Serotonin mediates food-odor associative learning in the nematode *Caenorhabditis elegans*

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We demonstrate that *Caenorhabditis elegans* is able to form an association between the presence of the odorant benzaldehyde and the food content of its environment. When exposed to 100% benzaldehyde for 1 h in the absence of food the naive attractive response is reduced, and we have found that this olfactory adaptation is attenuated by the presence of food. Contrary to nonassociative (single stimulus) learning theory, this response is not a function of the total time of exposure to benzaldehyde but rather an associative function of the ability of benzaldehyde to predict a nutrient-deficient environment. Genetic and pharmacological evidence revealed that the effects of food in this learning paradigm are mediated by serotonergic signaling.

Food is a powerful motivator for animals, and many behaviors depend on food availability and recent nutritional history. For the nematode *Caenorhabditis elegans*, the presence of *Escherichia coli*, the standard laboratory food source, modulates multiple behaviors including locomotion, egg laying, and pharyngeal pumping (1, 2). In addition to these acute modulatory effects, food deprivation has been shown to alter more complex behavioral phenomena such as thermotaxis (3) and chemotaxis (CTX; ref. 4), and prolonged starvation can lead to the expression of an alternative developmental pathway leading to a period of relative metabolic stasis, the dauer larva stage (5). Food also has been used as an unconditioned stimulus (US) in associative-learning paradigms. Differential pairing of food with water-soluble chemoattractants (e.g., Na^+ or Cl^-) leads to associative conditioning such that ions recently paired with food (CS^+ ion) are preferred to ions that have not been paired recently with food (CS^- ion; ref. 6).

Food is clearly a salient environmental cue for *C. elegans*, and it is likely that nematodes use odor cues to locate food and avoid detrimental environments. *C. elegans* utilizes six primary sensory neurons to respond to more than 40 different volatile attractants and repellents (7). Prolonged exposure to some odorants leads to a decrease in the level of attraction to that specific odorant because of a phenomenon called olfactory adaptation (8). For example, exposure to benzaldehyde for 1 h causes a significant decrease in subsequent chemotactic scores. Adaptation in this context is used to refer to a behavioral response rather than its mechanisms. Previous investigations into this behavioral response have demonstrated that the reduction of CTX is susceptible to dishabituation, confirming that it is not the result of fatigue in sensory or motor systems but rather the result of a learning process (9). Indeed, prolonged exposure to a high concentration of benzaldehyde leads to the development of an aversive response to the odorant such that the animals will actively avoid a point source of benzaldehyde. This behavioral switch is produced by the dynamic interaction of two separate (attractive and aversive) benzaldehyde-responsive motivational systems (9).

We investigated the relationship between food and the plasticity of chemotactic responses. We have found that benzaldehyde adaptation is suppressed in the presence of *E. coli*, and that this suppression is mediated by serotonergic signaling. Furthermore, behavioral experiments indicate that the magnitude of the response decrement is not simply reflective of the amount of exposure to benzaldehyde as nonassociative (single-stimulus) learning accounts would predict. Rather, the level of attraction to benzaldehyde is a function of the probability that benzaldehyde will co-occur with food. This predictive power, determined by the contingent relationship between benzaldehyde and food, is a central feature of contemporary associative-learning theories (10, 11). This behavior reveals a surprising degree of sophistication for such a simple organism and demonstrates that the modulation of single-stimulus learning (olfactory adaptation) by salient environmental cues (food availability) provides a simple mechanism for expression of associative-learning capabilities. Moreover, this learning depends on serotonergic neurotransmission.

Methods

Strains and General Methods. Nematode strains were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota (St. Paul). All experiments used well fed adults cultivated at 20°C on standard nematode growth medium (NGM plates) seeded with *E. coli* strain OP50 (12).

CTX Assays. CTX assays were performed with standard 100-mm Petri dishes containing 6 ml of CTX medium (10 mM Mops, pH 7.2/0.25% (vol/vol) Tween 20/15 g/liter agar). Where necessary, odorants were diluted in ethanol and reported as percentages by volume. Plates were sealed with a strip of parafilm around the edge during all odorant exposures. Standard 1-h CTX assays were performed as described previously (7). Briefly, 15 min before the assay 1 μl of 1 M NaN_3 was applied to the centers of two test spots that were 6 cm apart. This acts as an anesthetic to immobilize any animals that reach the spot during the assay. Individuals (100–300) then were placed at the center of the plate between the two spots, 1 μl of the test odorant was placed at one spot, and 1 μl of ethanol was applied to the control spot. After 1 h of CTX (or 15 min for approaches to 100% benzaldehyde; ref. 9), animals within a 2-cm radius of either spot were counted, and a chemotactic index (CI) was calculated as the number of animals at the test spot minus the number of animals at the control spot and divided by the total number of animals on the plate. A positive CI indicates an attraction to the odor, and a negative CI indicates an aversion.

For preexposure treatments, 500–1,000 animals were placed on a CTX plate, and then 2 μl of the odorant were placed on a piece of parafilm on the lid of the plate. The plate was sealed with parafilm and then left inverted for 1 h. The animals then were rinsed from the test plate in ≈2 ml of water and transferred to a conical centrifuge tube, where they were allowed to settle to the bottom of the tube. Within 5 min, the concentrated animals were collected and transferred to a fresh CTX plate for testing. For assays including food on the CTX plates, cells from a logarith-

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Abbreviations: US, unconditioned stimulus; CS, conditioned stimulus; CTX, chemotaxis; CI, chemotactic index.

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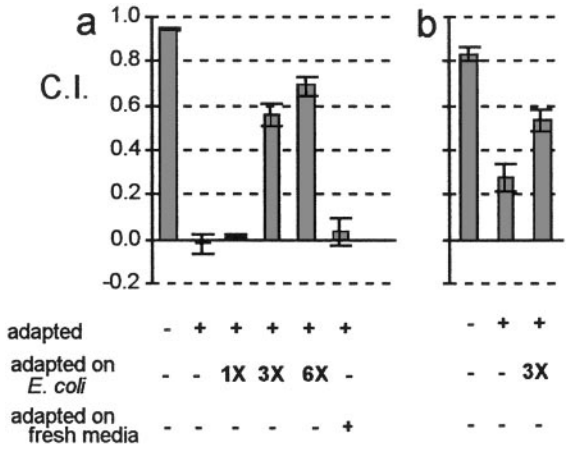

Fig. 1. Olfactory adaptation is suppressed in the presence of *E. coli*. (*a*) Well fed adult animals were tested for their 1-h chemotactic response to 1% benzaldehyde immediately after various conditioning treatments. Conditioned animals received a 1-h preexposure to 2 μl of 100% benzaldehyde. Adaptations were performed with no additions or after the addition of *E. coli*, 200 μl of fresh *E. coli* growth medium, or sephadex beads to the adaptation plates. (*b*) Well fed adult animals were tested for their 1-h chemotactic response to 0.1% trimethyl thiazole immediately after various conditioning treatments. Conditioned (adapted) animals received a 1-h preexposure to 2 μl of 100% trimethyl thiazole in the presence or absence of 3× *E. coli*.

mically growing *E. coli* culture were spread onto the plate and allowed to dry immediately before testing. A 1× concentration of *E. coli* was defined as an amount equivalent to 100 μl of culture diluted to an OD_{600} of 0.5.

For liquid preexposure treatments the animals were suspended in 10 ml of S-basal medium (0.1 M NaCl/0.05M potassium phosphate, pH 6.0/5 mg/liter cholesterol) containing 0.006% (vol/vol) benzaldehyde and incubated for 1 h at room temperature in a 15-ml conical centrifuge. These conditions have been shown to result in a response decrement that is equivalent to that obtained with 2 μl of 100% benzaldehyde in the traditional 1-h preexposure on plates (13). The animals then were allowed to sediment to the bottom of the tube, rinsed with 2 ml of fresh S-basal, collected by gravity sedimentation, and transferred to chemotactic test plates.

All CI values are the means and standard errors of the means calculated from at least six chemotactic tests. Student's *t* tests were used for two-way comparisons between groups for statistical significance ($P < 0.01$). Multiple group comparisons were performed with ANOVAs followed by Bonferroni corrected *t* tests.

Results

Olfactory Adaptation Is Suppressed in the Presence of *E. coli*. Olfactory adaptation in *C. elegans* is defined as an altered response to volatile chemicals after prolonged exposure to the same odorant (7). Wild-type nematodes show a strong attractive response to a 1% solution of benzaldehyde in standard tests of chemotactic acuity as evidenced by a high population CI (ref. 7; Fig. 1*a*, no treatment). When the animals were tested immediately after 1 h of conditioning to a 2-μl drop of 100% benzaldehyde on a CTX plate, this attractive response was eliminated (Fig. 1*a*, second bar, adapted on benzaldehyde). Analysis of the behavior of the nematodes during the preexposure training revealed that the animals first approach and then retreat from the point source of benzaldehyde. The behavior observed on tests to 1% benzaldehyde is therefore indicative of a decreased positive response and the concurrent development of an aversive response to the training source (9). The expression of an aversion after prolonged odorant exposure, the stimulus specificity of olfactory adaptation (8), and the susceptibility of benzaldehyde adaptation to dishabituation (9) indicate that olfactory adaptation in *C. elegans* is a learned response and not the result of fatigue or toxicity resulting from the odorant exposure. If odors indeed indicate food sources (4, 14), then these observations suggest that this learning may reflect a food-seeking behavior, where animals approach a source of benzaldehyde in search of food but leave the area if none is found. This is supported by the observation that the presence of food suppresses CTX toward some volatile attractants [1% benzaldehyde CI = 0.87 ± 0.02 on food and 0.07 ± 0.05 off food; 1% trimethyl thiazole CI = 0.84 ± 0.08 on food and 0.09 ± 0.07 off food; 1% diacetyl CI = 0.91 ± 0.01 on food and 0.34 ± 0.04 off food; ANOVA $F(2,47) = 8.1$, $P < 0.01$, showing a main effect with all the on-food groups significantly lower than the off-food groups]. Additionally, if the animals encounter a colony of *E. coli* during the initial attraction to 100% benzaldehyde, they will stay at the food rather than retreat from the odorant (data not shown).

Because odorant responses can be affected by the prolonged absence of food (4), we tested the effect of performing the odorant training in the presence of food. When the benzaldehyde preexposure was performed on the standard growth medium (NGM plates containing a lawn of *E. coli*) no reduction of the attraction to benzaldehyde was observed when tested immediately afterward in the absence of *E. coli* (data not shown). Furthermore, when small amounts of *E. coli* (see *Methods*) were added directly to the CTX plates during the preexposure to benzaldehyde, there was a significant suppression of adaptation [Fig. 1*a*, ANOVA $F(6,131) = 89.2$, $P < 0.01$, adapted (CI = −0.02 ± 0.04) vs. adapted on 3× *E. coli* (CI = 0.56 ± 0.04; $t_{59} = 111.2$, $P < 0.01$) or 6× *E. coli* (CI = 0.69 ± 0.06; $t_{37} = 63.6$, $P < 0.01$)]. This suppression of olfactory learning required the actual presence of *E. coli*, because no inhibition was seen when 200 μl of fresh growth medium without *E. coli* was added during the initial conditioning (CI = 0.03 ± 0.06; $t_{53} = 0.75$, $P > 0.01$ vs. adapted). Taken together, these results demonstrate that the adaptive response to benzaldehyde is inhibited in the presence of *E. coli*, the food source used for the cultivation of nematodes. A similar effect of food was observed with a second odorant, trimethyl thiazole. Nematodes display a strong naive approach to a test spot of trimethyl thiazole that is attenuated after preexposure to 100% trimethyl thiazole for 1 h. Inclusion of 3× *E. coli* on the preexposure plate resulted in a significant suppression of this olfactory adaptation [Fig. 1*b*, ANOVA $F(2,42) = 19.4$, $P < 0.01$, adapted (CI = −026 ± 0.06) vs. adapted on 3× *E. coli* (CI = 0.53 ± 0.05; $t_{32} = 3.1$, $P < 0.01$]. Therefore, the presence of food can suppress both the chemotactic approach and the adaptation of the chemotactic approach to some volatile odorants.

Possible explanations for the suppression of adaptation when conditioned in the presence of food are that the *E. coli* blocks the ability of the nematodes to sense odorants, or it blocks their ability to move toward the distant source of benzaldehyde. To assess the effect of *E. coli* (at levels that inhibited adaptation) on the mobility of the animals, we determined the rate of body bends performed on blank CTX plates, in the presence of *E. coli*, or on plates containing 200 μl of 30 mg/ml sephadex G-200 beads. Sephadex beads induce a dopamine-mediated mechanical response that results in reduced locomotion (15). We found no difference between the mobility of animals in the absence or presence of *E. coli* at a 3× concentration (Fig. 2*a*), a dose that significantly reduced the degree of adaptation to benzaldehyde (Fig. 1, adapted on 3× *E. coli*).

The ability of the nematodes to sense odorants in the presence of *E. coli* was tested by measuring CTX on CTX plates containing *E. coli*. As shown in Fig. 2*b*, with the data normalized to percent CTX relative to controls to facilitate between group compari-

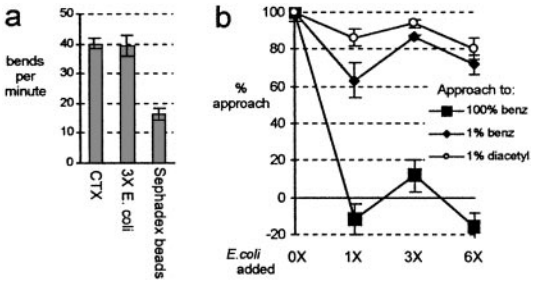

Fig. 2. Food does not block mobility or the ability to detect odors. (*a*) The rate of body bends was determined for wild-type animals on CTX plates with no additions, 3× *E. coli*, or sephadex beads. (*b*) CTX on CTX plates was measured after the addition of *E. coli* to the CTX plate. Chemotactic scores were determined after 15 min in the presence of 100% benzaldehyde or after 1 h in the presence of 1% benzaldehyde or diacetyl. Data are expressed as percent approach relative to the mean approach score of same day controls performed in the absence of *E. coli*.

sons, the animals showed a strong approach to a 1% point source of either benzaldehyde or diacetyl in the presence of 1×, 3×, or 6× *E. coli*. A one-way ANOVA revealed no significant suppression of diacetyl CTX [$F(3,39) = 3.6$; $P > 0.01$], confirming that the animals can sense and respond to the odorant under these conditions. Although the approach scores to 1% benzaldehyde were slightly depressed, a two-way ANOVA comparing group (100% vs. 1% benzaldehyde) vs. treatment (amount of *E. coli* added) revealed a significant interaction, confirming that the inhibition of the approach to 100% benzaldehyde was greater than the inhibition of the approach to 1% benzaldehyde [$F(3,193) = 12.8$; $P < 0.01$]. Therefore, the animals maintained an ability to sense and respond to odorants in the presence of low amounts of *E. coli*. The observation that the response to 100% benzaldehyde is more sensitive to food inhibition than the responses to lower odorant concentrations indicates that the lack of approach to 100% benzaldehyde is not caused by an inability to sense the odorant source but rather to a failure of this stimulus to induce a positive chemotactic response under these conditions. This behavior can be contrasted to the chemotactic abilities of animals carrying a mutation of the guanylyl cyclase encoding gene *odr-1*. *Odr-1(n1936)* mutant animals are deficient in their approach to low concentrations of benzaldehyde (8, 13). We studied the unconditioned response to 1% benzaldehyde in a 30-min test and confirmed that the *odr-1(n1936)* mutation causes a significant reduction in the approach score relative to wild-type controls (wild-type CI = 0.80 ± 0.04, *odr-1* CI = 0.14 ± 0.06; $t_{17} = 10.2$, $P < 0.01$). Notably, the *odr-1* and wild-type animals achieved equivalent approach scores in tests to 100% benzaldehyde (wild-type CI = 0.40 ± 0.02, *odr-1* CI = 0.39 ± 0.03; $t_{28} = 0.3$, $P > 0.01$). The double dissociation of the abilities to approach point sources of 1 and 100% benzaldehyde (food preferentially suppresses the approach to 100% benzaldehyde, whereas the *odr-1(n1936)* mutation preferentially suppresses the approach to 1% benzaldehyde) indicates the existence of at least two mechanisms for the detection of benzaldehyde. A high-affinity system dependent on the AWC sensory neurons (7) directs CTX to dilute odorant sources of 1% benzaldehyde or less. A second, lower affinity system would direct CTX to higher odorant concentrations, which saturate the high-affinity system. The low-affinity system presumably mediates adaptation, consistent with the observation that adaptive responses to benzaldehyde can be seen within 20 min of being exposed to high levels of the odorant, whereas exposure to a dilute (1%) source of benzaldehyde produced no adaptation even after prolonged exposures of up to 90 min (9).

The Adaptive Response to Benzaldehyde Shows the Contingent Predictive Properties Characteristic of Associative Learning. The suppression of olfactory learning and memory by food suggests that this behavior may represent a simple form of associative learning in that the animals do not approach a test spot of benzaldehyde (the conditioned stimulus or CS) after odorant preexposure, because they have learned that benzaldehyde predicts food deprivation (the US is the absence of food). It is therefore the ability of benzaldehyde to predict the presence of food, based on recent experience, that determines the level of attraction to the odorant (10). This associative-learning explanation predicts that a group that received benzaldehyde paired with food for the second hour would show an increased attraction to benzaldehyde on subsequent tests despite the longer preexposure because they would have learned that benzaldehyde and food occur together. In contrast, a nonassociative explanation of this behavior predicts that the length of time of preexposure to benzaldehyde will be proportional to the degree of adaptation and therefore the additional hour of exposure should increase benzaldehyde adaptation regardless of the presence of food. An experiment comparing the level of adaptation between groups that all were conditioned to 100% benzaldehyde for 1 h and then exposed to various treatments for a second hour before testing pits these two learning-process interpretations against each other.

We exposed animals to benzaldehyde (the CS) under conditions of food deprivation (the US) for 1 h and then tested their response immediately or after a second hour of CS–US pairing (benzaldehyde and no food), recovery with neither the CS nor the US present (no odorant + food), a CS-only trial (benzaldehyde + food), a control CS-only trial with a second odorant, trimethyl thiazole (tmt), or a 1-h US only trial (no odorant and no food). The results from these experiments are shown in Fig. 3. An ANOVA confirmed that there were significant differences between the treatment groups [$F(7,162) = 118$, $P < 0.01$], and therefore Bonferroni *t* tests were used to compare the effects of the various second-hour treatments. Animals exposed to benzaldehyde with no food (CS–US pairing) for the second hour displayed an aversion to 1% benzaldehyde upon testing that was greater than the slight aversion observed after 1 h of preexposure (1-h adapted CI = −0.06 ± 0.04, 2-h benzaldehyde no food CI = −0.35 ± 0.09; $t_{43} = 19.3$, $P < 0.01$). This behavior demonstrates the transition from an attractive to an aversive response that occurs with prolonged exposure to benzaldehyde (9) and also confirms that the low CIs seen after 1 h of exposure are not caused by toxic effects resulting from the exposure that leave the animals unable to perform in chemotactic assays. In the group where the animals received an additional hour of conditioning on their regular growth plates with food but no benzaldehyde (no CS or US present), they displayed a slight but significant degree of recovery of the attractive response on the subsequent test to 1% benzaldehyde (Fig. 3, second hour no odorant + food, CI = 0.30 ± 0.04; $t_{60} = 52.2$, $P < 0.01$ vs. 1-h adapted). Indeed, it has been shown that benzaldehyde adaptation in *C. elegans* lasts ≈3–4 h before full recovery (7), reminiscent of the decay of short-term memory that is observed in other organisms. Most important, in the treatment that paired benzaldehyde with food during the second hour of conditioning (a CS-only exposure), the animals displayed a full recovery to their naive level of attraction to benzaldehyde (CI = 0.82 ± 0.02, $t_{57} = 5.0$, $P > 0.01$, vs. naive CI = 0.93 ± 0.01). Thus, the inclusion of benzaldehyde during the 1-h recovery on food significantly accelerated the reestablishment of the attractive response relative to the conditioning with food alone during the second hour ($t_{57} = 104$, $P < 0.01$, comparing food exposure with no odorant vs. food exposure with benzaldehyde). It is this increased attraction to benzaldehyde, resulting from the simultaneous exposure to food and benzaldehyde during the second hour of training that indicates the associative properties of olfactory adaptation, because a non-

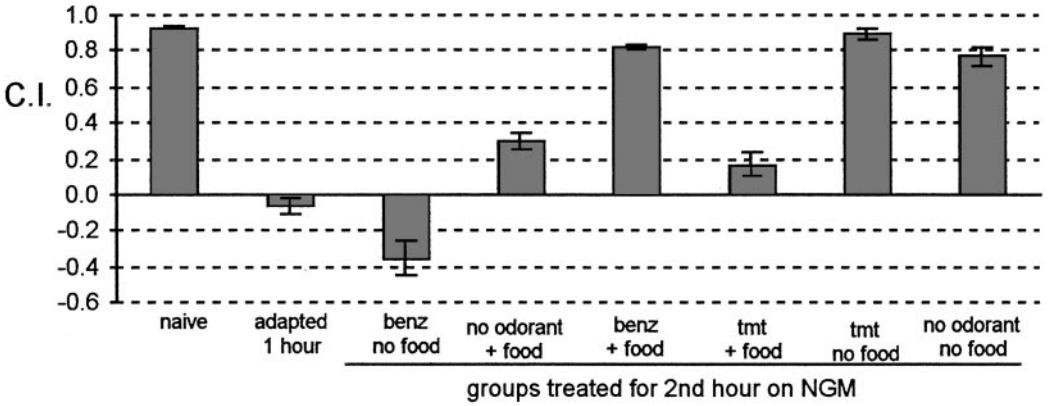

Fig. 3. Associative properties of olfactory learning. A second hour of training on NGM plates was added after the initial 1-h preexposure. Animals were tested without further treatment after the first hour of benzaldehyde exposure (adapted 1 h = primary CS–US pairing), exposed to benzaldehyde for the second hour in the absence of food (benz, no food = further CS–US pairing), returned to food containing culture plates in the absence of benzaldehyde for the second hour (no odorant + food), returned to food containing plates for the second hour in the presence of 2 μl of 100% benzaldehyde (benz + food = CS only), returned to food containing plates for the second hour in the presence of 2 μl of 100% trimethyl thiazole (tmt + food = control CS), or returned to plates with no food for the second hour (no odorant no food = US only). One-hour chemotactic approaches to 1% benzaldehyde were determined immediately after the second hour of training.

associative account simply predicts greater benzaldehyde adaptation with the longer time of benzaldehyde preexposure as seen in the absence of food.

The full recovery to naive levels of attraction after exposure to benzaldehyde on food is understood within an associative framework as a reversal of the initial CS–US learning (1-h adaptation) resulting from unpaired presentations of the CS alone, an extinction trial that degrades the usefulness of benzaldehyde to predict a food-deficient environment. This associative interpretation predicts that there would be stimulus specificity (just any odorant would not facilitate recovery) and that US-only presentations also would reduce the effectiveness of benzaldehyde as an indicator of the absence of food. To test for stimulus specificity we used trimethyl thiazole, an odorant that is sensed by both the AWA and AWC sensory neurons and therefore is expected to cause widespread activation of the nematode olfactory system [AWA and AWC are the primary amphid neurons used for the detection of volatile chemical (7)]. Inclusion of trimethyl thiazole during the incubation in the presence of food for the second hour of training had no effect on the subsequent CTX test scores (tmt + food CI 0.17 ± 0.07, $t_{37} = 2.9$, $P > 0.01$ vs. no odorant + food). For the US-only presentation, animals were incubated for the second hour without the benzaldehyde CS but under conditions of food deprivation (the US). After the 1-h US-only presentation, the animals again experienced a recovery to naive levels of attraction to benzaldehyde (CI = 0.77 ± 0.05, $t_{48} = 8.0$, $P > 0.01$ vs. naive). Once again, the inclusion of trimethyl thiazole during the second hour of conditioning had no effect, relative to the US-only group, on the subsequent approach to benzaldehyde (CI = 0.89 ± 0.03, $t_{21} = 1.0$, $P > 0.01$ vs. no odor + no food).

Food-Odor Associative Learning Depends on Serotonergic Signaling. Serotonergic signaling has been shown to be involved in a number of food-induced behavioral changes in *C. elegans*. We therefore tested the effect of performing the benzaldehyde preexposure in the presence of serotonin rather than *E. coli*. The addition of 5, 25, 100, or 400 μl of 100 mM serotonin to the agar plates used for benzaldehyde-adaptation training resulted in a significant inhibition of olfactory adaptation. This inhibition was proportional to the amount of serotonin added to the conditioning plates, such that animals preexposed to benzaldehyde on plates that had received 400 μl of serotonin showed test approach scores similar to those of naive animals (Fig. 4*a*). To determine the actual concentration of serotonin required to inhibit adaptation, we used a modified training procedure (13) in which the animals were preexposed to a solution of 0.006% (vol/vol) benzaldehyde in isotonic S-basal medium. Under these conditions, the preexposure to benzaldehyde resulted in adaptation of the olfactory response as indicated by the decrement of the subsequent CI after testing the 1-h approach to 1% benzaldehyde (Fig. 4*b*). Furthermore, this level of adaptation was indistinguishable from that obtained with a 1-h exposure to the vapors from a 100% point source of benzaldehyde (after liquid

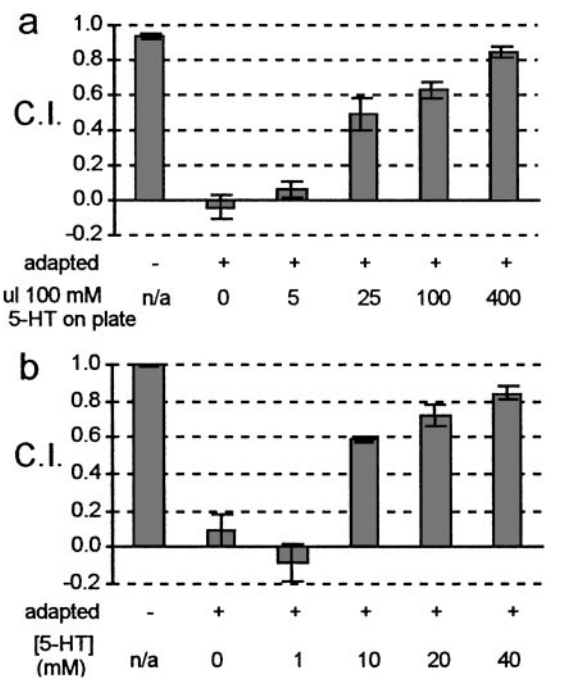

Fig. 4. Serotonin mimics the effect of food on olfactory learning. (*a*) Animals were incubated with or without benzaldehyde for 1 h on CTX plates containing varying amounts of 100 mM serotonin (5-HT). (*b*) Preexposure was performed in liquid medium containing 0.006% (vol/vol) benzaldehyde and varying concentrations of serotonin.

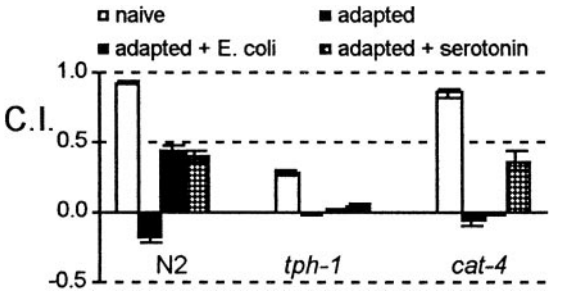

Fig. 5. Olfactory learning in serotonin-deficient mutant stains is not inhibited by the presence of *E. coli*. Wild-type N2 animals and the serotonin-deficient strains carrying the *tph-1(mg280)* or *cat-4(e1141)* alleles were exposed to 100% benzaldehyde for 1 h either alone or in the presence of 3× *E. coli* or 100 μl of 100 mM serotonin. Chemotactic responses were assessed immediately afterward by 1-h test approaches to 1% benzaldehyde.

adaptation CI = 0.09 ± 0.09, after plate adaptation CI = −0.04 ± 0.07; $t_{15} = 0.7$, $P > 0.01$). Serotonin was found to lead to a dose-dependent inhibition of adaptation under these solution conditions with an EC_{50} of ≈20 mM.

Mutations in Genes Required for Serotonergic Signaling Impair Food-Odor Associative Learning. Previous genetic studies have identified mutant strains that are defective in biogenic amine synthesis (16, 17). The *tph-1* gene encodes the tryptophan hydroxylase that catalyses the penultimate step in serotonin biosynthesis, whereas the *cat-4* gene product is required for the synthesis of the biopterin cofactor that is required for TPH-1 (18). Both of these mutations result in severely depleted levels of serotonin, which causes deficiencies in a number of food-induced effects that are mediated by serotonergic signaling. We therefore tested strains carrying these mutations for their olfactory behavior. An ANOVA confirmed significant differences between test groups [$F(6,166) = 12.7$; $P < 0.01$], allowing post hoc pairwise comparisons.

The GR1321 strain, which carries the *tph-1(mg280)* deletion allele, showed a significant reduction in the naive approach levels to benzaldehyde (CI = 0.28 ± 0.02; $t_{46} = 162$, $P < 0.01$ vs. naive CI = 0.92 ± 0.01), and this attraction was eliminated after a 1-h conditioning preexposure to 100% benzaldehyde (Fig. 5, *tph-1*). The level of adaptation was unaffected by the addition of *E. coli* to the preexposure plate, consistent with a role for serotonin in mediating the food-induced suppression of adaptation. The addition of exogenous serotonin during the preexposure caused a slight but statistically insignificant (CI = 0.05 ± 0.02, $t_{17} = 0.5$, $P > 0.01$) inhibition of adaptation.

Strain CB1141, which carries the *cat-4(e1141)* allele, also has very low levels of endogenous serotonin (16). This strain, however, does perform well on naive chemotactic tests, achieving CI scores equivalent to wild-type animals (Fig. 5, *cat-4*; CI = 0.85 ± 0.03; $t_{45} = 2.0$, $P > 0.01$ vs. N2 naive). This attractive response was susceptible to adaptation, but this learning was not inhibited by the inclusion of *E. coli* on the preexposure plate (adapted CI = −0.06 ± 0.03, adapted + *E. coli* CI = 0.00 ± 0.02; $t_{22} = 0.8$, $P > 0.01$). The addition of exogenous serotonin, however, did inhibit adaptation to a degree equivalent to the inhibition seen with wild-type animals (N2 CI = 0.39 ± 0.04, *cat-4* CI = 0.36 ± 0.07; $t_{32} = 0.2$, $P > 0.01$). Exogenous serotonin is known also to rescue the male mating-behavior defect of *cat-4* mutants, consistent with a requirement for *cat-4* activity for serotonin synthesis (19). Therefore, serotonin rescues the mutant phenotypes by acting in the food-sensing (US) pathway downstream of the mutation.

Discussion

The nematode *C. elegans* shows a surprising degree of behavioral complexity for an animal with a relatively simple nervous system of 302 neurons. Many nematode behaviors are modulated by the presence of food. This modulation presumably allows the animals to respond appropriately to changing environmental conditions and to efficiently explore their surroundings in search of food. Olfactory adaptation is highly specific (8), and olfactory cues are likely to be important for locating distant food sources. We have shown that the modulation of olfactory behavior by food shows characteristics of associative learning and memory. Most important in this regard is that the degree of adaptation of the naive attraction to the odorant benzaldehyde is determined by the probability that benzaldehyde will colocalize with a food source, based on recent experience. After preexposure to benzaldehyde in the absence of food (CS–US pairing), the pairing of benzaldehyde with food (CS-only presentation) or food deprivation in the absence of benzaldehyde (US-only presentation) serve as extinction trials that restore the naive attraction to benzaldehyde by eliminating the learned correlation of the food and odorant. This predictive ability (degree to which the CS predicts the US) is a defining feature of associative learning (10, 11).

The presence of food has been shown also to modulate other exploratory behaviors displayed by *C. elegans*. The modulation of thermotactic behavior depends on previous experience where the animals learn to associate specific temperatures with the presence or absence of food, indicating an associative component to thermotactic behavior (3, 20). Preferences displayed to water-soluble chemoattractants have been shown also to be modulated by differential training with *E. coli*, and this learning has also been interpreted as an example of associative conditioning (6, 21). The involvement of serotonergic signaling has not been investigated in these other examples where a food US is used for conditioning. It is important to realize that food deprivation causes many physiological and behavioral changes in *C. elegans* (1, 2, 22) and can even induce an alternative developmental pathway leading to dauer larvae formation (5). These responses to food deprivation indicate that it is an important environmental cue, making food availability a salient US with which to associate additional cues. Additionally, the finding that habituation to mechanical stimuli is context-dependent (23) suggests that the environmental modulation of "simple" learning processes may be more common than previously appreciated, and that signals other than food can serve as secondary cues in such multistimulus learning paradigms.

Two lines of evidence confirmed that the suppression of benzaldehyde adaptation by food is a specific response rather than a lack of responding because of an inability to perceive the benzaldehyde stimulus. First, adaptation by *tph-1* and *cat-4* mutants was resistant to suppression by the presence of food, indicating that a specific, serotonergic-dependent mechanism is involved in learning that benzaldehyde predicts food. Second, the observation that the animals can still chemotax toward 1% point sources of benzaldehyde or diacetyl shows that they can detect and respond to odorants in the presence of *E. coli*. It is noteworthy that the animals will still approach a source of 1% benzaldehyde under conditions that inhibit the approach to a 100% point source of the odorant. This sensitivity profile suggests the existence of at least two distinct mechanisms for benzaldehyde detection. The first utilizes a high-affinity system that depends on the AWC sensory neurons and directs CTX to dilute sources of benzaldehyde (7). A second, lower affinity system directs CTX to higher concentrations of benzaldehyde and mediates olfactory adaptation. The low-affinity system is apparently more sensitive to inhibition by the presence of food. The fact that *odr-1(n1936)* mutants are deficient in their re-

sponse to 1% benzaldehyde (7) but will still approach a point source of 100% benzaldehyde (our present results) provides a clear double dissociation of the concentration-dependent behavioral response to benzaldehyde through two separate mechanisms. A similar situation seems to exist for the perception of diacetyl, because *odr-10* (the high-affinity diacetyl receptor) mutants still approach high concentrations of the odorant by means of a low-affinity diacetyl receptor (24). Furthermore, behavioral studies have shown that high and low concentrations of diacetyl induce distinct learning mechanisms (25).

A number of neurotransmitters are used by *C. elegans*, and they are involved in many diverse behaviors. The ability of serotonin to mimic the effects of food on adaptation and the blocking of the food-induced suppression in the serotonin-deficient mutant strains indicate that serotonin plays an important role in this process *in vivo*. Food-odor associative learning is therefore another example of a food-dependent behavior that requires serotonergic signaling in *C. elegans* along with locomotion rate, and the frequencies of pharyngeal pumping, egg laying, and defecation (1, 2, 22). It therefore seems that serotonin levels broadcast the food status of the environment to the nervous system such that the animals act in a manner suited to their nutrient environment, which includes maintaining a high level of attraction to olfactory stimuli that have been encountered in the presence of food while muting the attractive response to, or actively avoiding, odorants encountered in the absence of food.

Associative learning has been described in a number of organisms. Although *C. elegans* may use different cellular learning strategies than organisms with more complex nervous systems, the essential features of the associative-learning process are apparent in the behavior we have described. The animals display an innate attraction to benzaldehyde, and it is only when benzaldehyde is encountered in a nutrient-deficient environment (the absence of *E. coli*) that their chemotactic response to this stimulus becomes attenuated. Furthermore, this learned suppression of the attractive response to benzaldehyde is extinguished when the animals encounter benzaldehyde paired with *E. coli*, because they learn that benzaldehyde predicts the presence of a food source. Indeed, the data presented in Fig. 3 show that the animals developed either an attraction or aversion to benzaldehyde depending on whether the odorant was encountered in the absence or presence of food in the final hour of training and whether the absence of food was encountered in the presence or absence of the odor. Such a change in the value of the response to benzaldehyde indicates that this behavior is not the result of the modulation of a single, nonassociative-learning process but rather can be taken as clear evidence of associative learning (26).

Our present data, as well as previous analyses of benzaldehyde-responsive behavior (9, 27), clearly demonstrate that *C. elegans* possesses multiple mechanisms that support both attractive and aversive responses to the odorant. We propose that the simultaneous strengthening and/or weakening of these "core" response mechanisms, dependent on presence or absence of additional environmental stimuli, endows this relatively simple nervous system with the ability to extract and remember important environmental relationships. Accordingly, associative-learning capabilities represent an emergent property of a hierarchical network in which these systems interact and contribute to whole animal behavior. The tractability of *C. elegans* as an experimental system should facilitate the elucidation of the molecular/genetic mechanisms underlying such multistimulus learning by simple cellular ensembles. These mechanisms are likely to contribute to the plasticity demonstrated by the nervous systems of more behaviorally complex animals.

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