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PLASTICITY OF CHEMOTAXIS REVEALED BY PAIRED PRESENTATION OF A CHEMOATTRACTANT AND STARVATION IN THE NEMATODE *CAENORHABDITIS ELEGANS*

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Summary

While the basic functioning of the nervous system of *Caenorhabditis elegans* has been extensively studied, its behavioural plasticities have not been fully explored because of the limited availability of assay systems. We report here a simple form of chemotaxis plasticity in this organism: when worms are starved on plates that contain NaCl, their chemotaxis towards NaCl falls dramatically. This conditioning requires both the presence of NaCl and the absence of a bacterial food source, indicating that it is not merely adaptation or habituation, but that it is likely to be a form of associative learning. While chemotaxis towards volatile chemoattractants does not change significantly after conditioning with NaCl, chemotaxis towards other water-soluble attractants does decrease. This suggests that an altered response of a cell or a group of cells specifically involved in chemotaxis towards water-soluble chemoattractants is responsible for the behavioural alteration. The decrease in chemotaxis occurred slowly over 3–4 h of conditioning and returned quickly to the original level when either of the conditioning stimuli, NaCl or starvation, was removed. The application of serotonin partially blocked this reduction in chemotaxis, consistent with the proposed function of this neurotransmitter in food signalling. Using this assay, we have isolated three mutants with reduced plasticity. This assay system expands the opportunities for studying the molecular and cellular mechanisms of behavioural plasticity in *C. elegans*.

Key words: chemotaxis, water-soluble chemoattractant, starvation, serotonin, associative learning, *Caenorhabditis elegans*.

Introduction

The nematode *Caenorhabditis elegans* is an ideal model organism for functional analysis of the nervous system because so much genetic information is available and the structure of its simple nervous system, which consists of 302 neurons in an adult hermaphrodite, has been well-described. To date, various ‘uncoordinated’ (Unc) mutants involving defects in locomotion have been isolated, and more than 100 genetic loci have been defined. While the analysis of these *unc* genes can provide substantial information about the essential functioning of the nervous system and musculature (Riddle et al., 1997), this nematode species should also be useful for studying behavioural plasticity using genetics and molecular biology.

Several forms of behavioural plasticity have been reported in *C. elegans*. For example, the tap withdrawal reflex, in which animals retreat in response to a tap on the culture plate, is known to be subject to habituation, dishabituation and sensitization (Rankin et al., 1990). The neural circuits underlining this response have been examined (Wicks and Rankin, 1996), but the molecular mechanism involved is only now starting to be understood. A recent report shows that EAT-4, an inorganic phosphate transporter supposedly essential for glutamate release, plays a critical role in this form of plasticity (Rankin and Wicks, 2000). Another form of plasticity, namely sensory adaptation, has been observed for several volatile chemoattractants (Colbert and Bargmann, 1995; Colbert and Bargmann, 1997; L’Etoile and Bargmann, 2000). Animals exposed to these odorants for a certain period show decreased chemotaxis towards them. The *adp-1*, *osm-9* and *odr-1(op)* strains have been identified as showing a decreased ability to adapt to overlapping subsets of odorants.

Higher-order behavioural plasticities have also been reported. When animals are placed in a temperature gradient, they move towards the temperature at which they were raised. However, if animals are starved, they gradually learn to avoid that temperature (Hedgecock and Russell, 1975). Mutants showing altered thermotactic behaviour have been isolated (Hedgecock and Russell, 1975; Mori and Ohshima, 1995). Some of these have defects in temperature sensing (e.g. Komatsu et al., 1996), and others may be defective in temperature memory. Recently, an associative learning paradigm based on chemotactic behaviour towards water-soluble chemoattractants was reported. Two mutants, *lrn-1* and

lrn-2, defective in this form of learning were isolated (Morrison and van der Kooy, 1997; Wen et al., 1997). All these assay systems show limitations, e.g. the change in behaviour may be small, the variability may be large or the assay may be incompatible with a population assay. Here, we report another assay system for associative learning that is simpler and more reliable than those used previously. Using this assay system, we have isolated three mutants defective in behavioural plasticity.

Materials and methods

Strains and media

Caenorhabditis elegans wild-type strain var. Bristol N2 was used for behavioural assays. Nematodes were cultivated on NGM plates (3 g l^{-1} NaCl, 2.5 g l^{-1} polypeptone, 5 mg l^{-1} cholesterol, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 25 mmol l^{-1} potassium phosphate, pH 6.0, 17 g l^{-1} agar) on the *Escherichia coli* strain NA22 (as described previously; Brenner, 1974). The NGM plates were also used for conditioning. The NGM(NaCl–) medium used for mock-conditioning was NGM lacking NaCl.

Chemotaxis assay

Chemotaxis assays were based on those described by Bargmann and colleagues (Bargmann and Horvitz, 1991; Bargmann et al., 1993) with some modifications. Assay plates contained 5 mmol l^{-1} potassium phosphate, pH 6.0, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$ and 20 g l^{-1} agar. For simple NaCl chemotaxis assays, an agar plug was excised from a NaCl plate (made up as above but with the addition of 100 mmol l^{-1} NaCl, unless noted otherwise) with a cork borer and placed on the surface, off-centre, of an assay plate, which was then left overnight (14–24 h). Shortly before the chemotaxis assay, the NaCl plug was removed and 1 µl of 0.5 mol l^{-1} sodium azide was spotted onto the same position to anaesthetize the animals at the centre of the gradient. As a control, sodium azide was also spotted at a position approximately 4 cm away from the centre of the NaCl gradient. Approximately 100 animals (either naive or conditioned) were then placed equidistant (approximately 3 cm) from these two spots (see Fig. 1C) and left to move freely on the assay plate for 30 min at 20 °C. The assay plates were then chilled to 4 °C, and the number of worms around each spot was counted. The chemotaxis index (CI) was calculated as $CI=(N_+-N_-)/$(total number of animals on the the plate), where N_+ is the number of animals within 1.5 cm of the centre of the NaCl gradient and N_- is the number within 1.5 cm of the control spot. Assays were typically performed in triplicate (animals were placed on three chemotaxis plates), and the CI value was averaged. The numbers of independent experiments performed, represented by *N*, are indicated in the figure legends.

Chemotaxis assays for water-soluble chemoattractants other than NaCl were performed exactly as those for NaCl, except that the NaCl in the agar plug was replaced with a chemoattractant. The chemicals used and their concentrations in the agar plugs were 200 mmol l^{-1} cAMP-NH_4 (simply designated cAMP), 100 mmol l^{-1} biotin-NH_4 (biotin), 500 mmol l^{-1} lysine acetate (lysine), 200 mmol l^{-1} sodium acetate (Na^+) and 250 mmol l^{-1} ammonium chloride (Cl^-). In the case of cAMP, biotin and lysine, the pH of the stock solution was adjusted to 6.0 with ammonium hydroxide (as described by Bargmann and Horvitz, 1991).

Chemotaxis towards volatile chemoattractants was assayed using the same format as water-soluble chemotaxis assays. In this case, however, 1 µl of diluted odorant was spotted directly onto the surface of the assay plate, together with sodium azide, just before placing the washed animals onto the plate (Bargmann et al., 1993). Isoamylalcohol was diluted with either water or ethanol. Diacetyl and 2,4,5-trimethylthiazole were diluted in ethanol.

For the counterattractant assay, a NaCl gradient was formed, and sodium azide was spotted as described above. In addition, 1 µl of isoamylalcohol, diluted one thousandfold in water, was spotted just before the chemotaxis assay on the side opposite the NaCl. The chemotaxis index was calculated as above. In this case, N_+ was the number of animals within 1.5 cm of the centre of the NaCl gradient and N_- was the number within 1.5 cm of the centre of the isoamylalcohol gradient.

Conditioning procedure

The procedure for the learning assay was as follows. Four to six adults were transferred to a seeded 6 cm plate and incubated at 20 °C for 4 days. In this time, the F_1 progeny grew to young adults under well-fed conditions. Plates on which the bacterial lawn had been exhausted at this stage were discarded. The animals were washed off the plates with wash buffer (5 mmol l^{-1} potassium phosphate, pH 6.0, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 0.5 g l^{-1} gelatin) and transferred to a test tube. The animals were washed three times by allowing the adults to fall through the wash buffer by gravity and replacing the supernatant, including floating small larvae, with fresh wash buffer. For the naive chemotaxis assay, washed animals were placed directly onto chemotaxis plates, and excess fluid was absorbed with a Kimwipe. For conditioning, washed animals were placed on a conditioning plate, and excess fluid was absorbed with a Kimwipe. An NGM plate was normally used for conditioning. For some control experiments, an NGM(NaCl–) plate was used instead, and this was designated mock-conditioning. After incubation at 20 °C for an appropriate time (4 h under the standard assay conditions), the animals were collected again with wash buffer and chemotaxis was assayed as described above.

Mutant screening

Caenorhabditis elegans N2 hermaphrodites at the L4 stage were mutagenized with ethyl methanesulfonate (EMS) (as described by Brenner, 1974) and allowed to self-fertilize for two generations. In principle, mutations that occurred in the germline of P_0 animals are transmitted to the F_1 generation in a heterozygous state. One-quarter of the F_2 animals from these F_1 animals are expected to be homozygous for each mutation.

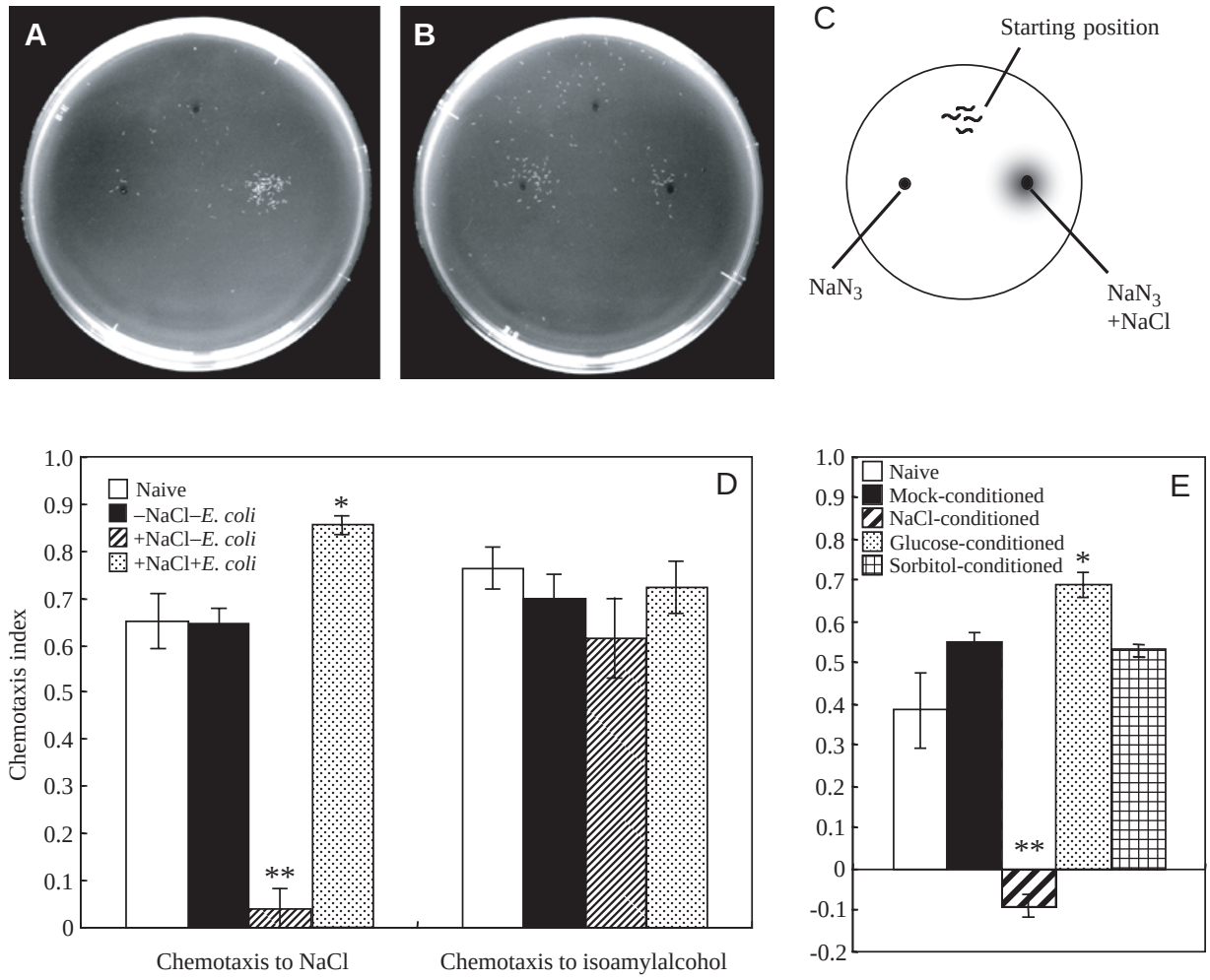

Fig. 1. Starvation-induced decrease in chemotaxis to NaCl. (A–C) A typical result of conditioning. Young adult hermaphrodites were collected, washed and assayed for NaCl chemotaxis directly (A) or placed on a bacteria-free NGM plate for 4 h and assayed for NaCl chemotaxis (B). Pictures were taken 30 min after placing the animals at the starting position. The configuration of the assay plates is shown schematically in C. (D) Young adult hermaphrodites were collected, washed and assayed for chemotaxis directly (naive) or placed on an NGM plate (which includes 50 mmol l^{-1} NaCl) without bacteria (+NaCl–*E. coli*), an NGM(NaCl–) plate without bacteria (–NaCl–*E. coli*) or an NGM plate with bacteria (+NaCl+*E. coli*) for 4 h, and chemotaxis towards 100 mmol l^{-1} NaCl (left) or to a one thousandfold dilution of isoamylalcohol (right) was assayed. Asterisks indicate a significant difference (**P*<0.05; ***P*<0.005; *N*=6) from the naive value. (E) Young adult hermaphrodites were collected, washed and assayed for NaCl chemotaxis directly (naive) or placed on an NGM(NaCl–) plate without bacteria (mock-conditioned; identical to –NaCl–*E. coli* in D), an NGM plate without bacteria (NaCl-conditioned; identical to +NaCl–*E. coli* in D) or an NGM plate in which NaCl had been replaced with either 100 mmol l^{-1} glucose (glucose-conditioned) or 100 mmol l^{-1} sorbitol (sorbitol-conditioned) without bacteria for 4 h, and chemotaxis towards 100 mmol l^{-1} NaCl was assayed. Asterisks indicate significant difference from the mock-conditioned value (**P*<0.05; ***P*<0.005; *N*=3). Values are means ± S.E.M.

Therefore, F_2 progeny of mutagenized animals were conditioned as a population and subjected to the counterattractant assay; the animals attracted to NaCl were collected and cultured, and their offspring were conditioned and tested again as a population. This operation was repeated 4–5 times. To omit mutants defective for chemotaxis towards isoamylalcohol, intervening steps were also included in which only animals attracted to isoamylalcohol were collected. Finally, individual animals were chosen and their progeny tested.

Egg-laying behaviour was observed by counting the number of eggs laid by each mutant during a 3 h period on either seeded or bacteria-free NGM plates. Locomotory behaviour was observed on seeded NGM plates by counting the number of body bends in 30 s.

Results

The learning assay system based on the plasticity of chemotactic behaviour

We found that when *C. elegans* adult hermaphrodites are kept on bacteria-free NGM plates, the standard culture plates for *C. elegans*, for approximately 4 h, chemotaxis towards NaCl falls dramatically (Fig. 1A–C). This decrease in chemotaxis is due to the presence of NaCl (at approximately

50 mmol l^{-1}) in the NGM medium, because the decrease in chemotaxis was not observed when animals were starved on NaCl-free NGM (Fig. 1D, left). The absence of bacteria must be another critical factor for this behavioural modulation, because naive animals, which served as controls (Fig. 1A), were cultivated on NGM plates with bacteria as a food source. In fact, animals harvested and transferred to NGM plates with bacteria in parallel with the other conditioning plates did not show the reduction in chemotaxis (Fig. 1D, left). These results indicate that the presentation of NaCl coupled with starvation causes the suppression of chemotaxis towards NaCl. This is not the result of a general defect in locomotion or other functions essential for chemotaxis in general, because conditioned animals still showed chemotaxis towards isoamylalcohol, a volatile chemoattractant for *C. elegans* (Fig. 1D, right). To determine whether the effect is specific to NaCl or is a nonspecific effect of high osmolarity, we replaced NaCl in the conditioning plate with either glucose or sorbitol at the same osmolarity (100 mmol l^{-1}). Sorbitol had no effect on NaCl chemotaxis, while glucose caused a slight increase in chemotaxis (Fig. 1E), indicating that the effect of NaCl cannot be attributed to its osmolarity.

The amphid sensilla, located bilaterally on the anterior end of the body, are major chemosensory organs mediating chemotaxis in *C. elegans*. Among amphid neurons, the ASE neuron pair is believed to be responsible for sensing both Na^+ and Cl^-, because destroying the ASE neurons greatly impairs chemotaxis towards these chemoattractants, while the ADF, ASG and ASI neurons are responsible for the residual response (Bargmann and Horvitz, 1991). The ASE neurons also play a major role in mediating chemotaxis towards other water-soluble chemoattractants such as cAMP, biotin and lysine. It is therefore assumed that many different chemoreceptors are expressed in the ASE chemosensory neurons.

We examined the specificity of the conditioning by testing chemotaxis towards these other chemoattractants after conditioning with NaCl (Fig. 2A). Interestingly, chemotaxis towards these water-soluble chemoattractants was also reduced after conditioning with NaCl. In contrast, chemotaxis towards volatile chemoattractants, which are sensed by AWC (isoamylalcohol), AWA (diacetyl) or both (trimethylthiazole) (Bargmann et al., 1993), was essentially unaffected. The effects of NaCl conditioning on the response to volatile chemoattractants were further tested with higher dilutions of the odorants, because chemotaxis towards these odorants was generally high at the dilution shown in Fig. 2A (10^{-3}), and a reduction in chemotaxis may have been overlooked. At higher dilutions, NaCl-conditioned animals did not show any reduction in chemotaxis towards volatile odorants, while chemotaxis towards NaCl was reduced at all the concentrations of NaCl tested (Fig. 2B).

Up to this point, we had used NaCl for conditioning. It is known that both Na^+ and Cl^- are chemoattractants for *C. elegans*, so we also tested these ions separately by using sodium acetate or ammonium chloride. Acetate and ammonium ions are known not to be chemoattractants (Ward,

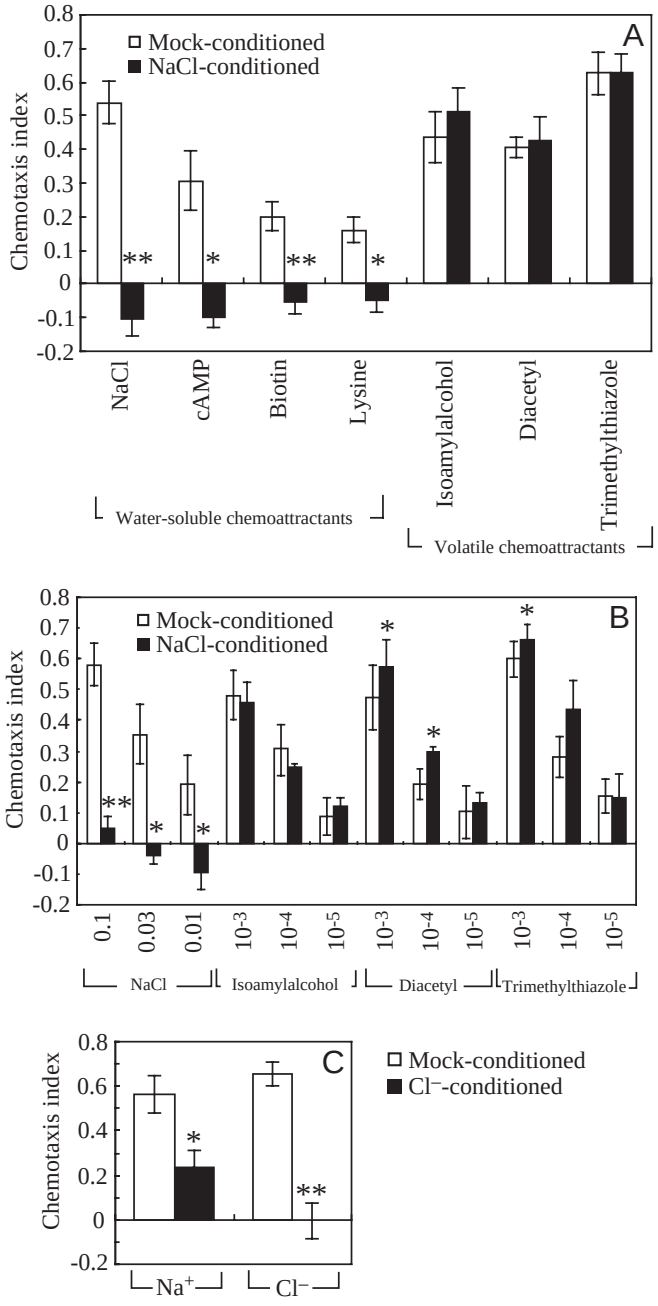

Fig. 2. Cross conditioning. (A) Animals were collected, washed and placed on NGM(NaCl–) plates without *E. coli* (mock-conditioned) or NGM plates without *E. coli* (NaCl-conditioned) for 4 h, and chemotaxis towards water-soluble or volatile chemoattractants, as shown in the figure, was assayed. The concentration of NaCl was 100 mmol l^{-1}, and the dilution of volatile chemoattractants was one thousandfold. Asterisks indicate that the NaCl-conditioned value is significantly lower than the mock-conditioned value (*P<0.05; **P<0.005; N=5). (B) As for A except that chemotaxis towards various concentrations (in mol l^{-1}) of NaCl and volatile chemoattractants was tested (N=5). (C) Animals were collected, washed and placed on an NGM(NaCl–) plate without *E. coli* (mock-conditioned) or an NGM plate in which NaCl had been replaced with ammonium chloride (Cl^--conditioned) for 4 h, and chemotaxis towards sodium acetate (Na^+) or towards ammonium chloride (Cl^-) was assayed (N=5). Values are means ± S.E.M.

1973). Animals conditioned with ammonium chloride showed a reduction in chemotaxis towards sodium acetate as well as towards ammonium chloride, although the reduction in chemotaxis towards sodium acetate was smaller (Fig. 2C). When sodium acetate was used for conditioning, the effect was variable and, if a response was seen, it was small.

These results show that cross conditioning generally occurs among ASE-sensed chemoattractants, which suggests that the modulation occurs at the cellular (or higher) level rather than at the receptor level. However, chemotaxis towards Cl^- was more affected by Cl^- conditioning than chemotaxis towards Na^+, suggesting the presence of a chemical-specific component as well (discussed below).

For the rest of the experiments, we used NaCl for conditioning because NaCl was easier to handle. To detect the change in chemotaxis more easily, we introduced a counterattractant assay involving a choice between NaCl at one end of the assay plate and isoamylalcohol at the other end acting as a counterattractant. The concentrations of NaCl and isoamylalcohol were set so that naive animals slightly preferred NaCl over isoamylalcohol. This assay should reflect the change in chemotaxis towards NaCl because chemotaxis towards isoamylalcohol does not change significantly, as shown above. In this assay, naive animals showed positive chemotaxis indices (CIs), which indicates that the animals preferred NaCl over isoamylalcohol, while animals starved in the presence of 50 mmol l^{-1} NaCl showed a negative CI value, indicating that they now preferred isoamylalcohol over NaCl (Fig. 3). The reduction in CI was not observed when animals were starved in the absence of NaCl or when animals were kept on the conditioning plate with or without NaCl in the presence of bacteria (Fig. 3).

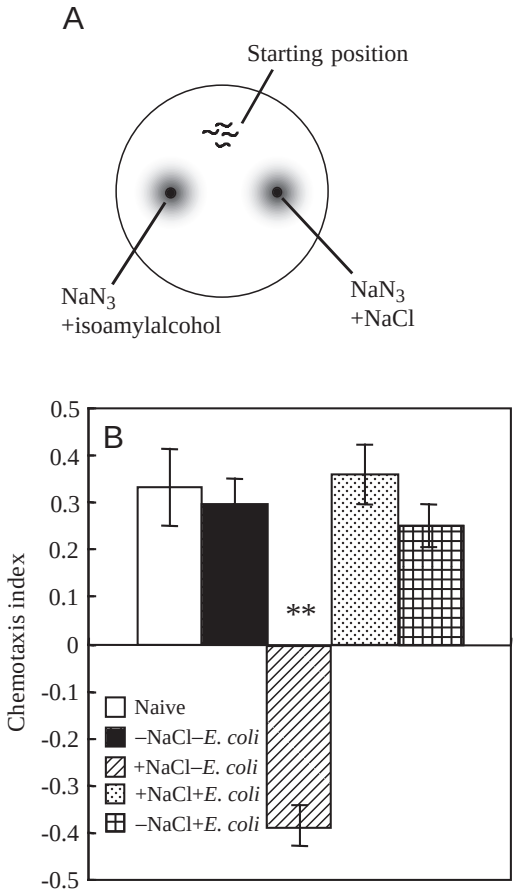

Fig. 3. Counterattractant assay for conditioning. (A) The format of the assay plate. (B) Animals were collected, washed and assayed for chemotaxis directly (naive) or placed on an NGM plate with (+) or without (–) NaCl and with (+) or without (–) *E. coli* for 4 h. Chemotaxis was then assayed using NaCl and isoamylalcohol as depicted in A and described in Materials and methods. Values are means ± S.E.M. (N=10). A double asterisk indicates a significant difference from the naive value (P<0.005).

Time course of acquisition and reversal of conditioned status

We further characterized this conditioning and assay system. First, we determined the time course of conditioning by keeping the animals on bacteria-free NGM plates for various times and testing their chemotaxis in the counterattractant assay. The change in chemotactic preference occurred gradually over approximately 4 h of starvation and reached a plateau thereafter (Fig. 4A and data not shown). This result suggests that the molecular or cellular event underlying the behavioural alteration occurs gradually in a cumulative fashion during the 4 h starvation period.

We next determined the time course of reversal of conditioning under two different conditions. First, animals conditioned by 4 h of starvation in the presence of NaCl were transferred to a plate without NaCl for various times, and their chemotactic preference was assayed. The reduction in CI caused by starvation in the presence of NaCl was rapidly (within 1 h) reversed under these conditions (Fig. 4B). Second, conditioned animals were transferred to a plate containing both NaCl and bacteria. The reduction in CI was reversed even more rapidly (within 10 min) under these conditions (Fig. 4C). Thus, the removal of either of the conditions required for behavioural change, namely the absence of bacteria or the presence of NaCl, rapidly abolished the conditioned status.

Effects of serotonin on the plasticity of chemotaxis

The monoamine neurotransmitters serotonin and dopamine are thought to be involved in the transmission of nutritional signalling in *C. elegans* (Avery and Horvitz, 1990; Horvitz et al., 1982; Sawin et al., 2000; Segalat et al., 1995). For example, animals on a bacterial lawn show a slower rate of locomotion than animals in the absence of bacteria. The addition of serotonin or dopamine slows locomotion. In addition, serotonin stimulates egg-laying and pharyngeal pumping, mimicking the presence of bacteria. To determine whether these neurotransmitters are also involved in the transmission of the food signal whose absence is responsible for the alteration in chemotaxis in our assay, we added these neurotransmitters to the medium and tested their effects on conditioning. Dopamine had no effect in our assay. In contrast, when serotonin was added to the conditioning plate, the change in chemotaxis was reduced (Fig. 5, compare C with B). Furthermore, if serotonin was added after conditioning, the

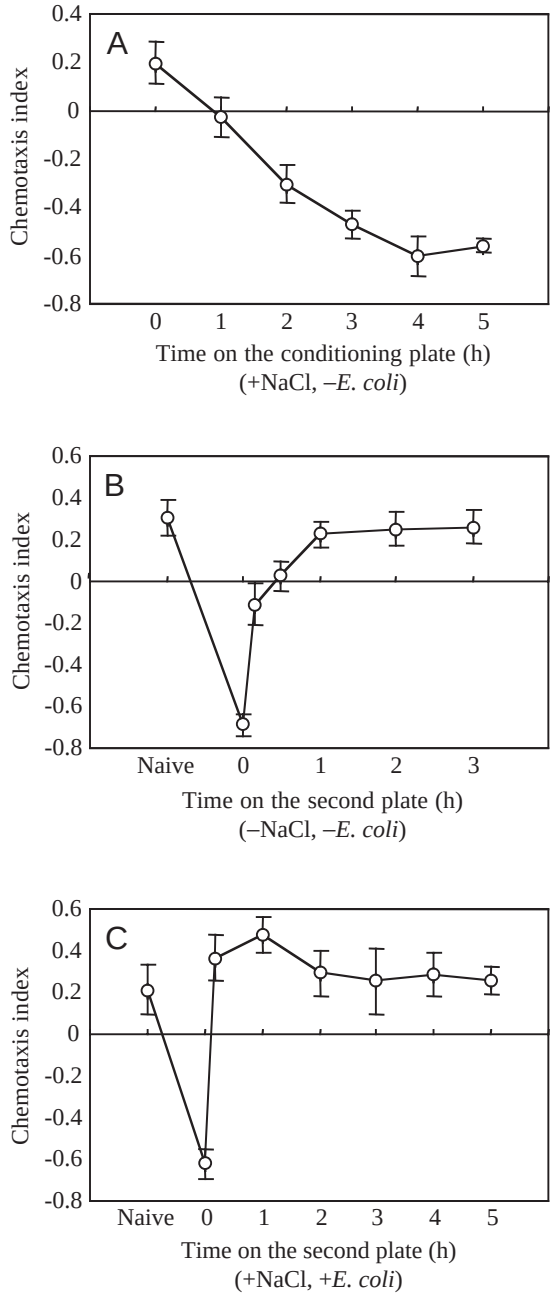

Fig. 4. Time course of conditioning and reversal. (A) Animals were collected, washed and transferred to NGM plates (with NaCl, without *E. coli*), kept on the plates for the times indicated (conditioned), and chemotaxis was assayed. (B) Animals were collected, washed and placed on an NGM plate without *E. coli* for 4 h (conditioned) and then transferred to NGM(NaCl–) plates without *E. coli*, kept on the plates for the times indicated (reversal), and chemotaxis was assayed. (C) Animals were collected, washed and placed on an NGM plate without *E. coli* for 4 h (conditioned) and then transferred to NGM plates with *E. coli*, kept on the plates for the times indicated (reversal), and chemotaxis was assayed. In A–C, chemotaxis was assayed using the counterattractant assay. Values are means ± S.E.M. (N=5).

reduction in chemotaxis was partially reversed (Fig. 5, compare E with D). As stated above, serotonin is known to slow locomotion. However, the above observation is not a

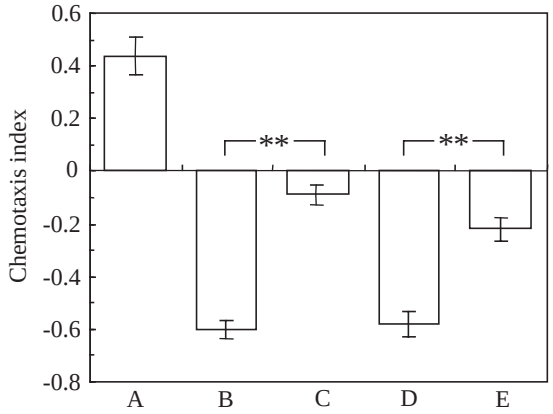

Fig. 5. Effects of serotonin application on conditioning. (A–C) Animals were collected, washed and assayed for chemotaxis directly (A) or placed on an NGM plate (B) or an NGM plate with 5 mg ml^{-1} serotonin (C) without *E. coli* for 4 h and assayed for chemotaxis. (D,E) Animals were collected, washed and placed on an NGM plate without *E. coli* for 4 h (conditioned), and then transferred to an NGM plate (D) or an NGM plate with 5 mg ml^{-1} serotonin (E) without *E. coli*, kept on the plate for 30 min, and then assayed for chemotaxis. All the chemotaxis assays were the counterattractant assay. Values are means ± S.E.M. (N=5). A double asterisk indicates a significant difference from the value in the absence of serotonin (P<0.005).

simple reflection of a general effect on locomotion, because most of the serotonin-treated animals moved towards either the NaCl or isoamylalcohol rather than staying at or around the starting point. Therefore, these results indicate that the suppression of NaCl chemotaxis by conditioning is partially blocked or reversed by the addition of serotonin and are compatible with the possibility that serotonin mimics the presence of food in this assay system.

Isolation of learning-deficient mutants

Our ultimate goal is to identify genes involved in behavioural plasticity in *C. elegans*. To this end, we attempted to isolate mutants that showed defects in plasticity in our assay system, as described in Materials and methods. We obtained five mutants whose chemotaxis index after conditioning was greater than that of the wild-type when tested in the counterattractant assay (with NaCl and isoamylalcohol). They were further tested with the NaCl-only assay, because sensitivity to isoamylalcohol may be altered in the mutants. Three mutants still showed the defect in this assay, as illustrated in Fig. 6. One of these mutants (JN693) showed an elevated naive chemotaxis index and may have an altered sensitivity to NaCl. The other two showed normal naive chemotaxis, but a smaller reduction in chemotaxis after conditioning compared with the wild type. To gain insights into the nature of the defects, egg-laying behaviour was observed in these mutants because mutants affected in terms of starvation signalling may also show altered egg-laying behaviour, which is under starvation control (see above). Normal egg-laying was observed in all mutants under both the

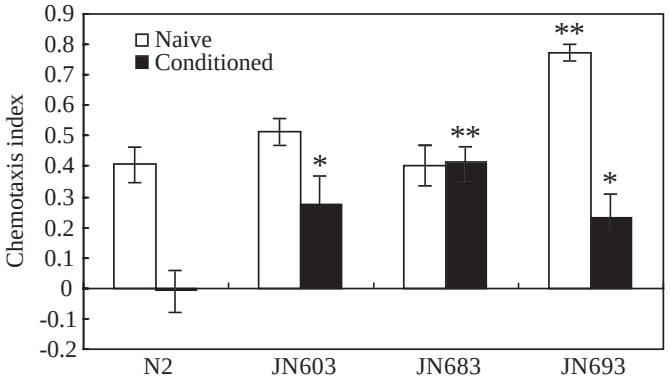

Fig. 6. Learning assays for newly isolated mutants. Young adult hermaphrodites of the wild type (N2) or of each mutant were collected and assayed for NaCl chemotaxis directly (naive) or placed on an NGM plate without *E. coli* for 4 h (conditioned) and assayed for NaCl chemotaxis. Asterisks indicate that the chemotaxis index of the mutants is significantly different from the wild-type value under the same conditions (*P<0.05; **P<0.005). Chemotaxis assays were simple NaCl chemotaxis. Values are means ± S.E.M. (N=9).

satiated and starved conditions. In addition, we examined locomotory behaviour, which is also under food control. One mutant, JN603, showed slow locomotion (data not shown), but this phenotype segregated from the learning defect in outcrosses. These mutants might therefore be defective in the basic mechanisms of learning.

Discussion

We have devised a simple system for assaying behavioural plasticity in *C. elegans*. This assay system is based on the observation that, when animals are starved on NaCl, their chemotaxis towards NaCl decreases. This behavioural change requires the simultaneous presentation of NaCl and starvation: no reduction in chemotaxis was observed in the absence of either factor. In one control experiment, animals were precultured on NGM (with NaCl) and then starved in the absence of NaCl, which resulted in no detectable change in chemotaxis (Figs 1D, 3B). The unpaired presentation of these stimuli therefore has no effect. These observations appear to meet the criteria for associative learning. The effect of NaCl is specific because neither glucose nor sorbitol had a similar effect. When the animals were starved in the presence of NaCl, chemotaxis towards other water-soluble chemoattractants, such as cAMP and biotin, was also reduced. In the reverse situation, when either cAMP or biotin was used for conditioning, chemotaxis towards NaCl was reduced (Y. Iino, unpublished observations). This is probably because all these chemoattractants are sensed by the ASE chemosensory neurons, although they are likely to bind to different cell-surface receptors, and because the observed behavioural change reflects alterations occurring at the cellular level rather than the receptor level. A further complication comes from the recent finding that the pair of ASE neurons is not functionally equivalent and that Cl^- is sensed by the right member of the ASE neurons, ASER, and Na^+ is mainly sensed by the left member, ASEL (Pierce-Shimomura et al., 2000). This probably explains our observation that conditioning with Cl^- affects chemotaxis towards Na^+ only modestly, whilst its effect on Cl^- chemotaxis is stronger (Fig. 2C). In a previous report, Wen et al. (Wen et al., 1997) paired either Na^+ or Cl^- with food and the other ion with the absence of food. Worms thus conditioned showed a preference for the ion paired with food over the other ion when tested in a counterattractant assay in which Na^+ was placed on one side of the assay plate and Cl^- was placed on the other side. This observation is probably another manifestation of the above-mentioned differential effect of Cl^- conditioning on Na^+ and Cl^- chemotaxis in our assay.

The change in chemotactic behaviour in our conditioning paradigm was manifest gradually over 4 h of conditioning. This time course is similar to that observed for the change in thermotactic preference in response to a change in the cultivation temperature (Hedgecock and Russell, 1975). Sensory adaptation to dopamine also requires several hours (Schafer and Kenyon, 1995). The reversal, however, occurs very quickly in our paradigm. Animals that have been starved show an altered response to the presence of food (Sawin et al., 2000). In our assay, the presence of food after starvation-induced conditioning appeared to cause an overshoot in the chemotaxis index (Fig. 4C). This is reminiscent of the enhanced effect of food on locomotion in starved animals, which is mediated by serotonin (Sawin et al., 2000). In combination with our observation that the application of serotonin partially blocks and reverses conditioning with NaCl and starvation, these results make it likely that serotonin does play a role in conditioning, although it is not likely to be the sole mediator of the food signal. The effect of starvation has also been reported for adaptation to odorants (Colbert and Bargmann, 1997), which is similar to, but different from, our observation on chemotaxis towards water-soluble attractants. Colbert and Bargmann (Colbert and Bargmann, 1997) observed that animals pre-starved for 1 h or more show an increased adaptation to some odorants compared with well-fed animals. Interestingly, the addition of serotonin reversed the effect of starvation, as it did in our assay. Whether similar cellular and molecular mechanisms underlie these two forms of plasticity is an interesting issue to be addressed in the future.

In spite of the various advantages of *C. elegans* as a model organism in which to study the nervous system and behaviour, it has not been as extensively used to study behavioural plasticity as other organisms such as *Drosophila melanogaster*. This is partly because of the poor repertoire of assay systems in *C. elegans*. The system we have reported here is very simple and robust compared with other assays used in this organism (Colbert and Bargmann, 1995; Wen et al., 1997) but, nevertheless, has the characteristics of associative learning. It remains to be determined whether this form of plasticity employs molecular mechanisms that are common to higher animals. Our system is also compatible with population assays,

which is another advantage compared with some other assays (Rankin et al., 1990). These advantages have allowed us to isolate with relative ease mutants that show defects in their behavioural plasticity: we repeatedly enriched for mutants in a population of mutagenized animals rather than examining each clonal population. We have isolated three mutants that are conditioned less efficiently than the wild type. There are several possible primary steps that could be affected by the mutations, including food signalling, the association between NaCl and starvation and a suppression of chemotaxis, although some food responses were observed to be intact in these mutants. By repeating this kind of screen, it will be possible to obtain more mutants. By testing whether these mutants also show defects in other learning assays and by molecularly cloning the affected genes, we should be able to gain further insights into the cellular and molecular mechanisms of learning in *C. elegans*.

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