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Olfactory Behavior of Swimming *C. elegans* Analyzed by Measuring Motile Responses to Temporal Variations of Odorants

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Luo L, Gabel CV, Ha H-I, Zhang Y, Samuel AD. Olfactory behavior of swimming *C. elegans* analyzed by measuring motile responses to temporal variations of odorants. *J Neurophysiol* 99: 2617–2625, 2008. First published March 26, 2008; doi:10.1152/jn.00053.2008. *Caenorhabditis elegans* responds to chemical cues using a small number of chemosensory neurons that detect a large variety of molecules in its environment. During chemotaxis, *C. elegans* biases its migration in spatial chemical gradients by lengthening (/shortening) periods of forward movement when it happens to be moving toward (/away) from preferred locations. In classical assays of chemotactic behavior, a group of crawling worms is placed on an agar plate containing a point source of chemical, the group is allowed to navigate for a period of time, and aggregation of worms near the source is quantified. Here we show that swimming worms exhibit acute motile responses to temporal variations of odor in their surrounding environment, allowing our development of an automated assay of chemotactic behavior with single-animal resolution. By placing individual worms in small microdroplets and quantifying their movements as they respond to the addition and removal of odorized airstreams, we show that the sensorimotor phenotypes of swimming worms (wild-type behavior, the effects of certain mutations, and the effects of laser ablation of specific olfactory neurons) are consistent with aggregation phenotypes previously obtained in crawling assays. The microdroplet swimming assay has certain advantages over crawling assays, including flexibility and precision in defining the stimulus waveform and automated quantification of motor response during stimulus presentation. In this study, we use the microdroplet assay to quantify the temporal dynamics of the olfactory response, the sensitivity to odorant concentration, combinations, and gradients, and the contribution of specific olfactory neurons to overall behavior.

INTRODUCTION

The olfactory system of the nematode *Caenorhabditis elegans* enables the worm to detect odors in its environment to mediate a range of developmental, physiological, and behavioral responses (Sengupta 2007). The worm's genome encodes as many as 1,000 G-protein-coupled receptor molecules that may serve as chemoreceptors to mediate the perception of a large odor space (Bargmann 1998). In insects and vertebrates, most chemosensory neurons express only one type of chemoreceptor molecule, facilitating the molecular and physiological analysis of the first stages of sensory processing as labeled lines (Hallem et al. 2006; Wilson and Mainen 2006). The worm, however, has only 32 neurons with ciliated endings exposed to the environment, morphological features consistent with chemosensation (Ward et al. 1975). Each worm chemosensory neuron may express several different chemoreceptor genes and a single chemoreceptor gene can be expressed in multiple neurons (Troemel et al. 1995). Many chemosensory neurons are interconnected by synapses, and downstream interneurons may integrate synaptic inputs from multiple chemosensory neurons (de Bono and Maricq 2005; Hobert 2003). Thus the worm is equipped with a sophisticated system for processing chemosensory input in a compact neural circuit, enabling it to meet the challenges of navigating chemical gradients in its natural environment, an inherently large behavioral task. As any animal moves toward or away from a chemical source, it must employ mechanisms for adapting to the local chemical concentration as well as mechanisms for calculating the direction of chemical gradients. To travel great distances toward chemical sources, an olfactory system must maintain sensitivity over a wide range of chemical concentrations, possibly spanning several orders of magnitude.

In classical chemotactic behavioral assays, a point source of a particular chemical is placed in the animal's environment, and aggregation at that source is quantified after some period of time. In this way, crawling *C. elegans* was discovered to aggregate near point sources of various soluble chemical attractants (Ward 1973). *C. elegans* performs chemotaxis toward or away from point sources of a variety of volatile organic molecules (Bargmann et al. 1993). A particular difficulty in using volatile odorants in aggregation assays is that the chemical gradients are difficult to characterize. Over the course of each experiment, odorant molecules are continuously evaporating from the source, moving in turbulent plumes, and diffusing back into the agar surface at all locations. Moreover, because each worm in each experiment takes its own trajectory, each worm has a unique olfactory experience in each experiment. Whereas successful aggregation at the source may indicate the proper workings of the entire chemosensory system, different problems with the chemosensory, decision-making, or motor systems can hinder aggregation, making it difficult to use classical behavioral assays to discriminate the roles of molecules and cells in chemotactic performance.

Important technical developments have occurred in analyzing worm chemotactic behavior. By tracking the movements of individual worms during chemotaxis in salt gradients, Pierce-Shimomura et al. showed that worms utilize a biased random walk strategy, alternating periods of forward movement (runs) and reorientation maneuvers (turns and pirouettes), extending runs that are pointed up chemical gradients and shortening runs that are pointed down chemical gradients (Pierce-Shimomura

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et al. 1999). Miller et al. showed that freely crawling worms respond to temporal variations in chemosensory input by monitoring the behavior of animals as they moved on substrates that were subjected to stepwise changes in chemical concentration, showing that approach and avoidance motile responses were evoked by increases and decreases in attractant concentration, respectively (Miller et al. 2005). Dusenbery and Faumont et al. invented "tethered worm" assays by holding individual worms by their heads or tails, quantifying forward and reverse movements during the delivery of chemical flows, also suggesting that worms respond to purely temporal changes in the concentration of ambient chemicals (Dusenbery 1980; Faumont et al. 2005). Chronis et al. recently invented microfluidic devices to simultaneously restrain individual worms and deliver chemical flows (Chronis et al. 2007). The advantage of tethered worm assays or microfluidic behavioral assays is that they allow extended observation of individual worms as the chemical environment is changed at will.

We have used a single-worm microdroplet assay to probe thermosensory behavior, putting individual worms in small microdroplets within an experimental chamber, monitoring their swimming movements using video microscopy, and detecting the rate of occurrence of reorientation maneuvers (Chung et al. 2006; Clark et al. 2007; Ryu and Samuel 2002). An advantage of the microdroplet assay is that worms swim freely within each microdroplet (a shortcoming of using tethers or microfluidic devices is that the worms might directly sense and respond to partial immobilization). Because worms never leave the field of view in the microdroplet assay, the behavior of each unrestrained animal can be recorded over an extended period of time. Because a grid of microdroplets can be used, a large number of worms can be simultaneously monitored as they respond to the same sensory input.

Here we show that the microdroplet assay can be used to quantify the behavior of *C. elegans* in response to olfactory inputs, delivered in airstreams carrying defined vapor concentrations of specific odorants. We use the microdroplet assay to quantify patterns in wild-type olfactory behavior as well as quantify the effects of mutations or cellular ablation of individual olfactory neurons.

METHODS

Strains

C. elegans strains were cultivated at 20°C following standard procedures. The wild-type N2 and mutant strain CX4 *odr-7(ky4)*X were obtained from Piali Sengupta (Brandeis University, Waltham, MA). The mutant strain CX5893 *ceh-36(ky646)*X and the CX3831 transgenic strain expressing the toxic gain-of-function degenerin gene *mec-4(d)* as well as GFP in the AWB neuron were obtained from Cori Bargmann (Rockefeller University, New York, NY). For AWA laser killing, we used a transgenic strain expressing green fluorescent protein (GFP) under the control of the *odr-10* promoter, obtained from Piali Sengupta. For AWC laser killing, we used a transgenic strain expressing GFP under control of the *odr-1* promoter. For AWB laser killing, we used a transgenic strain expressing GFP under control of the *str-1* promoter.

Laser ablation

Laser ablation of individual neurons was carried out using standard procedures (Bargmann and Avery 1995) except that a cavity-dumped Ti:sapphire femtosecond laser (KMLabs, Boulder, CO) was used to deliver the laser pulses instead of the more commonly used nanosecond pulsed dye laser (Chung et al. 2006). Neurons were killed in larvae anesthetized with sodium azide. The behavior of surgically operated worms was tested at the young adult stage. Destruction of the targeted neuron was double-checked by microscopy after behavioral assays.

Behavioral assay

We monitored the movements of individual worms swimming inside droplets of NGM buffer inside an experimental chamber that allowed computer-controlled olfactory stimulation (Fig. 1*A*). A computer captured images of the swimming worm at a rate of 10 Hz with a CCD camera. Video was analyzed by a computer algorithm that detects reorientation maneuvers on the basis of body extension (Chung et al. 2006; Clark et al. 2007). During forward swimming, the end-to-end distance of the worm oscillates around large values. During turns, the end-to-end distance drops sharply and briefly. The algorithm measured body extension in each video frame and flagged each dip in body extension below a threshold set to ~60% of maximum extension, thus differentiating sudden deep bends and turns from periods of forward swimming.

Olfactory stimulation was delivered in humidified, odorized airstreams at a flow rate of 2 l/min. The data-acquisition computer controlled solenoid valves to exchange airstreams flowing through one of two flasks before entering the experimental chamber. The flasks contained mineral oil with different dilutions of specific odorants. Nonodorized airstreams were produced by passing air through clean mineral oil. The vapor concentration of odorants in the airstream was controlled by varying the vol/vol odorant dilution in the mineral oil in the range 10^{-7} to 10^{-2}, in the manner described by Meister and Bonhoeffer (2001). The final vapor concentration was measured with a flame ionization detector (MicroFID, Photovac, Waltham, MA), and found to be nearly proportional to the dilution ratio D of either benzaldehyde or isoamyl alcohol. To obtain the approximate vapor concentration in ppm, multiply D by 70,000 for isoamyl alcohol, and 6,000 for benzaldehyde (for example, when $D = 10^{-4}$, the vapor concentration of isoamyl alcohol is ~7 ppm, the vapor concentration of benzaldehyde is ~0.6 ppm). Each trial lasted no more than 12 min, after which we replaced the mineral oil in each flask, replaced the tubing (Tygon tubing with chemically inert Teflon liner, VWR, West Chester. PA), and cleaned the experimental chamber by rinsing with EtOH and dH2O and placing it in a vacuum bell jar to remove all volatiles. We found that trials >12 min could lead to measurable odorant depletion and reductions in odorant concentration as measured using the flame-ionization detector. Within each 12-min trial, there was negligible variation in behavioral response between cycles of odorant inflow and outflow.

RESULTS

Swimming worms exhibit a bidirectional olfactory response to the additional and removal of attractive odors

C. elegans motility may be characterized as a random walk with successive periods of forward movement (runs) that are punctuated by the stochastic occurrence of reorientation maneuvers (turns and reversals) (Pierce-Shimomura et al. 1999; Ryu and Samuel 2002). During chemotaxis and thermotaxis, temporal variations in sensory inputs are transformed into variations in the stochastic rate of reorientation maneuvers, evoking (/suppressing) reorientation maneuvers when worms happen to be crawling toward worsening (/improving) conditions, allowing worms to migrate toward better environments over time. The sensorimotor behaviors of swimming worms

may be summarized in terms of reorientation rate, and quantified using a computer algorithm that automatically measures the body extension of individual worms within video frames (Chung et al. 2006; Clark et al. 2007).

First we asked whether our swimming assay reproduces the basic sensorimotor patterns that characterize crawling behavior. We subjected swimming worms to the addition and removal of airborne isoamyl alcohol (an attractant in crawling assays) provided in cycles of alternating streams of odorized air and clean air. Swimming worms responded to the inflow (/outflow) of airborne isoamyl alcohol by lowering (/raising) their turning rate. The variations in turning rate evoked by stepwise changes in the airstream are monophasic, reaching peak values at ~15–20 s following step changes before gradual adaptation to prestimulus turning rates (Fig. 1*B*). Thus the chemotactic response to isoamyl alcohol is bidirectional and exhibits short-term adaptation, making the worm sensitive to temporal gradients in olfactory inputs, and would allow crawling worms to bias their random walks up (/down) spatial gradients of attractive odor by shortening (/lengthening) each run.

In earlier experiments on thermotactic behavior, we quantified the turning response of worms to sudden temperature steps but found that the transient increase (/decrease) in turning rate evoked by upsteps (/downsteps) in temperature reach their peak values at ~5 s following the stimuli (Clark et al. 2007). The difference in response times between olfactory and thermosensory inputs is likely to reflect slowness in delivering the odorant stimulus not necessarily slowness in olfactory sensory transduction. In our thermotaxis experiments, we directly heated the droplets with controlled exposure to infrared laser irradiation. In these experiments, although the inflow and outflow of the odorant airstreams are provided in a stepwise manner, the odorant molecules themselves must diffuse into and out of the microdroplets. Our microdroplets are ~0.1 mm in depth, and small molecules in water have diffusion coefficients of $\sim 10^{-3}$ mm^2 s^{-1}, so the odorant concentration within the microdroplet requires ~10 s to equilibrate with the odorant concentration in the airstream, which coincides with the slowing of the response to stepwise changes of odorized and clean airstreams.

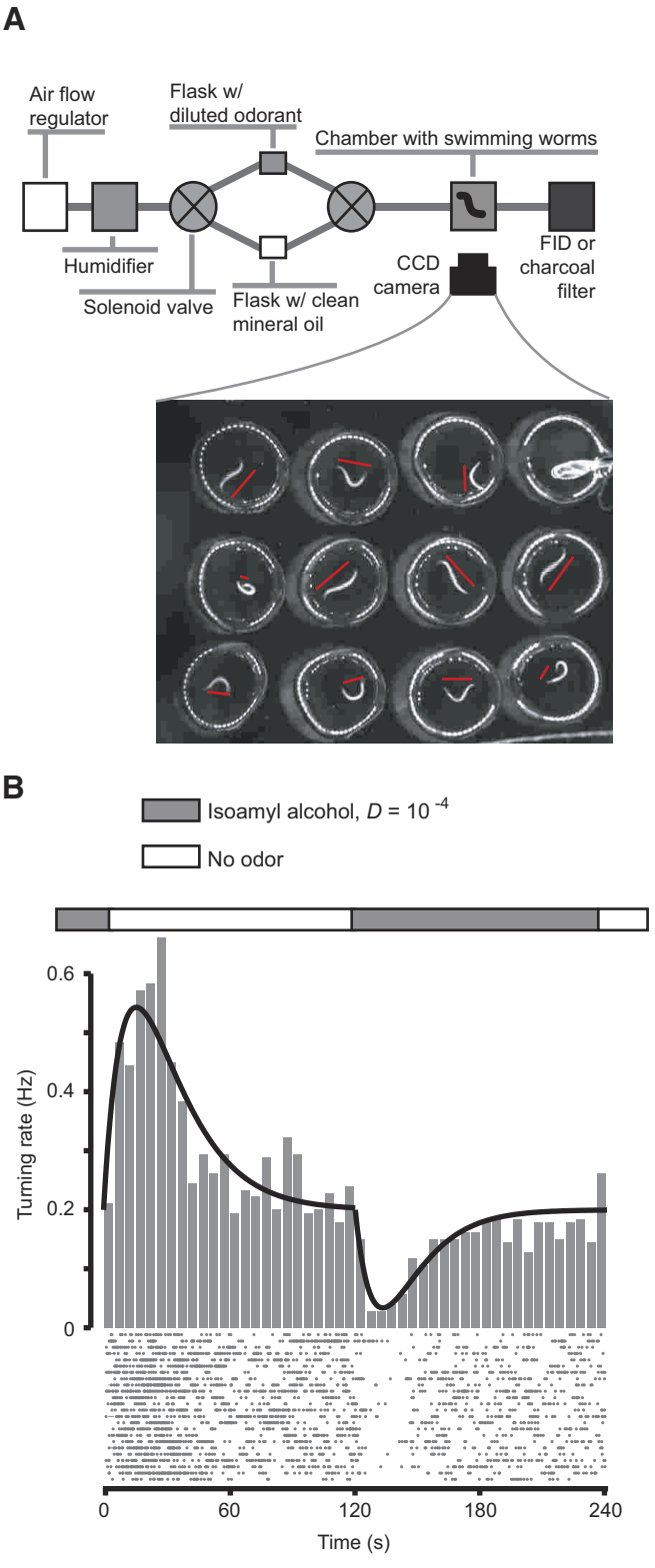


FIG. 1. Olfactory responses of swimming worms analyzed in a microdroplet assay. *A*: schematic of the microdroplet system to quantify the motile response of swimming worms subjected to defined olfactory inputs. Individual worms are placed in microdroplets within the experimental chamber. Humidified clean or odorized airstreams are directed into the experimental chamber using computer-controlled solenoid switches. The airstreams were odorized by passage through a flask containing mineral oil with a defined dilution of an odorant. Nonodorized airstreams were produced by passage through a flask containing clean mineral oil. The swimming movements of individual worms are recorded by CCD camera. A representative image is shown of a grid of microdroplets containing swimming worms (the top right droplet contains a microthermocouple, to monitor the temperature in each experiment). A machine-vision algorithm to measure the head-to-tail distance of each worm within each video frame (illustrated by red lines). During forward swimming, the head-to-tail distance oscillates around large values. When the head-to-tail distance dips below a threshold value, the algorithm flags a turn. *B*: the turning response of individual wild-type (N2) worms subjected to the alternation of 2 airstreams, 1 clean airstream and 1 airstream odorized with isoamyl alcohol. The vapor concentration in the odorized airstream is set by the dilution of isoamyl alcohol in the mineral oil, $D = 10^{-4}$, which corresponds to ~7 ppm, verified using a flame-ionization detector. Each raster plot shows the results of 24 independent representative experimental trials arranged vertically, each trial showing the turning response of 1 worm (1 dot represents 1 turn) during 1 cycle within a longer experiment. The histogram of turning events shows the continuous modulation of turning rate within each cycle. Removal of airborne isoamyl alcohol evokes an elevation of turning rate before adaptation to the prestimulus value. Addition of airborne isoamyl alcohol evokes a similar but inverted response. The responses to odor removal and to odor addition were separately fit by least-squares to the sum of 2 exponentials, shown by lines, $y = A(e^{-t/\tau_1} - e^{-t/\tau_2})$. The exponential time constants for adaptation to odor removal and addition (that is, the longer of τ_1 and τ_2 in each fit) are 14 s for odor removal and 10 s for odor addition.

Attractive odors at low concentrations are repellants at high concentrations

In our setup, we used a flame-ionization detector to verify that the absolute vapor concentration of odorant molecules in the odorized airstreams is roughly proportional to the concentration of dissolved odorant in the mineral oil used to scent the airstreams (see METHODS). We focused our investigation on isoamyl alcohol (IA) and benzaldehyde (BZ), attractive odorants that were shown to be sensed by the AWC olfactory neurons through laser killing and behavioral analysis (Bargmann et al. 1993). We subjected worms to alternating airstreams of odorized air and nonodorized air in 30-s intervals, an interval chosen to capture the behavioral response to odor removal and addition prior to short-term adaptation within each cycle (Fig. 1*B*). To quantify the olfactory response, we use an olfactory index based on the contrast in mean turning frequency during the 30-s inflow (f_{in}) and 30-s outflow (f_{out}) phases of the stimulus waveform

$$\text{Index} = (f_{out} - f_{in})/(f_{out} + f_{in}) \quad (1)$$

Thus when the index is positive, the worm tends to turn more (/less) frequently when the odor is removed (/added). When the index is zero, the worm is indifferent to odor removal or addition. When the index is negative, the worm tends to turn more (/less) frequently when the odor is added (/removed).

We found that at the lowest vapor concentrations of IA or BZ, just above the threshold of detection, inflow (/outflow) of each airborne odor lowered (/raised) the turning rate (Fig. 2). We interpret this behavioral pattern as an attractive response to each odorant because inflow (/outflow) would lengthen (/shorten) the runs of crawling worms. The attractive response to each odorant has a relatively constant strength over a range of vapor concentration spanning three to four orders of magnitude above the threshold of detection. However, above a second and sharper threshold at high vapor concentration, IA and BZ become repellants, as inflow (/outflow) of either odorant raised (/lowered) the turning rate. Thus whether any odorant is perceived as attractive, neutral, or repulsive depends on the type and amount of odorant. This observation is consistent with the original discovery using crawling assays that odorants can be attractive when spotted in small amounts in petri plates but less attractive or repulsive when spotted in large amounts (Bargmann et al. 1993).

Effects of cell-specific genetic lesions and laser ablation of olfactory neurons

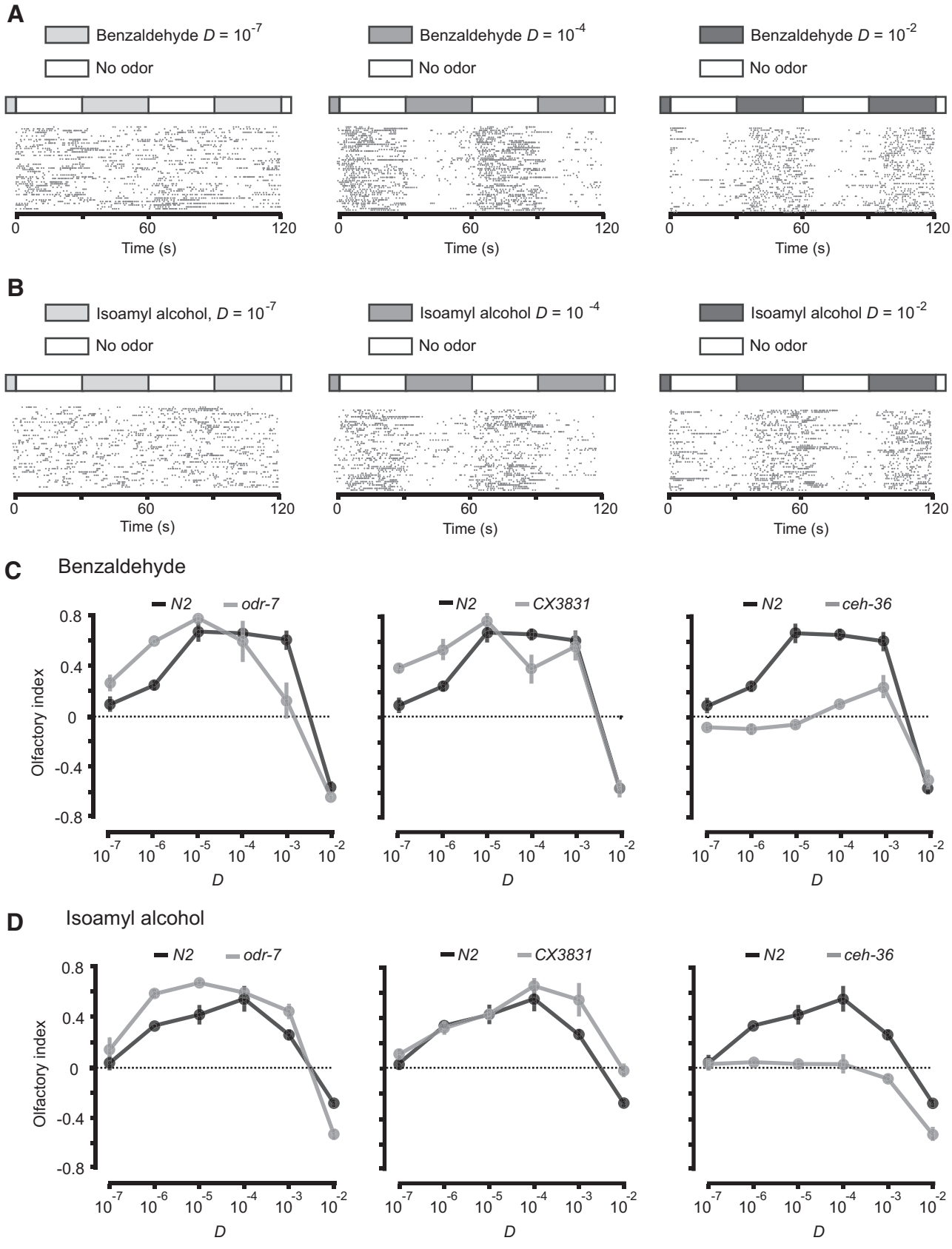
Of the 11 pairs of amphid chemosensory neurons, the AWA, AWB, and AWC neuronal pairs are specialized for detecting volatile odorants (Bargmann et al. 1993; Sengupta 2007). Laser killing of AWA, AWB, and AWC has been shown to weaken chemotactic responses in crawling assays toward point sources of a variety of odorants. So far, only AWC has been implicated in the detection of IA and BZ. As another test of the microdroplet assay, we sought to determine whether genetic disruption or laser killing of AWA, AWB, and AWC would reveal their predicted contributions in mediating the responses to IA or BZ.

The Otx-like homeodomain transcription factor *ceh-36* is required for specifying the identities of the AWC and ASE amphid chemosensory neurons, and mutation in *ceh-36* abolishes chemotaxis toward point sources of AWC-sensed volatile odorants in crawling assays (Lanjuin et al. 2003). ASE detects a variety of salts and small soluble molecules and has not been implicated in the detection of volatile odorants. We quantified the response of *ceh-36(ky646)* mutant worms across the full range of wild-type sensitivity to IA and BZ. We found that the *ceh-36(ky646)* mutation nearly abolished the detection of IA and BZ over the range of concentrations that IA and BZ are ordinarily perceived as attractants but did not affect the repellant responses at the highest concentration (Fig. 2, *B–D*). We confirmed that laser killing the AWC neurons nearly abolished the attractant response to BZ at a low vapor concentration but did not affect the repellant response to BZ at the highest concentration (Fig. 3*B*). Interestingly, laser killing the AWC neurons did not eliminate the attractant response to IA at a low vapor concentration nor did it affect the repellant response at the highest concentration (Fig. 3*A*). It is likely that the effects of the *ceh-36(ky646)* mutation on the olfactory sensory periphery are broader than laser ablation of AWC, so perhaps IA can be detected by another neuron besides AWC at low vapor concentrations, which may compensate for IA detection after AWC removal. Taken together, our results suggest that the worm's attractant response to IA or BZ is largely or entirely mediated by AWC at low concentrations, which is consistent with the results of original laser ablation experiments utilized with point-source aggregation assays (Bargmann et al. 1993). However, the repellant responses at the highest concentrations of either chemical appears to be entirely mediated by another neuron.

The AWA neuron has been implicated in the detection of attractive odorants such as diacetyl and pyrazine but has not been implicated in the direct sensation of IA or BZ (Sengupta et al. 1994, 1996). The *odr-7* gene encodes a nuclear receptor that is specifically required for function of the AWA neurons. As expected, we found that mutation in *odr-7* did not have significant effects on the attractant or repellant responses to IA or BZ across the normal range of sensitivity (Fig. 2, *B–D*). These experiments suggest that AWA alone is not responsible for the attractant or repellant responses to IA or BZ.

The AWB neuron has been implicated in the detection of repulsive odorants such as 2-nonanone (Troemel et al. 1997). To test the contribution of the AWB neurons to the detection of IA and BZ, we analyzed the behavioral response of worms

FIG. 2. Olfactory responses of wild-type and mutant worms. *A* and *B*: the turning response of wild-type (N2) worms subjected to the introduction and removal of airborne benzaldehyde (*A*) or isoamyl alcohol (*B*), odorized using different dilutions of each chemical in mineral oil. Each raster plot shows the result of independent representative experimental trials arranged vertically, each trial showing the turning response of 1 worm during 2 cycles within a longer experiment. At low dilutions, the worms do not modulate their turning rate in response to removal or addition of either odor. At moderate dilutions, inflow of either odor lowers the turning rate and outflow raises the turning rate. At high dilutions, inflow of either odor raises the turning rate and outflow lowers the turning rate. *C* and *D*: the olfactory index as a function of benzaldehyde (*C*) and isoamyl alcohol (*D*) odorant dilution for wild-type and mutant worms, calculated using *Eq. 1* (see RESULTS and DISCUSSION) using cycles with 60-s period as shown in *A* and *B*. At least 240 measurements (≥20 worms, each subjected to 12 cycles) were used to calculate each olfactory index at each odorant dilution. Each data point and error bar represents the mean olfactory index ±1 SE.



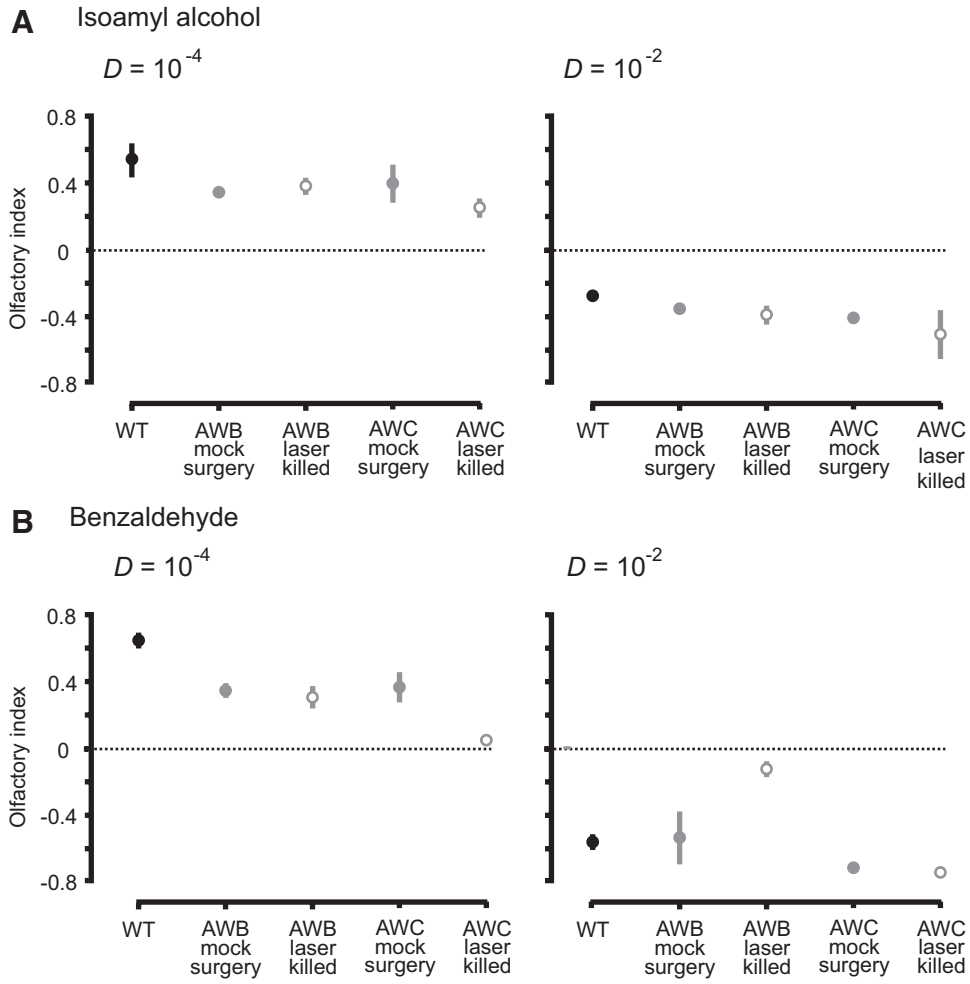


FIG. 3. Effects of AWB or AWC laser killing on isoamyl alcohol and benzaldehyde responses. *A* and *B*: each panel shows the olfactory indices, calculated using *Eq. 1*, for worms subjected to alternating airstreams, 1 clean airstream and 1 airstream that is odorized with isoamyl alcohol (*A*) or benzaldehyde (*B*) in the same manner as depicted in Fig. 2*A*. Laser killing of AWA and AWC were performed using transgenic animals that express GFP in these neurons, which facilitated target identification. Mock control measurements represent worms that were prepared for surgery without undergoing laser irradiation. Two odorant dilutions were used for isoamyl alcohol (IA) and benzaldehyde (BZ): $D = 10^{-4}$, which elicits an attractive response in normal worms (i.e., positive olfactory index) and $D = 10^{-2}$, which elicits a repulsive response in normal worms (i.e., negative olfactory index). At least 144 measurements (≥12 worms, each subjected to 12 cycles) were used to calculate each olfactory index. Each data point and error bar represents the mean olfactory index ±1 SE.

without the AWB neurons. For this, we used CX3831 transgenic worms that express the cell killing degenerin MEC-4D specifically in AWB (Driscoll and Chalfie 1991; Troemel et al. 1997). We also used transgenic worms that express GFP in the AWB neurons, allowing us to identify the neurons for laser ablation at the larval stage. We found that the AWB-degenerin-killed (Fig. 2, *C* and *D*) or AWB-laser-killed worms (Fig. 3) exhibited nearly normal attractant responses to IA and BZ at lower concentrations. The repellant response to BZ at high concentrations is significantly weakened by AWB-laser killing (Fig. 3*B*) but not significantly affected by AWB-degenerin killing (Fig. 2*C*). The repellant response to IA at high concentrations is eliminated by AWB-degenerin killing (Fig. 2*D*) but only slightly weakened by AWB-laser killing (Fig. 3*A*). It is unclear why AWB-degenerin and AWB-laser killings produce different results. One possibility is that the rest of the nervous system adapts differently to the loss of a specific neuron depending on how that neuron was removed. Nevertheless, taken together, these results suggest that AWB does not affect the attractant response to IA or BZ at low concentrations, but AWB partly contributes to the repellant response at the highest concentrations.

Olfactory response to steepness in odorant gradients

In many experimental setups to test physiological responses to olfactory cues, odorants are provided in discrete packets of odorized air. All-or-none presentation of olfactory inputs may be relevant to animals that live at high Reynolds numbers, a physical quantity that describes the relative importance of inertial forces and viscous forces in the animal's environment. For larger animals that live at high Reynolds numbers, turbulence moves packets of airborne odors from an emitting object to the animal's nose (Hopfield 1991). At high Reynolds numbers, simple diffusion does not significantly contribute to the dispersion of odorants, allowing sharp boundaries in the presence or absence of odorant molecules in olfactory experience. The millimeter-sized *C. elegans*, however, lives at Reynolds numbers near one or lower, where smooth chemical gradients are undisrupted by turbulence (Purcell 1977). Thus the chemosensory system of *C. elegans* should be able to encode the direction and steepness of smooth chemical gradients.

In our setup, we can manipulate the steepness of odorant gradients by alternating odorized airstreams carrying different odorant concentrations. In this way, the difference of the odorant concentrations in the two airstreams (ΔD) defines the steepness of temporal gradients in the olfactory input. In most biological sensory systems, the perceived intensity of a stimulus input depends directly on fractional changes in the physical magnitude of the stimulus. The well-known Weber-Fechner Law states that perceived changes in stimulus intensity are proportional to the fractional change in the size of the physical stimulus. We assume that changes in turning behavior caused

by alternation of airstreams, which we quantify using an olfactory index, reflect changes in perception. We quantified the size of the turning response of swimming worms subjected to fractional changes in odorant concentrations ($\Delta D/D$), near concentrations of IA and BZ at which, based on genetic disruption and laser ablation analysis, we knew that the AWC olfactory neurons were principally responsible for mediating the attractant response. We found that the strength of the olfactory response quantified using our olfactory index (*Eq. 1*) was roughly proportional to the fractional change in odorant concentration, reminiscent of the Weber-Fechner Law (Fig. 4). Our results suggest that AWC does not only serve to detect the presence or absence of odorant molecules at different concentrations, but that AWC is also capable of graded responses, allowing the worm to respond to the steepness of odorant gradients.

Quantifying behavioral responses mediated by single olfactory neurons to multiple odorants

Because individual neurons sense multiple odors, it is interesting to know how worms respond to combinations of odors that are sensed by the same neuron. Colbert and Bargmann discovered a form of habituation: long-term exposure to AWC-sensed odors reduces the worm's ability to aggregate near those odors in crawling assays (Colbert and Bargmann 1995). Cross-habituation can occur between certain AWC-sensed odors but not between other AWC-sensed odors, suggesting that AWC has multiple sensory transduction pathways for sensing distinct groups of odors. Cross-habituation has been shown to occur for IA and BZ; that is, long-term exposure to IA reduces chemotaxis toward BZ and vice versa.

The microdroplet assay provides a straightforward way to quantify the response of unhabituated worms to combinations of AWC-sensed odors. In this study, we subjected worms to alternating airstreams, one airstream containing both IA and BZ, each at odorant concentrations that AWC mediates an attractant response; the other airstream containing only IA or only BZ at the same concentration. Thus worms were subjected to the same change in each odorant as when we alternated an airstream odorized with a single odorant and a clean airstream, but now the worms were subjected to the change in each odorant in an invariant background of the other odorant. We found that the background of IA (/BZ) diminishes the worm's olfactory response to the addition and removal of BZ (/IA; Fig. 5). The simplest interpretation is that the AWC neuron detects the summed amounts of IA and BZ and responds to changes in the

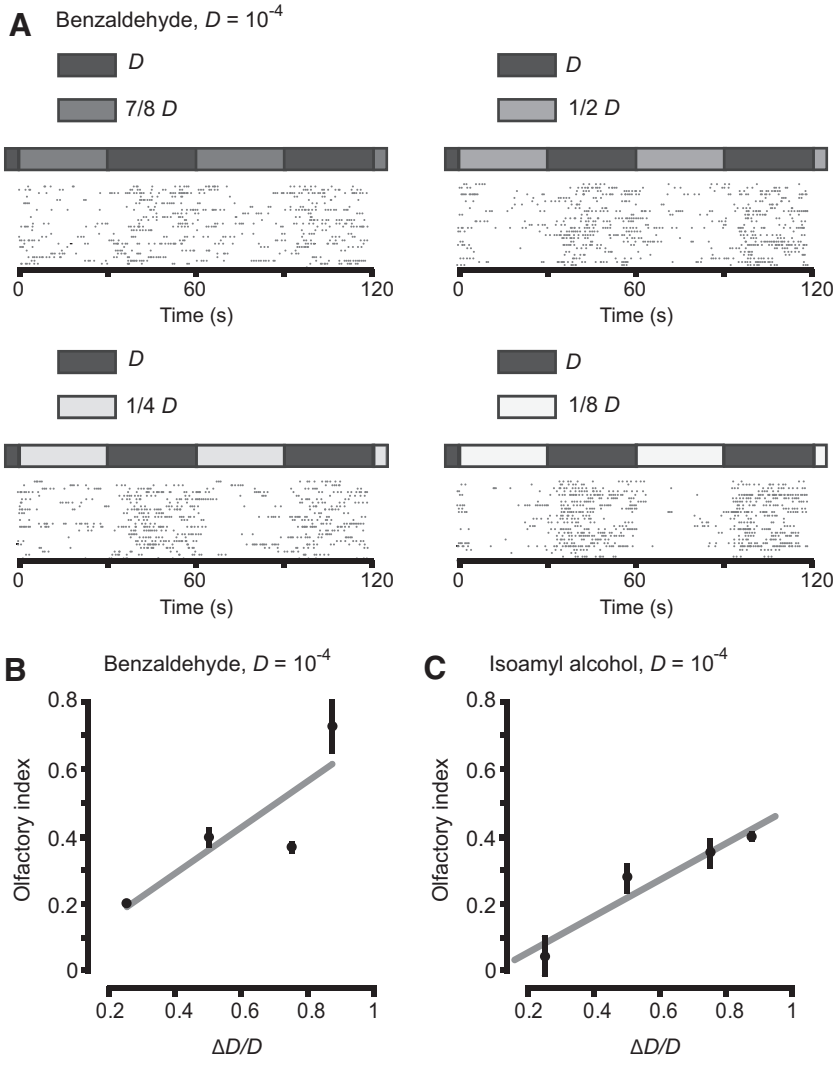


FIG. 4. Olfactory responses to fractional changes in odorant concentration. *A*: the turning response of wild-type (N2) worms subjected to the alternation of 2 airstreams, 1 airstream odorized with BZ at a dilution of $D = 10^{-4}$ and 1 airstream odorized with BZ at a dilution of a certain fraction of *D*. Each raster plot shows the result of independent representative experimental trials arranged vertically, each trial showing the turning response of 1 worm during 2 cycles within a longer experiment. *B* and *C*: olfactory indices showing the contrast in turning rate as a function of the fractional difference between the odor dilutions of 2 alternating airstreams as shown in *A*. Lines are least-squares fits to the olfactory index as a function of fractional change in odorant dilutions. For BZ, the slope is 0.68, intercept is 0.01, and R^2 is 0.72. For IA, the slope is 0.54, intercept is 0.066, and R^2 is 0.93. At least 240 measurements (≥20 worms, each subjected to 12 cycles) were used to calculate each olfactory index at each odorant dilution.

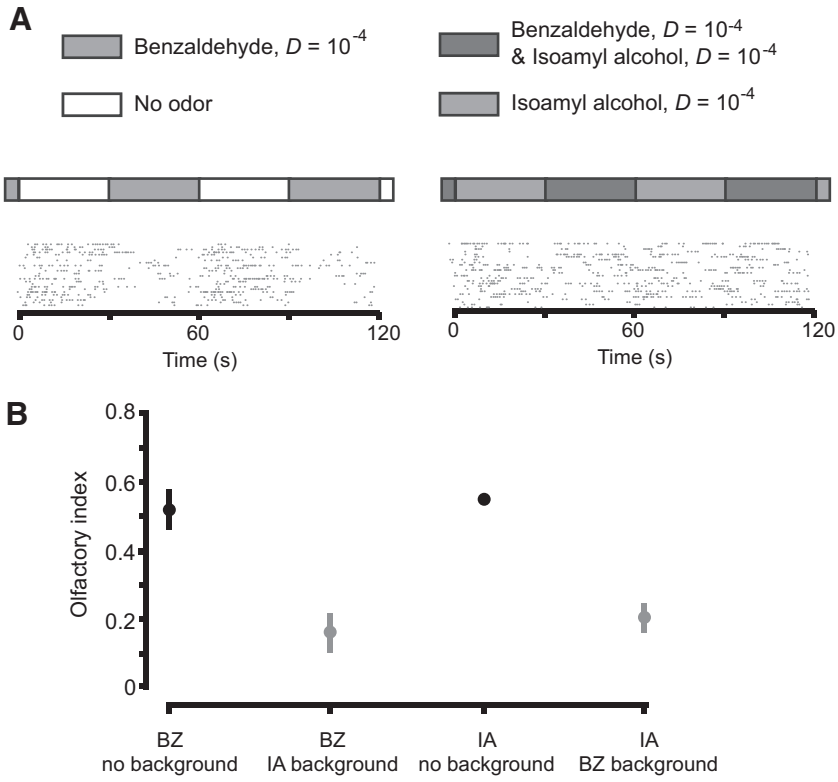


FIG. 5. Olfactory responses to one odor in the background of another odor. *A*: the turning response of wild-type worms subjected to the alternation of 2 airstreams. *Left*: 1 clean airstream and 1 airstream odorized with BZ at a dilution of $D = 10^{-4}$. *Right*: 1 airstream odorized with BZ using a dilution of $D = 10^{-4}$, but both airstreams were odorized with IA using a dilution of $D = 10^{-4}$. Each raster plot shows the result of independent representative experimental trials vertically, each trial showing the turning response of one worm during 2 cycles within a longer experiment. *B*: the olfactory indices show the relative olfactory response to the odor of BZ with no background (as in *A, left*), the odor of BZ in an IA background (as in *A, right*), the odor of IA with no background, and the odor of IAl with BZ background. At least 240 measurements (≥20 worms, each subjected to 12 cycles) were used to calculate each olfactory index at each odorant dilution. Each data point and error bar represents the mean olfactory index ±1 SE.

sum. Roughly speaking, when the IA-odorized airstream is alternated with a clean airstream, the percent change in AWC detection is 100%, eliciting a strong olfactory index, but when the IA+BZ-odorized airstream is alternated with an IA-odorized airstream, the percent change in AWC detection is ~50%, eliciting a weaker olfactory index.

DISCUSSION

To perform chemotaxis, the worm must detect a wide variety of molecules across a broad range of concentrations using a small number of sensory neurons, calculate the direction of odorant gradients, and modulate its motor system accordingly. Dissecting this sophisticated olfactory system requires new assays that are robust, precise, and quantitative. Significant technological developments are rapidly occurring in the experimental analysis of the worm olfactory system. For example, optical imaging now allows the measurement of calcium dynamics within individual chemosensory neurons as they are subjected to the addition and removal of dissolved volatile compounds using microfluidic devices. Using calcium imaging, Chalasani et al. recently showed that the AWC neuron is activated by the removal of odorants not by addition of odorants (Chalasani et al. 2007).

Technological developments are also occurring in the quantitative analysis of chemosensory behavior. Ward et al. originally used aggregation at a point source to quantify chemotaxis toward or away from soluble chemicals (Ward 1973). In the original work on olfactory behavior, Bargmann et al. used point sources of volatile odorants to quantify olfactory detection, attraction, and repulsion to specific molecules (Bargmann et al. 1993). Recently Chalasani et al. showed that flowing odorized airstreams of volatile odorants into and out of a petri plate can alter the turning rate of a population of crawling worms, but this assay did not allow extended observation of single worms (Chalasani et al. 2007). Chronis et al. showed that the behavior of individual worms can be quantified within microfluidic devices, as the animal attempts to crawl forward or backward inside narrow chambers when subjected to defined chemical flows, but it is possible that worm behavior is affected by the partial immobilization itself (Chronis et al. 2007).

The microdroplet assay for swimming worms has certain advantages that should complement other assays for olfactory behavior. First, the microdroplet assay allows us to more rapidly extract quantitative information about olfactory phenotypes than with traditional crawling assays. Instead of scoring success or failure to aggregate near an odorant source after 30–60 min in a crawling assay, we quantify the turning rate over time, allowing us to measure the temporal dynamics of the sensorimotor response. Second, all of the worms in the array of microdroplets within each experiment have the same olfactory experience, increasing the robustness of comparative analysis between different types of worms, and each worm is unrestrained, avoiding possible artifacts due to tethers or partial immobilization. Third, arrays of microdroplets confer high-throughput while maintaining single-worm resolution, which is a particular advantage when performing laser ablation analysis on small numbers of surgically operated worms. Finally, the microdroplet assay provides great flexibility in defining the stimulus, allowing us to systematically vary the type and concentration of each odorant, the steepness of odorant gradients, and mixtures of multiple odorants.

In this study, we validated the microdroplet assay by showing that olfactory phenotypes using AWC-sensed odorants are largely consistent with previous results obtained using crawling assays. Without AWC neurons, crawling worms are unable

to aggregate near point sources of IA or BZ. In the microdroplet assay, worms without AWC are unable to exhibit an attractant response to IA or BZ at any concentration, but the microdroplet also uncovered a repellant response to IA or BZ at very high concentrations. These data suggest that IA and BZ may be detected exclusively by AWC within a range of very low odorant concentrations. In general, the response of a particular olfactory cell should span a range of odorant concentrations near the dissociation constant between its molecular olfactory receptors and the odorant ligand. One possibility is that AWC has several chemoreceptors for IA and BZ, spanning a broad range of dissociation constants, allowing the detection of several orders of magnitude of odorant concentration. We also showed that the worm responds to fractional changes in odorant concentration, which would be vital to the navigation of smooth spatial gradients of odorant concentrations in its environment. Finally, we showed that at high concentrations, a repellant response is mediated by another set of neurons (possible including AWB) that must have chemoreceptors for IA and BZ with, presumably, a very high dissociation constant.

Finally, the time course of the behavioral response to step changes in odorant concentration shown in Fig. 1*B* reveals the behaviorally relevant computation for navigating smooth gradients in the animal's environment. A stepwise increase (/decrease) in attractant concentration produces a transient decrease (/increase) of turning rate. Taking stimulus input as attractant concentration and motor output as turning rate and viewing the mathematical transformation between input and output, the output resembles the first time derivative of the input averaged over a time interval corresponding to the total duration of the step response. A crawling worm in chemical gradients exhibits a biased random walk with average periods of forward movement lasting 20–30 s. Thus the behaviorally relevant computation to optimally execute the worm's biased random walk strategy is the first-time derivative of chemical concentration averaged over the duration of each period of forward movement. As we have previously argued for our step response measurements in relation to the biased random walk behavior of *C. elegans* thermotaxis, and as Block et al. argued for their impulse and step response measurements in relation to the biased random walk behavior of *Escherichia coli* chemotaxis, the mechanisms underlying the worm's olfactory response are matched to the behavioral task that the worm is required to perform (Block et al. 1982; Clark et al. 2007).

In summary, the microdroplet assay allows us to quantify the sensorimotor response of individual worms to well-defined olfactory inputs across a broad range of parameter space, presenting a new tool for rigorous investigation of the compact and sophisticated olfactory system of *C. elegans*.

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