors A–F, respectively, and an animal was given a score based on the sum of scores of the sectors in which it had traveled. For example, a score of −6 means the animal migrated into sectors D, E, and F but not A, B, or C while a score of 0 could represent an animal that crawled in all sectors, or in B, C, D, and E, or in C and D. These data sets were compared statistically using a Mann-Whitney rank sum test.

After the repulsion assays, laser kills were confirmed by behavioral tests (AWA) and by direct examination after DiO filling of the amphid neurons (AWB, ADL, ASH, ASI, ASK, ASJ). Only those animals with the appropriate neurons killed were included in the data sets for ablations in the wild-type background.

Population chemotaxis assays for attraction to volatile odorants were performed on round plates as described (Bargmann et al., 1993). Odorant dilutions were 1:1000 (diacetyl, 2,3-pentanedione, 2-butanone, 2,4,5-trimethylthiazole), 1:100 (isoamyl alcohol), 1:200 (benzaldehyde), and 10 mg/ml (pyrazine). These concentrations were chosen to maximize the response to each odorant while ensuring that the response is due to a single type of olfactory neuron, either AWA or AWC (except for 2,4,5-trimethylthiazole, which is sensed by both AWA and AWC). Population assay data for both attraction and repulsion assays were compared using the two-tailed t test.

Strain Construction

odr-10(B) and odr-10(AB) strains containing other mutations were constructed using single-animal PCR to confirm the presence of the *odr-10(ky225)* allele and the *str-1::odr-10* transgene, and the following behavioral assays to confirm the presence of the other

mutations: osmotic repulsion assays for *osm-9* and *odr-3* and benzaldehyde chemotaxis for *tax-2*.

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Chapter 4

Activity regulates stochastic, asymmetric expression of the *C. elegans* candidate odorant receptor, *str*-2.

Summary

C. elegans detects several attractive odorants with a bilaterally symmetric pair of neurons called AWC. The two AWC neurons differ in their ability to express an olfactory receptor gene: the candidate odorant receptor, *str-2*, is expressed in only one of the AWC neurons. Each animal expresses *str-2* in either the left AWC neuron or the right AWC neuron, but never in both. When the precursor to one AWC neuron is killed, the other neuron never expresses *str-2*, suggesting that the AWC neurons compete for alternative fates defined by *str-2* expression. This competition requires normal axon guidance as well as calcium signalling via a voltage-dependent Ca^{++} channel and CaM kinase II, *unc-43*. *unc-43(lf)* mutants express *str-2* in both AWC neurons, and *unc-43(gf)* mutants express *str-2* in neither, indicating that CaM kinase activity can act as a switch in the AWC fate choice. cGMP signalling is subsequently required to maintain *str-2* expression. These results uncover a novel, activity-regulated pathway that functions to differentiate initially equivalent neurons into distinct fates.

Introduction

The developing nervous system creates an enormous variety of neurons through multiple rounds of specialization. Cells destined to be neurons must be distinguished from non-neuronal cells, then be subdivided into classes such as sensory, motor or interneurons, and further differentiated within these classes to express the appropriate repertoire of signalling molecules. These cell fate decisions can be made through a variety of pathways, including both expression of cell intrinsic determinants and cell-cell signalling.

The olfactory systems in worms, flies and mice contain neurons of many distinct types. Olfactory neurons are differentiated by the olfactory receptors they express, which determine the odorants that those neurons can detect. In both vertebrates and invertebrates, olfactory receptors are encoded by large families of seven-transmembrane G protein-coupled receptors (Buck and Axel, 1991; Troemel et al., 1995; Clyne et al., 1999; Vosshall et al., 1999). The expression of olfactory receptors is regulated in interesting ways, but the mechanisms underlying their regulation are poorly understood. In mammals, an individual neuron likely expresses only one receptor, and receptors are subject to allelic exclusion such that only one of the two alleles of a receptor is expressed in a particular neuron (Buck and Axel, 1991; Chess et al., 1994; Malnic et al., 1999). Expression of other signalling molecules such as guanylyl cyclases also differentiates subclasses of mammalian olfactory neurons (Juilfs et al., 1997). Thus, based on patterns of receptor expression, the olfactory receptor epithelium may be a fine mosaic of thousands of different cell types. The expression of mammalian receptors has been suggested to include a stochastic step, but an interesting alternative possibility is that cells within the epithelium communicate with each other to regulate receptor expression.

The nematode *C. elegans* detects hundreds of volatile and water-soluble chemicals using a nervous system of only 302 neurons, 22 of which are known to be chemosensory (Bargmann and Mori, 1997). These 22 neurons are present in two symmetric olfactory organs on the left and the right side of the head. Based on their morphologies, the neurons can be divided into 11 different classes, with one member of each class on the left side and one

member on the right side. Each left/right pair of neurons has a particular function. For example, two pairs of olfactory neurons called AWA and AWC are used to detect volatile attractants, while one pair of neurons called ASE detects water-soluble attractants (Bargmann and Horvitz, 1991; Bargmann et al., 1993). Each of these neurons responds to several different attractants. The receptor for one attractive odorant, diacetyl, is a G protein-coupled receptor encoded by the *odr-10* gene that is expressed in the two AWA neurons.

C. elegans distributes a large number of chemosensory receptors among a few chemosensory neurons. The genome sequence of *C. elegans* encodes about 1000 potential G protein-coupled receptors (GPCRs), over 700 of which are in a large superfamily related to the diacetyl receptor, *odr-10.* Expression studies using promoter fusions to the green fluorescent protein (GFP) suggest that most of the receptors related to *odr-10* as well as several other large gene families are expressed predominantly in chemosensory neurons. *C. elegans* therefore has more receptors than neurons; individual neurons have been shown to express multiple receptor genes (Troemel et al., 1995). Many predicted GPCRs, including ODR-10, are expressed in both neurons of a pair (Sengupta et al., 1996). In contrast, three receptor guanylyl cyclases are expressed asymmetrically in the ASE neurons: two are only expressed in ASER, while one is expressed only in ASEL, suggesting that these two neurons are distinct from one another (Yu et al., 1997). Here we describe a different type of asymmetric expression, in this case affecting *str-2*, which codes for a predicted GPCR in the AWC neurons. We find that *str-2* is expressed in a stochastic manner, with about half of the animals in a population expressing *str-2* in AWCL and the other half in AWCR. This asymmetric expression is regulated by calcium/CaM kinase II and cGMP

signalling, suggesting that molecules which affect neuronal activity can differentiate equivalent cells into distinct cell fates.

Results

str-2 encodes a candidate odorant receptor expressed in the AWC neurons

str-2 is a predicted GPCR with sequence similarity to the diacetyl receptor *odr-10*. To ask if *str-2* might also encode an odorant receptor, the expression pattern of *str-2::GFP* fusion genes was examined. A transgene with the *str-2* promoter fused to GFP (*str-2::GFP*) was first expressed in the late embryo, where GFP expression could be detected in approximately 10 cells (Figure 1A). Expression became more restricted after hatching and by late L1 was only seen strongly in the AWC olfactory neurons and faintly in the ASI chemosensory neurons (Figure 1B). Other odorant receptors, like ODR-10, are localized to the cilia of olfactory neurons, where odorant detection occurs. To examine whether STR-2 also localizes to the cilia, GFP was fused to its C terminus to create a fluorescently tagged STR-2 protein (STR-2::GFP). Transgenic animals containing the STR-2::GFP fusion gene had strong expression in the AWC cilia, with faint expression in the cell body, excluded from the nucleus (Figure 1C). Expression in AWC and localization to AWC cilia suggests that STR-2 could be a receptor for attractive odorants.

str-2 expression is bilaterally asymmetric

C. elegans chemosensory neurons including the olfactory neurons AWA, AWB and AWC, exist in bilaterally symmetric pairs. ODR-10 and STR-1 are

receptors expressed in the AWA and AWB neurons, respectively. Using GFP fusions, their expression is seen in both the left and right neurons of a pair. For example, in a transgenic strain containing a *str-1::GFP* fusion, 100% of the animals in a population have both AWB left (AWBL) and AWB right (AWBR) expression, as seen in a dorsal view of the head in Figure 1D. By contrast, we found that *str-2* receptor expression in the AWC neurons is bilaterally asymmetric. In a strain with *str-2::GFP* integrated on chromosome I (*kyIs140*), 42% of the animals had expression in AWCL, and the other 58% of the animals had expression in AWCR (n= 198, Figure 1E and F). No animals had expression in both AWC neurons. Therefore, *str-2* appears to be stochastically expressed in AWCL or AWCR, but is invariably restricted to just one of the two neurons.

Although it has not been possible to examine endogenous *str-2* RNA or protein, the potential to express these GFP fusions is likely to reflect a reproducible difference between the two AWC neurons. *str-2* expression is asymmetric regardless of the chromosomal location. Four independent *str-2::GFP* strains with the transgene integrated on chromosome I, IV, V, and X were characterized. All strains had *str-2* expression restricted to just one AWC neuron, with about half of the animals in a population showing expression in AWCL and the other half showing expression in AWCR. These *str-2::GFP* integrants were used to monitor *str-2* expression in all experiments; subsequent references to *str-2* expression refer to *str-2::GFP* expression. Although asymmetric regulation only requires the *str-2* promoter, the STR-2::GFP fusion also had asymmetric expression, with transgenic animals showing expression only in the AWCL cilia, or the AWCR cilia, and never in both. Furthermore, the *str-2* promoter could

confer asymmetric expression onto other genes. A *str-2*::ODR-10::GFP fusion was expressed and localized to the AWC cilia in an asymmetric fashion similar to *str-2* (in an extrachromosomal array line, 35% of transgenic animals had expression in AWCL, 56% in AWCR and 9% had no expression, n=179). Thus the *str-2* promoter reveals an intrinsic difference between the two AWC neurons.

The asymmetric expression of *str-2* is reminiscent of other developmental events involving cells that are initially equivalent and then achieve distinct cell fates. In many systems, including *C. elegans*, these cell fate decisions are mediated by a pathway involving the Notch receptor. To ask if Notch signalling regulates the decision for an AWC neuron to express *str-2*, expression of the *str-2::GFP* transgene was examined in several mutants affecting the Notch pathway. *C. elegans* has two Notch homologs, *glp-1* and *lin-12* (Yochem et al., 1988; Austin and Kimble, 1989; Yochem and Greenwald, 1989). *str-2* expression was examined in *lin-12(lf)* and *(gf)*, *glp-1* null, *glp-1(ts)* mutants and in a mutant for the Notch ligand, *lag-2* (see Experimental Procedures for strain details). In all cases, *str-2* expression in the AWC neurons was asymmetric, with a wild-type ratio of AWCL and AWCR cells. These results suggest that Notch signalling is not required for *str-2* asymmetry. However, it was not possible to examine whether *glp-1* and *lin-12* are required redundantly for this decision because *glp-1 lin-12* double mutants die before the stage when *str-2* is first upregulated in AWC.

The default state for *str-2* expression in a single AWC neuron is off

To determine the default state for *str-2* expression in an AWC neuron without its contralateral partner, we killed precursors of AWCL and AWCR early in development. The operated adult animals were then examined for *str-2* expression. The AWCL precursor (ABplpaa) and the AWCR precursor (ABprpaa) were laser killed in embryos at about the 50 cell stage. At this point in development, each precursor cell gives rise to 15 cells including AWC. When either the AWCL (n=17) or the AWCR (n=11) precursor was killed, the remaining AWC neuron failed to express *str-2* (Table 1). Control cell ablations in a *tax-2Δ::GFP* strain were performed to confirm that killing AWC precursors resulted in elimination of the expected cells. *tax-2Δ::GFP* is expressed in 6 chemosensory neurons, including AWC (Coburn and Bargmann, 1996). Based on the *tax-2Δ::GFP* expression pattern, killing AWC precursors led to the death of expected cells, and not others (n=7). These results suggest that in the absence of its contralateral partner, the default state for an AWC neuron is off; an AWC neuron requires a signal from the other AWC neuron or a closely related cell in order to express *str-2*. For simplicity, we will hereon refer to an *str-2*-expressing AWC cell as AWC^{ON}, and a non-*str-2*-expressing AWC cell as AWC^{OFF}. When AWC^{ON} was killed in L1 after strong *str-2* expression appeared, the other AWC did not express *str-2* (n=60, Table 1). Similarly, if AWC^{OFF} was ablated after *str-2* was strongly expressed in AWC^{ON}, expression of *str-2* in AWC^{ON} didn't change (n=3).

Axon guidance mutants are defective in *str-2* expression

The AWC cell bodies are distant from one another, but their axons contact one another on the dorsal side of the circumferential nerve ring (Figure 2A, B, C). Therefore, if the decision to express *str-2* is made through cell-cell

interactions between AWCL and AWCR, the most likely place for this communication to occur is the AWC axons. To ask if axon contact is required for the decision to express *str-2*, expression of *str-2* was examined in four different axon guidance mutants: *unc-76*, *sax-5*, *vab-3*, and *sax-3*. These mutants all display premature axon termination of chemosensory axons in the nerve ring (Hedgecock et al., 1985; Zallen et al., 1998; Zallen et al., submitted). For example, an AWC axon fails to reach the dorsal midline in the *unc-76* animal shown in Figure 2D. In all four mutants, around half of the animals had *str-2* expression in one AWC neuron, and the other half had no *str-2* expression in AWC (Table 2, Figure 2D, E). These results suggest that normal axon guidance, perhaps to allow contact between the two AWC axons, is required for asymmetric expression of *str-2*. Since AWC^{OFF} is the default state, contact with the contralateral AWC neuron may induce *str-2* expression and the AWC^{ON} fate.

The AWC neurons make several synapses onto one another that were observed in electron micrographs of the nerve ring (White et al., 1986). Mutants that affect several aspects of synaptic transmission were examined for *str-2* expression. *snt-1*, *unc-104* and *unc-13* are mutants that are required for chemical neurotransmission: *snt-1* encodes a synaptotagmin homolog implicated in endocytosis, *unc-104* encodes a kinesin-related protein that transports vesicles to synapses and *unc-13* encodes a novel conserved protein that can stimulate synaptic release (Nonet et al., 1993; Hall and Hedgecock, 1991; Maruyama and Brenner, 1991). *unc-7* encodes a membrane protein that is a possible gap junction component (Starich et al., 1993). In all four mutants, asymmetry of *str-2* was unaffected (see Experimental Methods for details).

Therefore, classical synaptic transmission may not be involved in the decision to express *str-2*.

A genetic screen was performed to identify mutants that express *str-2* in both AWC neurons. One of the mutants identified in this screen was in the known axon guidance gene, *unc-44* (Hedgecock et al., 1985). Interestingly, *str-2* could be expressed in 0, 1 or 2 AWC cells in *unc-44* mutants (Table 2). A similar effect was observed in *unc-33* mutants, which share many phenotypes with *unc-44* (Hedgecock et al., 1985) (Table 2). *unc-44* encodes a *C. elegans* ankyrin, which may have a general role in cell polarity and the cytoskeleton (Hedgecock et al., 1985; Otsuka et al., 1995). In addition to axon guidance defects, both *unc-33* and *unc-44* mutants have abnormal microtubule structures in sensory cilia. The defective *str-2* expression in these mutants could be a result of altered axon guidance or of other changes caused by cytoskeletal defects.

Ca^{++} signalling inhibits *str-2* expression

The screen for animals that express *str-2* in both AWC neurons yielded mutations in the known genes *unc-2* and *unc-36*, which encode the alpha1 and alpha2 subunits of a voltage-gated calcium (Ca^{++}) channel (Schafer and Kenyon, 1995). To confirm this result, *str-2* expression was examined in the canonical *unc-2(e55)* and *unc-36(e251)* alleles. In all cases, many animals expressed *str-2* in both AWC neurons (Table 2, Figure 3A). *unc-2(e55)* had the mildest defect, perhaps because it is a partial loss of function mutation. These results indicate that the *unc-2/36* Ca^{++} channel is required to repress *str-2* in

one AWC neuron; in *unc-2/36* mutants neither AWC is able to become AWC^{OFF}.

In wild-type animals, laser ablations indicated that the AWC neurons communicate and that a single AWC neuron always becomes AWC^{OFF} (Table 1). The presence of two AWC^{ON} cells in Ca^{++} channels mutants could result from signalling between AWC neurons or from an intrinsic change in the AWC default state. To distinguish between these models, ablations of AWC precursors were performed in an *unc-36* background (Table 1). In three animals in which the AWCR precursor was killed, the remaining cell expressed *str-2*. This result suggests that *unc-2/36* affect *str-2* expression in an isolated AWC neuron so that the default state is changed to AWC^{ON} in these mutants.

The Ca^{++} and calmodulin-dependent kinase CaMKII converts an increase in Ca^{++} levels into protein phosphorylation of many targets, including transcription factors (Braun and Schulman, 1995). *unc-43* encodes a *C. elegans* CaMKII homolog that is expressed in neurons and affects many processes, including movement and egg-laying (Reiner, D. and Thomas, J., submitted). To ask if *unc-43* could mediate Ca^{++} channel effects in AWC, expression of *str-2* was examined in *unc-43* gain of function (gf) and *unc-43* loss of function (lf) mutant backgrounds. These experiments revealed that *unc-43* acts as a switch for *str-2* expression: *str-2* was expressed in both cells in an *unc-43(lf)* background, while it was often expressed in neither cell in an *unc-43(gf)* background (Table 2, Figure 3B). Thus, the level of *unc-43* CaMKII activity is sufficient to specify either the AWC^{ON} or AWC^{OFF} fate.

unc-43/CaMKII could be either a target or a regulator of the *unc-2/36* Ca^{++} channel. If Ca^{++} entry through the channel increases *unc-43* kinase activity, then the *unc-43(gf)* mutation should be epistatic to the *unc-36(lf)* phenotype. Conversely, if *unc-43* kinase phosphorylates and regulates Ca^{++} channel activity, Ca^{++} channel mutations should be epistatic to *unc-43(gf)*. Double mutant analysis indicates that *unc-43(gf)* is epistatic to *unc-36*, suggesting that Ca^{++} from the *unc-3/36* channel stimulates *unc-43* to inhibit *str-2* expression in one neuron (Table 2).

egl-2(n693) encodes an ERG-type potassium channel that opens at a lower voltage than the wild-type channel, resulting in defects in egg-laying and other behaviors (Weinshenker and Thomas, personal communication). In *egl-2(n693)* mutants, *str-2* was expressed in both AWC neurons (Table 2). Hyperpolarization of the AWC neurons in *egl-2(n693)* mutants might prevent the voltage-dependent Ca^{++} channel from opening and repressing *str-2* expression.

Olfactory signalling components are required for *str-2* expression

Within the olfactory neuron, Ca^{++} channels could potentially respond to depolarization at the sensory cilia, depolarization at neuronal synapses, or second messenger pathways activated independent of depolarization. To explore these possibilities, *str-2* expression was examined in mutants that affect olfactory signal transduction. AWC olfactory signalling appears to involve GPCRs such as STR-2 that signal through the G alpha protein ODR-3 to regulate the activity of the TAX-2/4 cGMP-gated channel, probably via guanylyl cyclases (Birnby, D. and Thomas, J., personal communication;

Coburn and Bargmann, 1996; Komatsu et al., 1996; Roayaie et al., 1998). *str-2* expression was normal in *odr-3* mutants (Table 2), but several other G alpha proteins are expressed and may play a role in AWC olfaction. Lack of a phenotype in *odr-3(lf)* mutants may thus be due to G protein redundancy. *str-2* was not expressed in either of the AWC neurons in the guanylyl cyclase mutants *daf-11* and *odr-1* (Figure 3C, Table 2). Similarly, mutations in either the *tax-2* or *tax-4* subunit of the cyclic nucleotide-gated channel resulted in lower *str-2* expression (Table 2). *tax-2/4* mutants occasionally expressed *str-2* in both AWC neurons, but one neuron was usually much fainter than the other, unlike Ca^{++} signalling mutants, where two AWC neurons expressed *str-2* at equivalent levels. The channel mutants had a low but visible level of *str-2* expression in most animals, in contrast to the guanylyl cyclase mutants where no *str-2* could be detected. This result suggests that the guanylyl cyclases are more important for *str-2* regulation than the *tax-2/4* channel, perhaps because cGMP regulates multiple targets. *tax-4;odr-1* double mutants had no *str-2* expression in the AWC neurons, a result consistent with this hypothesis.

Surprisingly, the signal transduction mutants had the opposite effect on *str-2* expression from the Ca^{++} channel mutants, suggesting that these genes affect distinct aspects of *str-2* expression. Indeed in *odr-1*, *daf-11* and *odr-1;tax-4* mutants, the initial decision to express *str-2* was mostly unaffected: *str-2* expression was seen early in one AWC neuron in the L1-L2 stage and that expression disappeared later (Table 2, Figure 3D). Other mutants that lack *str-2* expression as adults, such as *unc-76* and *unc-43gf*, did not have a higher percentage of *str-2* expression in L1-L2 than in adults. These results suggest

that olfactory signalling is required to maintain *str-2* expression, but is not required to create the initial asymmetry.

Because signalling through *odr-3*, *daf-11/odr-1* and *tax-2/4* is likely initiated by GPCRs like STR-2, we asked whether misexpression of STR-2 might affect its own expression. A *str-2* cDNA was fused to the *odr-3* promoter, which should drive expression of *str-2* at high levels in both AWC neurons (Roayaie et al., 1998). *str-2::GFP* expression was examined in transgenic animals that expressed the *odr-3::str-2* transgene. Of nine transgenic lines, five lines expressed *str-2::GFP* in 0, 1 or 2 cells, two lines expressed *str-2::GFP* in 0 or 1 cell, and two lines expressed *str-2::GFP* in 1 cell (n>20 for each line). To ask if this effect was receptor-specific, another receptor was overexpressed in AWC. An *odr-3::odr-10* transgene also affected expression of the *str-2::GFP* reporter gene in AWC. Three of five transgenic lines had expression in 0 or 1 cell and one line had expression in 0, 1 or 2 cells (n>20 for each). Transgenic lines carrying the *odr-3* promoter alone did not affect *str-2::GFP* expression (eight lines examined). Inappropriate receptor expression is therefore able to affect expression from the *str-2* promoter.

A requirement for the STR-2 receptor in regulating expression from its own promoter could be addressed by examining a *str-2* mutant. Although a *str-2* mutant has not been obtained, a requirement for STR-2 signalling in the cilia could be examined in *odr-4* mutants, which fail to localize STR-2 to the AWC cilia (Dwyer et al., 1998). *str-2* expression was normal in *odr-4(n2144)* mutants (data not shown), suggesting that STR-2 signalling in the cilia is not required for *str-2* asymmetric expression.

Double mutant analysis of genes required for *str-2* expression

str-2::GFP expression appears to be regulated by genes that fall into four different categories; 1) axon guidance mutants in which there are 2 AWC^{OFF} cells, 2) cytoskeleton/axon guidance mutants that can have 2 AWC^{OFF} cells or 2 AWC^{ON} cells, 3) Ca^{++} signalling mutants in which there are 2 AWC^{ON} cells, and 4) cGMP signalling mutants that can have 2 AWC^{OFF}. For all mutant classes, some animals exhibited the normal 1 AWC^{ON}/1 AWC^{OFF} pattern. To determine the relationship among these different pathways with regard to their effects on *str-2* expression, epistasis analysis was performed by constructing double mutants with genes from different pathways (Table 3). Ca^{++} and cGMP signalling mutants were epistatic to the axon guidance mutant, *unc-76,* suggesting that these signalling mutants override the initial information provided by axon contact. These results are consistent with the laser killing experiment showing that AWC cell contact is not required for *str-2* expression in *unc-36* mutants. The Ca^{++} signalling mutants were epistatic to the cytoskeletal mutants, *unc-33/44,* which affect axon guidance. The cGMP pathway was epistatic to the Ca^{++} signalling mutants, revealing that the maintenance pathway is needed even when *str-2* expression comes on via abnormal Ca^{++} signalling. Paradoxically, the cytoskeletal mutants were epistatic to cGMP signalling mutants. A possible resolution for this result is that cytoskeletal factors act both in axon guidance and in another process. Perhaps they act in axon guidance upstream of Ca^{++} signalling to promote expression of *str-2* in one cell (leading to the 2 AWC^{OFF} phenotype) and also at a later step to inhibit *str-2* expression in one cell (leading to the 2 AWC^{ON} phenotype).

Discussion

Activity-related pathways regulate *str-2* expression.

The *C. elegans* putative olfactory receptor*str-2* is expressed in a stochastic, asymmetric fashion in the AWC olfactory neurons. Half the animals in a population express *str-2* in the left AWC neuron, while the other half express it in the right AWC neuron. Thus, *str-2* is regulated to restrict expression to a single neuron of this pair. A model for *str-2* regulation is proposed in Figure 4B. Early in development before AWC axon outgrowth, the default state for *str-2* expression in the AWC neurons is off (Step 1). This default state is likely maintained by Ca^{++}, which enters the *unc-2/36* voltage-gated Ca^{++} channel and activates the *unc-43*/CaMKII. Following axon outgrowth, the axons of the two AWC neurons communicate either directly or via another axon in the nerve ring, and *str-2* expression is specified in one of the two cells. The initial signal for asymmetry between the two AWC neurons is unknown, but an essential aspect of their communication involves the *unc-2/36* calcium channel and *unc-43*/CaMKII. At some point one cell takes on the AWC^{OFF} fate (shown as the left cell in Figure 4B) and instructs the other cell to take on the AWC^{ON} fate (Step 2). The AWC^{ON} fate is then maintained by the cGMP signalling pathway (Step 3).

The two AWC neurons are likely to communicate with each other to mediate the decision to express *str-2*. Ablations indicate that both AWC neurons are required for *str-2* expression: when one AWC precursor was killed early in development, *str-2* expression was never observed in the other AWC neuron, although control experiments showed that it was still alive. These

experiments suggest that the unablated AWC always became AWC^{OFF}, and that a descendent of the ablated AWC precursor was required to induce *str-2* expression and the AWC^{ON} fate. AWC itself is the best candidate to be the descendent that provides the inductive signal. The two AWC axons contact one another along the dorsal portion of their trajectory, and in mutants in which AWC axons fail to reach the midline *str-2* is often not expressed. The decision does not appear to require downstream interneuron targets of AWC, based on analysis of *str-2* expression in mutants that affect these cells (data not shown). Although it is possible that a third cell or set of cells may be involved, the simplest explanation is that the two AWC axons interact at the dorsal midline to specify permanent *str-2* expression in one neuron. There are synapses between the two AWC axons, but genes required for classical neurotransmission do not appear to be involved in *str-2* asymmetry.

The decision to express *str-2* appears to be fixed by the end of the L1 stage. Ablation of AWC^{ON} at that time did not cause AWC^{OFF} to express *str-2*, nor did ablation of AWC^{OFF} cause AWC^{ON} to turn off *str-2*. The course of *str-2::GFP* expression during embryonic and larval development bears interesting parallels to the transition from cell communication to irreversible decision. *str-2::GFP* is first expressed in a number of cells during late embryonic development and expression becomes progressively more restricted until only one AWC shows expression in larvae. Perhaps *str-2::GFP* expression reflects a progressive restriction in the competence to achieve the AWC^{ON} fate, with many cells that are initially competent, then just two AWCs, and finally just one AWC.

Cell-cell signalling can occur via inductive signals, where one cell instructs another, or via lateral signalling pathways, where cells of equivalent potential interact with each other to generate two distinct cell fates, as appears to be the case for *str-2* expression. Whereas instructive cell-cell signalling has been shown to be mediated by a number of different pathways, all lateral signalling decisions examined so far appear to involve the Notch signalling pathway. Notch signalling has been implicated in a broad spectrum of cell fate decisions in animal development (Robey, 1997; Greenwald, 1998). In most cases, these cell fate decisions involve cells of equivalent potential that have a default state in the absence of signalling and require Notch activity to specify an alternative fate. While Notch signalling has not been definitively ruled out as playing a role in the *str-2* decision, the fact that *str-2* expression is not altered in many mutants of this pathway makes its involvement seem unlikely. Moreover, our direct mutant screens have not yet revealed any genes in the *C. elegans* Notch pathway.

Ca^{++} signalling appears to be directly involved in the decision to express *str-2*. Analysis of *str-2* expression in CaMKII *unc-43lf* and *unc-43gf* mutants indicates that CaMKII acts as a switch for *str-2* expression, probably as a result of Ca^{++} entering the *unc-2/36* Ca^{++} channel. Ablations of an AWC precursor in *unc-36* Ca^{++} channel mutants indicate that the default state for a single AWC was changed to AWC^{ON} in this mutant. These results suggest that Ca^{++} signalling is required intrinsically for each AWC cell to repress *str-2* and take on the AWC^{OFF} fate. Ca^{++} signalling could be directly involved in the lateral signalling event between these two neurons, with one cell stochastically acquiring an advantage that is then amplified into an irreversible decision. Developing neurons have been shown to display a

large Ca^{++} influx and change their Ca^{++} setpoint in response to target contact (Zoran et al., 1993). Alternatively, these two cells could use another pathway for communication, and only use Ca^{++} to execute the decision. In Notch signalling the lateral signalling decision is thought to be triggered by a stochastic difference in expression of the receptor and ligand used for communication. This initial asymmetry is then amplified by both positive and negative feedback loops to yield two distinct cell types. An analogous ligand/receptor set might function in the *str-2* lateral signalling decision and use Ca^{++} signalling to reinforce the decision.

cGMP signalling may play a maintenance role in *str-2* expression. Direct observation and double mutant analysis suggests that cGMP signalling acts after Ca^{++} signalling to increase *str-2* expression. In contrast to other mutants defective in the AWC^{ON} fate, the initial decision to express *str-2* asymmetrically appears to occur normally in cGMP signalling mutants but expression is not maintained. It is possible that cGMP signalling also has an ancillary role in the decision to express *str-2*, since *tax-2/4(lf)* mutants sometimes have 2 AWC^{ON} cells. Overexpression experiments with STR-2 suggest that the STR-2 receptor itself can play a role in this decision. However, these experiments should be interpreted with caution, since inappropriate STR-2 expression might artefactually affect Ca^{++}, cGMP or some other signalling pathway to disrupt *str-2* asymmetry. Analysis in *odr-4* mutants argues against a requirement for STR-2 signalling in the cilia.

Interestingly, both the *unc-2/36* Ca^{++} channel and *tax-2/4* cGMP channel can be Ca^{++} permeable and yet they appear to have opposite effects on *str-2* expression. These channels might be differentially localized within AWC,

with the cGMP channel enriched in the cilia and the Ca^{++} channel in the axons. Another explanation could be a difference in the time at which these channels are active, either due to their own expression or other regulatory pathways.

The *unc-33/44* cytoskeletal mutants are likely to act at multiple places in the *str-2* decision. *unc-33/44* animals can have either 2 AWC^{OFF} cells, 1 AWC^{ON} cell/1 AWC^{OFF} cell or 2 AWC^{ON} cells. The 2 AWC^{OFF} phenotype suggests that one function of *unc-33/44* is to promote *str-2* expression. Double mutants between *unc-33/44* and Ca^{++} signalling mutants have 2 AWC^{ON}, suggesting that *unc-33/44* act in the axon guidance pathway that can be suppressed by Ca^{++} signalling. The 2 AWC^{ON} phenotype of *unc-33/44* mutants suggests that *unc-33/44* also function to keep *str-2* expression off. *unc-33/44* mutants suppress the maintenance defects in guanylyl cyclase mutants, so *unc-33/44* also act in later steps of *str-2* asymmetry. Because *unc-33/44* affect the cytoskeleton in sensory cilia, these later defects could be due to mislocalization of molecules important for maintenance of *str-2* repression.

A role for *str-2*-like signalling in other systems?

Our studies of *str-2* expression show that alternative olfactory cell fates can be specified by activity-related signalling molecules, such as Ca^{++} channels. It will be interesting to examine the roles of Ca^{++} channels in other developmental systems where fine patterning occurs via neuronal interactions. Cells interact to create numerous cell types in the Drosophila retina, where a similar phenomenon of restricting and coordinating receptor expression has been observed. Drosophila has two photoreceptor cells called

R7 and R8 that detect UV light - R7 can express either the rh3 or rh4 rhodopsin, while R8 can express either the rh5 or rh6 rhodopsin (Papatsenko et al., 1997; Chou et al., 1999). Neighboring R7/R8 pairs coordinately express rh3/rh5 or rh4/rh6 and the choice made by R7 instructs the choice made by the neighboring R8. In the absence of signalling from R7, R8 always expresses rh6 by default. Although the reason for this regulation is unknown, the stochastic and coordinated nature of this phenomenon is reminiscent of *str-2* regulation and could involve similar signalling pathways.

C. elegans has an overall left-right body plan; within this context, *str-2* expression creates an additional stochastic bilateral asymmetry. Other examples of stochastic bilateral asymmetry established through cell-cell interactions and activity can be found in leeches and lobsters. In the leech nerve cord, individual AS neurons lie on alternate left and right sides of successive ganglia (Blair et al., 1990). An individual AS neuron develops from bilaterally paired embryonic homologs that likely interact through their axons to specify one to adopt the AS fate and the other the non-AS fate. Like the *str-2* decision, this patterning thus appears not to be predetermined, but rather specified through interactions among initially equivalent neurons during development. Lobsters provide another example of bilateral asymmetry that is both stochastic and activity-regulated (Govind, 1992). Lobsters develop claws that are bilaterally asymmetric; one side forms as a major (crusher) claw, while the other side becomes a minor (cutter) claw, with the sidedness randomly chosen. Activity has been shown to be important during a critical period of development in which the more active side develops as a crusher. As with *str-2*, this decision therefore seems to

involve comparison of activity between two initially equivalent sides, which then results in bilateral asymmetry.

The differentiation of the two initially equivalent AWC neurons into distinct sub-types may represent a simplified model for the differentiation of vertebrate olfactory neurons. The vertebrate olfactory system needs to specialize millions of otherwise equivalent neurons by the receptors they express. These receptors are distributed over a wide surface area, and there are suggestions that local interactions among neighboring neurons prevent them from expressing the same receptor (Baier et al., 1994). While all receptor expression is not likely to be controlled by local interactions, this mechanism might be coupled with others to refine expression and create the appropriate distribution of receptors.

Asymmetric expression of the *str-2* candidate odorant receptor reveals an intrinsic difference in the transcriptional ability of the two AWC neurons. We hypothesize that other endogenous receptors could be regulated so that they are expressed in only one of two AWC cells, and AWC^{OFF} expresses an alternate (unknown) subset of receptors. What is the significance of this regulation? One possibility is that it creates two independent AWC neurons that each have a distinct olfactory specificity. Alternatively, the asymmetric expression of receptors may allow for comparison of input between the two AWC neurons. Perhaps this organization increases discrimination between odors, or creates a system for signal integration. These questions cannot be addressed in the existing mutants with 2 AWC^{OFF} cells or 2 AWC^{ON} cells, because these mutants all have global sensory defects. Further understanding of the importance of this pathway could be provided by analysis of a *str-2*

mutant, identification of a *str-2* ligand, or isolation of other AWC receptors and their ligands.

Experimental Procedures

Plasmid Construction

str-2 corresponds in part to the predicted gene C50C10.7, but cDNA clones of *str-2* isolates by RT-PCR had a 3' exon distinct from that predicted by the Genome Sequencing Consortium. The authentic STR-2 protein is 26% identical to *odr-10* at the amino acid level. An *str-2::GFP* fusion gene was prepared by using the polymerase chain reaction (PCR) to amplify 3.7 kb of sequence upstream of the predicted start site of *str-2* and the first 4 amino acids of its coding region. A PstI site and a BamHI site engineered into the PCR primers were used to insert the amplified product into the GFP vector pPD95.75 (A. Fire, S. Xu, N. Ahnn, and G. Seydoux, personal communication). The STR-2::GFP (C terminal tag) construct was prepared by using PCR to amplify 3.7 kb of sequence upstream of the predicted start site of *str-2* and the entire coding region except for the last 6 amino acids. A PstI site and a BamHI site engineered into the PCR primers were used to insert the amplified product into the GFP vector pPD95.75. The *str-2*::ODR-10-GFP fusion gene was generated by PCR using 3.7 kb upstream region without any of the *str-2* coding region. A PstI site and a BamHI site engineered into the PCR primers were used to insert the amplified product into a vector containing an *odr-10* cDNA fused to GFP at its carboxy-terminus (Sengupta et al., 1996). The *odr-3::str-2* transgene was generated by placing the *odr-3* promoter, which drives expression in both neurons of the AWC, AWB,

AWA, ASH and ADF pairs, into the SmaI site upstream of the *str-2* cDNA expression vector. The *str-2* cDNA was obtained by RT-PCR in two pieces, which were ligated together to create a full-length cDNA. A *str-2* cDNA expression vector was then created by replacing GFP with the *str-2* cDNA in the pPD95.77 vector (A. Fire, S. Xu, N. Ahnn, and G. Seydoux, personal communication).

Transgenic Strains

Germline transformation was carried out as described (Mello et al., 1991). The *lin-15* clone pJM23 (50 ng/µl)(Huang et al., 1994) and *str-2::GFP*, *str-2*::ODR-10-GFP or STR-2::GFP (50 ng/µl) were injected into *lin-15(n765)* animals. Transgenic animals were identified by rescue of the *lin-15(n765ts)* multivulval phenotype at 20°C. At least three independent lines were characterized for each transgene. Transgenes were integrated into *lin-15(n765ts)* by psoralen/UV mutagenesis. The *str-2::GFP* transgene was integrated onto chromosomes I, IV, V, and X to generate four independent integrated strains. Similar results were obtained with all integrated strains; in all cases, *str-2* is expressed strongly in only one AWC neuron, and weakly in both ASI neurons. Cell identification was based on the characteristic morphology and position of GFP-positive cell nuclei viewed by simultaneous fluorescence and Nomarski differential interference microscopy. Most crosses were performed with the integrant on chromosome I, *kyIs140*. A subset of mutants were analyzed with both *kyIs140* and the integrant on chromosome X, *kyIs136*, and yielded similar results in both strains. The *odr-3::str-2* transgene was injected at 175 ng/µl or 250 ng/µl, while the *odr-3::odr-10*

transgene (Sengupta et al., 1996) was injected at 120 ng/µl or 175 ng/µl. Both were co-injected with the dominant marker *rol-6*.

Other genetic strains

Expression of *str-2::GFP* was examined in a *lin-12* loss of function mutant (*n676n930* at 25°C, n=120) and two *lin-12* gain of function mutants (*n137n460* at 15°C, n=160 and *n676n930* at 15°C, n=117). A zygotic requirement for *glp-1* was examined in two mutants (*q339*, n=36, *q175*, n=52). A maternal requirement for *glp-1* was also examined with temperature shift experiments using the *glp-1* temperature-sensitive allele, *q224*. *glp-1* is essential for early embryonic inductive events, so shifts to the restrictive temperature were performed after this time period (n=79). In all cases expression was normal (i.e. asymmetric expression in AWC, with about half the animals showing expression in AWCL and the other half in AWCR). Expression of *str-2::GFP* was also normal in four synaptic transmission mutants: *unc-13(e450)*, n=50, *unc-104(e1265)*, n=68, *snt-1(md172)*, n=150, and *unc-7(e1390)*, n=30.

Screen

str-2::GFP(kyIs140); rol-6(e187) worms were mutagenized with EMS according to standard protocols and screened for ectopic expression of GFP under a fluorescence dissecting scope in the F2 generation (Anderson, 1995). Eleven independent mutants that express *str-2::GFP* in both AWC cells were identified that fall into seven complementation groups. New alleles of previously identified genes include: *unc-2(ky385* and *ky390)*, *unc-44(ky394)*, *unc-36(ky386)*, and *unc-43(ky398)*.

Laser killing of AWC neurons and AWC precursors

AWC precursors were killed in embryos at approximately the 50 cell stage. Embryos were prepared by cutting *str-2::GFP* gravid adult hermaphrodites and transferring their embryos to a 5% agar pad in M9 solution. Cells were identified in embryos at the 28 cell stage, which were allowed to go through one more cell division before either ABplpaa (AWCL precursor) or ABprpaa (AWCR precursor) was irradiated with a laser beam. Embryos were then recovered on an NGM plate at 20°C, allowed to hatch and grow for 3 days. Killing ABplpaa leads to a loss of the left amphid socket cell, while killing ABprpaa leads to a loss of the right amphid socket cell. Socket cells are required for uptake of the fluorescent DiI, so the death of these cells was assessed by lack of dye-filling. For example, in ABplpaa kills, if the left side failed to fill with DiI while the right side filled successfully, ABplpaa and therefore AWCL was considered to be killed, and AWCR was then examined for *str-2* expression. Occasionally, the ablation appeared to damage but not kill the precursor cell, because the operated animals lost socket cell function while retaining the ipsilateral AWC neuron. In these cases, GFP was observed in AWC on a side that failed to fill with DiI. None of these animals expressed *str-2::GFP* on the contralateral side, so these results do not affect the conclusion that the default state for *str-2* expression in a single AWC is off. In most animals without *str-2::GFP* expression in AWC, faint ASI expression was still visible, indicating that the effect on *str-2::GFP* expression was specific to AWC. Mock-killed animals were exposed to the same conditions as the successfully killed animals, but DiI filling occurred on both sides indicating that the kill was not successful. Killing AWC in the L1 stage was performed

as described (Bargmann and Horvitz, 1991). Briefly, L1 animals were mounted on a 5% agar pad, the AWC neuron was identified by its through characteristic morphology and position, or by the use of cell-specific GFP markers, and then laser irradiated.

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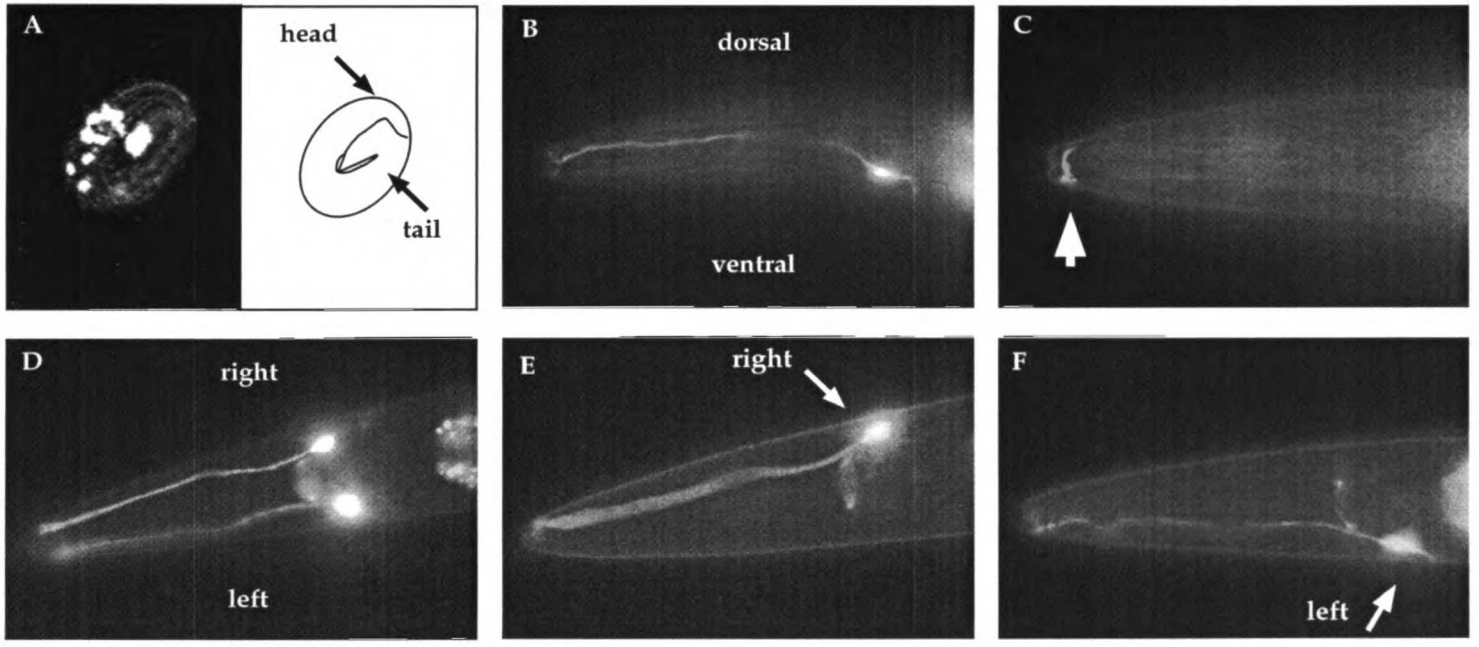


Figure 4-1. *str-2* encodes a candidate odorant receptor that is asymmetrically expressed in the AWC olfactory neurons. A. A *str-2::GFP* fusion is first expressed in about 10 cells in the late embryo. The *str-2::GFP* fusion contains 3.7 kb of upstream region and the first seven amino acids fused to GFP. Figure shows a confocal Z series image collapsed into one plane. B. *str-2::GFP* drives strong expression in the AWC neurons of an adult. C. A GFP-tagged full-length STR-2::GFP fusion protein localizes to the AWC cilia of an adult. D. All animals with an integrated *str-1::GFP* transgene show expression in both AWB neurons. E. and F. About half the animals with an integrated *str-2::GFP* transgene show expression in the right AWC neuron and the other half show expression in the left AWC neuron. B, C. Lateral views. Anterior is left, dorsal is up. D-F. Dorsal views. Anterior is left, right is up.

Table 1. Effects of embryo and L1 cell ablations on *str-2* expression in AWC

Embryonic ablations

	% with *str-2::GFP* on in:		Number of animals
	AWCL	AWCR	
mock kill	47	53	n=17
kill AWCR precursor	0	N/A	n=11
kill AWCL precursor	N/A	0	n=17
kill AWCR precursor in *unc-36*	100*	N/A	n=3

AWCR precursor is ABprpaa, AWCL precursor is ABplpaa

* Statistically different from control, p=0.003.

L1 ablations

AWC killed	% with *str-2::GFP* in remaining neuron	
AWC^{ON}	0	AWCR, n=35 AWCL, n=27
AWC^{OFF}	100	AWCR, n=1 AWCL, n=2

Table 4-2. Genes required for *str-2* asymmetry.

Strain	%animals with: 2 AWC^{OFF}	1 AWC^{ON} 1 AWC^{OFF}	2 AWC^{ON}	n
Wild-type	0	100	0	1000
Axon guidance mutants				
unc-76(e911)	43	57	0	114
sax-5(ky118)	52	47	1	79
vab-3(e648)	51	47	2	126
sax-3(ky123)	56	44	0	126
Axon guidance/cytoskeleton mutants				
unc-33(e204)	14	59	27	195
*unc-44(e362)**	21	62	18	195
Ca^{++} signalling mutants				
*unc-2(e55)**	13	39	48	158
*unc-36(e251)**	0	3	97	121
unc-43(n1186)	3	5	92	101
unc-43(n498gf)	80	20	0	133
unc-43(gf);unc-36	65	25	10	166
egl-2(n693gf)	0	1	99	166
Olfactory signalling mutants				
odr-3(n1605)	0	100	0	150
odr-1(n1933)	100	0	0	150
daf-11(sa195),15°C	100	0	0	150
daf-11, L1/L2, 15°C	0	100	0	98
odr-1, L1/L2	21	79	0	114
tax-4(p678)	13	81(56)	6	162
tax-2(p691)	4	80(10)	16	69
odr-1; tax-4	100	0	0	126
odr-1; tax-4 L1/L2	4	96	0	140

Table 4-2. Genes required for *str-2* asymmetry.

All animals scored as adults at 20°C, except *daf-11* animals, which were scored at 15°C to allow non-dauer development. Numbers in parentheses for *tax-2* and *tax-4* are percentages of animals that have 1 AWC^{ON} with dim expression. Asterisks indicate genes also identified in screen for 2 AWC^{ON} animals.

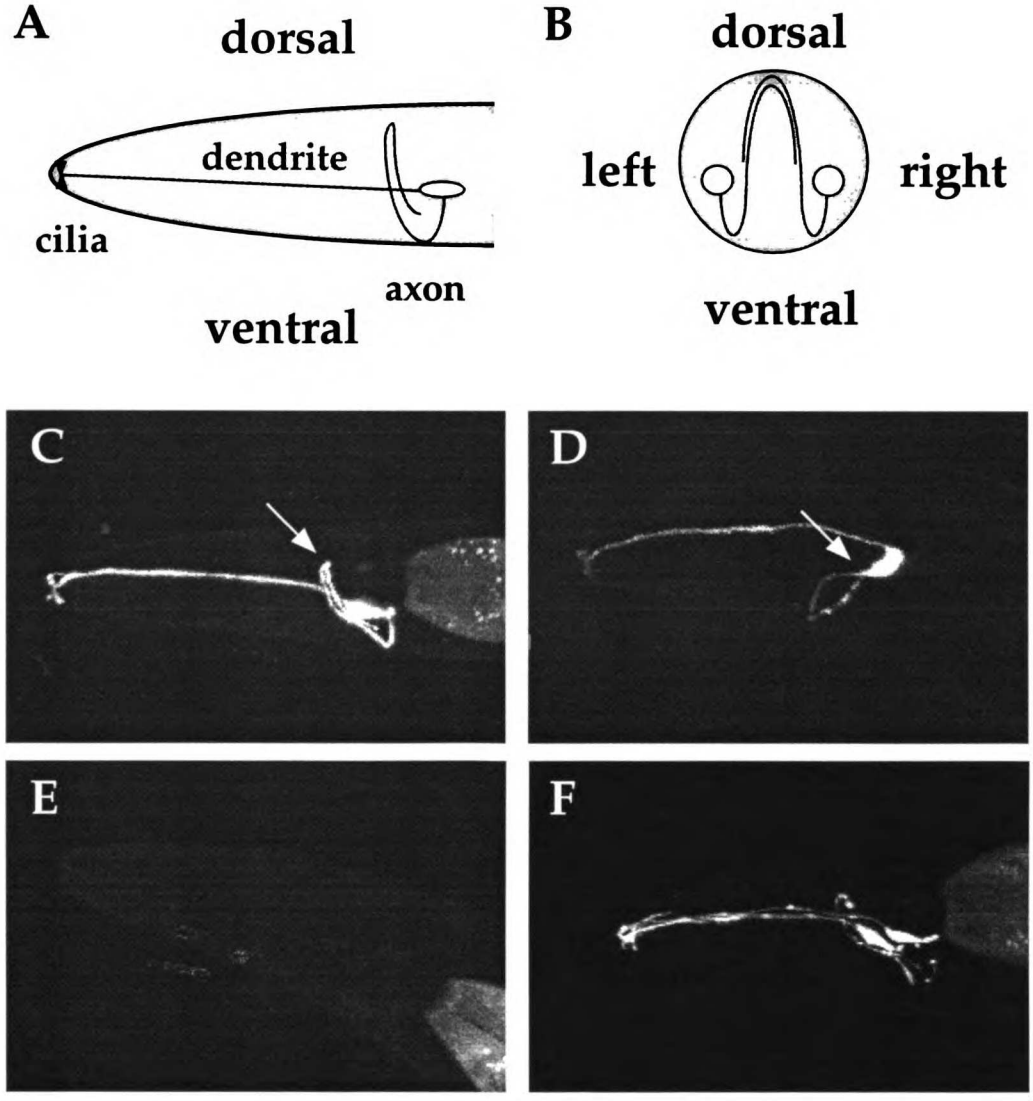


Figure 4-2. Axon guidance mutants are defective in *str-2* asymmetry. A. A lateral diagram showing the morphology of one AWC neuron. B. A cross-sectional diagram of both AWC axons. The two neurons are in contact at the dorsal portion of their trajectory, and synapse on each other there. C. A lateral view of the AWC neuron as visualized by *str-2::GFP* expression. The axon projects dorsally past the cell body and then wraps around the contralateral side. D. *str-2::GFP* expression in an *unc-76* mutant animal. The AWC axon often terminates prematurely and fails to reach the dorsal midline. E. *str-2::GFP* is not expressed in this *unc-76* mutant animal. F. *str-2::GFP* can be expressed in both AWC neurons in *unc-33* mutants. C. through F. are confocal z series images collapsed into a single plane; anterior is left and dorsal is up.

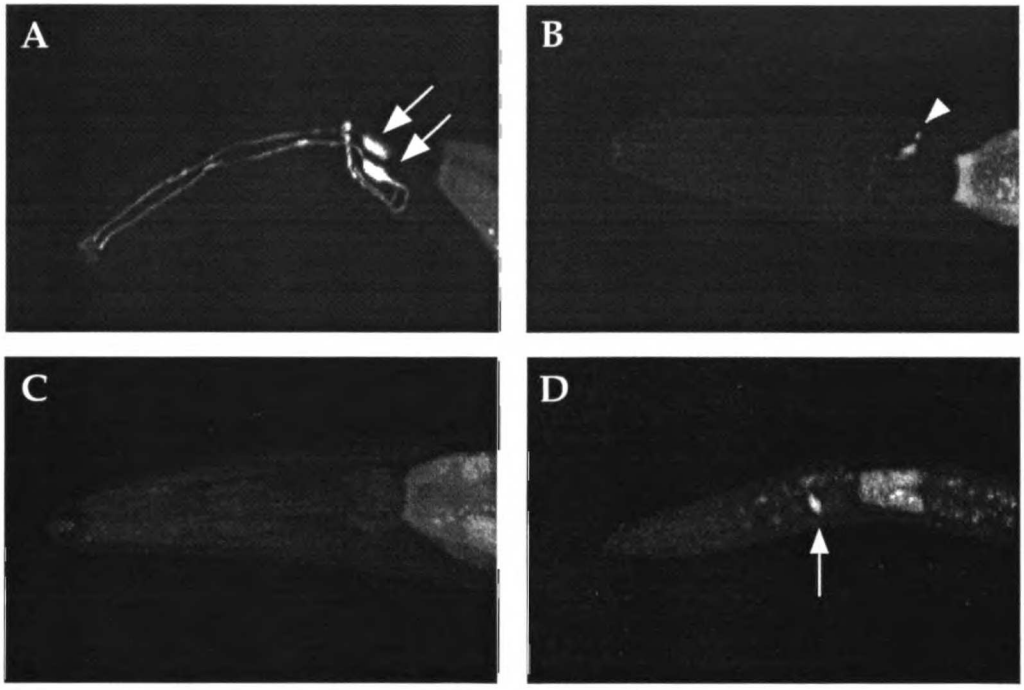


Figure 4-3. Ca^{++} and cGMP signalling regulate *str-2* expression.
A. *str-2::GFP* is expressed in both AWC neurons in an *unc-36* mutant animal.
B. *str-2::GFP* is expressed in neither AWC neuron in an *unc-43(gf)* mutant animal. Arrowhead indicates faint ASI expression.
C. *str-2::GFP* is not expressed in an *odr-1* adult animal.
D. *str-2::GFP* is expressed in one AWC neuron in an *odr-1* L1 animal.
Images are confocal z series collapsed into one plane; anterior is left and dorsal is up.

Table 4-3. *str-2* expression in double mutants.

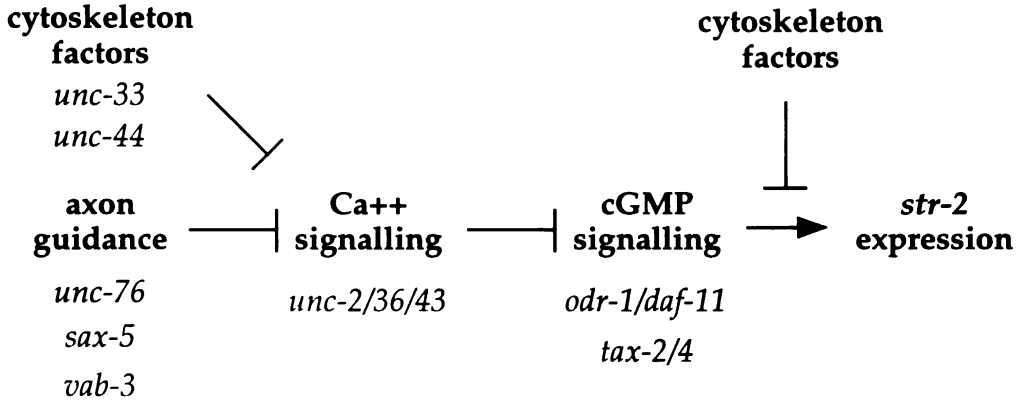
Strain		% of animals with: 2 AWC^{OFF}	1 AWC^{ON} 1 AWC^{OFF}	2 AWC^{ON}	n
Wild-type		0	100	0	1000
Double mutants:					
axon guidance with:					
alone	*unc-76*	43	57	0	114
Ca^{++} signalling	*unc-76;unc-2*	19	43	38	150
	unc-76;unc-36	10	23	67	136
	unc-76;unc-43lf	2	5	93	116
cGMP signalling	*unc-76;odr-1*	97	3	0	116
Ca^{++} signalling with:					
alone	*unc-2*	13	39	48	158
	unc-36	0	3	97	121
	unc-43 lf	3	5	92	101
cGMP signalling	*unc-2;daf-11*	100	0	0	82
	unc-36;daf-11	98	2	2	131
	unc-36;odr-1	98	2	0	81
	unc-43lf;odr-1	100	0	0	125
cytoskeleton factors	*unc-2;unc-33*	1	21	78	114
	unc-2;unc-44	1	40	59	81
	unc-36;unc-33	0	0	100	82
	unc-36;unc-44	1	5	94	121
cGMP signalling with:					
alone	*odr-1*	100	0	0	150
	daf-11	100	0	0	150
cytoskeleton factors	*odr-1;unc-33*	15	33	52	115
	odr-1;unc-44	17	38	45	196
	daf-11;unc-33	28	65	8	219
	daf-11;unc-44	23	58	19	150

Table 4-3. *str-2* expression in double mutants.

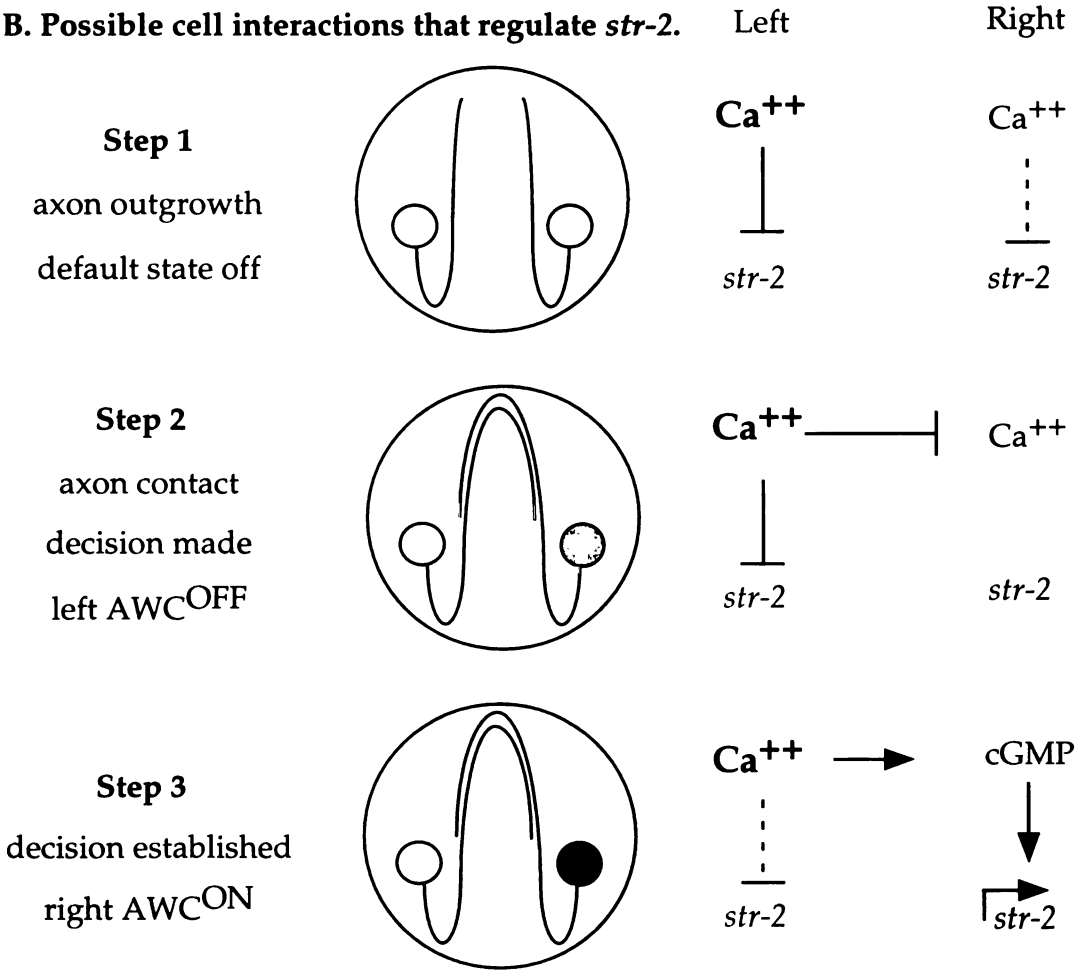
Mutant alleles as in Table 2, except *unc-43lf; odr-1(n1936)*. All animals scored at 20°C, except *daf-11* mutants, which were scored at 15°C to allow non-dauer development.

Figure 4-4. Models for *str-2* regulation

A. Genetic pathway for *str-2::GFP* expression



B. Possible cell interactions that regulate *str-2*.



Chapter 5

Chemosensory receptor regulation in response to changing environmental and developmental conditions

Abstract

The role of olfactory receptors in plasticity of olfactory behavior is poorly understood. Here we describe changes in chemosensory receptor expression in the nematode, *C. elegans*, that result from specific sensory experience. Three putative chemosensory receptor genes are regulated before and during the alternative dauer larval phase in response to harsh environmental conditions. The *srd-1* gene is expressed in the ASI neurons in healthy well-fed animals but is unobservable in starved, pheromone-treated or dauer animals. In contrast, *str-2* expression in the ASI neurons is faint in healthy well-fed animals, further repressed by pheromone, but then upregulated in dauer animals. Expression of a third ASI receptor, *str-3*, is similar in non-dauer and dauer animals but is repressed by pheromone. Thus, each of these receptors has a specific, dynamic expression pattern in response to different environmental conditions. Genes important for mediating dauer entry and exit regulate these changes in receptor expression. We propose that altering chemosensory receptor gene expression may be a strategy for modifying olfactory behavior.

Introduction

Animals sense and respond to their environment using several sensory modalities. For each modality, an animal uses several classes of cells and receptors to detect the stimulus. For example, the retina consists of distinct photoreceptor cell types that can be further distinguished by their choice of opsin expression to detect different wavelengths of light. Likewise, vertebrate somatosensory cells fall into several distinct classes, with anatomies and

response properties specialized for sensing touch, temperature, or pain. The olfactory systems in flies, mice and worms contain many different cell types that can be further distinguished by the receptors they express (Clyne et al. 1999, Vosshall et al. 1999, Mombaerts 1999, Bargmann and Mori 1997). The stimuli detected by all these systems can change over time and an animal must alter its processing of these signals to respond appropriately. While alterations in higher processing centers can underlie such plasticity, the role of the detection machinery in mediating these changes is poorly understood.

Olfactory cues can elicit multiple behaviors, including attraction, repulsion and mating. Animals display changes in their olfactory behavior both in response to short-term changes in their environment, and as part of a hard-wired developmental program. For example, *Drosophila* temporarily changes its preference for odors that have been paired with a repellent shock, which is known to involve higher processing centers (Heisenberg et al., 1985; de Belle and Heisenberg 1994; de Belle and Heisenberg 1996). *Drosophila* also demonstrates olfactory preferences that differ from the larval to the adult stage (Dubin et al. 1997, Shaver et al. 1998). The contribution of specific olfactory neurons and their receptors to these short and long-term behavioral modifications is unknown. Changes in receptor expression could alter both the stimuli detected as well as the perception of those stimuli. Thus, distinct behavioral responses could be generated under different conditions.

The nematode *C. elegans* demonstrates behavioral plasticity in response to its environment and as part of a developmental program. *C. elegans* is exquisitely sensitive to chemical, thermal and mechanical stimuli, and based on its experience will rapidly alter its responses to those stimuli. *C. elegans*

also displays predictable behavioral changes through its lifetime. One strikingly distinct life period is the dauer larval phase. When the nematode encounters harsh environmental conditions such as high temperature, limited food, and high population density it will arrest normal development and enter the dauer phase (Albert et al. 1981, Swanson and Riddle 1981, Albert and Riddle 1983). An animal is thought to assess high population density by detecting dauer pheromone, an uncharacterized chemical cue constitutively secreted by the nematode (Golden and Riddle 1984). As animals enter the dauer stage, they undergo morphological changes and become resistant to harsh environmental conditions (Albert and Riddle 1983). Dauer larvae also display markedly different locomotory, chemosensory and thermosensory behaviors that allow them to disperse and seek out better conditions.

The chemosensory amphid organ contains 12 pairs of ciliated chemosensory and thermosensory neurons that can be reproducibly identified from individual to individual (Bargmann et al. 1993, Bargmann and Mori 1997). Each of these cells has a distinct function, including sensation of temperature, volatile or water-soluble chemicals. For example, the AWA and AWC neurons detect volatile chemicals that elicit attraction (Bargmann et al. 1993). The receptor for one of these attractants has been identified: *odr-10* is expressed in the AWA neurons and detects the attractant diacetyl (Sengupta et al. 1996). The ASI neurons appear to direct two kinds of output. They contribute to detection of water-soluble attractants and also signal to prevent dauer entry during non-dauer stages (Bargmann and Horvitz 1991). Ablation and genetic studies suggest that when ASI (and ASG and ADF) detects pheromone, it reduces its non-dauer signal to allow dauer development. In dauers, the amphid alters its structure, withdrawing the dendrite endings of

the ASI neurons from the pore through which chemical stimuli are sensed. However, dauer animals still can sense improved environmental conditions that allow them to resume normal development, mostly via the ASJ sensory neurons.

C. elegans detects its chemical environment with a large number of chemosensory receptors. The fully sequenced *C. elegans* genome contains 1000 predicted G protein-coupled receptors (GPCRs), 700 of which are related to the diacetyl receptor, *odr-10* (Troemel et al. 1995, Sengupta et al. 1996, *C. elegans* sequencing consortium, 1998). These GPCR genes are predominantly expressed in chemosensory neurons and estimates are that *C. elegans* may have 500 different functional kinds of chemosensory receptors. The identification of these receptors provides an opportunity to investigate their contribution to plasticity of olfactory behavior under different environmental conditions, such as during the dauer stage.

Many of the genes that mediate dauer entry have been described, and studies to date support the following model. Dauer entry is due in part to a loss of the dauer-preventing transforming growth factor (TGF)-ß homolog, DAF-7, which is thought to be secreted from the ASI neurons and to signal through the heteromeric TGF-ß receptor complex of DAF-1 and DAF-4 (Georgi et al. 1990, Estevez et al. 1993, Ren et al. 1996, Schackwitz et al. 1996). This signal is propagated by a group of proteins called SMADs (for Sma/decapentaplegic (dpp)/mothers against dpp) that are thought to translocate to the nucleus upon activation and regulate dauer-specific genes, resulting in anatomical and behavioral changes (Malone and Thomas 1994, Patterson et al. 1997. Thatcher et al. 1999). Other genes and molecules including an insulin

receptor pathway also contribute to dauer formation (Riddle and Albert, 1997),

What is the basis for the behavioral changes seen in dauer larvae? Each *C. elegans* chemosensory neuron is believed to control a specific set of behavioral responses. An animal could utilize different neurons during dauer and non-dauer stages to mediate dauer-specific behaviors. An alternate explanation is that neurons regulate gene expression to effect differences in non-dauer and dauer behaviors. Previous work in the nematode has shown that by altering expression of the *odr-10* receptor, the behavioral response to diacetyl can be changed from attraction to repulsion (Troemel et al. 1997). Thus, changes in receptor expression can cause significant behavioral changes. Here we describe altered expression of three putative chemosensory receptors in the ASI neurons, both in dauer larvae and in response to dauer pheromone. *srd-1* is detectable in the ASI neurons of healthy well-fed animals and is undetectable in starved or dauer animals. *str-2* is expressed only in AWC neurons in healthy well-fed animals, and only in ASI neurons in dauer animals. Expression of another receptor in the ASI neurons, *str-3*, is not altered in dauer animals but is lost in response to pheromone. We have determined that some genes important for regulating dauer entry and exit also mediate chemosensory receptor expression. Changes observed in the spatial pattern of chemosensory receptor gene expression may underlie the behavioral plasticity observed between dauer and non-dauer animals.

Results

srd-1 and *str-2* receptor expression is altered in dauer stage

Visualizing promoter-GFP fusion genes is a common method for assessing expression from a specific promoter. We have used this technique to determine the expression pattern and levels of different chemosensory receptors. When we refer to alterations in receptor expression, we are describing the pattern and intensity of GFP expression from a promoter-GFP fusion (e.g. *srd-1* expression = *srd-1::GFP* expression).

Using promoter-GFP fusions, we monitored the expression of three candidate receptors from the *odr-10* superfamily, *srd-1*, *str-2* and *str-3*, during dauer and non-dauer development (Table 5-1) (Troemel et al. 1995, Peckol et al. 1999, Troemel et al., in preparation). Two of these genes, *srd-1* and *str-2*, show a regulated pattern of expression. In well-fed larvae and adults, *srd-1* is expressed in the ASI neurons and *str-2* is expressed strongly in the AWC neurons and weakly in the ASI neurons. However, in animals that have entered the dauer stage, these expression patterns change such that *str-2* is expressed in ASI but not AWC neurons and *srd-1* expression is undetectable (Fig. 5-1). A third ASI-specific fusion gene, *str-3* is expressed strongly in both dauer and non-dauer larvae and adults (Fig. 5-1). Expression of other candidate receptors found in the repellent-sensing ADL and AWB neurons is also unchanged in dauers (data not shown). To ask if dauer-induced changes in expression are permanent, *str-2* expression was examined in animals that had recovered from dauer. Animals that expressed *str-2* strongly in the ASI neurons were recovered onto food to allow resumption of normal development, and within 24 hours, all animals had downregulated *str-2* expression in ASI (n=15).

Sensory activity regulates *srd-1* receptor expression

The decision to enter the dauer phase is regulated by chemicals from food and a pheromone, which *C. elegans* detects using ciliated sensory neurons. To investigate whether detection of chemicals regulates *srd-1* or *str-2* expression, we examined receptor expression in mutant backgrounds that deprive animals of the ability to detect these cues (Table 5-1). A class of mutants that have defects in the ciliated endings of their dendrites are unable to respond to water-soluble attractants and retain a limited ability to sense volatile odorants. In *osm-6(p811)* and *che-3(e1124)*, two mutants with severe truncations of their sensory cilia (Perkins et al. 1986), *srd-1* is no longer expressed while *str-2* remains strongly expressed in the AWC neurons and faintly expressed in the ASI neurons (Fig. 5-2). Expression of the third ASI-specific receptor, *str-3*, is unaltered in mutants with cilia defects. Thus, external chemosensory cues sensed through the cilia may regulate *srd-1* expression.

If chemosensory input is indeed important for *srd-1* expression, then molecules important in transducing that signal should also regulate *srd-1* expression. To investigate this possibility, we examined expression of the three receptor genes in mutants with defects in TAX-2/TAX-4, subunits of a cyclic nucleotide-gated channel required for chemosensation of many water soluble and volatile odorants (Coburn and Bargmann 1996, Komatsu et al. 1996, Mori and Ohshima 1997). As observed in the cilia mutants, *srd-1* but not *str-2* or *str-3* expression is altered in *tax-2(p691)* and *tax-4(p678)* mutants. *srd-1* expression in *tax-2/4* mutants is only weakly detectable in the ASI

neurons, *str-3* is strongly expressed in the ASI neurons, and *str-2* remains weakly expressed in the ASI neurons (Fig. 5-2 and data not shown). Sensory signaling via TAX-2/TAX-4 appears important for *srd-1* expression, but not *str-2* or *str-3* expression. This result implicates a subset of ciliated neurons in *srd-1* expression - those that express *tax-2/4*. ASI itself is one of the *tax-2/4* -expressing neurons.

To test the hypothesis that ASI activity affects *srd-1* expression, we expressed a rat delayed rectifying potassium channel, Kv1.1, in ASI neurons expressing either *srd-1::gfp* or *str-3::gfp*. This channel should open in response to depolarization of the ASI neuron and allow for potassium efflux to repolarize the cell (Grissmer et al. 1994, Peckol et al., 1999). In neurons expressing Kv1.1, *str-3* was highly expressed while *srd-1::GFP* was barely visible (data not shown). Thus, *srd-1* expression in ASI might be responsive to ASI neuron activity.

Sensory neurons in *C. elegans* are thought to amplify sensory signals by opening voltage-gated Ca^{++} channels and allowing Ca^{++} influx. If a sensory signal regulating *srd-1* expression downstream of the TAX-2/4 channel is amplified via voltage-gated Ca^{++} channels, mutations in these channels could reduce GFP expression. Mutations in three different calcium channel subunits, *unc-36(e251)*, *egl-19(n582)*, and *unc-2(e55)* (Schafer and Kenyon 1995, Schafer et al. 1996, Lee et al. 1997), do not alter *srd-1*, *str-2*, or *str-3* expression in the ASI neurons (data not shown). These results suggest that the regulation of *srd-1* expression does not require the opening of voltage-gated Ca^{++} channels.

Pheromone regulates *srd-1*, *str-2* and *str-3* receptor expression

The changes in *srd-1* and *str-2* expression in dauer could result from developmental changes that occur with dauer entry or from environmental conditions that lead to dauer entry. To distinguish between these possibilities, we examined expression of *srd-1*, *str-2*, and *str-3* in mutants under different conditions that regulate dauer entry. High temperature can enhance dauer formation, yet exposing both larval and adult animals to high temperature (27-28°C) did not alter GFP expression of any of the fusion genes in ASI (*srd-1::gfp*, 95% ON; *str-2::gfp*, 8% ON, *str-3::gfp*, 100% ON (n>25 for each value)). Thus, temperature does not appear to regulate receptor expression in non-dauer stages.

Absence of food and abundance of pheromone are the two major stimuli that induce *C. elegans* to enter the dauer stage. Adult animals in crowded conditions reduce both *srd-1* and *str-3* expression (*srd-1::gfp*, 32% ON; *str-3::gfp*, 67% ON (n>25 for each value)). To determine whether crowding-induced alterations in receptor expression were due to the dauer pheromone, we exposed animals to different concentrations of a dauer pheromone preparation and examined receptor expression (Golden and Riddle 1982, Golden and Riddle 1984). We let animals with different receptor promoter-GFP fusions lay eggs onto plates with or without pheromone and examined receptor expression in larval and adult progeny. Effects of pheromone on receptor gene expression were at concentrations below those that induced dauer development (see Materials and Methods). Thus, receptor expression can be directly modulated by chemical signals independent of the development of the dauer stage.

Interestingly, a decrease in expression of all three receptor genes was observed with increasing pheromone concentration (Fig. 5-3). Expression of *srd-1* decreased with increasing pheromone in both larvae and adults, and on 100 µl pheromone, was completely undetectable (Fig. 5-3A). Similarly, *str-3* expression decreased in response to increasing pheromone in both larvae and adults (Fig. 5-3C). Expression of *str-2* is very faint but detectable in ASI neurons of well-fed wild-type animals. The percent of animals expressing *str-2* in the ASI neurons also decreases with pheromone exposure (Fig. 5-3B). To examine recovery of receptor expression after pheromone exposure, we transferred animals that failed to express *srd-1* or *str-3* from 100 µl pheromone to no pheromone. Gene expression was restored in most animals within 24 hours of removal from pheromone, and in all animals within 48 hours of removal (24 hours: *srd-1::gfp*, 81% (n=27), *str-3::gfp*, 100% (n=10); 48 hours: *srd-1::gfp*, 100% (n=24), *str-3::gfp*, 100% (n=26)). No correlations were observed between receptor expression and larval stage, either for pheromone-induced decrease in expression or for recovery of expression (data not shown).

These levels of pheromone were not sufficient to reduce expression another ASI-specific gene, *daf-7*, which has previously been shown to be down-regulated by pheromone (Fig. 5-3) (Ren et al. 1996, Schackwitz et al. 1996). Pheromone preparations are crude, uncharacterized worm extracts, so the discrepancy could be due to a qualitatively different preparation. Another explanation is that receptor expression is more sensitive to pheromone than *daf-7* expression. Because we used non-dauer-inducing concentrations of pheromone, it may not have had sufficient activity to repress *daf-7*

expression. It is not surprising that it is easier to decrease chemosensory receptor expression than *daf-7* expression. One might expect that coordinating dauer entry, which is signaled by repressing *daf-7* expression, would require tighter control than altering receptor expression, given that the whole animal must alter its morphology in concert during the dauer phase.

A pheromone production mutant has altered *str-2* expression

To confirm the repressive effects of pheromone on receptor expression, we examined receptor expression in *daf-22* mutants, which are unable to produce pheromone. *daf-22(m130)* mutants have a defect in dauer entry (Daf-d) that can be rescued by providing exogenous pheromone. As might be expected, *daf-22* mutants express *srd-1* and *str-3* strongly in their ASI neurons (Fig. 5-4 and data not shown). Surprisingly, they also express *str-2* at high levels in ASI, instead of the low levels observed in wild-type animals. *str-2* expression can be reduced in *daf-22* mutant animals by exposing them to pheromone-producing wild-type worms (Fig. 5-4A). Pheromone could be a signal to keep *str-2* off in the ASI neurons.

Dauer pheromone potentially inhibits expression of *str-2* and *str-3*, yet both are expressed strongly in dauer larvae that have been exposed to high levels of pheromone. One possible explanation for this paradox involves the dauer-specific changes that occur in ASI morphology. During the dauer stage, ASI cilia retract from the amphid pore, changing their access to the environment. ASI remodeling was first observed by electron microscopy, but we were also able to score this change by the ability of the ASI neurons to take up the vital dye DiI. DiI fills six pairs of amphid neurons including ASI; in many dauers

we found that five pairs of neurons would fill with DiI, while ASI would not (Fig. 5-4B). Interestingly the extent of dye-filling in dauers correlated with the extent of ASI-specific *str-2* expression, such that animals with retracted ASI neurons and no dye-filling express *str-2* strongly in their ASI neurons (Fig. 5-4), whereas ASI neurons that filled well with DiI failed to express *str-2*. Control experiments demonstrated that DiI filling was not affected by GFP expression in other contexts (data not shown). These results suggest that pheromone represses *str-2* and *str-3* expression in ASI, but relief from this repression occurs when the ASI neurons remodel in the dauer stage. *srd-1* expression is also reduced by pheromone, but it remains low when the ASI neurons remodel.

TGF-ß ligand and receptor regulate chemosensory receptor expression

The TGF-ß signaling mutants *daf-7*, *daf-1*, and *daf-4* are temperature-sensitive mutants that enter the dauer stage constitutively (Daf-c) at 25°C (Georgi et al. 1990, Estevez et al. 1993, Ren et al. 1996, Schackwitz et al. 1996). *daf-7* is expressed in the ASI neurons, and its expression is decreased by pheromone. The receptor genes could be regulated in parallel to *daf-7*, or they could be targets of *daf-7*. To distinguish between these models, we examined the expression of *srd-1*, *str-2*, and *str-3* in *daf-7* and *daf-4* mutants. At 15°C, *daf-7(e1372)* and *daf-4(e1364)* mutant animals develop as non-dauers. In *daf-7* and *daf-4* mutants at 15°C, *srd-1* and *str-3* were undetectable but *str-2* was present in ASI neurons (Fig. 5-5).

The SMADs are putative transcriptional regulators that act downstream of the DAF-7/1/4 TGF-ß signal; four SMADs are known in the dauer pathway.

The SMADs DAF-8 and DAF-14 appear to act in concert to prevent dauer formation, by inhibiting the ability of the SMADs DAF-3 and DAF-5 to induce dauer formation. *daf-8* and *daf-14* mutants are Daf-c, while *daf-3* and *daf-5* mutants are dauer-defective (Daf-d) (Georgi et al. 1990, Estevez et al. 1993, Ren et al. 1996, Schackwitz et al. 1996, Patterson et al. 1997, Thatcher et al. 1999). To investigate whether *daf-7* regulation of *srd-1* and *str-3* occurs via SMAD signaling, we examined receptor expression in SMAD mutants. In *daf-14(m77)*, and *daf-5(e1386)* mutants, GFP expression was wild-type, with strong *srd-1* and *str-3* expression in ASI (Fig. 5-5 and data not shown). In *daf-8(e1393)* mutants, *srd-1* expression was normal, but *str-3* expression was faint in the ASI neurons. These results suggest that DAF-7 signals through DAF-4 to promote *srd-1* and *str-3* expression in the ASI neurons. Signaling through the DAF-8 SMAD is required for *str-3* expression, but *srd-1* expression does not require the SMADs (DAF-8, DAF-14, and DAF-5) through which DAF-7 signals to prevent or induce dauer formation. The SMADs signal via the steroid hormone receptor homolog, DAF-12, to induce dauer formation (Larsen PL et al. 1995, Antebi A et al. 1998). In *daf-12(m20)* mutants, GFP expression also appeared wild-type (Fig. 5-6). Chemosensory receptor expression patterns observed in the *daf* mutants and activity mutants are outlined in Table 1.

Discussion

Dauer entry, pheromone and activity regulate receptor expression

C. elegans responds to lack of food, abundance of pheromone, and high temperature by entering the dauer stage. Dauer animals exhibit different chemosensory behaviors from adult animals, responding differently to volatile odorants known to be attractive to adults. Structural changes in the amphid chemosensory neurons may prevent them from detecting the same odors that adults detect, yet dauers must be able to sense cues signaling favorable environmental conditions. We have found that dauers alter their chemosensory receptor expression, which may contribute to changes in dauer behavior. Two putative chemosensory receptor genes, *srd-1* and *str-2*, display altered expression in dauer animals. Dauer entry suppresses *srd-1* expression in the ASI neurons while inducing *str-2* expression in the ASI neurons.

One of the environmental conditions that contributes to dauer entry is population density, which is measured by detection of the constitutively secreted dauer pheromone. We have found that non-dauer inducing levels of pheromone are sufficient to repress ASI neuron expression of *srd-1*, *str-2*, and *str-3*. The reduction of *str-3* and *str-2* expression in the presence of high pheromone levels is somewhat paradoxical, because their expression is high in dauer animals when pheromone should be abundant. The upregulation of *str-3* and *str-2* expression in dauer may result from a retraction of the ASI cilia from the amphid pore, relieving the inhibitory effects of pheromone. Some other mechanism maintains the repression of *srd-1*, which is also down-regulated by pheromone, and remains low in the dauer stage. One possibility is that the activity-dependent regulation of *srd-1* expression accounts for its different behavior. An activity-regulated pathway involving detection of chemoattractants and channel activity regulates *srd-1*, but not *str-2* or *str-3*. One model for the dynamics of receptor expression are shown in

Figure 5-6. Initially, *srd-1* and *str-3* expression are high, while *str-2* expression is low. As pheromone concentrations increase, expression of all three receptors decrease, and if harsh conditions persist, dauer development occurs. One consequence of dauer development is the retraction of the ASI cilia from the pore, relieving pheromone repression of these genes. While this derepression is sufficient for *str-2* and *str-3* to be expressed, a reduction in ASI activity maintains repression of *srd-1*.

C. elegans turns off *str-3* expression in the presence of pheromone, only to turn it back on in dauer. Dauer entry occurs in two steps: a pre-dauer L2d phase that is distinct from normal L2 animals is followed by the dauer phase characterized by gross morphological and behavioral changes. *str-3* downregulation in the L2d phase could sensitize the animal and prepare it for the transition into actual dauers. For example, a sensitized L2d animal may be more responsive to cues that represent abundant food and less responsive to subtle cues about food characteristics.

Dauer pathway genes regulate receptor expression

TGF-ß signaling is thought to regulate dauer entry via the TGF-ß DAF-7, its receptor DAF-1/DAF-4, as well as downstream effectors, including the SMADs, DAF-8, DAF-14, DAF-3, and DAF-5 (Georgi et al. 1990, Estevez et al. 1993, Thomas JH et al. 1993, Malone and Thomas 1994, Ren et al. 1996, Schackwitz et al. 1996, Patterson et al. 1997). Mutations in *daf-7* and *daf-4* lead to altered expression of *srd-1*, *str-2*, and *str-3*: *srd-1::gfp* and *str-3::gfp* are dim and *str-2::gfp* is bright in the ASI neurons. Surprisingly, mutations in *daf-8*, *daf-14*, and *daf-12* fail to affect *srd-1::gfp* expression. These results suggest that

regulation of *srd-1* expression may occur through a novel pathway independent of the known dauer signaling pathway.

By contrast, *str-3* expression is reduced in *daf-8* mutants but bright in *daf-14* mutants. This observation provides evidence that these two molecules are not completely redundant in function. DAF-8 and DAF-14 may function partly in concert and partly independently to orchestrate different components of dauer entry. Independent regulation of the chemosensory receptor genes is consistent with the model that dauer entry involves two steps. Perhaps DAF-8 activity is important earlier and this SMAD moves the animal into the sensitized L2d phase where *str-3* expression is low. The other SMAD mutants may be more critical in the later entry into the dauer stage.

Strategies for olfactory plasticity in invertebrates

Olfactory sensitivity can change as a consequence of developmental stage. *Drosophila* larvae and adults display different olfactory behaviors, suggesting that olfactory plasticity occurs as the animal develops. For example, *Scutoid* mutant adults display only weak responses to the odorants acetone and 2-butanone, but *Scutoid* larvae respond to these odors normally (Dubin et al. 1997). During metamorphosis, many parts of the larval nervous system degenerate and new neurons are born to subserve adult fly behaviors. It is not clear whether different olfactory repertoires of larvae and adults result from changes in existing structures or development of new structures during metamorphosis (Tissot et al. 1997). Larvae do display different responses to odorants, for example, responses to alcohols decrease as the larvae age (Cobb et al. 1992). To date, alterations in olfactory behavior are thought to be

primarily due to changes in synaptic connectivity or efficacy. Changes in chemosensory receptor gene expression in the nematode present a possible alternate strategy for plasticity of olfactory behavior.

Functional implications of altering chemosensory receptor gene expression

Altering circuitry and altering gene expression could be two separate strategies for accomplishing the same task: changing response to a specific odorant. The structures of the vertebrate vs. the nematode olfactory systems provide distinct advantages for using either a rewiring or a gene expression strategy of olfactory plasticity. In vertebrates, a single olfactory neuron expresses a single olfactory receptor, so the function of that neuron is always to sense the corresponding odor (Mombaerts 1999). By contrast, *C. elegans* olfactory neurons express many odorant receptors and the identity of the neuron that expresses a receptor defines the animals response to that receptor's odor (Troemel et al. 1997). Changing the synaptic strength of a single vertebrate neuron could alter responses to one odor, while altering connectivity of *C. elegans* olfactory neurons would alter responses to many odors. Specificity of olfactory plasticity in the nematode may better achieved by altering gene expression.

Additionally, altering olfactory afferent gene expression in vertebrates could substantially impact the guidance of olfactory axons to their targets. Vertebrates olfactory receptor proteins have been implicated in guiding projections of primary olfactory axons to their specific target glomeruli in the olfactory bulb (Vassar et al. 1994, Mombaerts et al. 1996, Bulfone et al. 1998, Wang et al. 1998). The process of using the receptor as a guidance molecule

may impose a constraint on primary afferent wiring that altering gene expression could severely disrupt. In the nematode, olfactory receptor proteins appear unimportant in the development of the chemosensory nervous system (Sengupta et al. 1996). Manipulating receptor expression in the nematode could alter olfactory responses to a single odor without affecting neural development or connectivity. In contrast, individual vertebrate neurons are thought to express a single receptor. Thus, vertebrate olfactory plasticity may result from changes in the number of cells expressing a certain receptor or the responsiveness of higher processing centers.

Materials and Methods

Strains and Genetics

Wild-type nematodes were *C. elegans* variety Bristol, strain N2 grown under standard uncrowded and well-fed conditions at 20°C unless otherwise noted (Brenner, 1974). A set of GFP fusion genes was used to track chemosensory receptor expression. Integrated version of *srd-1::GFP* I, *str-2::GFP* X, and *str-3::GFP* X fusion genes were examined for expression in the ASI neurons (Troemel et al. 1995, Peckol et al. 1999, Troemel et al, in preparation). Animals were examined as adults unless otherwise noted. Mutants include the following: *che-3(e1124)*, *daf-4(m63)unc-32(e189)*, *daf-5(e1386)*, *daf-7(e1372)*, *daf-8(m85)unc-13(e51)*, *daf-12(m20)*, *daf-14(m85)dpy-13(e184)*, *daf-14(m85)unc-22(e66)*, *egl-19(n582)*, *egl-19(ad695)*, *osm-6(p811)*, *tax-2(p691)*, *tax-4(p678)*, *unc-2(e55)*, and *unc-36(e251)*. In graphs, animals were scored as GFP positive if any GFP labeling was visible. Statistical analysis was conducted using Primer of Biostatistics software (Stanton A. Glantz, McGraw Hill publishers). Epifluorescence images were acquired using an Axioplan 2 (Zeiss).

Pheromone preparation and experimentation

Dauer pheromone was prepared according to Golden and Riddle (1984). Two mls of non-glucose medium (NGM) agar, containing either 0 µl, 50 µl, or 100 µl of pheromone, was poured into 3 cm plates. Plates were allowed to dry overnight, then were seeded with UV-killed concentrated OP50 bacteria. Adult *srd-1::GFP*, *str-2::GFP*, *str-3::GFP*, or *daf-7::GFP* (kindly provided by P Ren and DL Riddle) animals were allowed to lay eggs onto the plates at 25° for

3-5 hours, yielding approximately 80 eggs per plate. The adults were then removed and the plates were stored at 25°. Larval animals were removed at each stage for examination of GFP expression. Larvae that expressed no GFP were transferred to non-pheromone plates and scored for GFP expression 24 and 48 hours later. Adult animals grown entirely in the presence of pheromone were also examined for GFP expression. Experiments described in Figure 5-4A with *daf-22* mutants and exogenous animals were performed as follows: For *daf-22* alone controls, four *daf-22; str-2::GFP* L4 animals were placed on a well-seeded NGM plate, allowed to lay eggs and four days later these progeny were examined as adults for *str-2* expression in the ASI neurons. For *daf-22* mutants treated with wild-type animals, two *daf-22;str-2::GFP* L4 and two wild-type (or *dpy-20*) animals were placed on a well-seeded NGM plate, allowed to lay eggs and four days later the *daf-22;str-2::GFP* adult progeny were examined for ASI *str-2::GFP* expression.

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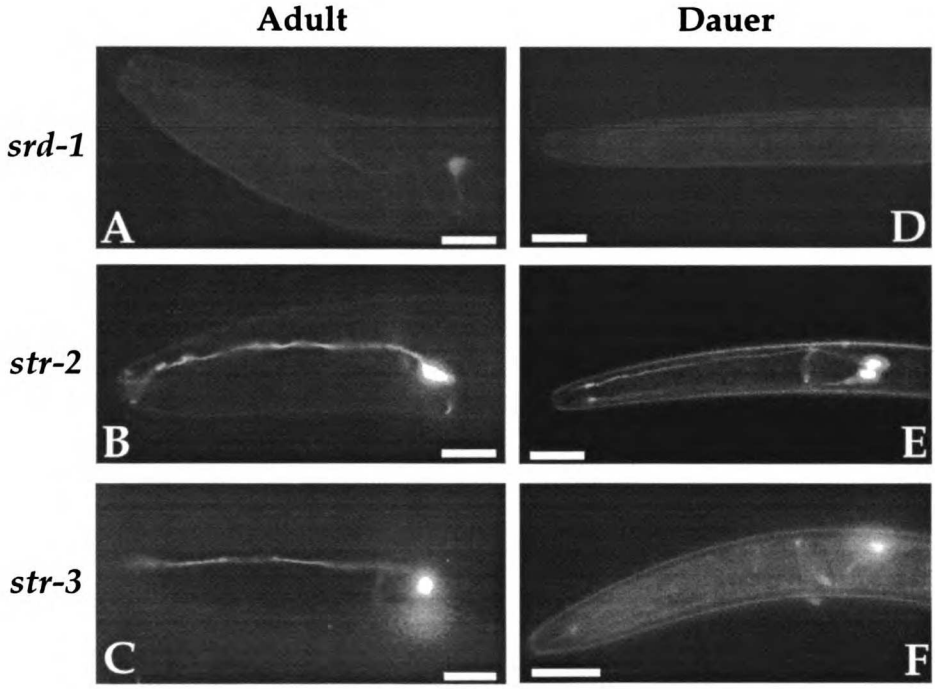

Figure 5-1. Chemosensory receptor expression is altered in the dauer stage. Promoter-GFP fusions were used to monitor the expression of three chemosensory receptors: *srd-1, str-2,* and *str-3* in adult (A,B,C) and dauer (D,E,F) animals. The heads of the animals are visible, with anterior to the left and dorsal up, except E, where anterior is left and right is up. Each neuron extends a dendrite to the tip of the nose and a single U-shaped axon. Scale bar=10 μm for all panels. *srd-1* and *str-3* expression was bright in the ASI neurons of adult animals (A,C), while *str-2* expression was dim or absent in ASI neurons yet bright in AWC neurons (B). In dauer animals, *srd-1* (D) expression was absent, but *str-2* and *str-3* expression was bright in the ASI neurons (E,F).

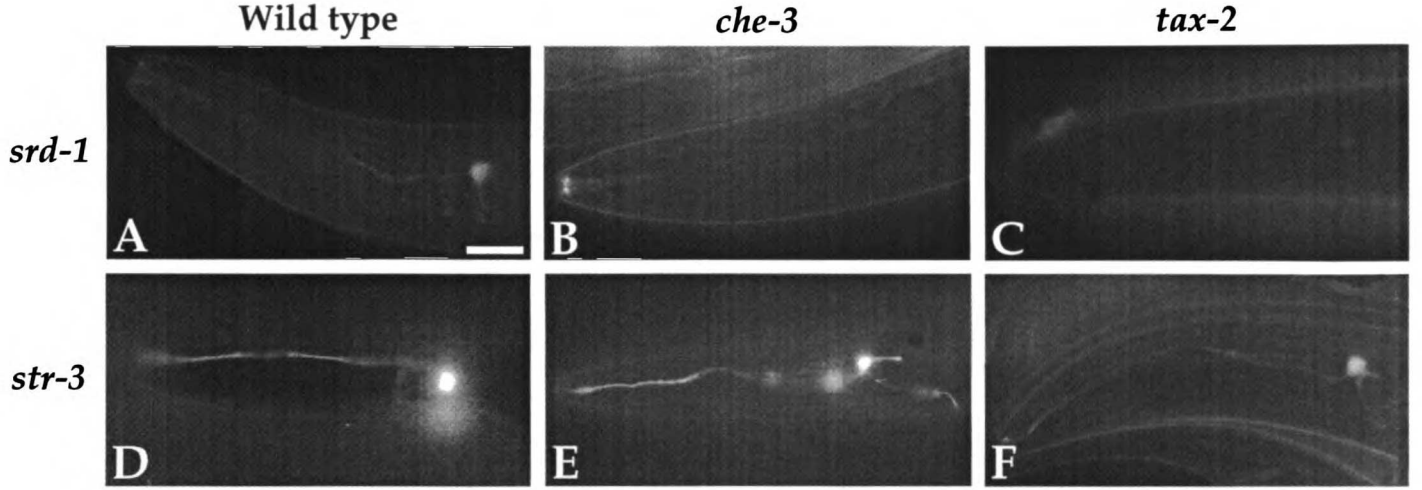

Figure 5-2. Sensory activity regulates *srd-1* receptor expression. Animals with defects in sensation were examined for alterations in chemosensory receptor expression. Their *srd-1::GFP* and *str-3::GFP* expression patterns are displayed. Scale bar=10 μm for all panels. *srd-1* was not expressed in *che-3* mutant animals, which have defects in their sensory cilia (B), or in *tax-2* mutants, which are defective in sensory signal transduction (C). *str-3* expression displayed wild-type levels (D) in both *che-3* (E) and *tax-2* (F) mutant animals. *str-2* expression also resembled that observed in wild-type animals (data not shown).

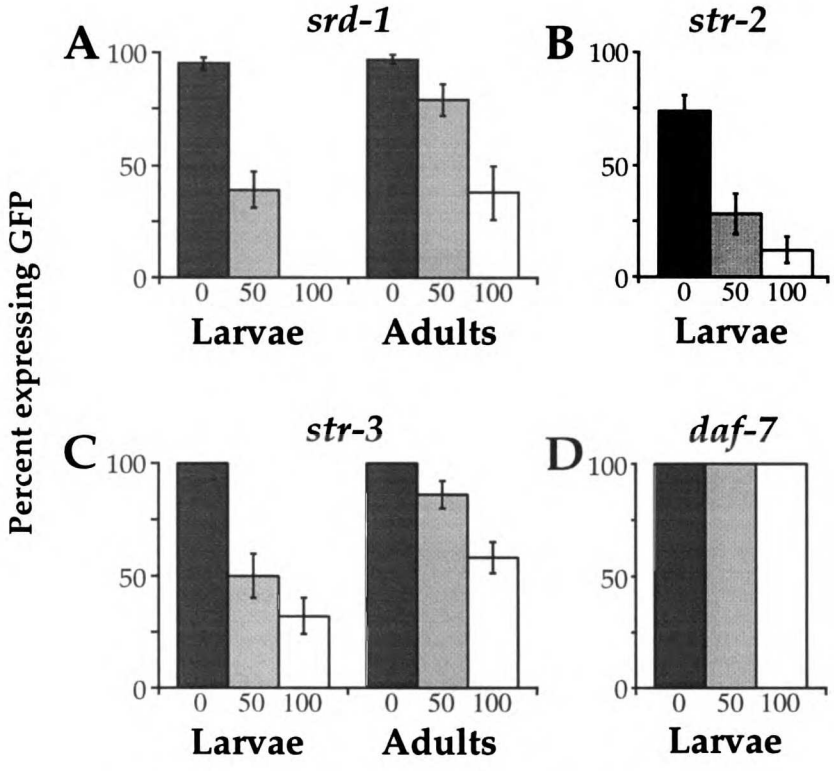

Figure 5-3. Pheromone suppresses *srd-1, str-2,* and *str-3* expression. The expression of GFP fusions with *srd-1* (A), *str-2* (B), *str-3* (C), and *daf-7* (D) was examined in animals exposed to different concentrations of pheromone: 0 µl (dark bars), 50 µl (gray bars), 100 µl (white bars) in a 2 ml plate (see Materials and Methods). At all concentrations, animals did not enter the dauer phase. Pheromone suppressed chemosensory receptor expression (A, B, C) but not *daf-7* expression (D) in both larvae and adults, and this suppression was dose-dependent with 100 µl suppressing more than 50 µl. The percentages of animals expressing any GFP, regardless of brightness, are tabulated for each strain. For each column, at least 50 animals were scored and error bars represent standard error of proportion. Adults were not examined for *str-2* or *daf-7* expression.

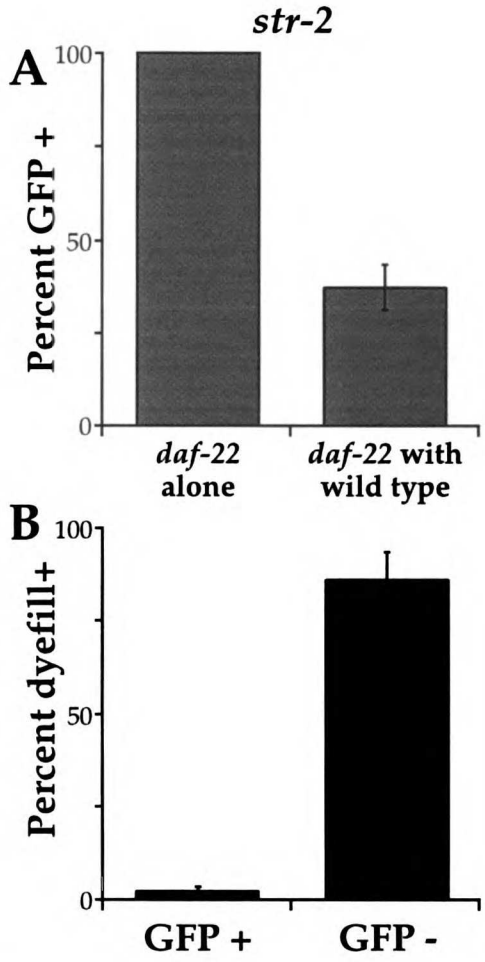

Figure 5-4. *daf-22* mutants, which fail to produce pheromone, display altered *str-2* expression. *str-2* expression was increased in *daf-22* mutants (A). The percentage of animals expressing *str-2* in ASI was determined (*daf-22* alone, n=89) and was decreased by exposing *daf-22;str-2::GFP* animals to wild-type, pheromone-producing animals (*daf-22* with wild type, n=55). Thus, pheromone could be the signal to repress *str-2* in ASI. To test whether dauer animals could have access to the pheromone signal, dauers were exposed to the vital dye DiI, which stains exposed neurons. *str-2* was highly expressed in dauer animals that did not stain with DiI (n=132) and not expressed in dauers that filled with DiI (n=42) (B). *srd-1* and *str-3* expression were unaltered in *daf-22* mutants compared to wild type (data not shown).

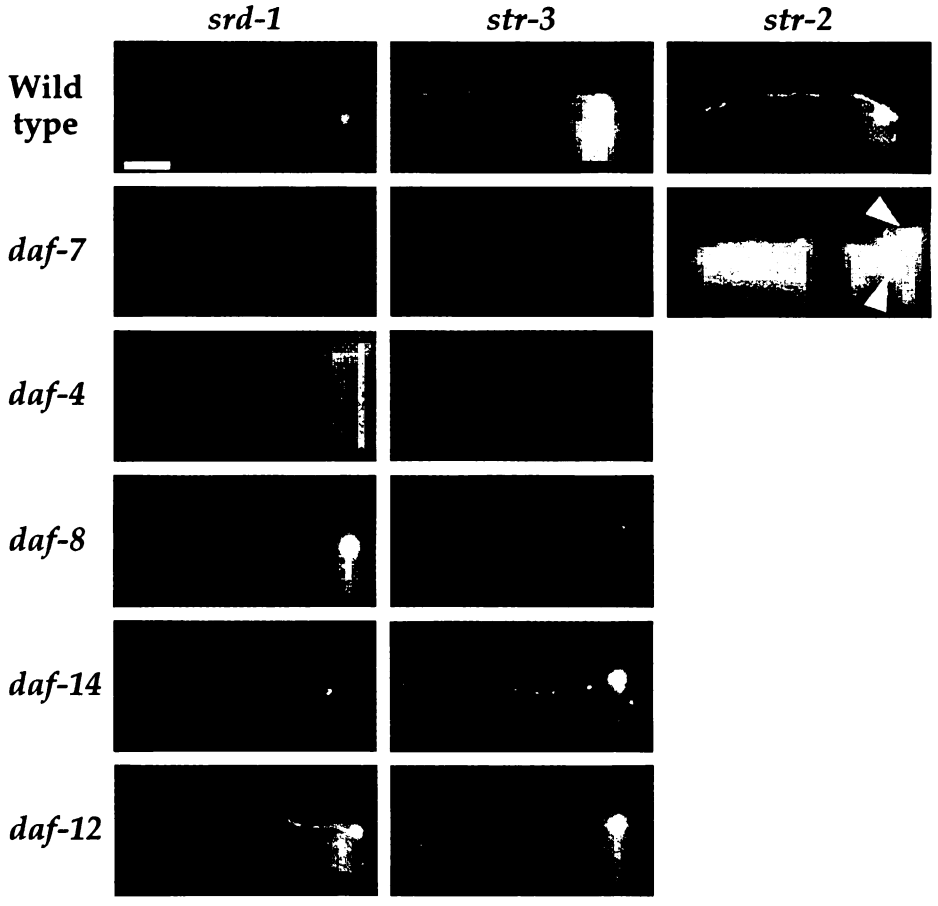

Figure 5-5. TGF-B ligand and receptor regulate chemosensory receptor expression. *srd-1* (left panel), *str-3* (middle panel), and *str-2* (right panel and data not shown) expression was examined in dauer pathway mutants (labeled on the left). Scale bar=10 μm for all panels. *srd-1* and *str-3* expression was low or absent in *daf-7* and *daf-4* mutants, which encode a TGF-B homolog and its receptor important for preventing dauer formation. *str-2* expression was bright in ASI neurons in these mutants (*daf-7*, two neurons are visible, ASI and AWC; *daf-4*, data not shown). *srd-1* expression was unaltered in *daf-8, daf-14, daf-5,* and *daf-12* mutants (bottom three panels). *str-3* expression was low in *daf-8* but not *daf-14* mutants, suggesting distinct roles for the two SMAD proteins. *str-2* expression was unaltered in *daf-8* and *daf-12* mutants (data not shown); *daf-5* and *daf-14* were not examined for *str-2* expression.

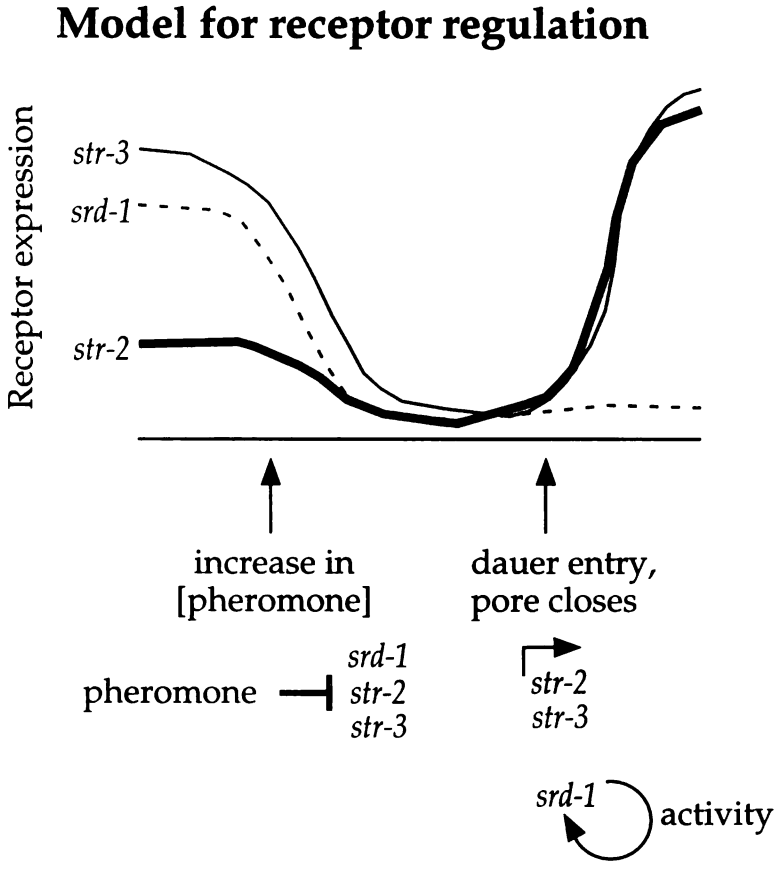

Figure 5-6. Model for chemosensory receptor regulation. Initially, ASI expression of *srd-1* and *str-3* is high, while *str-2* is low. As pheromone concentrations increase, expression of all three receptors decreases, and if harsh conditions persist, dauer development occurs. One consequence of dauer development is the retraction of the ASI cilia from the pore, relieving pheromone repression of these genes. While this derepression is sufficient for *str-2* and *str-3* to be expressed, a reduction in ASI activity prevents upregulation of *srd-1*.

Table 1. Genes required for receptor regulation in ASI

Strain	ASI expression		
	srd-1::GFP	***str-2::GFP***	***str-3::GFP***
Wild-type adults	+	-	+
Wild-type dauers	-	+	+
Activity mutants			
osm-6	-	-	+
che-3	-	-	+
tax-2	-	-	+
tax-4	-	-	+
Dauer mutants			
daf-22 (dafd)	+	+	+
daf-7 (dafc)	-	+	-
daf-4 (dafc)	-	+	-
daf-8 (dafc)	+	-	+
daf-14 (dafc)	+	n.d.	+
daf-5 (dafd)	+	n.d.	+
daf-12 (dafd)	+	-	+

(n.d., not done)

Chapter 6

Perspectives and future directions

I. The *C. elegans* chemosensory receptor repertoire (Chapter 2a,b)

C. elegans uses a simple nervous system to detect many chemicals in its environment. Ablation and genetic studies have demonstrated that 11 pairs of neurons are chemosensory, with each pair serving a distinct function (Bargmann and Mori, 1997). Using information from the genome sequencing project and expression studies, we have found that these neurons express a surprisingly large number of receptors. The genome contains about 1000 predicted G protein-coupled receptors (GPCRs) that fall into four large families, and our studies with promoter-GFP fusions suggest that half of these are expressed in chemosensory neurons. Therefore, *C. elegans* may have approximately 500 different functional kinds of chemosensory receptors. This large number of receptors highlights the importance of chemosensation for the nematode. Since *C. elegans* does not have vision or hearing, it uses detection of chemicals as the major source of information about the environment and thus dedicates a large portion of its genome toward encoding chemosensory receptors.

At this point our assessment of the extent of *C. elegans* chemosensation is fairly biased: chemicals are only known to be detected by *C. elegans* if they elicit a change in behavior. By simply testing more chemicals in different kinds of behavioral assays, the known chemosensory repertoire of *C. elegans* is likely to increase. However, an assay that measures events upstream of the behavioral response is needed to obtain a more complete picture of the *C. elegans* chemosensory repertoire. Electrophysiology and Ca^{++} imaging are two techniques that could be used to assess chemical detection by *C. elegans*.

These techniques can assess changes in the activity of a chemosensory neuron in response to different chemicals. The use of these techniques in *C. elegans* is in the early stages, but if developed further would be valuable additions to the study of *C. elegans* chemosensation.

The *sr*, *str* and other families of predicted GPCRs have many characteristics that suggest they encode chemosensory receptors. However, functional evidence is lacking for all but *odr-10*, the receptor for diacetyl. One straightforward approach to examining the function of these genes is to generate deletion mutants and test their responses to different chemicals. Such mutants could be obtained by screening deletion libraries. Two deletion mutants have been isolated from deletion libraries in our lab, and dozens have been isolated in other labs. While the success rates with deletion libraries can be variable, it would be feasible to generate deletion mutants for at least a few receptors.

What might the phenotype of these receptor mutants be? Based on results with *odr-10*, a receptor that is required for responses to only one odorant at low concentrations, one might expect that these animals would be defective in response to only one odorant, perhaps at low concentrations. Alternatively, it is possible that these receptors have broad specificity, and mutants will have many chemosensory defects. Another possibility is that they will have no phenotype, perhaps because of redundancy with other receptors. If this is the case, double mutants could be made between different receptors. Another way to knock out multiple receptors is by selecting for deletion of multiple receptors that are clustered together in the genome. Even if knock-outs of individual receptors demonstrate a phenotype, it will

be interesting to generate double mutants between receptors to determine if they are redundant for response to another odorant.

An alternative to the reverse genetics strategy described above is a forward genetics approach, as was successful in isolating the *odr-10* mutant. The screen that yielded *odr-10* was based on the assumption that a receptor mutation would cause a defect in response to one odorant, but not others sensed by the same neuron. For the case of *odr-10*, mutants defective in diacetyl, but not pyrazine chemotaxis were selected. A reciprocal screen was performed by Kayvan Roayaie to identify mutants defective in pyrazine, but not diacetyl chemotaxis (Roayaie, 1996). Theoretically, this screen could identify mutants defective in a pyrazine receptor. Pyrazine-specific mutants identified in this screen are being further characterized by Rob Rutherford. A similar screen could be performed to identify receptors for AWC-sensed odorants, such as a screen for mutants that respond to isoamyl alcohol, but not benzaldehyde. However, extensive benzaldehyde screens have yet to produce benzaldehyde-specific mutants (C.I.B., personal communication). This observation suggests that certain odorants, like benzaldehyde, may be sensed by multiple receptors. A mutation in one of these receptors might not cause obvious chemotaxis defects.

The odorant ligands for these receptors could also be identified through misexpression studies, either in *C. elegans* neurons or heterologous cell lines. By misexpressing an attractant-sensing receptor in a repellent-sensing neuron, responses to the attractant sensed by that receptor can be compromised (Chapter 3). I used this approach to search for a *str-2* ligand, with negative results (see Appendix B). However, many more odorants could

have been tested. Recently, improvements in heterologous expression of olfactory receptors have made possible the identification of ligands for rodent receptors (Krautwurst et al., 1998). In these studies, chimeras were made between olfactory receptors and other membrane receptors, which showed much better cell surface expression than normal olfactory receptors. Panels of odorants were applied to cells expressing these receptors to identify those that stimulated signalling and thus were ligands for the receptors. Similar experiments could be done with *C. elegans* receptors. I made a fusion between the *str-2* receptor and the 5HT3 receptor and sent it to Kai Zinn at Caltech for analysis. However, this fusion was expressed poorly. Fusions with the rhodopsin receptor might be more successful, as were used with rodent olfactory receptors in the Krautwurst et al study.

Some *sr* and *str* receptors were found in interneurons, making it unlikely that they are chemosensory receptors. Perhaps they perform a role in cell communication, such as neurotransmitter reception. Analysis of the subcellular localization of their gene products could provide insight into the types of pathways they serve.

II. Chemosensory signalling and information coding in *C. elegans* (Chapter 3)

Chapter 3 describes experiments in which the ODR-10 diacetyl receptor was misexpressed in the AWB neurons of *odr-10* null mutants, causing them to be repelled by diacetyl. Thus, ODR-10 expression could confer diacetyl sensitivity onto the AWB neurons. ODR-10 signalling in AWB required the same G protein as in AWA, but a different channel. The ODR-3 G protein is

expressed in both AWA and AWB, and ODR-10 signalling required ODR-3 in both cell types. However, the OSM-9 protein is expressed in AWA, but not AWB, and the TAX-2/4 channel is expressed in AWB, but not AWA. Based on these expression patterns, it is therefore not surprising that ODR-10 signalling in AWB required the TAX-2/4 channel instead of the OSM-9 channel.

Many chemosensory neurons, including the AWC neurons, express both the TAX-2/4 and OSM-9 channels (Coburn and Bargmann, 1996; Komatsu et al., 1996; Colbert et al., 1997). ODR-10 appears to be capable of signalling in AWC to elicit attraction to diacetyl (Sengupta et al., 1996; P. Sengupta, personal communication; Appendix B). It would be interesting to examine the channel requirements for ODR-10 signalling in the AWC neurons. Mutant analysis suggests that TAX-2/4 is the primary transduction channel in AWC, while OSM-9 has a modulatory role. Therefore, it is likely that ODR-10 signalling in AWC is mediated by the TAX-2/4 channel, rather than its normal downstream channel, OSM-9. However, this hypothesis has not been tested directly. This question could be addressed by examining diacetyl responses of *odr-10(ky225); str-2::odr-10* animals (see Appendix B for details) in a *tax-2*, *tax-4* or *osm-9* mutant background. These experiments could determine which channel is required for diacetyl attraction as mediated by ODR-10 signalling in AWC. If ODR-10 signalling in AWC requires TAX-2/4, it suggests that all receptors in AWC use TAX-2/4 as their transduction channel. On the other hand, if ODR-10 signalling in AWC requires the OSM-9 channel, it suggests that not all receptors in a cell are equivalent, and that receptors can contribute specificity to downstream signalling pathways.

Since the *str-2::odr-10* transgene only partially rescues the diacetyl defects of *odr-10* mutants, it might be easier to assess loss of diacetyl responses in the *odr-7;odr-3::odr-10* strain generated by Piali Sengupta. This strain has wild-type diacetyl responses, but has *odr-10* expression in other cells as well. It would be important to first determine that diacetyl attraction in this strain is mediated by AWC signalling. Laser ablations of the AWC neurons could be performed in this strain, or analysis in an AWC-defective background, like *odr-1*. Once it has been determined that diacetyl attraction of *odr-7;odr-3::odr-10* animals is due to ODR-10 signalling in AWC, this strain could be also be examined for loss of diacetyl response in a *tax-2*, *tax-4* or *osm-9* mutant background to determine which channel is required for ODR-10 signalling in AWC.

Laser ablation experiments had suggested that different neurons direct distinct behavioral outputs, but misexpression of ODR-10 in the AWB neurons tested this hypothesis in a more direct manner. Misexpression of ODR-10 in AWB instead of AWA caused diacetyl to elicit repulsion rather than attraction. This result indicates that sensory neurons and not receptors specify behavioral responses. However, the AWA and AWB pairs are each known to direct only one kind of behavioral response, while other cells, like the ASI and ASH neurons, probably direct multiple outputs. In these neurons, receptors are likely to play a role in specifying the output of a neuron. Rob Rutherford has been studying ODR-10 misexpression in ASI and ASH to explore signalling and information coding in these neurons.

The odr-10(AB) transgenic strain expresses *odr-10* in AWA endogenously and in AWB under the control of the *str-1* promoter. This strain had neutral

responses to diacetyl, possibly because it receives both attractive and repulsive signals from diacetyl that interfere with each other. Simultaneous activation of *odr-10* in both the AWA and AWB neurons might mimic the effect of an attractant and repellent mixture. Because chemosensation in the wild involves simultaneous detection of multiple chemicals, this strain might provide an interesting model for studies of signal integration. One problem with the odr-10(AB) strain was that it became more attracted to diacetyl over many generations, even though the *str-1::odr-10* transgene was integrated. A progressive reduction in transgene expression over multiple generations has been observed in other integrated strains as well (e.g. the *kyIs140 I str-2::GFP* strain, E.T. unpublished observations). One possible improvement on this strain might be to remake it in an *odr-10* null background, using two different *odr-10* transgenes, one with an AWA promoter and one with an AWB promoter. In this way, expression from both transgenes might decline similarly over time. While not a perfect solution, at least the relative contributions of each neuron should not change.

III. Asymmetry of *str-2* expression in the AWC neurons

(Chapter 4)

Chemosensory neurons in *C. elegans* exist in bilaterally symmetric pairs. In the past it was thought that each neuron in a pair was probably equivalent and functionally redundant. However, it was recently shown that the ASE neurons can be distinguished from one another molecularly (Yu et al., 1997). Expression of three receptor guanylyl cyclases in the ASE neurons is asymmetric: one is only expressed in the right ASE neuron and two are only expressed in the left ASE neuron. We have found that the two AWC

neurons can be distinguished from one another by expression of the *str-2* candidate odorant receptor. *str-2* expression is stochastically asymmetric; half of the animals in a population express *str-2* in the left AWC while the other half express *str-2* in the right AWC neuron.

A model for regulation of *str-2* asymmetry is described in detail in the discussion section of Chapter 4. Briefly, the default state for *str-2* expression is off before outgrowth of the AWC axons. Once contact is made between the two AWC neurons, *str-2* expression can be specified in one of them. This specification involves Ca^{++} signalling early, which turns off *str-2* expression in one cell, and cGMP signalling later, which keeps *str-2* expression on in one cell.

The cell autonomy of molecules mediating changes in *str-2* expression is unknown. Among the players so far identified in Ca^{++} signalling are the *unc-2/36* voltage-gated Ca^{++} channel and the *unc-43* CaMKII, while those involved in cGMP signalling are the *daf-11* and *odr-1* guanylyl cyclases and the *tax-2/4* cGMP-gated channel. *unc-2/36/43* are widely expressed (Schafer, W., Thomas, J., personal communications), so it would be interesting to examine whether they act in the AWC neurons or elsewhere to affect *str-2* expression. This question could be addressed with misexpression of these genes under the *odr-3* or *odr-1* promoters, which would restore wild-type function in just a few chemosensory neurons. If these constructs rescued the effects on *str-2* expression, it would suggest that they are acting within the AWC neurons. Alvaro Sagasti has plans to conduct such experiments.

Because no promoters other than the *str-2* promoter are known to be asymmetrically expressed in the AWC neurons, it is not possible to use misexpression analysis to determine whether these genes act in AWC^{ON} or AWC^{OFF}. In order to address this question, mosaic analysis could be performed. To this end, I have generated reagents for a two-color fluorescent protein system. I have made *str-2::CFP* integrated strains (three independent *str-2::CFP* integrated strains were obtained, all of which express CFP asymmetrically in the AWC neurons) and an *odr-3::YFP* transgene. Mosaics could be generated in a *str-2::CFP* background by co-injecting genes to be analyzed with *odr-3::YFP* to make unstable extrachromosomal array lines. Loss in the gene of interest could be assessed by loss of YFP expression and the resulting changes in *str-2* expression could be assessed with CFP expression. Alvaro will continue this work.

Many of the players in regulation of *str-2* asymmetry have yet to be identified. Most notably, the molecules that mediate communication between the two AWC axons and the transcription factor(s) that affect *str-2* expression are as yet unknown. The screen for *str-2* asymmetry mutants performed by Alvaro yielded mutants in seven complementation groups, four of which are in known genes, as described in Chapter 4. The remaining three complementation groups might represent genes that serve in communication and transcription.

The screen performed by Alvaro was designed to identify mutants in which both AWC cells express *str-2*. This screen would have missed molecules important for activating *str-2* expression. If the lateral signalling pathway regulating *str-2* expression is analogous to the Notch lateral signalling

pathway, this might include molecules used for communication between the two AWC neurons. If Notch signalling is defective, it prevents the signalling between two equivalent cells that allows one of them to achieve a fate other than the default state. Since the default state for *str-2* expression is off, a mutation in analogous signalling molecules might lead to neither cell expressing *str-2*. Therefore, a screen for mutants in which both AWC neurons are off might identify such molecules as well as provide further insight into this signalling pathway.

It is interesting to speculate about the universality of the *str-2* regulation pathway. All lateral signalling events examined so far in other systems are regulated by Notch signalling, whereas *str-2* expression does not appear to be. Perhaps regulation of *str-2* expression is representative of a class of cell fate decisions that are mainly determined by molecular instead of morphological criteria. Most cell fate decisions studied in the past were those that resulted in obvious differences of cell appearance. A new class of cell fate decisions are now being recognized with the advent of molecular markers, which create distinctions between cells previously thought to be identical. An example of this is the mammalian olfactory epithelium, which contains millions of neurons that appear similar but can be distinguished by olfactory receptor expression (Buck and Axel, 1991). Perhaps cell fate decisions that result in fine distinctions between cells will use an activity-regulated signalling pathway similar to the one used for *str-2* expression.

Although we have gained insights into the mechanism of *str-2* asymmetry, the reasons for its regulation are unclear. We speculate that other AWC receptors will be expressed asymmetrically, although no other AWC receptors

have yet been identified. One reason for asymmetric receptor expression might be to create two different AWC neurons that each have a distinct olfactory specificity. Two preliminary experiments described in Appendix C argue against co-regulation of all AWC receptors with *str-2* into either AWC^{ON} or AWC^{OFF}, to create two AWC neurons with distinct specificity. However, both experiments could have been pursued further and one has a flaw. An alternative possibility is that every AWC receptor is regulated independently, which would create a distinct olfactory specificity for each AWC neuron that is unique in every animal. To determine if this is the case, individual transgenic or ablated animals could be assayed multiple times to identify trends in their olfactory defects. However, a third possibility is that asymmetric receptor expression does not lead to two AWC neurons with distinct olfactory specificities. Differential receptor expression in the two AWC neurons might then serve some comparative function, perhaps for odorant discrimination or adaptation. It would be interesting to test animals that only have a single AWC neuron for their ability to discriminate or adapt to different odorants.

IV. Regulation of chemosensory receptor expression (Chapter 5)

We have found that chemosensory receptor expression is regulated by pheromone and entry into dauer, an alternative larval phase. For example, *str-2* is only expressed at low levels in the ASI neurons in well-fed larvae and adults, but is upregulated upon entry into dauer. These effects appear to be mediated by some of the *daf* genes, which regulate entry into the dauer phase. Two of these, *daf-7* and *daf-4*, encode a TGF-ß ligand and receptor,

respectively, required to repress dauer formation (Estevez et al., 1993; Ren et al., 1996). In *daf-7* and *daf-4* mutants, *str-2* is expressed at inappropriately high levels in the ASI neurons of well-fed animals. Genes that function downstream of *daf-7* and *daf-4* are known, so it would be interesting to determine whether they also affect *str-2* expression in the ASI neurons (Thomas et al., 1993). For example, *daf-5* and *daf-12* are both genes that suppress *daf-7* and *daf-4* function in dauer formation. It would be interesting to determine if they also suppress the effects on *str-2* expression in the ASI neurons.

The substantial changes seen in chemosensory receptor expression suggest that dauer chemosensory behavior is also altered. Dauer locomotory and thermosensory behavior is significantly different from well-fed animals (Riddle and Albert, 1997), but chemosensory behavior has not been extensively analyzed. It would be interesting to determine the extent of chemosensory responses in the dauer phase. One potential problem with measuring dauer chemosensation is that dauer behavior can be erratic, so it might be difficult to design a reproducible behavioral assay. Assays that measure the activity of chemosensory neurons would be very useful for these experiments. Previous studies had shown that the chemosensory neurons of dauer larvae undergo significant morphological changes (Albert and Riddle, 1983), and we find that they undergo several molecular changes as well. Correlating these changes with behavioral modifications could provide insights into the organization of the chemosensory system, as well as the manner in which animals adapt to a changing chemical environment.

Overview

Understanding how animals sense and respond to their chemical environment is a complex and challenging problem. Over the last few years it has become apparent that *C. elegans* chemosensory signalling bears many similarities to vertebrate signalling; both systems use receptors of similar structure and number, which activate effectors of a common type. One of the advantages of studying chemosensation in *C. elegans* instead of vertebrates is that the nematode provides a tractable system where one can study not only the molecules, but also the behavior of an organism. Work in this thesis describes a receptor misexpression experiment that provided insight into the specification of *C. elegans* behavioral responses to odorants. By misexpressing the diacetyl receptor in another neuron, diacetyl elicited repulsion instead of attraction, suggesting that sensory neurons and not receptors encode behavioral responses. Taking these studies further downstream to the level of sensory to interneuron signalling could provide a more complete understanding of how odorant detection leads to a behavioral response. Perhaps one day it will be possible to define an entire behavioral circuit, including odorant detection, input processing and behavioral response.

Chemosensation in the natural environment involves processing multiple cues under a variety of conditions. Again, the simple nervous system and defined behavioral responses of *C. elegans* provide a good model in which to explore how animals process multiple inputs in different contexts. Our studies of receptor regulation in response to pheromone and dauer entry suggest that long-term regulation of olfactory behavior might occur on the level of receptor expression. It will be important to correlate these changes in receptor expression with alterations in behavior, which should be easier

when more receptors are matched with ligands. In addition, the use of techniques like microarray analysis could provide a more comprehensive picture of the changes in receptor repertoire that occur under different conditions.

Investigations with the *str-2* receptor suggest that studies of *C. elegans* chemosensation may also provide insights into sensory neuron development. The two superficially similar AWC neurons are actually distinct from one another, as visualized by *str-2* receptor expression. A novel, activity-regulated pathway involving cell communication appears to underlie this regulation. Perhaps a similar pathway will be found to regulate vertebrate olfactory receptor expression or even neuronal specification in other systems as well.

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Appendix A

cDNA isolation of *str-2* and F10D2.4, the *odr-10* twin.

Two of the *str* genes identified in Chapter 2b had features that made them sufficiently interesting to warrant cDNA isolation. The *str-2* gene encodes a candidate olfactory receptor expressed in the AWC neurons that has interesting regulation of its expression (see Chapters 4 and 5). F10D2.4 is the closest relative to the diacetyl receptor *odr-10*: they are 83% identical by amino acid sequence (see Chapter 2b). The high similarity between these two receptors might make them an interesting pair for structure-function studies. I therefore isolated the cDNAs of both *str-2* and F10D2.4 (the *odr-10* twin) by RT-PCR.

Total RNA was prepared from mixed stage wild-type N2 animals by Trizol extraction (GIBCO). Both cDNAs were obtained in two pieces, which were ligated together to create a full-length cDNA. First-strand cDNA was reverse transcribed from RNA using a gene-specific primer for the first piece and an oligo-dT adaptor primer for the second piece. The first piece was then amplified in a first reaction with SL1 and a gene-specific primer and then subjected to a second round of amplification with nested primers, while the second piece was amplified in a first reaction with a gene-specific primer and the oligo-dT adaptor primer and then subjected to a second round of amplification with nested primers. These two pieces were ligated together into the PCRII vector (Invitrogen) using a restriction site in the overlapping region between the two.

PCR:	1st reaction	2nd reaction
str-2 cDNA:		
Piece #1	SL1+ET16	ET15+C50.7-A
Piece #2	ET15+AP	C50.7-D+ET22

F10D2.4 cDNA:

Piece #1	SL1+ET20	ET19+F10.1-A
Piece #2	ET19+AP	F10.1-D+ET20

The constructs that have *str-2* and F10D2.4 cDNAs in the PCRII vector are called pET87 (TA+C) and pET80 (TA+F), respectively.

The F10D2.4 cDNA corresponds exactly to the Genefinder prediction. The third exon of the C50C10.7 cDNA differs slightly from the updated version of the Genefinder prediction made by Hugh Robertson. The predicted nucleotide annotation of this exon was complement(join(18304..18931)) on the C50C10 cosmid, while the cDNAs isolated have complement(join(18313...18931)).

A *str-2* expression vector (pET88) was created by replacing GFP with the *str-2* cDNA in the Fire vector, pPD95.77. The *str-1* and *odr-10* promoters were placed upstream of the *str-2* cDNA in pET88 to create *AWB::str-2* (pET91) and *AWA::str-2* (pET90), respectively. Results with these misexpression constructs are described in Appendix B.

Appendix B

Receptor misexpression and behavioral analysis

Misexpression experiments with *odr-10* in the AWB neurons indicated that chemosensory receptors could function in heterologous neurons. I therefore performed several receptor misexpression experiments to address questions about signalling and receptor specificity. The following experiments gave mostly negative results.

I. The search for a *str-2* ligand with *AWB::str-2* and *AWA::str-2* transgenic animals.

Chapter 3 describes results with misexpression of the *odr-10* diacetyl receptor in the AWB neurons. *odr-10* is normally expressed in the AWA neurons, where it directs attraction to diacetyl. *odr-10* expression in AWB conferred diacetyl sensitivity onto these neurons, and directed repulsion, rather than attraction to diacetyl. Simultaneous expression of *odr-10* in both AWA and AWB led to a reduced attraction to diacetyl. Thus, misexpressing a receptor for an attractant in the AWB neurons could interfere with responses to that attractant. *str-2* is likely to detect attractive odorants based on its endogenous expression pattern. To identify odorants that might be ligands for *str-2*, I misexpressed *str-2* in the AWB neurons with the idea that this might lead to reduced attraction toward these odorants. Transgenic animals with the *AWB::str-2* transgene were analyzed for chemotaxis defects to volatile attractants known or likely to be detected by the AWC neurons. Construction of the *AWB::str-2* transgene is described in Appendix A.

Four independent *AWB::str-2* transgenic lines were obtained and a list of the odorants tested in chemotaxis assays with these animals is listed in Table B-1. Odorants known to be detected by the AWC neurons were the first candidates

to be tested, namely benzaldehyde, isoamyl alcohol, and butanone. Other odorants known to be attractive to *C. elegans* were also tested. No odorants elicited significantly different responses from wild-type and *AWB::str-2* animals. In initial assays, 1-pentanol elicited less attraction from *AWB::str-2* transgenic animals than wild-type animals (Figure B-1). Subsequent re-testing failed to reproduce significant differences.

odr-10 expression from the *odr-3* promoter is sufficient to rescue the diacetyl defect of the AWA-defective *odr-7(ky4)* animals. The *odr-3* promoter drives expression in a number of neurons, but *odr-3::odr-10* is likely to be acting in the AWC neurons in these animals to mediate attraction to diacetyl (Sengupta et al., 1996; P. Sengupta, personal communication). These experiments suggest that an AWA receptor can function in the AWC neurons. If the converse were true, *str-2* should be able to function in the AWA neurons. AWA expression of *str-2* in an AWC-defective mutant might then restore chemotaxis to the odorant sensed by *str-2*, but not other odorants. An *AWA::str-2* construct was injected into the AWC-defective *odr-1(ky29)* mutant, and responses of animals carrying this transgene were tested to two odorants (benzaldehyde and 1-pentanol). Two independent *odr-1;AWA::str-2* transgenic lines were isolated, and no significant differences were seen between their responses and the responses of wild-type animals (see Figure B-1 for 1-pentanol). Construction of the *AWA::str-2* transgene is described in Appendix A.

II. Does expression of *str-2* in both AWC neurons interfere with chemotaxis?

str-2 is normally expressed in only one of the two AWC neurons. This regulation may be important for chemotaxis, odorant discrimination or adaptation. To ask if *str-2* expression in both AWC neurons could affect chemotaxis, animals carrying an *odr-3::str-2* transgene were tested for their chemotaxis responses. Four independent lines were tested, and all showed normal responses to benzaldehyde and isoamyl alcohol. The *odr-3::str-2* construct was able to disrupt expression of the *str-2::GFP* promoter in other transgenic lines, suggesting that this transgene has functional activity. However, the *odr-3::str-2* lines that disrupted *str-2::GFP* expression could not be tested for chemotaxis, because they carried the dominant *rol-6* marker, which prevents normal movement. It would be interesting to test the responses of *odr-3::str-2* animals in cross-saturation or adaptation assays.

III. Can expression of F10D2.4 (the *odr-10* twin) in AWA rescue the *odr-10* diacetyl defect?

The high similarity in sequence between the diacetyl receptor *odr-10* and F10D2.4 led to the question of whether the F10D2.4 receptor might also be able to detect diacetyl. The F10D2.4 cDNA (described in Appendix A) was fused to the *odr-10* promoter, which drives expression in the AWA neurons. The AWA::F10D2.4 construct was then injected into *odr-10(ky225)* animals and their responses to diacetyl were tested. Four independent transgenic lines were tested and none showed substantial rescue of the diacetyl defect.

IV. Can *odr-10* function in the AWC neurons?

Experiments described in Part I suggested that *odr-10* can function in the AWC neurons. To confirm this, I generated a transgene that should drive strong expression of *odr-10* only in the AWC neurons. The *str-2* promoter was fused to *odr-10*, transformed into *odr-10(ky225)* animals and their responses to diacetyl were assayed. Three independent lines were assayed for their response to diacetyl, and all three showed partial rescue of the diacetyl defect (Figure B-2). The partial rescue does not appear to be due to low level expression of *odr-10* in AWC: an *str-2::ODR-10-gfp* fusion is expressed at high levels and localizes to the cilia of one AWC neuron. Why was there not more complete rescue, as was seen for *odr-3::odr-10*? The *str-2* promoter only drives expression in a single AWC neuron - perhaps efficient diacetyl detection requires expression in both neurons. Alternatively, expression in neurons other than AWC may contribute to the diacetyl response mediated by *odr-3::odr-10*. Another possible explanation for the discrepancy could lie in a difference between the *odr-7* and *odr-10* mutant backgrounds. Assays with the *str-2::odr-10* transgene were done in an *odr-10* mutant background, while *odr-3::odr-10* rescue was assessed in an *odr-7* mutant background. *odr-7* mutants can express AWC markers ectopically (Sagasti et al, submitted), so perhaps *odr-7* mutants have increased AWC function.

V. Can a rat odorant receptor function in worms?

Heterologous and misexpression studies with rodent receptors have led to the isolation of odorant ligands for a few receptors. One of these receptors is the rat I7 receptor, which detects the odorant heptaldehyde (Krautwurst et al., 1998; Zhao et al., 1998). To determine if expression of I7 in the AWA neurons could mediate attraction to heptaldehyde in *C. elegans*, I expressed the I7

cDNA under the *odr-10* promoter and assayed transgenic animals carrying the AWA::I7 transgene. Wild-type animals were neither attracted nor repelled by heptaldehyde, and animals with the AWA::I7 construct had a similar neutral response to heptaldehyde, suggesting that I7 was not able to function in the AWA neurons.

To determine whether the I7 receptor is expressed and properly localized in the AWA neurons, an AWA::I7-GFP transgene was made, with GFP at the C terminus. A rotation student, Levente Egry, PCR amplified the AWA::I7 construct with BglII and HindIII sites on the end. I cut out this AWA::I7 piece and placed it into the BamHI and HindIII sites of pPD95.79. AWA::I7 was injected into wild-type animals and four independent lines were examined for expression. GFP expression was seen rarely, and only in the cell body, excluded from the nucleus. Therefore, the failure of I7 to mediate attraction to heptaldehyde is likely due at least in part to poor receptor expression and localization.

Materials and methods

All transgenic animals were generated by injecting the transgene of interest at 50 ng/µl with the *lin-15* clone pJM23 at 50 ng/µl (Huang et al., 1994) into *lin-15(n765)* animals. Transgenic animals were identified by rescue of the *lin-15(n765ts)* multivulval phenotype at 20°C. Standard chemotaxis assays were performed as described (Bargmann et al., 1993).

Table B-1. Odorants tested in chemotaxis assays with *AWB::str-2* transgenic animals.

benzaldehyde (1:100, 1:200)^
benzonitrile*
beta-mercaptoethanol
1-butanol*
butanone (1:1000)
ethyl propionate
fufurol*
2-hexanone
isoamyl alcohol
2-octanone
1-pentanol (1:100, 1:1000)^
2-pentanol
2-pentanone
2-acetyl thiazole*
2,4 dimethyl thiazole
2,4,5 trimethyl thiazole (1:10, 1:100)
p-tolualdehyde

All odorants tested at 1:100 dilution in ethanol, unless otherwise noted. All odorants tested with at least two different *AWB::str-2* transgenic lines, except asterisked odorants, which were only tested in two assays with one transgenic line. ^ indicates odorants tested with *AWA::str-2* transgenic strains as well.

Figure B-1. Response of *AWB::str-2* and *odr-1;AWA::str-2* transgenic animals to 1-pentanol.

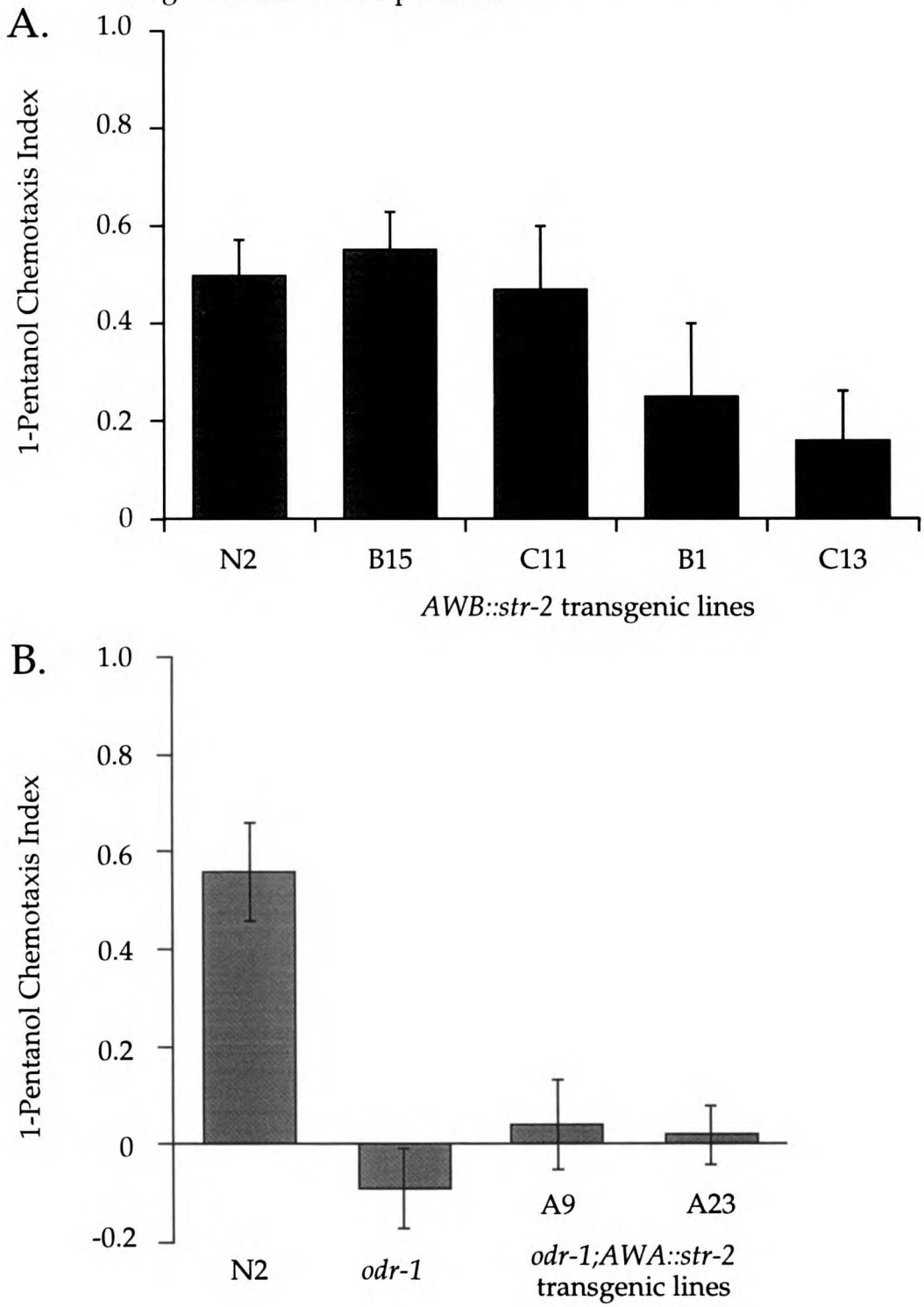
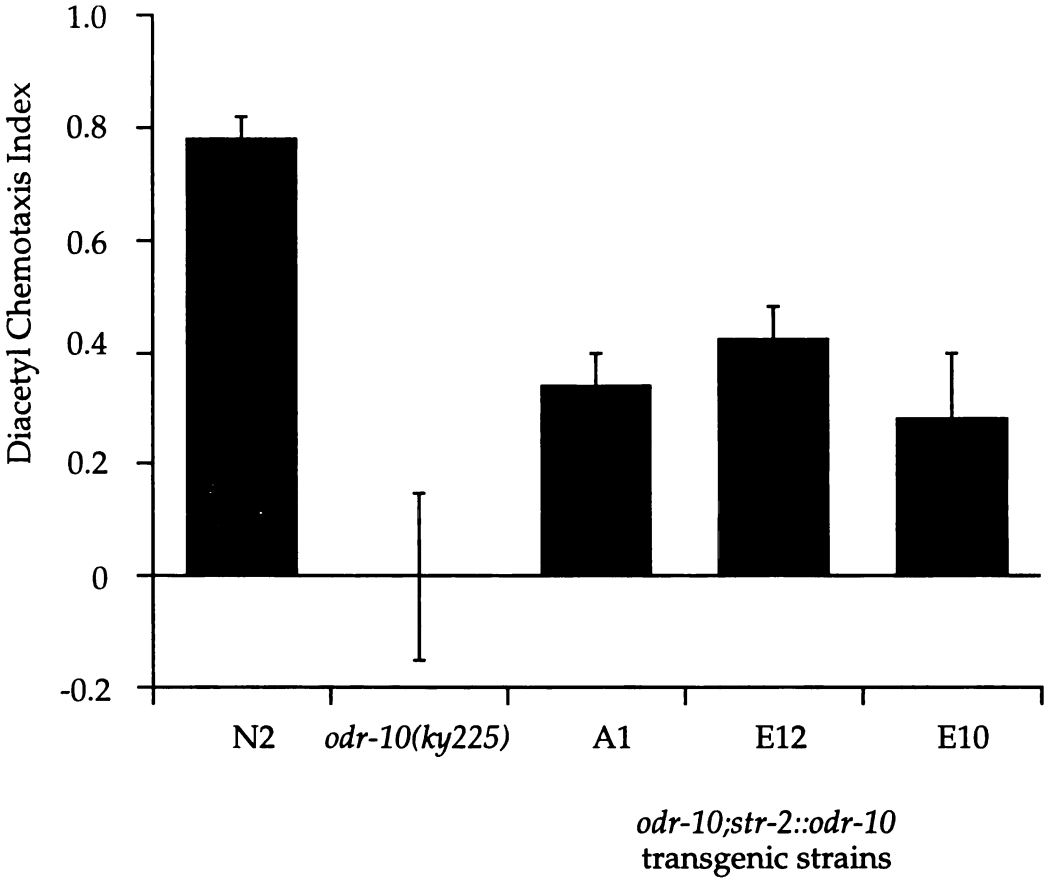


Figure B-1. Response of *AWB::str-2* and *odr-1;AWA::str-2* transgenic animals to 1-pentanol. 1-pentanol was diluted 1:100 in ethanol. At least six different assays were performed on three different days for *AWB::str-2 strains*, and four different assays were performed for *odr-1;AWA::str-2* strains. Error bars are SEM.

Figure B-2. Diacetyl chemotaxis is partially restored in *odr-10;str-2::odr-10* transgenic strains. Diacetyl was diluted 1:1000 in ethanol. At least eight assays were performed on three separate days for each transgenic strain. Error bars are SEM.



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Appendix C

Analysis of AWC olfactory specificity

The AWC pair of neurons are known to detect at least five different attractive odorants by laser ablation studies (Bargmann et al., 1993, P. Sengupta and C.I.B., personal communication). These experiments were performed by killing both of the AWC neurons and testing the chemotaxis responses of operated adults. However, asymmetric expression of the *str-2* receptor in these neurons reveals an underlying difference between the two cells. This observation suggested that perhaps all AWC receptors are expressed asymmetrically, resulting in two AWC neurons with distinct olfactory capabilities. I conducted two preliminary experiments to ask whether *str-2*-expressing AWC neurons (AWC^{ON}) have different olfactory specificity than non-*str-2* expressing neurons (AWC^{OFF}). The first experiment was designed to ask what odorants AWC^{ON} was sufficient to detect. The second experiment was designed to ask what odorants AWC^{ON} was required to detect.

I. Sufficiency experiment: Does *str-2::tax-2* expression in a *tax-2* background restore a subset of the AWC defects in *tax-2* mutants?

The TAX-2 cyclic nucleotide-gated channel subunit is required for AWC function and is expressed in both AWC neurons (Coburn and Bargmann, 1996). By expressing *tax-2* under the *str-2* promoter in a *tax-2* mutant background, function might be restored to just AWC^{ON}. Testing the odorant responses of these animals might therefore indicate the odorants detected by AWC^{ON}. For example, if AWC^{ON} detects benzaldehyde, while AWC^{OFF} detects isoamyl alcohol, *tax-2;str-2::tax-2* animals would respond to benzaldehyde, but not isoamyl alcohol.

str-2::tax-2 was co-injected with the widely expressed *sax-1::GFP* transgene (Zallen et al., in prep.) into *tax-2(p691)* animals. The chemotaxis responses of two independent lines were compared to a control *tax-2* strain that had been injected with *sax-1::GFP* only (Figure C-1). Responses to four attractive odorants were tested, three of which are known to be detected by the AWC neurons (butanone, isoamyl alcohol and benzaldehyde) and the fourth (isoamyl acetate) is likely to be detected by the AWC neurons. *str-2::tax-2;tax-2* mutants had wild-type chemotaxis responses to all four odorants, suggesting that *tax-2* function was rescued in the AWC neurons. Responses to the ASE-sensed attractant NaCl were also tested to determine whether the effects were cell-specific. *str-2::tax-2;tax-2* animals behaved like *tax-2* alone, indicating that ASE function was not restored with this transgene.

The results of this experiment suggest that AWC^{ON} is sufficient to detect all four odorants tested. However, many other odorants are likely to be sensed by the AWC neurons and responses to those odorants could be tested with this strain, as long as *tax-2* is defective in response to them. If responses to all AWC-sensed odorants are restored in this strain, it suggests that AWC^{ON} expresses all the receptors necessary for the normal repertoire of AWC responses. Assuming that all AWC receptors are expressed asymmetrically, two possible explanations could account for this result. One possibility is that AWC^{ON} expresses all the receptors used by AWC for chemotaxis and AWC^{OFF} expresses none of them, and is therefore not a chemosensory neuron. This idea seems unlikely, being that AWC^{ON} and AWC^{OFF} have almost identical morphologies and downstream targets (also, see Part II). A more likely explanation is that there is redundancy in the receptors used for AWC-sensed odorants (see Perspectives and future directions, part I). The

repertoire expressed by AWC^{ON} and AWC^{OFF} would then be different, but each neuron would have the full detection capabilities of the entire AWC repertoire.

The two possibilities for receptor segregation discussed above assume that asymmetric expression of all receptors will be coordinated with *str-2*, with one group of receptors expressed in AWC^{ON} and a separate group in AWC^{OFF}. However, a third possibility is that receptors might be regulated independently of *str-2*, leading to many different patterns of receptor segregation in the two AWC neurons across a population of animals. For example, the benzaldehyde receptor might sometimes be expressed in the same cell as *str-2*, and sometimes not. If this were true, restoring function to AWC^{ON} could restore different odorant responses in different animals. Then, population chemotaxis assays of *str-2::tax-2;tax-2* animals would show partial responses to different odorants. Responses to isoamyl acetate were not fully restored, consistent with this possibility.

One major flaw with this experiment is that *str-2* expression is affected in *tax-2* mutants, and thus expression of *tax-2* may not be asymmetric in every animal. Expression from the *str-2* promoter is down and sometimes on in both cells of *tax-2* mutants (see Chapter 4), and it is unclear if the *str-2::tax-2* transgene can rescue that defect. I constructed a *str-2::tax-2-GFP* transgene to assess whether *tax-2* expression from the *str-2* promoter was asymmetric. However, expression of the *str-2::tax-2-GFP* transgene was too weak to confidently determine that it is always asymmetric. When the *str-2::tax-2* transgene was crossed into *tax-2; str-2::GFP (kyIs136X)* animals to assess expression from the *str-2* promoter, expression from the *str-2::GFP* transgene

actually became weaker. This effect may be due to promoter squelching, since there are two separate multi-copy arrays with the *str-2* promoter.

It would be better to do this experiment in an AWC mutant background where *str-2* asymmetry is not affected. For example, *str-2::GFP* asymmetry is normal in *odr-4* mutants, but AWC function, including localization of STR-2::GFP is defective. By introducing a *str-2::odr-4* transgene into *odr-4* mutants, *odr-4* function and therefore STR-2 localization should only be restored in AWC^{ON}. This strain could be tested for its responses to various AWC odorants. However, *odr-4* mutants are not completely defective in AWC function, so there would not be as many responses to test. Another possibility would be to do this experiment in an *odr-3* mutant background, since *odr-3* mutants have broader defects and *str-2::GFP* asymmetry is also normal in this background. However, overexpression of *odr-3* results not only in chemotaxis defects, but also misregulation of the *str-2* promoter, so expression levels from the *str-2::odr-3* transgene would have to be carefully regulated to rescue chemotaxis and not affect the *str-2* promoter.

II. Requirement experiment: Does killing AWC^{ON} lead to chemotaxis defects?

A more direct way to assess AWC^{ON} and AWC^{OFF} function is to perform ablations of these cells and determine which odorant responses are lost. Because it is technically easier to ablate AWC^{ON}, I performed such ablations in the L1 stage, and then analyzed adult animals for their responses to AWC-sensed odorants (Table C-1). Ablations and assays were done as described, with numbers reported in Table C-1 as the fraction of animals with positive

assays (Bargmann et al., 1993). The results of these experiments suggest that AWC^{ON} is not required for responses to isoamyl alcohol or benzaldehyde and thus AWC^{OFF} is sufficient for them. Taken together with the results from the *str-2::tax-2;tax-2* experiment (which has flaws), it suggests that either AWC^{OFF} or AWC^{ON} alone are sufficient to sense both isoamyl alcohol or benzaldehyde. This result supports the second model suggested above, that AWC^{ON} and AWC^{OFF} each express a repertoire of receptors sufficient to accommodate all AWC responses. Identification of more AWC receptors is needed to determine whether that repertoire is the same, or different. However, responses to benzaldehyde in AWC^{ON}-ablated animals appeared reduced compared to wild-type, which might be due to regulation of the benzaldehyde receptor(s) that is independent of the *str-2* receptor (see possibility three in Part I). More data is needed to make these results significant.

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Coburn, C. M., and Bargmann, C. I. (1996). A putative cyclic nucleotide-gated channel is required for sensory development and function in *C. elegans*. Neuron *17*, 695-706.

Zallen, J., Peckol, E., and Bargmann, C. I., in preparation.

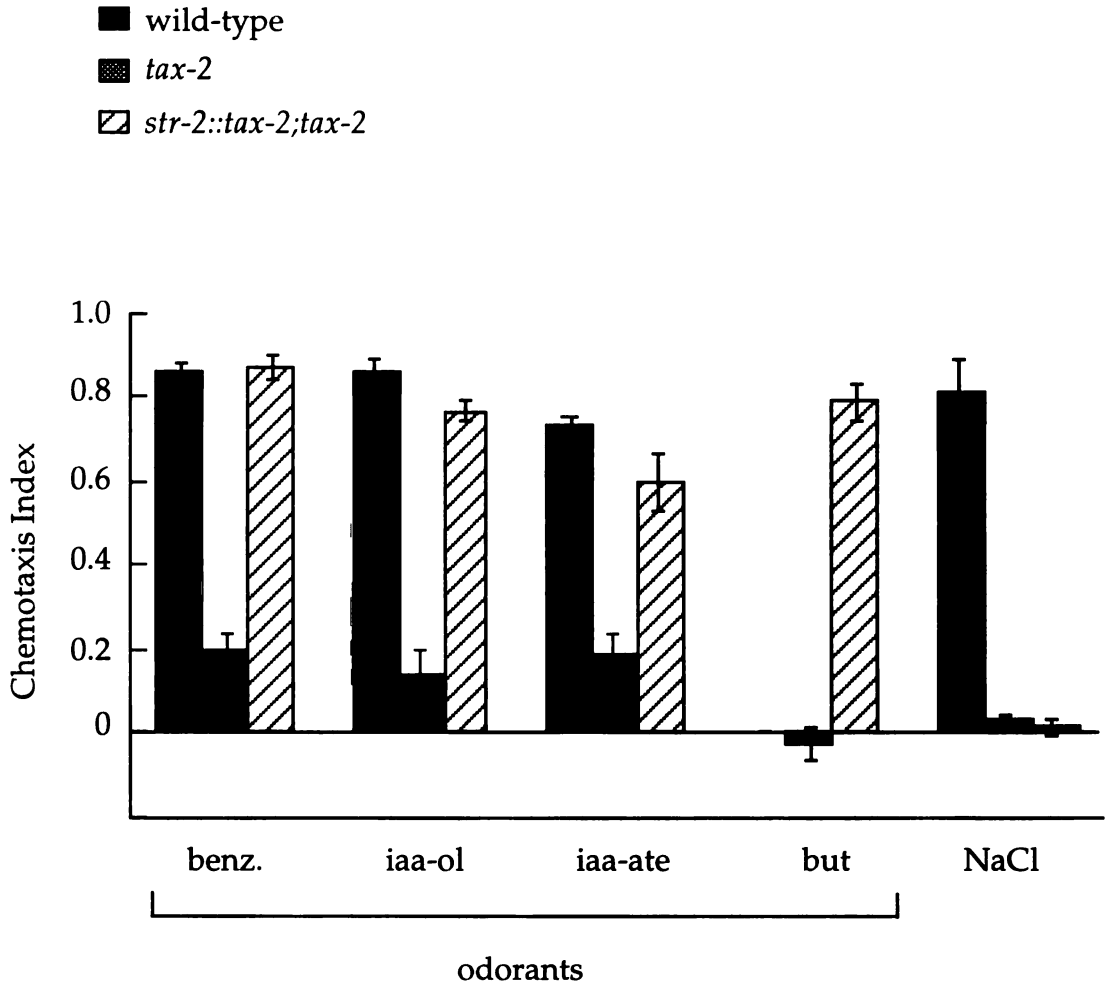
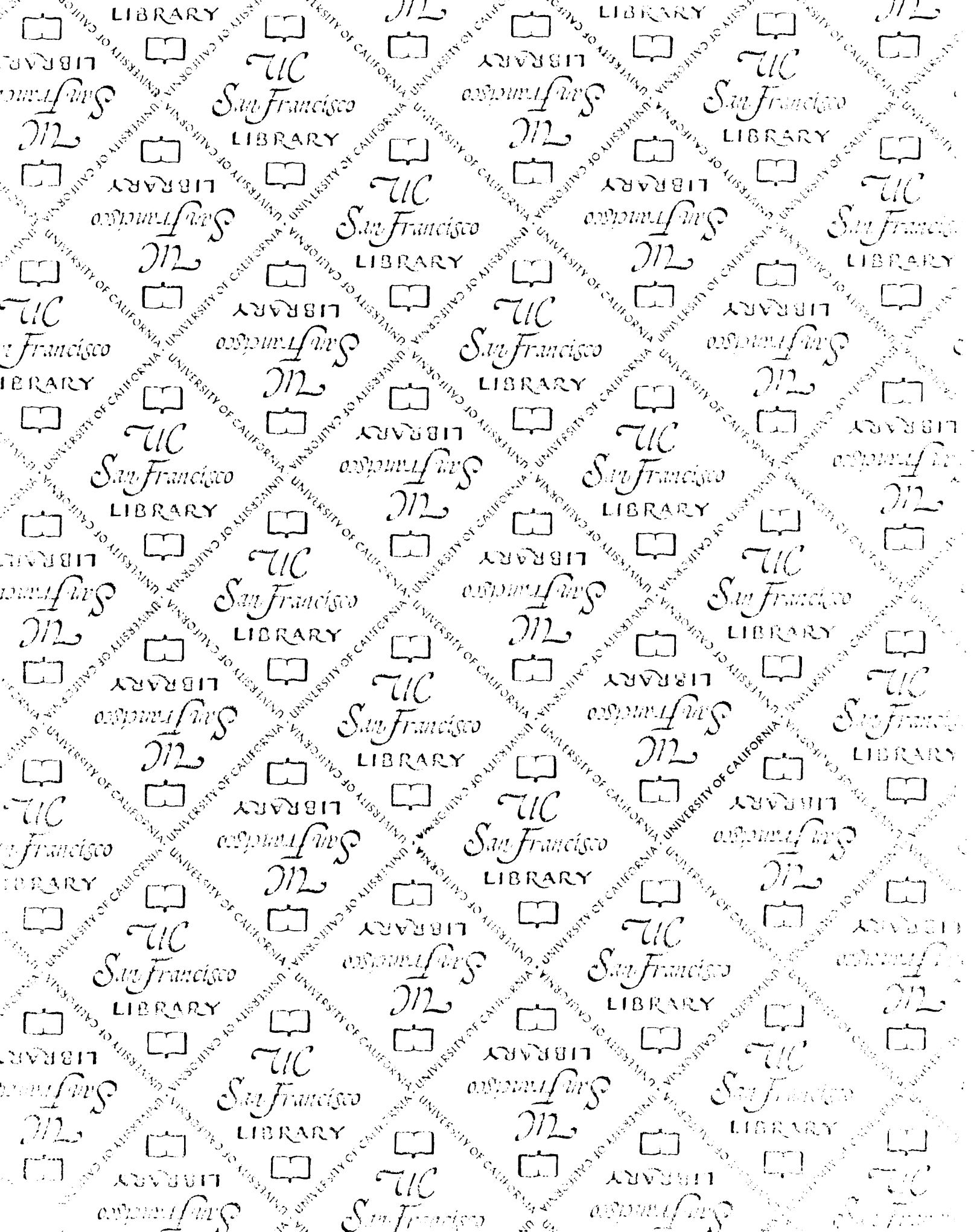


Figure C-1. Expression of *tax-2* from the *str-2* promoter rescues the AWC defect, but not the ASE defect of *tax-2* mutants. The *str-2* promoter was introduced into a plasmid containing the *tax-2* cDNA and this *str-2::tax-2* transgene was injected into *tax-2(p691)* mutants. Two independent transgenic lines were isolated and transformed animals were tested in standard chemotaxis assays. The data points for *str-2::tax-2;tax-2* animals are the combination of results from these two transgenic lines. The *tax-2* negative control carries the same co-injection marker used for *str-2::tax-2* transgenic animals. N2 animals were not tested for butanone. Error bars are SEM. Concentrations of chemicals tested: benzaldehyde (benz.) 1:200, isoamyl alcohol (iaa-ol) 1:100, isoamyl acetate (iaa-ate) 1:100, butanone (but) 1:1000, NaCl 0.4M plug.

	pyrazine	n	benzaldehyde	n	isoamyl alcohol	n
Wild-type	0.91	22	0.86	22	0.72	18
Cells killed:						
AWC^{ON}	0.90	21	0.50	20	0.94	17
right			0.50	12		
left			0.50	8		
AWC^{OFF}	1.0	4	0.67	3	0.67	3
both AWC's	.80	5	0.00	5	0.00	5

Table C-1. Chemotaxis of wild-type and AWC ablated animals. Wild-type numbers include both unablated, unmounted *str-2::GFP* animals, as well as unablated, mounted animals. AWC^{ON} cells express *str-2::GFP*, while AWC^{OFF} cells do not express *str-2::GFP*. n is the number of individual assays performed.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