

G Protein-Coupled Receptor Kinase Function Is Essential for Chemosensation in *C. elegans*

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Summary

G protein-coupled receptors (GPCRs) mediate diverse signaling processes, including olfaction. G protein-coupled receptor kinases (GRKs) are important regulators of G protein signal transduction that specifically phosphorylate activated GPCRs to terminate signaling. Despite previously described roles for GRKs in GPCR signal downregulation, animals lacking *C. elegans* G protein-coupled receptor kinase-2 (*Ce-grk-2*) function are not hypersensitive to odorants. Instead, decreased *Ce-grk-2* function in adult sensory neurons profoundly disrupts chemosensation, based on both behavioral analysis and Ca²⁺ imaging. Although mammalian arrestin proteins cooperate with GRKs in receptor desensitization, loss of *C. elegans* arrestin-1 (*arr-1*) does not disrupt chemosensation. Either overexpression of the *C. elegans* G α subunit *odr-3* or loss of *eat-16*, which encodes a regulator of G protein signaling (RGS) protein, restores chemosensation in *Ce-grk-2* mutants. These results demonstrate that loss of GRK function can lead to reduced GPCR signal transduction and suggest an important role for RGS proteins in the regulation of chemosensation.

Introduction

Signal transduction through G protein-coupled receptors (GPCRs) is conserved from yeast to mammals and mediates cellular processes as diverse as odorant detection, hormonal signaling, vision, and drug tolerance and addiction (Nestler and Aghajanian, 1997; Pierce et al., 2002; Prasad and Reed, 1999). Elucidating GPCR

regulation is, therefore, essential for understanding numerous biological processes. GPCR signaling occurs when an agonist binds to and induces a conformational change in the GPCR, causing activation and dissociation of the heterotrimeric G protein complex. The G α subunit exchanges GDP for GTP and dissociates from the G β and G γ (G $\beta\gamma$) subunits. The G α and G $\beta\gamma$ subunits then activate distinct signaling effectors, which in turn regulate the intracellular concentration of “second messenger” molecules (e.g., cAMP) that mediate the cellular response to the bound agonist. GTP hydrolysis allows the subunits to reassociate with the GPCR and await reactivation by agonist (Neer, 1995).

Desensitization of GPCRs by G protein-coupled receptor kinases (GRKs) and arrestin proteins protects cells against receptor overstimulation (Ferguson, 2001; Pitcher et al., 1998). This desensitization is critical; it allows cells to terminate signaling, integrate information from multiple signaling inputs, and respond to new stimuli. GRKs are a specialized family of serine/threonine kinases that specifically recognize and phosphorylate the activated (agonist bound) conformation of GPCRs. Proteins of the arrestin family can then recognize and bind to the phosphorylated GPCR, sterically “uncouple” it from G proteins, and block reactivation. Arrestin association can also target the activated receptor for internalization and recycling to the cell membrane, but internalization is not always required for desensitization. In addition to downregulating GPCR signaling, arrestin proteins can couple to and activate additional signaling cascades (Perry and Lefkowitz, 2002; Pierce and Lefkowitz, 2001).

The mammalian GRK family currently consists of seven members (GRK1-7) that are divided into three subfamilies based on sequence, subcellular localization, and regulation: GRK1 and GRK7, GRK2 and GRK3, and GRK4 through 6 (Ferguson, 2001; Pitcher et al., 1998). GRK2 and GRK3 are both ubiquitously expressed, but GRK3 is present at much higher levels in mouse olfactory epithelia (Benovic et al., 1989, 1991; Schleicher et al., 1993). GRK3 knockout mice are viable but have defects in olfactory signal transduction (Peppel et al., 1997; Schleicher et al., 1993).

Underscoring the importance of GRKs in receptor desensitization, loss of GRK function often results in hypersensitivity to GPCR stimulation. For example, although GRK2^{-/-} homozygous knockout mice are embryonic lethal due to cardiac failure, GRK2^{+/-} heterozygous mice show enhanced cardiac contractility in response to isoproterenol (Jaber et al., 1996; Rockman et al., 1998). Furthermore, both homozygous and heterozygous GRK6 knockout animals are hypersensitive to psychostimulant drugs, including cocaine (Gainetdinov et al., 2003), while mice lacking GRK5 show supersensitivity to muscarinic receptor stimulation (Gainetdinov et al., 1999).

The nematode *Caenorhabditis elegans* responds to chemical, thermal, and mechanical stimuli (Bargmann and Mori, 1997; Troemel, 1999). Animals move toward odorants that indicate a food source and away from odorants that indicate a less desirable environment. In

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C. elegans, five pairs of the chemosensory neurons (AWA, AWC, ASH, ADL, and AWB) have been shown to detect volatile chemicals (Bargmann et al., 1993). The AWA and AWC chemosensory neurons detect attractive volatile odorants that animals chemotax toward. In contrast, the ASH, ADL, and AWB chemosensory neurons detect aversive stimuli, which animals avoid by rapidly initiating backward locomotion upon stimulus detection. The polymodal ASH sensory neurons respond to a broad range of aversive stimuli, including volatile (e.g., octanol) and soluble (e.g., quinine) chemicals, high osmolarity, and touch to the nose (Bargmann et al., 1990; Hilliard et al., 2004; Kaplan and Horvitz, 1993; Troemel et al., 1995). The ASH sensory neurons synapse onto the command interneurons that regulate both spontaneous and evoked locomotion through their connections with motor neurons.

Many of the proteins that play critical roles in chemosensation in diverse species are conserved in *C. elegans* (Prasad and Reed, 1999; Troemel, 1999). Chemosensation is generally mediated by odorant receptors that are members of the 7-transmembrane GPCR family. Approximately 500 functional chemosensory GPCRs are predicted in the *C. elegans* genome (Troemel, 1999), and the *odr-10* gene encodes a diacetyl receptor (Sengupta et al., 1996). Other key signaling proteins were also identified in classical genetic screens. ODR-3, a G α with similarity to the G α /G β family, is required for response to all stimuli detected by the AWA, AWC, and ASH neurons (Roayaie et al., 1998). The TAX-2/TAX-4 subunits comprise a cyclic nucleotide gated channel required in AWC neurons (Coburn and Bargmann, 1996; Komatsu et al., 1996), while OSM-9/OCR-2 form a putative TRP-related channel required for AWA- and ASH-mediated detection of stimuli (Colbert et al., 1997; Tobin et al., 2002). ODR-1 is a guanylyl cyclase required for AWC-mediated chemotaxis (L'Etoile and Bargmann, 2000). Although some of these signaling molecules function in different chemosensory neurons, Ca²⁺ influx is likely downstream of signaling in all of these neurons.

To identify additional proteins critical for chemosensation and GPCR signaling, we conducted a forward genetic screen for *C. elegans* genes whose function is required for octanol avoidance. A loss-of-function allele of a G protein-coupled receptor kinase gene (*grk-2*) was identified. For clarity, *C. elegans grk-2* is referred to as *Ce-grk-2* in this paper. Although previous results describe a role for GRKs in GPCR downregulation, we find that a severe reduction in Ce-GRK-2 function renders animals unable to respond to a wide range of chemical stimuli. In contrast, the *C. elegans arrestin-1* gene is not required for chemosensation, a surprising result since GRK and arrestin proteins cooperate to regulate signal transduction in most mammalian systems.

Results

Ce-grk-2 Is Required for Chemosensation

A classical genetic screen was undertaken to identify genes required for response to octanol, an aversive chemical stimulus. A recessive mutant allele, *rt97*, was isolated. *rt97* mutant animals are severely defective in both octanol avoidance and chemotaxis to attractive

compounds (Figures 1A and 1B). Octanol is detected by the ASH, ADL, and AWB neurons (M.Y. Chao et al., submitted; Troemel et al., 1995), and attractive odorants are detected by the AWA and AWC neurons (Bargmann et al., 1993). Although chemosensory responses are impaired in *rt97* animals, response to some sensory stimuli remains intact. The ASH neurons also play a critical role in response to nose touch (Kaplan and Horvitz, 1993). This mechanosensory response is normal in *rt97* animals (Figure 1C). Response to body touch, which is mediated by the ALM, AVM, and PLM mechanosensory neurons (Chalfie et al., 1985), is also retained (data not shown). This relative specificity indicates that many neuronal functions remain intact in *rt97* animals and that the corresponding gene may play a specific role in chemosensation.

Using standard strategies, the *rt97* mutation was linked to a small interval on the left arm of chromosome III. A single cosmid from this region, W02B3, restored octanol response in *rt97* animals. A 5 kb long-range PCR product containing only *Ce-grk-2* (W02B3.2), including ~3 kb of 5' and ~0.3 kb of 3' flanking genomic DNA, was sufficient for the rescue (data not shown). The full-length *Ce-grk-2* cDNA, expressed using the ~3 kb 5' *Ce-grk-2* promoter, also completely restored octanol response in *rt97* animals (Figure 3C, column 4). The entire coding region of *Ce-grk-2* was sequenced and a single missense mutation was identified in *rt97* animals. The mutant allele changes amino acid residue 354 from threonine to isoleucine (T354I) (Figure 2A). When the T354I mutation was introduced into the *Ce-grk-2* cDNA construct, its rescuing activity was nearly eliminated (Figure 3C, columns 3 and 5). We conclude that *rt97* corresponds to the T354I change and that normal *Ce-grk-2* function is essential for chemosensation.

The *C. elegans* genome contains two predicted *grk* genes, *Ce-grk-1* (F19C6.1) and *Ce-grk-2* (W02B3.2). The predicted GRK proteins are remarkably similar to mammalian GRKs and contain related protein motifs. Ce-GRK-1 shares 56% overall identity with human GRK5. Ce-GRK-2 shares 66% overall identity with human GRK3 and 65% identity with human GRK2 (β -adrenergic receptor kinase-2 and 1, respectively). Importantly, both Ce-GRK-1 and Ce-GRK-2 contain the sequence DLG (Asp Leu Gly) in their predicted ATP binding region, characteristic of the GRK family of kinases. Most other serine/threonine kinases contain DFG (Asp Phe Gly) in this region (Figure 2B; Brenner, 1987; Hanks et al., 1988). Ce-GRK-2 is also structurally similar to GRK2 and GRK3 (Figure 2A). All three proteins contain an N-terminal RGS (regulator of G protein signaling) homology domain and a C-terminal PH (pleckstrin homology) domain. The RGS domain of mammalian GRK2 has only weak GTPase-activating activity (Carman et al., 1999) and instead may regulate signaling sterically through its interactions with GPCRs and G α proteins (Lodowski et al., 2003; Pao and Benovic, 2002). The PH domain may mediate interactions with G $\beta\gamma$ subunits and contribute to membrane/receptor targeting (Lodowski et al., 2003; Pitcher et al., 1998).

Ce-GRK-2 Is Broadly Expressed in the Nervous System

To determine where *Ce-grk-2* is expressed, the ~3 kb upstream promoter and ~0.3 kb downstream of the pre-

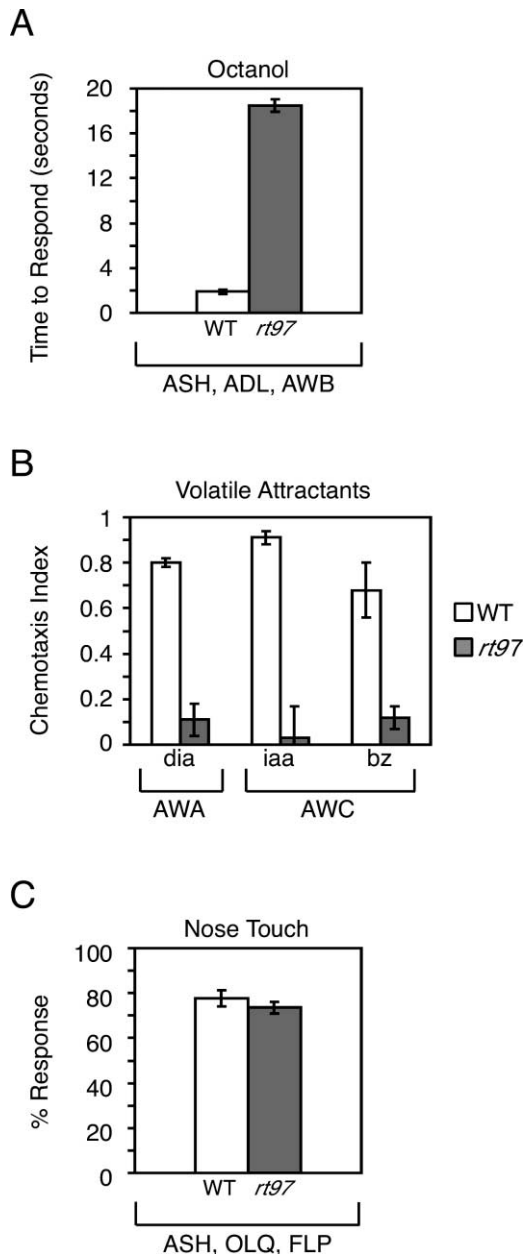


Figure 1. *Ce-grk-2(rt97)* Mutation Perturbs Chemosensory Responses

(A) *Ce-grk-2* animals fail to respond to octanol. Time to reverse (seconds) for wild-type (wt) and *Ce-grk-2(rt97)* mutant animals in response to octanol. $n \geq 30$ animals tested for each genotype. The sensory neurons mediating each response are indicated under each graph.

(B) *Ce-grk-2(rt97)* animals are defective for chemotaxis. Chemotaxis index = (number of animals at odorant - number of animals at control)/total number of animals on the assay plate. dia = 1:1000 dilution of diacetyl; iaa = 1:1000 dilution of isoamyl alcohol; bz = 1:100 dilution of benzaldehyde. Each bar represents the average of ≥ 4 assays with 50–150 animals per trial.

(C) *Ce-grk-2(rt97)* animals respond to nose touch. Percent response of wild-type and *Ce-grk-2(rt97)* mutant animals to touch to the nose is indicated. Each animal was tested 10 times; ≥ 34 animals were tested for each genotype.

Error bars indicate the standard error of the mean (SEM).

dicted *Ce-grk-2* coding region was used to express GFP. *Ce-grk-2::gfp* reporter expression was observed in embryos as early as the 20–30 cell stage and persisted throughout development and into adulthood. The *Ce-grk-2::gfp* reporter was expressed in many neurons of adult animals, including the ASH neurons and other sensory neurons, many interneurons, and motor neurons of the ventral nerve cord (Figures 2C and 2D). Expression was also observed in vulval muscles. A monoclonal antibody raised against mammalian GRK2/3 was used to confirm the putative *Ce-GRK-2* cellular expression pattern. Although endogenous *Ce-GRK-2* was below the level of detection using this antibody, *Ce-GRK-2* was detected in transgenic animals overexpressing *Ce-GRK-2* from the genomic rescue fragment (Figures 2E and 2F). The GFP reporter and antisera expression patterns were nearly identical, but no immunoreactivity was observed in the vulval muscles. These data show that *Ce-GRK-2* is broadly expressed in the *C. elegans* nervous system at all stages.

T354I Both Decreases *Ce-GRK-2* Protein Levels and Perturbs Function

The *Ce-grk-2(rt97)* mutant allele is recessive and changes a single conserved amino acid in *Ce-GRK-2*. This residue (T354) is conserved in all known serine/threonine kinases (Figure 2B; Hanks et al., 1988). To determine whether *Ce-grk-2(rt97)* is a loss-of-function allele, *Ce-GRK-2* protein levels were compared in wild-type (N2) and *Ce-grk-2* mutant animals. On Western blots, the anti-GRK2/3 antibody recognized a single band of ~ 81 kDa in wild-type *C. elegans*, the predicted molecular weight for *Ce-GRK-2*. However, in *Ce-grk-2* mutant animals, only $11\% \pm 4\%$ of the *Ce-GRK-2* protein was observed compared to wild-type animals (Figure 3A), indicating that the T354I amino acid change results in a dramatic decrease in *Ce-GRK-2* protein levels.

To determine if T354 is also critical in other GRKs, site-directed mutagenesis was used to incorporate the corresponding change (T353I) into bovine GRK2 (bGRK2). When equal amounts of DNA encoding wild-type bGRK2 and bGRK2(T353I) were transfected into either HEK293 or COS-7 cells, a dramatic decrease in bGRK2(T353I) protein levels was observed compared to wild-type bGRK2 (Figure 3B, data not shown). This decrease was seen in both cell lines and across a wide range of DNA concentrations (data not shown).

A small amount of *Ce-GRK-2* protein remains in *Ce-grk-2* mutant animals and the residual protein could retain some biological activity. Due to the extremely low expression levels of both *Ce-GRK-2*(T354I) and bGRK2 (T353I) in tissue culture cells (Figure 3B, data not shown), it was not possible to directly assay GPCR phosphorylation by these proteins using previously developed assays (Freedman et al., 1995). Therefore, functional activity of the T354I mutant protein was tested in *C. elegans* using behavioral assays.

Both wild-type and the T354I mutant *Ce-GRK-2* proteins were overexpressed in *Ce-grk-2* mutant animals using the *Ce-grk-2* promoter. *Ce-GRK-2* expression levels and octanol response were assessed. Octanol response of *Ce-grk-2* mutant animals expressing the wild-type *Ce-grk-2* cDNA was completely restored (2 ± 0.2 s, Figure 3C, column 4). To express *Ce-GRK-2*(T354I) at

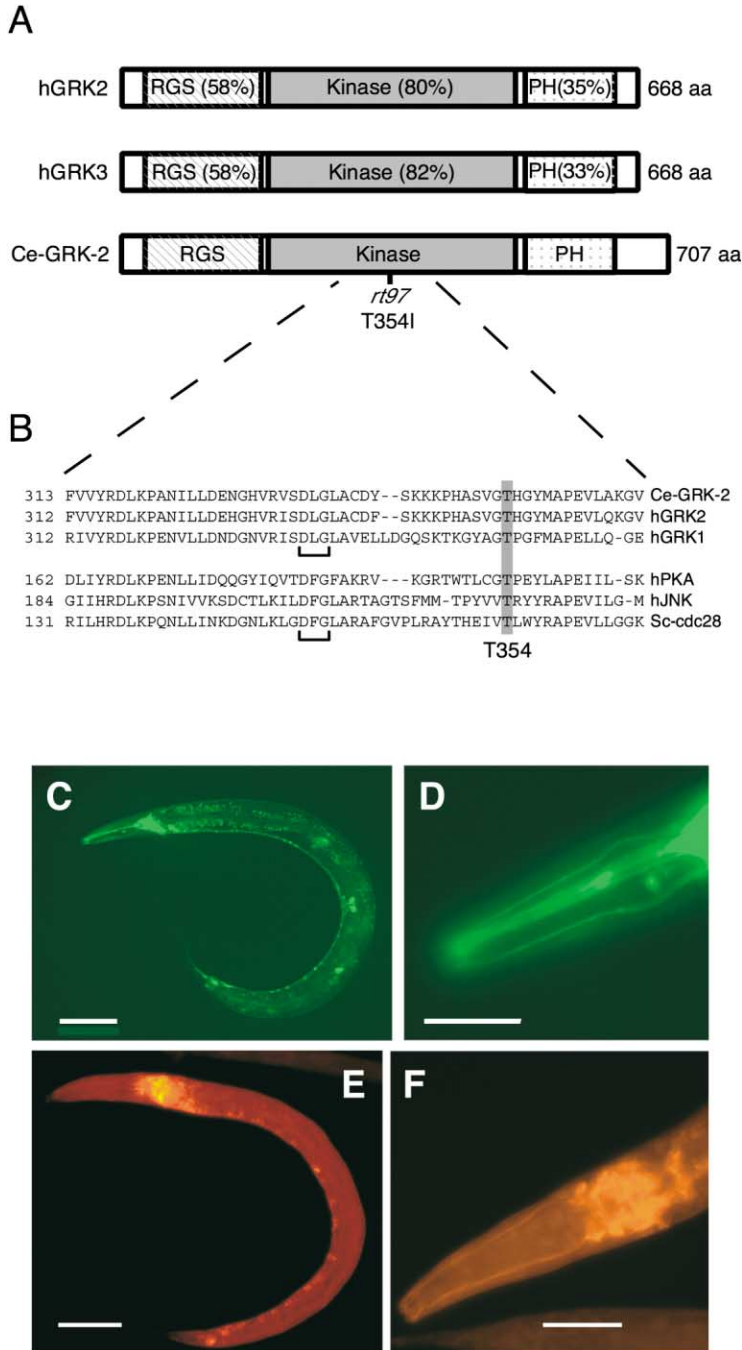


Figure 2. *Ce-grk-2* Encodes a G Protein-Coupled Receptor Kinase

(A) Predicted domain structures of Ce-GRK-2, human GRK2 (hGRK2), and human GRK3 (hGRK3) proteins. Ce-GRK-2 is 66% and 65% identical overall to hGRK3 and hGRK2, respectively. RGS, regulator of G protein signaling protein homology domain; PH, pleckstrin homology domain. The amino acid identity (%) between each human GRK and Ce-GRK-2 domain is indicated. The *rt97* mutation corresponds to a T354I change in the predicted kinase domain of Ce-GRK-2.

(B) Threonine 354 is conserved among serine/threonine kinases. Predicted kinase domains were aligned using the ClustalW program (Thompson et al., 1994) in the MegAlign software package (DNASTAR). The threonine residue equivalent to T354 of Ce-GRK-2 is indicated in the gray rectangle. The signature DLG motif of GRKs (top three sequences) and the DFG motif of other kinases (bottom three sequences) are indicated by the brackets. h, human; Sc, *S. cerevisiae*.

(C and D) A *Ce-grk-2::gfp* transcriptional reporter is expressed in neurons in the body (C) and head (D). Broad expression in the nervous system and the vulval muscles is observed. Scale bar equals 100 μ m in (C) and 25 μ m in (D).

(E and F) Ce-GRK-2 overexpressed using its own promoter can be detected in the nervous system with anti-mammalian GRK2/3 antibody. The entire animal (E) or just the head of the animal (F) are shown. Scale bar equals 100 μ m in (E) and 25 μ m in (F). No overt localization to sensory neuron cilia was observed, although overexpression of Ce-GRK-2 may mask or interfere with normal subcellular localization.

levels equivalent to wild-type Ce-GRK-2 in the *Ce-grk-2* rescued lines, the transgene encoding the T354I mutation was introduced at 10-fold higher concentration (Figure 3C, compare lanes 4 and 5). However, the T354I mutant protein expressed at the same level had only a small impact on octanol response in *Ce-grk-2* mutant animals (14 ± 0.5 s, Figure 3C, column 5) compared to the mutant control animals (18 ± 1 s, Figure 3C, column 2). This indicates that the mutant protein has little residual biological function.

To address whether Ce-GRK-2(T354I) has neomorphic or dominant-negative properties, wild-type and T354I Ce-GRK-2 overexpression transgenes were

crossed into wild-type animals. Neither transgene perturbed octanol response nor chemotaxis to diacetyl (data not shown), suggesting that the mutant protein does not interfere with normal chemosensory pathways. We conclude that *Ce-grk-2(rt97)* is a severe loss-of-function allele.

***Ce-grk-2* Mutant Animals Are Not Hypersensitive**

Mammalian GRKs are involved in receptor desensitization and the termination of GPCR signaling. Thus, loss of GRK function often results in hypersensitivity or loss of desensitization. It was perhaps surprising that loss of Ce-GRK-2 function abolished *C. elegans* chemosensory

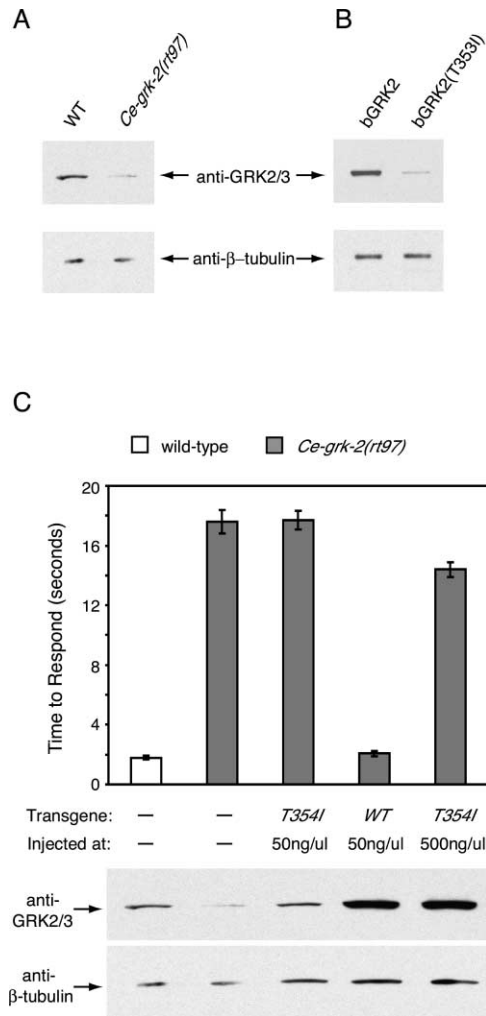


Figure 3. *Ce-grk-2(rt97)* Is a Loss-of-Function Allele
 (A) The T354I mutation decreases *Ce-GRK-2* protein levels. Samples were analyzed by Western blotting using monoclonal anti-GRK2/3 and anti- β -tubulin antibodies. Positions of *Ce-GRK-2* (wt and T354I) and β -tubulin are indicated by arrows.
 (B) The T353I mutation decreases bovine GRK2 (bGRK2) levels. HEK293 cells were transfected with either wild-type or T353I mutant bGRK2 plasmid DNA. The positions of bGRK2 (wt and T353I) and β -tubulin are indicated by arrows.
 (C) *Ce-GRK-2*(T354I) retains little biological activity. The white bar represents the response of wild-type animals. The gray bars represent the *Ce-grk-2(rt97)* animals, without or with transgene injected at the indicated concentrations. A Western blot of animals of the corresponding genotypes is shown below the bars. *Ce-grk-2(rt97)* animals expressing the wild-type *Ce-grk-2* transgene responded normally to octanol (column 4). *Ce-grk-2(rt97)* animals overexpressing *Ce-GRK-2*(T354I) were still defective (lane 5). Each column represents the combined data of ≥ 2 trials of ≥ 3 independent transgenic lines. In all cases ≥ 38 animals were assayed.

responses. One hypothesis is that decreases in *Ce-grk-2* function can lead to increased sensitivity to odorants only at decreased concentrations. To test for hypersensitivity, we assayed response to dilute concentrations of odorants (Figure 4). Heterozygous *Ce-grk-2*⁺ animals were indistinguishable from control animals in response to diacetyl and octanol (Figures 4A and 4B). In addition, homozygous *Ce-grk-2* mutant animals never responded

as well as wild-type animals and were not hypersensitive to diacetyl, octanol (Figures 4C and 4D), isoamyl alcohol, or benzaldehyde (data not shown) at any concentration tested. These results indicate that reduced *Ce-GRK-2* function does not lead to an enhanced response to weaker chemosensory stimuli.

Arrestin Is Not Required for Chemosensation in *C. elegans*

In mammals and *Drosophila*, arrestins bind GPCRs that have been phosphorylated by GRKs to mediate physical uncoupling of receptors from downstream signaling components and promote receptor internalization. Arrestins can also activate the Src and MAP kinase pathways (Perry and Lefkowitz, 2002; Pierce and Lefkowitz, 2001). In the *C. elegans* genome, *arrestin-1* (*arr-1*, F53H8.2) is the only predicted gene that has significant overall similarity to arrestins in other organisms (Figure 5A). ARR-1 is 54% and 52% identical overall to human β -arrestin 1 and β -arrestin 2, respectively.

Consistent with a regulatory role in signal transduction in the nervous system, an *arr-1::gfp* promoter reporter construct is expressed throughout the *C. elegans* nervous system, including the octanol-detecting ASH sensory neurons (Figure 5B). To determine if loss of *arr-1* function would cause defects similar to loss of *Ce-grk-2* function, the deletion allele *arr-1(ok401)* was obtained from the *C. elegans* gene knockout consortium for analysis. *arr-1(ok401)* is predicted to be a severe or complete loss-of-function allele (Figure 5A). Interestingly, *arr-1(ok401)* animals responded as well as wild-type animals to octanol and volatile attractants (Figure 5C, data not shown). *Ce-grk-2; arr-1* double mutant animals were as defective in octanol avoidance as animals lacking only *Ce-grk-2* function (Figure 5C). However, *Ce-grk-2; arr-1* double mutant animals are unhealthy and grow slowly, suggesting that these genes may have additional roles in other tissues. We conclude that primary chemosensory signaling in *C. elegans* does not require ARR-1 function. Our results do not rule out a role for *Ce-grk-2* or *arr-1* in olfactory adaptation or discrimination.

Ce-grk-2 Is Required Only in Adult Stages for Chemosensation

To assess when *Ce-grk-2* function is required for chemosensation, the full-length *Ce-grk-2* cDNA was placed under the control of a heat shock inducible promoter (Stringham et al., 1992) and introduced into *Ce-grk-2* mutant animals. Induction of *Ce-grk-2* expression by heat shock in adult animals significantly restored response to octanol (5.5 ± 0.7 s) when assayed 4 hr later (Figure 6A). Without heat shock induction, no rescue was observed (19 ± 0.6 s). We also examined the morphology, development, and survival of sensory neurons in *Ce-grk-2* mutant animals using a variety of cellular differentiation markers, including ODR-3, ODR-10::GFP, and the lipophilic dye DiD (Perkins et al., 1986; Roayaie et al., 1998; Sengupta et al., 1996). No changes in sensory cilia, cell fate, or axonal projections were observed (Figure 8B, data not shown). Combined, these results demonstrate that *Ce-grk-2* is only required in adult stages for normal chemosensory response, after cell

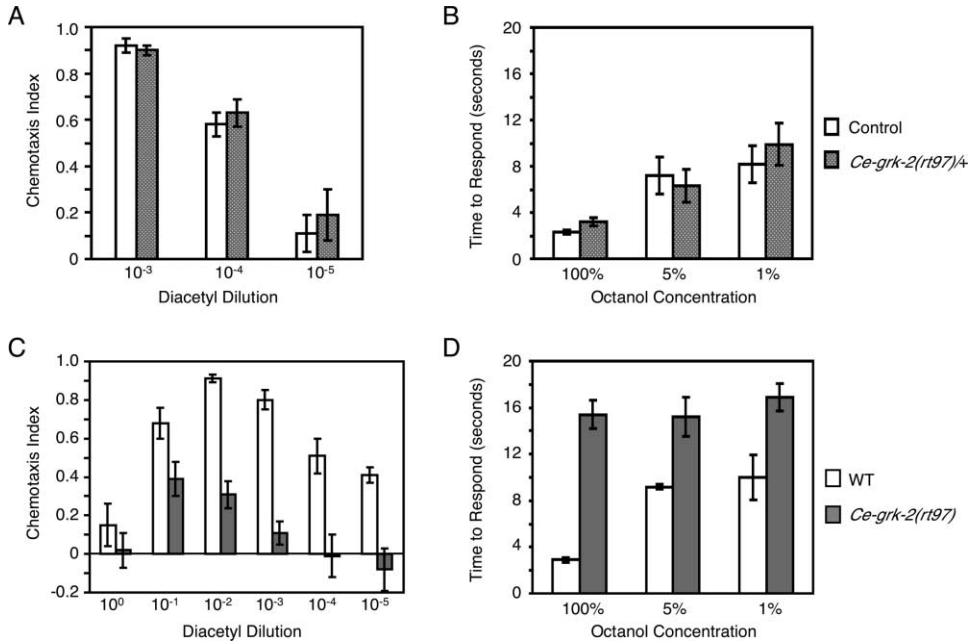


Figure 4. *Ce-grk-2(rt97)* Animals Are Not Hypersensitive to Dilute Odorants

(A and B) *Ce-grk-2(rt97)/+* animals are not hypersensitive to dilute diacetyl nor dilute octanol.

(A) Dose-response curves of control and *Ce-grk-2(rt97)/+* heterozygous animals to dilute concentrations of diacetyl are shown. The genotypes of control and *Ce-grk-2/+* animals are *dpy-11/+ him-5/+* and *Ce-grk-2(rt97)/+; dpy-11/+ him-5/+*. Each bar represents the average of ≥ 3 assays with 50–150 animals per trial.

(B) Time to respond (seconds) to octanol is shown. The genotypes of control and *Ce-grk-2/+* animals are *dpy-11/+* and *Ce-grk-2(rt97)/+; dpy-11/+*. Each bar represents the average of 3 independent trials, $n \geq 19$ animals.

(C and D) *Ce-grk-2(rt97)* animals are defective in their response to dilute diacetyl and dilute octanol.

(C) Each bar represents the average of 3 assays with 50–150 animals per trial.

(D) Each bar represents the combined data of ≥ 2 independent trials, $n \geq 17$ animals.

fate determination and neuronal connectivity is complete.

Ce-GRK-2 Functions in the Sensory Neurons

As Ce-GRK-2 is broadly distributed in the nervous system, it may function in the sensory neurons, the interneurons, or both during chemosensation. To determine where Ce-GRK-2 functions, various promoters were used to drive expression of Ce-GRK-2 in subsets of neurons. The *srb-6* promoter was used to express Ce-GRK-2 in two of the chemosensory neurons that detect octanol, ASH and ADL (Troemel et al., 1995). Expression of the *srb-6::Ce-grk-2* transgene restored response to octanol in a dose-dependent manner (Figure 6B). Injection of *srb-6::Ce-grk-2* into *Ce-grk-2* mutants at a low concentration partially restored octanol response, whereas 10-fold more completely restored octanol response (Figure 6B). In addition, expression of *Ce-grk-2* in ASH neurons using the *osm-10* promoter (Hart et al., 1999) (injected at a low concentration, 50 ng/ μ l) also partially restored octanol response in *Ce-grk-2* mutant animals (12 ± 1 s, data not shown). These results demonstrate that Ce-GRK-2 can function in the sensory neurons.

To address whether Ce-GRK-2 is also required in the interneurons that control forward and backward locomotion, the *glr-1* promoter was used to express *Ce-grk-2*. The *glr-1* promoter drives expression in 17 classes of neurons, including the command interneurons, but

not in the chemosensory neurons (Hart et al., 1995; Maricq et al., 1995). The *glr-1::Ce-grk-2* transgene, expressed at either low or high concentrations, had no effect on octanol response (Figure 6B). Furthermore, co-expression of *srb-6::Ce-grk-2* and *glr-1::Ce-grk-2* restored octanol response no more than expression of *srb-6::Ce-grk-2* alone (Figure 6B). Taken together, these results indicate that the primary site of Ce-GRK-2 function in chemosensation is the chemosensory neurons but do not rule out a contribution from other tissues.

Since Ce-GRK-2 acts in sensory neurons, odorant receptors may be phosphorylation targets of Ce-GRK-2. Given that phosphorylation of GPCRs by GRKs is required for receptor endocytosis/recycling in other systems, a mutation in *Ce-grk-2* could change the expression or localization of odorant receptors such as the diacetyl receptor ODR-10 (Sengupta et al., 1996). However, no overt change in ODR-10::GFP (Sengupta et al., 1996) localization nor expression levels was observed in control versus *Ce-grk-2* mutants, even when animals were pre-exposed to diacetyl (data not shown). Therefore, changes in receptor localization are unlikely to account for the chemosensory defects of *Ce-grk-2* mutant animals.

Loss of *Ce-grk-2* Function Perturbs Stimulus-Evoked Ca²⁺ Influx

If *Ce-grk-2* is required for response to sensory stimuli in the chemosensory neurons, then perhaps signaling

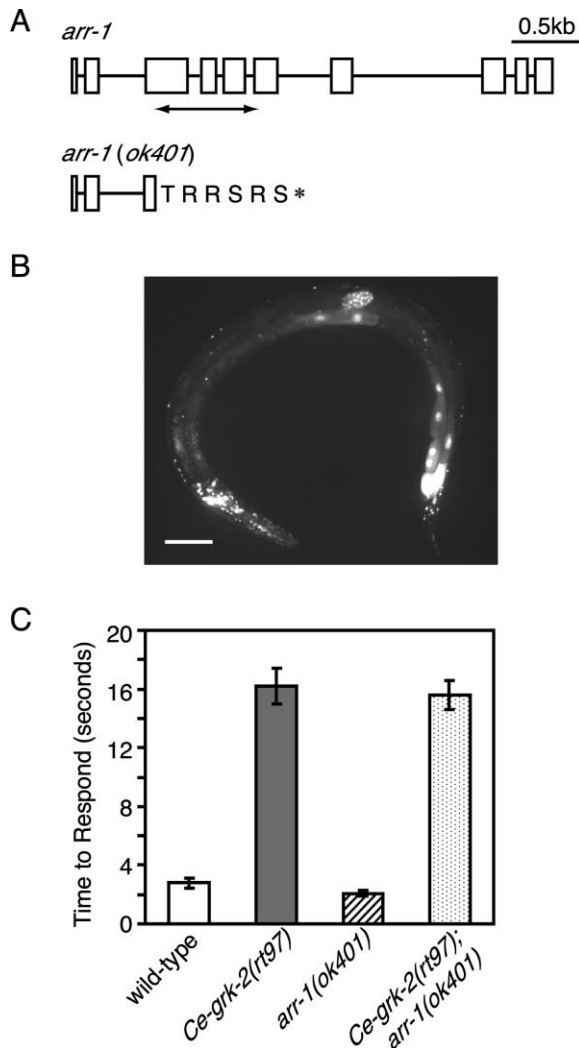


Figure 5. *arr-1* Is Not Required for Chemosensation

(A) Predicted gene structure of *arr-1*. Exons and introns are shown as boxes and lines, respectively. The *ok401* mutation creates a 0.7 kb deletion, as indicated by the arrowheads, eliminating ARR-1 amino acids 68 through 236 and introducing a frame shift and a premature stop codon (asterisk).

(B) Expression of an *arr-1::gfp* transcriptional reporter. Broad expression in the nervous system and intestine is observed. Scale bar equals 100 μ m.

(C) *arr-1(ok401)* animals respond normally to octanol, but *Ce-grk-2(rt97); arr-1(ok401)* animals do not. ≥ 30 animals were tested for each genotype.

events downstream of chemosensory receptors are perturbed in *Ce-grk-2* mutant animals. Recently, techniques have been developed to measure calcium influx into the ASH sensory neurons in intact *C. elegans* using a genetically encoded calcium indicator, cameleon (M.A. Hilliard et al., submitted; Miyawaki et al., 1997). The relative increase in the intracellular calcium concentration is measured as an increase in the YFP/CFP fluorescence ratio of the cameleon protein. To assess whether sensory signal transduction is perturbed in *Ce-grk-2* mutant animals, we measured stimulus-evoked calcium influx into the ASH neurons.

Volatile chemicals are immiscible with the perfusion buffer used for optical recordings. Therefore, soluble

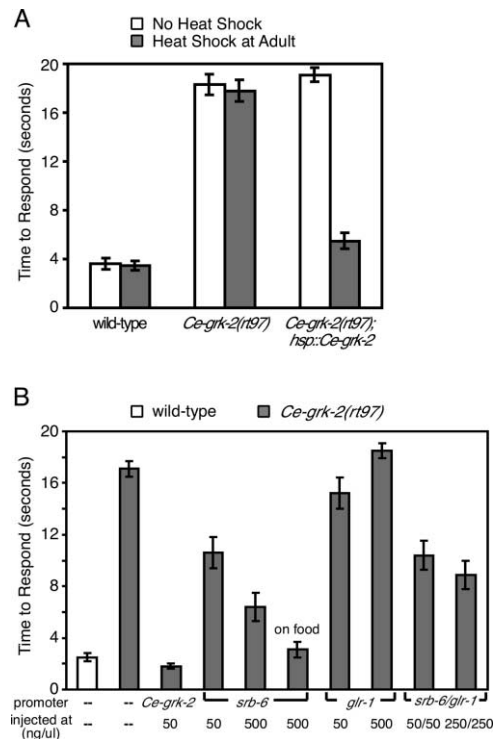


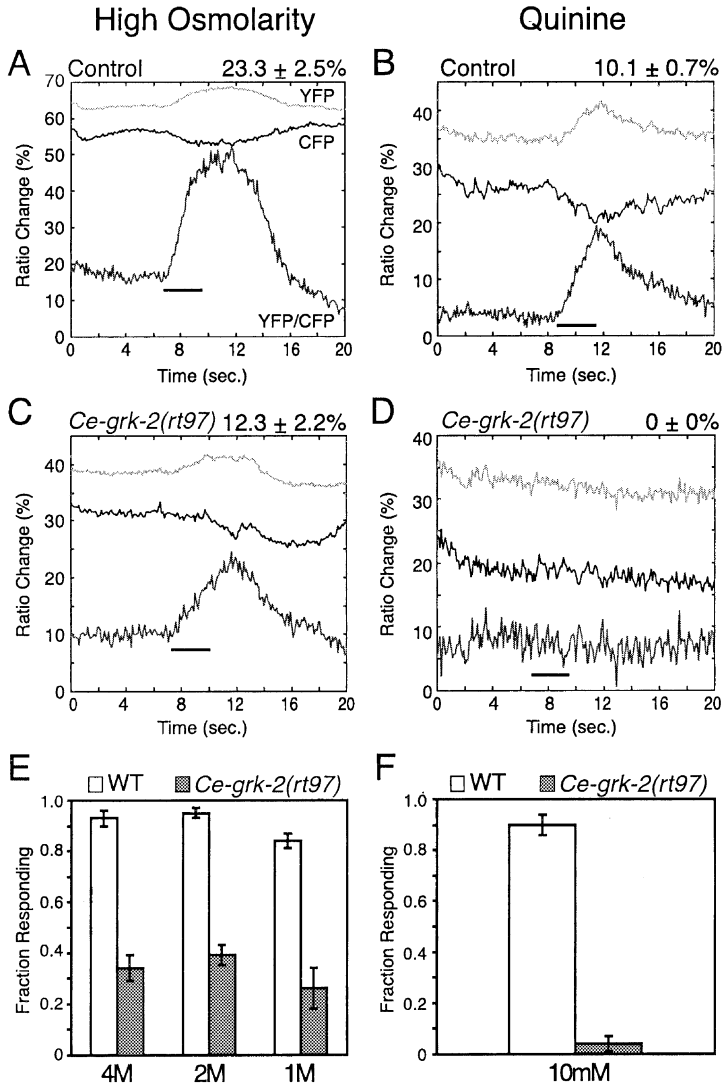
Figure 6. Ce-GRK-2 Is Required in Adult Sensory Neurons for Chemosensation

(A) Ce-GRK-2 is required in adult stages for octanol response. Time to respond to octanol for wild-type, *Ce-grk-2(rt97)*, and *Ce-grk-2(rt97)* animals expressing full-length *Ce-grk-2* cDNA under the control of a heat shock promoter (*hsp::Ce-grk-2*) is shown. Animals were tested as adults grown without heat shock treatment (white) or after heat shock (gray). Octanol response of *Ce-grk-2* mutant animals carrying *hsp::Ce-grk-2* was restored significantly 4 hr after heat shock. Results represent the combined data of 2 independent trials of 2 transgenic lines. $n \geq 28$ animals.

(B) Ce-GRK-2 is required in the sensory neurons for octanol response. The white bar represents octanol response of wild-type animals. Gray bars represent octanol response of *Ce-grk-2(rt97)* mutant animals without or with *Ce-grk-2* cDNA transgenes. The promoter used, as well as the concentration at which the transgene was introduced, is indicated below each column. Ce-GRK-2 expressed using the *Ce-grk-2* or *srb-6* promoter restored octanol response of *Ce-grk-2(rt97)* animals. The partial rescue by *srb-6::Ce-grk-2* (columns 4 and 5) suggested that Ce-GRK-2 might act in neurons other than ASH and ADL to mediate octanol response. The ASH sensory neurons are required for octanol response when animals are on food, but the ADL and AWB sensory neurons both play critical roles when animals are off food (M.Y. Chao et al., submitted). *Ce-grk-2* mutant animals are equally defective both on and off food (data not shown). However, the *srb-6::Ce-grk-2* transgene is not expressed in AWB, and all of the assays described herein took place off food. Expression in the ASH neurons using the *srb-6::Ce-grk-2* transgene effectively restored octanol response on food, when animals utilize only the ASH neurons to detect octanol (3 ± 1 s, column 6).

Expression via the *glr-1* promoter had no effect on octanol avoidance off food (columns 7 and 8) or on food (data not shown). Results represent the combined data of two independent trials of 3 transgenic lines. $n \geq 33$ animals tested for each genotype.

aversive stimuli detected by the ASH neurons (high osmolarity and quinine) were used to stimulate the ASH neurons (Bargmann et al., 1990; Hilliard et al., 2004). *Ce-grk-2* mutants are partially defective in their behavioral response to the osmotic stimulus glycerol (Figure 7E)



and completely defective in their response to quinine (Figure 7F). A large calcium influx was observed in ASH neurons in response to 1 M glycerol and to 10 mM quinine in control animals (Figures 7A and 7B). In contrast, evoked calcium influx into the ASH neurons was significantly impaired in *Ce-grk-2* mutant animals (Figures 7C and 7D). While the YFP/CFP ratio increased by 23% in control animals following the application of 1 M glycerol, the ratio increased by only 12% in *Ce-grk-2* mutants (Figures 7A and 7C). This is consistent with the partial defect in the behavioral response to high osmolarity observed in *Ce-grk-2* mutant animals (Figure 7E). Whereas the YFP/CFP ratio increased by 10% in response to quinine in control animals, no change was seen in *Ce-grk-2* mutant animals (Figures 7B and 7D), also in agreement with the behavioral results (Figure 7F). We conclude that loss of *Ce-grk-2* function perturbs signal transduction upstream of calcium influx in the ASH sensory neurons.

Overexpression of *odr-3* Restores Octanol Response in *Ce-grk-2* Mutant Animals

The behavioral defects and reduced neuronal Ca^{2+} influx both suggest that G protein signaling is decreased in *Ce-*

Figure 7. Stimulus-Evoked Calcium Transients in the ASH Neurons Are Reduced in *Ce-grk-2(rt97)* Animals

A genetically encoded calcium indicator, cameleon, was expressed in the ASH neurons. Soluble stimuli were delivered to the nose of an adult animal for 3 s (black horizontal bar), and the change in YFP (top line) and CFP (middle line) fluorescence intensities were recorded. The YFP/CFP ratio (bottom line) is shown. The average ratio change \pm SEM is indicated above each graph. ≥ 6 animals were assayed for each panel.

(A and B) The fluorescence ratio change of cameleon in an ASH sensory neuron of control animals in response to 1 M glycerol (A) and 10 mM quinine (B). A sharp increase in YFP/CFP fluorescence ratio was observed following stimulation.

(C and D) The fluorescence ratio change in ASH sensory neurons of *Ce-grk-2(rt97)* animals in response to 1 M glycerol (C) and 10 mM quinine (D). The calcium transient was diminished in response to glycerol and quinine.

(E and F) *Ce-grk-2(rt97)* animals are defective in their response to high osmolarity and quinine. The fraction of animals that responded to 4, 2, or 1 M glycerol (E) and 10 mM quinine (F) is shown. *Ce-grk-2(rt97)* animals are partially defective for response to the osmotic stimulus glycerol and completely defective for response to quinine.

grk-2 mutant animals. If GPCR signaling is decreased in *Ce-grk-2* mutants, then overexpression of other signaling components in the pathway might restore chemosensory response.

When stimulated by activated G proteins, cyclases produce the second messengers cAMP or cGMP. ODR-1 is a guanylyl cyclase required for AWC-mediated chemotaxis (L'Etoile and Bargmann, 2000). To determine if increased ODR-1 cyclase levels could restore chemotaxis, *Ce-grk-2* mutant animals overexpressing an *odr-1* transgene were tested for chemotaxis to the AWC-detected stimulus isoamyl alcohol; no improvement was observed. This may be because the signaling deficit occurs upstream of second messenger production in *Ce-grk-2* mutant animals, or because cyclase activity may be regulated (Hurley, 1998) and *odr-1* overexpression alone does not increase signaling.

As an alternative, we examined whether overexpression of a critical $G\alpha$ protein, *odr-3*, could restore chemosensation in *Ce-grk-2* mutant animals. *odr-3* loss-of-function mutant animals have defects in both chemotaxis and odorant avoidance, reminiscent of the *Ce-grk-2* mutant phenotype (Roayaie et al., 1998). As shown in Figure 8A, introduction of *odr-3* at 25 ng/ μ l significantly restored

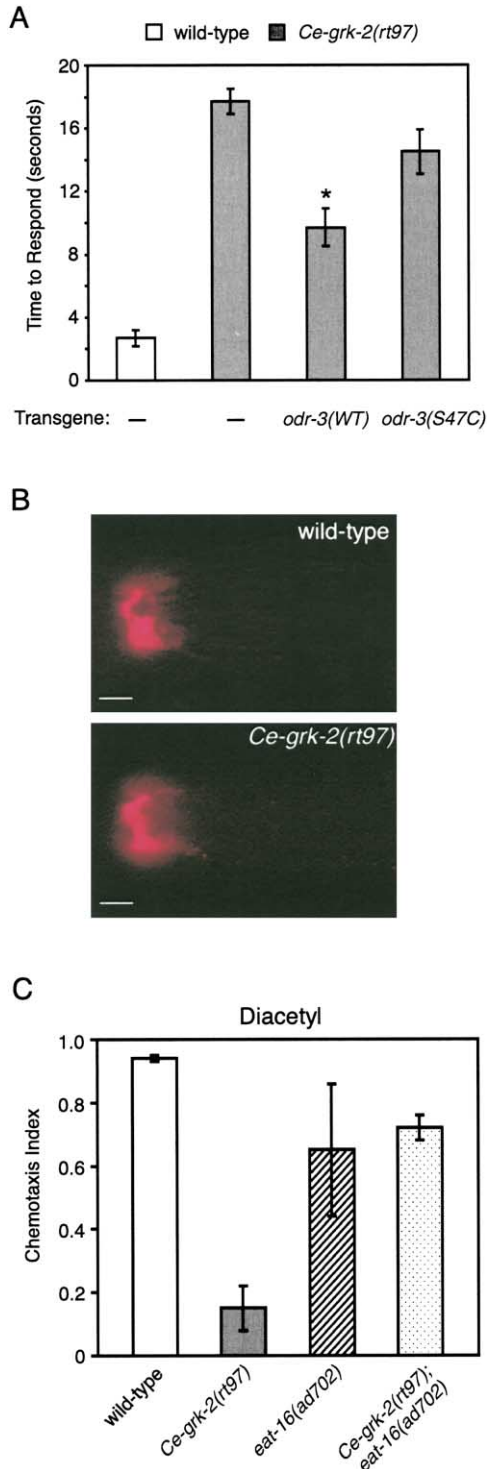


Figure 8. Overexpression of *odr-3* or Loss of *eat-16* Function Restores Chemosensory Response in *Ce-grk-2(rt97)* Animals

(A) The white bar represents wild-type animals. The gray bars represent *Ce-grk-2(rt97)* animals without or with transgene, as indicated. The wild-type *odr-3* transgene restored octanol response (asterisk, $p < 0.6 \times 10^{-5}$), while *Ce-grk-2(rt97)* animals expressing the *odr-3(S47C)* transgene responded less robustly ($p > 0.05$). Each column represents the combined data of 2 independent trials of 3 transgenic lines, \pm SEM. In all cases, ≥ 36 animals were assayed. The Student's two-tailed t test was utilized for statistical analysis.

(B) ODR-3 protein levels are not altered in *Ce-grk-2(rt97)* animals. Expression of endogenous ODR-3 protein in the AWC sensory cilia

response to octanol (10 ± 1 s, $p < 0.6 \times 10^{-5}$). The incomplete rescue may be due to a requirement for additional positively acting G_{α} proteins in chemosensory signaling (Jansen et al., 1999; H. Lans et al., submitted; Roayaie et al., 1998). Responses to AWA- or AWC-mediated attractive odorants could not be determined in these animals as *odr-3* overexpression at this level rendered the *Ce-grk-2* mutant animals too lethargic for chemotaxis assays. Increasing *odr-3* transgene levels further did not restore octanol avoidance (data not shown). However, high-level overexpression of *odr-3* blocks chemosensation even in wild-type animals (Roayaie et al., 1998). Thus, proper ODR-3 signaling levels may be crucial for chemosensation and behavioral responses. To determine whether ODR-3 protein levels are decreased in *Ce-grk-2* mutants, the endogenous level of ODR-3 was compared to that of wild-type animals, but no significant difference was observed (Figure 8B). This suggests that ODR-3 activity may be altered in *Ce-grk-2* mutant animals.

odr-3 overexpression may have restored octanol response in *Ce-grk-2* mutants by increasing signaling through ODR-3 G_{α} or by interfering with $G_{\beta\gamma}$ subunits that are overstimulated due to the loss of *Ce-grk-2*. To determine if normal ODR-3 activity is required for restoration of octanol response, a mutant *odr-3* transgene, *odr-3(S47C)*, was expressed in *Ce-grk-2* mutant animals. ODR-3(S47C) converts the conserved serine at residue 47 to cysteine. Mammalian G_{α} proteins (G_o and G_i) with the equivalent mutation have severely reduced affinity for GTP, but they retain the ability to bind $G_{\beta\gamma}$ subunits (Slepek et al., 1993, 1995). As such, the S47C change confers dominant-negative activity to the G_{α} proteins when they are overexpressed (Roayaie et al., 1998; Slepek et al., 1993, 1995). In *C. elegans*, the S47C change in ODR-3 nearly eliminates its ability to restore chemosensation in *odr-3* mutant animals and blocks chemotaxis when expressed at high levels in wild-type animals (Roayaie et al., 1998). The *odr-3(S47C)* transgene also had significantly reduced rescuing activity in *Ce-grk-2* mutant animals (Figure 8A; $p > 0.05$), suggesting that interaction with $G_{\beta\gamma}$ subunits is not sufficient for restoration of chemosensation in *Ce-grk-2* mutants. The ability of *odr-3* overexpression, but not *odr-3(S47C)*, to appreciably restore response to octanol is also consistent with a decrease in chemosensory signal transduction in the sensory neurons of *Ce-grk-2* mutant animals.

Loss of EAT-16 RGS Protein Function Suppresses a *Ce-grk-2* Chemosensory Defect

Chemosensory signaling in *Ce-grk-2* mutant animals may be decreased because a compensatory G protein regulatory pathway dampens signaling. EGL-10 and EAT-16 are two RGS (regulator of G protein signaling) proteins with broad neuronal expression that negatively

of wild-type (top) and *Ce-grk-2(rt97)* (bottom) animals was detected with anti-ODR-3 antisera. Chemosensory stimulus detection occurs in the cilia. No staining was observed in *odr-3(n1605)* mutant animals (data not shown). Scale bar equals 5 μ m.

(C) *eat-16; Ce-grk-2* double mutant animals respond to diacetyl. Chemotaxis index for a 1:1000 dilution of diacetyl is shown. Each bar represents the average of 4 trials of 50–150 animals per trial.

regulate the activity of distinct $G\alpha$ proteins involved in *C. elegans* locomotion and egg laying (Hajdu-Cronin et al., 1999; Koelle and Horvitz, 1996). *Ce-grk-2*; *egl-10* double mutant animals were as defective as *Ce-grk-2* single mutants in their response to diacetyl (AWA), isoamyl alcohol (AWC), and octanol (ASH) (data not shown), although the *Ce-grk-2*; *egl-10* double mutant animals were also very lethargic. *eat-16*; *Ce-grk-2* double mutant animals were defective in their response to isoamyl alcohol and octanol, but their response to diacetyl was significantly restored (Figure 8C). This suggests that RGS proteins may be important negative regulators of signal transduction in chemosensory neurons and that EAT-16 may downregulate AWA chemosensory signaling in the absence of Ce-GRK-2.

Discussion

Ce-GRK-2 Is a G Protein-Coupled Receptor Kinase Required for Chemosensation

GRKs play critical roles in signaling processes ranging from sensory transduction to hormonal response. We describe the identification of *Ce-grk-2*, a *C. elegans* gene encoding a putative G protein-coupled receptor kinase. A loss-of-function mutation in *Ce-grk-2* causes profound defects in AWA-, AWC-, and ASH-mediated chemosensory responses, suggesting a broad and essential role for *Ce-grk-2* in chemosensation. *Ce-grk-2* is required in sensory neurons for chemosensory response and functions in the adult neurons rather than in cell fate determination or morphogenesis. Ce-GRK-2 is expressed broadly in the nervous system, suggesting that *Ce-grk-2* may play additional roles in other *C. elegans* neuronal circuits and behaviors.

A T354I Mutation in Ce-GRK-2 Decreases Protein Levels and Function

The T354I mutation in *Ce-grk-2(rt97)* animals changes a conserved residue in the kinase domain of Ce-GRK-2. This residue is conserved in all known serine/threonine kinases (Hanks et al., 1988). A threonine to alanine change at the corresponding residue (T201) of the catalytic subunit of cAMP-dependent protein kinase (PKA) results in a kinase-dead protein but does not affect protein stability (Moore et al., 2002). Thus, the T354I mutation in Ce-GRK-2 was predicted to drastically reduce the protein's kinase activity or function by disrupting the active site and the floor of the peptide binding cleft (J. Tesmer, personal communication; Lodowski et al., 2003). Surprisingly, the T354I mutation severely reduced Ce-GRK-2 protein levels in *Ce-grk-2(rt97)* animals. An isoleucine at this position may result in greater structural disruption than the alanine replacement in the mutant PKA. In addition, the T354I mutant protein was unable to restore octanol response in *Ce-grk-2* animals, even when overexpressed at a level similar to wild-type Ce-GRK-2. This, along with the predicted structural disruption, suggests that the T354I mutation likely reduces the kinase activity of Ce-GRK-2 as well as the protein levels. Our Ce-GRK-2(T354I) overexpression analysis in wild-type animals indicates that even if its kinase activity is impaired, Ce-GRK-2(T354I) does not interfere with normal chemosensation by acting as a dominant-nega-

tive or a neomorph. Taken together, the *Ce-grk-2(rt97)* mutation is most likely a severe loss-of-function mutation.

Ce-grk-2 Animals Are Not Hypersensitive

In mammalian systems, GRKs downregulate GPCR signaling, and loss of GRKs often results in hypersensitivity or loss of desensitization (Chen et al., 1999; Cideciyan et al., 1998; Gainetdinov et al., 1999, 2003; Jaber et al., 1996; Lyubarsky et al., 2000; Peppel et al., 1997; Rockman et al., 1998; Walker et al., 1999). Surprisingly, neither *Ce-grk-2(rt97)* homozygous nor *Ce-grk-2/+* heterozygous animals are hypersensitive to dilute odorants. These results suggest that chemosensory signaling is not enhanced when Ce-GRK-2 function is decreased.

tax-6 calcineurin mutant animals have defects in response to AWC-detected odorants due to hyperadaptation to olfactory stimuli (Kuhara et al., 2002). Since the OSM-9 cation channel is required for adaptation in AWC neurons (Colbert et al., 1997), chemotaxis to isoamyl alcohol (AWC) was completely restored in *tax-6* mutant animals by a second mutation in *osm-9* (Kuhara et al., 2002). However, response to isoamyl alcohol was not restored in *Ce-grk-2*; *osm-9* double mutant animals (data not shown), suggesting that the AWC chemosensory defect in *Ce-grk-2* mutant animals is not due to hyperadaptation.

Loss of Ce-GRK-2 May Result in Decreased G Protein Signaling

Both the decrease in stimulus-induced neuronal Ca^{2+} influx, as well as the ability of ODR-3 $G\alpha$, but not ODR-3(S47C), to restore octanol response in *Ce-grk-2* mutant animals suggests that loss of *Ce-grk-2* function leads to decreased G protein signal transduction. Loss of function in the stimulatory $G\alpha$ protein ODR-3 also results in chemosensory defects and decreased Ca^{2+} signaling similar to *Ce-grk-2(rt97)* animals (M.A. Hilliard et al., submitted; Roayaie et al., 1998). Therefore, in the absence of Ce-GRK-2 function, there may be a compensatory downregulation of G protein signal transduction. This could occur at the level of ODR-3 $G\alpha$ protein or, more generally, by decreasing the overall efficiency of the G protein signaling cascade. For example, a $G\alpha$ that inhibits chemosensation could be upregulated (Jansen et al., 1999; H. Lans et al., submitted). However, deletion of the inhibitory $G\alpha$ proteins that function in AWA (GPA-5) and AWC (GPA-2) does not restore chemotaxis in *Ce-grk-2* mutant animals (H.L. and G.J., unpublished results). Alternatively, the activity of ODR-3 could be downregulated in *Ce-grk-2* mutant animals, perhaps by a GTPase activating protein (GAP). Consistent with this hypothesis, mutation of the RGS protein EAT-16 restores the ability of *Ce-grk-2* mutant animals to respond to diacetyl. At least 12 *rgs* genes are predicted in the *C. elegans* genome (Hajdu-Cronin et al., 1999); different RGS proteins may regulate signaling in different neurons.

In mice, GRK3 plays an important role in the desensitization of olfactory signal transduction (Dawson et al., 1993; Peppel et al., 1997; Schleicher et al., 1993). Odorant stimulation causes a transient increase in cAMP levels that quickly returns to basal levels due to desensitization by GRK3 (Boekhoff et al., 1994; Dawson et al.,

1993; Schleicher et al., 1993). Surprisingly, olfactory epithelia isolated from GRK3 knockout mice showed a significantly reduced production of cAMP in response to odorants in addition to a lack of desensitization (Peppel et al., 1997). Direct activation of G proteins and adenylyl cyclase with GTP γ S or forskolin, respectively, could not stimulate cAMP production in the absence of GRK3 (Peppel et al., 1997). Combined, these data suggest that loss of Ce-GRK-2 or mammalian GRK3 may activate similar mechanisms to downregulate signaling in olfactory/chemosensory neurons.

An alternative possibility is that Ce-GRK-2 may function positively in chemosensory signal transduction. Although mammalian GRKs and arrestins normally cooperate to dampen signaling, accumulating evidence suggests that they can also function together to have a positive role in signaling through arrestin-mediated activation of the Src and MAP kinase pathways (Perry and Lefkowitz, 2002; Pierce and Lefkowitz, 2001). However, loss of *arr-1* does not cause chemosensory defects in *C. elegans*. The roles of GRKs and arrestins may be distinct in *C. elegans* chemosensation, or there could be other signaling molecules (besides ARR-1) that couple to Ce-GRK-2 or to the phosphorylated receptors to act positively in chemosensory signal transduction. Our data also does not rule out the possibility that Ce-GRK-2 could have a positive role in chemosensory signal transduction on its own, an intriguing possibility as GRKs have not yet been shown to have a positive signaling role independent of arrestins in any system.

GRK activity and/or regulation has been implicated in several human diseases, including cancer, opiate addiction, hypertension, and chronic heart failure (Gros et al., 1997; Metaye et al., 2002; Terwilliger et al., 1994; Ungerer et al., 1994). Additionally, mutations in GRK1 or visual arrestin result in Oguchi disease, a hereditary form of night blindness (Cideciyan et al., 1998; Yamamoto et al., 1997). In these patients, the inability to desensitize photoreceptors can ultimately lead to retinal degeneration. It is therefore of great importance to address the mechanisms by which GRKs function and regulate GPCR signaling. Further characterization of *C. elegans* GRKs may lead to therapeutic intervention in disease states where disruption or enhancement of GPCR signaling causes altered cellular physiology.

Experimental Procedures

Strains

Strains were maintained under standard conditions (Brenner, 1974). The strains include: N2 Bristol wild-type, CB4856 Hawaiian, HA865 *Ce-grk-2(rt97)* III, GE24 *pha-1(e2123)*, HA1203 *rtIs25* X, RB660 *arr-1(ok401)* X, CB1467 *him-5(e1467)*, CB0224 *dpy-11(e224)*, HA1379 *Ce-grk-2(rt97); mIs11; him-5(e1467)*, SP529 *unc-45(e286) dpy-1(e1)*, KP623 *ncl-1(e1865) glr-1(n2461)*, CX3390 *odr-3(n1605)*, CX10 *osm-9(ky10)*, DA702 *eat-16(ad702)*, and MT8504 *egl-10(md176)*. Also see Supplemental Data at <http://www.neuron.org/cgi/content/full/42/4/581/DC1>.

Genetic Analysis

ncl-1(e1865) glr-1(n2461) animals were mutagenized with EMS (ethyl methanesulfonate) (Brenner, 1974). F2 animals that wandered off the bacterial lawn were assayed for octanol avoidance; those that failed to respond within 20 s were selected. 7400 F2 animals were screened; 6 mutant strains were isolated including *rt97*. The *rt97* mutation was outcrossed 4 times, and *ncl-1 glr-1* mutant alleles

were removed prior to analysis. *rt97* animals are mildly egg-laying defective, slightly short and lethargic.

rt97 was linked to III L using RW7000 (Williams et al., 1992) and SP529 *unc-45 dpy-1*. Recombination mapping utilized *unc-45 rt97 dpy-1* and the Hawaiian SNP strain. W02B3 was injected at 8 ng/ μ l. The *Ce-grk-2* genomic rescue fragment was obtained by amplifying nt6400-nt13479 of W02B3 using the Expand Long Template PCR Kit (Roche) and was injected at 5–10 ng/ μ l.

The *arr-1(ok401)* deletion allele was obtained from the *C. elegans* Gene Knockout Consortium and outcrossed 6 times by N2. The deletion removes nt13626-nt14317 of F53H8. Only the predicted truncated *arr-1* transcript was detected in *arr-1(ok401)* animals by RT-PCR.

Behavioral Assays

Well-fed, young adult animals were used for behavioral assays. Octanol avoidance assays were performed as described (Troemel et al., 1995). Animals were tested off food, 10–20 min after transfer to NGM plates lacking bacteria, unless indicated. For on food octanol assays, 300 μ l of an overnight OP50 bacterial culture was spread onto NGM plates and dried overnight. Animals were assayed 10–20 min after transfer onto these plates.

Drop tests for osmotic avoidance and quinine avoidance were performed as described (Hilliard et al., 2002), except that the drop was placed directly in front of the animal. Nose touch assays and volatile chemotaxis assays were performed as previously described (Bargmann et al., 1993; Kaplan and Horvitz, 1993). All behavioral assays were repeated on at least two separate days in parallel with controls.

For heat shock experiments, animals were raised to young adulthood, then shifted to 33°C for 2 hr. They were allowed to recover at 25°C for 4 hr and tested.

Supplemental Data

For information on transgenic strains, immunohistochemistry, microscopy, Western blotting, plasmid construction, and calcium imaging, see Supplemental Data at <http://www.neuron.org/cgi/content/full/42/4/581/DC1>.

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