The Signaling Pathway of *Caenorhabditis elegans* Mediates Chemotaxis Response to the Attractant 2-Heptanone in a Trojan Horse-like Pathogenesis*  

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The nematode *Caenorhabditis elegans* exhibits behavioral responses to a wide range of odorants associated with food and pathogens. A previous study described a Trojan Horse-like strategy of pathogenesis whereby the bacterium *Bacillus natto*roida* B16 emits the volatile organic compound 2-heptanone to trap *C. elegans* for successful infection. Here, we further explored the receptor for 2-heptanone as well as the pathway involved in signal transduction in *C. elegans*. Our experiments showed that 2-heptanone sensing depended on the function of AWC neurons and a GPCR encoded by str-2. Consistent with the above observation, the HEK293 cells expressing STR-2 on their surfaces showed a transient elevation in intracellular Ca2+ levels after 2-heptanone applications. After combining the assays of RNA interference and gene mutants, we also identified the Gα subunits and their downstream components in the olfactory signal cascade that are necessary for responding to 2-heptanone, including Gi subunits of egl-30 and gpa-3, phospholipase C of plc-1 and egl-8, and the calcium channel of cmk-1 and cal-1. Our work demonstrates for the first time that an integrated signaling pathway for 2-heptanone response in *C. elegans* involves recognition by GPCR STR-2, activation by Gα subunits of egl-30/gpa-3 and transfer to the PLC pathway, indicating that a potentially novel olfactory pathway exists in AWC neurons. Meanwhile, since 2-heptanone, a metabolite from the pathogenic bacterium *B. natto*roida* B16, can be sensed by *C. elegans* and thus strongly attract its host, our current work also suggested coevolution between the pathogenic microorganism and the chemosensory system in *C. elegans*.

Because *Caenorhabditis elegans* lives in soil inhabited by various microorganisms, the capability to sense environmental stimuli is essential for the survival of the animal, including differentiating pathogens from food bacteria (1, 2). Generally, the discrimination of pathogens mainly depends on the 12 pairs of amiphid chemosensory neurons with cilia structures (3, 4). Among the *C. elegans* chemosensory systems, the olfactory system is presumed to be one of the most sensitive and effective tools because of its specificity in detecting volatile chemicals at a distance. Furthermore, it has also been shown that subtle alterations in chemical structures among different odorants can result in substantial differences in perceived olfactory quality, which helps animals navigate in their environment and allows them to generate diverse behavioral responses to odorants. To achieve this remarkable discriminatory power of the olfactory system, *C. elegans* employs at least three pairs of olfactory neurons, including the AWA, AWB, and AWC neurons, to detect olfactory stimuli (5). In most cases, the worms detect attractive compounds emitted by food mainly using the olfactory neurons AWA and AWB (6, 7); while AWB neurons are responsible for sensing repulsive volatile chemicals that may be produced by pathogens (8, 9). Though part of the odorant specificity might be due to the odorant differences detected by AWA and AWC, or the functional asymmetry between the left and the right AWC, these two factors cannot account for all the odorant specificity, suggesting that additional intracellular mechanisms exist to establish odorant specificity (10, 11).

Intracellular components are thought to account for the majority of discriminatory power, and some of them have been identified through the genetic analysis of mutations that affect olfactory sensing of specific chemicals. However, until now, ODR-10 and the attractant diacetyl are the only known G protein-coupled receptor (GPCR)2 and its specific ligand in *C. elegans*. Worms with the odr-10 mutation have a 100-fold-reduced sensitivity to the volatile attractant diacetyl, but retain a normal chemotaxis to other odorants detected by the AWA or AWB neuron (7, 12). Despite the involvement of different molecules, a common pathway for olfaction transduction in either AWA or AWB has been proposed, which consists of recognition by specialized GPCRs, activation of the G protein, and transduction of the odorant signal to channel proteins (5). However, the integration of the specific signaling molecules, the individual sensory receptor that responds to its ligands and the signal cascade still require further elucidation.

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2 The abbreviations used are: GPCR, G protein-coupled receptor; CNG, cyclic nucleotide-gated channel; PLC, phospholipase C.
The chemotaxis of *C. elegans* toward food bacteria has been described by detecting both water-soluble and volatile clues. Consistently, the volatile compounds that have been found to be attractive to worms are mostly natural products of bacterial metabolism, including alcohols, ketones, aldehydes, esters, amines, sulfides, sulfuric acids, and aromatic and heterocyclic compounds (6, 13). Our previous work reported a bacterial strain, *B. nematocida* B16, which can infect and kill *C. elegans* using the strategy of a “Trojan horse” (14). Potent volatile organic compounds produced by this bacterium are more attractive to worms than those from ordinary dietary bacteria, luring the nematode and permitting successful infection. Among volatile organic compounds, 2-heptanone belonging to methyl ketone, a compound found widely in nature, has been reported to serve as a biological pheromone in some species (15). Our previous work has revealed an interesting phenomenon: the fatal pathogenic bacterium *B. nematocida* 16 can initiate this beneficial signal to trap animals. The interaction that the bacterial pathogens produce the potent signal to lure its hosts and the worms sense this attractant to be trapped suggests an interesting coevolution between the pathogenic microorganism and the chemosensory system in *C. elegans*.

The volatile compounds of 2-heptanone has also been shown as one of the most important attractants since the absence of 2-heptanone would significantly weaken the capability to attract nematodes (35). In our current investigation, we explored the receptor and transductory pathway in *C. elegans* using 2-heptanone as a ligand. Our experiments showed that the detection of 2-heptanone depended on the function of a GPCR encoded by *str-2* that expressed on the AWC neuron (36). We also identified the Gα subunits and their downstream components in the olfactory signal cascade that are necessary for responding to 2-heptanone. The olfactory pathway in the chemosensory neuron AWC includes Gα subunits of *egl-30* and *gpa-3*, phospholipase C of *plc-1* and *egl-8*, and the calcium channel of *cnc-1* and *cal-1*, which differs from the previously reported transductory pathway in AWC mediated by the cyclic nucleotide-gated channel (CNG) consisting of TAX-4 and TAX-2 subunits. Therefore, our current investigation proposes a potentially novel olfactory signal pathway for 2-heptanone sensing in AWC olfactory neurons.

### Results

**C. elegans Detects 2-Heptanone Depending on Functions of AWC Olfactory Neurons**—Chemotaxis of *C. elegans* was measured by establishing a gradient of attractant from a point source and observing the accumulation of animals at the attractant source. Because the volatile organic chemical 2-heptanone diluted by ethanol was spotted on the surface of the agar, we spotted ethanol on the other side of the agar as a control for the effect of ethanol. Since *C. elegans* adapts to an attractant after prolonged exposure, the steady-state number of animals at an attractant reflected the result of both attraction and adaptation processes. To minimize the effects of adaptation, we did pre-experiments to calculate the time of the process. It was found that the worms immediately oriented their movements and then they congregated at the 2-heptanone, and the accumulation of worms at 2-heptanone would be complete within 1 h (Fig. 1A). The rapid response of *C. elegans* to 2-heptanone suggested that 2-heptanone diffused into the air where it could be detected by the nematodes. To verify this possibility, 2-heptanone was spotted on the lid of the Petri plate so that the attractant was not in direct contact with the agar. The animals in the assays moved toward the source of 2-heptanone immediately, confirming that 2-heptanone was sensed by olfactory chemosensory neurons as a volatile signal. To further characterize the responses to volatile attractants, dose assays for 2-heptanone were performed. It was indicated that the animals responded well to 1 μl of attractants at either $10^{-1}$ dilutions or $10^{-2}$ dilutions ($7.2 \times 10^{-1}$ mol/liter or $7.2 \times 10^{-2}$ mol/liter).

In *C. elegans*, two pairs of olfactory neurons, AWA and AWC, were in charge of detecting the attractive odors (6, 7, 10). Therefore, to identify which olfactory neurons were essential for chemotaxis to 2-heptanone, we first tested the chemotaxis to 2-heptanone using the RNAi method of knocking down the genes *odr-7* and *osm-9* required for the function of AWA, and the genes *odr-1, tax-2*, and *tax-4* required for AWC. Our results showed that genes responsible for AWC olfactory neurons, including *odr-1, tax-2*, and *tax-4*, decreased the chemotaxis to 2-heptanone (data not shown). Then we further validated the chemotaxis to 2-heptanone using the mutants of *odr-1* (n1936) and *odr-7* (ky4). Our results showed that the *odr-1* mutation animals were defective in sensing 2-heptanone at dilutions of $10^{-1}$ and $10^{-2}$, while the *odr-7* mutant retained the capability of sensing 2-heptanone (Fig. 1B). Furthermore, the mutant of *celh-36* (ks86), which lacked AWC chemosensory neurons, was also assayed for the chemotaxis to 2-heptanone. The result from the *celh-36* (ks86) mutation was similar to that from *odr-1* (n1936), suggesting that the defect in AWC neurons would impede the sensing of 2-heptanone (Fig. 1E).

**GPCR STR-2 of AWC Neuron Is Required for Sensing 2-Heptanone**—GPCRs have been reported as the most common olfactory receptors in *C. elegans* and mammalians. The genome of *C. elegans* encodes more than 500 putative GPCRs (2, 8, 16). Because some GPCRs that express asymmetrically on the chemosensory neurons are usually localized in the cilia, possess variable expression levels, and are highly divergent between *C. elegans* and other species, it is reasonable to hypothesize that GPCRs enable the different chemotaxis responses for different chemical molecules in *C. elegans* (2, 5).

Because two known GPCRs, including *STR-2* and *SRA-13*, have been demonstrated to be expressed in AWC olfactory neurons, the worms with the mutation of *str-2* (ok3148) and *sra-13* (zh13) were tested for chemotaxis to 2-heptanone. It was shown that the animals with *str-2* (ok3148) mutation were defective in sensing 2-heptanone; but the animals with *sra-13* (zh13) mutation retained a normal response to 2-heptanone (Fig. 1C). To further confirm the specificity between the receptor *STR-2* and its signal molecule 2-heptanone, the mutant of *str-2* (ok3148) was tested for chemotaxis to benzaldehyde, the other attractant sensed by AWC olfactory neurons, and to 2-ethyl alcohol, an attractant sensed by AWA. Consistently, our data illustrated that mutation of *str-2* had no effects on worms sensing benzaldehyde and 2-ethyl alcohol, suggesting that the response to 2-heptanone mediated by the receptor *STR-2* is specific (Fig. 1D).
STR-2 has been reported to be asymmetrically expressed in one of AWC neurons (AWCon) (17, 18). Thus, the mutants of *nsy-1* (ok593) and *inx-19* (ky634), which had two AWCon or two AWCoff, respectively, were employed to clarify the roles of STR-2 involved in 2-heptanone sensing. Compared with the wild-type worms, the animals with *nsy-1* (ok593) mutation, which possessed two AWCon neurons, represented comparable sensitivity to 2-heptanone (*p* > 0.05) (Fig. 1E). Contrarily, the worms with *inx-19* (ky634) mutation lost the ability to respond to the odor of 2-heptanone because of the lack of expression of STR-2 (*p* < 0.01) (Fig. 1E). On the other hand, to determine whether the expression of STR-2 in AWC neurons could be induced by its signal 2-heptanone, we used the worms of *kyIs140 [str-2::GFP + lin-15(+)]* to observe the changes in STR-2 expression. It was found that the STR-2 indeed was asymmetrically strongly expressed in one AWC neuron and weakly expressed in ASI neurons that sense the situations such as crowding and starvation during dauer formation, which is consistent with the previous report (Fig. 2B) (19). Furthermore, the fluorescence of the STR-2::GFP in AWC off neuron was enhanced after adding the signal molecule 2-heptanone (Fig. 2C).

Finally, to confirm STR-2 protein is an odorant receptor for 2-heptanone, it was necessary to express this GPCR on the membrane of mammalian cell line HEK293T and then observe the cell response. Therefore, we transiently transfected mammalian cells with a plasmid encoding STR-2 protein with a myc-epitope tag at its N terminus (*str-2*-pCMV-Tag 3C), which was
expected to be exposed to the external face of the plasma membrane. To evaluate STR-2 expression, immunofluorescent staining was performed, and the result demonstrated that myc-STR-2 was heterologously expressed and was mainly located on the cell membrane (Fig. 3A). To assay STR-2 function, we measured intracellular Ca\(^{2+}\) levels using fura-2-based Ca\(^{2+}\) imaging. Upon perfusion of 2-heptanone into HEK293T cells that had been transfected with the plasmids encoding myc-STR-2, a transient increase in intracellular Ca\(^{2+}\) was observed in cells (Fig. 3B). The graphs of time-dependent changes in intracellular Ca\(^{2+}\) suggested that the Ca\(^{2+}\) elevation response was maximal about 3.5 min after the onset of 2-heptanone application (at 90 s) and then decayed within 1.5 min; but such responses were never seen in the negative control transfected with the blank vector (Fig. 3C). Quantitative analysis of 800 cells having myc-STR-2 expression or the blank vector revealed statistically significant changes in fluorescence when the cells with myc-STR-2 sensed 2-heptanone (Fig. 3D). Similarly, to further confirm the specificity between the receptor STR-2 and its signal transduction, we replaced 2-heptanone with benzaldehyde. As expected, the cells expressing myc-STR-2 exhibited no changes of intracellular Ca\(^{2+}\) under the stimulation of benzaldehyde (Fig. 3E).

The G Protein \(\alpha\) Subunits of GPA-3 and EGL-30 Mediate the Signal Pathway Sensing 2-Heptanone—The olfaction in \(C.\) \textit{elegans} is transduced from environmental stimuli to intracellular by the heterotrimeric G protein-coupled signal pathway. The genome of \(C.\) \textit{elegans} encodes 21 \(G\) a subunits that play an active role in the G protein signaling cascades, and therefore we tested the changes of chemotaxis to 2-heptanone with the 21 \(G\) a gene mutations. Our results showed that three \(G\) a mutations have effects on the chemotaxis to 2-heptanone, including \(odr-3\) (n2130), \(gpa-3\) (pk35), and \(egl-30\) (ad805) (Fig. 4A).

\textit{ODR-3} has been thought of as the main \(G\) a mediating the olfactory signal pathway, and it has been described to disturb the development of cilia in chemosensory neurons. The proper structure of cilia is required for chemosensory neurons to detect most odors, hence we assayed the chemotaxis of the mutant \(odr-3\) (n2130) to some other attractive molecules of benzaldehyde and 2-ethyl alcohol besides 2-heptanone. The mutation of \(odr-3\) indeed disturbed the detection of the other two odors (Fig. 4B), consistent with the results previously described. However, it was noticed that \(odr-3\) RNAi had a normal chemotaxis to 2-heptanone as well as to the other two tested odors, benzaldehyde and 2-ethyl alcohol (Fig. 4B). Since the different results we had obtained from the mutant \(odr-3\) (n2130) and \(odr-3\) RNAi, we employed qPCR experiment to validate the effect of \(odr-3\) knock-down. Our data of qPCR obviously demonstrated that, compared with the negative control, the expression level of \(odr-3\) was retained less than half in the animals of \(odr-3\) RNAi (Fig. 4C). Considering that \(odr-3\) RNAi was performed after L1 stage, and it should have little effect on cilia development, it is reasonable to speculate that, besides the potential role in transducing the signal for sensing 2-heptanone, the disturbance of cilia development may be responsible for the defect in chemotaxis for all the tested odors.

To further confirm the involvement of \(egl-30\), we rescued the expression of \(egl-30\) under the control of the \(sr1-3\) promoter, which initiated gene expression specifically in AWC neurons. Most of the chemotaxis to 2-heptanone was rescued in the transgenic animals (Fig. 4D), suggesting that the \(G\) a subunits \(EGL-30\) participated in mediating the olfactory signal of 2-heptanone.

We tested the chemotaxis for the \(gpa-3\) (pk35) mutation together with \(egl-30\) RNAi or \(egl-30\) (ad805) mutation together with \(gpa-3\) RNAi, and the change in the chemotaxis was cumulative (Fig. 4E), suggesting that \(GPA-3\) and \(EGL-30\) can activate the downstream molecules independently.

PLC Pathway Functions in the Olfactory Signal Pathway—Stimulation of a GPCR and its downstream G protein can activate some classical signaling cascades, including PKA, PLC, and cGMP pathways. Next, we tested the effects on chemotaxis to 2-heptanone of the genes located in the three pathways.

In our tested genes in PKA, PLC, and cGMP pathways, we first excluded the involvement of PKA because the mutation of genes, such as \(kin-2\) (ce179), had no defect to sense 2-heptanone. In PLC pathway, we screened the mutant genes \(egl-8, plc-1, plc-2, plc-3, itr-1, cal-1, cal-2,\) and \(cmk-1\). Our experimental results showed that, among all genes tested, the mutants or RNAi of genes \(egl-8\) (n488), \(plc-1, itr-1\) (sa73), \(cal-1,\) and \(cmk-1\) (oy21) affected the sensing of 2-heptanone (Fig. 5A).

Downstream of the PLC pathway, the \(Ca^{2+}\) influx can be observed since the endoplasmic reticulum releases \(Ca^{2+}\) to the cytoplasm. To further confirm that PLC pathway mediated the olfactory signal transduction for sensing 2-heptanone, we determined \(Ca^{2+}\) release using the strain carrying \(Ca^{2+}\) reporter GCaMP3 under the control of the AWC-specific promoter of \(str-2\) gene. After the addition of 2-heptanone, our results showed that the AWC neurons responded to 2-heptanone by a change in intracellular \(Ca^{2+}\) concentration, suggesting the molecule of 2-heptanone could be transduced by...
the olfactory signal pathway of PLC as well as the intracellular Ca\(^{2+}\) (Fig. 5B).

However, to our surprise, the animals with odr-1, daf-11, tax-2, and tax-4 mutations, which belong to the genes in cGMP pathway, were also defective in chemotaxis to 2-heptanone (Fig. 6A). The cyclic nucleotide-gated channel encoded by the tax-4 and tax-2 genes has been reported to be essential for the chemotaxis of AWC to a variety of volatile odors (20-22). The other two genes, daf-11 and odr-1, encoding the receptor guanylate cyclase in AWC olfactory neurons, likely function as heterodimers to synthesize cGMP for the opening of the TAX-4/TAX-2 channel (23-25). Hence, another possibility was raised because it had been reported that the genes odr-1/daf-11 and tax-2/tax-4 were necessary for maintaining the expression of the gene str-2 in *C. elegans* (26, 27). To test the hypothesis, we first determined if the expression level of odr-1 could be induced by the signal molecule of 2-heptanone. Compared with the negative control without signal molecule, it was found that the addition of 2-heptanone could not enhance the changes of odr-1 expression in AWC neurons (Fig. 6B). Then we further assayed the changes of STR-2 in the worms with odr-1 RNAi. It was observed that str-2::GFP expressed weakly both with and without the addition of 2-heptanone (Fig. 6C). However, when animals of odr-1 RNAi were cultured on media with extra cGMP addition, the str-2::GFP expression was rescued and the animals also presented a relatively normal chemotaxis to 2-heptanone (Fig. 6D). Finally, we expressed str-2 using the srsx-3 promoter in odr-1 (n1936), and the chemotaxis to 2-heptanone was rescued in the transgenic animals (Fig. 6E). Collectively, our current data demonstrated that those genes, including guanylyl cyclases odr-1 and daf-11 as well as cGMP gated cation subunits tax-2 and tax-4, seemed to play more important roles in maintaining str-2 expression to influence the olfactory signal transduction of sensing 2-heptanone.

**Discussion**

It is well known that *C. elegans* has chemotaxis to a variety of volatile compounds, which can mainly be mediated by the olfactory neurons. The olfactory responses for the different odors are associated with the most powerful behaviors in worms, such as pursuing food and escaping pathogens. Among those olfactory responses, *C. elegans* mainly employs four cells (left and right AWA or AWC cells) to sense and discriminate among a variety of volatile attractants (11). Previous studies also have revealed that two distinct pathways of olfactory signal transduction are employed in these two types of neurons based
on the analyses of gene mutants of *C. elegans*. Besides the shared GPCRs and Go subunits, the opening of a cyclic nucleotide-gated channel (CNG) in AWC and a transient receptor potential vanilloid (TRPV) channel in AWA ultimately triggers the olfaction.

Regardless of whether either AWC or AWA is involved, the binding of odorant molecules (ligands) to specific receptors on the external surface of cilia is generally the first step for initiation of olfactory transduction. The *str-2* gene encodes a seven-transmembrane domain protein, a member of the *str* family of chemosensory receptors, asymmetrically expressed at the top of cilia in AWCon neurons and weakly expressed in the ASI neurons. Mutations in the *str-2* gene obviously affect chemotaxis toward 2-heptanone, but had little effect on sensing benzaldehyde whose recognition also requires AWC function. Certainly, our data have shown that the seven-transmembrane receptor STR-2 was responsible for sensing the volatile odorant 2-heptanone when heterologously expressed in human HEK293 cells, and the behavioral response of STR-2 to 2-heptanone should be highly selective in vivo.

Heterotrimeric G protein-coupled signaling cascades are used to transduce olfactory signals varying from intercellular mediators to environmental stimuli. It has been revealed that a variety of Go subunits, including odr-3, gpa-3, gpa-5, and gpa-6 in AWA neurons as well as odr-3, gpa-2, gpa-3, and gpa-13 in AWC neurons, play either positively or negatively in chemosensation (16, 28–30). Among them, *gpa-3* gene was expressed in cilia, cell bodies and axons of AWA and AWC neurons. Since loss of gpa-3 activity exacerbated the chemotaxis defect of *odr-3* (lf) mutants to all odorants, its function has been suggested to be redundant to ODR-3 (30, 32). In our current study, genes *gpa-3* and *egl-30* influenced animals’ response to 2-heptanone, and the double mutant worms almost lost all chemotaxis to 2-heptanone. Compared with *gpa-3*, *egl-30* was more widely expressed. However, when we specifically rescued the expression of *egl-30* in AWC neurons, the chemotaxis of transgenic animals was restored, confirming that *egl-30* in AWC neurons was required for chemotaxis to 2-heptanone. Therefore, two Go subunits, *gpa-3* and *egl-30*, were sufficient for the olfactory transduction pathway of detecting 2-heptanone.

**FIGURE 4. G protein α subunit regulates the chemotaxis to 2-heptanone.** A, Go-mutant animals have different chemotaxis to 2-heptanone, and the genes of *gpa-3*, *odr-3*, and *egl-30* seriously decreased the chemotaxis to 2-heptanone. B, Odr-3 mutants were defective in chemotaxis to 2-heptanone, but animals of odr-3RNAi responded to 2-heptanone normally. C, qPCR validated the decreased the expression of *odr-3* in the worms of odr-3RNAi. D, most of the chemotaxis to 2-heptanone was rescued in the transgenic animals of *Psrx-3:egl-30*. E, effects of gene *gpa-3* and *egl-30* were cumulative and affected the chemotaxis for 2-heptanone more severely. The statistical differences were analyzed using one-way ANOVA, n.s., *p* > 0.05; **, <0.05; ***, *p* < 0.01.
The gene odr-3 has commonly been considered the major Gα gene for olfactory transduction in either AWC or AWA, but its role in detecting 2-heptanone seemed different. Though the method of RNAi in neurons was believed to have less obvious effects than in other organs of *C. elegans*, we think, the intact response of animals treated with ord-3 RNAi was not due to the inadequately knocked-down because using the RNAi method of knocking down the genes odr-1, tax-2, and tax-4 decreased the chemotaxis to 2-heptanone. Additionally, the gene of odr-3 is involved in cilia morphogenesis in olfactory neurons, and its amount determined the extent of ciliary outgrowth in AWA and AWC neurons. For example, it has been proposed that ODR-3 expression defined the unique fan-like extension of the AWC olfactory cilia (29). Thus it is reasonable for us to speculate that the ODR-3’s function for maintaining the AWC cilia structure should be partially responsible for the deficiency to sense 2-heptanone besides its potential role in transducing the signal.

In the olfactory signal cascade in *C. elegans*, the ion channel is the key component in the last step to sense and transduce receptor activity into electrical activity in olfactory cells (20, 33). Here, a series of signal molecules, such as egl-8, plc-1, itr-1, cmk-1, and cal-1 have been shown to influence the chemotaxis to 2-heptanone, and therefore the PLC pathway, instead of the individual sensory neuron AWC<sup>on</sup> sensing 2-heptanone. So far, the integration of a specific signaling pathway, including the individual sensory receptor that responds to its ligands as well as the downstream signal cascade, was suggested for sensing 2-heptanone (Fig. 7), which might be a potentially novel olfactory signal pathway existing in AWC olfactory neurons. Additionally, since 2-heptanone, a metabolite from the pathogenic bacterium *B. nematocida* B16, can be sensed by *C. elegans* and thus strongly attract its host (5), our current work also suggested coevolution between the pathogenic microorganism and the chemosensory system in *C. elegans*.

**Experimental Procedures**

**Strains and Culture**—All strains were derived from the wild-type *C. elegans* variety Bristol, strain N2. Mutant strains used in this study, including odr-1(n1936), odr-7(ky4), nsy-1(ok593), inx-19(ky634), ceh-36(ks86), daf-11(m47), tax-2(ks10), tax-4(ks28), str-2(ok3418), sra-13(zh13), gpa-1(pk15), gpa-2(pk16), gpa-3(pk35), gpa-4(pk381), gpa-5(pk376), gpa-6(pk480), gpa-7(pk61), gpa-8(pk345), gpa-9(pk348), gpa-10(pk362), gpa-11(pk349), gpa-12(pk322), gpa-13(pk1270), gpa-14(pk342), gpa-15(pk477), gpa-16(ks2349), gpa-17(ok2334), egl-30(ks805), odr-3(n2130), itr-1(sa73), kin-2(ce179), egl-8(n488), cmk-1(oy21), and kyls140 [str-2::GFP + lin-15(+)], were purchased from the Caenorhabditis Genetics Center at the University of Minnesota, Minneapolis, MN. *CX11935KyEx3252* was a generous gift from Professor C. I. Bargmann.

The wild type N2 and mutant strains were grown to adulthood on NGM (50 mM NaCl, 20 g/liter of agar, 2.5 g/liter of peptone, 1.0 mM cholesterol, 1.0 mM CaCl<sub>2</sub>, 1.0 mM MgSO<sub>4</sub>, and 25 mM potassium phosphate at pH 6.0) seeded with *Escherichia coli* OP50 under well-nourished conditions at 20 °C using standard methods.

**Chemotaxis Assays**—All of the attractants were obtained from Sigma Chemicals. Chemotaxis assays were modified based on the assays of Ward (34) and Bargmann and Horvitz (31). Assay plates were 10-cm tissue culture dishes containing...
FIGURE 6. The genes in the cGMP pathway have effects on the olfactory signal transduction of sensing 2-heptanone via maintaining the expression level of GPCR gene str-2. A, gene mutations located on the cGMP pathway, including odr-1, daf-11, tax-2, and tax-4, led to the deficiencies in response to 2-heptanone. B, result from the fluorescence of odr-1::RFP showed that the addition of the molecule of 2-heptanone could not induce the expresional increase of gene odr-1. C, expression of GPCR STR-2 was dependent on the intact of gene odr-1. The fluorescence of str-2::GFP was fairly weak under the background of odr-1 RNAi regardless of the level of 2-heptanone. D, cGMP pathway has effect on sensing 2-heptanone via maintaining the expression level of GPCR gene str-2. Additional cGMP rescued the expression level of gene str-2 under the background of odr-1 RNAi. The changes of fluorescence were taken at 45 min after 2-heptanone application. The statistical differences were analyzed using one-way ANOVA, ***,$p < 0.01$.

FIGURE 7. A potentially novel olfactory signal pathway existing in AWC olfactory neurons. The pathway of sensing 2-heptanone was affected by genes located on the PLC and cGMP pathways. Between them, the genes in the PLC pathway, such as phospholipase C of plc-1 and egl-8, calcium channel of cmk-1 and cal-1, were expected to be responsible for transducing the specific olfactory signals of 2-heptanone. However, the genes of cGMP pathway seemed to play more important roles in maintaining str-2 expression to influence the olfactory signal transduction.
Chemotaxis Response to the Attractant 2-Heptanone

10 ml of 1.6% agar. Two marks were made on the back of the plate at opposite sides of the plate about 0.5 cm from the edge of the agar. About 1 µl of attractant diluted in ethanol was placed on the agar over one mark, and 1 µl of ethanol was placed as the counter-attractant over the opposite mark. One microliter sodium azide with the concentration of 1 M was also placed at both the attractant source and the counter-attractant source. This drug could anesthetize animals within about a 0.5-cm radius of the attractant. Since it was found that the chemotaxis index of worms would reach the maximum and stable after 45 min, the chemotaxis assays were generally finished within 1 h.

During the assay, the well-fed adult animals were washed three times with M9 Buffer and once with water to free them of bacteria. Then the worms were placed near the center of a plate equidistant from the attractant and the counter-attractant. At 1 h after the assay began, the numbers of animals at the attractant and the counter-attractant areas were determined. The chemotaxis index was calculated as (animal numbers at attractant/counter-attractant area) × (radius of attractant). Since it was found that the chemotaxis index of worms would reach the maximum and stable after 45 min, the chemotaxis assays were generally finished within 1 h.

RNA-mediated Interference—RNAi bacterial strains containing targeted genes were obtained from the Arrhinger RNAi library. RNAi feeding experiments were performed on synchronized L1 larvae at 20 °C until L4 larvae or young adult worms that were used in the chemotaxis assay.

Mammalian Cell Culture and Immunofluorescence—HEK293T cell lines were maintained in DMEM with 10% fetal calf serum and lacking pyruvate. Cells were plated onto poly-D-lysine-coated 15-mm glass coverslips in 6-well cell culture plates for 24 h before transfection. Lipofectamine 2000 (ThermoFisher Scientific) was used as the DNA carrier. Two micrograms of plasmid DNA (str-2-pCMV-Tag 3C or the negative control of blank vectors) were added to each culture dish individually, and then were incubated with the cells for 10 h at 37 °C. Two days after transfection, the cells were fixed and permeabilized with methanol at −20 °C for 20 min. Anti-c-myc was used at 1:500 dilution. The secondary antibody (goat anti-mouse H+L, PE conjugate) was used at a 1:100 dilution. Staining and visualization were carried out according to standard procedures.

Fura-2 Ca²⁺ Imaging—After 48 h of cell transfection, the cells were harvested and incubated in HBSS buffer (137 mM NaCl, 5.4 mM KCl, 4.5 mM glucose, 0.4 mM KH₂PO₄, 0.3 mM Na₂HPO₄, and 0.1% PluronicF-127, pH 7.2) containing 0.005 mM Fura-2-AM (Molecular Probes Life Tech) for 45 min at 37 °C. The mixture of HBSS buffer with Fura was then removed. Following three washes in HBSS buffer, the cells were incubated in HBSS buffer for another 4.5 h at room temperature. After the cells were washed again, the compounds for visualization were dissolved in dimethyl sulfoxide and finally mixed in HBSS buffer. Fluorescence imaging was carried out according to standard procedures.

Construction of Transgenic Worm Lines—The genes str-2 and egl-30 under the control of the srx-3 promoter were expressed in animals odr-1 (n1936) and egl-30 (ad805) respectively to generate rescue lines. The full encoding frames along with their 3’-UTR were obtained by PCR using N2 genomic DNA as a template. The resulting PCR products were purified from agarose gel by using a QIAquick Gel Extraction Kit (Qiagen). Then the purified PCR product, 10 ng/ml, was co-injected with pPD95.79. Two days after DNA injection, four worms expressing GFP were allowed to self-fertilize. The genotype of the transgenic lines was confirmed by PCR amplification of a portion of the gene using oligonucleotide primers.

Imaging Sensory-evoked Ca²⁺ Transients in C. elegans—To confirm that 2-heptanone was detected by the AWC olfactory neuron, we used the worm strain CX11935KyEx3252 (str-2: GCaMP3 10 ng/µl, coel:GFP 10 ng/µl) to observe transient Ca²⁺ in the AWC neuron in C. elegans. Animals were placed on the agar plate for the assay of chemotaxis, and 1 µl of 2-heptanone at 100× dilution was placed on the center of the plate. After 5 min, we picked signal animals to capture the image using fluorescence microscopy (Olympus, Melville, NY).

Quantitative Real-time PCR (qPCR)—The total RNA samples of worms, including the odr-3 RNAi and the negative control HT115 that was fed on the E. coli with blank vector, were extracted using the Trizol reagent from Invitrogen (Carlsbad, CA) at young adult. Random primed cDNA were obtained by reverse transcription of the total RNA samples with TransScript®Reverse Transcriptase from TransGen Biotech (Beijing, China). A real-time PCR analysis was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems) using SYBR® Premix Ex Taq™ II (Takara, Dalian, China). The primers for the target gene odr-3 used as follows: odr-3F: 5’-TATCTGAACCTGGAATCCG-3’ and odr-3R: 5’-GCTATGAGACCTCGTGAA-3’. The partial sequence of β-actin was used for an internal control, whose primers were 5’-GGATTCTCGAGAATCTCGGA-3’ and 5’-CATCCAGTGTCGCCATGT-3’. PCR amplification followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s on ABI PRISM 7000 Real-Time PCR. The real-time PCR experiments were repeated three times for each reaction using independent RNA sample.

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