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The Cyclic GMP-Dependent Protein Kinase EGL-4 Regulates Olfactory Adaptation in *C. elegans*

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Summary

Prolonged odor exposure causes a specific, reversible adaptation of olfactory responses. A genetic screen for negative regulators of olfaction uncovered mutations in the cGMP-dependent protein kinase EGL-4 that disrupt olfactory adaptation in *C. elegans*. G protein-coupled olfactory receptors within the AWC olfactory neuron signal through cGMP and a cGMP-gated channel. The cGMP-dependent kinase functions in AWC neurons during odor exposure to direct adaptation to AWC-sensed odors, suggesting that adaptation is a cell intrinsic process initiated by cGMP. A predicted phosphorylation site on the beta subunit of the cGMP-gated channel is required for adaptation after short odor exposure, suggesting that phosphorylation of signaling molecules generates adaptation at early time points. A predicted nuclear localization signal within EGL-4 is required for adaptation after longer odor exposure, suggesting that nuclear translocation of EGL-4 triggers late forms of adaptation.

Introduction

Sensory systems respond to ongoing changes in sensory information by adjusting their sensitivity through adaptation, using a variety of strategies and molecular mechanisms. Adaptation can be specific to one receptor or stimulus, or it can affect the sensitivity of the entire sensory system; adaptation can be extremely rapid or it can integrate information over time scales of hours or days. Numerous pathways of rapid sensory adaptation and desensitization have been described in the visual system, including rapid receptor phosphorylation, arrestin binding to receptors, and attenuation of G protein signaling by RGS proteins and phosphodiesterase (reviewed in Pugh et al., 1999). Slower mechanisms of gain control include calcium regulation of the retinal guanylyl cyclase that allows adjustment to ambient light levels over a few minutes, while very slow adjustments are controlled by circadian rhythms (Kramer and Molokanova, 2001). The olfactory system also adjusts to stimulation by adaptation. Rapid adaptation in vertebrate olfactory neurons occurs by calcium/calmodulin binding to, and inhibition of, the cAMP-gated olfactory transduction channels (Munger et al., 2001; Bradley et al., 2001; Chen and Yau, 1994). Additional mechanisms may also be utilized, including a cGMP pathway (Zufall and Leinders-Zufall, 1997). Unlike the visual system, which usually coordinates its properties across all photoreceptor neurons, the olfactory system adapts to ambient odors by specifically modifying the activity of a small number of odorant receptors or olfactory receptor neurons. This is most widely observed as a gradual reduction in sensitivity to the ambient odor (Zufall and Leinders-Zufall, 2000; Dalton, 2000). The mechanisms for long-term regulation of olfactory behavior are not well understood.

The nematode *Caenorhabditis elegans* is well suited for understanding olfactory behavior and its regulation. *C. elegans* has a compact nervous system consisting of 302 neurons with stereotyped morphologies and functions (White et al., 1986). Genetic analysis can be used to find molecules involved in specific behaviors and the functions of these molecules mapped within the neural circuits. *C. elegans* detects a rich variety of odors and tastes using specific chemosensory neurons that have been defined by cell ablation (Bargmann and Mori, 1997). As is true in other animals, odors are sensed by a large group of G protein-coupled receptors. The proposed odorant receptors are encoded by several gene families that encompass over 1,000 of the 19,959 predicted genes in *C. elegans*. The largest odorant receptor gene family is defined by its similarity to the known odorant receptor ODR-10, which senses diacetyl, and it consists of 831 genes and pseudogenes (Sengupta et al., 1996; Robertson, 1998, 2000). Many members of the *odr-10* gene family are expressed in olfactory neurons, consistent with a role in odor detection (Troemel et al., 1995; Troemel, 1999). This vast array of receptors is accommodated in a small number of chemosensory neurons. The adult hermaphrodite has only 32 chemosensory neurons associated with its chemosensory organs, and each neuron expresses many predicted odorant receptor genes. Most individual odorant receptor genes are reproducibly expressed in a small number of neurons (usually one pair), although their expression can vary based on the animal's sex, developmental stage, or experience (Troemel et al., 1995; Peckol et al., 2001).

Many attractive odors, including benzaldehyde, butanone, isoamyl alcohol, and 2,3-pentanedione, are sensed by the two AWC olfactory neurons. Although each of these four odors is thought to have its own receptor in AWC, they all converge on a common cGMP signaling pathway. This signaling pathway consists of

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two G_i-like G α proteins (ODR-3 and GPA-2), two receptor-type guanylyl cyclases (DAF-11 and ODR-1), and two subunits of a cGMP-gated cation channel (TAX-2 and TAX-4) (Roayaie et al., 1998; Birnby et al., 2000; L'Etoile and Bargmann, 2000; Coburn and Bargmann, 1996; Komatsu et al., 1996, 1999). The G_i proteins have some odor selectivity, but the two guanylyl cyclases and cGMP-gated channel are essential for all AWC-mediated odor responses, suggesting that all odors modulate cGMP levels and the opening of the cation-permeable cGMP-gated channel.

The activity of the olfactory system is modulated in several ways. First, rapid adjustments to rising or falling odorant levels are made in seconds to mediate chemotaxis in an odorant gradient (Pierce-Shimomura et al., 1999). Second, exposure to a field of odor results in a loss of responsiveness to that odor in a few minutes, permitting odor discrimination and detection of a second odor. The downregulation that occurs in odor discrimination is rapidly reversible and relies in part on segregation of odorant receptors between the two AWC neurons (Wes and Bargmann, 2001). Third, prolonged exposure to one AWC odor leads to a reversible, specific loss of the response to the adapting odor, while sparing responses to other AWC-sensed odors (Colbert and Bargmann, 1995). Long-lasting adaptation develops slowly over a period of minutes to hours and recovers on a similar time scale. Two genes have been shown to act in adaptation of AWC-mediated olfaction, the predicted TRP-family ion channel *osm-9* and the uncloned gene *adp-1*. Both *osm-9* and *adp-1* mutants are defective in adaptation to a subset of odors sensed by AWC (Colbert and Bargmann, 1995). The mechanisms that they use to affect adaptation are unknown. Adaptation also has the interesting property that it is modulated by experience. Animals in food do not adapt to an odor, and starved or stressed animals adapt more quickly than recently fed animals (Colbert and Bargmann, 1997). The odor-specific nature of adaptation and its modulation by starvation cause an animal to prefer food-associated odors and new odors to those that it encountered in adverse conditions.

Here, we describe a new AWC adaptation mutant *ky95*, which revealed a specific olfactory adaptation function of cGMP-dependent protein kinase EGL-4. Our results suggest a molecular model in which cGMP mediates olfactory transduction in AWC through the cGMP-gated channel and mediates adaptation through the cGMP-dependent kinase.

Results

Isolation of an Adaptation Mutant in a *daf-11* Suppressor Screen

The receptor guanylyl cyclases DAF-11 and ODR-1 both contribute to chemotaxis to all odors detected by the AWC olfactory neurons (Birnby et al., 2000; L'Etoile and Bargmann, 2000). This redundancy suggested a genetic strategy for isolating negative regulators of cGMP signaling. In a *daf-11* mutant, ODR-1 cannot direct robust olfactory chemotaxis, perhaps because the cGMP levels it produces are below a threshold for olfactory signaling. A second mutation that removes a negative regulator of cGMP signaling could potentiate the weak activity of ODR-1, suppressing the signaling defect and restoring chemotaxis in a *daf-11* mutant.

The screen for *daf-11* suppressors was facilitated by the easily scored dauer-constitutive phenotype of *daf-11*. The dauer larva is an arrested third larval stage that develops in adverse environmental conditions of high temperature, high nematode density, and limited food (Riddle and Albert, 1997). *daf-11* mutants grown at 25°C arrest as dauer larvae and fail to reach adulthood due to altered activity of their ASI and ASJ sensory neurons (Schackwitz et al., 1996). This defect appears to be due to diminished cGMP production, because it can be rescued by supplying exogenous 8-Bromo cGMP. *odr-1* is expressed in ASI and ASJ neurons (L'Etoile and Bargmann, 2000), but in wild-type animals it cannot prevent dauer formation. Mutations that potentiate the function of *odr-1* might suppress the dauer-constitutive phenotype of *daf-11* as well as its AWC olfactory defect. We mutagenized *daf-11* and sought rare F2 animals that grew to adulthood at 25°C (see Experimental Procedures). The progeny of these animals were examined in the next generation to determine the penetrance of the suppressed phenotype, and later generations were subsequently tested for chemotaxis to benzaldehyde. 50 independent suppressors of *daf-11* were isolated in 24,000 mutagenized genomes (see Experimental Procedures). Four of these suppressed strains were able to chemotax to benzaldehyde. One recessive mutant, *ky95,* which strongly suppressed the constitutive dauer formation and chemotaxis defects in *daf-11* mutants, was chosen for further study (Figure 1A).

The *ky95* mutation was separated from the original *daf-11* mutation to examine its effects on AWC function. We first asked whether *ky95* affected normal odor sensation in chemotaxis assays. *ky95* mutants exhibited normal chemotaxis to the AWC-sensed odors benzaldehyde, butanone, and isoamyl alcohol across a range of odor concentrations (Figure 1B).

ky95 mutants also behaved normally in an olfactory discrimination assay where animals must find a point source of one odor in a uniform field of a second odor (diagram in Figure 1E). Discrimination requires the animal to ignore the pervasive odor, which is present at much higher concentrations than the odor at the point source. This response has a rapid onset (about 2 min), and it recovers rapidly when the pervasive odor is removed (Bargmann et al., 1993, and data not shown). Some forms of discrimination require segregation of olfactory receptors into different AWC neurons, whereas others appear to be intracellular in AWC (Wes and Bargmann, 2001). *ky95* performed normally in both kinds of discrimination, as assessed by its successful chemotaxis in discrimination assays in both directions between benzaldehyde and butanone (Figure 1D and data not shown).

ky95 mutants were also tested for adaptation of AWC olfactory responses. In this assay, animals are exposed to an odor for an hour, washed, and then challenged with a point source of the same odor (diagram in Figure 1E). Adaptation represents a slow, reversible inhibition of the chemotaxis response. Wild-type animals had diminished olfactory chemotaxis following odor exposure, but *ky95* mutants continued to chemotax to odors al-

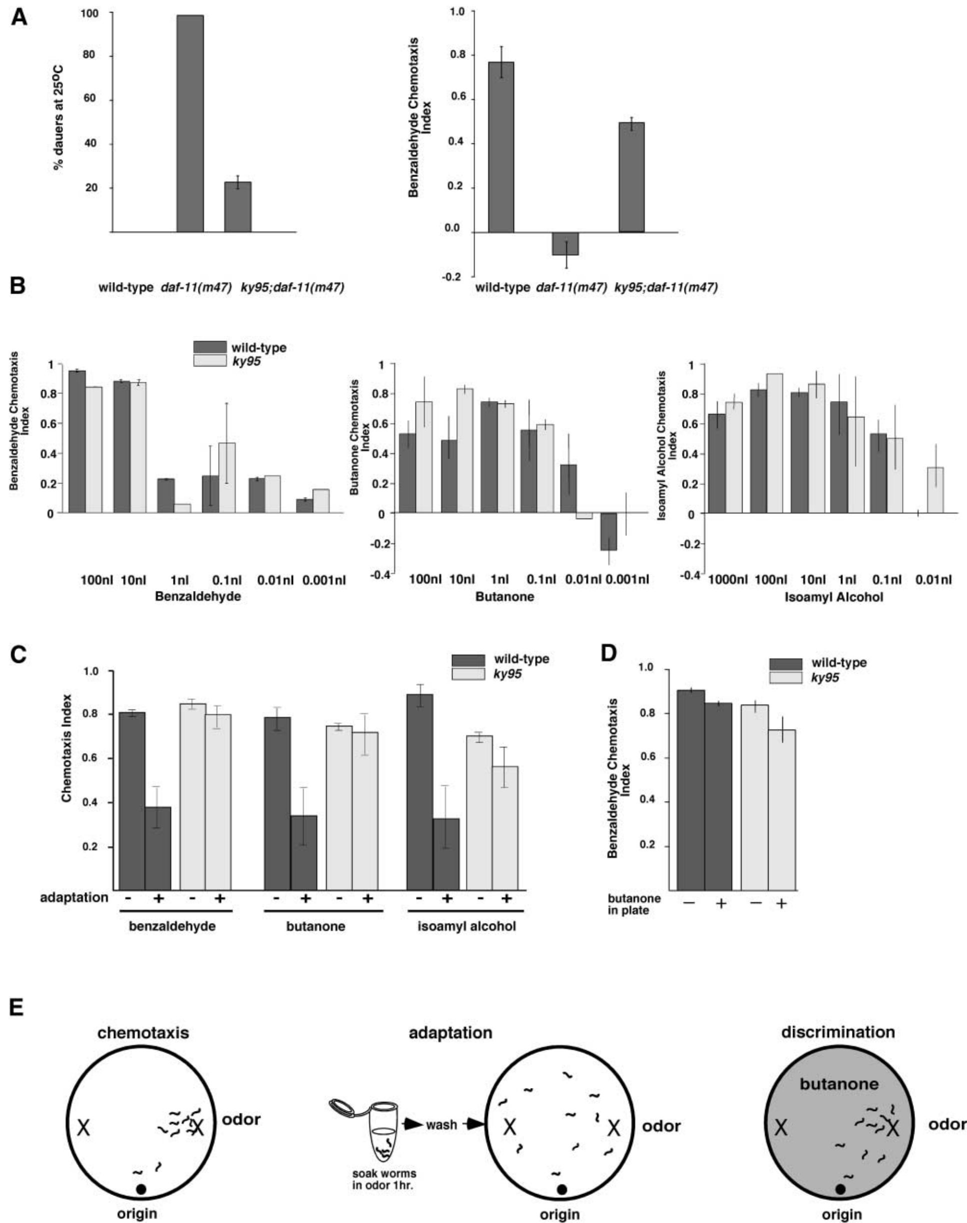

Figure 1. *ky95* Mutants Are Defective for Adaptation to AWC-Sensed Odors

(A) The *ky95* mutation suppresses *daf-11(m47)* dauer constitutivity and chemotaxis defects. Left, percentage of animals that formed dauer larvae at 25°C. Each data point represents the mean of at least four separate experiments. Right, benzaldehyde chemotaxis of wild-type animals, *daf-11(m47)* mutants and *ky95;daf-11(m47)* double mutants. Each data point represents the mean value from three separate experiments with ~100 animals per experiment.

(B) *ky95* animals respond normally to AWC-sensed odors. Chemotaxis of wild-type (dark gray) and *ky95* mutants (light gray) to three AWC-sensed odors: benzaldehyde, butanone, and isoamyl alcohol. Quantity of each odor at the point source is given for dose-response curves. Each data point is the mean of at least three independent assays with ~100 animals per experiment.

(C) *ky95* mutants are defective for adaptation to AWC-sensed odors. Wild-type animals responded differently to all odors after adaptation ($p < 0.01$), whereas responses of *ky95* mutant animals were unchanged after adaptation. Results are the average of at least four separate assays per data point with ~100 animals per experiment.

(D) *ky95* mutant animals discriminate between two AWC-sensed odors, butanone and benzaldehyde. Results are the mean values from four separate assays with ~100 animals per experiment.

(E) Behavioral assays. Left, chemotaxis assay; animals placed at the origin of a plate (black spot) are assayed for chemotaxis to a point source of the test odor. Middle, adaptation assay; animals are soaked in buffer with or without odor for 1 hr, washed, placed at the origin of a chemotaxis assay plate, and tested for chemotaxis to a point source of the adapting odor. In the discrimination assay at right, a standard chemotaxis assay is performed except that second saturating odor (butanone) is added uniformly to the agar in the assay plate.

In (A)–(D), error bars represent the standard error of the mean.

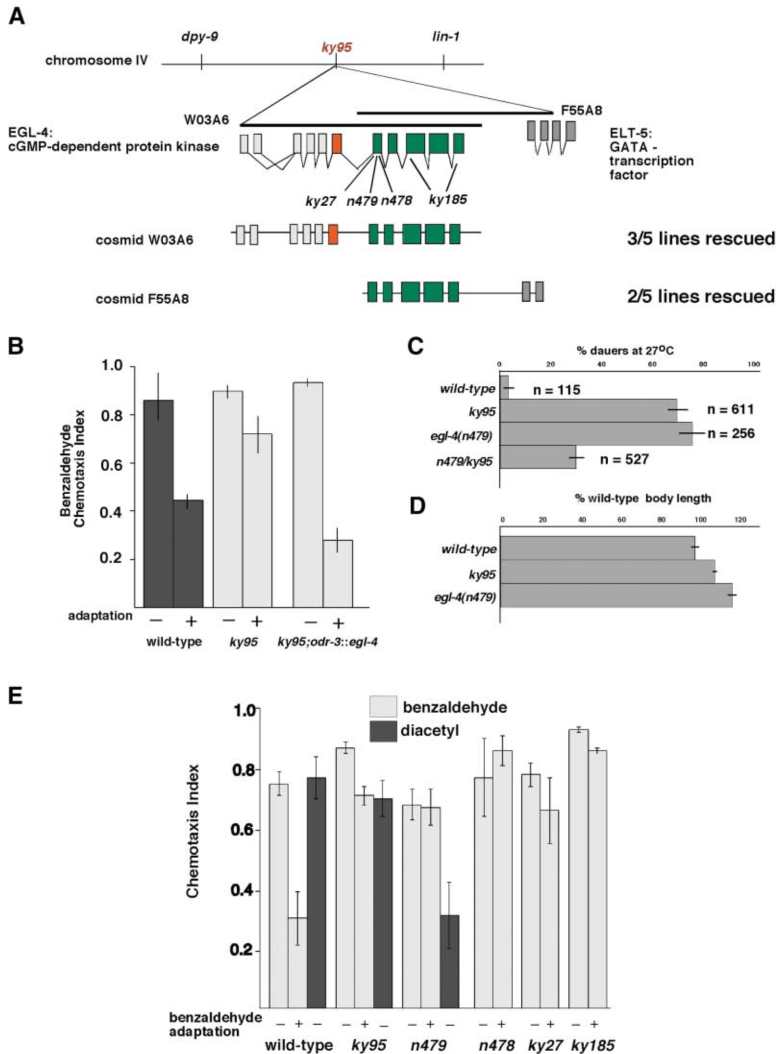

Figure 2. *ky95* Affects the cGMP-Dependent Protein Kinase EGL-4

(A) Mapping and cloning strategy. *ky95* was mapped between *dpy-9* and *lin-1*, and the cosmids W03A6 and F55A8 both rescued the axon defects of *ky95*. The gene structure for EGL-4 and ELT-5, the two proteins encoded by these cosmids, are depicted. Unshaded exons encode the N-terminal regions of EGL-4, red exons encode the negative regulatory cGMP binding domain, and green exons encode the kinase domain. The positions of mutations within *egl-4* were determined by Manabi Fujiwara and Steve McIntire (Fujiwara et al., 2002). The mutations are: *ky27*; Q to STOP at position 406, *n479*; R458STOP, *n478*; G481E, *ky185*, a deletion that removes the last two exons of *egl-4*.

(B) Full-length EGL-4 cDNA restores adaptation to *ky95* mutants. Adaptation assays to benzaldehyde were performed on wild-type animals, *ky95* mutants, and *ky95* mutants carrying an extrachromosomal array with the full-length EGL-4 cDNA under the *odr-3* promoter. The *odr-3* promoter is expressed primarily in AWC and weakly in AWB, AWA, ASH, and ADL. *ky95* animals carrying the *odr-3::egl-4* transgene responded differently from *ky95* animals to benzaldehyde after adaptation ($p < 0.05$ by t test). These experiments were performed on three separate days, and the data are the mean from three separate lines with ~100 animals per experiment.

(C) *ky95* and *egl-4(n479)* partially complement for dauer constitutivity at 27°C. The error bars represent the standard error of the proportion.

(D) *ky95* mutant animals, like *egl-4(n479)* mutant animals, are longer than wild-type animals. Animals were scored in the L4 stage. The error bars represent the standard error of the proportion. Wild-type and *ky95* animals are significantly different in length ($p < 0.05$ by t test). $N = 20$ for each genotype examined.

(E) *egl-4* mutants are adaptation defective. Light gray bars, benzaldehyde chemotaxis without (−) or with (+) prior exposure to benzaldehyde. Dark gray bars, chemotaxis to the AWA-sensed odor, diacetyl. Each assay was performed on three separate days with ~100 animals per experiment.

most as well as untreated animals (Figure 1C). This defect was observed in response to all AWC-sensed odors tested. Thus, *ky95* mutants have a defect in olfactory adaptation, a long-term regulation of olfactory behavior, but they are proficient in short-term chemotaxis and discrimination assays.

ky95 Is Rescued by the cGMP-Dependent Protein Kinase *egl-4*

The *ky95* mutation was mapped to an interval of chromosome IV between the genes *dpy-9* and *lin-1*. Cosmids in this region were injected into *ky95* mutant animals, and rescue was observed in three of five transgenic lines bearing the cosmid W03A6 (Figure 2A). This cosmid contains one gene encoding a cGMP-dependent protein kinase (Stansberry et al., 2001). F55A8, an overlapping cosmid that contains the open reading frame for the kinase domain of the cGMP-dependent protein kinase as well as the first two exons of the transcription factor ELT-5, also rescued a mutant axon phenotype of *ky95* in two of five transgenic lines (Figure 2A and see Experimental Proceedures). The lines carrying F55A8 were not tested for their ability to rescue the behavioral defects of *ky95*. No other predicted coding regions are shared between the two cosmids. To confirm that the cGMP-dependent protein kinase was likely to be affected by *ky95,* a full-length cDNA for the predicted kinase was expressed under the *odr-3* olfactory promoter and tested for rescue of *ky95* adaptation phenotype. Three of three transgenic lines were rescued for olfactory adaptation (Figure 2B). We conclude that *ky95* is likely to affect the activity of the cGMP-dependent protein kinase.

The EGL-4 kinase is 46% identical over its C-terminal 714 amino acids to the mouse cGMP-dependent protein kinase (PKG) 1β isoform. Like the mammalian PKGs, EGL-4 consists of an amino-terminal regulatory domain with two cGMP binding motifs followed by a carboxy-terminal serine/threonine kinase domain. This gene was previously recognized as a potential *C. elegans* PKG,

and the recombinant protein was shown to have cGMP-dependent serine/threonine kinase activity in vitro (Stansberry et al., 2001).

During the course of this work, Manabi Fujiwara and Steve McIntire discovered that the cGMP-dependent protein kinase on W03A6 corresponded to the *egl-4* gene (Fujiwara et al., 2002 [this issue of *Neuron*]). Their sequence of *egl-4* alleles strongly suggests that the null phenotype of the cGMP-dependent kinase is the *egl-4* phenotype. *egl-4* mutants have many defects in the function of the nervous system including an egg laying defect, chemotaxis defects to many odors, large body size, a neurotransmission defect, and a high propensity to develop as dauer larvae at 27°C (Trent et al., 1983; Daniels et al., 2000). The *egl-4* olfactory defect in AWC is odorant specific; *egl-4* mutants have reduced chemotaxis to butanone and isoamyl alcohol but exhibit normal chemotaxis to benzaldehyde across a range of concentrations (Daniels et al., 2000).

We compared the mutant phenotype of known *egl-4* null mutants to *ky95* to ask whether they affect similar biological processes. Like *egl-4*, *ky95* animals had an increased proportion of dauer larvae at 27°C (Figure 2C), and their body size was slightly larger than wild-type, though not as large as that of *egl-4* null mutants (Figure 2D). Since *egl-4* null mutants can chemotax to benzaldehyde, it was possible to examine adaptation to this odor in *egl-4* animals. Like *ky95* mutants, animals bearing the *egl-4* mutations *n478, n479, ky27,* and *ky185* were defective in adaptation to benzaldehyde (Figure 2E). The shared phenotypes of *egl-4* and *ky95* and their rescue by the same cosmid suggest that they may be mutations in the same gene.

Several phenotypes, however, distinguished *egl-4* loss-of-function mutations from *ky95*. *ky95* suppressed the dauer-constitutive phenotype of *daf-11* mutants at 25°C, but *egl-4* mutations enhanced *daf-11,* leading to a nonconditional, fully penetrant dauer formation phenotype at all temperatures (Daniels et al., 2000). *ky95* and *egl-4* null mutants partly complemented each other for the dauer-constitutive phenotype at 27°C (Figure 2C). In addition, *ky95* mutants exhibited normal chemotaxis to butanone and isoamyl alcohol, two AWC odors, and diacetyl, an odorant sensed by the AWA olfactory neurons; these responses are defective in strong *egl-4* mutants (Figures 1 and 2; Daniels et al., 2000). These results indicate that *ky95* is not a strong loss-of-function mutation in the *egl-4* gene. No mutations in the coding region of the cGMP-dependent protein kinase were detected in *ky95,* but the T at position 127 after the stop codon was changed to a G, and the T at position 129 after the stop codon was changed to an A. The coding region of *egl-4* spans over 31 kb, so there may be additional mutations in *ky95* that contribute to its defect. Since *ky95* is recessive and shares at least three phenotypes with *egl-4* null mutants, it is most likely that it represents an alteration in *egl-4* function. *ky95* may be a regulatory mutation that alters *egl-4* function in specific times or places.

egl-4 Acts in the AWC Neurons at the Time of Adaptation

To gain a further mechanistic understanding of the role of *egl-4* in adaptation, we focused on the benzaldehyde adaptation phenotype. To minimize the issues of interpreting non null mutants, we examined adaptation in *egl-4* null mutants.

egl-4 is widely expressed in neurons, including sensory neurons, and is also present in nonneuronal cells (Fujiwara et al., 2002; Stansberry et al., 2001). To ask whether *egl-4* functions in AWC during adaptation, a full-length *egl-4* cDNA was fused to two different promoters, the *odr-3* promoter and a short *odr-1(gcy-10)* promoter. The *odr-3* promoter drives strong expression in AWC olfactory neurons and weak expression in AWB, AWA, ADF, and ASH sensory neurons (Roayaie et al., 1998). A 2.5 kb fragment of the *odr-1/gcy-10* upstream region drives expression only in AWC and AWB sensory neurons (Yu et al., 1997). These fusion genes were introduced into *egl-4(n479)* mutants, and the resulting trangenic animals were tested for adaptation. Both *odr-3::egl-4* and *odr-1::egl-4* rescued the benzaldehyde adaptation defects of *egl-4* mutants (Figure 3A). These results indicate that *egl-4* functions in sensory neurons, most likely the AWC olfactory neurons, to control AWC adaptation.

The AWC neurons are born in the embryo and reach their mature form by the first larval stage. Chemotaxis to AWC odorants is usually assayed in adult animals. To ask when *egl-4* acts to promote adaptation, we fused the full-length *egl-4* cDNA to a heat shock promoter that is expressed in neuronal cells (Stringham et al., 1992). When grown at low temperature, *egl-4(n479)* animals bearing the *hsp::egl-4* transgene were defective in adaptation like *egl-4* control animals (Figure 3B). By contrast, *hsp::egl-4* animals were rescued for adaptation if they were subjected to heat shock for 2 hr immediately before the beginning of the adaptation assay (Figure 3B). Heat shock had no effect on the adaptation of *egl-4* control animals or wild-type animals. These results show that *egl-4* activity supplied in the adult near the time of adaptation is sufficient for normal adaptation to benzaldehyde.

Phosphorylation of the cGMP-Gated Channel β Subunit, TAX-2, May Contribute to Short-Term Adaptation

The requirement for the cGMP-dependent protein kinase EGL-4 in AWC neurons suggests that olfactory signaling in AWC is regulated by protein phosphorylation. EGL-4 has been demonstrated to have protein kinase activity on mammalian PKG substrates in vitro (Stansberry et al., 2001). We scanned the known signal transduction components in AWC for predicted cGMP-dependent protein kinase phosphorylation sites using Prosite (http://us.expasy.org/prosite) and identified one strong candidate, serine 727 of the cGMP-gated channel β subunit, TAX-2. This site is in the extended cytoplasmic C terminus of the channel, immediately after the conserved cGMP binding domain (Figure 4A). Three additional potential phosphorylation sites were found, one in TAX-2 and two in TAX-4, the α subunit of the cGMP-gated channel. No obvious phosphorylation sites were present in the G protein subunits ODR-3 and GPA-2, or the G protein-coupled receptor STR-2. To ask whether the predicted serine 727 site in TAX-2 might be a target of EGL-4 phosphorylation in adaptation, we

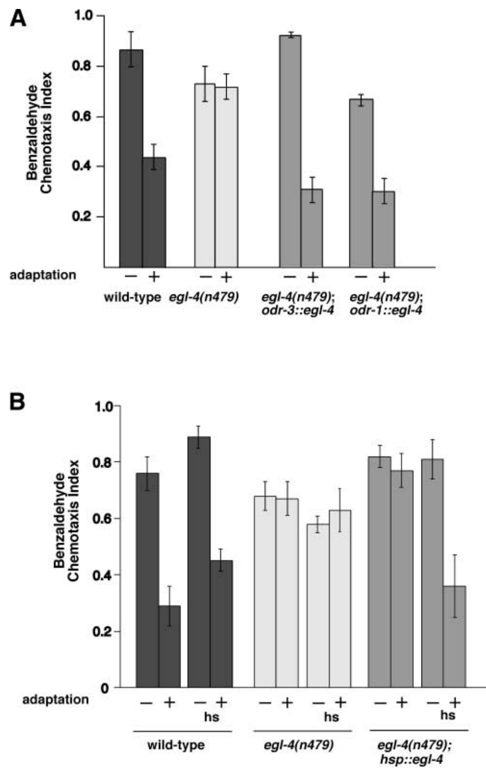

Figure 3. EGL-4 Is Required in the AWC Neurons at the Time of Odorant Exposure

(A) Adaptation of wild-type animals, *egl-4(n479)* mutant animals and *egl-4(n479)* mutant animals bearing extrachomosomal arrays with the full-length *egl-4* cDNA under the control of either the *odr-3* promoter (*odr-3*::*egl-4*) or a short form of the *odr-1* promoter *(odr1*::*egl-4)*. *egl-4(n479)* animals carrying either the *odr-3::egl-4* or the *odr-1::egl-4* transgene responded differently from *egl-4(n479)* animals to benzaldehyde after adaptation ($p < 0.005$, t test). All assays were performed on three separate days with ~100 animals per experiment.

(B) Adaptation of wild-type animals, *egl-4(n479)* mutant animals and *egl-4(n479)* mutant animals bearing an extrachromosomal array with the full-length *egl-4* cDNA under the control of the heat shock promoter (*hsp*::*egl-4*) either under standard conditions or after heat shock for 2 hr at 33°C. Heat shocked animals carrying the *hsp::egl-4* transgene adapted to benzaldehyde after adaptation, unlike their untreated counterparts ($p < 0.01$, t test). All assays were performed on three separate days with ~100 animals per experiment.

mutated serine 727 to alanine in a genomic clone of the *tax-2* gene, then introduced this mutant transgene into a strong *tax-2(p691)* mutant. The resulting animals were fully rescued for the chemotaxis defects of *tax-2(p691)* and showed wild-type sensitivity to benzaldehyde and butanone, suggesting that the point mutation did not eliminate TAX-2 function (Figures 4B and 4C and data not shown). This transgenic strain will be described as *tax-2(S727A)*.

tax-2(S727A) animals had a subtle but reproducible defect in adaptation at early time points. After 30 min of benzaldehyde treatment, wild-type animals had a partial reduction in olfactory responses, but the *tax-2(S727A)* animals did not adapt (Figure 4D). After 1 hr of benzaldehyde treatment, wild-type animals and *tax-2(S727A)* adapted to similar extents (Figure 4D). *ky95* mutations and strong *egl-4* mutations were defective at adaptation after either 30 min or 1 hr of benzaldehyde exposure. Thus, *tax-2(S727A)* animals have a defect that is only evident at short times of adaptation. Similar phenotypes were observed when *tax-2(S727A)* animals were tested for adaptation to butanone or isoamyl alcohol; for both odors, *tax-2(S727A)* animals were defective in adaptation at intermediate time points (1 hr of adaptation) but proficient in adaptation at longer time points (2 hr of adaptation) (data not shown). Control *tax-2(p691)* animals bearing a wild-type *tax-2* transgene adapted to the same extent as wild-type animals (data not shown).

Parallel experiments were conducted for the other predicted phosphorylation site in TAX-2 and for two predicted phosphorylation sites in TAX-4. Serine or threonine to alanine mutations at these three positions did not result in adaptation defects (data not shown). These results suggest that phosphorylation of serine 727 in TAX-2 could contribute to olfactory adaptation at short times.

A Conserved Nuclear Localization Signal within EGL-4 Contributes to Long-Term Adaptation

Odor adaptation generally persists for a period that correlates with the length of odor exposure. Adaptation to an hour-long odor exposure can persist for 2 hr, whereas adaptation after half an hour of odor persists for only 30 min. This difference suggests that prolonged odor exposure elicits long-term changes within the AWC neurons.

cGMP-dependent protein kinases can regulate gene expression after translocation to the nucleus, providing one possible model for long-term adaptation in AWC (Gudi et al., 1997). EGL-4 exhibits homology to the human cGMP-dependent protein kinase-1β (PKG-1β) throughout the protein, including a short stretch of positively charged amino acids with homology to the nuclear localization signal (NLS) of the interleukin 1α precursor (Figure 5A). When lysine 407 within this domain of human PKG-1β is mutated to the negatively charged glutamate, PKG-1β is no longer able to enter the nucleus (Gudi et al., 1997). This PKG-1β NLS lies within the catalytic ATP binding domain defined by the crystal structure of PKA, but lysine 407 does not contribute to the ATP binding pocket, is not absolutely conserved among kinases (Zheng et al., 1993), and was not required for PKG-1β kinase activity (Gudi et al., 1997).

Lysine 459 within EGL-4 corresponds to lysine 407 of PKG-1β. We mutated lysine 459 to glutamate and expressed this form of the protein in *egl-4(n479)* mutants. This strain, *egl-4(K459E)*, was able to adapt at wild-type levels after a 30 min exposure to benzaldehyde (Figure 5B). However, the level of adaptation plateaus at the 30 min time point, and adaptation after an hour exposure was defective (Figure 5B). *egl-4(K459E)* rescued butanone chemotaxis of *egl-4* null mutants, indicating that this mutant has biological activity in olfaction (data not shown). The requirement for a potential NLS suggests that nuclear translocation of EGL-4 could contribute to adaptation after long odor exposures.

Discussion

A cGMP-Dependent Protein Kinase Acts in Olfactory Adaptation

The cGMP-dependent protein kinase encoded by *egl-4* mediates slow, long-lasting behavioral adaptation to

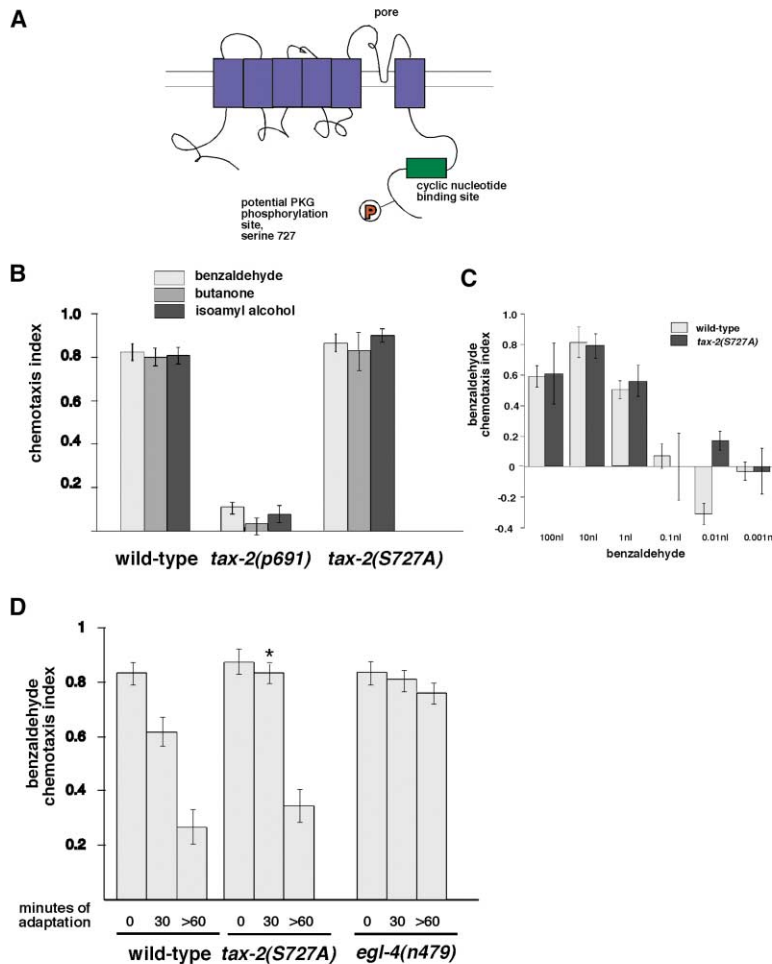

Figure 4. The Cation Channel β Subunit, TAX-2, Affects Adaptation after Brief Odor Exposure

(A) Predicted topology for TAX-2 with serine 727, the potential cGMP-dependent protein kinase target within TAX-2, indicated by a red P.

(B) Chemotaxis to three AWC-sensed odors of wild-type, *tax-2(p691)*, and *tax-2(p691)* mutants bearing an extrachromosomal array of a genomic region *tax-2* clone with a ser 727 to ala mutation. The mutated form of TAX-2 rescues *tax-2(p691)* mutant animals for AWC-mediated chemotaxis. All assays were performed on three separate days with ∼100 animals per experiment.

(C) Chemotaxis of wild-type and *tax-2(S727A)* to different amounts of benzaldehyde. Each experiment was performed on three separate days with ∼100 animals per experiment.

(D) Time course for adaptation of wild-type animals, *tax-2(S727A)* mutant animals, and *egl-4(n479)* mutant animals. The asterisk indicates points at which the *tax-2(S727A)* response is significantly different from wild-type ($p = 0.021$, t test). Each experiment was performed on seven separate days with >100 animals per assay.

odors. EGL-4 promotes adaptation within the mature AWC sensory neurons at the time of olfaction, suggesting that adaptation could respond to changes in cGMP that occur during olfactory signaling. The odorant specificity of adaptation suggests that olfactory adaptation occurs within the sensory neurons; the cell autonomy of *egl-4* in AWC provides direct evidence that the sensory neuron is a site of adaptation.

The identification of numerous molecules involved in cGMP signaling in genetic screens and the analogy with vertebrate vision and olfaction support the idea that odors regulate cGMP levels in AWC (L'Etoile and Bargmann, 2000; Birnby et al., 2000; Coburn and Bargmann, 1996; Komatsu et al. 1996, 1999). However, analysis of the signaling networks in *C. elegans* olfaction is limited by the inability to record from these neurons during signaling. We suggest that the cGMP-gated channel mediates rapid changes in AWC activity in response to odors, while the cGMP-dependent protein kinase responds to cGMP to cause slower, long-lasting changes in sensory properties. In one model, these two activities could be directly linked with EGL-4 responding to the cGMP modulation that occurs in response to odors. However, this simple possibility is not the only explanation consistent with our results. For example, adaptation only occurs when food is absent (Saeki et al., 2001), so *egl-4* could be required to sense the lack of food that is paired with the adapting odor.

The failure of adaptation in *egl-4* mutants suggests that cGMP-dependent protein phosphorylation reduces the activity of the signaling network after sustained exposure to odors. This result is straightforward for benzaldehyde responses, where only adaptation is affected. A more complex situation exists for butanone and isoamyl alcohol, where *egl-4* functions both in olfaction and in olfactory adaptation. For these two odors, EGL-4 must have a positive olfactory function as well an inhibitory adaptation function. The chemotaxis defects in *egl-4* mutants are fully suppressed by mutations in *daf-3,* a CoSMAD transcriptional regulator in the *daf-7* TGF-β pathway (Daniels et al., 2000). Therefore, EGL-4 is not essential for butanone and isoamyl alcohol chemotaxis in all conditions and is likely to be a regulator rather than an essential component of olfactory signaling pathways. The CoSMAD pathway senses starvation and crowding in the decision to form dauer larvae; disruption of the starvation-sensing pathway by the *daf-3* and *egl-4* mutations could contribute to the complex effects that these genes have on chemotaxis. It is possible that these regulatory functions also contribute to the *egl-4* adaptation defect. The near-complete loss of adaptation in *egl-4* mutants would be consistent with *egl-4* contributions to multiple aspects of adaptation.

A number of biochemical and electrophysiological approaches have been used to study desensitization in vertebrate and invertebrate photoreceptors and desen-

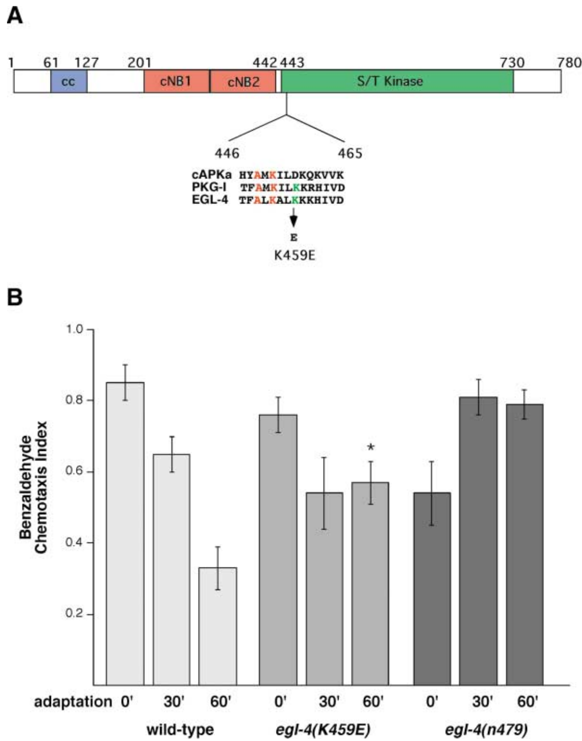

Figure 5. A Predicted Nuclear Localization Signal in EGL-4 Affects Adaptation after Prolonged Odor Exposure

(A) Block diagram of the predicted domain structure of EGL-4 (Smart protein analysis http://smart.embl-heidelberg.de). A short region is aligned to the corresponding region of PKA and PKG-1β. Red residues face into the ATP binding pocket of PKA. The lysine to glutamate change is shown in green. The K407E mutation in PKG-1β blocked its nuclear translocation (Gudi et al., 1997).

(B) Time course for adaptation of wild-type animals, *egl-4(K459E)* mutant animals, and *egl-4(n479)* mutant animals. The asterisk indicates the point at which the *egl-4(K459E)* response is significantly different from wild-type ($p < 0.05$, t test). Each experiment was performed on five separate days with ~100 animals per experiment.

sitization in vertebrate olfactory neurons (reviewed in Pugh et al., 1999; Munger et al., 2001; Bradley et al., 2001; Chen and Yau, 1994; Zufall and Leinders-Zufall, 1997). In all cases, the desensitization pathways that were analyzed are extremely rapid compared to the slower behavioral adaptation that is affected by *egl-4,* and their behavioral significance is probably quite distinct from that of *egl-4*. Defects in light adaptation lead to blindness or retinal degeneration because rapid adjustments are essential for the basic signaling properties of a sensory system. Rapid desensitization is likely to be a feature of the *C. elegans* olfactory system, but a defect in this process would be likely to generate a chemotaxis defect in a gradient or a discrimination defect rather than a behavioral adaptation defect. The behavioral experiments performed here provide access to the pathways that store information over relatively long time periods.

The cGMP-dependent protein kinase has been previously implicated in several interesting forms of behavioral regulation. In *Drosophila* larvae, anoxic conditions lead to activation of nitric oxide synthase (NOS), production of NO gas, and activation of guanylyl cyclases that stimulate the cGMP-dependent kinase (Wingrove and O'Farrell, 1999). PKG mediates a behavioral withdrawal from food that facilitates oxygen uptake. Another behavioral response, the rover/sitter locomotion pattern of *Drosophila* larvae, is associated with a natural polymorphism in the cGMP-dependent protein kinase (Osborne et al., 1997). These phenotypes represent only a subset of PKG functions that are revealed by special assays or alleles. Null mutations of PKG are lethal in *Drosophila* and they have many defects in *C. elegans,* consistent with the widespread expression of the kinase in neuronal and nonneuronal tissues.

TAX-2(S727) Affects Adaptation at Short Times

In the vertebrate olfactory system, one form of olfactory adaptation occurs by modulation of the cAMP-gated olfactory channel. Calcium/calmodulin binds to the α2 subunit of the vertebrate cAMP-gated channel to inhibit its opening (Chen and Yau, 1994); the α4 subunit and the β subunit of the channel are required for this modulation (Munger et al., 2001; Bradley et al., 2001). A potential PKG phosphorylation site is present in the TAX-2 subunit of the cGMP-gated channel; mutation of this serine to alanine results in a mild, time-dependent adaptation defect. This site could be phosphorylated by EGL-4 to enhance adaptation. Alternatively, it is possible that another kinase phosphorylates the site, that the serine has a function in adaptation that is unrelated to phosphorylation, or that the phosphorylation at this site is permissive rather than instructive for adaptation to occur. Regardless of the exact mechanism, this result indicates that the cGMP-gated channel can affect early adaptation in *C. elegans*.

All odors sensed by AWC use the same cGMP-gated channel, so the idea that TAX-2 is a site of adaptation does not explain why adaptation is specific to a subset of odors sensed by AWC. This paradox could be explained if different pools of TAX-2/TAX-4 channels are functionally segregated to different odor receptors within one cell so that each channel is actually regulated by the presence a single odor. The idea of precoupled signaling pathways was developed in *Drosophila* phototransduction and has support in other systems as well (Tsunoda et al., 1997; Huber et al., 1996). For example, in hippocampal cells, the β(2) adrenergic receptor, Gs, adenylyl cyclase, the cAMP-dependent protein kinase, and a protein phosphatase and its ultimate effector, the voltage-gated calcium channel, can all be immunoprecipitated in a single complex (Davare et al., 2001). However, this is not the only model that could explain odor specificity. Even if general proteins like EGL-4 and TAX-2 are essential for adaptation, they could act in a context defined by other proteins that give more specific responses. For example, adaptation might require both the modification of TAX-2 and a simultaneous modification of a particular odorant receptor. The receptor is not the only protein that could convey specificity, since several signaling proteins in AWC including *adp-1*, the *gpa-2* G protein, and the *osm-9* channel have relatively odorant-selective effects on signaling and adaptation (Colbert and Bargmann, 1995, 1997; Roayaie et al., 1998). Odorant specificity could be explained if a shared protein like TAX-2 and a specific protein like GPA-2 act together during adaptation.

Adaptation Is Regulated and Is Temporally Complex

Our analysis reveals a temporal discontinuity in adaptation, with different mechanisms that contribute at different times of odor exposure. *egl-4* mutants are defective at all times of adaptation. The *tax-2(S727A)* strain is defective in adaptation but only at short times of odor exposure. The *egl-4(K479E)* strain, by contrast, is defective only after prolonged odor exposure. This mutation disrupts a predicted nuclear localization sequence in EGL-4, suggesting that nuclear translocation may contribute to long-lasting forms of adaptation. Activated mammalian PKG translocates from the cytoplasm to the nucleus to regulate gene expression, providing a potential mechanism for linking short-term and long-term forms of adaptation (Gudi et al., 1997). Like the odor-specificity of signaling changes, odor-specificity of transcriptional changes could result either from regulation of odor-specific molecules like receptors or from regulation of shared signaling molecules that are interpreted in the context of the odors present at a given time.

A biological change that appears homogenous can actually incorporate many changes over different times. For example, in hippocampal LTP, the enhanced activity in synapses is slowly converted from a phosphorylation-based form, to a protein-synthesis dependent form, to a form that requires new gene expression (Bear and Linden, 2001). We suggest that adaptation has similar forms, with different temporal stages of information storage. Early adaptation may involve phosphorylation of proteins like TAX-2, whereas later stages of adaptation could involve changes in newly synthesized proteins or gene expression. A recent characterization of the *C. elegans tax-6* mutant demonstrated that calcineurin has a negative role in adaptation (Kuhara et al., 2002): calcineurin mutants hyperadapt, resulting in decreased odor sensitivity even without prior odor exposure. The integration of positive and negative adaptation pathways during discrete temporal windows could provide a subtle and flexible response to odor fluctuations in the environment.

Experimental Procedures

Strains and Genetics

Strains were maintained at 20–25°C under standard conditions (Brenner, 1974). The following strains were used: DR47 *daf-11(m47)* V, DR87 *daf-11(m87)* V, MT6025 *lin-31(n301)* II; *dpy-9(e12)* IV; *unc-51(e369)* V, MT6996 *dpy-9(e12) ced-2(e1752) lin-1(e1275)* IV, MT1073 *egl-4(n478)* IV, MT1074 *egl-4(n479)* IV, *egl-4(ky185)* IV, *egl-4(ky27)* IV, PR691 *tax-2(p691)* I, CB1376 *daf-3(e1376)* X, *egl-4(n478)* IV; *daf-3(e1376)* X, CB1467 *him-5(e1490)* V.

daf-11 Suppressor Screen

Fourth larval stage (L4) *daf-11(m47)* or *daf-11(m87)* hermaphrodites were mutagenized with EMS, and three to five mutagenized animals were grown per 10 cm plate at 15°C until the F1 progeny reached the L4/young adult stage, shifted to 25°C, and screened 3–5 days later for nondauer F2 adults. Progeny from approximately 12,000 F1 animals were screened (24,000 genomes), about half from each *daf-11* allele. F2 animals were placed on single plates at 25°C and rescreened for the dauer phenotype in the F3 generation; 53 animals that had >80% nondauer progeny were kept for further study. The mutants represented at least 50 independent suppressors from different initial plates. Suppressors were characterized for dye-filling of sensory neurons with the vital dye DiO, since mutations that affect sensory cilium structure disrupt dye-filling and suppress *daf-11* dauer formation. Nine suppressors had a dye-filling defective phenotype. The remaining strains were screened for suppression of the *daf-11* benzaldehyde chemotaxis defect. Three strains including *ky95; daf-11(m47)* exhibited robust chemotaxis to benzaldehyde and were chosen for further study; a fourth mutant with robust chemotaxis was probably an intragenic revertant of *daf-11*. Dye-filling with DiO reveals defects in the outgrowth and pathfinding of amphid axon neurons. *daf-11* has a weak axon defect, which was strongly enhanced in 15 suppressed strains including *daf-11(m47); ky95* and the other two chemotaxis-proficient suppressors. This phenotype was used to separate *ky95* from the *daf-11* mutation. The *ky95* mutant was separated from *daf-11* by crossing to *him-5(e1490),* which is closely linked to *daf-11*, screening in the F2 generation for nondauers with amphid axon defects, and screening for homozygosity of *him-5* in the F3 generation. *ky95* had an axon phenotype that was strong enough that it was recovered using this strategy, but the other suppressors did not have axon phenotypes that were recovered in the absence of *daf-11* mutations, and they were not studied further. The *ky95* mutations was outcrossed six additional times by N2 using the axon phenotype to separate it from other mutations.

Cloning of *ky95*

Mapping and Rescue Based on Dye-Filling

The axons of ASJ and five other pairs of anterior sensory neurons can be visualized by staining with the vital fluorescent dye DiI. L4 animals were soaked in 40 mg/ml DiI (Molecular Probes) in M9 buffer with food for >16 hr at 25°C, washed, and allowed to recover on a seeded plate before examination by fluorescence microscopy. The ASJ sensory neurons send their axons anteriorly from their cell bodies into the nerve ring. In *ky95* mutants grown at 25°C, the ASJ neurons have posterior axons, either due to a failure of termination of the primary axon or due to the sprouting of secondary axons from the cell body. This defect is characteristic of reduced sensory activity of the ASJ neurons and is also seen in mutants for *daf-11*, *tax-2*, and *tax-4* (Coburn and Bargmann, 1996; Coburn et al., 1998). This ASJ dye-filling phenotype served as the standard phenotype that was scored during mapping and cosmid rescue of *ky95* mutants (Figure 2A). *ky95; him-5(e1490)* males were mated into *lin-31(n301)* II; *dpy-9(e12)* IV; *unc-51(e369)* V strain, and the F3 progeny of individual F2s were examined: 0/7 F2 with aberrant axons had Dpy progeny, 0/6 Dpy F2s had progeny with aberrant axons. The map position was refined by mating *ky95; him-5* into *dpy-9(e12) ced-2(e1752) lin-1(e1275)* IV. 2/2 Lin nonDpys had aberrant axons and 1/4 Dpy non-Lins had aberrant axons. The interval between *dpy-9* and *lin-1* contained 20 cosmids. Initially, the cosmids F55A8 and W03A6 were injected into *ky95* mutants with the coinjection marker pRF4 *rol-6(su1006)*. Subsequently, *ky95; lin-15(n765)* double mutants were injected with F55A8 or W03A6, and in this case, the coinjection marker *lin-15(+)* was used. Transgenic lines carrying in which >30% of animals had an aberrant ASJ axon were scored as rescued (n > 50). After the initial identification of the coding region that rescued *ky95* axon defects, behavioral rescue of benzaldehyde adaptation was used to examine rescue in subsequent experiments (e.g., Figure 2B).

Allele Sequencing

Genomic DNA was isolated from *ky95* and N2 (Sulston and Hodgkin, 1988); *egl-4* regions were amplified by PCR; and coding regions, intron/exon boundaries, and predicted untranslated regions were sequenced.

Plasmid Construction

odr-3::egl-4

2.5 kb of the *odr-3* promoter (from the EcoRV site to the first ATG; Roayaie et al., 1998) were cloned into the pPD49.26 expression vector (a gift from Andy Fire). The 3 kb *egl-4* cDNA was subcloned from the pBluescriptSKII yk410a4 clone (a generous gift from Yuji Kohara) by digestion with XbaI and Asp718. This fragment was cloned into the *odr-3* vector using the NheI and Asp718 sites in the polylinker following the *odr-3* promoter.

odr-1::egl-4

The *odr-3* promoter from *odr-3*::*egl-4* was replaced by the *odr-1* promoter. 2.5 kb of the *odr-1* promoter was released from *odr-1*::GFP (L'Etoile and Bargmann, 2000) by digestion with Asp718, blunting with T4 DNA polymerase, and digesting with BamHI. This fragment was ligated into the *odr-3*::*egl-4* clone at the BamHI and EcoRI (blunted) sites that flank the *odr-3* promoter.

hsp16-2::egl-4

The 3 kb EGL-4cDNA fragment liberated by XbaI and Asp718 digestion of yk410a4 was placed under the hsp16-2 heat shock promoter in pPD49.78 (a gift from Andy Fire).

tax-2(S727A)

The genomic rescuing fragment of *tax-2* (Coburn and Bargmann, 1996) was used as template for site-directed mutagenesis (Quick Change, Stratagene).

odr-3::egl-4(K459E)

odr-3::egl-4 (see above) was used as a template for site-directed mutagenesis (Quick Change, Stratagene).

Transgenic Strains

Germ-line injections to make transgenic strains were as described (Mello and Fire, 1995). *ky95* mutants were injected with 50 ng/ml of *odr-3::egl-4* or *odr-1::egl-4* and 100 ng/ml *sur-5*::GFP used as a coinjection marker (Gu et al., 1998). *egl-4(n479)* mutants were injected with the same mixture. *hsp16-2::egl-4* was injected into *egl-4(n479)* at 5ng/ml with *sur-5*::GFP as a coinjection marker at 100 ng/ml. 50 ng/ml *tax-2(S727A)* was injected with *sur-5*::GFP into *tax-2(p691*) mutants.

Heat Shock Experiments

Transgenic strains were grown at 25°C to young adulthood, then shifted to 33°C for 2 hr (Stringham et al., 1992). Animals were allowed to recover for an additional 2 hr before using them in adaptation assays.

Behavioral Assays and Statistical Methods

Chemotaxis assays were performed as described (Bargmann et al., 1993). Briefly, animals were grown on *E. coli* HB101 on NGM plates at 25°C to young adulthood, washed free from food with S Basal, placed onto chemotaxis assay plates, and scored after 2 hr. Chemotaxis assays are conducted on 10 cm Petri plates containing 10 ml of 1.6% agar, 5mM KPO_4 (pH 6.0), 1 mM $CaCl_2$, and 1 mM $MgSO_4$. Animals are placed at an origin equidistant from a point source of attractant and a point source of control diluent (ethanol), with sodium azide at each point source to anesthetize animals as they arrive. The chemotaxis index is calculated as {[Number of animals at attractant] − [Number of animals at control diluent]} / [Total number of animals]. Discrimination (cross-saturation) assays were adapted from Bargmann et al. (1993). Adaptation assays were as described (L'Etoile and Bargmann, 2000). Briefly, animals were washed free of food, placed in Eppendorf tubes, and incubated in liquid with 7 ml benzaldehyde, 11 ml butanone, or 1 ml isoamyl alcohol/100 ml S Basal for a given period of time. They were then washed free of odor and assayed in a standard chemotaxis assay. Control animals were soaked in S Basal without odor for the equivalent period of time. At least 100 animals were used in each assay. Error bars are all the standard error of the mean unless indicated. Data were analyzed using a two-tailed Student t test (Statview, Stanton Glantz).

Length Assessment

The length of 20 animals of each genotype was determined by placing NaN_3-anesthetized Christmas tree stage L4 larvae under a dissecting scope and measuring length with the eyepiece micrometer.

Dauer Constitutivity at 27°C

The percent of each brood that enters the dauer stage at 27°C was determined by allowing animals of each genotype to lay eggs for an hour on a seeded plate, removing the mother, placing the plate at 27°C for 3 days, counting the number of dauer animals (scored by failure to pump), and dividing by the total number of animals.

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