Title
Odorant receptor localization to olfactory cilia is mediated by ODR-4 and UNC-101

Author
Dwyer, Noelle Diane

Publication Date
1998

Peer reviewed|Thesis/dissertation

ODORANT RECEPTOR LOCALIZATION TO OLFACTORY CILIA IS MEDIATED BY ODR-4 AND UNC-101

by

Noelle Diane Dwyer

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

this thesis is dedicated to my parents

Acknowledgements

I would like to thank the many people that have helped me to successfully finish my Ph.D. My advisor Cori Bargmann has been an outstanding mentor. Not only has she created a wonderful environment and niche for a graduate student, but she has taught me how to be a good scientist. My wonder at her breadth of knowledge and effusion of ideas will never cease. I deeply appreciate her effort and time spent thinking about my project and helping me with publications.

The members of the Bargmann Lab, past and present, have provided expertise, ideas, advice, and a lot of fun besides. I am indebted to them all, whether it be for reagents, fun times, or a sympathetic ear. The CCD imaging in Chapter 4 would not have been possible without the generous help of Tim Yu. I also thank rotation students Levente Egry and Brenda Ng. I would also like to thank the members of the Tessier-Lavigne and Martin Labs for creating a fun and stimulating scientific environment.

I would like to thank the members of my qualifying exam and thesis committees: Lily Jan, Cynthia Kenyon, Mark von Zastrow, Jon Scholey, Peter Sargent, David Copenhagen, and Lou Reichardt for their advice, support, enthusiasm, and helpful discussions.

Without the support of my friends and family, I would probably not have made it through graduate school. I thank every person who ever gave me a word of encouragement or advice. It was those little offerings of "good job" or "You can do it!" that got me through the tough times of frustration and self-doubt. My parents and my two brothers Patrick and Sean have helped me more than they realize just by having faith in me. Certain people provided encouragement or support at crucial moments when it was

desperately needed: Piali, Tracy, Penny, Katja, Emily, Jen, Lee, Elena, Shelley, James, Elly, and Julia. I would also like to thank the students of the Neuroscience Program, especially my classmates, for providing a supportive environment. I look forward to many years of collegiality with all of them.

dwyer@cgl.ucsf.EDU, 10:53 PM 5/4/98 , No Subject

Date: Mon, 4 May 1998 19:53:11 -0700 (PDT)
From: dwyer@cgl.ucsf.EDU
To: editor@cell.com

Monday May 4, 1998

Cell
Cell Press
1050 Massachusetts Avenue
Cambridge, Massachusetts 02138

To Whom it may concern:

I would like to request permission to include in my thesis dissertation a copy of the papers cited below:

Dwyer, N.D., Troemel, E.R., Sengupta, P., and Bargmann, C.I., 1998. Odorant Receptor Localization to Olfactory Cilia is Mediated by ODR-4, a Novel Membrane-Associated Protein. Cell , vol. 93, No.3, pp. 455-466.

Troemel, E.R.*, Chou, J.H.*, Dwyer, N.D.*, Colbert, H.A.*, and Bargmann, C.I., 1995. Divergent Seven Transmembrane Receptors are Candidate Chemosensory Receptors in C. elegans. Cell, vol. 83, No. 2, pp. 207-218.

The dissertation will be microfilmed by University Microfilms Incorporated and they request permission to supply single copies upon demand. Since I must submit my thesis May 23, I would greatly appreciate it if you could respond within the next two weeks by fax to (415) 476-3493.

Thank you very much.

Sincerely,

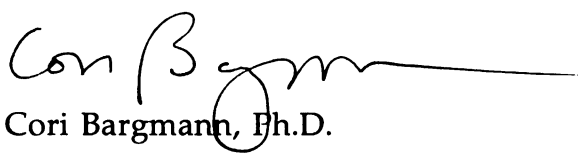
Noelle Dwyer
Neuroscience Program
UC San Francisco

MAY 14 1998
"Permission granted subject to citation of the original manuscript, and notation that copyright is held by Cell Press. (Our permission is contingent on permission of the author.)"
for Cell Press

Advisor Statement

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

Chapter 3 was published as Cell, Vol. 93, No. 3, pp. 455-466, 1998. Piali Sengupta isolated the *odr-8* alleles and Emily Troemel made the STR-2-GFP tag, performed the RT-PCR experiment, and tested the *odr-4* requirement for diacetyl avoidance. All other experiments were performed by Noelle Dwyer.



Cori Bargmann, Ph.D.
Associate Professor
HHMI, Department of Anatomy UCSF

Odorant Receptor Localization to Olfactory Cilia is Mediated by ODR-4 and UNC-101

Noelle D. Dwyer

Abstract

How cells localize proteins to specific plasma membrane domains is a fundamental cell biological question. This thesis describes an effort to understand how G protein-coupled (seven transmembrane domain) receptors are localized in neurons, taking a genetic approach and using the *C. elegans* chemosensory system as a model. The identification of two genes with different roles in the localization of the odorant receptor ODR-10, and the discovery that ODR-10 can be transported in rapidly-moving dendritic vesicles shows that the *C. elegans* chemosensory neurons provide a valuable new system for the study of protein trafficking and localization in neurons.

For sensitive and efficient olfactory transduction, chemosensory neurons concentrate odorant receptors and other signalling machinery in the olfactory cilia. *C. elegans* appears to possess hundreds of putative chemoreceptors. The diacetyl receptor ODR-10 localizes to the cilia of the AWA neurons. The *str* genes are a family of seven transmembrane receptors related to *odr-10*, while the *sr* genes form several independent subfamilies.

The genes *odr-4* and *odr-8* are required for the localization of a subset of odorant receptors to the olfactory cilia. In *odr-4* or *odr-8* mutants, ODR-10-GFP is not visible in the AWA cilia, but only in the cell body. These mutants

show defective responses to diacetyl, benzaldehyde, and trimethylthiazole. The requirement for the *odr-4/odr-8* system shows cell and sequence specificity. *odr-4* encodes a novel membrane protein that is expressed exclusively on intracellular membranes of chemosensory neurons, where it acts cell-autonomously to facilitate odorant receptor folding or localization.

unc-101 is an AP-1 clathrin adaptor that is also required for localization of ODR-10 to olfactory cilia. When expressed in the AWB neurons, ODR-10-GFP is seen in the cilia and in rapidly moving vesicles in the dendrites. In *unc-101* mutants, these organelles are not seen and the ODR-10-GFP is spread over the entire surface of the neuron. Thus, ODR-10 localization to cilia may be accomplished through recruitment by *unc-101* into a special population of cilium-targeted vesicles.

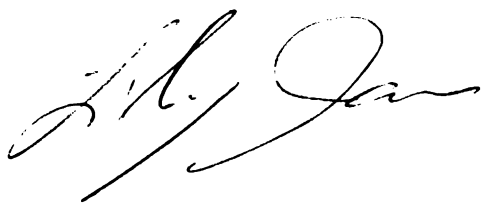


Table of Contents

LIST OF TABLES AND FIGURES

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER 1

INTRODUCTION:

Asymmetric Protein Localization in Neurons

ABSTRACT:

This introduction will review the steps involved in asymmetric protein localization on cell surfaces. We will focus on neurons, but will take examples from non-neuronal cells when informative. We will also particularly address these questions with regards to G protein-coupled receptors, which are the largest class of cell-surface receptors, but are not well-characterized in terms of their folding, trafficking, and maintenance of localization. Section I of this introduction will explain the functional significance of asymmetric protein localization in neurons. The second section of the introduction will discuss what is known about the steps and molecular mechanisms for localization of membrane proteins. The third section will describe the anatomy and function of the *C. elegans* chemosensory neurons, and explain why they are a useful system in which to study the problem of asymmetric protein localization in neurons.

SECTION I: FUNCTIONAL SIGNIFICANCE OF MEMBRANE PROTEIN LOCALIZATION IN NEURONS

Proper development and function of neurons requires that signalling machinery such as receptors and channels be distributed on the cell surface in strict spatial patterns. Signaling machinery is concentrated in specialized domains so that transduction is sensitive, efficient, and local. For example, many CNS neurons both release the neurotransmitter glutamate and express glutamate receptors. If such a neuron were to release glutamate at random sites from its plasma membrane, and expressed excitatory glutamate receptors all over its surface, it might stimulate itself to release more glutamate in a neverending positive feedback loop. The neuron solves this problem by

localizing glutamate release at the axonal terminal, and localizing glutamate receptors in post-synaptic densities in dendrites. Further functional complexity could result if the neuron localized AMPA receptors to some synapses, and inhibitory metabotropic glutamate receptors to other synapses, so that it could be excited by some inputs and inhibited by others. The neuron localizes neurotransmitter receptors to dendrites, high concentrations of voltage-gated sodium and potassium channels to the axon hillock, and voltage-gated calcium channels to the axon terminal for mediating synaptic vesicles release. This complex parcellation of functions demands special mechanisms for precise transport and targeting of surface proteins.

Interestingly, proteins can be localized to just one particular part of an axon or a dendritic arbor. Specialized sub-domains in axons and dendrites include axon hillocks, nodes of Ranvier, presynaptic active zones, and postsynaptic densities. The nodes of Ranvier on myelinated axons provide one characterized example of strict protein localization. The evenly spaced nodes contain a high concentration of sodium and potassium channels, allowing saltatory propagation of action potentials, while internode regions that rely on passive propagation are channel-poor. Cell culture experiments suggest that a glial-derived protein is permissive for the periodic spacing of nodes, but direct contact of glia is not required, indicating that the spacing is determined by intrinsic neuronal factors (Kaplan et al., 1997).

Specific distributions of cell surface molecules are critical for proper development of the nervous system. Axon guidance receptors such as Robo in *Drosophila* are concentrated in the growth cones of growing neurites at appropriate times in their development to inhibit growth on the nerve cord midline (Kidd et al., 1998). Some adhesion receptors may be localized to specific segments of growing axons to allow fasciculation and defasiculation

along different points in their trajectories. TAG-1 and L1 are adhesion molecules that appear to be segregated on different segments of the same embryonic spinal axons, and removal of axonin-1 (the avian homolog of TAG-1) from growth cones resulted in a blockage of neurite outgrowth on both Ng-CAM (L1) and axonin-1 substrata (Dodd et al., 1988; Stoeckli et al., 1996). In Drosophila, the fasciclin molecules also show restricted localization to neurite segments where they mediate fasciculation (Bastiani et al., 1987; Patel et al., 1987). In addition to molecules that help set up initial nervous system wiring, it is possible that recruitment of signalling receptors to specific parts of axonal or dendritic arbors plays an important role in synaptic plasticity. A possible mechanism for plasticity mediated by rapid recruitment of receptors is suggested by the recent finding that insulin stimulation causes translocation of $GABA_A$ receptors from an intracellular compartment to the plasma membrane (Wan et al., 1997).

G protein-coupled receptors play crucial roles in neuronal function. These receptors have seven transmembrane domains, and include neurotransmitter receptors, peptide receptors, opsins, and chemoreceptors. G protein-coupled receptors can provide amplification of signals and longer-lasting signals than are possible from ion channels. One activated receptor can activate many G proteins, which then act on downstream effectors. This allows for high sensitivity to chemical messengers and long-lasting changes in neuronal responses.

While the seven transmembrane domain receptors comprise the largest family of signalling receptors, control of their biogenesis and localization is not well-understood. Having so many membrane-spanning domains may make membrane threading and folding a challenge, perhaps especially because most G protein-coupled receptors do not have a signal

peptide. Many G protein-coupled receptors are localized asymmetrically within neurons. The mu opioid receptor 1 appears to be targeted to dendrites in CNS neurons, but to axons in primary afferent neurons (Arvidsson et al., 1995). One receptor, mGluR7, can even be differentially clustered at two synapses within a single nerve terminal (Brandstatter et al., 1996; Shigemoto et al., 1996). The mechanisms and molecules that generate these complex protein localization patterns are poorly understood.

While neurons are the most geometrically complex of polarized cells, an often-used system for studying the problem of asymmetric protein targeting is the Madin-Darby canine kidney (MDCK) cell. These cells maintain polarity in culture, having an apical side that is separated by tight junctional complexes from a basolateral side. Many proteins are enriched in either the apical or basolateral surface. Neurons will undoubtedly share some targeting mechanisms with polarized epithelial cells, so data from trafficking studies in MDCK cells will be discussed below when relevant.

SECTION II: STEPS AND MECHANISMS OF MEMBRANE PROTEIN DISTRIBUTION TO ASYMMETRIC AND RESTRICTED DOMAINS

In thinking about how neurons might create patterns of proteins on their surfaces, it is useful to consider all the steps that a protein must take on its way to the plasma membrane. We will focus on membrane proteins and seven-transmembrane proteins in particular. Determination of a protein's fate can be viewed in five steps: 1) folding, 2) sorting into the proper secretory pathway, 3) transport of secretory vesicles to the proper cell domain, 4) targeting of secretory vesicles to the membrane by selective fusion, and 5) maintenance of restricted localization. Implicit in this view is

the assumption that secretion of plasma membrane proteins is accomplished by targeting of transport vesicles. It is worth noting, however, that this is only the prevailing model, and it is possible that some proteins are localized by non-vesicular transport. For example, mRNAs could be transported, and proteins could be translated locally (see below). For each of these steps, sequences within the receptors may function as signals to determine the outcome of a step.

Step 1: Folding

Protein folding generally occurs in the endoplasmic reticulum (ER). Molecular chaperones such as Hsp70 and chaperonins may help proteins achieve their correct fate in the cell by assisting folding, oligomeric assembly, transport to subcellular compartments, or degradation (Rassow et al., 1997).

Studies of opsin suggest that there may be special foldases for seven transmembrane domain proteins. The *Drosophila ninaA* gene encodes a membrane-associated cyclophilin required for localization of Rh1 rhodopsin to the rhabdomeres of R1-R6 photoreceptors (Colley et al., 1991; Stamnes et al., 1991). Cyclophilins are specialized peptidyl-prolyl *cis-trans* isomerases that catalyze the *cis-trans* isomerization of prolyl-peptide bonds, a rate-limiting step in protein folding. In *ninaA* mutants, the steady-state levels of functional Rh1 is decreased and the ER expands from retention of this rhodopsin. NinaA is required for production of functional Rh1 (blue) and Rh2 (violet), but not Rh3 (UV) rhodopsins (stamnes). In addition, since NinaA is found in transport vesicles, it may have a role in later steps during secretion (Colley et al., 1991). The vertebrate RanBP2 protein has both a GTPase binding domain (the RBD4 domain), and a cyclophilin domain. The RBD4/cyclophilin supradomain of RanBP2 forms a complex with red/green

opsin but not with blue-cone or rod opsins, displaying substrate specificity, just as NinaA does (Ferreira et al., 1996). Co-expression of human red opsin and RanBP2 in COS cells increased the production of functional opsin as measured by light absorbance of red pigment purified from the cells. The cyclophilin domain seems to perfom both a chaperone function, stabilizing formation of the opsin-RBD4 complex, and a foldase function (Ferreira et al., 1997). These opsin cyclophilins are the only chaperones yet identified for a G protein-coupled receptor.

Proper folding may be a prerequisite for protein access to the cell surface. Receptors with mutations that cause de-stabilization are often not sent to the cell surface but are retained in the ER (Gudermann et al., 1997). The importance of quality control suggests the existence of "folding monitors" that recognize fully folded proteins and allow them to leave the ER (Rothman and Wieland, 1996). Furthermore, efficient export from the ER may require a sorting signal: a di-acidic signal (Asp-X-Glu) on the cytoplasmic tail of vesicular stomatitis virus glycoprotein (VSV-G) and lysosomal acid phosphatase was required for efficient recruitment to vesicles mediating export from the ER in baby hamster kidney cells (Nishimura and Balch, 1997; Bannykh et al., 1998).

Step 2: Sorting

As they travel through various secretory pathways, proteins are sorted at each step, being retained or segregated into vesicles that will carry them to the next step. It is possible that some proteins are not specifically sorted and follow a default or "bulk flow" pathway to the cell surface.

There are many possible mechanisms for sorting. Most involve some kind of "address code" on the protein or on an associated protein. Some

proteins are sorted by interacting with "sorting receptors" or "cargo receptors" that bind to protein motifs or epitopes, or to carbohydrate or lipid modifications. Other proteins may be sorted not by interacting with proteinaceous receptors, but by preferential association with membranes of different lipid compositions. Again, as was the case with folding, the mechanisms for sorting G protein-coupled receptors are poorly understood. Integral membrane proteins that ultimately reside in different compartments all have their origins in the ER, so presumably certain sorting signals remain "silent" until the proteins encounter the appropriate coat components that bind and sort these proteins to their correct site of function.

Sorting of some proteins may be determined by lipid associations. For example, palmitoylation and myristoylation of heterotrimeric G proteins may allow them to be plasma membrane-associated. Mutations that prevented palmitoylation of $G\alpha_z$ allowed mistargeting to intracellular membranes, while removal of the myristoylation site produced a mutant α_z that was located in the cytosol (Morales et al., 1998). Glycosyl phosphatidlyinositol (GPI) anchors are on many apically-targeted proteins in MDCK cells, and are sufficient to redirect basolaterally-targeted proteins to the apical surface (Lisanti et al., 1988; Lisanti et al., 1989), It is thought that GPI anchors may allow proteins to associate with cholesterol-rich membrane domains, such as caveolae or "lipid rafts", that could function in targeting to apical domains of epithelial cells (Simons and Ikonen, 1997). Another sorting model holds that the length of the transmembrane domain of some proteins determines the compartment of residence, based on the different bilayer thicknesses (Munro, 1998).

Carbohydrate-mediated sorting probably involves protein receptors that bind the sugar modifications. The best characterized carbohydrate-based

sorting mechanism is the mannose-6-phosphate receptor (VPS protein in yeast), which sorts proteins into vesicles destined for lysosomes (Pfeffer, 1991; Hille-Rehfeld, 1995; Stack et al., 1995). Other relevant sugar modifications may include N-glycans, which have also been proposed to function in apical targeting in epithelial cells (Scheiffele et al., 1995).

Other proteins contain peptide sequences that determine their sorting by binding to sorting receptors. These receptors can be residents of secretory pathway compartments (e.g. the KDEL receptor for retention of ER resident proteins (Lewis et al., 1990; Semenza et al., 1990)), or the receptors can be vesicle coat components, discussed below.

Sorting by vesicles

For some proteins, cells may solve the problem of subcellular targeting by sorting cargo into different types of transport vesicles at the *trans*-Golgi network. There are two mechanisms for vesicle-mediated localization to a particular surface: direct delivery or indirect delivery. Direct delivery means the vesicle is permitted to fuse only with the correct membrane, and denied fusion with the incorrect membrane. Indirect delivery involves either fusion with the "wrong" surface and transcytosis to the correct surface, or random fusion followed by selective retention (see Step 5).

Sorting and transport of proteins through the secretory pathway depends on the existence of distinct vesicle populations for various transport steps (for general review see (Schekman and Orci, 1996). A variety of vesicle types have been observed, distinguished by their coats. The first and structurally best characterized coat is the clathrin coat, found on vesicles budding from the plasma membrane, *trans*-Golgi network, and endosomes. Two other types of coated vesicles, COPI and COPII vesicles are involved in

transport between the ER and Golgi. COPI may retrieve ER proteins by binding to KKXX motifs in their tails, but is also found on anterograde-directed vesicles (Cosson and Letourner, 1994; Orci et al., 1997). In addition to clathrin and the COPs, there are other less well-characterized coats seen by EM: a striated coat on caveolae and a lace-like coat at the TGN (Traub and Kornfeld, 1997).

Critical vesicle sorting decisions are made upon exiting the *trans*-Golgi network (TGN) that will largely seal the localization fate of a protein. Proteins can be sorted into a variety of vesicle types, destined for different intracellular and plasma membrane locations (see Traub and Kornfeld, 1997) for review). While some proteins are sorted into vesicles that are delivered directly to the plasma membrane (the constitutive pathway), others are sent through the selective endosomal pathway. There may be multiple types of vesicles for the constitutive pathway out of the TGN. Several non-clathrin coatlike proteins, including p62, p200, p230 have been identified cycling on and off the TGN. Furthermore, for some proteins there may be additional sorting steps after the TGN, as seems to be the case for transcytosed proteins such as the polymeric immunoglobulin receptor, which ends up on the apical surface of MDCK cells via the basolateral surface first and then endocytosis and re-sorting in an apical recycling compartment (Mostov and Cardone, 1995). The class of TGN vesicles that are routed to the endosome are coated with clathrin (Traub and Kornfeld, 1997).

While several types of coats have been identified on TGN vesicle buds, clathrin coats are the best-studied, in part because clathrin-coated vesicles (CCVs) are also involved in endocytosis from the plasma membrane (Schmid, 1997). Clathrin triskelions self-assemble into a polyhedral lattice that forms a framework for cargo recruitment into budding vesicles. The

assembly of clathrin-coated buds on the Golgi requires the heterotetrameric AP-1 adaptor complex. The clathrin associated protein (AP) complexes provide a membrane-binding site for clathrin and also interact with trafficking membrane proteins to serve a cargo-selective function (Marks et al., 1997; Robinson, 1997; Schmid, 1997). AP-1 consists of four subunits: two large chains, ß' and γ, one medium chain, μ1 or AP47, and one small chain, σ1 or AP19. The clathrin coats at the plasma membrane are involved in receptor endocytosis and include the AP-2 adaptor complex. Similar to AP-1, AP-2 is comprised of two large chains, α and ß, one medium chain, μ2, and one small chain σ2. AP's may sort or retain some transmembrane proteins by μ2 binding to tyrosine motifs in their tails (Ohno et al., 1995; Marks et al., 1997; Schmid, 1997). These motifs can be YXXØ or NPXY that are predicted to lie within a structure called a "tight turn". YXXØ motifs have been implicated in directing localization to endosomes, lysosomes, the TGN, and the basolateral plasma membrane of polarized epithelial cells (Marks et al., 1997).

Step 3: Transport of vesicles to the correct cell domain

Once coated pits at the TGN are loaded with proteins to be placed on the cell surface, they bud off, a process that may be aided by vesicle coats and by a homolog of dynamin (Henley and McNiven, 1996). Dynamin is a GTPase that is required at the plasma membrane during endocytosis, localizing to the neck of budding vesicles (Herskovits et al., 1993; van der Bliek et al., 1993).

Once vesicles have budded off, motor proteins haul vesicles full of cargo proteins to the proper vicinity. Using microtubules as tracks, kinesins and dyneins move anterogradely and retrogradely. Myosins moving on actin filaments may also transport some organelles (Langford, 1995). It may be that

there are specific motors for dendrites versus axons, or for differently "addressed" vesicles For review see (Hirokawa, 1998). However, while many motor proteins have been identified, little is known about their cargo specificity, or how they bind to different vesicles. Even less is known about motors acting in dendrites, where microtubules have mixed orientations (Baas et al., 1988). KIFC2, an unusual kinesin with a C-terminal motor domain, has been proposed to act specifically to carry multivesicular bodies anterogradely in dendrites (Saito et al., 1997), but immunolocalization and nerve ligation experiments suggest that it may play a role in retrograde axonal transport (Hanlon et al., 1997).

Step 4: Targeting of secretory vesicles to the correct membrane by selective fusion

After vesicle arrival at the proper "neighborhood", mechanisms are still needed to allow vesicle fusion with the proper target membrane and protect against fusion with inappropriate membrane domains. Moreover, fusion of some receptor-containing vesicles with the plasma membrane may be temporally regulated: insulin stimulation causes rapid translocation of $GABA_A$ receptors from intracellular compartments to the plasma membrane (Wan et al., 1997). Specificity of vesicle targeting to appropriate membrane compartments is ensured by interactions of proteins on the transport vesicle with cognate receptors on the target membrane. SNARE complexes and rabs may mediate these interactions (Rothman and Wieland, 1996; Hanson et al., 1997; Novick and Zerial, 1997).

SNAREs are membrane-anchored proteins residing on both vesicles (v-SNAREs) and on target membranes (t-SNAREs) that may function to mediate vesicle docking or fusion with its appropriate target membrane (for

review see Hanson et al., 1997). While many SNAREs have been found to localize to specific membranes, including syntaxin for synapses, SNAREs for other plasma membrane domains have not yet been identified. Syntaxin-3 is restricted to the apical domain of epithelial cells, while syntaxin-4 is restricted to the basolateral domain, indicating that these syntaxin isoforms are in correct positions to play a role in targeting specificity (Low et al., 1996). However, the functions of syntaxins-3 and -4 are not defined.

Rab proteins, related to the Ras superfamily of small GTPases, are implicated in vesicle docking and regulation of SNARE pairing. While specific rabs may be dedicated to specific transport steps, at the moment more rabs than steps have been identified in mammalian cells (Novick and Zerial, 1997). rab8 may be important in both basolateral transport in MDCK cells and dendritic transport in cultured hippocampal neurons. Transport of VSV-G from the TGN to the basolateral plasma membrane was found to be significantly inhibited by a peptide derived from the hypervariable COOH-terminal region of rab8, while transport of the influenza HA from the TGN to the apical surface and ER to Golgi transport were unaffected (Huber et al., 1993b). Similarly, antisense oligonucleotides to rab8 resulted in a significant decrease in transport of SFV-E2 to the dendrites, but not in axonal transport of influenza HA (Huber et al., 1993a). rab6 and rab8 have been found in post-Golgi membranes in rods, and are candidates to mediate rhodopsin transport to the outer segment (Deretic and Papermaster, 1993; Deretic et al., 1995).

One group has proposed that targeting to the basolateral surface of epithelial cells may be mediated by the Rab-NSF-SNAP-SNARE mechanism, but targeting to the apical surface is mediated by lipid interaction mechanisms (the "lipid raft" mentioned above), since it was not abolished by agents that disrupt these proteins (Ikonen et al., 1995). However, another group showed

that both receptor-mediated transcytosis of IgA to the apical surface as well as recycling to the basolateral surface required NSF and were inhibited by botulinum E toxin, which may cleave a SNAP-25 homologue (Apodaca et al., 1996).

Step 5: Maintenance of receptor localization

The steady-state distribution of a protein depends not only on vectorial delivery, but also on retention. Receptors that have been properly targeted to the appropriate plasma membrane domain may still require additional factors to keep them on that surface or to keep them tethered near other signalling components. For example, a protein could be randomly targeted to an entire dendrite, then maintained at specific synapses by a clustering mechanism, or by endocytosis from areas outside the synapses. The α 2B adrenergic receptor achieves 90% basolateral localization in MDCK II cells at the steady-state, but does so by initial random delivery to both the apical and basolateral surface followed by selective retention on the basolateral surface (Wozniak and Limbird, 1996).

Examples of clustering proteins for several types of channels and receptors have recently been identified. Some have been shown compellingly to play a role in localization *in vivo*, others have been shown to function in clustering in cell culture experiments, while others have only been co-localized with specific receptors or channels. (For review, see Sheng and Wyszynski, 1997). An axonal isoform of ankyrin ($ankyrin_G$) may cluster voltage-gated sodium channels at nodes of Ranvier (Kordeli et al., 1990). Rapsyn is required for clustering of nicotinic acetylcholine receptors *in vivo* (Gautam et al., 1995) and clusters $GABA_A$ receptors in cultured cells (Yang et al., 1997), while gephyrin could cluster both glycine receptors and $GABA_A$

receptors (Kirsch et al., 1993; Sassoe-Pognetto et al., 1995; Sassoe-Pognetto and Wassle, 1997). A growing family of PDZ-containing proteins interact with various types of cation channels, glutamate receptors, and receptor tyrosine kinases, and some are required for localization (Simske et al., 1996; Chevesich et al., 1997; Sheng and Wyszynski, 1997; Tejedor et al., 1997). An S/TXV motif in the C-terminal tail is required for PDZ interactions. Recently a new type of PDZ protein called Homer was identified that is dendritic and binds specifically to the carboxy terminus of metabotropic glutamate receptors mGluR1α and mGluR5 (Brakeman et al., 1997). Interestingly, expression of Homer is responsive to synaptic activity. Homer could be the first example of a protein that clusters a G protein-coupled receptor by direct binding, but it is not yet clear whether Homer is actually required for mGluR clustering, or whether it functions in modulating signalling. Some other seven transmembrane domain receptors have PDZ binding motifs in their tails (Kornau et al., 1995), but none have yet been shown to be required for localization, or to interact with PDZ proteins.

In addition to clustering, maintenance of membrane protein localization can be affected by endocytosis. Specific localization can be achieved by random targeting followed by selective retention or by transcytosis. Most endocytosis is mediated by clathrin-coated vesicles, but there seem to be nonclathrin-mediated pathways as well. Clathrin-mediated endocytosis requires AP-2 and dynamin (for review see Robinson et al., 1996). G protein-coupled receptors are endocytosed after activation by ligand, phosphorylation, and arrestin binding (Goodman et al., 1996; Koenig and Edwardson, 1997). Tyrosine motifs may play a role in endocytosis of the neurokinin-1 receptor (Bohm et al., 1997), and ubiquitination is required for endocytosis of the α factor receptor in yeast (Hicke and Riezman, 1996).

Protein Localization by RNA transport

The above discussion presents only pathways and mechanisms of localization dependent on control of protein trafficking. One other possible mechanism of localized protein expression is RNA transport and localized translation (for review see Wilhelm and Vale, 1993). RNA localization plays an important role in *Drosophila* embryonic development (Campos-Ortega, 1997), and could have a role in localization of neuronal proteins as well. mRNAs for MAP2 and α-CAM kinase II have been found in dendrites, and mRNAs for tau, vasopressin, and oxytocin may localize to axons. Moving RNA granules have been observed in neurons in culture (Knowles et al., 1996). It is unclear how and why these mRNAs are transported into dendrites, and how general this mechanism is. It could be energetically economical: only one mRNA need be transported for the translation of hundreds of copies of the encoded protein. For some proteins such as cytoskeletal proteins that spontaneously self-assemble after translation, controlling the location of translation may be necessary. For several genes whose mRNAs are localized, a *cis*-element in the 3' untranslated region (3' UTR) is required, but there is no conserved motif. There is no evidence thus far for mRNA localization for any seven transmembrane domain protein.

Polarized epithelial cells as a model for asymmetric localization of plasma membrane proteins

Many of the concepts discussed in this section have arisen from studies in MDCK cells, especially some of the models for sorting and maintenance. Simons has postulated that targeting to the apical and basolateral domains is mediated by completely different mechanisms. Apical proteins could be sorted by lipid interactions with sphingolipid-cholesterol rafts that function as

platforms for carrying proteins in post-Golgi trafficking. Apical proteins, for example the influenza hemaglutinin protein, GPI-anchored proteins, and some glycosphingolipids, would preferentially associate with these rafts through hydrogen bonds. Basolateral proteins would be sorted separately by virtue of cytoplasmic sorting signals that interact with sorting receptors in the coats of vesicles that are targeted by an NSF/SNARE-dependent mechanism (Simons and Ikonen, 1997). How transcytosed proteins fit into this model is not clear. The polymeric immunoglobulin receptor is targeted to the basolateral surface by virtue of a cytoplasmic signal that forms a type I beta turn, and then endocytosed and transcytosed to the apical surface. Tyrosine sorting signals may also form type I beta turns (Mostov and Cardone, 1995).

Can neurons be viewed as modified MDCK cells? The VSV-G protein is a basolateral protein in MDCK cells, and is localized to the dendritic domain in cultured hippocampal neurons, while influenza HA is apically sorted in MDCK cells and is targeted preferentially to the axon in cultured hippocampal neurons (Dotti and Simons, 1990). In addition, the GPI protein Thy-1 is delivered preferentially to the apical surface in MDCK cells, and is located exclusively on axons of cultured hippocampal neurons (Dotti et al., 1991). These data have led to a model that axons are equivalent to the apical surface while the somatodendritic compartment approximates the basolateral surface of MDCK cells. However, since a given receptor type can display different localization patterns in different types of neurons (Arvidsson et al., 1995), there are likely to be a variety of different factors that determine the behavior of membrane proteins in different neuron types.

Limbird and colleagues have done the most work on the problem of G protein-coupled receptor targeting in MDCK cells (summarized in Wozniak et al., 1997), and have found that even closely-related receptors can display

very different trafficking itineraries. Through immunocytochemistry and metabolic labelling studies, they have found that α 2A, 2B, and 2C adrenergic receptors all achieve basolateral localization at the steady-state, but that the 2A and 2C subtypes are targeted directly, while the α 2B subtype is initially randomly targeted but is selectively retained basolaterally. Furthermore, the α 2C adrenergic receptor also resides on an intracellular compartment which may be the endoplasmic reticulum or TGN. Another type of G protein-coupled receptor, the A1 adenosine receptor, is enriched apically (~75% at steady-state) in MDCK cells. The *cis*-acting signals on these receptors that determine their trafficking remain mysterious: mutation of N-terminal glycosylation sites, C-terminal acylation sites, a conserved aspartate in TM2, tyrosine motifs in TM7, or deletion of the third cytoplasmic loop or the C-terminal tail of the α 2A adrenergic receptor did not disrupt basolateral targeting, even when all these insults were combined. They conclude from these negative results that motifs in or near the bilayer must determine targeting. Recent work suggests that there may be two distinct trafficking pathways to the apical surface: microtubule-disrupting agents caused mis-localization of the A1 adenosine receptor to the basolateral surface, but did not disrupt the random apical delivery of the α 2B adrenergic receptor (Saunders and Limbird, 1997).

SECTION III. C. ELEGANS CHEMOSENSORY NEURONS AS A USEFUL MODEL SYSTEM FOR STUDYING G PROTEIN-COUPLED RECEPTOR FUNCTION AND LOCALIZATION

Most studies of protein localization thus far have used biochemical and cell biological approaches. There is much to be gained from a genetic

approach to the problem: an ability to focus in endogenous membrane proteins and their in vivo interactions, an ease of introduction of mutated molecules, and an unbiased approach that yields novel molecules involved in localization. We have taken a genetic approach in *C. elegans* to identify molecules involved in the proper localization of odorant receptors. The chemosensory system of *C. elegans* may provide a new model for studying how proteins are localized to sub-regions of the plasma membrane in neurons. This system has been successfully used by the lab to confirm the role of G protein-coupled receptors in olfactory transduction (Chapter 2 and Sengupta et al., 1996), and to identify new molecules involved in olfaction, such as OSM-9 (Colbert et al., 1997), a capsaicin receptor-like channel. A genetic screen for olfaction-defective mutants has the potential to identify mutants in any one of the five steps discussed above; alternatively, an investigator could focus on one specific step by performing a visual screen for mutants with specific mis-localization phenotypes. This approach in *C. elegans* has so far provided a new molecule, ODR-4, that may act as a chaperone for a subset of odorant receptors (Chapter 3), and new insight into the sorting function of UNC-101, a previously identified AP-1 clathrin adaptor (Chapter 4). Potential exists to find additional proteins acting in these two pathways, and mediators of other steps as well.

Chemosensory neuron anatomy and function

C. elegans has fourteen types of chemosensory neurons, of which eleven pairs are included in the amphid sensory organs. *C. elegans* chemosensory amphid neurons have a simple bipolar morphology, consisting of an axon, a cell body a few microns in diameter, a single non-branching dendrite approximately 100 μm long, and a non-motile cilium at

the tip of the dendrite (see Chapter 2, Figure 3 and Chapter 3, Figure 2 for diagrams). The anterior process is defined as the dendrite functionally, because it receives information, while the posterior process joining the nerve ring is defined as the axon because it contains synapses. However, these axons have both pre- and postsynaptic sites. The cilia are exposed to the environment through an opening in the worm's cuticle called the amphid channel. Thus these cells receive information about the world in the form of chemicals in the environment. The neurons transmit information through *en passant* synapses in the axon.

Using this handful of neurons, *C. elegans* are able to sense hundreds of compounds. The nematodes respond to some chemicals by chemotaxis toward them, but avoid others. The sensory functions of individual types of amphid neurons have been defined by laser ablation and behavioral experiments (bargmann 91). The three types of "wing" neurons, AWA, AWB, and AWC, sense volatile chemicals, the ADL neurons sense volatile repellents, and the "exposed" neurons, ASE, ADF, ASG, ASI, ASJ, and ASK sense water-soluble compounds. The ASH neurons mediate osmotic avoidance, volatile avoidance, and nose-touch avoidance (Kaplan and Horvitz, 1993). Different cells have distinctly shaped cilia (Ward et al., 1975; Ware, 1975). The wing cells have larger and more complex cilia than the exposed neurons, presumably for better reception of volatile chemicals, which can exist at very low concentrations in the air (see Chapter 3, Figure 3). Mutants have been isolated with general or specific defects in the functions of these ciliated neurons: the Che, Osm, Odr, and Tax mutants.

When I entered the lab, these neurons had been well-characterized functionally, and many chemosensory mutants had been isolated, but the molecular underpinnings of olfactory transduction in *C. elegans* were still

unknown. Candidate odorant receptors had recently been discovered in mammals (Buck and Axel, 1991), and these were members of the G protein-coupled receptor superfamily. A major focus of the Bargmann Lab was to identify the odorant receptors in *C. elegans*. Several people in the lab, including myself, attempted to clone nematode odorant receptors by homology to the candidate mammalian receptors. These efforts were fruitless. Later, the cloning of *odr-10*, and the discovery of clusters of seven transmembrane domain proteins in the genome revealed that *C. elegans* indeed uses G protein-coupled receptors for olfactory transduction.

G protein-coupled receptor mediated olfactory transduction in *C. elegans*

The first candidate odorant receptors (the OR's) were identified in rat by Buck and Axel in 1991 (Buck and Axel, 1991). It had been suspected that the odorant receptors would be G protein-coupled receptors because odorant stimulation resulted in increased cyclic AMP in olfactory cilia, and adenylyl cyclase is dependent on the presence of GTP (Pace et al., 1985; Sklar et al., 1986; Boekhoff et al., 1990; Breer et al., 1990). Therefore degenerate primers to conserved sequences in seven transmembrane domain proteins were used in PCR from an olfactory neuron library, yielding the OR's. The OR's were believed to be the long-sought odorant receptors for several reasons. First, their expression was limited to the olfactory epithelium. Second, there were many of them and they were highly divergent, as might have been expected since mammals can detect and discriminate thousands of odors. Since the discovery of this family of receptors by Buck and Axel, many related receptors have been cloned in various vertebrate species, including fish and humans (Selbie et al., 1992; Ngai et al., 1993; Barth et al., 1996; Crowe et al., 1996; Barth et al., 1997; Rouquier et al., 1998). Recently, two other families of candidate

vertebrate chemoreceptors have been cloned, the V1R's and the V2R's (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997). These receptors are expressed in the vomeronasal organ and may mediate responses to sex pheromones. The V2R's are larger than the OR's and V1R's, and are most closely related to metabotropic glutamate receptors (Reviewed in Bargmann, 1997).

Many questions remain for the vertebrate chemoreceptors. To date, none of these receptors has been proven to be required for an animal's sensation of a specific chemical. Odorant ligands have been identified for only one vertebrate OR (Zhao et al., 1998). Defining odorant-receptor specificity would perhaps be most easily accomplished by heterologous cell expression and physiology experiments. However, such experiments have proven difficult because receptor expression on the cell surface of heterologous cell types is poor. Furthermore, it has not yet been shown that these receptors are localized to the olfactory cilia, where transduction takes place. In fact, the most pursued function of these receptors has been in axon guidance. These receptors seem to play at least a permissive, and perhaps an instructive role in targeting of olfactory neuron axons to specific glomeruli in the olfactory bulb. This work is reviewed in (Mombaerts et al., 1996) , and will not be discussed further here.

The unbiased approach of forward genetics finally proved that olfactory reception of a specific odorant could indeed be mediated by a G protein-coupled receptor in *C. elegans*. Through genetic screening for a mutant specifically defective in diacetyl chemotaxis, Piali Sengupta cloned *odr-10*. The predicted protein encoded by *odr-10* is a 339 amino acid, seven membrane spanning protein (Sengupta et al., 1996). It displays no homology to any of the vertebrate candidate olfactory receptors, but provided the founding member

of a large family of *C. elegans* candidate chemoreceptors, the *str* family (E. Troemel, unpublished results). Evidence has accumulated that *odr-10* is an odorant receptor, directly responsible for binding to the odorant diacetyl and transducing a signalling response. First, loss of *odr-10* causes a specific defect in chemotaxis to the odorant diacetyl, but not to other odorants. Secondly, ODR-10 is expressed in the AWA neuron (which mediates diacetyl sensation), localizes to the cilia, and requires cilium localization for function (Sengupta et al., 1996; Dwyer et al., 1998). Third, expression of *odr-10* in the AWB avoidance neuron causes animals to avoid diacetyl (Troemel et al., 1997). Fourth, expression of *odr-10* in HEK293 cells confers upon them the ability to respond to diacetyl (Zhang et al., 1997).

Another large family of candidate chemoreceptors, the *sr* family, was found by inspection of genomic sequence that had been deposited in databases by the *C. elegans* Genome Sequencing Consortium. While sharing no primary sequence similarity, the *str* and *sr* gene families share many hallmarks of the putative mammalian odorant receptors. 1. They are large families of extremely divergent serpentine receptors. 2. They are relatively short for seven transmembrane domain receptors, in the neighborhood of 340 amino acids. 3. They reside in genomic clusters (for reasons that are not yet understood). Further supporting the idea that these genes represent chemoreceptors was the finding that two of these receptors, *srd-1* and *srg-2*, localize to chemosensory cilia (Chou et al., 1996). The preliminary characterization of the *sr* family is described in Chapter 2. The finding that a given *C. elegans* chemosensory neuron can express more than one *sr* receptor provided an explanation for the finding from behavioral experiments that one neuron mediates responses to several odorant classes.

Upon ligand binding, G protein-coupled receptors become activated

and initiate a signalling cascade. In *C. elegans*, multiple receptors in AWA, AWB, and AWC probably converge on the Gα protein, ODR-3 (Troemel et al., 1997; Roayaie et al., 1998). Another Ga proteins, *gpa-2*, may also function in AWC (Roayaie et al., 1998), and other *gpa*'s are expressed in chemosensory neurons (Zwaal et al., 1997). The capsaicin receptor-like channel, OSM-9, may be used as the transduction channel in AWA, while the cyclic nucleotide-gated channel encoded by *tax-2* and *tax-4* is required for function of AWC and AWB (Coburn and Bargmann, 1996; Komatsu et al., 1996; Troemel et al., 1997). It is not yet known whether ODR-3 directly activates OSM-9 and TAX-2/TAX-4. There are also several receptor guanylyl cyclases expressed in amphid neurons that may function in olfactory transduction, including gcy-10 in AWC (Yu et al., 1997).

This array of transduction machinery must be localized and clustered in the sensory cilia in order to make odorant detection sensitive and efficient. Several putative chemoreceptors including ODR-10 have been shown to localize subcellularly to the chemosensory cilia (Chou et al., 1996; Sengupta et al., 1996). ODR-3, OSM-9, and TAX-2/TAX-4 are also localized to cilia (Coburn and Bargmann, 1996; Komatsu et al., 1996; Colbert et al., 1997). It is not known how these molecules are each localized, although they may use independent mechanisms, since ODR-10 still appears in the cilia even in the absence of ODR-3 (Roayaie et al., 1998).

Chapters 3 and 4 of this thesis describe the effort to elucidate the mechanisms involved in odorant receptor localization to the cilia, taking a molecular genetic and cell biological approach. While the mechanisms of localization of G proteins and channels will be mentioned, the focus of this thesis has been on odorant receptors, and specifically on *odr-10*, since this is the best-characterized receptor, and the one for which functional behavioral

assays are possible. Chapter 3 describes the discovery of a novel protein, ODR-4, that may be involved in the folding or trafficking of odorant receptors. Chapter 4 describes the observation of rapidly-moving ODR-10-GFP-containing vesicles in the dendrite of AWB, and reports that an AP-1 clathrin adaptor, *unc-101*, is required for the specific localization of ODR-10-GFP in these vesicles and to the cilia. Chapter 5 summarizes where these studies have led us, and suggests directions for future study of the molecular mechanisms of protein targeting in neurons.

REFERENCES

Apodaca, G., M. H. Cardone, S. W. Whiteheart, B. R. DasGupta, and K. E. Mostov. 1996. Reconstitution of transcytosis in SLO-permeabilized MDCK cells: existence of an NSF-dependent fusion mechanism with the apical surface of MDCK cells. *Embo J* 15: 1471-81.

Arvidsson, U., M. Riedl, S. Chakrabarti, J. Lee, A. Nakano, R. Dado, H. Loh, P. Law, M. Wessendorf, and R. Elde. 1995. Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. *Journal of Neuroscience* 15: 3328-41.

Baas, P. W., J. S. Deitch, M. M. Black, and G. A. Banker. 1988. Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. *Proc Natl Acad Sci U S A* 85: 8335-8339.

Bannykh, S. I., N. Nishimura, and W. E. Balch. 1998. Getting into the Golgi. *Trends Cell Biol.* 8: 21-25.

Bargmann, C. I. 1997. Olfactory receptors, vomeronasal receptors, and the organization of olfactory information. *Cell* 90: 585-7.

Barth, A. L., J. C. Dugas, and J. Ngai. 1997. Noncoordinate expression of odorant receptor genes tightly linked in the zebrafish genome. *Neuron* 19: 359-69.

Barth, A. L., N. J. Justice, and J. Ngai. 1996. Asynchronous onset of odorant receptor expression in the developing zebrafish olfactory system. *Neuron* 16: 23-34.

Bastiani, M. J., A. L. Harrelson, P. M. Snow, and C. S. Goodman. 1987. Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. *Cell* 48:

745-55.

Boekhoff, I., E. Tareilus, J. Strotmann, and H. Breer. 1990. Rapid activation of alternative second messenger pathways in olfactory cilia from rats by different odorants. *EMBO J.* 9: 2453-2458.

Bohm, S. K., L. M. Khitin, S. P. Smeekens, E. F. Grady, D. G. Payan, and N. W. Bunnett. 1997. Identification of potential tyrosine-containing endocytic motifs in the carboxyl-tail and seventh transmembrane domain of the neurokinin 1 receptor. *J Biol Chem* 272: 2363-72.

Brakeman, P. R., A. A. Lanahan, R. O'Brien, K. Roche, C. A. Barnes, R. L. Huganir, and P. F. Worley. 1997. Homer: a protein that selectively binds metabotropic glutamate receptors [see comments]. *Nature* 386: 284-288.

Brandstatter, J. H., P. Koulen, R. Kuhn, H. van der Putten, and H. Wassle. 1996. Compartmental localization of a metabotropic glutamate receptor (mGluR7): two different active sites at a retinal synapse. *J Neurosci* 16: 4749-56.

Breer, H., I. Boekhoeff, and E. Tareilus. 1990. Rapid kinetics of second messenger formation in olfactory transduction. *Nature* 345: 65-68.

Buck, L., and R. Axel. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65: 175-187.

Campos-Ortega, J. A. 1997. Asymmetic division: dynastic intricacies of neuroblast division. *Curr. Biol.* 7: R726-R728.

Chevesich, J., A. J. Kreuz, and C. Montell. 1997. Requirement for the PDZ domain protein, INAD, for localization of the TRP store-operated channel to a signaling complex. *Neuron* 18: 95-105.

Chou, J. H., E. R. Troemel, P. Sengupta, H. A. Colbert, L. Tong, D. M. Tobin, K. Roayaie, J. G. Crump, N. D. Dwyer, and C. I. Bargmann. 1996. Olfactory recognition and discrimination in *Caenorhabditis elegans. Cold Spring*

Harb Symp Quant Biol 61: 157-64.

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

Colbert, H. A., T. L. Smith, and C. I. Bargmann. 1997. OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptaion in *Caenorhabditis elegans*. *J. Neurosci.* 17: 8259-8269.

Colley, N., E. Baker, M. Stamnes, and C. Zuker. 1991. The cyclophilin homolog ninaA is required in the secretory pathway. *Cell* 67: 255-263.

Cosson, P., and F. Letourner. 1994. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science* 263: 1629-1631.

Crowe, M. L., B. N. Perry, and I. F. Connerton. 1996. Olfactory receptor-encoding genes and pseudogenes are expressed in humans. *Gene* 169: 247-9.

Deretic, D., L. A. Huber, N. Ransom, M. Mancini, K. Simons, and D. S. Papermaster. 1995. rab8 in retinal photoreceptors may participate in rhodopsin transport and in rod outer segment disk morphogenesis. *J Cell Sci* 108: 215-24.

Deretic, D., and D. S. Papermaster. 1993. Rab6 is associated with a compartment that transports rhodopsin from the trans-Golgi to the site of rod outer segment disk formation in frog retinal photoreceptors. *J Cell Sci* 106: 803-13.

Dodd, J., S. B. Morton, D. Karagogeos, M. Yamamoto, and T. M. Jessell. 1988. Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. *Neuron* 1: 105-16.

Dotti, C. G., R. G. Parton, and K. Simons. 1991. Polarized sorting of glypiated

proteins in hippocampal neurons. *Nature* 349: 158-61.

Dotti, C. G., and K. Simons. 1990. Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell* 62: 63-72.

Dulac, C., and R. Axel. 1995. A novel family of genes encoding putative pheromone receptors in mammals. *Cell* 83: 195-206.

Dwyer, N. D., E. R. Troemel, P. Sengupta, and C. I. Bargmann. 1998. Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. *Cell* 93: 455-466.

Ferreira, P. A., T. A. Nakayama, W. L. Pak, and G. H. Travis. 1996. Cyclophilin-related protein RanBP2 acts as chaperone for red/green opsin. *Nature* 383: 637-40.

Ferreira, P. A., T. A. Nakayama, and G. H. Travis. 1997. Interconversion of red opsin isoforms by the cyclophilin-related chaperone protein Ran-binding protein 2. *Proc Natl Acad Sci U S A* 94: 1556-61.

Gautam, M., P. G. Noakes, J. Mudd, M. Nichol, G. C. Chu, J. R. Sanes, and J. P. Merlie. 1995. Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* 377: 232-6.

Goodman, O., Jr., J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen, and J. L. Benovic. 1996. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* 383: 447-50.

Gudermann, T., T. Schoneberg, and G. Schultz. 1997. Functional and structural complexity of signal transduction via G-protein-coupled receptors. *Annu Rev Neurosci* 20: 399-427.

Hanlon, D. W., Z. Yang, and L. S. Goldstein. 1997. Characterization of KIFC2, a neuronal kinesin superfamily member in mouse. *Neuron* 18: 439-51.

Hanson, P. I., J. E. Heuser, and R. Jahn. 1997. Neurotransmitter release - four years of SNARE complexes. *Curr Opin Neurobiol* 7: 310-315.

Henley, J. R., and M. A. McNiven. 1996. Association of a dynamin-like protein with the Golgi apparatus in mammalian cells. *J Cell Biol* 133: 761-75.

Herrada, G., and C. Dulac. 1997. A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. *Cell* 90: 763-73.

Herskovits, J. S., C. C. Burgess, R. A. Obar, and R. B. Vallee. 1993. Effects of mutant rat dynamin on endocytosis. *J Cell Biol* 122: 565-78.

Hicke, L., and H. Riezman. 1996. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell* 84: 277-287.

Hille-Rehfeld, A. 1995. Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. *Biochim Biophys Acta* 1241: 177-94.

Hirokawa, N. 1998. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science* 279: 519-526.

Huber, L. A., M. J. de Hoop, P. Dupree, M. Zerial, K. Simons, and C. Dotti. 1993a. Protein transport to the dendritic plasma membrane of cultured neurons is regulated by rab8p. *J Cell Biol* 123: 47-55.

Huber, L. A., S. Pimplikar, R. G. Parton, H. Virta, M. Zerial, and K. Simons. 1993b. Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. *J Cell Biol* 123: 35-45.

Ikonen, E., M. Tagaya, O. Ullrich, C. Montecucco, and K. Simons. 1995. Different requirements for NSF, SNAP, and Rab proteins in apical and basolateral transport in MDCK cells. *Cell* 81: 571-80.

Kaplan, J. M., and H. R. Horvitz. 1993. A dual mechanosensory and chemosensory neuron in Caenorhabditis elegans. *Proc Natl Acad Sci U*

S A 90: 2227-31.

Kaplan, M. R., A. Meyer-Franke, S. Lambert, V. Bennett, I. D. Duncan, S. R. Levinson, and B. A. Barres. 1997. Induction of sodium channel clustering by oligodendrocytes. *Nature* 386: 724-8.

Kidd, T., K. Brose, K. J. Mitchell, R. D. Fetter, M. Tessier-Lavigne, C. S. Goodman, and G. Tear. 1998. Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* 92: 205-15.

Kirsch, J., I. Wolters, A. Triller, and H. Betz. 1993. Gephyrin antisense oligonucleotides prevent glycine receptor clustering in spinal neurons. *Nature* 366: 745-8.

Knowles, R. B., J. H. Sabry, M. E. Martone, T. J. Deerinck, M. H. Ellisman, G. J. Bassell, and K. S. Kosik. 1996. Translocation of RNA granules in living neurons. *J. Neurosci.* 16: 7812-7820.

Koenig, J. A., and J. M. Edwardson. 1997. Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol. Sci.* 18: 276-287.

Komatsu, H., I. Mori, J. S. Rhee, N. Akaike, and Y. Ohshima. 1996. Mutations in a cyclic nucleotide-gated channel lead to abnormal thermosensation and chemosensation in *C. elegans*. *Neuron* 17: 707-18.

Kordeli, E., J. Davis, B. Trapp, and V. Bennett. 1990. An isoform of ankyrin is localized at nodes of Ranvier in myelinated axons of central and peripheral nerves. *J Cell Biol* 110: 1341-52.

Kornau, H. C., L. T. Schenker, M. B. Kennedy, and P. H. Seeburg. 1995. Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* 269: 1737-40.

Langford, G. M. 1995. Actin- and microtubule-dependent organelle motors: interrelationships between the two motility systems. *Curr Opin Cell*

Biol 7: 82-8.

Lewis, M. J., D. J. Sweet, and H. R. Pelham. 1990. The ERD2 gene determines the specificity of the luminal ER protein retention system. *Cell* 61: 1359-63.

Lisanti, M. P., I. W. Caras, M. A. Davitz, and E. Rodriguez-Boulan. 1989. A glycophospholipid membrane anchor acts as an apical targeting signal in polarized epithelial cells. *J Cell Biol* 109: 2145-56.

Lisanti, M. P., M. Sargiacomo, L. Graeve, A. R. Saltiel, and E. Rodriguez-Boulan. 1988. Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line. *Proc Natl Acad Sci U S A* 85: 9557-61.

Low, S. H., S. J. Chapin, T. Weimbs, L. G. Komuves, M. K. Bennett, and K. E. Mostov. 1996. Differential localization of syntaxin isoforms in polarized Madin-Darby canine kidney cells. *Mol Biol Cell* 7: 2007-18.

Marks, M., H. Ohno, T. Kirchhausen, and S. Bonifacino. 1997. Protein sorting by tyrosine-based signals: Adapting to the Ys and wherefores. *Trends in Cell Biology* 7: 124-128.

Matsunami, H., and L. B. Buck. 1997. A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell* 90: 775-84.

Mombaerts, P., F. Wang, C. Dulac, R. Vassar, S. K. Chao, A. Nemes, M. Mendelsohn, J. Edmondson, and R. Axel. 1996. The molecular biology of olfactory perception. *Cold Spring Harb Symp Quant Biol* 61: 135-45.

Morales, J., C. S. Fishburn, P. T. Wilson, and H. R. Bourne. 1998. Plasma membrane localization of G alpha z requires two signals. *Mol Biol Cell* 9: 1-14.

Mostov, K. E., and M. H. Cardone. 1995. Regulation of protein traffic in polarized epithelial cells. *Bioessays* 17: 129-38.

Munro, S. 1998. Localization of proteins to the Golgi apparatus. *Trends Cell Biol.* 8: 11-15.

Ngai, J., M. Dowling, L. Buck, R. Axel, and A. Chess. 1993. The family of genes encoding odorant receptors in the channel catfish. *Cell* 72: 657-666.

Nishimura, N., and W. E. Balch. 1997. A di-acidic signal required for selective export from the endoplasmic reticulum. *Science* 277: 556-8.

Novick, P., and M. Zerial. 1997. The diversity of Rab proteins in vesicle transport. *Curr Opin Cell Biol* 9: 496-504.

Ohno, H., J. Stewart, M. C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J. S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* 269: 1872-5.

Orci, L., M. Stamnes, M. Ravazzola, M. Amherdt, A. Perrelet, T. H. Sollner, and J. E. Rothman. 1997. Bidirectional transport by distinct populations of COPI-coated vesicles. *Cell* 90: 335-49.

Pace, U., E. Hanski, Y. Salomon, and D. Lancet. 1985. Odorant-sensitive adenylate cyclase may mediate olfactory reception. *Nature* 316: 255-258.

Patel, N. H., P. M. Snow, and C. S. Goodman. 1987. Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell* 48: 975-88.

Pfeffer, S. R. 1991. Mannose-6-phosphate receptors and their role in protein sorting along the pathway to lysosomes. *Cell Biophys* 19: 131-40.

Rassow, J., O. vonAhsen, U. Bomer, and N. Pfanner. 1997. Molecular chaperones: Towards a characterization of the heat-shock protein 70 family. *Trends Cell Biol.* 7: 129-133.

Roayaie, K., J. G. Crump, A. Sagasti, and C. I. Bargmann. 1998. The Gα Protein ODR-3 Mediates Olfactory and Nociceptive Function and Controls

Cilium Morphogenesis in C. elegans Olfactory Neurons. *Neuron* 20: 55-67.

Robinson, M. S. 1997. Coats and vesicle budding. *Trends Cell Biol.* 7: 99-102.

Robinson, M. S., C. Watts, and M. Zerial. 1996. Membrane dynamics in endocytosis. *Cell* 84: 13-21.

Rothman, J. E., and F. T. Wieland. 1996. Protein sorting by transport vesicles. *Science* 272: 227-34.

Rouquier, S., S. Taviaux, B. J. Trask, V. Brand-Arpon, G. van den Engh, J. Demaille, and D. Giorgi. 1998. Distribution of olfactory receptor genes in the human genome. *Nat Genet* 18: 243-50.

Saito, N., Y. Okada, Y. Noda, Y. Kinoshita, S. Kondo, and N. Hirokawa. 1997. KIFC2 is a novel neuron-specific C-terminal type kinesin superfamily motor for dendritic transport of multivesicular body-like organelles. *Neuron* 18: 425-38.

Sassoe-Pognetto, M., J. Kirsch, U. Grunert, U. Greferath, J. M. Fritschy, H. Mohler, H. Betz, and H. Wassle. 1995. Colocalization of gephyrin and GABAA-receptor subunits in the rat retina. *J Comp Neurol* 357: 1-14.

Sassoe-Pognetto, M., and H. Wassle. 1997. Synaptogenesis in the rat retina: subcellular localization of glycine receptors, GABA(A) receptors, and the anchoring protein gephyrin. *J Comp Neurol* 381: 158-74.

Saunders, C., and L. E. Limbird. 1997. Disruption of microtubules reveals two independent apical targeting mechanisms for G-protein-coupled receptors in polarized renal epithelial cells. *J Biol Chem* 272: 19035-45.

Scheiffele, P., J. Peranen, and K. Simons. 1995. N-glycans as apical sorting signals in epithelial cells. *Nature* 378: 96-8.

Schekman, R., and L. Orci. 1996. Coat proteins and vesicle budding. *Science* 271: 1526-33.

Schmid, S. L. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu Rev Biochem* 66: 511-48.

Selbie, L. A., A. Townsend-Nicholson, T. P. Iismaa, and J. Shine. 1992. Novel G protein-coupled receptors: a gene family of putative human olfactory receptor sequences. *Brain Res Mol Brain Res* 13: 159-63.

Semenza, J. C., K. G. Hardwick, N. Dean, and H. R. Pelham. 1990. ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* 61: 1349-57.

Sengupta, P., J. H. Chou, and C. I. Bargmann. 1996. *odr-10* encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl. *Cell* 84: 899-909.

Sheng, M., and M. Wyszynski. 1997. Ion Channel Targeting in Neurons. *BioEssays* 19: 847-853.

Shigemoto, R., A. Kulik, J. D. Roberts, H. Ohishi, Z. Nusser, T. Kaneko, and P. Somogyi. 1996. Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. *Nature* 381: 523-5.

Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature* 387: 569-72.

Simske, J. S., S. M. Kaech, S. A. Harp, and S. K. Kim. 1996. LET-23 receptor localization by the cell junction protein LIN-7 during *C. elegans* vulval induction. *Cell* 85: 195-204.

Sklar, P. B., R. R. H. Anholt, and S. H. Snyder. 1986. The odorant-sensitive adenylate cyclase of olfactory receptor cells: differential stimulation by distinct classes of odorants. *J. Biol. Chem.* 261: 25538-25543.

Stack, J. H., B. Horazdovsky, and S. D. Emr. 1995. Receptor-mediated protein sorting to the vacuole in yeast: roles for a protein kinase, a lipid kinase and GTP-binding proteins. *Ann Rev Cell Dev Biol* 11: 1-33.

Stamnes, M. A., B. H. Shieh, L. Chuman, G. L. Harris, and C. S. Zuker. 1991. The cyclophilin homolog *ninaA* is a tissue-specific integral membrane protein required for the proper synthesis of a subset of *Drosophila* rhodopsins. *Cell* 65: 219-27.

Stoeckli, E. T., U. Ziegler, A. J. Bleiker, P. Groscurth, and P. Sonderegger. 1996. Clustering and functional cooperation of Ng-CAM and axonin-1 in the substratum-contact area of growth cones. *Dev Biol* 177: 15-29.

Tejedor, F. J., A. Bokhari, O. Rogero, M. Gorczyca, J. Zhang, E. Kim, M. Sheng, and V. Budnik. 1997. Essential role for dlg in synaptic clustering of Shaker K+ channels *in vivo*. *J Neurosci* 17: 152-9.

Traub, L. M., and S. Kornfeld. 1997. The trans-Golgi network: a late secretory sorting station. *Curr Opin Cell Biol* 9: 527-33.

Troemel, E. R., B. E. Kimmel, and C. I. Bargmann. 1997. Reprogramming chemotaxis responses: sensory neurons define olfactory preferences in *C. elegans*. *Cell* 91: 161-169.

van der Bliek, A. M., T. E. Redelmeier, H. Damke, E. J. Tisdale, E. M. Meyerowitz, and S. L. Schmid. 1993. Mutations in human dynamin block an intermediate stage in coated vesicle formation. *J Cell Biol* 122: 553-63.

Wan, Q., Z. G. Xiong, H. Y. Man, C. A. Ackerley, J. Braunton, W. Y. Lu, L. E. Becker, J. F. MacDonald, and Y. T. Wang. 1997. Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin. *Nature* 388: 686-90.

Ward, S., N. Thomson, J. G. White, and S. Brenner. 1975. Electron microscopical reconstruction of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*.. *J Comp Neurol* 160: 313-37.

Ware, R. W. 1975. Three-dimensional reconstruction from serial sections. *Int*

Rev Cytol 40: 325-440.

Wilhelm, J. E., and R. D. Vale. 1993. RNA on the move: the mRNA localization pathway. *J Cell Biol* 123: 269-74.

Wozniak, M., J. R. Keefer, C. Saunders, and L. E. Limbird. 1997. Differential targeting and retention of G protein-coupled receptors in polarized epithelial cells. *J. Recept. Signal Transduct. Res.* 17: 373-383.

Wozniak, M., and L. E. Limbird. 1996. The three alpha 2-adrenergic receptor subtypes achieve basolateral localization in Madin-Darby canine kidney II cells via different targeting mechanisms. *J. Biol. Chem.* 271: 5017-5024.

Yang, S. H., P. F. Armson, J. Cha, and W. D. Phillips. 1997. Clustering of GABAA receptors by rapsyn/43kD protein in vitro. *Mol Cell Neurosci* 8: 430-8.

Yu, S., L. Avery, E. Baude, and D. L. Garbers. 1997. Guanylyl cyclase expression in specific sensory neurons: a new family of chemosensory receptors. *Proc Natl Acad Sci U S A* 94: 3384-7.

Zhang, Y., J. H. Chou, J. Bradley, C. I. Bargmann, and K. Zinn. 1997. The *Caenorhabditis elegans* seven-transmembrane protein ODR-10 functions as an odorant receptor in mammalian cells. *Proc. Natl. Acad. Sci. USA* 94: 12162-12167.

Zhao, H., L. Ivic, J. M. Otaki, M. Hashimoto, K. Mikoshiba, and S. Firestein. 1998. Functional expression of a mammalian odorant receptor. *Science* 279: 237-42.

Zwaal, R. R., J. E. Mendel, P. W. Sternberg, and R. H. Plasterk. 1997. Two neuronal G proteins are involved in chemosensation of the *Caenorhabditis elegans* dauer-inducing pheromone. *Genetics* 145: 715-27.

CHAPTER 2

Divergent Seven Transmembrane Receptors are Candidate Chemosensory Receptors in *C. elegans*

(published in Cell, Vol. 83, No. 2, pp. 207-218, 1995)

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 aldenydes, 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).

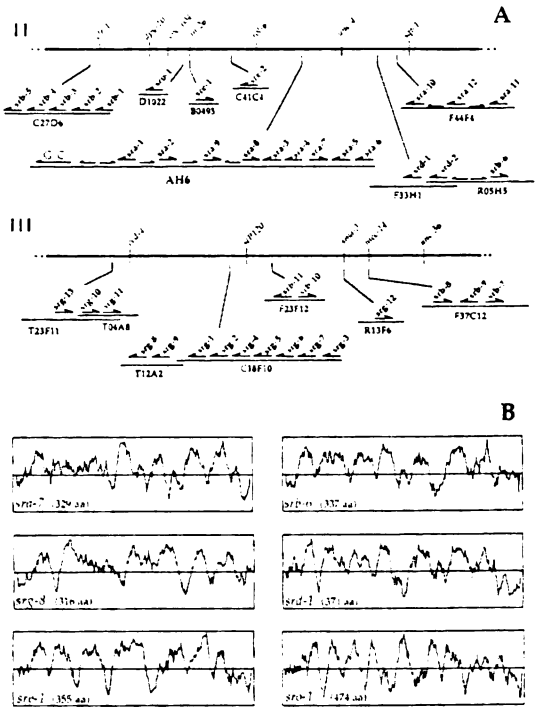


Figure 1. Genomic Organization and Structure of Predicted Receptor Genes

(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-

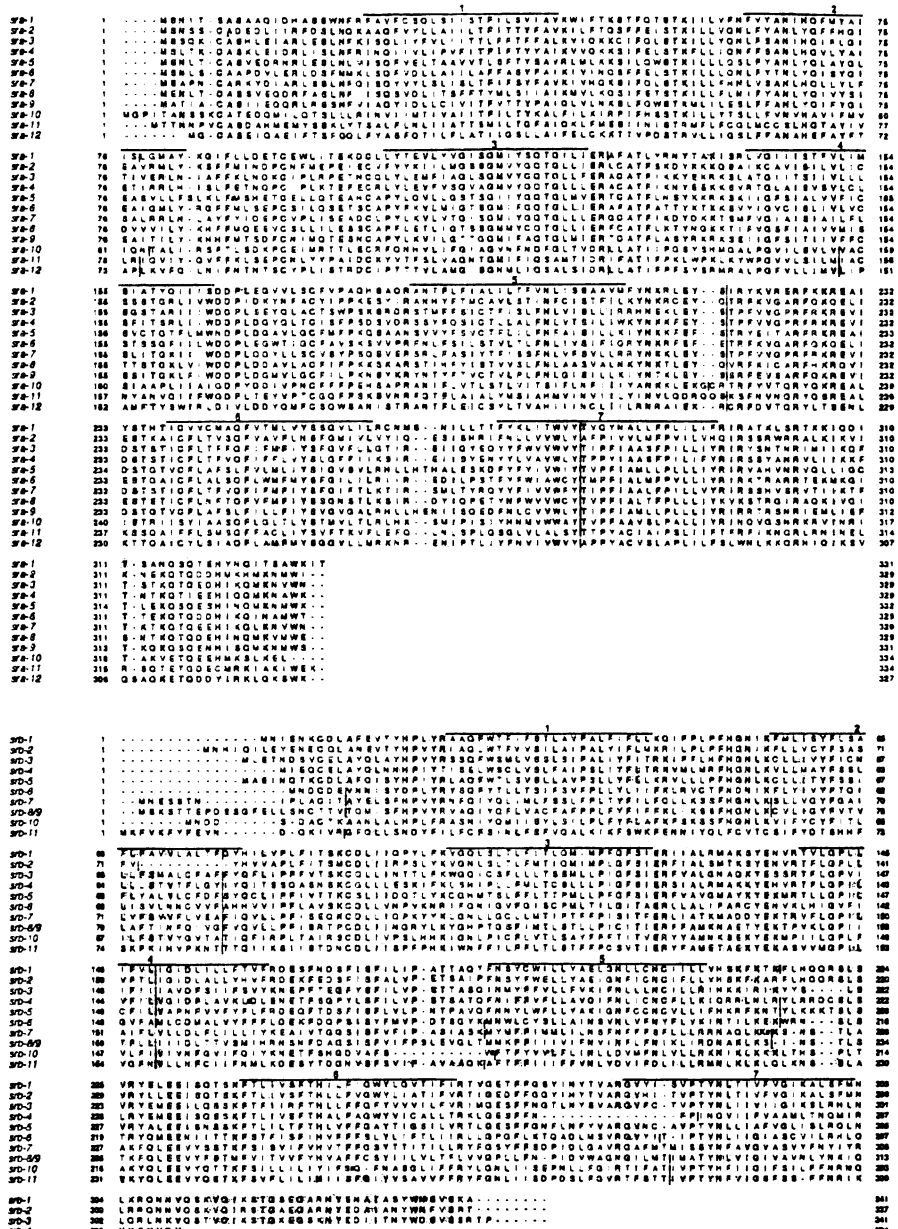


Figure 2. Sequence Alignments of Predicted Seven Transmembrane Receptors

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).

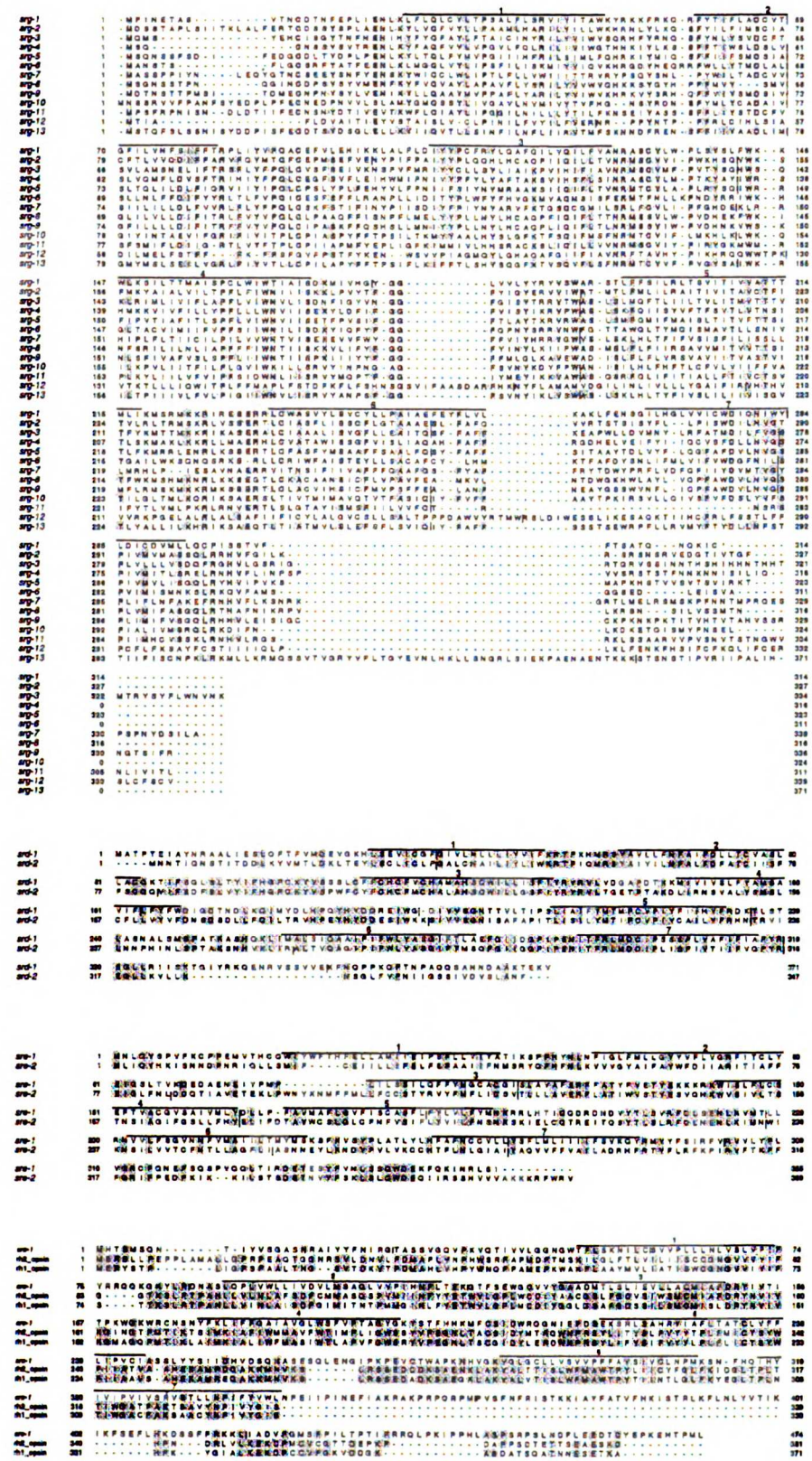
The *srb* family: *srb-1*, *srb-2*, *srb-3*, *srb-4*, and *srb-5* were all different from the previously predicted genes. *srb-8* and *srb-9* were also different from the previously predicted genes; these two genes are identical at the amino acid level. *srb-4*, *srb-7*, and *srb-10* could only be aligned 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.



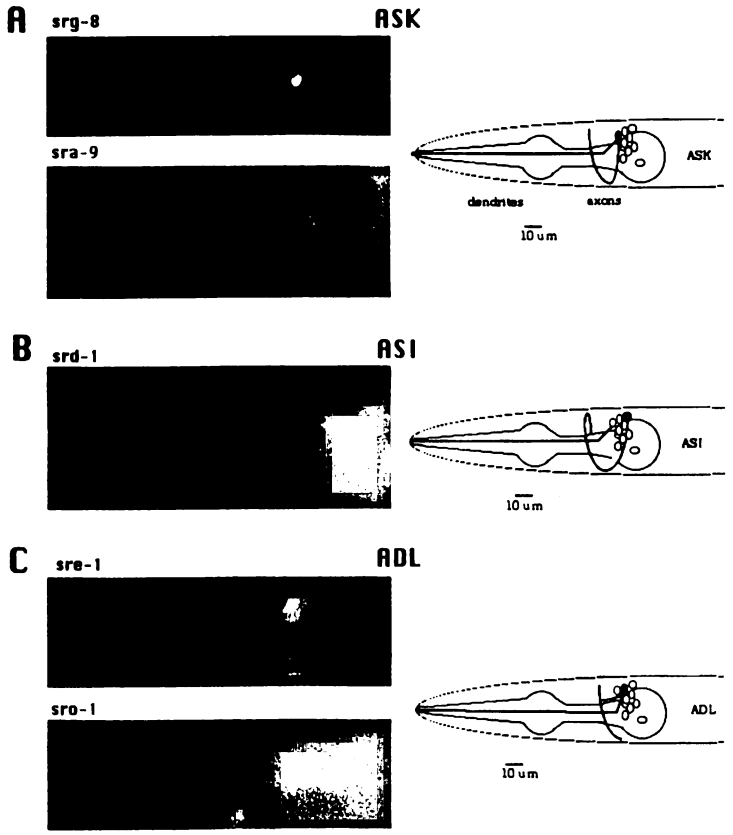


Figure 3. Expression of Reporter Gene Constructs in the ASK, ADL, and ASI Neurons

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.

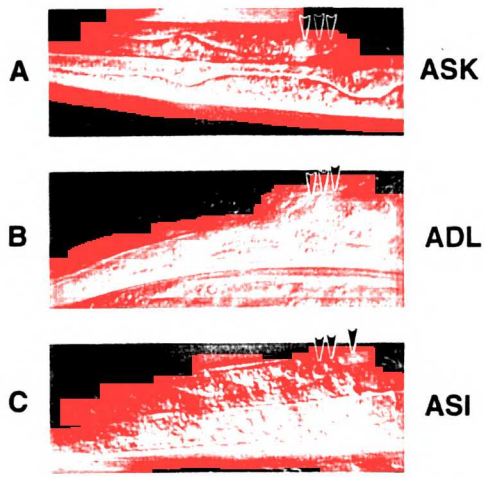


Figure 4. Alignment of Fluorescence and Nomarski Images

(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)			Number of Assays (number of animals)
	Median	Mean	SEM	
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.

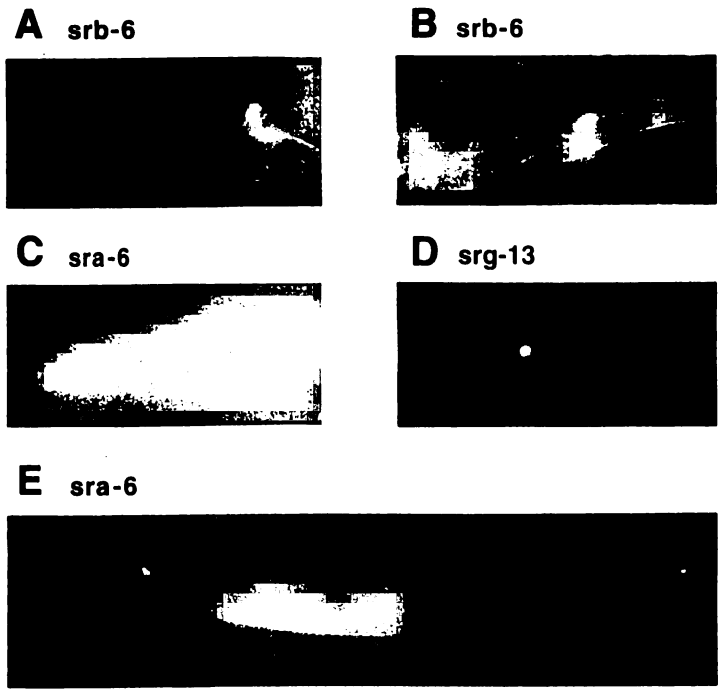


Figure 5. Expression of Additional Reporter Gene Constructs in Sensory and Nonsensory Cell Types

(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

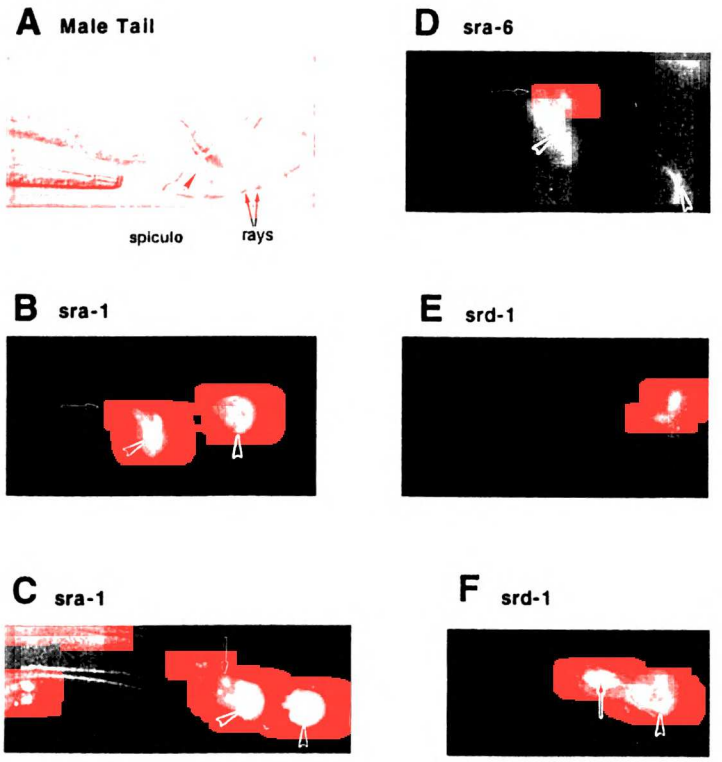


Figure 6. Male-Specific Expression of Fusion Genes

(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 framesnifts 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).

Acknowledgments

We thank Piali Sengupta and Kayvan Roayaie for their advice and assistance; Liqin Tong for technical assistance; Erik Sonnhammer, Steve Jones, Richard Zwaal, Ronald Plasterk, Jane Mendel, Paul Sternberg, Beth Sawin, Catherine Dulac, Richard Axel, and especially the C. elegans genome sequencing consortium for sharing unpublished data; and Piali Sengupta, Marc Tessier-Lavigne, and Richard Axel for their comments on this manuscript. This work was supported by Public Health Service grant DC01393. C. I. B. is a Markey Scholar and a Searle Scholar, and this work was supported in part by grants from these foundations. C. I. B. is an Assistant Investigator of the Howard Hughes Medical Institute.

Received July 21, 1995; revised August 21, 1995.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Avery, L., and Horvitz, H.R. (1987). A cell that dies during wild-type C. elegans development can function as a neuron in a *ced-3* mutant. Cell *51*, 1071–1078.

Avery, L., and Horvitz, H.R. (1990). Effects of starvation and neuroactive drugs on feeding in *Caenorhabditis elegans*. J. Exp. Zool. *253*, 263–270.

Bargmann, C.I., Hartwieg, E., and Horvitz, H.R. (1993). Odorant-

selective genes and neurons mediate olfaction in C. elegans. Cell *74*, 515–527.

Bargmann, C.I., and Horvitz, H.R. (1991a). Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in *C. elegans*. Neuron *7*, 729–742.

Bargmann, C.I., and Horvitz, H.R. (1991b). Control of larval development by chemosensory neurons in *Caenorhabditis elegans*. Science *251*, 1243–1246.

Bargmann, C.I., Thomas, J.H., and Horvitz, H.R. (1990). Chemosensory cell function in the behavior and development of *Caenorhabditis elegans*. Cold Spring Harbor Symp. Quant. Biol. *55*, 529–538.

Ben-Arie, N., Lancet, D., Taylor, C., Khen, M., Walker, N., Ledbetter, D., Carrozzo, R., Patel, K., Sheer, D., Lehrach, H., and North, M.A. (1994). Olfactory receptor gene cluster on human chromosome 17: possible duplication of an ancestral receptor repertoire. Hum. Mol. Genet. *3*, 229–235.

Boekhoff, I., Michel, W., Breer, H., and Ache, B. (1994). Single odors differentially stimulate dual second messenger pathways in lobster olfactory receptor cells. J. Neurosci. *14*, 3304–3309.

Breer, H., Boekhoeff, I., and Tareilus, E. (1990). Rapid kinetics of second messenger formation in olfactory transduction. Nature *345*, 65–68.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71–94.

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell *65*, 175–187.

Carulli, J.P., and Hartl, D.L. (1992). Variable rates of evolution among *Drosophila* opsin genes. Genetics *132*, 193–204.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802–805.

Chess, A., Simon, I., Cedar, H., and Axel, R. (1994). Allelic inactivation regulates olfactory receptor gene expression. Cell *78*, 823–834.

Colbert, H.A., and Bargmann, C.I. (1995). Odorant-specific adaptation pathways generate olfactory plasticity in C. elegans. Neuron *14*, 803–812.

Culotti, J.G., and Russell, R.L. (1978). Osmotic avoidance defective mutants of the nematode *Caenorhabditis elegans*. Genetics *90*, 243–256.

Dulac, C., and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. Cell *83*, this issue.

Dusenbery, D.B. (1974). Analysis of chemotaxis in the nematode *Caenorhabditis elegans* by countercurrent separation. J. Exp. Zool. *188*, 41–47.

Golden, J.W., and Riddle, D.L. (1984). A *Caenorhabditis elegans* dauer-inducing pheromone and an antagonistic component of the food supply. J. Chem. Ecol. *10*, 1265–1280.

Hodgkin, J. (1983). Male phenotypes and mating efficiency in *Caenorhabditis elegans*. Genetics *103*, 43–64.

Horvitz, H.R., Chalfie, M., Trent, C., Sulston, J.E., and Evans, P.D. (1982). Serotonin and octopamine in the nematode *Caenorhabditis elegans*. Science *216*, 1012–1014.

Huang, L.S., Tzou, P., and Sternberg, P.W. (1994). The *lin-15* locus encodes two negative regulators of *Caenorhabditis elegans* vulval development. Mol. Biol. Cell *5*, 395–412.

Huber, A., Smith, D.P., Zuker, C.S., and Paulsen, R. (1990). Opsin of *Calliphora* peripheral photoreceptors R1–6: homology with *Drosophila* Rh1 and posttranslational processing. J. Biol. Chem. *265*, 17906–17910.

Kaplan, J., and Horvitz, H. (1993). A dual mechanosensory and chemosensory neuron in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA *90*, 2227–2231.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. *157*, 105–132.

Lawless, H.T. (1987). Gustatory psychophysics. In Neurobiology of Taste and Smell, T.E. Finger and W.L. Silver, eds. (New York: John Wiley and Sons), pp. 401–420.

Liu, K.S., and Sternberg, P.W. (1995). Sensory regulation of male mating behavior in Caenorhabditis elegans. Neuron *14*, 79–89.

Lynch, A.S., Briggs, D., and Hope, I.A. (1995). A screen of developmental expression patterns for genes predicted in the *C. elegans* genome sequencing project. Nature Genet., in press.

McBurney, D.H., Smith, D.V., and Shick, T.R. (1972). Gustatory cross acaptation: sourness and bitterness. Percept. Psychophys. *11*, 228–232.

Mello, C.C., Kramer, J.M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. *10*, 3959–3970.

Probst, W.C., Snyder, L.A., Schuster, D.I., Brosius, J., and Sealfon, S.C. (1992). Sequence alignment of the G-protein coupled receptor superfamily. DNA Cell Biol. *11*, 1–20.

Ressler, K., Sullivan, S., and Buck, L. (1993). A zonal organization of odorant receptor gene expression in the olfactory epithelium. Cell *73*, 597–609.

Ressler, K.J., Sullivan, S.L., and Buck, L.B. (1994). Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. Cell *79*, 1245–1256.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sulston, J., Du, Z., Thomas, K., Wilson, R., Hillier, L., Staden, R., Halloran, N., Green, P., Thierry-Mieg, J., Qiu, L., Dear, S., Coulson, A., Craxton, M., Durbin, R., Berks, M., Metzstein, M., Hawkins, T., Ainscough, R., and Waterston, R. (1992). The *C. elegans* genome sequencing project: a beginning. Nature *356*, 37–41.

Sulston, J.E., Albertson, D.G., and Thomson, J.N. (1980). The *Caenorhabditis elegans* male: postembryonic development of nongonadal structures. Dev. Biol. *78*, 542–576.

Thomas, D.D., and Stryer, L. (1982). Transverse location of the retinal chromophore in rod outer segment disc membranes. J. Mol. Biol. *154*, 145–157.

Thomas, J.H. (1990). Genetic analysis of defecation in *Caenorhabditis elegans*. Genetics *124*, 855–872.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. *22*, 4673–4680.

Vanderhaeghen, P., Schurmans, S., Vassart, G., and Parmentier, M. (1993). Olfactory receptors are displayed on dog mature sperm cells. J. Cell Biol. *123*, 1441–1452.

Vassar, R., Ngai, J., and Axel, R. (1993). Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. Cell *74*, 309–318.

Vasser, R., Chao, S.K., Sitcheran, R., Nuñez, J.M., Vosshall, L.B., and Axel, R. (1994). Topographic organization of sensory projections to the olfactory bulb. Cell *79*, 981–992.

Ward, S. (1973). Chemotaxis by the nematode *Caenorhabditis elegans*: identification of attractants and analysis of the response by use of mutants. Proc. Natl. Acad. Sci. USA *70*, 817–821.

Ward, S., Thomson, N., White, J.G., and Brenner, S. (1975). Electron microscopical reconstruction of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*. J. Comp. Neurol. *160*, 313–337.

Ware, R.W., Clark, D., Crossland, K., and Russell, R.L. (1975). The nerve ring of the nematode *Caenorhabditis elegans*: sensory input and motor output. J. Comp. Neurol. *162*, 71–110.

White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. Phil. Trans. Roy. Soc. (Lond.) B *314*, 1–340.

Zorio, D., Cheng, N., Blumenthal, T., and Spieth, J. (1994). Operons as a common form of chromosomal organization in *C. elegans*. Nature *372*, 270–272.

CHAPTER 3

Odorant Receptor Localization to Olfactory Cilia is Mediated by ODR-4, a Novel Membrane-Associated Protein

(published in Cell, Vol. 93, No. 3, pp. 455-466, 1998)

Odorant Receptor Localization to Olfactory Cilia Is Mediated by ODR-4, a Novel Membrane-Associated Protein

Noelle D. Dwyer, Emily R. Troemel, Piali Sengupta,† 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

Seven transmembrane domain receptors can be localized to different parts of the plasma membrane or to different intracellular compartments in a receptor-specific and cell type–specific fashion. We show here that the *C. elegans* genes *odr-4* and *odr-8* are required for localization of a subset of seven transmembrane domain odorant receptors to the cilia of olfactory neurons. Other cilia-signaling proteins, including ion channels, a Gα protein, and even other receptor types, are localized via an *odr-4*/*odr-8*–independent pathway. *odr-4* encodes a novel membrane protein that is expressed exclusively on intracellular membranes of chemosensory neurons, where it acts cell-autonomously to facilitate odorant receptor folding or localization.

Introduction

Polarized cells such as epithelial cells and neurons create functional compartments by localizing signaling machinery to specialized regions on the cell surface. Molecules such as channels and receptors are targeted to and maintained at distinct sites whose spatial arrangement is crucial for cell function. Although much is known about the spatial distribution patterns of signaling molecules on cells, little is known of the mechanisms by which proper targeting is achieved and regulated. For example, the localization pathways for seven transmembrane domain G protein–coupled receptors, the largest known class of cell surface receptors, are mysterious.

Seven transmembrane domain receptors display a wide variety of receptor-specific and cell-specific localization patterns. In neurons, seven transmembrane domain receptors are often localized to either axons or dendrites and are sometimes further localized to specific synapses (Shigemoto et al., 1996). The identical receptor can display different behaviors in different cell types: the μ-opioid receptor 1 is localized to dendrites in CNS neurons and to axons in peripheral neurons (Arvidsson et al., 1995). Polarized epithelial cells, long used as a model for protein trafficking, localize some seven transmembrane receptors, such as the A1 adenosine receptor, to the apical surface, but they localize others, such as the α-adrenergic receptors, to the basolateral surface (Saunders et al., 1996; Wozniak and Limbird, 1996; Wozniak et al., 1997). In these cells, multiple mechanisms seem to be used to produce this distribution, including localized delivery and ubiquitous delivery with selective retention (Wozniak and Limbird, 1996). Even within one cell type, the localization patterns of seven transmembrane receptors can be dynamic. G protein–coupled receptors can be mobilized to the cell surface in response to activation of signal transduction pathways (Shapiro et al., 1996; Ng et al., 1997), or they can be removed from the surface after ligand binding (Bohm et al., 1997; Koenig and Edwardson, 1997).

Both *cis*-acting and *trans*-acting factors may be involved in the localization of G protein–coupled receptors. Many mutations resulting in blindness are mutations in rhodopsin itself that cause improper localization (Sung et al., 1991, 1994). One specific *trans*-acting factor implicated in opsin transport is *ninaA*, a *Drosophila melanogaster* photoreceptor cyclophilin required for localization of a subset of opsins to the rhabdomeres (Colley et al., 1991). *ninaA* and its mammalian homolog RanBP2 bind specific opsins during their biogenesis and may act as chaperones for folding or transport (Colley et al., 1991; Baker et al., 1994; Ferreira et al., 1996). A requirement for cell-specific *trans*-acting localization factors may explain why cloned seven transmembrane receptors expressed in heterologous cell types often are not present on the cell surface (von Zastrow et al., 1993; Hein and Kobilka, 1995; Yu and Hinkle, 1997).

Caenorhabditis elegans and mammalian olfactory receptors are seven transmembrane domain receptors that are also inefficiently transported to the surface of heterologous cell types, suggesting that olfactory neurons may have specialized systems for receptor localization (Zhang et al., 1997; Y. Zhang, J. Bradley and K. Zinn, personal communication). Olfactory receptor proteins in *C. elegans* reside in sensory cilia of olfactory neurons where they are exposed to odorant molecules from the environment (Chou et al., 1996; Sengupta et al., 1996). Mammalian olfactory receptors have proposed localizations in sensory cilia and perhaps in growing axons (Mombaerts et al., 1996).

In *C. elegans*, mutants with abnormal cilium structure have poor chemosensation, indicating that cilia are critical for efficient sensory transduction (Perkins et al., 1986). The cilia increase the surface area for odorant detection and provide a compartment for concentration of the sensory transduction machinery. For example, the AWA olfactory neurons express a seven transmembrane domain receptor (ODR-10), a cation channel (OSM-9), and a Gα protein (ODR-3) that are all localized to the cilia (Sengupta et al., 1996; Colbert et al., 1997; Roayaie et al., 1998). These neurons mediate attraction to the odorants diacetyl, 2,4,5-trimethylthiazole, and pyrazine (Bargmann et al., 1993). The diacetyl receptor, ODR-10, is necessary and sufficient for diacetyl responses but not for responses to other odorants (Sengupta et al., 1996; Troemel et al., 1997; Zhang et al., 1997). The downstream Gα protein and cation channel are required

*To whom correspondence should be addressed.
†Present address: Department of Biology, Brandeis University, Waltham, Massachusetts 02254.

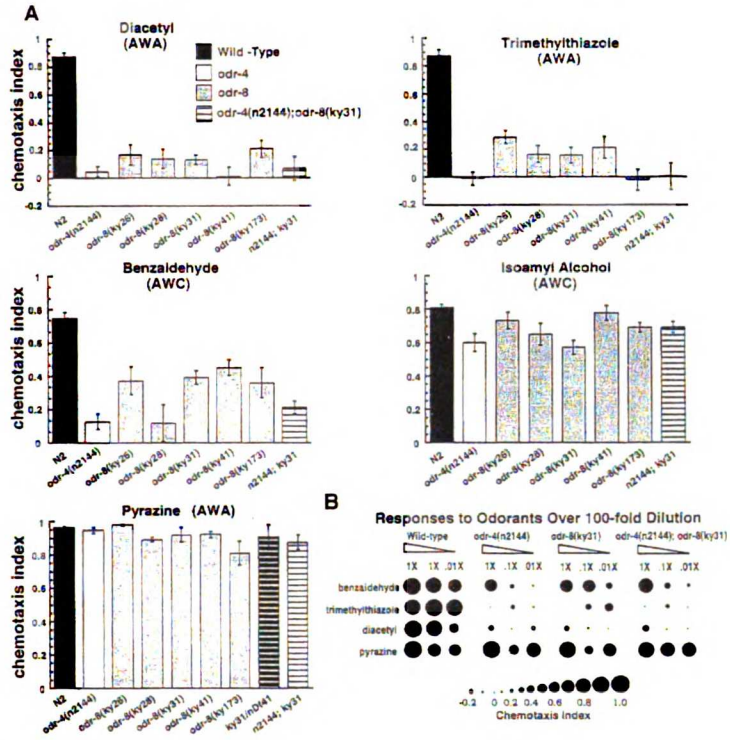


Figure 1. *odr-4* and *odr-8* Mutants Have Defective Responses to a Subset of AWA-Sensed and a Subset of AWC-Sensed Odorants

(A) Animals were raised at 25°C and tested for chemotaxis to a point source of 1 μl of odorant. Chemotaxis Index (C.I.) = (number of animals at odorant − number of animals at control) / total number of animals on the plate. A C.I. of 1.0 indicates complete attraction, while a C.I. of −1.0 indicates complete repulsion. Each data point represents the average of at least six independent chemotaxis assays using ~100 animals per assay. Error bars equal SEM. Dilutions of odorants (in ethanol) were the following: 1/1000 benzaldehyde, 1/1000 diacetyl, 1/10 trimethylthiazole, 1/100 isoamyl alcohol, and 10 mg/ml pyrazine. Chemotaxis to trimethylthiazole at a 1/1000 dilution can be mediated by either AWA or AWC (Bargmann et al., 1993), but only AWA mediates chemotaxis at a 1/10 dilution (data not shown).

(B) Chemotaxis responses of wild-type (N2), *odr-4(n2144)*, *odr-8(ky31)*, and the double mutant *odr-4(n2144); odr-8(ky31)* were compared over a series of 10-fold odorant dilutions. Each data point represents the average of at least five independent assays. Highest concentrations (1×) for each odorant were 1/10 benzaldehyde, 1/10 trimethylthiazole, 1/100 diacetyl, and 10 mg/ml pyrazine.

for all AWA-mediated responses (Colbert et al., 1997; Roayaie et al., 1998). Hence, multiple receptors probably converge on the Gα protein and channel. Several gene families that encode seven transmembrane domain receptors including the *odr-10*-related genes (*str* genes), are candidate olfactory receptors of *C. elegans*. Expression studies of these receptors revealed that an individual olfactory neuron can express several different candidate receptor genes (Troemel et al., 1995; data not shown), consistent with the observation that an individual neuron responds to multiple odorants.

Here, we examine the mechanisms involved in odorant receptor localization to olfactory cilia in *C. elegans*. A previous screen for *C. elegans* mutants with defective olfactory behaviors led to the isolation of mutations in the *odr-4* gene (Bargmann et al., 1993). We show that *odr-4* mutants as well as *odr-8* mutants are defective in a subset of AWA-mediated olfactory responses: they fail to respond to diacetyl and trimethylthiazole, but they respond normally to pyrazine. *odr-4* and *odr-8* are required for localization of some odorant receptors to the cilia, including the diacetyl receptor ODR-10. A comparison of *odr-4* and *odr-8* effects on different signaling molecules, receptors, and in different cell types implicates receptor localization to the cilia as the critical function disrupted by these mutations. *odr-4* encodes a novel type II membrane protein expressed in chemosensory neurons that is localized to intracellular membranes in the cell body and dendrites. We propose that *odr-4* may function in the folding or transport of odorant receptors to mediate their efficient targeting to olfactory cilia.

Results

odr-4 and *odr-8* Mutants Have Specific Olfactory Defects

odr-4 and *odr-8* mutants were isolated in behavioral screens for chemotaxis-defective animals. *odr-4(n2144)* was previously found in a screen for mutants defective in benzaldehyde chemotaxis (Bargmann et al., 1993), and five *odr-8* mutants (*ky26*, *ky28*, *ky31*, *ky41*, and *ky173*) were isolated in screens for mutants defective in diacetyl chemotaxis. All of these mutants displayed defective responses to benzaldehyde, diacetyl, and 2,4,5-trimethylthiazole, but they responded normally to isoamyl alcohol and pyrazine (Figure 1). The sensory neurons that respond to these odorants have been defined by cell ablation experiments (Bargmann et al., 1993) and genetic ablations; the AWA neurons detect diacetyl, trimethylthiazole, and pyrazine, and the AWC neurons detect benzaldehyde, isoamyl alcohol, and trimethylthiazole. Thus, *odr-4* and *odr-8* mutants lack a subset of AWA-mediated responses and a subset of AWC-mediated responses. Chemosensory responses mediated by other neurons were intact in *odr-4* and *odr-8* mutants, including chemotaxis to water-soluble compounds, dauer formation, and avoidance of high osmolarity and volatile repellents (data not shown).

To explore the apparent odorant specificity of the *odr-4* and *odr-8* mutations, a double mutant between *odr-4(n2144)* and *odr-8(ky31)* was characterized. These mutants showed no new chemotaxis or behavioral defects compared to either single mutant. Moreover, placing *odr-8(ky31)* in *trans* to the deficiency *nDf41*, a

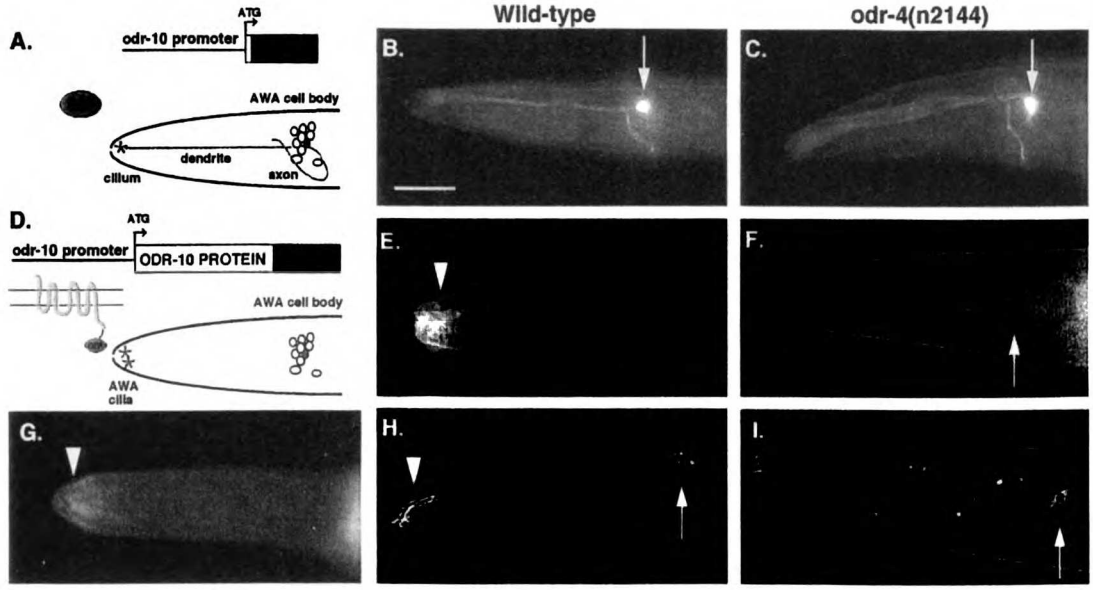


Figure 2. *odr-4* and *odr-8* Are Required for Localization of the Odorant Receptor ODR-10 to Cilia

(A) An *odr-10::GFP* fusion gene, *kyIs37* (Sengupta et al., 1996), with the *odr-10* promoter and the first six amino acids of ODR-10 fused to GFP is expressed in AWA neurons.

(B) Expression in an *odr-4(+)* animal.

(C) Expression in *odr-4(n2144)* mutant. Similar results were obtained for *odr-8* mutant animals (data not shown).

(D) A GFP-tagged full-length ODR-10 protein (*kyIs53*) is localized to the AWA cilia.

(E) ODR-10-GFP in the cilia of a wild-type animal (arrowhead).

(F) ODR-10-GFP in the cell body of an *odr-4(n2144)* animal (arrow). Similar results were observed in *odr-8(ky31)* animals and in animals stained with anti-GFP antisera to enhance ODR-10 detection (data not shown).

(G) Rescue of ODR-10-GFP cilium localization by injection of *odr-4* DNA (pODR-4-1) into *odr-4(n2144)* mutants.

(H) Wide-field deconvolution image of ODR-10-GFP in wild-type animal, showing AWA cilia (arrowhead) and faint cell body (arrow).

(I) Wide field deconvolution image of ODR-10-GFP in *odr-4* mutant, showing accumulation in cell body (arrow). Scale bar, 30 μm.

deletion that spans the *odr-8* region, caused no new olfactory defects (data shown for pyrazine, Figure 1).

odr-4 and *odr-8* Mutants Fail To Localize the Diacetyl Receptor ODR-10 to the AWA Sensory Cilia

Genetic data indicate that all AWA receptors converge on the same ODR-3 Gα protein, so the odorant-specific defects in *odr-4* and *odr-8* mutants suggested that these genes would affect functions upstream of the Gα protein, such as odorant receptors. To investigate this possibility, we examined the effect of *odr-4* and *odr-8* mutations on the diacetyl receptor ODR-10.

To ask whether *odr-4* and *odr-8* affect transcription of *odr-10*, a fusion gene between the *odr-10* upstream region and the green fluorescent protein (GFP) reporter was examined in *odr-4* and *odr-8* mutants. This transgene includes the first six amino acids of ODR-10 and is expressed at high levels in the AWA olfactory neurons (Sengupta et al., 1996 and Figures 2A and 2B). *odr-4 (n2144)* and *odr-8(ky31)* mutants expressed this transgene at normal levels, demonstrating that *odr-4* and *odr-8* are not required for transcription from the *odr-10* promoter (Figure 2C).

By contrast, *odr-4* and *odr-8* mutations had striking effects on the localization of a tagged ODR-10 protein. A protein fusion of GFP to the extreme C terminus of the ODR-10 protein was localized to the cilia of AWA in wild-type animals (Sengupta et al., 1996 and Figures 2D, 2E, and 2H). In *odr-4(n2144)* or *odr-8(ky31)* mutants, the same fusion protein was only visible in the cell body and very faintly in the dendrite (Figures 2F and 2I; Table 1). This result indicates that *odr-4* and *odr-8* are required for ODR-10 localization to the sensory cilia.

The level of ODR-10-GFP also appeared to be decreased compared to wild-type, but this difference is not sufficient to explain the missing cilium expression. In wild-type animals in which the ODR-10-GFP was weakly expressed, the GFP was seen exclusively in the cilia; punctate GFP was seen in the cell body only when the cilia were very bright. In *odr-4* and *odr-8* mutants, however, the highest expressing animals had GFP only in the cell body, indicating that the pool of ODR-10 protein was distributed differently from wild-type (Table 1). Similar results were obtained in animals stained with anti-GFP antisera to enhance ODR-10 detection. The decrease in ODR-10-GFP protein levels in the mutants may be secondary to receptor misfolding or mislocalization.

The overall structure of the AWA neurons revealed by the *odr-10::GFP* fusion was normal in *odr-4* and *odr-8* mutant animals, including the axons, dendrites, and cell bodies (Figure 2C, and data not shown). The AWA cilia were also examined in detail using an OSM-9-GFP fusion protein that localizes to the AWA cilia (Colbert et

Table 1. GFP-Tagged ODR-10 Is Never Seen in the Cilia in *odr-4* or *odr-8* Mutants

Strain	 Cilia only	 Cilia and Soma	 Soma only	 No visible GFP	n
ODR-10-GFP *(kyIs52)* in *odr-4(+)*	27%	70%	0%	4%	n=56
ODR-10-GFP *(kyIs53)* in *odr-4(+)*	7%	85%	0%	7%	n=54
ODR-10-GFP *(kyIs52)* in *odr-4(n2144)*	0%	0%	69%	31%	n=52
ODR-10-GFP *(kyIs53)* in *odr-4(n2144)*	0%	0%	71%	29%	n=59
ODR-10-GFP *(kyIs53)* in *odr-8(ky31)*	0%	0%	54%	46%	n=61

The GFP-tagged ODR-10 fusion gene was integrated into the genome in two different locations, *kyIs52* and *kyIs53*. These were crossed into *odr-4* and *odr-8* mutant strains. Animals were scored for visibly detectable GFP in the AWA neurons at 100× magnification on a Zeiss Axioplan.

al., 1997). This fusion protein showed that the AWA cilia had normal morphologies in *odr-4* and *odr-8* mutants (Figure 3B). Electron microscopy previously showed that the AWC cilia were normal in *odr-4* mutants (Bargmann et al., 1993). To ask whether receptor localization was disrupted in malformed cilia, we examined ODR-10-GFP localization in the cilium-structure mutant *che-3(e1124)*, which has stunted AWA cilia (Perkins et al., 1986). In this mutant, robust localization of ODR-10-GFP to the cilia was observed despite the severe malformation of the AWA cilia (Figure 3C). Thus, ODR-10 protein localization can occur even if AWA morphology is altered.

The effects of *odr-4* and *odr-8* on other proteins in the AWA and AWC cilia were examined using GFP fusion proteins and antibody staining. The Gα protein ODR-3 (Roayaie et al., 1998), the cyclic nucleotide-gated channel subunit TAX-2 (Coburn and Bargmann, 1996), and the capsaicin receptor-like channel OSM-9 (Colbert et al., 1997) were all correctly localized to the AWA or AWC cilia in *odr-4* or *odr-8* mutants (data shown for OSM-9-GFP in AWA, Figure 3B; ODR-3 in AWC, Figure 3D). These results indicate that *odr-4* and *odr-8* are not generally required for protein localization to sensory cilia.

odr-4 Encodes a Novel Membrane Protein

The *odr-4* gene was cloned by positional mapping and transformation rescue of the *n2144* mutant olfactory defect. A 5.4 kb subclone of the cosmid C28A6 rescued both the olfactory defect and the ODR-10-GFP localization defect of *odr-4* mutants (Figure 4B, pODR-4-1; Figure 2G). Two different full-length cDNA clones from this region were isolated, both with SL1 *trans*-spliced leaders and poly(A) tails. These cDNAs differ by the presence or absence of a noncoding exon, and both encode a predicted protein of 445 amino acids (Figures 4B and 4C).

The predicted ODR-4 protein does not share homology with any described proteins. A Kyte-Doolittle plot (Figure 4D) showed a strong hydrophobic peak of 22 amino acids at the extreme C terminus of ODR-4 that could act as a membrane association domain, but there is no predicted signal peptide. Two tests confirmed that the gene we had isolated was the *odr-4* gene. First, an *odr-4* clone with a frameshift in the first exon of the putative *odr-4* open reading frame failed to rescue the behavioral phenotype of *odr-4(n2144)* mutants (Figure 4B, pODR-4-2). Second, sequencing of the *odr-4* gene in the *n2144* mutant strain revealed two mutations, an A to C transition changing Met372 to Leu, and a G to A transition in the splice acceptor for the sixth coding exon (Figure 4C).

Splice acceptor mutations typically cause a decrease in mRNA levels, suggesting that the *odr-4(n2144)* mutation could alter the level of *odr-4* mRNA. Indeed, RT-PCR on wild-type and *odr-4(n2144)* mutant mRNA under semiquantitative conditions revealed that *odr-4* mRNA was reduced in *n2144* mutants (Figure 4E). A GFP-tagged ODR-4 protein bearing the *n2144* mutations was also poorly expressed (data not shown). Thus, it is likely that the splice site mutation results in a reduction of ODR-4 protein levels.

odr-4 Acts Cell-Autonomously in AWA Neurons

The expression of *odr-4* was examined using GFP reporter constructs (Figure 4B and Figure 5). An *odr-4* fusion gene that included the entire *odr-4* coding region fused to GFP was expressed only in twelve types of chemosensory neurons (Figure 5A), including AWA and AWC neurons; this fusion gene rescued all behavioral defects of *odr-4* mutants (Figure 4B, pODR-4-4). ODR-4-GFP expression was seen in all neurons of the chemosensory amphid and phasmid organs except the ASE chemosensory neurons and the AFD thermosensory neurons. To date, seven transmembrane domain receptors from the *str* and *sr* receptor gene families have not been found to be expressed in ASE or AFD neurons (Troemel et al., 1995, and our data not shown).

Expression in the AWA neurons alone was sufficient to rescue *odr-4* AWA function in an *odr-4* mutant. ODR-4-GFP was expressed solely in the AWA neuron using the *odr-10* promoter (Figure 5D), introduced into *odr-4* mutant animals, and the transgenic animals were tested for rescue of diacetyl chemotaxis (mediated by AWA olfactory neurons) and benzaldehyde chemotaxis (mediated by AWC olfactory neurons). The *odr-10*::ODR-4-GFP fusion gene rescued diacetyl chemotaxis but not benzaldehyde chemotaxis, suggesting that *odr-4* acts cell-autonomously in olfactory neurons (Figure 4B, p[odr-10p::ODR-4-GFP]).

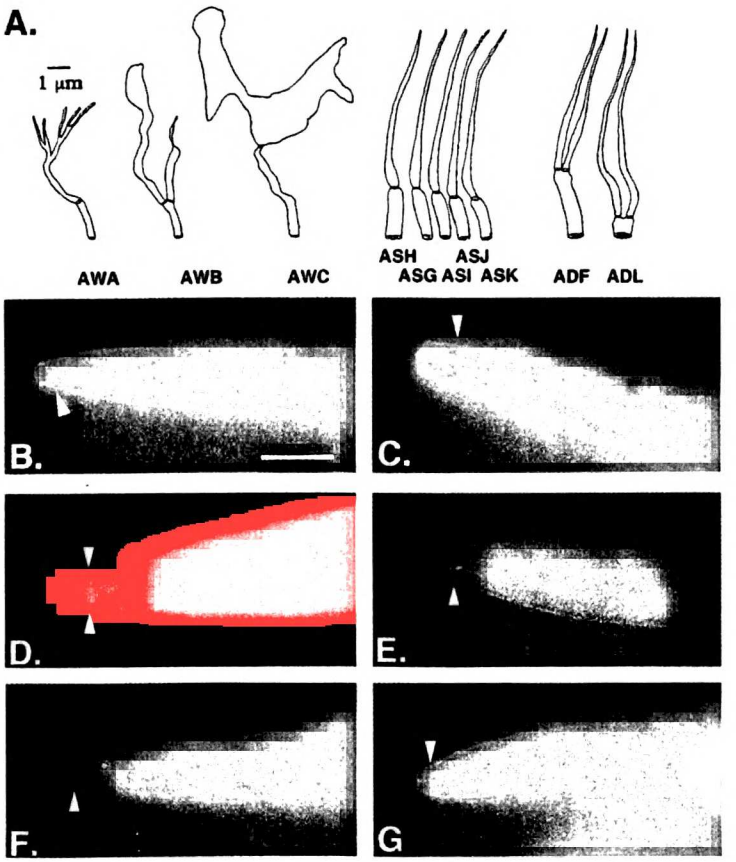


Figure 3. *odr-4* Is Not Required for Localization of Many Cilia Proteins

(A) Drawings of chemosensory cilia depicted in Figures 3 and 6 (adapted from Perkins et al., 1986).

(B) *odr-4* mutants have apparently normal AWA cilia and localize the channel-like protein OSM-9 to AWA cilia. OSM-9-GFP expression in the AWA cilia (arrowhead) reveals a normal morphology in an *odr-4(n2144)* animal. OLQ cilia are visible dorsal and ventral to the AWA cilia.

(C) Deformed AWA cilia are competent to localize ODR-10-GFP. ODR-10-GFP expressed in the AWA neuron of a *che-3(e1124)* cilium structure mutant, showing localization to the aberrant AWA cilium (arrowhead).

(D) The Gα protein ODR-3 is localized to AWC cilia in *odr-8(ky31)* mutants (arrowheads) and in *odr-4(n2144)* mutants (data not shown). Animals were fixed and stained with rabbit anti-ODR-3 IgG antibodies (Roayaie et al., 1998) and Cy-3-conjugated secondary antibodies. This dorsal view shows the left and right AWC cilia.

(E) The cyclic nucleotide-gated channel subunit TAX-2-GFP is localized to cilia in *odr-4(n2144)* mutants (arrowhead). TAX-2-GFP is enriched in cilia of eight types of sensory neurons in the amphid (Coburn and Bargmann, 1996).

(F) The seven transmembrane domain receptor SRG-2-GFP (Chou et al., 1996) is localized to the ASK cilia in *odr-4(n2144)* mutants (arrowhead).

(G) The seven transmembrane domain receptor SRD-1-GFP, expressed ectopically in AWA using the *odr-10* promoter, is present in the AWA cilia in *odr-4(n2144)* mutants. SRD-1 is also independent of *odr-4* for its normal localization in ASI neurons (data not shown). In all cases shown above, similar results were observed in wild-type, *odr-4(n2144)*, and *odr-8(ky31)* animals. Scale bar, 30 μm.

ODR-4 Protein Is Associated with Intracellular Membranes and Is Not Present in the Sensory Cilia

odr-4 could be involved in odorant receptor folding, trafficking, or clustering. To help distinguish among these models for *odr-4* function, the subcellular localization of ODR-4 was examined using a functional ODR-4-GFP protein (see Figure 4B, pODR-4-4).

ODR-4-GFP was present at a high level in the cell body, in the dendrites, and to a lesser extent in the axons of the chemosensory neurons in a discrete punctate pattern (Figure 5B). It was not observed in the sensory cilia, making a receptor clustering role less likely. In the cell body, ODR-4-GFP was excluded from the nucleus but was present in the cytoplasm in interconnecting ribbons or tubules (Figure 5C). Three-dimensional rendering and rotation of a stack of high-resolution images suggested that ODR-4-GFP was not present on the plasma membrane. This subcellular localization of ODR-4 is consistent with residence in the endoplasmic reticulum, Golgi apparatus, and transport vesicles.

The topology of ODR-4 protein was explored using β-galactosidase hybrid proteins (Froshauer et al., 1988); β-galactosidase is active only in the cytoplasm. The reporter genes GFP and LacZ were fused to *odr-4* in tandem either replacing the hydrophobic domain or following it (see Figure 4B). The GFP sequence served as an internal control for expression of the fusion protein, while β-galactosidase activity was assessed to monitor protein localization. When GFP and LacZ were fused to *odr-4* after amino acid 421, replacing the hydrophobic domain, the triple fusion protein exhibited both robust GFP fluorescence and β-galactosidase activity (Figures 5E and 5G) (12/12 transgenic lines stained). However, when GFP and LacZ were fused to ODR-4 just C-terminal to the hydrophobic domain, the triple fusion protein exhibited GFP fluorescence but no β-galactosidase activity (0/6 transgenic lines stained) (Figures 5F and 5H). This fusion protein also rescued the diacetyl response of *odr-4* mutants, indicating that ODR-4 function was not disrupted by the addition of GFP and β-galactosidase (Figure 4B). These results do not prove, but are consistent with, a topology in which the bulk of the ODR-4 protein is located in the cytoplasm, its C-terminal hydrophobic tail anchoring it into a subcellular membrane.

The *odr-4*/*odr-8* Requirement Is Specific for a Subset of Chemoreceptors Expressed in AWA and AWC Neurons

Since *odr-4* and *odr-8* have defective responses to several odorants, we asked whether other olfactory receptors require *odr-4* and *odr-8* for localization to cilia. *odr-10* is the only receptor for which an odorant ligand has been determined, but a large number of candidate *C. elegans*

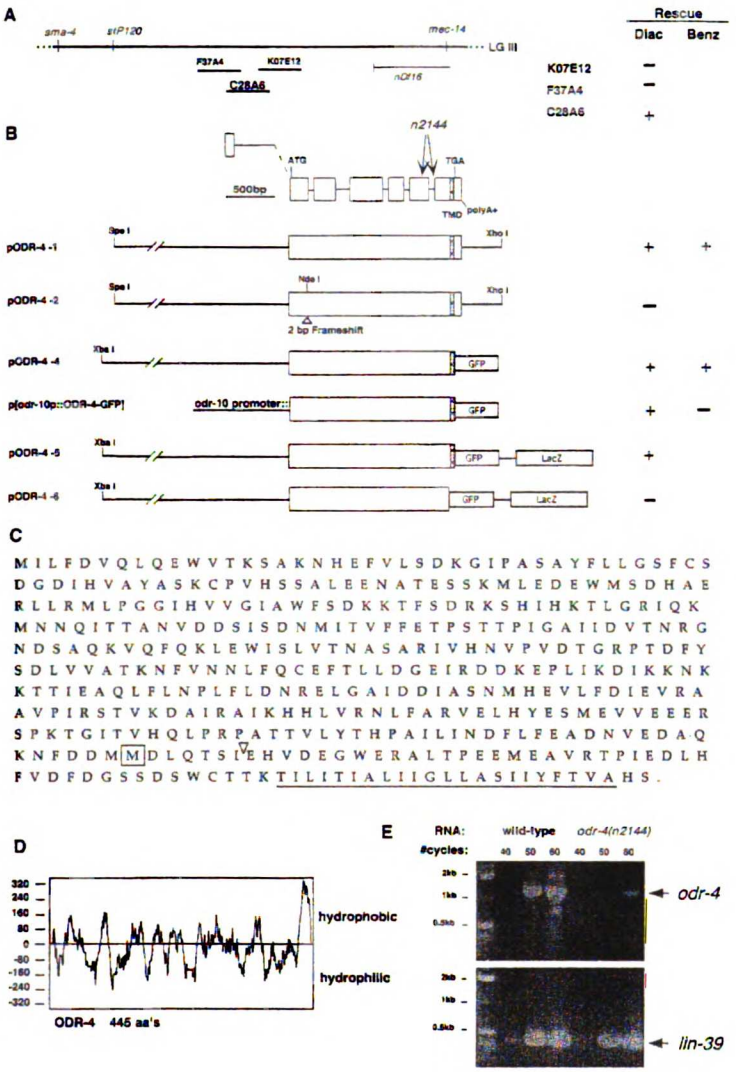


Figure 4. *odr-4* Encodes a Novel Membrane-Associated Protein

(A) Genetic and physical map position of *odr-4* on Linkage Group III.

(B) *odr-4* gene structure, expression constructs, and rescue data. The *odr-4* open reading frame was fully contained on a 5.4 kb SpeI-XhoI fragment of the cosmid C28A6. The gene structure is shown, along with the location of start and stop codons, transmembrane domain (stippled), and mutations in *n2144*. Boxes depict exons. Two different classes of full-length *odr-4* cDNAs with SL1 *trans*-splice leaders at the 5′ end were found; one contained an extra 5′ noncoding exon (raised box linked by dashed line) that was not required for function. Subclones and fusion genes were injected into *odr-4(n2144); lin-15(n765ts)* together with *lin-15* marker DNA. For each clone at least three independent transgenic lines were tested for chemotaxis to 1/1000 diacetyl or 1/1000 benzaldehyde after raising at 20°C with a minimum of four assays per individual line. A chemotaxis index of 0–0.5 was scored as negative; indices of 0.5–1.0 were scored as positive. *lin-15* DNA alone did not rescue chemotaxis (data not shown).

(C) The predicted amino acid sequence of ODR-4 deduced from *odr-4* cDNAs. The protein is 445 amino acids long. Methionine 372 altered to a leucine in *n2144* is boxed, and the location of the altered splice acceptor in *n2144* is indicated by an inverted triangle. Residues predicted to form the transmembrane domain are underlined.

(D) Hydrophobicity plot of ODR-4 shows a potential transmembrane domain at the C terminus. The plot was derived from Kyte-Doolittle hydropathic analysis of the predicted amino acid sequence of *odr-4* (Kyte and Doolittle, 1982).

(E) Semiquantitative RT-PCR of total wild-type and *odr-4(n2144)* mRNA for 40, 50, or 60 cycles with *odr-4* primers (top) or *lin-39* primers (bottom). *odr-4* mRNA was reduced in the *odr-4* mutant.

chemosensory receptors have been identified through examination of genome sequence (Troemel et al., 1995, 1997; E. R. T. and C. I. B., data not shown). These seven transmembrane receptors fall into six families based on sequence similarity: the *str* gene family, for which *odr-10* is the founding member, and the *sra*, *srb*, *srg*, *srd*, and *sre* gene families. There is distant sequence similarity between the *sra* and *srb* gene families and between the *srd* and *str* gene families, but the other families are essentially unrelated.

Analysis of additional receptor-GFP fusions revealed that *odr-4* and *odr-8* are required for correct localization of some, but not all, receptor proteins. STR-2 is a candidate chemoreceptor that is localized to the cilia of AWC neurons in wild-type animals (Figure 6A), with a lower level of protein apparent in the cell body excluding the nucleus. In *odr-4* and *odr-8* mutants, the STR-2-GFP fusion protein was absent from the cilia but appeared in the cell body and faintly in the dendrite (Figure 6B, and data not shown). Examination at high magnification using wide-field deconvolution revealed that GFP fluorescence in *odr-4* mutants was diffuse throughout the cytoplasm (Figure 6C), with lower levels in the nucleus.

Other candidate chemoreceptor proteins were localized to cilia in an *odr-4*– and *odr-8*–independent fashion. SRD-1 and SRG-2 are candidate chemoreceptors that localize to the ASI and ASK cilia, respectively (Troemel et al., 1995; Chou et al., 1996). The localization of SRD-1-GFP and SRG-2-GFP proteins was normal in *odr-4(n2144)* and *odr-8(ky31)* mutants. Thus, while they express ODR-4-GFP protein, the ASI and ASK neurons are able to transport receptors to the cilia by an alternative pathway (data shown for SRG-2-GFP in *odr-4*, Figure 3F).

By altering the cell that expressed ODR-10-GFP, we found that *odr-4*-dependence was defined by a combination of receptor sequence and sensory cell type. Since ODR-4-GFP expression was observed in the AWB and ASI neurons, an ODR-10-GFP fusion protein was expressed using either the AWB-specific *str-1* promoter or the ASI-specific *str-3* promoter. The ODR-10 fusion

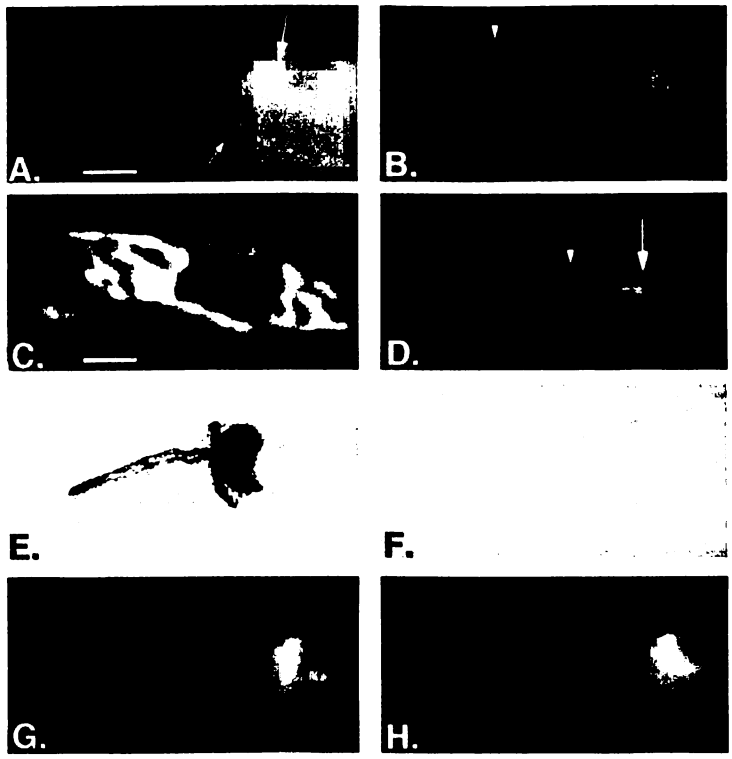


Figure 5. ODR-4 Is Expressed on Intracellular Membranes in Chemosensory Neurons

(A) GFP-tagged ODR-4 (pODR-4-4 in Figure 4B) is expressed in ten types of amphid neurons: AWA, AWC, AWB, ADF, ADL, ASG, ASH, ASI, ASJ, and ASK, and in the two types of phasmid neurons, PHA and PHB. Expression is seen from the 3-fold embryonic stage onward. The amphid neurons are shown in the head of an adult (lateral view, large arrow points to ADL). The phasmid neurons are visible in the tail of an adjacent larva (small arrow, dorsal view). Scale bar, 30 μm.

(B) GFP-tagged ODR-4 appears punctate in the dendrites (arrowhead) and axons but is not seen in the cilia. This C-terminal tag rescues the *odr-4* olfactory defect (pODR-4-4, Figure 4B). Similar localization was observed in animals stained with anti-GFP antisera to enhance ODR-4 detection (data not shown). The expression pattern was not altered in *odr-4(n2144)* or *odr-8(ky31)* mutants.

(C) GFP-tagged ODR-4 labels intracellular membranes in the cell body reminiscent of endoplasmic reticulum. Wide-field deconvolution image of a neuronal cell body expressing this protein shows fluorescence on intracellular structures. Three-dimensional rendering and rotation of a stack of images revealed interconnected sheets and tubules; surface invaginations suggested that the labeled membranes are cytoplasmic, not on the surface. Scale bar, 2 μm.

(D) Expressing ODR-4 only in AWA neurons was sufficient to rescue diacetyl chemotaxis (see Figure 4B). GFP-tagged ODR-4 expressed from the *odr-10* promoter was expressed only in the AWA neuron (arrow). The arrowhead points to a puncta in the dendrite.

(E–H) Double fusions of ODR-4 to GFP and β-galactosidase, which is functional only in the cytosolic compartment. When the transmembrane domain of ODR-4 was deleted and replaced by GFP-LacZ, robust β-galactosidase activity was observed (E), but olfaction was not rescued. Conversely, when GFP-lacZ was fused after the transmembrane domain, olfactory function was restored, but no β-galactosidase activity was observed (F). GFP was expressed well in both fusion proteins (G and H). See Figure 4B for constructs and rescue data. Scale bar, 30 μm.

protein was strongly localized to AWB and ASI cilia in wild-type animals (Figure 6D, arrowhead shows AWB cilium; Figure 6I, arrowhead shows ASI cilium). However, in *odr-4* and *odr-8* mutants, far less ODR-10-GFP appeared in these cilia (Figure 6E and 6J, arrowheads; and data not shown). Moreover, *odr-4* mutants accumulated ODR-10-GFP in the AWB cell body (arrow in Figure 6E), suggesting that some receptors are retained there. Thus, ASI and AWB neurons do use *odr-4* and *odr-8* to localize the ODR-10 protein, but unlike AWA neurons, these heterologous neurons can also use a different mechanism to partly substitute for *odr-4* and *odr-8* (e.g., ODR-10 may take advantage of the mechanism that ASI neurons use to localize SRD-1). The partial effects of *odr-4(n2144)* in AWB were not enhanced by placing it in *trans* to a deficiency of the *odr-4* locus (Figure 6H).

An intermediate requirement for *odr-4* was also observed when assessing the behavioral function of *odr-10* in the AWB neurons. When *odr-10* is expressed under the AWB-specific *str-1* promoter to create a strain called odr-10(B), it causes diacetyl avoidance instead of the diacetyl attraction usually mediated by AWA neurons (Troemel et al., 1997). *odr-4* mutations decreased but did not eliminate diacetyl avoidance in odr-10(B) animals (Figure 6F). These results show that the *odr-4* requirement for *odr-10*–mediated behaviors is closely correlated with ODR-10 localization: in AWA neurons, *odr-4* is essential for both, and in AWB neurons, *odr-4* is partly required for both.

In a converse experiment, when the *odr-10* promoter was used to express an SRD-1-GFP fusion protein in the AWA neurons, the fusion protein was localized to the AWA cilia to an equal extent in wild-type and *odr-4 (n2144)* mutant animals (Figure 3G). Although the expression of this protein was poor, this result suggests that the AWA neurons can export some receptor proteins to the cilia using an *odr-4*–independent mechanism, even though ODR-10 in AWA is wholly dependent on *odr-4*.

Discussion

odr-4 and *odr-8* Are Required for the Function of a Subset of Odorant Receptor Proteins

ODR-4 is a novel protein with an essential function in the biogenesis of seven transmembrane domain receptors. *odr-4* and *odr-8* are required for the localization of some odorant receptors in the AWA and AWC olfactory neurons. The odorant-specific behavioral phenotypes of *odr-4* and *odr-8* mutants implicate them in olfactory signal transduction, and their cellular phenotypes reveal specific defects in receptor localization to the sensory cilia. *odr-4::GFP* fusions were expressed only in chemosensory neurons, including the AWA and AWC neurons

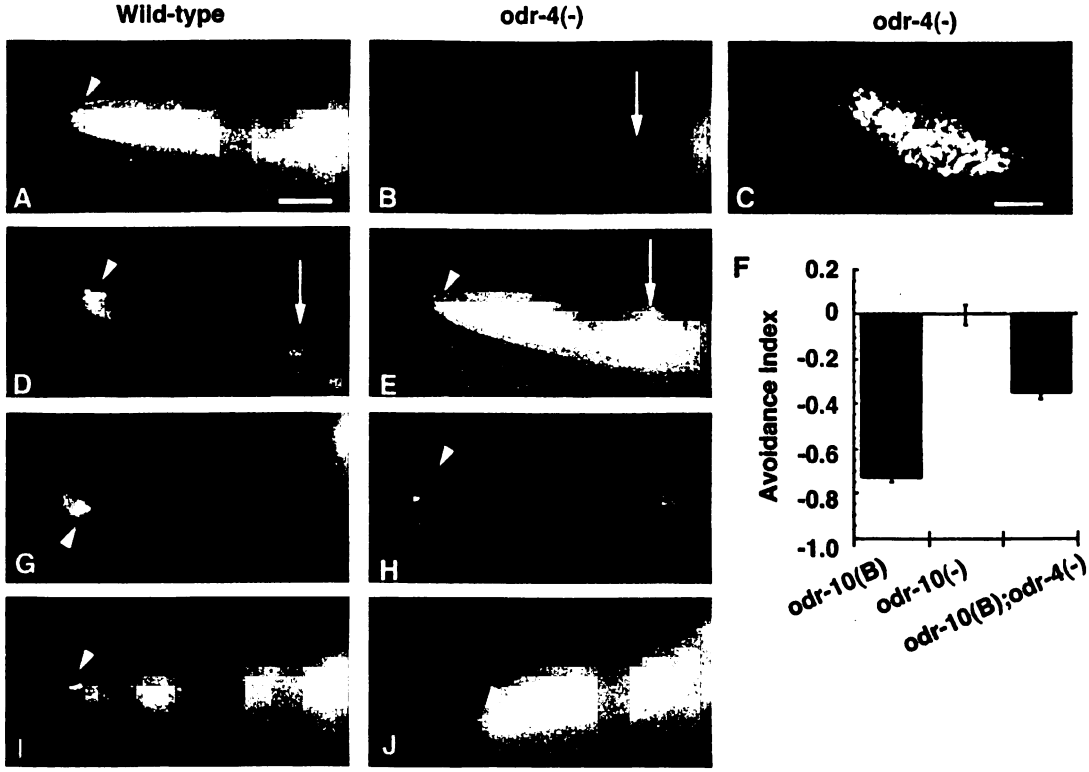


Figure 6. *odr-4* Affects Receptor Localization in AWC, AWB, and ASI Neurons

(A–C) STR-2, a seven transmembrane domain protein expressed in AWC neurons, requires *odr-4* and *odr-8* for localization to the AWC cilia. (A) STR-2-GFP expressed in *odr-4(+)* animals is visible in an AWC cilium (arrowhead). Bar is 30 μm for all panels except (C). (B) STR-2-GFP is seen only in the AWC cell body (arrow) in *odr-4(n2144)* mutants and in *odr-8(ky31)* mutants (data not shown). (C) Wide-field deconvolution image of one plane of AWC in an *odr-4* mutant animal expressing STR-2-GFP shows GFP in the cytoplasm. The diffuse GFP extends into the dendrite and axon (not shown). Bar, 2 μm.

(D–H) ODR-10 expressed ectopically in AWB neurons shows partial dependence on *odr-4* for localization and function. (D) The *str-1*::ODR-10-GFP (*kyIs156*) protein is expressed strongly in the AWB cilia (arrowhead) in *odr-4(+)* animals. (E) In *odr-4(n2144)* and *odr-8(ky31)* (not shown) mutants less protein reaches the cilia (arrowhead), while more GFP appears in the cell body (arrow). (F) ODR-10 expression in AWB (the odr-10(B) strain) causes animals to avoid diacetyl at a 1/1000 dilution (Troemel et al., 1997). This avoidance is reduced but not eliminated in *odr-4(n2144)* mutants. Error bars represent the SEM. (G) Normal localization of *str-1*::ODR-10-GFP in *sDf127/+* heterozygous animals. (H) Partial defect in AWB localization in *odr-4(n2144)/sDf127* animals, comparable to *odr-4* homozygotes in (E) (n = 50 animals each for [G] and [H]).

(I and J) ODR-10 expressed ectopically in ASI neurons shows partial dependence on *odr-4* for localization to ASI cilia. The *str-3* promoter was used to express ODR-10-GFP in the ASI neurons. (I) In *odr-4(+)* animals ODR-10-GFP is very bright in an ASI cilium (arrowhead). (J) In *odr-4(n2144)* or *odr-8(ky31)* animals less ODR-10-GFP appears to reach the cilium (arrowhead); this photo shows the brightest example seen in *odr-4* mutants.

as well as several additional classes of neurons that are not known to be defective in *odr-4* and *odr-8* mutants. All of these neurons terminate in specialized sensory cilia that are in contact with the environment. Mechanosensory and thermosensory neurons in *C. elegans* also terminate in sensory cilia, but *odr-4* expression was observed only in neurons with chemosensory functions, suggesting involvement in chemosensory function and not general cilium function.

Even within the chemosensory neurons, the *odr-4/odr-8* system is not a general cilium transport system, since a Gα protein and two putative cation channels are localized properly to AWA or AWC cilia in *odr-4* and *odr-8* mutants. Nor is it an essential factor for seven transmembrane receptor localization, since SRD-1-GFP can find its way to the AWA cilia independently of *odr-4*. It is likely that some odorant receptors expressed in AWA and AWC require *odr-4* for their localization, while others in the same cells do not. Single olfactory neurons in *C. elegans* can detect multiple odorants (Bargmann et al., 1993) and express multiple candidate chemoreceptor genes (Troemel et al., 1995). Since *odr-4* and *odr-8* mutants respond to pyrazine, which is sensed by AWA neurons (Bargmann et al., 1993), the pyrazine receptor presumably makes use of a non–*odr-4* localization system in AWA neurons. Non–*odr-4* localization systems are probably present in other chemosensory neurons as well, since ODR-10 in AWB or ASI can still be transported to the cilia in *odr-4* and *odr-8* mutants, albeit less efficiently.

The *cis*- and *trans*-acting factors that control folding and localization of G protein–coupled receptors are

poorly understood. Some deletion studies suggest that sequences within the transmembrane segments determine targeting (Keefer et al., 1994; Unson et al., 1995), while others suggest that the C-terminal tail may be required for efficient localization (Konopka et al., 1988; Sung et al., 1994). Our results suggest that the receptor sequences may be recognized by overlapping or competing systems like the *odr-4*/*odr-8* system. The requirements for receptor folding and localization may differ depending on the complement of accessory pathways that exist in a given cell.

The existing allele in *odr-4* probably reduces *odr-4* function, but it may not be a null allele. It is possible that a null mutation in *odr-4* would have more severe phenotypes. However, genetic evidence argues that there is a discrete *odr-4*/*odr-8* function required for the activation of benzaldehyde, diacetyl, and trimethylthiazole receptors. All five *odr-8* alleles disrupt the same odorant responses that are affected by *odr-4*, and double mutants and deficiency heterozygotes maintain this specificity. Even if these genes ultimately affect other odorant receptors as well, they appear to be absolutely essential in a subset of responses.

odr-4 Is Likely To Act during Receptor Folding, Sorting, or Transport

odr-4 encodes a novel 445 amino acid protein with a C-terminal transmembrane domain. Relatively few proteins have the membrane orientation proposed for ODR-4, and many of those that are known are proteins such as syntaxin that are involved in vesicle transport or targeting. ODR-4 has no sequence or structural similarity to ninaA or RanBP2, the cyclophilins that interact with opsins, but those proteins also end in a single hydrophobic membrane attachment domain (Colley et al., 1991; Ferreira et al., 1996). The sequence of ODR-4 is acidic and hydrophilic, including 15% acidic residues (Asp/Glu) and 9% basic residues (Lys/Arg). The acidic charge distribution is interesting because the intracellular sequences of predicted *C. elegans* odorant receptors are highly basic, including ~20% basic residues and only 6%–8% acidic residues, while their extracellular sequences are neutral or slightly acidic. A similar systematic charge bias is seen in other G protein–coupled receptors, including mammalian olfactory receptors. One possibility is that recognition of the basic intracellular loops of the receptor by the acidic ODR-4 protein leads to a stable interaction between the two proteins that assists folding or transport.

Transcription from the *odr-10* promoter was not visibly affected by *odr-4*, but levels of ODR-10-GFP protein appear to be reduced in *odr-4* mutants. Protein degradation may occur as a secondary effect of ODR-10 misfolding or mislocalization.

Figure 7 represents three simple models for *odr-4* function in the olfactory neurons. ODR-4 might be a chaperone required to help fold or stabilize some olfactory receptors. Proper folding is required for receptors to exit from the endoplasmic reticulum (Gudermann et al., 1997), and unfolded receptors would not be allowed into the secretory pathway and might be degraded. Olfactory receptors and most seven transmembrane domain proteins lack a classical signal sequence, so membrane

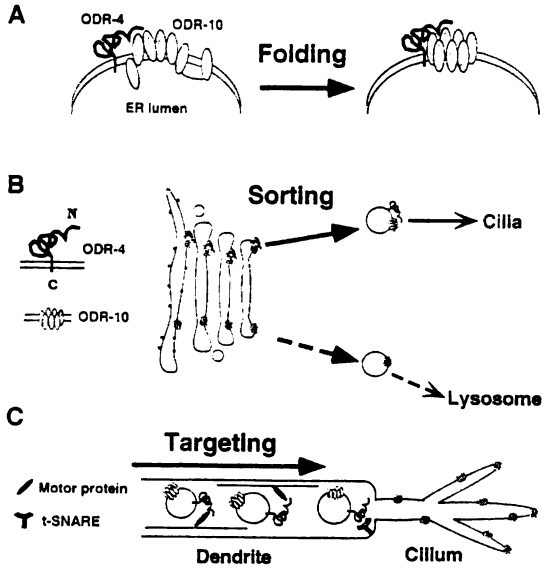


Figure 7. Models for *odr-4* Function

(A) ODR-4 could serve as a chaperone aiding or monitoring receptor folding. In the absence of *odr-4*, ODR-10 or STR-2 cannot fold or cannot exit the ER. ODR-4 could also aid initial membrane insertion of receptors into the ER.

(B) ODR-4 might function as a cargo receptor sorting odorant receptors into the correct secretory vesicles. In *odr-4*'s absence, ODR-10 and STR-2 cannot enter the secretory organelles "addressed" to the cilia.

(C) ODR-4 could be involved in targeting receptor-containing vesicles to the cilia, either by interactions with motor proteins or by mediating specific fusion at the base of the cilium. In the absence of *odr-4*, vesicles containing ODR-10 or STR-2 cannot enter the dendrite or fuse with the cilium and so return to the cell body.

insertion itself may be inefficient without accessory factors. *odr-4* and *odr-8* could also be involved in recognition of the folded receptor as a hypothetical "folding monitor" (Rothman and Wieland, 1996). In the absence of such a monitor, even a correctly folded receptor might not be recognized as such and so could be banished to lysosomes or proteasomes.

Second, ODR-4 could function as a cargo receptor, packaging odorant receptors into vesicles destined for the cilia (Rothman and Wieland, 1996). The diffuse cytoplasmic appearance of the ODR-10-GFP and STR-2-GFP in *odr-4* mutants could result from the receptors being sorted erroneously into small vesicles or lysosomes.

Third, ODR-4 could function in targeting vesicles to the cilia. It might act as a vesicle coat protein that facilitates vesicle trafficking (Kirchhausen et al., 1997) or as a factor that aids motor-driven transport of the vesicles down the dendrite (Hirokawa, 1998). Alternatively, at a later step, ODR-4 could mediate vesicle fusion specificity, allowing vesicles containing receptors to fuse with the cilium membrane. Vesicles that were denied fusion access at the cilium might be sent back to the cell body, explaining the observation of receptor-GFP enrichment in the cell bodies of *odr-4* mutants.

Although there are many other possible models for

posttranscriptional regulation of receptors, some combination of those listed above is the most likely for *odr-4* function. *odr-4* and *odr-8* are probably not required for translation initiation, since the mutants are able to express high levels of a GFP fusion protein containing the first six amino acids of ODR-10. However, *odr-4* and *odr-8* might act as mRNA transporters if ODR-10 can only be synthesized at the base of the cilium. They are probably not required for maintenance of receptor protein in the cilia, since the ODR-4 protein is absent from this cellular compartment.

How widespread are accessory systems like *odr-4*/*odr-8* and *ninaA*? It is likely that similar systems remain to be discovered. Attempts to express the mammalian olfactory receptors in heterologous cell systems have been largely unsuccessful, suggesting that *trans*-acting factors may be required for efficient targeting of those odorant receptors to the plasma membrane. Similarly, the α2C adrenergic receptor and some peptide receptors are poorly transported to the surface of cultured cells but are thought to be mainly surface-associated in their normal context (von Zastrow et al., 1993; Yu and Hinkle, 1997). The importance of transport systems as possible regulatory pathways may also be exploited in some cell types, allowing receptors to be mobilized between the surface and intracellular compartments (Wan et al., 1997).

The complex anatomy of neurons, with different functions parceled out in different cell domains, requires strict control of protein traffic to the surface. The cell can exert control at many steps in this trafficking: protein folding, cargo sorting, vesicle transport, and vesicle fusion. *odr-4* and *odr-8* may be representative of a variety of proteins involved in traffic control for seven transmembrane domain receptors.

Experimental Procedures

Strains and Genetics

Wild-type nematodes were *C. elegans* variety Bristol, strain N2. Strains were maintained using standard methods (Brenner, 1974). Animals were grown at 20°C for most experiments and at 25°C for chemotaxis assays. Many strains were provided by the Caenorhabditis Genetics Center.

Isolation of Mutants

odr-4(n2144) was isolated previously (Bargmann et al., 1993). *odr-8* alleles were isolated in behavioral screens for animals that could not chemotax to diacetyl. The mutagenesis and screens were performed as described previously, using either NaCl or pyrazine as the second odorant in a choice (Bargmann et al., 1993; Sengupta et al., 1996). All mutants were backcrossed at least four times before behavioral testing. *odr-4* and *odr-8* were identified as two different genes by complementation testing and by Joe Chou by mapping *odr-8* to chromosome IV. *odr-4; odr-8* double mutants were made by eliminating markers linked to both mutations and confirmed by complementation testing.

Mapping of *odr-4*

odr-4(n2144) had been previously mapped near the marker *sma-3* (Bargmann et al., 1993). We used three-factor, four-factor, and PCR mapping to refine the *odr-4* position to the interval between the Tc1 polymorphism *stP120* (Williams et al., 1992) and the left breakpoint of the deficiency *nDf16*. From heterozygotes of the genotype *dpy-17(e164) odr-4(n2144) unc-32(e189)/stP120*, 12/25 Dpy non-Unc recombinants were Odr$^-$stP120$^+$, 2/25 were Odr$^+$stP120$^-$, and 11/25 were Odr$^-$stp120$^-$; 12/28 Unc non-Dpy recombinants were Odr$^-$stP120$^-$, 1/28 was Odr$^-$stP120$^+$, and 15/28 were Odr$^-$stP120$^+$. The presence or absence of *stP120* was determined by PCR. Thus, three recombinants separated *odr-4* from *stP120* and indicated that it was to the right of this polymorphism.

Defining the Left Breakpoint of *nDf16*

odr-4(n2144) was complemented by the deficiency *nDf16*, contrary to previous report (Bargmann et al., 1993). Since the left breakpoint of *nDf16* lies in the *stP120* to *mec-14* interval, we determined the breakpoint in relation to the physical map. PCR primers were generated to unique sequences from cosmids in the region and used to assay for the presence of those sequences in *nDf16* homozygous arrested embryos. The left breakpoint of *nDf16* lay between the cosmids F37A4 and R13F6.

Chemotaxis Assays

Population chemotaxis assays on well-fed adult animals were performed as described (Bargmann et al., 1993; Troemel et al., 1997).

Molecular Biology Methods

All general molecular biology manipulations were performed using standard methods (Sambrook et al., 1989). Sequencing was performed using the fmol sequencing system (Promega) or by automated sequencing using a LiCor automated sequencer. Sequence analysis was performed using GeneWorks (Intelligenetics). Preliminary sequence of the *odr-4* genomic region was provided courtesy of the *C. elegans* sequencing consortium at Washington University (St. Louis) (Wilson et al., 1994).

RT-PCR

Total RNA was prepared from mixed stage wild-type N2 and *odr-4(n2144)* mutant animals by Trizol extraction (GIBCO). First-strand cDNA was reverse transcribed from N2 or *odr-4(n2144)* RNA using primers specific for *lin-39* or *odr-4*. This cDNA was used for 30 cycles of PCR amplification with primers to *odr-4* and *lin-39*, mixed in the same reaction tube. One microliter of each reaction was used as a template in a new reaction with nested primers to *odr-4* and *lin-39* in separate reaction tubes. Following 10, 20, or 30 cycles, 10 μl of the reaction was removed for viewing on an agarose gel stained with ethidium bromide.

Germline Transformation

Rescue experiments were performed by injecting cosmids from the *odr-4* genomic region at concentrations of 10–30 ng/μl or GFP constructs at 30–50 ng/μl (Mello et al., 1991) together with pJM23 *lin-15* marker DNA (Huang et al., 1994) at 50 ng/μl into the gonads of adult *n2144;lin-15(n765ts)* worms. Transgenic lines were identified by rescue of the *lin-15(n765ts)* multivulva phenotype at 20°C, tested for chemotaxis to diacetyl or other odorants, and/or examined for GFP expression. Integrated arrays of transgenes were made by inducing chromosomal breaks in animals carrying extrachromosomal arrays of test DNA + pJM23 with either γ rays from a ^{137}Cs source (6000 rads) for *kyIs37*, or psoralen + UV irradiation, cloning out two generations of progeny, and identifying animals in the F2 which had no *lin-15* mutant progeny and expressed the transgene.

Isolation and Characterization of cDNAs

The 5.4 kb SpeI-XhoI genomic fragment of cosmid C28A6 was used as a probe to screen approximately 1 × 10^6 plaques of a mixed-stage *C. elegans* cDNA phage library (a gift of Bob Barstead; Barstead and Waterson, 1989). Twenty-one positive clones were identified and partially sequenced, and nine clones were sequenced completely on both strands. Six clones were identical and were full-length as judged by the presence of the SL1 *trans*-spliced leader at their 5′ ends. One clone had an additional 5′ noncoding exon and an SL1 splice leader.

Generation of Frameshift Mutation

A two base pair frameshift in the 5.4 kb SpeI-XhoI rescuing fragment was introduced by filling in the NdeI site in the second exon of the *odr-4* gene. This results in a frameshift after Ala48 of ODR-4 and a stop codon after eight more amino acids.

Sequencing of the *odr-4(n2144)* Mutant Allele

Genomic DNA was isolated from N2 and *n2144* single animals, and at least one strand of all exons, splice junctions, and 3′ UTRs was sequenced. The regions containing the mutations were sequenced on both strands.

Generation of Expression Constructs

The sequences of all PCR products and of all plasmid junctions were verified by sequencing. GFP and LacZ vectors were generously supplied by Andrew Fire. PCR amplification was carried out using the Expand Long Template PCR kit (Boehringer-Mannheim) and an MJ Research Thermal Cycler.

Microscopy

Most photos were taken using standard fluorescence microscopy. Images in Figures 2H, 2I, 5C, and 6C were acquired with a scientific grade cooled CCD camera on a multiwavelength wide-field three-dimensional microscopy system (Hiraoka et al., 1991) in which the shutters, filters, and XYZ stage are all computer-driven. Samples were imaged using a 60 × 1.4 NA lens (Olympus) and n = 1.1518 immersion oil. Three dimensional data stacks of GFP-expressing samples were acquired in the FITC channel by moving the stage in successive 0.25 μm focal planes through the sample, and out-of-focus light was removed with a constrained iterative deconvolution algorithm (Agard et al., 1989). Maximum intensity projections of the three-dimensional data stacks were generated using IVE software (Chen et al., 1996).

Acknowledgments

We thank Orion Weiner for taking the high resolution images in Figures 2H, 2I, 5C, and 6C; David Tobin for the SRD-1-GFP transgene; J. G. Crump for the slide of *kyIs53*; Joe Chou for the initial mapping of *odr-8*; Liqin Tong and Shannon Grantner for technical support; Levente Egry for integrating the *str-1*::ODR-10-GFP transgene; and Sue Kirch, Mark von Zastrow, Katja Brose, Jen Zallen, and Peter Walter for comments on the manuscript. This work was supported by grants from the American Cancer Society and the National Institutes of Health. C. I. B. is an Assistant Investigator of the Howard Hughes Medical Institute. P. S. was supported by fellowships from the American Cancer Society, California Division. E. R. T. is a National Science Foundation predoctoral fellow.

Received January 27, 1998; revised March 27, 1998.

References

Agard, D.A., Hiraoka, Y., Shaw, P., and Sedat, J.W. (1989). Fluorescence microscopy in three dimensions. Methods Cell. Biol. *30*, 353–377.

Arvidsson, U., Riedl, M., Chakrabarti, S., Lee, J., Nakano, A., Dado, R., Loh, H., Law, P., Wessendorf, M., and Elde, R. (1995). Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. J. Neurosci. *15*, 3328–3341.

Baker, E.K., Colley, N.J., and Zuker, C.S. (1994). The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. EMBO J. *13*, 4886–4895.

Bargmann, C.I., Hartwieg, E., and Horvitz, H.R. (1993). Odorant-selective genes and neurons mediate olfaction in *C. elegans*. Cell *74*, 515–527.

Barstead, R.J., and Waterston, R.H. (1989). The basal component of the nematode dense-body is vinculin. J. Biol. Chem. *264*, 10177–10185.

Bohm, S.K., Khitin, L.M., Smeekens, S.P., Grady, E.F., Payan, D.G. and Bunnett, N.W. (1997). Identification of potential tyrosine-containing endocytic motifs in the carboxyl-tail and seventh transmembrane domain of the neurokinin 1 receptor. J. Biol. Chem. *272*, 2363–2372.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71–94.

Chen, H., Hughes, D.D., Chan, T.A., Sedat, J.W., and Agard, D.A. (1996). IVE (Image Visualization Environment): a software platform for all three-dimensional microscopy applications. J. Struct. Biol. *116*, 56–60.

Chou, J.H., Troemel, E.R., Sengupta, P., Colbert, H.A., Tong, L., Tobin, D.M., Roayaie, K., Crump, J.G., Dwyer, N.D., and Bargmann, C.I. (1996). Olfactory recognition and discrimination in Caenorhabditis elegans. Cold Spring Harb. Symp. Quant. Biol. *61*, 157–164.

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.

Colbert, H.A., Smith, T.L., and Bargmann, C.I. (1997). OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptation in Caenorhabditis elegans. J. Neurosci. *17*, 8259–8269.

Colley, N., Baker, E., Stamnes, M., and Zuker, C. (1991). The cyclophilin homolog ninaA is required in the secretory pathway. Cell *67*, 255–263.

Ferreira, P.A., Nakayama, T.A., Pak, W.L., and Travis, G.H. (1996). Cyclophilin-related protein RanBP2 acts as chaperone for red/green opsin. Nature *383*, 637–640.

Froshauer, S., Green, G.N., Boyd, D., McGovern, K., and Beckwith, J. (1988). Genetic analysis of the membrane insertion and topology of MalF, a cytoplasmic membrane protein of Escherichia coli. J. Mol. Biol. *200*, 501–511.

Gudermann, T., Schoneberg, T., and Schultz, G. (1997). Functional and structural complexity of signal transduction via G-protein-coupled receptors. Annu. Rev. Neurosci. *20*, 399–427.

Hein, L., and Kobilka, B.K. (1995). Adrenergic receptor signal transduction and regulation. Neuropharmacology *34*, 357–366.

Hiraoka, Y., Swedlow, J.R., Paddy, M.R., Agard, D.A., and Sedat, J.W. (1991). Three-dimensional multiple-wavelength fluorescence microscopy for the structural analysis of biological phenomena. Semin. Cell. Biol. *2*, 153–165.

Hirokawa, N. (1998). Kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science *279*, 519–526.

Huang, L.S., Tzou, P., and Sternberg, P.W. (1994). The *lin-15* locus encodes two negative regulators of Caenorhabditis elegans vulval development. Mol. Biol. Cell *5*, 395–411.

Keefer, J.R., Kennedy, M.E., and Limbird, L.E. (1994). Unique structural features important for stabilization versus polarization of the alpha 2A-adrenergic receptor on the basolateral membrane of Madin-Darby canine kidney cells. J. Biol. Chem. *269*, 16425–16432.

Kirchhausen, T., Bonifacino, J.S., and Riezman, H. (1997). Linking cargo to vesicle formation: receptor tail interactions with coat proteins. Curr. Opin. Cell. Biol. *9*, 488–495.

Koenig, J.A., and Edwardson, J.M. (1997). Endocytosis and recycling of G protein-coupled receptors. Trends Pharmacol. Sci. *18*, 276–287.

Konopka, J.B., Jenness, D.D., and Hartwell, L.H. (1988). The C-terminus of the *S. cerevisiae* α-pheromone receptor mediates an adaptive response to pheromone. Cell *54*, 609–620.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. *157*, 105–132.

Mello, C.C., Kramer, J.M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in C. elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J. *10*, 3959–3970.

Mombaerts, P., Wang, F., Dulac, C., Vassar, R., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). The molecular biology of olfactory perception. Cold Spring Harb. Symp. Quant. Biol. *61*, 135–145.

Ng, G.Y., Varghese, G., Chung, H.T., Trogadis, J., Seeman, P., O'Dowd, B.F., and George, S.R. (1997). Resistance of the dopamine D2L receptor to desensitization accompanies the up-regulation of receptors on to the surface of Sf9 cells. Endocrinology *138*, 4199–4206.

Perkins, L.A., Hedgecock, E.M., Thomson, J.N., and Culotti, J.G. (1986). Mutant sensory cilia in the nematode Caenorhabditis elegans. Dev. Biol. *117*, 456–487.

Roayaie, K., Crump, J.G., Sagasti, A., and Bargmann, C.I. (1998).

The Gα protein ODR-3 mediates olfactory and nociceptive function and controls cilium morphogenesis in *C. elegans* olfactory neurons. Neuron *20*, 55–67.

Rothman, J.E., and Wieland, F.T. (1996). Protein sorting by transport vesicles. Science *272*, 227–234.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Press).

Saunders, C., Keefer, J.R., Kennedy, A.P., Wells, J.N., and Limbird, L.E. (1996). Receptors coupled to pertussis toxin-sensitive G-proteins traffic to opposite surfaces in Madin-Darby canine kidney cells. A1 adenosine receptors achieve apical and alpha 2A adrenergic receptors achieve basolateral localization. J. Biol. Chem. *271*, 995–1002.

Sengupta, P., Chou, J.H., and Bargmann, C.I. (1996). *odr-10* encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl. Cell *84*, 899–909.

Shapiro, M.J., Trejo, J., Zeng, D., and Coughlin, S.R. (1996). Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking: distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. J. Biol. Chem. *271*, 32874–32880.

Shigemoto, R., Kulik, A., Roberts, J.D., Ohishi, H., Nusser, Z., Kaneko, T., and Somogyi, P. (1996). Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature *381*, 523–525.

Sung, C.H., Schneider, B.G., Agarwal, N., Papermaster, D.S., and Nathans, J. (1991). Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. Proc. Natl. Acad. Sci. USA *88*, 8840–8844.

Sung, C.H., Makino, C., Baylor, D., and Nathans, J. (1994). A rhodopsin gene mutation responsible for autosomal dominant retinitis pigmentosa results in a protein that is defective in localization to the photoreceptor outer segment. J. Neurosci. *14*, 5818–5833.

Troemel, E.R., Chou, J.H., Dwyer, N.D., Colbert, H.A., and Bargmann, C.I. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. Cell *83*, 207–218.

Troemel, E.R., Kimmel, B.E., and Bargmann, C.I. (1997). Reprogramming chemotaxis responses: sensory neurons define olfactory preferences in *C. elegans*. Cell *91*, 161–169.

Unson, C.G., Cypess, A.M., Kim, H.N., Goldsmith, P.K., Carruthers, C.J., Merrifield, R.B., and Sakmar, T.P. (1995). Characterization of deletion and truncation mutants of the rat glucagon receptor: seven transmembrane segments are necessary for receptor transport to the plasma membrane and glucagon binding. J. Biol. Chem. *270*, 27720–27727.

von Zastrow, M., Link, R., Daunt, D., Barsh, G., and Kobilka, B. (1993). Subtype-specific differences in the intracellular sorting of G protein-coupled receptors. J. Biol. Chem. *268*, 763–766.

Wan, Q., Xiong, Z.G., Man, H.Y., Ackerley, C.A., Braunton, J., Lu, W.Y., Becker, L.E., MacDonald, J.F., and Wang, Y.T. (1997). Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin. Nature *388*, 686–690.

Williams, B.D., Schrank, B., Huynh, C., Shownkeen, R., and Waterston, R.H. (1992). A genetic mapping system in Caenorhabditis elegans based on polymorphic sequence-tagged sites. Genetics *131*, 609–624.

Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., et al. (1994). 2.2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans. Nature *368*, 32–38.

Wozniak, M., and Limbird, L.E. (1996). The three alpha 2-adrenergic receptor subtypes achieve basolateral localization in Madin-Darby canine kidney II cells via different targeting mechanisms. J. Biol. Chem. *271*, 5017–5024.

Wozniak, M., Keefer, J.R., Saunders, C., and Limbird, L.E. (1997). Differential targeting and retention of G protein-coupled receptors in polarized epithelial cells. J. Recept. Signal Transduct. Res. *17*, 373–383.

Yu, R., and Hinkle, P.M. (1997). Effect of cell type on the subcellular localization of the thyrotropin-releasing hormone receptor. Mol. Pharmacol. *51*, 785–793.

Zhang, Y., Chou, J.H., Bradley, J., Bargmann, C.I., and Zinn, K. (1997). The Caenorhabditis elegans seven-transmembrane protein ODR-10 functions as an odorant receptor in mammalian cells. Proc. Natl. Acad. Sci. USA *94*, 12162–12167.

GenBank Accession Number

The GenBank accession number for ODR-4 is AF055911.

CHAPTER 4

ODR-10 Localization to Cilia and in Rapidly Transported Vesicles Requires UNC-101, a μ1 clathrin adaptor

Summary

The function of polarized cells such as neurons requires specific patterns of protein expression on the cell surface. Sorting of proteins into specific transport vesicle populations has been proposed as a mechanism for ensuring targeting specificity to specialized membrane domains. However, protein sorting into specific vesicle types is poorly understood, especially in neurons. Here we show that an odorant receptor tagged with green fluorescent protein is transported in rapidly moving vesicles in the dendrites of chemosensory neurons. The AP-1 clathrin adaptor protein, UNC-101, is required for receptor sorting into these vesicles. UNC-101 is also required for receptor localization to the olfactory cilia; in *unc-101* mutants, receptor is present over the entire plasma membrane. These results suggest that sorting into a special dendritic vesicle population by UNC-101 allows specific targeting of receptor to the sensory cilia.

Introduction

Polarized cells such as epithelial cells and neurons must localize protein machinery to specialized surface domains in order to spatially segregate functions such as secretion and signal reception. Neurons localize some proteins to axons, others to dendrites, and further localize proteins to specific parts of axonal or dendritic arbors (Craig et al., 1994; Arvidsson et al., 1995; Brandstatter et al., 1996; Shigemoto et al., 1996). How neurons target proteins to specific regions with such exquisite control is a matter of great interest, but it is difficult to study since neuronal cells in culture may lose some aspects of cell polarity, and expressing mutated proteins in neurons has been technically difficult until recently.

For many proteins, cells solve this problem through precise packaging

of transport vesicles at the *trans*-Golgi network. Proteins in a particular vesicle type are transported to and fuse specifically with the correct target membrane (Rothman and Wieland, 1996). Alternatively, some proteins are localized through an indirect pathway, either by transcytosis or by random targeting and selective retention (Mostov and Cardone, 1995; Weimbs et al., 1997).

Upon exiting the *trans*-Golgi network (TGN), proteins can be sorted into a variety of vesicle types, destined for different intracellular and plasma membrane locations. While some proteins are sorted into vesicles that are delivered directly to the plasma membrane (the constitutive pathway(s)), others are sent through the selective endosomal pathway (Traub and Kornfeld, 1997). The class of TGN vesicles that are routed to the endosome are coated with clathrin (Traub and Kornfeld, 1997).

While several types of coats have been identified on TGN vesicle buds, clathrin coats are the best-studied, in part because clathrin-coated vesicles (CCVs) are also involved in endocytosis from the plasma membrane (Robinson, 1994). The assembly of clathrin-coated buds on the Golgi requires the heterotetrameric AP-1 adaptor complex (Traub et al., 1993; Traub et al., 1995; Schmid, 1997). The clathrin associated protein (AP) complexes provide a membrane-binding site for clathrin and also interact with trafficking membrane proteins to serve a cargo-selective function. (Marks et al., 1997; Robinson, 1997). AP-1 consists of four subunits: two large chains, β′ and γ, one medium chain, μ1 or AP47, and one small chain, σ1 or AP19. AP's may sort or retain some transmembrane proteins by binding to tyrosine motifs in their tails (Ohno et al., 1995; Ohno et al., 1996).

Vesicles loaded with their cargo are thought to be carried to their destinations by motor proteins that bind to the vesicles and move along

cytoskeletal tracks (Hirokawa, 1998). Vesicles travelling through the cell are permitted or denied fusion access at various intracellular or plasma membrane sites by virtue of the "addressing" proteins on their surfaces. A variety of protein families including coat proteins, small GTPases (rabs), motor proteins, and SNARE proteins may all play a role in vesicle targeting (Rothman and Wieland, 1996). However, how the vast array of cargo proteins are sorted into the vesicles containing the proper routing information (motors) and address information (SNAREs, rabs) is largely unknown.

Because neurons parcel different functions such as information reception and transmission into different compartments, accurate protein targeting is critical. It is not clear how well the mechanisms identified in simpler cell types will translate to solve the targeting problems of neurons. It is thought that synaptic vesicle proteins travel from the TGN to the synapse through several precursor compartments, undergoing multiple sorting steps, with final synaptic vesicle assembly occurring only at the nerve terminal (Mundigl and De Camilli, 1994). Cytoplasmic signals that mediate two distinct sorting steps for synaptobrevin (VAMP) targeting to synaptic vesicles in both PC12 cells and cultured hippocampal neurons have been identified (Grote et al., 1995; West et al., 1997). Most studies of vesicle transport in neurons have been in axons, but the sorting, transport, and targeting of proteins in dendrites might involve different compartments, sorting signals, or vesicle components.

Here we establish *C. elegans* chemosensory neurons as a new genetically accessible system for the study of protein targeting and vesicle trafficking. By expressing a GFP-tagged odorant receptor at high levels in a single type of chemosensory neuron, we can observe not only the restricted

localization of the receptor at the ciliated tips of dendrites, but also the rapid transport of receptor-containing organelles in the dendrites of living neurons. In addition, we have sought molecules involved in trafficking of this odorant receptor by using mutants that affect proteins involved in vesicle trafficking.

Many of the recently identified olfactory signalling molecules, including candidate odorant receptors, G proteins, and ion channels, are localized to chemosensory cilia (Troemel et al., 1995; Chou et al., 1996; Coburn and Bargmann, 1996; Sengupta et al., 1996; Colbert et al., 1997; Roayaie et al., 1998). Previously we identified a novel membrane protein, ODR-4, required for the localization of a subset of odorant receptors to the cilia of chemosensory neurons (Dwyer et al., 1998). Here we visualize the odorant receptor, ODR-10-GFP, being rapidly transported in vesicles in the dendrite of a chemosensory neuron. We demonstrate that the localization of *odr-10* into such vesicles and its restriction to the cilium is disrupted by mutations in a μ1 subunit of the AP-1 clathrin adaptor, *unc-101*. In the absence of UNC-101, the odorant receptor, normally localized exclusively to sensory cilia, is instead present on the entire plasma membrane.

Results

ODR-10-GFP is present in rapidly moving vesicles in the dendrites of AWB neurons

To study receptor localization we expressed ODR-10-GFP in the AWB chemosensory neuron. The odorant receptor ODR-10 is normally expressed in the AWA cilia, but it is also functional and localized to the cilia when expressed in the AWB neuron (Troemel et al., 1997). The very strong AWB expression of ODR-10-GFP using the *str-1* promoter revealed dots and blobs of

GFP fluorescence in the dendrite and cell body of the AWB neuron. These discrete punctate structures exhibited heterogeneity in shape and size. The smallest detectable dots by eye were less than 0.5 μm in diameter, while the largest moving organelles were tubules 2 μm or more in length (Figures 1, 2).

The AWB::ODR-10-GFP organelles are capable of rapid movement in the dendrite. These GFP-labelled vesicles are so bright that their movement is visible by eye using conventional fluorescence microscopy with a 63 X oil objective. Vesicles travelled rapidly, both toward the cilia (anterograde), and toward the cell body (retrograde) (Figures 1, 2). The movement was saltatory, involving rapid steps, rests, and renewal of movement. Single vesicles were often observed to change direction. Vesicles travelling in opposite directions were sometimes seen to pass one another, so the diameter of some these organelles may be less than half that of the dendrite. We observed speeds ranging from 0.5 to 1.3 μm/sec. The following speed estimates were made based on a low number of individual events. For wild-type animals, average anterograde speed was 1 μm/sec (n=2), and the average retrograde speed was 0.5 μm/sec (n=5). For *unc-104(e1265)* mutant animals, the average anterograde speed was 0.8 μm/sec (n=6), and the average retrograde speed was 0.8 μm/sec (n=13). Vesicles were seen to enter both the cilia and the cell body, and to emerge from these compartments as well. Vesicles were also observed to coalesce on a few occasions.

We suspected that these organelles might be transport vesicles responsible for transporting ODR-10 protein down the dendrite and to the cilium. Transport vesicles have been shown to move at rates in the 0.5 - 5 microns per second range in cells and neuronal processes (Hirokawa, 1998),

Figure 1. ODR-10-GFP is transported in rapidly moving vesicles in the dendrite of the AWB chemosensory neuron

Time-lapse images of rapid anterograde movement of an ODR-10-GFP vesicle (arrowhead) in the AWB dendrite. The time is shown in the lower right corner. Arrow indicates origin of dendrite from cell body. The cilium is the bright white spot in the upper right corners. This animal is an *unc-104(e1265)* mutant, which has vesicle movement indistinguishable from that of wild-type animals. Scale bar represents 20 μm. This vesicle moved 11 μm in 16.7 seconds, travelling at 0.7 μm/sec.

Figure 2. ODR-10-GFP is also transported retrogradely in rapidly moving vesicles in the AWB dendrite

Time-lapse images of a rapid retrograde movement of an ODR-10-GFP tubule (large arrowhead) that emerged from the cilium. The time is shown in the lower right corner. The large arrow points to the cell body. This animal is an *unc-104(e1265)* mutant, which has vesicle movement indistinguishable from that of wild-type animals. Scale bar represents 20 μm. This tubule moved 22 μm in 40 seconds, travelling at a speed of 0.6 μm/sec. The smaller arrow and arrowhead point to two vesicles that pass one another.

Figure 1

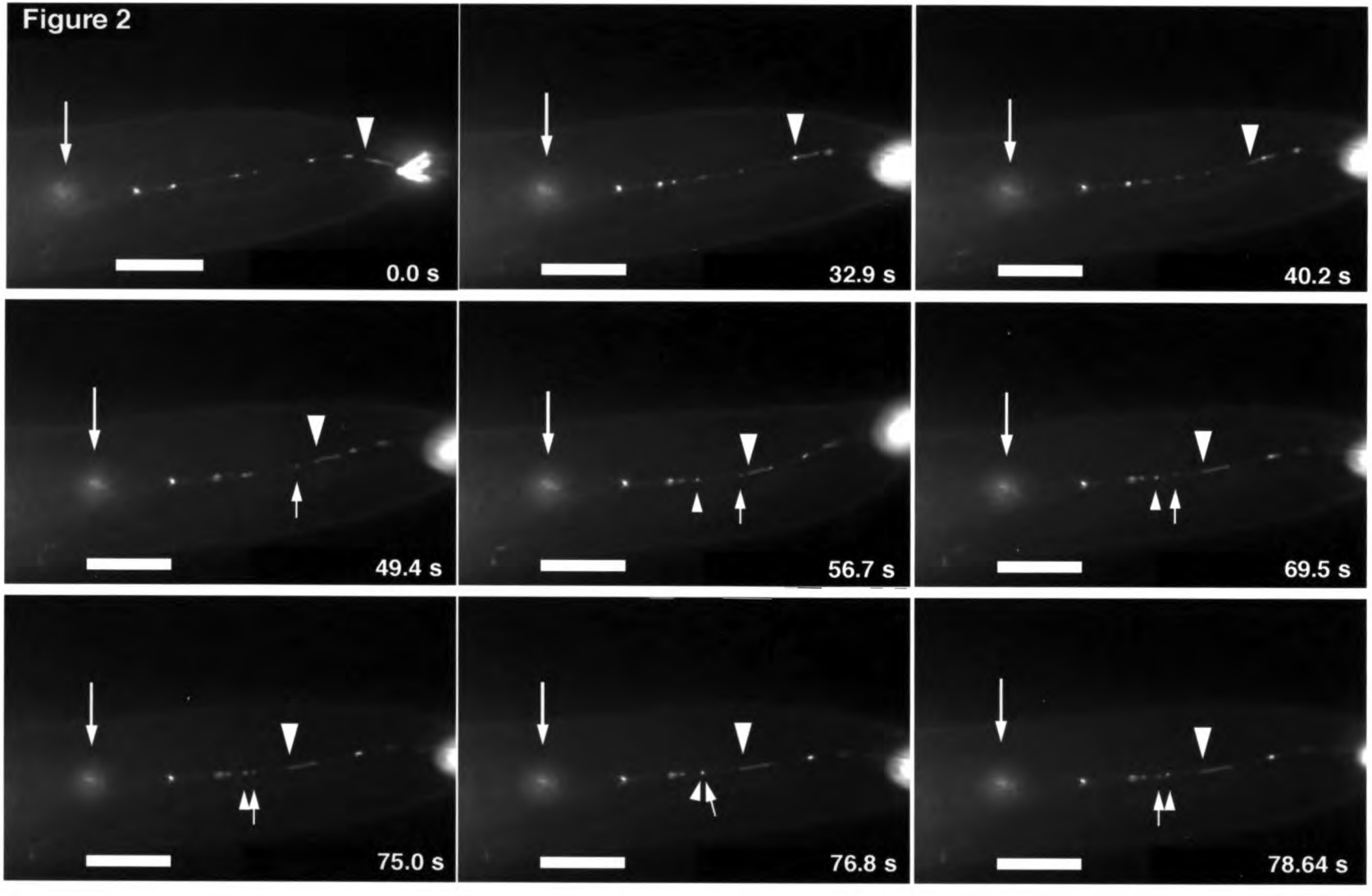


Figure 2
0.0 s
32.9 s
40.2 s
49.4 s
56.7 s
69.5 s
75.0 s
76.8 s
78.64 s

carried by ATP-dependent motor proteins. Since known motor protein movement is ATP-dependent, we tested whether the rapid movement of these dendritic vesicles was ATP-dependent. A commonly used anaesthetic for *C. elegans* is sodium azide, which uncouples oxidative phosphorylation in mitochondria, thereby disrupting ATP production. When animals were anaesthetized in 5mM sodium azide, no vesicle movement was observed, suggesting an ATP requirement for vesicle movement.

We typically observed vesicle movement in animals immobilized by the acetylcholine receptor agonist, levamisole. This drug causes hypercontraction of the animals' muscles. To control for the possibility that the levamisole was causing an artifactual movement of the ODR-10-GFP-containing vesicles, animals were examined using a different immobilization procedure. When piperazine, a $GABA_A$ agonist, was used as an anaesthetic, or when paralyzed animals (such as animals mutant for the muscle myosin *unc-54*) were examined without anaesthetic present, rapid vesicle movement was still observed (data not shown).

Low temperature has been shown to disrupt membrane trafficking at various steps in mammalian cells. For example, 20°C reversibly blocks exit from the TGN (Traub and Kornfeld, 1997) in epithelial cells. *C. elegans* is normally raised at 20°C, but can survive at temperatures ranging from 9°C to 27°C. To test whether low temperature could disrupt formation of these vesicles or localization of ODR-10 to the cilia, animals were raised at 10°C starting from the first larval stage, and ODR-10-GFP localization was examined when animals reached adulthood. Interestingly, despite severely retarding the growth of the animals, the low temperature had no overt effect on localization of ODR-10-GFP into vesicles or the cilia (data not shown).

In some animals no vesicle movement was observed, while in others

there was a great deal of transport observed. We wondered whether the animals' experience might regulate the amount of vesicle trafficking. Both starvation and exposure to high concentrations of odorant have been shown to modulate behavioral response to odorants (Colbert and Bargmann, 1995; Colbert and Bargmann, 1997). To examine whether these treatments would affect the localization or trafficking of odorant receptors, animals were starved for six hours, or exposed to high concentrations of the ODR-10 odorant ligand (diacetyl) for one hour. Neither of these manipulations visibly altered localization of ODR-10-GFP to the AWB cilia or anterograde and retrograde vesicle transport (data not shown).

Known motor protein mutations do not affect ODR-10-GFP localization or vesicle transport

Vesicle transport in neurons is generated by motor proteins such as kinesins and dyneins (Hirokawa, 1998). Two *C. elegans* kinesin heavy chain genes, *unc-104* and *unc-116*, are known to be expressed throughout the nervous system (Hall and Hedgecock, 1991; Patel et al., 1993) . A third kinesin heavy chain-like protein, *osm-3*, and a cytoplasmic dynein, *che-3*, function specifically in chemosensory neurons (Perkins et al., 1986; Shakir et al., 1993; T. Starich pers. comm.). We examined AWB::ODR-10-GFP in these animals to test for any changes in transport or targeting of ODR-10-GFP. Interestingly, none of these mutations affected ODR-10-GFP localization or vesicle movement (Table 1).

Table 1. Mutations Tested for ODR-10-GFP Localization to Cilia or ODR-4-GFP Localization in Dendrites

Gene	Gene Function	AWB::ODR-10-GFP localized?	ODR-10-GFP vesicle transport?	ODR-4-GFP localized?	References
unc-101	μ1 clathrin adaptor	NO	no vesicles	YES	Lee et al. 1994
odr-4	novel	YES, but less	no vesicles visible	YES	Dwyer et al. 1998
odr-8	in *odr-4* pathway	YES, but less	no vesicles visible	YES	Dwyer et al. 1998
unc-104	kinesin heavy chain	YES	normal	YES	Hall and Hedgecock, 1991; Otsuka et al., 1991
unc-116	kinesin heavy chain	YES	normal	YES	Patel et al., 1993
F56E3.3	kinesin-like	YES	normal	YES	G. Garriga, pers. comm.
che-3	dynein, cilium structure	YES	normal	YES	T. Starich, pers. comm.
osm-3	kinesin heavy chain	YES	normal	n.d.	Shakir et al., 1993
dyn-1	dynamin	n.d.	n.d.	n.d.	Clark et al., 1997
unc-11	AP-3 vesicle coat	YES	normal	n.d.	M. Nonet, pers. comm
unc-13	has C2 domains; gap junction formation?	YES	normal	n.d.	Maruyama and Brenner, 1991; Betz et al., 1997
dpy-23	μ2 clathrin adaptor	YES	normal	n.d.	G. Garriga, pers. comm.
osm-6	novel, cilium structure	YES	normal	n.d.	Collet et al., 1998
unc-14	interacts with UNC-51	YES	normal	n.d.	Ogura et al., 1997

unc-51	kinase for rac?	YES	normal	n.d.	Ogura et al., 1994
unc-33	cytoskeletal organization?	YES	normal	n.d.	Li et al., 1992
unc-44	ankyrin repeats	YES	normal	n.d.	Otsuka et al., 1995
unc-73	activates Rac, cytoskeletal regulation?	YES	normal	n.d.	Run et al., 1996 ; Steven et al., 1998
unc-76	novel, axon fasciculation?	YES	normal	n.d.	Bloom and Horvitz, 1997
unc-119	novel, axon fasciculation?	YES	normal	n.d.	Maduro and Pilgrim, 1995

Most strains were made by crossing an integrated array of AWB::ODR-10-GFP, *kyIs156* into the mutant strains or an extrachromosomal array of ODR-4-GFP into the mutants. GFP-tagged ODR-4 and *lin-15* marker DNA were injected into *che-3(e1124); lin-15(n765ts)* to create transgenic lines. Normal ODR-10-GFP localization means GFP brightly in the cilia of AWB, and vesicles in the denrite, cell body, and axon. Normal ODR-4-GFP localization means GFP on reticular structures in the cell body and a punctate/patchy distribution in the dendrite and axon. Normal transport of ODR-10-GFP vesicles means qualitatively, vesicles were moving in both the anterograde and retrograde direction in the micron/second range of speed. See Experimental Procedures for specific alleles used.

Mutations in *unc-44* and *unc-33* do not affect ODR-10-GFP localization or vesicle transport

UNC-44 is an ankyrin-related protein that is involved in axonal guidance, is thought to be part of the cortical cytoskeleton, and is required for localization of UNC-33, a putative microtubule-binding protein also involved in axonal guidance (Li et al., 1992; Otsuka et al., 1995 ; W. Li and J. Shaw pers. comm.). These mutants are known from electron microscopy to disrupt the cytoskeleton of amphid cilia and dendrites (Otsuka et al., 1995). Since the cytoskeleton plays an important role in both vesicle trafficking and protein localization, we examined mutants in *unc-33* and *unc-44* for effects on AWB::ODR-10-GFP localization. These mutants had ODR-10-GFP localization and vesicle movement that was indistinguishable from wild-type animals (Table 1), despite visible axon morphology defects (data not shown).

A clathrin adaptor, UNC-101, is required for localization of the odorant receptor ODR-10 to chemosensory cilia

Vesicle coat proteins are important both for the biogenesis of vesicles and for sorting of cargo proteins into vesicles. Clathrin coats are one of several types of vesicle coats found at the *trans*-Golgi network (TGN) (Schmid, 1997). To investigate the possible involvement of clathrin-coated vesicles in the transport and localization of ODR-10, AWB::ODR-10-GFP was examined in clathrin adaptor mutants. No clathrin mutants exist in *C. elegans*, but there are three known mutations in clathrin-associated proteins. The clathrin adaptor complexes mediate binding of clathrin to different membranes, and may play a role in recruiting cargo into the vesicle buds. DPY-23 is a clathrin adaptor most closely related to the μ2 (or AP50) subunit of AP-2, required for endocytosis at the plasma membrane in mammalian cells

(G. Garriga pers. comm.; Schmid, 1997). UNC-11 is a subunit of the AP-3 neuronal-specific adaptor (AP180), thought to play a role in synaptic vesicle biogenesis from endosomes (M. Nonet pers. comm.; Faundez et al., 1998). UNC-101 is most closely related to the μ1 (or AP47) subunit of AP-1, found at the TGN. *unc-101* was identified based on an uncoordinated phenotype originally, and exhibits a genetic interaction with the EGF receptor/ras signalling pathway (Lee et al., 1994).

While *unc-11* and *dpy-23* mutations had no effect on ODR-10-GFP transport or localization (Table 1), the pattern of AWB::ODR-10-GFP localization observed in *unc-101* mutants was dramatically different from that seen in wild-type animals. First, the receptor-containing vesicles normally seen in the dendrite and cell body were not visible in *unc-101(m1)* mutants. Second, rather than being strictly localized to the cilia, the ODR-10-GFP was present in a uniform distribution over the entire cell (Figure 3B). The protein appeared to be on the plasma membrane of the entire neuron, including but not exclusively the cilia. This complete delocalization of ODR-10-GFP in the absence of the AP-1 clathrin adaptor, UNC-101, suggests that ODR-10 may require sorting into clathrin-coated vesicles at the TGN in order to be specifically targeted to the cilia.

In addition to loss of specific localization, the *unc-101* mutants had highly penetrant defects in the overall morphology of the AWB neurons, as seen with the AWB::ODR-10-GFP. The AWB cilia (Figure 3B, arrowhead) appeared smaller in *unc-101* mutants than they did in wild-type animals, and one or more ectopic processes often protruded from the cell body. The ectopic processes were usually directed posteriorly and ranged in size from one to several microns long (Figure 3B, small arrowhead). This morphological

Figure 3. UNC-101 is required for the localization of ODR-10-GFP, but not for localization of synaptic vesicles to the axon

A. The AWB::ODR-10-GFP protein is localized strongly in the AWB cilia (arrowhead) in wild-type animals, and also in vesicles in the dendrite (small arrowheads) and cell body (arrow). Scale bar = 15 μm for all panels.

B. In an *unc-101(m1)* mutant animal, AWB::ODR-10-GFP is localized uniformly over the entire plasma membrane of the AWB neuron, including in the (thin) cilium (arrowhead), dendrite, cell body (arrow), and axon. There is an ectopic process protruding dorsally from the cell body (small arrowhead).

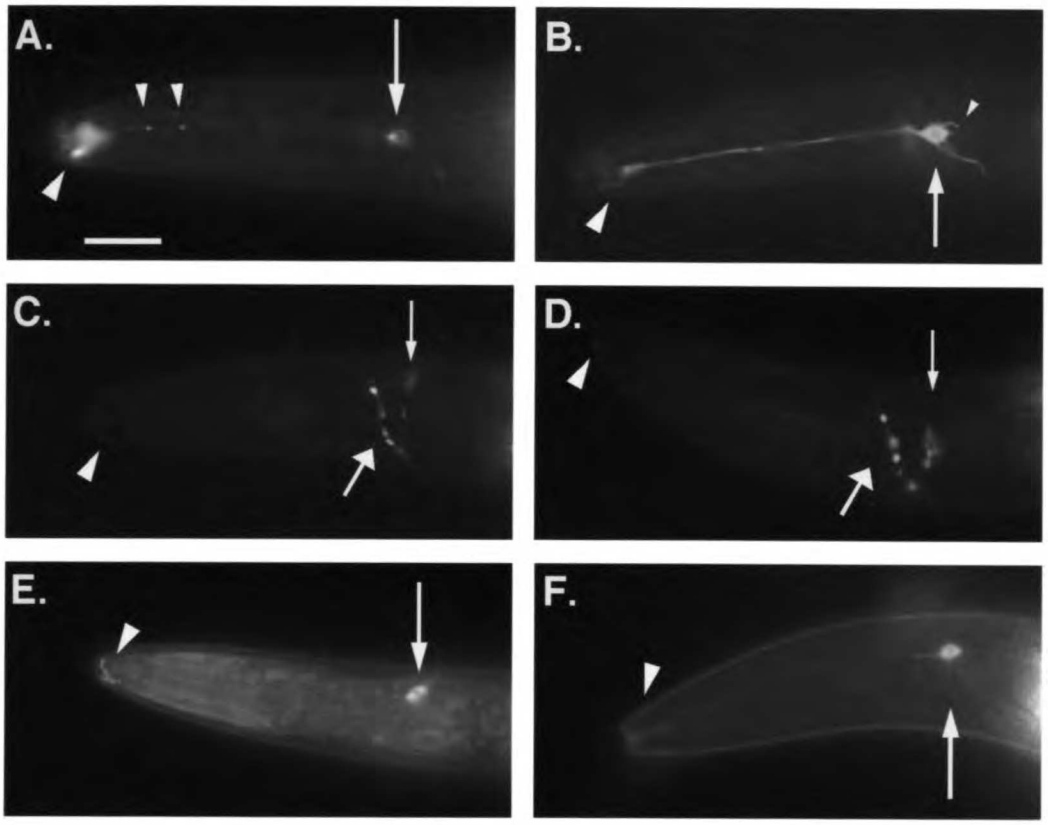
C. ASI::VAMP-GFP is localized strongly in the axon (wide arrow) and slightly in the cell body (arrow) of the ASI neuron in wild-type animals.

D. The *unc-101(m1)* mutation does not disrupt VAMP-GFP localization to the axon (wide arrow) of the ASI neuron.

E. ODR-10 expressed in AWB neurons shows partial dependence on *odr-4* for localization. In *odr-4(n2144)* mutants less protein reaches the cilia (arrowhead), while more GFP appears in the cell body (arrow).

F. A double mutant *unc-101(m1);odr-4(n2144)* shows a more severe AWB::ODR-10-GFP localization defect than either single mutant alone. No GFP is visible in the cilia (arrowhead), while low levels of GFP are seen in the cell body (arrow) and weakly in the dendrite.

Figure 3



defect could result from direct or indirect effects of *unc-101* mutations on the regulation of membrane or structural components.

unc-101 is not required for localization of VAMP to the axon of ASI

To test whether neuronal cell polarity was generally disrupted in *unc-101* mutants, the localization of a synaptic vesicle protein was examined. Synaptobrevin (VAMP) is a v-SNARE that is required in the membranes of synaptic vesicles for fusion with the presynaptic membrane. When expressed under the *str-3* promoter, VAMP-GFP localizes to synaptic vesicle clusters at synapses in the ASI axon (J. Crump, pers. comm.). The *unc-101* mutation did not disrupt this pattern (Figure 3 C, D). This result suggests that UNC-101 is not required for synaptic vesicle targeting to axons.

Mutations in a large number of other genes implicated in protein transport or localization were examined for effects on ODR-10-GFP localization and transport in the AWB neuron (Table 1). Although some of the other mutations tested caused axonal morphology defects, most of them did not disrupt ODR-10 localization (Table 1).

ODR-4 may function at a step prior to the TGN

The *C. elegans odr-4* gene is required for ODR-10 function and localization to AWA cilia. ODR-4 is a novel transmembrane protein that is involved in ODR-10 folding, sorting, or transport. ODR-4-GFP is present on internal membranes in the cell body, and punctate blobs in the dendrite (Dwyer et al., 1998). The punctate dendritic staining of ODR-4-GFP suggested that it might be present on transport vesicles. However, the ODR-4-GFP blobs do not move rapidly as the ODR-10-GFP blobs do (data not shown), leading us to the conclusion that ODR-4 and ODR-10 may not colocalize in dendritic

vesicles. Some of the mutations examined for effects on ODR-10-GFP localization were also analyzed for effects on ODR-4-GFP localization (Table 1). If ODR-4 were a vesicle protein present on ODR-10-containing vesicles, then ODR-4 localization might also be disrupted by *unc-101* mutations. We asked whether *unc-101* was required for the localization of ODR-4-GFP. In *unc-101* mutants, ODR-4-GFP had a completely normal expression pattern, including its characteristic reticular pattern in the cell body, punctate pattern in the processes, and absence from the cilia. This result is consistent with the hypothesis that the punctate ODR-4-GFP structures are not the same kind of compartment as the ODR-10-GFP vesicles and therefore that ODR-4 and ODR-10 do not colocalize in the dendrite.

odr-4 and *odr-8* mutations decreased but did not eliminate ODR-10-GFP in the AWB cilia. The dendritic vesicles were not visible in *odr-4* and *odr-8* mutants (Dwyer et al., 1998; Table 1; Figure 3E). This phenotype may reveal a defect in receptor packaging into vesicles, or the vesicles may be present but contain fluorescence below the level of visual detection. We used the *unc-101* phenotype to learn more about the role of *odr-4* in receptor localization.

Assessing the effect on ODR-10 localization of a double mutant between *odr-4* and *unc-101* suggested that *odr-4* and *unc-101* act at different steps in receptor biogenesis. While wild-type animals had a high level of ODR-10-GFP in the cilium and a low level in the cell body, *odr-4* mutants had less ODR-10-GFP overall, more in the cell body than the cilia, and none visible in the dendrite. In *unc-101* mutants, ODR-10-GFP was present at uniform brightness on the plasma membrane of the entire neuron, including the cilia. Thus lack of *odr-4* affects protein expression levels and shifts the GFP distribution to the cell body, while lack of *unc-101* causes an even protein distribution. When both the *odr-4* and the *unc-101* functions were disrupted,

the resulting appearance of AWB::ODR-10-GFP was different from either single mutant. The level of GFP expression was less than that seen in *unc-101* alone. The GFP was not visible in the cilia at all, but was seen in the cell body and weakly in the dendrite (Figure 3F). Taken together with the fact that ODR-4-GFP does not depend upon *unc-101* for its localization pattern, these data suggest that ODR-4 may act at a step before UNC-101. UNC-101 is likely to act at the TGN: it is more closely related to μ1 than μ2 (there is a distinct μ2 homolog in *C. elegans*), and vertebrate AP47 was able to rescue *unc-101* mutant phenotypes (Lee et al., 1994). Therefore, *odr-4* may act at a secretory step prior to the TGN, perhaps in a sorting or folding role.

Discussion

Selective vesicle targeting is one possible mechanism for asymmetric protein localization in neurons. Large families of motor proteins, rabs, coat proteins, and SNAREs have been identified as potential players in routing and addressing vesicles to various compartments, but their cargo- and target-specificities are not known in many cases. More investigation is needed to discover how most channels and receptors are sorted into vesicles with the correct routing and addressing information. This work describes a new genetically accessible system for the study of vesicle transport in dendrites. Additionally, we provide the first evidence implicating an AP-1 clathrin adaptor in the localization of a seven transmembrane domain protein.

A new genetic system for the study of vesicle trafficking

The diacetyl receptor ODR-10 is strictly localized to the cilia of *C. elegans* chemosensory neurons. By expressing a GFP-tagged *odr-10* cDNA

under the *str-1* promoter, such high levels of the fusion protein are produced that travelling vesicles containing ODR-10-GFP are visible with conventional fluorescence microscopy. The transparency of *C. elegans* and the long dendrite of these chemosensory neurons provide a 50 μm long "window" through which to observe vesicles moving along a straight path. The size of the GFP-labelled vesicles and tubules seen here may not represent the size of the actual vesicles that normally carry ODR-10, since these compartments may expand as a result of the presence of receptor overexpression.

The speed measured for the receptor-containing vesicles in the AWB neuron is too fast to be due to passive diffusion, and is in the range measured for motor proteins. Mutations in UNC-104, UNC-116, and OSM-3 kinesins and CHE-3 dynein did not disrupt the trafficking of these vesicles. These results could mean that transport of ODR-10 to the cilia is a non-motor-driven process, that redundancy exists in this tranpsport, or more likely, that the relevant motor has not yet been identified. Many other candidate motor protein genes exist in the *C. elegans* genome, but they have not been mutated. (Currently, 21 putative new kinesin-related genes, 7 dynein-related genes, and over 25 myosin-related genes exist in the *C. elegans* genomic database.) The ODR-10-GFP vesicles moved rapidly in both the anterograde and retrograde directions, and sometimes changed direction. How are these motions generated? It is not known whether the dendrites of *C. elegans* chemosensory neurons are like classical dendrites in having their microtubules in mixed orientations (Black and Baas, 1989). There are three possibilities for a microtubule motor, although an actin motor cannot be ruled out. If the dendrite contains uniformly oriented microtubules, there may be two different unidirectional motors, one plus-end directed and one minus end-directed; or there may be a bidirectional motor. Alternatively, if the dendrite

contains microtubules of mixed orientations, then the bidirectional vesicle motion could be produced by a single unidirectional motor. Future genetic screens for ODR-10 localization mutants will doubtless uncover the motor proteins and motor-associated proteins used in these dendrites.

UNC-101 may sort the odorant receptor ODR-10 into cilium-bound vesicles

We have demonstrated that the μ1 subunit of the AP-1 clathrin adaptor, UNC-101, is required for the localization of ODR-10 to the cilia. Since *unc-101* mutations also prevent the localization of ODR-10-GFP in vesicles, it may be that the sorting of ODR-10 into these vesicles is critical for its asymmetric targeting. Clathrin AP (adaptor protein) complexes are heterotetramers that are thought to couple clathrin coated pit assembly to the entrapment of membrane receptors (Marks et al., 1997; Robinson, 1997). Since mammalian μ1 (AP47) is primarily localized to the TGN, we propose that *unc-101* acts at the TGN to sort ODR-10 into transport vesicles addressed to the cilia. In *unc-101* mutants, sorting into this special vesicle population does not occur, and so the receptor is sent through a default plasma membrane pathway. In yeast, clathrin seems to be needed to sort or retain Kex2p or DPAP-A in the Golgi. In clathrin-deficient yeast, Kex2p, normally sequestered at the TGN, is expressed on the cell surface, resulting in secretion of some unprocessed alpha factor (Seeger and Payne, 1992). Loss of the yeast μ1 adaptin, Apm1p, enhances this defect (Stepp et al., 1995). Here, we show the first example *in vivo* that loss of the μ1 subunit alone is sufficient to cause mislocalization of a plasma membrane protein.

As an alternative to the model of specific vesicle targeting to the cilia, it is possible that localization of ODR-10 occurs by secretion all over the neuron, followed by endocytosis and re-sorting to the cilia. Deletion studies of the

transferrin receptor suggest that endocytosis and dendritic sorting may share signals (West et al., 1997). UNC-101 could function to sort receptor in a recycling endosome or a sorting endosome; clathrin-coated buds have been observed on endosmomes (Stoorvogel et al., 1996). AP-1 adaptors are not only associated with the TGN, but also with the early endosome (Le Borgne et al., 1996) and immature secretory granules in regulated secretory cells (Dittie et al., 1996).

The ODR-10 requirement for *unc-101* suggests that the ODR-10-GFP vesicles, whether budded from a secretory or an endocytic compartment, are clathrin-coated. However it is possible that AP-1 components could act in a non-clathrin pathway too: the fact that yeast μ1 (Apm1p) deletions also enhance the Kex2p sorting defect of clathrin null mutants suggests that the μ1 adaptin could act in a non-clathrin pathway in addition to a clathrin pathway (Stepp et al., 1995). However it could also be simply that AP-1 retains some trapping function even in the absence of clathrin.

UNC-101 is probably not specific for sorting of G protein-coupled receptors. A trafficking pathway involving UNC-101 may also play a role in localization of an EGF receptor during vulval development. *unc-101* mutations seem to promote vulval induction, suppressing weak alleles of the *let-23* EGF receptor. *unc-101* mutants have no vulval phenotype on their own, however. It was hypothesized that wild-type UNC-101 down-regulates the activity of LET-23 by altering its localization (Lee et al., 1994). Whether UNC-101 acts in the same trafficking pathway for both ODR-10, a seven transmembrane receptor, and LET-23, a single transmembrane tyrosine kinase receptor, remains to be seen. Considering the ubiquitous requirement for clathrin-coated vesicles in all cells, the *unc-101* mutant phenotype seems less severe than one might expect: although uncoordinated, the animals are

healthy and fertile, and the chemosensory neurons appear to develop relatively normally. The specific basis for the uncoordination is not known. The *m1* allele used in this study behaves as a null allele (Lee et al., 1994). Examination of the genomic sequence reveals another putative μ1 adaptin in *C. elegans* genome, so *unc-101* and this other μ1 may have different cargo- or cell-specificities.

Targeting of many integral membrane proteins to intracellular compartments is mediated by identified sorting signals contained within the cytoplasmic domain of the proteins (Rothman and Wieland, 1996; Marks et al., 1997). These sorting signals then interact with sorting proteins such as the clathrin adaptors. However, while some targeting sequences have been identified in soluble or single transmembrane proteins, the motifs and sorting proteins involved in targeting seven transmembrane domain proteins to different plasma membrane surfaces are not characterized.

If UNC-101 does indeed sort ODR-10 into vesicles, it may do so by direct interaction with the receptor. Through yeast two-hybrid and *in vitro* binding studies, μ1 adaptin was shown to interact with a tyrosine-based sorting signal, YXXØ, where Ø is an amino acid with a bulky hydrophobic side chain (Ohno et al., 1995; Marks et al., 1996; Ohno et al., 1996). Examination of the ODR-10 protein sequence reveals such a motif, YATW, in the third cytoplasmic loop. However, Keefer et al. found that the third cytoplasmic loop was not required for MDCK cell basolateral direct delivery of the α2A adrenergic receptor, and proposed that targeting signals were located in the transmembrane domains (Keefer et al., 1994). Alternatively, if ODR-4 acts as a chaperone for ODR-10, then ODR-4 could contain the tyrosine sorting signal that interacts with AP-1. While there are four potential YXXØ motifs in the cytoplasmic domain of ODR-4, the prediction from this model is that *odr-4* and *unc-101* mutations

would have the same effect on ODR-10 localization, which is not what was observed. Di-leucine motifs have been shown to mediate internalization and targeting to lysosomes, endosomes, and the basolateral surface of epithelial cells (Pond et al., 1995). ODR-10 does contains a di-leucine on the second cytoplasmic loop. ODR-10 does not contain any NPXY motifs (as required in the LDL receptor for early endosome targeting), nor does it contain a D/ExD/E motif thought to be required for exit from the ER (Bannykh et al., 1998). At any rate, seven transmembrane receptors may use motifs rather different than those that have been found so far for single transmembrane molecules (Keefer et al., 1994).

Protein targeting in neurons has been compared to that in MDCK epithelial cells. Based on correlated localization of VSV-G, HA, and GPI proteins, it has been suggested that the apical and baso-lateral surfaces are similar to the axonal and dendritic domains, respectively (Dotti and Simons, 1990; Dotti et al., 1991). Basolateral-directed vesicles with transferrin receptor appear to be clathrin-coated (Futter et al., 1998). The location of the *C. elegans* cilia at the tips of dendrites suggests that they represent a basolateral domain; however, cilia in epithelial cells are on the apical surface. The cilium and the dendrite are distinct compartments, separated by belt junctions (Perkins et al., 1986). It may be that the *C. elegans* sensory cilia, as well as those of vertebrate olfactory neurons, rod and cone cells, and hair cells, defy the apical/basolateral dichotomy.

ODR-4 may act at a step prior to UNC-101, facilitating odorant receptor folding or sorting

Aside from affording insight into the role of clathrin adaptors in ODR-10 localization, the *unc-101* phenotype has provided a tool with which to

further probe the function of *odr-4*, a novel gene also required for ODR-10 localization. AP-1 has been found on the TGN, but may also function in sorting on endosomes and immature secretory granules (Seaman et al., 1993; Traub et al., 1993; Dittie et al., 1996). It is reasonable to hypothesize that UNC-101 sorts ODR-10 into clathrin-coated vesicles at the TGN or a sorting endosome. If ODR-4 were acting in vesicle transport for ODR-10-containing vesicles, one would expect that ODR-4 might require UNC-101 for sorting into these vesicles as well. Since *unc-101* does not appear to be required for ODR-4 localization, and since the animals mutant for both *unc-101* and *odr-4* had a more severe phenotype than *unc-101* alone, these data are consistent with ODR-4 acting at a site prior to UNC-101. ODR-4 probably acts upstream of the compartment where UNC-101 resides. Of the three models (receptor folding, receptor sorting, or vesicle targeting) previously presented for *odr-4* function (Dwyer et al., 1998), these data refute a role for *odr-4* in vesicle transport, and are most consistent with a role for *odr-4* in folding or sorting in a compartment upstream of the TGN. Alternatively, it is possible that ODR-4 functions upstream of *unc-101* in protein stability, and downstream or parallel to *unc-101* for targeting.

Much remains to be learned about protein targeting in neurons, especially for G protein-coupled receptors. This work shows that *C. elegans* chemosensory neurons provide a valuable new system for dissecting transport and localization mechanisms for membrane proteins important for neuronal function and animal behavior.

Experimental Procedures:

Strains and genetics

Wild-type nematodes were *C. elegans* variety Bristol, strain N2. Strains were maintained using standard methods (Brenner, 1974). Animals were grown at 20°C for most experiments, and at 25°C for chemotaxis assays. Many strains were provided by the Caenorhabditis Genetics Center. The following strains were used or created in this work. CX3877*kyIs156X*, CX3878*unc-116(e2310)III;kyIs156X*, *CX2395dpy-5(e61)unc-101(m1)I*, *unc-101(m1)I*, *unc-101(m1)I;kyIs156X*, *FF41 unc116(e2310)III*, *PR802 osm-3(p802)IV*, *unc-104(e1265)II;kyIs156X*, *CX3882unc104(e1265)II;lin15X*, *che-3(e1124)I;lin15X* *CX3875 che-3(e1124)I;kyIs156X*, *fax-1(gm27) lon-2(e678)X*, *CB3253 dpy-23(e840) lon-2(e678)X*, *unc-76(e911)*, *unc-73(e936)*, *unc-44(e362)*, *unc-33(e204)IV*, *unc-119(e2498)III*, *unc-14(e57)*, *unc-51(e369)*, *CX3876 osm-6(p811) V; kyIs156 X*, *CX3880 dpy-17(e164)odr-4(n2144)unc-32(e189); kyIs156X*, *CX3883 dpy-17(e164)unc-32(e189); kyIs156X*, *CX3881 odr-8(ky31) unc-24(e138)IV; kyIs156X*. Paralytic agents used in agarose pads on slides were either 5 mM sodium azide or 1 mM levamisole.

Microscopy

Most photos were taken using standard fluorescence microscopy. Time-lapse images were captured using a cooled CCD camera based on Kodak 768 x 512 CCD (Princeton Instruments), a Princeton Instruments Micromax Controller, and using Metamorph software. 2 x 2 binning of pixels was used to increase the signal.

Quantitation of vesicle speeds

Vesicle speeds were estimated by the following method. Frames in a Metamorph time-lapse CCD sequence were imported into NIH Image and

stacked. Speeds were only measured during segments of time when the vesicle was in focus and when the animal did not move excessively. Speeds were measured for continuous movement segments, not stops or changes in direction. Speeds were not measured for vesicles less than about half a micron long becasue they were not well-resolved. Adjustments were made for any slight animal movement. Distances were calibrated in Metamorph by imaging a hemacytometer (VWR). The distance measured was a straight line between the positions of the vesicle in two different frames. (This may be an underestimate because the dendrite is not perfectly straight.) Time was measured using the time-stamp feature of Metamorph. Since exposure times for each image were close to one second in the examples used for these calculations, these speeds can only be taken as estimates. (Using the filter wheel, shorter exposure times are now possible.)

Acknowledgements

We thank Gage Crump for the ASI::VAMP-GFP strain, and especially thank Tim Yu for help with CCD imaging. This work was supported by grants from the American Cancer Society and the National Institutes of Health. C.I.B. is an Assistant Investigator of the Howard Hughes Medical Institute.

REFERENCES

Arvidsson, U., M. Riedl, S. Chakrabarti, J. Lee, A. Nakano, R. Dado, H. Loh, P. Law, M. Wessendorf, and R. Elde. 1995. Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. *Journal of Neuroscience* 15: 3328-41.

Bannykh, S. I., N. Nishimura, and W. E. Balch. 1998. Getting into the Golgi. *Trends Cell Biol.* 8: 21-25.

Betz, A., M. Okamoto, F. Benseler, and N. Brose. 1997. Direct interaction of the rat unc-13 homologue Munc13-1 with the N terminus of syntaxin. *J Biol Chem* 272: 2520-6.

Black, M. M., and P. W. Baas. 1989. The basis of polarity in neurons. *Trends Neurosci* 12: 211-4.

Bloom, L., and H. R. Horvitz. 1997. The Caenorhabditis elegans gene unc-76 and its human homologs define a new gene family involved in axonal outgrowth and fasciculation. *Proc Natl Acad Sci U S A* 94: 3414-9.

Brandstatter, J. H., P. Koulen, R. Kuhn, H. van der Putten, and H. Wassle. 1996. Compartmental localization of a metabotropic glutamate receptor (mGluR7): two different active sites at a retinal synapse. *J Neurosci* 16: 4749-56.

Brenner, S. 1974. The genetics of *Caenorhabditis elegans. Genetics* 77: 71-94.

Chou, J. H., E. R. Troemel, P. Sengupta, H. A. Colbert, L. Tong, D. M. Tobin, K. Roayaie, J. G. Crump, N. D. Dwyer, and C. I. Bargmann. 1996. Olfactory recognition and discrimination in *Caenorhabditis elegans. Cold Spring Harb Symp Quant Biol* 61: 157-64.

Clark, S. G., D. L. Shurland, E. M. Meyerowitz, C. I. Bargmann, and A. M. van der Bliek. 1997. A dynamin GTPase mutation causes a rapid and

reversible temperature-inducible locomotion defect in C. elegans. *Proc Natl Acad Sci U S A* 94: 10438-43.

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

Colbert, H., and C. Bargmann. 1997. Environmental signals modulate olfactory acuity, discrimination, and memory in *Caenorhabditis elegans*. *Learning & Memory* 4: 179-191.

Colbert, H. A., and C. I. Bargmann. 1995. Odorant-specific adaptation pathways generate olfactory plasticity in *C. elegans*. *Neuron* 14: 803-12.

Colbert, H. A., T. L. Smith, and C. I. Bargmann. 1997. OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptaion in *Caenorhabditis elegans*. *J. Neurosci.* 17: 8259-8269.

Collet, J., C. A. Spike, E. A. Lundquist, J. E. Shaw, and R. K. Herman. 1998. Analysis of *osm-6*, a Gene That Affects Sensory Cilium Structure and Sensory Neuron Function in *Caenorhabditis elegans*. *Genetics* 148: 187-200.

Craig, A. M., C. D. Blackstone, R. L. Huganir, and G. Banker. 1994. Selective clustering of glutamate and gamma-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitters. *Proc Natl Acad Sci U S A* 91: 12373-7.

Dittie, A. S., N. Hajibagheri, and S. A. Tooze. 1996. The AP-1 adaptor complex binds to immature secretory granules from PC12 cells, and is regulated by ADP-ribosylation factor. *J Cell Biol* 132: 523-36.

Dotti, C. G., R. G. Parton, and K. Simons. 1991. Polarized sorting of glypiated proteins in hippocampal neurons. *Nature* 349: 158-61.

Dotti, C. G., and K. Simons. 1990. Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell* 62: 63-72.

Dwyer, N. D., E. R. Troemel, P. Sengupta, and C. I. Bargmann. 1998. Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. *Cell* 93: 455-466.

Faundez, V., J.-T. Horng, and R. B. Kelly. 1998. A Function for the AP3 Coat Complex in Synaptic Vesicle Formation from Endosomes. *Cell* 93: 423-432.

Futter, C. E., A. Gibson, E. H. Allchin, S. Maxwell, L. J. Ruddock, G. Odorizzi, D. Domingo, I. S. Trowbridge, and C. R. Hopkins. 1998. In polarized MDCK cells basolateral vesicles arise from clathrin--adaptin-coated domains on Endosomal Tubules. *J. Cell Biol.* 141: 611-623.

Grote, E., J. C. Hao, M. K. Bennett, and R. B. Kelly. 1995. A targeting signal in VAMP regulating transport to synaptic vesicles. *Cell* 81: 581-9.

Hall, D. H., and E. M. Hedgecock. 1991. Kinesin-related gene *unc-104* is required for axonal transport of synaptic vesicles in *C. elegans*. *Cell* 65: 837-847.

Hirokawa, N. 1998. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science* 279: 519-526.

Huang, L. S., P. Tzou, and P. W. Sternberg. 1994. The *lin-15* locus encodes two negative regulators of Caenorhabditis elegans vulval development. *Mol Biol Cell* 5: 395-411.

Keefer, J. R., M. E. Kennedy, and L. E. Limbird. 1994. Unique structural features important for stabilization versus polarization of the alpha 2A-adrenergic receptor on the basolateral membrane of Madin-Darby canine kidney cells. *J. Biol. Chem.* 269: 16425-16432.

Le Borgne, R., G. Griffiths, and B. Hoflack. 1996. Mannose 6-phosphate receptors and ADP-ribosylation factors cooperate for high affinity interaction of the AP-1 Golgi assembly proteins with membranes. *J. Biol. Chem.* 271: 2162-70.

Lee, J., G. D. Jongeward, and P. W. Sternberg. 1994. *unc-101*, a gene required for many aspects of *Caenorhabditis elegans* development and behavior, encodes a clathrin-associated protein. *Genes Dev* 8: 60-73.

Li, W., R. K. Herman, and J. E. Shaw. 1992. Analysis of the Caenorhabditis elegans axonal guidance and outgrowth gene *unc-33*. *Genetics* 132: 675-689.

Maduro, M., and D. Pilgrim. 1995. Identification and cloning of *unc-119*, a gene expressed in the Caenorhabditis elegans nervous system. *Genetics* 141: 977-88.

Marks, M., H. Ohno, T. Kirchhausen, and S. Bonifacino. 1997. Protein sorting by tyrosine-based signals: Adapting to the Ys and wherefores. *Trends Cell Biol.* 7: 124-128.

Marks, M. S., L. Woodruff, H. Ohno, and J. S. Bonifacino. 1996. Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components. *J. Cell Biol.* 135: 341-54.

Maruyama, I. N., and S. Brenner. 1991. A phorbol ester/diacylglycerol-binding protein encoded by the *unc-13* gene of Caenorhabditis elegans. *Proc Natl Acad Sci U S A* 88: 5729-33.

Mostov, K. E., and M. H. Cardone. 1995. Regulation of protein traffic in polarized epithelial cells. *Bioessays* 17: 129-38.

Mundigl, O., and P. De Camilli. 1994. Formation of synaptic vesicles. *Curr Opin Cell Biol* 6: 561-7.

Ogura, K., M. Shirakawa, T. M. Barnes, S. Hekimi, and Y. Ohshima. 1997. The

UNC-14 protein required for axonal elongation and guidance in Caenorhabditis elegans interacts with the serine/threonine kinase UNC-51. *Genes Dev* 11: 1801-11.

Ogura, K., C. Wicky, L. Magnenat, H. Tobler, I. Mori, F. Muller, and Y. Ohshima. 1994. Caenorhabditis elegans *unc-51* gene required for axonal elongation encodes a novel serine/threonine kinase. *Genes Dev* 8: 2389-400.

Ohno, H., M. C. Fournier, G. Poy, and J. S. Bonifacino. 1996. Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. *J Biol Chem* 271: 29009-15.

Ohno, H., J. Stewart, M. C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J. S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* 269: 1872-5.

Otsuka, A. J., R. Franco, B. Yang, K. H. Shim, L. Z. Tang, Y. Y. Zhang, P. Boontrakulpoontawee, A. Jeyaprakash, E. Hedgecock, V. I. Wheaton, et al. 1995. An ankyrin-related gene (*unc-44*) is necessary for proper axonal guidance in *Caenorhabditis elegans*. *J. Cell Biol.* 129: 1081-92.

Otsuka, A. J., A. Jeyaprakash, J. Garcia-Anoveros, L. Z. Tang, G. Fisk, T. Hartshorne, R. Franco, and T. Born. 1991. The C. elegans *unc-104* gene encodes a putative kinesin heavy chain-like protein. *Neuron* 6: 113-122.

Patel, N., D. Thierry-Mieg, J. R. Mancillas, F. 1993. Cloning by insertional mutagenesis of a cDNA encoding *Caenorhabditis elegans* kinesin heavy chain. *Proc Natl Acad Sci U S A* 90: 9181-5.

Perkins, L. A., E. M. Hedgecock, J. N. Thomson, and J. G. Culotti. 1986. Mutant sensory cilia in the nematode *Caenorhabditis elegans*. *Dev. Biol.* 117:

456-87.

Pond, L., L. A. Kuhn, L. Teyton, M. P. Schutze, J. A. Tainer, M. R. Jackson, and P. A. Peterson. 1995. A role for acidic residues in di-leucine motif-based targeting to the endocytic pathway. *J Biol Chem* 270: 19989-97.

Roayaie, K., J. G. Crump, A. Sagasti, and C. I. Bargmann. 1998. The Gα Protein ODR-3 Mediates Olfactory and Nociceptive Function and Controls Cilium Morphogenesis in *C. elegans* Olfactory Neurons. *Neuron* 20: 55-67.

Robinson, M. S. 1994. The role of clathrin, adaptors and dynamin in endocytosis. *Curr Opin Cell Biol* 6: 538-44.

Robinson, M. S. 1997. Coats and vesicle budding. *Trends Cell Biol.* 7: 99-102.

Rothman, J. E., and F. T. Wieland. 1996. Protein sorting by transport vesicles. *Science* 272: 227-34.

Run, J. Q., R. Steven, M. S. Hung, R. van Weeghel, J. G. Culotti, and J. C. Way. 1996. Suppressors of the *unc-73* gene of *Caenorhabditis elegans*. *Genetics* 143: 225-36.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, New York. Pages.

Schmid, S. L. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu Rev Biochem* 66: 511-48.

Seaman, M. N., C. L. Ball, and M. S. Robinson. 1993. Targeting and mistargeting of plasma membrane adaptors *in vitro*. *J Cell Biol* 123: 1093-105.

Seeger, M., and G. S. Payne. 1992. Selective and immediate effects of clathrin heavy chain mutations on Golgi membrane protein retention in *Saccharomyces cerevisiae*. *J Cell Biol* 118: 531-40.

Sengupta, P., J. H. Chou, and C. I. Bargmann. 1996. *odr-10* encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl. *Cell* 84: 899-909.

Shakir, M. A., T. Fukushige, H. Yasuda, J. Miwa, and S. S. Siddiqui. 1993. *C. elegans osm-3* gene mediating osmotic avoidance behaviour encodes a kinesin-like protein. *Neuroreport* 4: 891-894.

Shigemoto, R., A. Kulik, J. D. Roberts, H. Ohishi, Z. Nusser, T. Kaneko, and P. Somogyi. 1996. Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. *Nature* 381: 523-5.

Stepp, J. D., A. Pellicena-Palle, S. Hamilton, T. Kirchhausen, and S. K. Lemmon. 1995. A late Golgi sorting function for *Saccharomyces cerevisiae* Apm1p, but not for Apm2p, a second yeast clathrin AP medium chain-related protein. *Mol. Biol. Cell.* 6: 41-58.

Steven, R., Kubiseski, T. J., Zheng, H., Kulkarni, S., Mancillas, J., Ruiz Morales, A., Hogue, C. W., Pawson, T., Culotti, J. 1998. UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in *C. elegans.* *Cell* 92 : 785-795.

Stoorvogel, W., V. Oorschot, and H. J. Geuze. 1996. A novel class of clathrin-coated vesicles budding from endosomes. *J. Cell Biol.* 132: 21-33.

Traub, L. M., and S. Kornfeld. 1997. The trans-Golgi network: a late secretory sorting station. *Curr Opin Cell Biol* 9: 527-33.

Traub, L. M., S. Kornfeld, and E. Ungewickell. 1995. Different domains of the AP-1 adaptor complex are required for Golgi membrane binding and clathrin recruitment. *J Biol Chem* 270: 4933-42.

Traub, L. M., J. A. Ostrom, and S. Kornfeld. 1993. Biochemical dissection of AP-1 recruitment onto Golgi membranes. *J Cell Biol* 123: 561-73.

Troemel, E. R., J. H. Chou, N. D. Dwyer, H. A. Colbert, and C. I. Bargmann.

1995. Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. *Cell* 83: 207-18.

Weimbs, T., S. H. Low, S. J. Chapin, and K. E. Mostov. 1997. Apical targeting in polarized epithelial cells: there's more afloat than rafts. *Trends Cell Biol.* 7: 393-399.

West, A. E., R. L. Neve, and K. M. Buckley. 1997. Targeting of the synaptic vesicle protein synaptobrevin in the axon of cultured hippocampal neurons: evidence for two distinct sorting steps. *J Cell Biol* 139: 917-27.

Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks, J. Bonfield, J. Burton, M. Connell, T. Copsey, J. Cooper, et al. 1994. 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans* . *Nature* 368: 32-8.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS:

C. elegans Chemosensory Neurons as a New Model System for the Study of Membrane Protein Targeting in Neurons

Overview

G protein-coupled receptors are critical for neuronal function and display varied patterns of subcellular localization. The mechanisms of biogenesis and localization of G protein-coupled (seven transmembrane domain) receptors are not well-understood. This thesis establishes the *C. elegans* chemosensory neurons as a valuable new system for the study of seven transmembrane receptor targeting in neurons.

The chemoreceptors of *C. elegans* include several families of seven transmembrane domain receptors. Some of these receptors have been shown to localize specifically to the cilia at the exposed dendritic endings of the chemosensory neurons (Troemel et al., 1995; Chou et al., 1996). The high concentration of these receptors on the cilia which have a large surface area enables the nematode to detect odorants at very low concentrations in the air. Since chemosensation is the nematode's primary sense used for finding food, this extreme sensitivity is critical for survival.

We have focused in particular on the mechanism of cilium localization of the diacetyl receptor ODR-10. Through this work, two new players have been added to the very small number of *trans*-acting factors known to be required for localization of G protein-coupled receptors. Mutants in *odr-4* and *unc-101* display ODR-10 localization defects. While *odr-4* mutants have decreased levels of receptor and show a shift in receptor distribution toward the cell body, *unc-101* mutants have receptor present over the entire cell surface. ODR-4 is a novel membrane-associated protein expressed exclusively in chemosensory neurons (Dwyer et al., 1998). It may play a role in folding or trafficking of ODR-10. UNC-101 is a μ1 clathrin adaptor (Lee et al., 1994) that could act to recruit ODR-10 into clathrin-coated

transport vesicles. While the requirement for *odr-4* seems specific for only a subset of odorant receptors, the specificity of the *unc-101* requirement has not yet been evaluated.

We have also shown that ODR-10-GFP is present in rapidly moving vesicles in the dendrite of the AWB neuron. The movement is reminiscent of that mediated by microtubule motors (J. Scholey, N. Pollock, pers. comm.). This finding suggests that dendritic transport of odorant receptors to the cilia is vesicle-mediated. The mechanism of this vesicle movement, although unknown at present, could be generated by motor proteins. Thus, this system could provide a valuable tool for discovering and characterizing motors and associated proteins.

The movement of ODR-10-GFP vesicles in the dendrite, and the requirement of the UNC-101 clathrin adaptor for localization suggest that the receptor is localized as a protein. In addition, while 3' UTR's or introns seem to play a role in localization of mRNAs (Wilhelm and Vale, 1993), the AWB::ODR-10-GFP construct that localizes to cilia was made using the *odr-10* cDNA without its 3' UTR. Therefore *odr-10* RNA localization seems less likely but has not been ruled out.

The *odr-4* and *unc-101* phenotypes show that genes with different roles in the trafficking pathway for ODR-10 can be identified through mutant hunts. Future screens could allow identification of more genes in the *odr-4* or *unc-101* pathways, or could provide genes involved in other steps of odorant receptor targeting to the cilia.

Models for ODR-4 and UNC-101 functions

One of the most striking features of *odr-4* function is its specificity. *odr-4* mutants appear essentially wild-type, but have defective chemotaxis to a

subset of odorants, and fail to localize a subset of odorant receptors to the cilia. Since ODR-4-GFP is expressed only in chemosensory neurons, it is likely that ODR-4 function is specific for chemoreceptors. The evidence in Chapter 3 suggests that ODR-4 is only required for the function of a subset of chemoreceptors. While more receptors need to be tested to draw a compelling conclusion, the trend after testing five receptors is that the requirement for *odr-4* may be family-specific. Three *str* family members, ODR-10, STR-1, and STR-2 required *odr-4* for their localization, while two *sr* family members, SRD-1 and SRG-2, did not (Dwyer et al., 1998 and E. Troemel, unpub. res.). It could be that the *str* family has "special needs" that other families do not. Alternatively, there could be an *odr-4*-like gene for the *sr* families and for other families of G protein-coupled receptors. Given the high degree of divergence of the chemoreceptors, additional *odr-4*-like genes might be expected to be extremely divergent as well.

The data presented in Chapters 3 and 4 are most consistent with a model in which ODR-4 acts in folding or sorting of odorant receptors at a step prior to UNC-101. The data is consistent with ODR-4 residence on internal membranes, facing the cytoplasm with its hydrophobic tail anchoring it in the membrane. ODR-4 does not display any recognizable structural motifs.

The exact cellular mechanism of ODR-4's function remains uncertain. While it may act at a step prior to the *trans*-Golgi network in folding, chaperoning, or sorting, it could act at multiple steps or have multiple roles. It could act as a foldase, actually helping fold receptors, or it could act as a "folding monitor" or "epitope receptor" (Rothman and Wieland, 1996), allowing only folded receptors to exit the ER. As a chaperone, it could be required for receptor stability or could be required to prevent inappropriate interactions between receptors and other proteins. If ODR-4 functions in

sorting prior to the TGN, it could do so by interactng with vesicle coat proteins. Alternatively, perhaps it acts to mask a late sorting signal until the receptor has passed through earlier sorting steps. It is possible that *odr-4* interacts directly with odorant receptors, but there is no compelling evidence yet for such an interaction. The best way to determine this would be to do *in vitro* binding studies with ODR-4 and ODR-10. Further elucidation of *odr-4* function awaits the cloning of homologs from other systems or of interacting proteins (see screens suggested below). ODR-8 probably acts in the ODR-4 pathway, but has not yet been cloned (see Appendix B).

Future Directions

Many experiments could shed light on the function of *odr-4*. It is possible that an *odr-4* homolog will be found in another system. If *odr-8* is cloned and proves to be similar to a previously described protein, its identity could provide insight into *odr-4* function. Finding proteins that interact with ODR-4 could be the most informative. A two-hybrid screen in yeast using ODR-4 as bait or a GFP screen for localization phenotypes similar to that of *odr-4* could allow identification of interacting molecules.

Since clathrin-coated vesicle trafficking is well-studied in other cell types, understanding the function of *unc-101* in localization of ODR-10 may prove to be more straightforward than that for *odr-4*. UNC-101 is likely, based on homology, to be acting in sorting at the TGN, but it remains possible that it acts in a sorting endosome or in endocytosis from the plasma membrane. Making a GFP tag of UNC-101 and examining its localization in the AWB neuron would be interesting. It might localize exclusively to the TGN, or it might be present in the dendrite or cilia, or in the ODR-10-GFP vesicles. The question of whether *unc-101* is required for endocytosis may be

answered by examining ODR-10-GFP localization in *dyn-1* mutants. *dyn-1(ky51)* animals have a temperature-sensitive mutation in dynamin that blocks endocytosis (Clark et al., 1997). Moreover, while μ1 adaptin in mammalian cells is thought to recruit cargo into clathrin-coated vesicles at the TGN (Schmid, 1997), it is possible that *unc-101* functions as part of a non-clathrin coat in these *C. elegans* neurons. It will also be interesting to find out whether *unc-101* is required generally for protein localization to the cilia. Since *unc-101* also regulates the EGF receptor/*let-23* signalling pathway (Lee et al., 1994), it may be required for localization of other classes of proteins besides odorant receptors. While chemotaxis assays that might reveal odorant- or cell-specificity cannot be performed because of the uncoordination of the mutants, this question can be addressed by examining the localization of other chemosensory signalling proteins in *unc-101* mutants with GFP tags or immunohistochemistry. It could be that *unc-101* is generally required for cilium proteins, or it could be specific for seven transmembrane domain receptors, or it could sort only proteins that function together in a complex with ODR-10, so that they would be on the same patch of membrane when they arrived at the cilium.

What are the *cis*-acting elements within ODR-10 that mediate its localization, and do these interact with ODR-4 or UNC-101? Comparing sequences of receptors dependent on *odr-4* with those that are *odr-4*-independent could reveal important motifs that determine a requirement for *odr-4*. However, based on the current small sample set, there are no obvious candidate localization motifs. The clearest trend is that the N-terminal tails of the three *str* receptors are shorter than those of *srd-1* and *srg*-2 (about 15 amino acids versus about 30). Deletion studies and swaps of tails, cytoplasmic domains, and transmembrane domains could provide clues as to the

domains that determine localization and interaction with the *odr-4* or *unc-101* systems. For example, Appendix A describes one experiment showing that deleting the tail of ODR-10 disrupts its localization to cilia, similar to the phenotype observed in *odr-4* mutants. There may be an *odr-10* mutation that would cause mislocalization similar to that seen in *unc-101* mutants.

The brightness of the AWB::ODR-10-GFP integrant(*kyIs156*) makes possible a powerful dissecting scope localization screen. Using the fluorescent dissecting microscope, green cilia are visible in *kyIs156* animals, while in *odr-4(n2144); kyIs156* animals no GFP is visible, and in *unc-101(m1); kyIs156* animals only the cell body is visible. Thus, by screening for animals in which the cilia are not visible, one would at least identify mutants with *odr-4*-like and *unc-101*-like phenotypes, and perhaps mutants with new phenotypes. Then a secondary screen under higher magnification would allow a more precise determination of GFP expression and localization. Further characterization of any mutants from these screens could involve dyefilling to check for the proper development of cilia, examination without sodium azide to check for vesicle transport, and diacetyl assays to determine whether the ODR-10 function has been lost. Mutants that showed no GFP expression at all might be set aside as likely mutants in gene expression rather than in localization. It is possible that aside from *odr-4*-like and *unc-101*-like phenotypes, a variety of other localizaton phenotypes might be seen. One might find mutations in other components of the sorting machinery, or in the molecules responsible for vesicle transport or targeting, or for receptor anchoring in the cilia. For example, mutations in a cilium-specific SNARE might cause an accumulation of GFP at the base of the cilium, or might produce normal-looking vesicles and transport, but absence of GFP in the cilium.

The yield of mutants from such a screen could be quite rich. At the very least, one would obtain more alleles of *unc-101*, *odr-4* and *odr-8*. More alleles of *odr-4* would be useful since a null allele does not exist, and new mutations could provide valuable information about the domains of *odr-4* important for function. It is likely that genes that interact with the *odr-4* system could also be pulled out, which would be extremely informative about the function of *odr-4*. The previous behavioral screens that resulted in identification of *odr-4* and *odr-8* were biased for coordinated animals that were able to chemotax to some chemicals. However, the *unc-101* phenotype indicates that localization mutants might be uncoordinated. In fact, it might be wise to enrich for uncoordinated animals in a localization screen.

Alternatively, if one were interested specifically in the function of clathrin vesicle-based sorting, one could perform an *unc-101* suppressor screen. The *m1* allele used in this thesis has an early stop and is probably a null allele, but the *sy161* allele changes a conserved arginine to a cysteine (Lee et al., 1994). This allele has not yet been examined for an ODR-10 localization phenotype, but if it shows defects it might be useful in a suppressor screen to identify UNC-101-interacting proteins. Since the *unc-101* mutants are severely paralyzed, a suppressor screen for animals that moved normally could be quite easy. Then a secondary screen could be done to see if the ODR-10-GFP localization phenotype was also suppressed.

By applying the genetic approach to the problem of neuronal protein localization, new molecules can be identified that will complement the cell biological and biochemical work on this problem being conducted in other systems. The identification of the *odr-4* and *unc-101* as genes required for ODR-10 localization provide two molecular points of departure for futher studies of the mechanisms of protein targeting in neurons. This thesis

demonstrates that *C. elegans* chemosensory neurons provide a valuable new system for dissecting transport and localization mechanisms for membrane proteins important in neuronal function and animal behavior.

REFERENCES

Chou, J. H., Troemel, E. R., Sengupta, P., Colbert, H. A., Tong, L., Tobin, D. M., Roayaie, K., Crump, J. G., Dwyer, N. D., and Bargmann, C. I. (1996). Olfactory recognition and discrimination in *Caenorhabditis elegans*. Cold Spring Harb Symp Quant Biol *61*, 157-64.

Clark, S. G., Shurland, D. L., Meyerowitz, E. M., Bargmann, C. I., and van der Bliek, A. M. (1997). A dynamin GTPase mutation causes a rapid and reversible temperature-inducible locomotion defect in *C. elegans*. Proc Natl Acad Sci U S A *94*, 10438-43.

Dwyer, N. D., Troemel, E. R., Sengupta, P., and Bargmann, C. I. (1998). Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. Cell *93*, 455-466.

Lee, J., Jongeward, G. D., and Sternberg, P. W. (1994). unc-101, a gene required for many aspects of *Caenorhabditis elegans* development and behavior, encodes a clathrin-associated protein. Genes Dev *8*, 60-73.

Rothman, J. E., and Wieland, F. T. (1996). Protein sorting by transport vesicles. Science *272*, 227-34.

Schmid, S. L. (1997). Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu Rev Biochem *66*, 511-48.

Troemel, E. R., Chou, J. H., Dwyer, N. D., Colbert, H. A., and Bargmann, C. I. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. Cell *83*, 207-18.

Wilhelm, J. E., and Vale, R. D. (1993). RNA on the move: the mRNA localization pathway. J Cell Biol *123*, 269-74.

APPENDIX A

The C-terminal Tail of ODR-10 is Required for its Localization

Appendix A: Truncation of the C-terminal Tail of ODR-10 Disrupts its Localization in a Manner Similar to that of *odr-4* and *odr-8* mutants

The requirement for the *odr-4/odr-8* system is in part sequence-specific. It is possible, but by no means certain, that the requirement may be for genes of the str family and not other seven transmembrane domain receptors, since *str-1*, *str-2*, and *odr-10* all require *odr-4/8*, while *srd-1* and *srg-2* do not. Therefore, there may be amino acid sequences or motifs in the receptors that determine the requirement for *odr-4* and perhaps even interact with *odr-4*. Studies in other systems have provided scant clues as to which domain(s) of seven transmembrane receptors might be involved in targeting. Deletion studies have prompted some to suggest N or C tails, others to suggest various transmembrane domains.

A student in the lab, Joe Chou, deleted the C-terminal tail of *odr-10* using a convenient restriction site, and found that this construct failed to rescue the diacetyl chemotaxis defect of *odr-10* mutants.

To ask whether the lack of rescue was due to defective localization of the ODR-10 protein, I made a construct in which the ODR-10 tail was truncated at the same position, but tagged with GFP.
This produced a truncated form of ODR-10 in which the 35 residues C-terminal to the seventh transmembrane domain were deleted and replaced by GFP. This fusion protein was not localized to the AWA cilia, but remained in the cell body in a pattern similar to that observed in *odr-4* and *odr-8* mutants (Figure 1). This result suggests that the C-terminus may contain signals important for targeting, and is a reasonable domain to begin looking for interactions with *odr-4*. An alternate explanation for this result is that the C-terminus is required for proper folding. To determine whether this C-

terminal tail does in fact determine dependence on the *odr-4* system, it would be necessary to test whether replacing the tail of ODR-10 with that from a receptor that does not require *odr-4* abolished ODR-10's dependence on *odr-4*.

Alternatively, the tail of *odr-10* might be sufficient to allow localization to the cilia of AWA, whether or not it interacts with *odr-4*. This could be tested by fusing it to the end of a non-ciliary protein and seeing if it caused localization to the cilia.

Another possibility is that it is the N-terminus that determines whether a receptor is dependent upon the *odr-4/odr-8* system. The N-terminal tails of *odr-10* and *str-2* are shorter than those of *srd-1* and *srg-2*. The *sr* genes may contain something in the N terminus that allows them to be independent of *odr-4*.

It should be noted that the construct represented by all of the ODR-10-GFP localization studies in this thesis does not contain the last two amino acids of the ODR-10 predicted protein. Therefore these residues must not be part of the critical tail domain required for cilium localization.

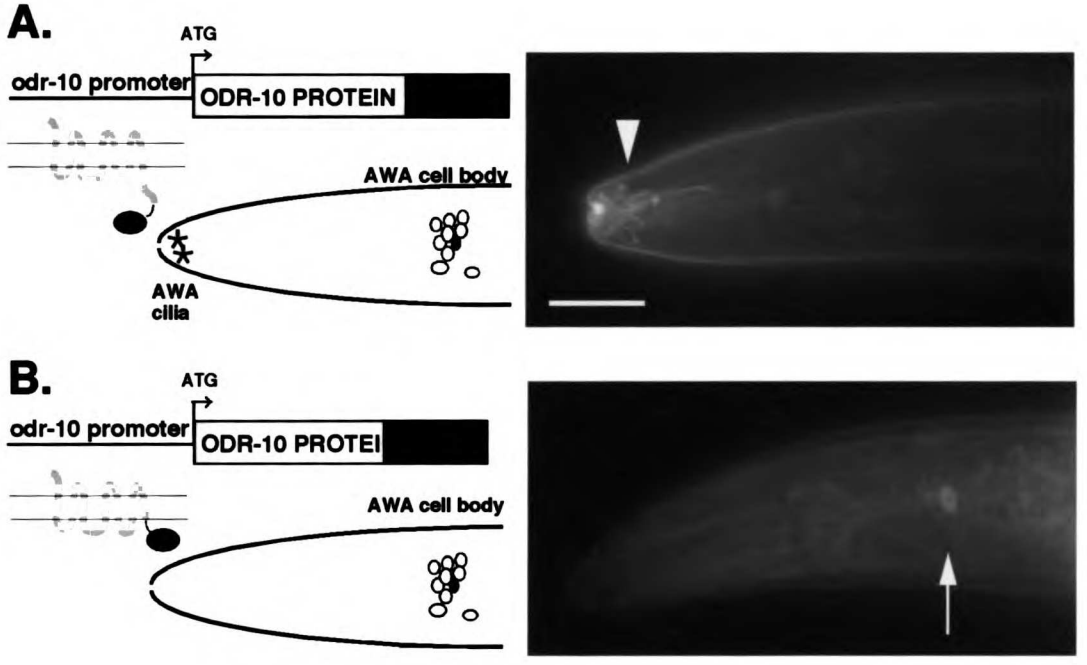
Figure 1

The C-terminal tail of ODR-10 is required for localization to the cilia.

A. (Left) Diagram showing the GFP-tagged ODR-10 and its localization in wild-type animals. (This construct does in fact delete the last two amino acids of ODR-10.) (Right) Photo shows localization of GFP-tagged ODR-10 to AWA cilia (arrowhead) in a wild-type animal. Scale bar, 30 μm.

B. (Left) The amino acids following the seventh transmembrane domain were deleted and replaced with GFP. (Right) This truncated fusion protein was localized to the AWA cell body in *odr-4(+)* animals (arrow).

Figure 1


A.
ATG
odr-10 promoter
ODR-10 PROTEIN
AWA cell body
AWA
cilia
B.
ATG
odr-10 promoter
ODR-10 PROTEI
AWA cell body

APPENDIX B

Efforts to Clone *odr-8*

Appendix B: Efforts to Clone *odr-8*, a gene likely to be in the same pathway as *odr-4*

As is shown in Chapter 3, *odr-8* is a gene that is likely to act in the same pathway as *odr-4*. Localization of ODR-10 and STR-2 requires both *odr-4* and *odr-8*. The five alleles of *odr-8* have the same spectrum of odorant defects as *odr-4(n2144)*. Furthermore, all of the proteins whose localization was examined in *odr-4(n2144)* were also examined in *odr-8(ky31)*. Thus far, every phenotype of *ky31* has been indistinguishable from *n2144*. A double mutant between these two alleles maintains the specificity of the olfactory defect. Overexpressing *odr-4* does not rescue the *odr-8* diacetyl chemotaxis phenotype. No conclusions can be drawn about epistasis.

Knowing the identity of the *odr-8* gene could provide insight into the function of *odr-4*. Furthermore, assaying function of ODR-10 in heterologous cell systems probably requires *odr-8* as well as *odr-4* for efficient cell surface expression. To this end, much effort has been expended in trying to clone *odr-8*.

odr-8 was mapped to the interval between *lin-45* and *deb-1* on LG IV using three-factor genetic mapping. The deficiency *nDf41* failed to complement *odr-8(ky31)*. From heterozygotes of the genotype *odr-8(ky31)/lin-45(sy96)unc-24(e138)*, 2/11 Unc non-Lin recombinants were Odr+. From heterozygotes of the genotype *odr-8(ky31)/unc-44(e362)deb-1(st555)*, 3/10 Unc non-Deb recombinants were Odr+.

Pools of cosmids from this region, along with *lin-15* DNA, were injected into *odr-8(ky31);lin15(n765ts)* mutants in an attempt to rescue the diacetyl phenotype. No rescue was obtained with any of the following clones.

Pool A:
F54E10
R07D10
C40G11
C44F12
C51D2
ZZC11!

Pool B:
R02H9
R13H7
D2043
T22D1
C02F1

Pool C:
C02F1
B0318
C25A8
F10A11
C17D5
C06G3

PoolD:
ZC477
C16G10
C34D4
R07A5

After part of the *odr-8* region had been sequenced, three genes were identified as candidates for *odr-8*. Clones containing these genes were injected for rescue attempts.

C06G3, injected previously as part of Pool C, was injected again by itself because it contains two candidates: a **kinesin-like protein**, and a Golgi protein **LDLC**. CO6G3 did not rescue by itself.

A **dynein intermediate chain** was found on **C17H12**, and because our cosmid clone was deleted, a 6.7kb and a 10kb fragment containing the gene were amplified by PCR, using the Expand Long Template PCR kit, and the PCR products purified with the Qiaquick PCR Purification Kit, and injected directly. The 6.7kb fragment was injected along with *rol-6* DNA into *odr-8(ky31); kyIs52* animals, and F1's were examined for rescue of ODR-10-GFP to the AWA cilia. The 10kb fragment was injected along with *lin-15* DNA into *odr-8(ky31); lin-15(n765ts)* animals, and F3's were assayed for rescue of diacetyl chemotaxis. No rescue was seen.

There were three "Yac gaps", gaps in the cosmid coverage, in the *odr-8* region. Since cosmid pools had failed to rescue, we predicted that *odr-8* might

lie in one of these gaps. A rotation student, Brenda Ng, tried to rescue *odr-8(ky31)* mutants by injecting whole yeast DNA from yeast strains containing Yacs covering the cosmid gaps. She purified DNA from yeast containing **Y6F5, Y8B6, Y53F3, Y45D2,** and **Y40D6**. She used Qiagen genomic prep columns, and ran out the yeast DNA on a Chef gel to confirm the presence of the Yac. She tried both to rescue the diacetyl phenotype and the GFP phenotype. That the Yacs did not rescue could be for any of several reasons. The Yac concentrations may have been too low, or the Yacs may have been sheared in the column purification. Alternatively, the *odr-8* function may be very sensitive to dosage, and so may be difficult to rescue with an extrachromosomal array containing multiple copies of the gene.

It remains possible that the *odr-8* gene does lie below a cosmid, but that the cosmids I chose to inject did not overlap enough or had small deletions. At this point in time, this region is almost entirely sequenced. It has been shown that there are only two Yac gaps rather than three, and there are many new candidates in the Yac gap regions. Smaller subclones of these Yacs exist as Fosmids, and rescue experiments may be attempted with these.

APPENDIX C

Details of Plasmid Construction

GFP and LacZ vectors were supplied by Andrew Fire (A. Fire et al, pers. comm.). PCRs were carried out using the Expand Long Template PCR kit (Boehringer-Mannheim) and an MJResearch Thermal Cycler. All constructs were verified by sequencing. ODR-4 constructs generated by PCR were thoroughly checked for PCR-generated mutations, which were corrected when found.

1. 5.4 kb Spe-Xho (pODR-4-1) This is the 5.4 kb rescuing fragment of C28A6. It was cut out of the cosmid using *Spe I* and *Xho I* and inserted into those sites in the MCS of pBluescript.

2. ODR-4 Xba-GFP The 6.5kb *Xba I* fragment of cosmid C28A6 was dropped into the XbaI site in the MCS of the GFP expression vector pPD95. 75. This contains about 4 kb upstream of *odr-4* and about half of the coding region of *odr-4*.

3. ODR-4 C tag (pODR-4-4) PCR using cosmid as template was used to create a translational fusion of ODR-4 with GFP. The downstream primer (GP3) was designed to end in a *Bam HI* site, and the upstream primer (GP1) in an SphI site, and this fragment was inserted into a special GFP expression vector ("95.85 - Xma-Age"). The *Xma I-Age I* fragment was removed from pPD95.85 (religated to self: *Xma I* and *Age I* are compatible ends) to create a vector with the S65T version of GFP in frame 2, without a secretion signal. (This was done because the other new GFP vectors were S65C, which is reputed not to work in lumenal compartments, and we suspected that the C terminus of ODR-4 might be in a lumenal compartment.) The *Xba I* fragment was then replaced with the genomic fragment from the cosmid, to replace any PCR-created mutations.

4. ODR-4 N tag (pODR-4-3) The sequence encoding GFP was amplified by PCR from pPD95.85 (S65T), using primers that created *Aat II* sites at the ends

(GP4 and GP5). This fragment was then cloned into the *Aat II* site in the 5.4kb *Spe I-Xho I* rescuing subclone of C28A6, six amino acids into ODR-4, maintaining the *odr-4* reading frame. This creates extra amino acids (in lower case): MILFDVvpvekGFPefdvQLQ...

5. ODR-4 N+C tag To tag ODR-4 at both the N- and C-termini with GFP, the *Xba I* fragment from ODR-4N tag replaced the *Xba I* fragment in ODR-4 C tag.

6. ODR-4 N+C mutant tag First, the identical procedure as was used to create "C tag" was performed again using *odr-4(n2144)* mutant worms as template. Then, the *Xba I* fragment from ODR-4 N tag was used to replace the *Xba I* fragment of this "C mutant tag", in order to replace PCR errors.

7. ODR-4-GFP-LacZ (pODR-4-5) First a "Frame 2" version of GFP-LacZ was made by cutting out GFP-LacZ from pPD116.21 (S65T,M153A) using *Age I* and *Eco RI*, and popping this fragment into pPD95.77. Then the 6.5kb *Sph I-Bam HI* fragment from ODR-4 C tag was inserted into this new GFP-LacZ expression vector called "GL2".

8. ODR-4DTM-GFP-LacZ (pODR-4-6) Primers GP-1 and GS-2-2 were used to amplify the *odr-4* promoter and whole coding sequence up to the transmembrane domain (TM). This *Sph I-Bam HI* fragment was then inserted into "GL2". Then the *Xba I* fragment was replaced with genomic DNA to get rid of any PCR errors.

9. ODR-10 no tail GFP The genomic *odr-10 Eco RV-Sca I* subclone (P.Sengupta) was cut with *Sal I* and *Bcl I* (*Bcl I* cuts immediately after TM7). This 2.5kb fragment was ligated to the GFP expression vector pPD95.79 cut with *Sal I* and *Bam HI*. (*Bcl I* and *Bam HI* sites have compatible ends.)

10. *str-3::odr-10*cDNA-GFP (Transcriptional fusion of *str-3* (M7.1) promoter (ASI) to the *odr-10* cDNA tagged with GFP). The M7.1 promoter was amplified by PCR using primers "M7.1-5'" and "M7.1-Xba". This product was

cut with *Xba I* and put into the *Xba I* site in the MCS of the *odr-10*cDNA-gfp construct (from P.S., but mutations replaced by N.D.) Later the *Sph I-Xba I* 0.7kb fragment was added to the 5' end of the promoter, (by replacing *Sph I-Sna BI* fragment with that from E.T.'s "pM7.1+TU61"), which greatly improved expression.

11. SRD-1 tag (D. Tobin) Approximately 3.5 kb of promoter and most of the *srd-1* coding region was amplified by PCR, using primers "F33H1.5 5' " and "F33H1.5 epitope", cut with *Pst I*, and inserted into the Fire GFP vector. This translational fusion does not contain most of the C-tail of *srd-1*.

12. p*odr-10*::SRD1-gfp (*odr-10* promoter fused to genomic SRD-1-GFP C tag) First the *srd-1* coding region was amplified from D. Tobin's *srd-1* tag using primers "SPH-SD-1" and "F33H1.5 epitope" (D.T.). This PCR product was cut with *Sph I* and *Pst I* and inserted into these sites in the GFP vector. This construct was then cut with *Hind III*, filled in with Klenow, and the *odr-10* promoter was inserted. It had been cut out of the "p*odr-10*Xho#3" construct (J. Chou) using *Eco RV* sites.

13. p*odr-10*::ODR-4-gfp (p[*odr-10*p::ODR-4-GFP]) (Transcriptional fusion of the *odr-10* promoter to the ODR-4 C tag.) First the *odr-10* promoter was cut out of J. Chou's "p*odr-10*Xho#3" with *Hind III* and *Xba I*. This was ligated into the ODR-4 C GFP tag cut with *Hind III* and *Xba I*. This created an intermediate vector that contained *odr-10* promoter:: second half of *odr-4*. The the first half of *odr-4* was amplified by PCR using primers "Pst o4" and "spx7R". The PCR product was cut with *Pst I* and *Xba I* and ligated with the intermediate vector cut with *Pst I* and *Xba I*.

14. Nde FS (*odr-4* Frameshift) (pODR-4-2) The 5.4 kb *Spe I-Xho I* rescuing fragment was subcloned into pcDNA3. A two base pair frameshift was introduced at the *Nde I* site in the first coding exon of the *odr-4* gene. The

Nde I site was cut, filled in with Klenow, and re-ligated. This results in a frameshift after Ala48 of ODR-4. and a stop after 8 more amino acids.

PRIMERS: 5' -> 3'

primers for *odr-4*:

GP-1 (*Sph I* site) : AGT CTA CTG GCA TGC ATG AGA AAC GGA AGG TGA GG

GP-2 (*Bam HI* site) : A TGT GAC CAG GAT CCA CAT CCA TTC GTC TTC CAG C

GP-3 (*Bam HI* site) : GAC TGT ACT GGA TCC GGA ATG TGC TAC AGT GAAA TA

GS-2-2 (*Bam HI* site) : TAA GTT ACT GGA TCC TTT CG TTG TGC ACC ATG AAT CG

Pst-o4 (*Pst I* site) : AGA TCT AAC TGC AGATG ATT CTG TTT GAC GTC CAA CTCC

o4s (start of odr-4) : GAC TGT ACT GTC GAC CAT GGA TTC TGT AAC TGG AAT TGC

spx7R : GTG TTCGCA CAG CTT CCA TC

SP3: TCA GAA AGT GCG CTC TGT CG

SP4: GTG ATG GGG AGA TCT CTA CG

primers for other genes:

GP-4 (5'end of GFP)(*Aat II* site) : CAT GAT CAG GAC GTC GTA CCG GTA GAA AAA ATG AG

GP-5 (3' end of GFP) (*Aat II* site): A GAC TGT ACT GAC GTC GAA TTC TTT GTA TAG TTC ATC CAT GCC

SPH-SD1 (to *srd-1*, with *Sph I* site) : CAA TTG AAT GCA TGC ATG GCG ACT CCA ACC GAG

F33H1.5 epitope (to *srd-1*) : TCTTCAGGGAGAGACTTTTAA (D. Tobin)

F33H1.5 5' (to *srd-1, Pst I* site) : GTCAAGTTCGACCATAAGGC (D. Tobin)

M7.1 Xba (to *str-3*, with *Xba I* site) : TAC TGAA TCT AGA GTT CC TTTT GAAA TT GAGGCAG

The HHMI request forms for other primers made by N.D. are in the back of notebook 4, "N.D. odr-4 post-rescue".