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DIET-INDUCED PLASTICITY IN THE TASTE SYSTEM OF AN INSECT: LOCALIZATION TO A SINGLE TRANSDUCTION PATHWAY IN AN IDENTIFIED TASTE CELL

JOHN I. GLENDINNING*, SONYA ENSSLEN, MICHAL E. EISENBERG AND PHOEBE WEISKOPF
Department of Biological Science, Barnard College, Columbia University, 3009 Broadway, New York, NY 10027, USA
*e-mail: jglendinning@barnard.edu

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Summary

We studied exposure-induced sensitivity changes in an identified taste cell from *Manduca sexta*, a herbivorous caterpillar. This taste cell occurs within the lateral styloconic sensillum and responds selectively to compounds that humans characterize as bitter (e.g. caffeine, salicin and aristolochic acid). We made extracellular recordings from several classes of identified taste cell within the lateral sensillum, both before and after dietary exposure (for 48 h) to a suprathreshold concentration of caffeine, salicin or aristolochic acid. Our results revealed (1) that dietary exposure to caffeine desensitized the bitter-sensitive taste cell to caffeine, whereas dietary exposure to salicin or aristolochic acid did not desensitize the same taste cell to salicin or to aristolochic acid; (2) that dietary exposure to caffeine failed to alter the responsiveness of the sugar-, salt- or inositol-sensitive taste cells within the same sensillum; (3) that the caffeine-induced desensitization phenomenon generalized to salicin, a compound that stimulates the same transduction pathway as caffeine, but not to aristolochic acid, a compound that stimulates a different pathway; and (4) that chronically stimulating the lateral sensillum with caffeine, in the absence of ingestion, was sufficient to induce desensitization. We conclude that caffeine causes desensitization through a direct effect on a single transduction pathway within the bitter-sensitive taste cell.

Key words: bitter-sensitive taste cell, taste, diet-induced plasticity, desensitization, caterpillar, *Manduca sexta*, Sphingidae.

Introduction

The responsiveness of animals to sapid and odoriferous compounds is not fixed. Chronic exposure to specific compounds can profoundly alter subsequent behavioral responsiveness to chemical stimuli in both vertebrates and invertebrates. In some cases, chronic exposure enhances the behavioral responsiveness of an animal to a chemical stimulus (Wysocki et al., 1989; Hepper, 1992; Wang et al., 1993; Bilkó et al., 1994; Renwick and Huang, 1995; Dalton and Wysocki, 1996; Preston and Hammond, 1998) and, in other cases, it diminishes responsiveness (Warren and Pfaffman, 1959; Szentesi and Bernays, 1984; Zellner et al., 1985; Jermy et al., 1987; Usher et al., 1988; Harder et al., 1989; Burghardt, 1992; Simpson and Raubenheimer, 1993; Colbert and Bargmann, 1995; Glendinning and Gonzalez, 1995; Renwick and Huang, 1995). These sensitivity changes usually develop over a period of hours to days, persist for a similar period following removal of the test chemical, and generalize to some but not all novel compounds.

The mechanistic basis for these sensitivity changes is difficult to determine because there are many sites in the nervous system that could mediate this phenomenon. Chronic exposure to specific chemical stimuli can activate associative and non-associative learning processes in the central nervous system, which alter subsequent behavioral responsiveness to sapid stimuli (Zellner et al., 1985; Whitney and Harder, 1986; Szentesi and Jermy, 1990; Sclafani, 1991; Swithers-Mulvey et al., 1991; Smith, 1996). The periphery can also contribute to the exposure-induced sensitivity changes. For example, chronic exposure to specific chemical stimuli can markedly increase (Wang et al., 1993; Nevitt et al., 1994; Semke et al., 1995) or decrease (Blaney et al., 1986; Vet et al., 1990; Simpson and Simpson, 1992; Lee et al., 1995) the sensitivity of taste and olfactory receptors to the same (and in some cases, novel) chemical stimuli. Little is known, however, about the mechanistic basis of these peripheral sensitivity changes. One study found that levels of nutrients (e.g. amino acids) in the blood can directly modulate the responsiveness of taste cells to the same nutrients in locusts (Simpson and Simpson, 1992).

We examined the cellular mechanisms underlying exposure-induced desensitization in the peripheral taste system of a caterpillar (*Manduca sexta*; Sphingidae). Caterpillars have three distinct advantages over most other model systems in this type of study. First, one can record neural responses from chemosensory cells in an intact preparation (Gothilf and Hanson, 1994). Second, one can distinguish action potentials from different classes of taste cell (e.g. bitter- *versus* sugar-

sensitive taste cells), and thereby look for sensitivity changes in identified taste cells (Glendinning and Hills, 1997). Third, because these recordings can be made non-destructively, one can record from the same taste cell before and after chronic exposure to specific sapid stimuli.

Previous studies have established that 2–3 days of dietary exposure to specific sapid stimuli can reduce the responsiveness of identified taste cells in caterpillars. While some of these studies used complex sapid stimuli (e.g. plant tissue extracts or artificial diets; Städler and Hanson, 1976; van Loon, 1990; Schoonhoven, 1969), others used diets treated with compounds that taste bitter to humans and inhibit feeding by caterpillars. In the latter studies, several days of dietary exposure to a bitter compound reduced the responsiveness of bitter-sensitive taste cells to the same compound (Schoonhoven, 1969, 1976; Simmonds and Blaney, 1983; Blaney and Simmonds, 1987), but did not alter the responsiveness of sugar-sensitive taste cells to sucrose (Simmonds and Blaney, 1983). There is also preliminary evidence that desensitization of the bitter-sensitive taste cell to one compound can generalize to other bitter compounds (Schoonhoven, 1978).

In the present study, we studied desensitization of the bitter-sensitive taste cell within the lateral sensillum of *M. sexta*. We focused on this taste cell because (1) its response properties are well characterized, (2) it contributes significantly to feeding inhibition on diets treated with bitter compounds (e.g. caffeine, salicin and aristolochic acid; Glendinning et al., 1999), and (3) previous work indicated that it is desensitized by chronic exposure to diets treated with caffeine or salicin (Schoonhoven, 1969, 1978). We addressed five inter-related questions. Can chronic dietary exposure to a suprathreshold concentration of any bitter compound induce desensitization? Is the desensitization phenomenon limited to the bitter-sensitive taste cell? What cellular processes mediate desensitization? Can chronic caffeine exposure, in the absence of ingestion, desensitize the bitter-sensitive taste cell? Finally, does desensitization of one bitter-sensitive taste cell transfer to the contralateral taste cell?

Materials and methods

Insects

We reared the caterpillars of *Manduca sexta* on a wheat-germ-based diet (77 % water), and maintained them in an environmental chamber at 25 °C with a 16 h:8 h L:D photoperiod (for details, see Bell and Joachim, 1976). We began all experiments with caterpillars in the first day of the fifth stadium. All caterpillars were naive to the bitter compounds prior to testing. To control for any potential differences among larvae from different egg batches, we interspersed individuals from each batch across experimental treatments. The number of caterpillars tested in each experiment is given in the figure legends.

Neural recording technique

In all the experiments described below, we recorded sensory responses from identified taste cells within the lateral styloconic sensillum using a non-invasive, extracellular tip-recording technique (Gothilf and Hanson, 1994). In brief, the recording procedure involved placing a stimulating/recording electrode over the tip of the sensillum and recording ensuing action potentials from a live and intact preparation. Using this technique, we could individually stimulate a single lateral sensillum, but not specific taste cells within the sensillum. To monitor responses of specific taste cells, we exploited the fact that each taste cell responds to its respective best stimulus with a characteristic temporal pattern of firing: that from the salt-sensitive taste cell is temporally irregular, that from the inositol-sensitive taste cell is strongly phasic-tonic, that from the sugar-sensitive taste cell is less strongly phasic-tonic and that from the bitter-sensitive taste cell is predominantly tonic, with a variable latency of onset (Peterson et al., 1993; Glendinning and Hills, 1997). These distinctive temporal patterns of firing enabled us to discriminate reliably between spikes from different taste cells.

We stimulated the lateral sensillum with a variety of compounds that are known to elicit vigorous sensory responses in at least one of the four taste cells within the lateral sensillum (see below for details). All compounds were dissolved in an electrolyte solution containing 100 mmol l^{-1} KCl in distilled H_2O. This concentration of KCl is commonly used in tip recording and does not appear to stimulate the bitter-sensitive taste cells (see, for example, Glendinning and Hills, 1997). To facilitate dissolution of one of the bitter compounds, aristolochic acid, we added 10 % ethanol to the electrolyte solution; this ethanol concentration does not alter firing of the taste cells (Peterson et al., 1993; Glendinning and Hills, 1997).

We processed neural recordings using a high-impedance preamplifier with a baseline-restoring circuit (George Johnson, Baltimore, Maryland, USA; see Frazier and Hanson, 1986), and an alternating-current-coupled amplifier-filter system with a band pass set at 130–1200 Hz. We digitized and stored neural recordings directly onto a computer using SAPID tools (Smith et al., 1990).

For each sensory recording, we stimulated a single sensillum for approximately 2 s, but only quantified action potentials generated 0.01–1.01 s after contact with the sensillum. We waited for at least 3 min between successive stimulations. To minimize the effects of solvent evaporation at the tip of the recording/stimulating electrode, we drew fluid from the tip with a piece of filter paper 7–10 s before each stimulation. We recorded from only one of the bilaterally paired lateral sensilla in each caterpillar in all experiments except the last. Whenever possible, we selected the right or left sensillum on a random basis; however, there were cases where only one sensillum was accessible. No caterpillar was used in more than one experiment.

Does dietary exposure to any bitter compound desensitize the bitter-sensitive taste cell?

This experiment (experiment 1) examined whether chronic exposure to diets treated with suprathreshold concentrations of caffeine, salicin or aristolochic acid would desensitize the

bitter-sensitive taste cell in the lateral sensillum to the same compound. To this end, we maintained caterpillars on one of these bitter diets for 48 h (e.g. the caffeine diet) and then determined the responsiveness of its bitter-sensitive taste cell to caffeine. For comparison, we maintained other caterpillars on a caffeine-free (i.e. control) diet for 48 h and then determined the responsiveness of their bitter-sensitive taste cell to caffeine. Because the exposure diets were the only source of nutrients and water for the caterpillars throughout this exposure period, they were strongly motivated to eat them. Direct observations revealed that all caterpillars investigated the diets extensively with their gustatory sensilla and fed on them to varying degrees (see Results for details). We used a 48 h exposure period because previous studies (and our own personal observations) indicated that it was the minimal period required to produce a robust change in taste cell sensitivity (Schoonhoven, 1969; Blaney et al., 1986).

Dietary exposure protocol

We used the rearing diet as a substrate for presenting the different bitter compounds during the exposure periods. We produced specific concentrations of each compound by heating the agar-containing diet to approximately 60 °C, adding the appropriate quantity of compound, stirring vigorously for 3 min, and then pouring the diet into acrylic molds (2 cm×3 cm×1.5 cm). One diet block contained sufficient food to sustain the caterpillars for an entire day.

We prepared three bitter diets. Each diet contained one of the bitter compounds at the following concentration (in mmol kg^{-1} diet): 0.4 for aristolochic acid, 5 for caffeine, and 157 for salicin. These diets contain the lowest concentration of each compound that rapidly inhibits feeding in *M. sexta* (Glendinning et al., 1999).

We also prepared three control diets, which had the same concentration of nutrients as their corresponding bitter diet. For example, the salicin diet contained 6 % salicin (fresh mass) and, as a result, its corresponding control diet contained 6 % alphacel (a non-nutritive form of cellulose; ICN Biochemicals). Other than replacing the bitter compound with an equal mass of alphacel, the control diets were identical to their corresponding bitter diet. All control diets were ingested readily by the caterpillars.

The exposure protocol involved placing a caterpillar in a sealed plastic deli-cup (160 ml volume with a vented lid), offering it one of the exposure diets, and then returning it to the environmental chamber. After 24 h, we gave it a fresh diet block (of the same type) and removed the accumulated frass. To assess how well the caterpillars adjusted to each diet, we weighed them before and after the 48 h exposure period.

Data analysis

To determine whether any of the bitter diets (e.g. the one containing salicin) desensitized the bitter-sensitive taste cell, we compared neural responsiveness to salicin between caterpillars exposed to the salicin diet and those exposed to the control diet. We made this comparison using the Mann–Whitney *U*-test ($P \leqslant 0.05$). We used a non-parametric statistical test (in this and all subsequent experiments) because the data were not normally distributed.

Is the caffeine-induced desensitization localized to the bitter-sensitive taste cell?

This experiment (experiment 2) examined whether dietary exposure to caffeine desensitizes all four classes of taste cell within the lateral sensillum (i.e. the inositol-, sugar-, salt- and bitter-sensitive taste cells). Our methodology differed in several respects from that of the previous experiment. We evaluated the responsiveness of all taste cells within a lateral sensillum both before and after the 48 h exposure period. To stimulate the inositol-sensitive taste cell, we used 10 mmol l^{-1} inositol; to stimulate the sugar-sensitive taste cell, we used 200 mmol l^{-1} sucrose; to stimulate the salt-sensitive taste cell, we used 100 mmol l^{-1} KCl; and to stimulate the bitter-sensitive taste cell, we used 5 mmol l^{-1} caffeine. Given that each taste cell has a unique temporal pattern of firing, we were able to identify spikes from the same taste cell across both recording sessions.

Test protocol

On day 1, we tested the responsiveness of all four classes of taste cell within a single lateral sensillum to the sapid stimuli indicated above. Once the recordings had been completed, we transferred the caterpillar to a deli-cup containing control diet, and waited (1–2 h) until it recovered (i.e. began locomoting and/or feeding on the control diet). At that point, we began the 48 h exposure period on the caffeine or control diet. After this exposure period (i.e. on day 3), we repeated the neural recording procedure with the same four taste cells.

To determine whether the caffeine (or control) diet changed the responsiveness of a particular taste cell, we compared the firing rate of the cell before and after the dietary exposure period using the Wilcoxon matched-pairs signed-rank test. We made these comparisons separately for each taste cell and exposure diet ($P \leqslant 0.05$).

What mechanism(s) underlie the caffeine-induced desensitization phenomenon?

This experiment (experiment 3) investigated the etiology of the desensitization phenomenon in two ways. First, we determined the response of the bitter-sensitive taste cell to an ascending series of caffeine concentrations (0.1, 1, 5, 10, 50 and 100 mmol l^{-1}) both before and after exposure to the control or caffeine diet. We reasoned that a comparison of these concentration–response (C/R) curves would show whether the desensitization phenomenon involved (a) shifting the C/R curve to the right, (b) a reduction in the slope of the C/R curve at 50 % of the maximal response point, and/or (c) a reduction in the maximal firing rate.

We also determined how the bitter-sensitive taste cell responded to an ascending series of salicin and aristolochic acid concentrations (1, 5, 10, 50 and 100 mmol l^{-1}; and 0.001, 0.04 and 0.1 mmol l^{-1}, respectively) both before and after exposure

to the control or caffeine diet. We selected these compounds because salicin and caffeine are thought to act through the same transduction pathway, and aristolochic acid through another independently regulated pathway (Glendinning and Hills, 1997). If the responses to caffeine, salicin and aristolochic acid were all diminished by dietary exposure to caffeine, then this would indicate that the desensitization was mediated by a downstream site in the signal transduction system of the taste cell, which is used by both the caffeine- and aristolochic-acid-activated transduction pathways (e.g. the spike-generating mechanism). Alternatively, if the response to caffeine and salicin, but not to aristolochic acid, were diminished by dietary exposure to caffeine, then this would indicate that the desensitization was localized to the transduction pathway activated by caffeine or salicin.

To determine whether 48 h of dietary exposure to the caffeine or control diet changed the responsiveness of the bitter-sensitive taste cell to any of the bitter compounds, we compared the firing rate of the cell before and after the dietary exposure period, using the Wilcoxon matched-pairs signed-rank test. We made these comparisons separately for each compound, concentration and diet treatment ($P \leqslant 0.05$). To control for the use of multiple paired comparisons on different concentrations of the same compound, we made Bonferroni corrections to the alpha level by dividing it by the number of concentrations tested.

Can caffeine induce desensitization through gustatory stimulation alone?

In the previous experiments, we desensitized the bitter-sensitive taste cell by placing caffeine in the diet. We used this method because it mimics the conditions under which caterpillars would naturally experience prolonged gustatory stimulation by a bitter compound. However, this method has a major drawback: it does not enable one to ascertain whether the ingested caffeine desensitized the bitter-sensitive taste cell by acting directly on the taste cell itself or by acting indirectly through a systemic effect after the caffeine had been ingested. In this experiment (experiment 4), we investigated whether direct stimulation of the bitter-sensitive taste cell, in the absence of ingestion, was a sufficient condition for inducing the desensitization phenomenon. To our knowledge, no previous studies of exposure-induced sensitivity changes in taste or olfactory cells have addressed this question.

Our suspicion that caffeine could desensitize the bitter-sensitive taste cell through a systemic mechanism stemmed from two independent observations. First, ingested methylxanthines readily cross the gut barrier of *M. sexta* caterpillars and accumulate in the blood and central nervous system (Nathanson, 1984). Second, high concentrations of amino acids in the blood of caterpillars and locusts appear to reduce the responsiveness of amino-acid-sensitive taste cells through a peripheral mechanism (Simmonds et al., 1992; Simpson and Simpson, 1992).

Our overall approach involved assessing the responsiveness of the bitter-sensitive taste cell (to caffeine, salicin and aristolochic acid) and the inositol-sensitive taste cell (to inositol) both before and after dripping a caffeine solution directly onto the lateral sensillum. If this stimulation regime were a sufficient condition for desensitizing the bitter-sensitive taste cell, then we would expect (on the basis of results from the previous experiments) it to desensitize the bitter-sensitive taste cell to caffeine and salicin, but not to desensitize the same taste cell to aristolochic acid or the inositol-sensitive taste cell to inositol.

Method for directly stimulating the lateral sensillum

We inserted a caterpillar backwards into a glass vial and left its head protruding though a latex seal. Then, we immobilized the lateral sensillum and mandibles by wrapping thin strips of Parafilm around the head, maxillae and vial. Care was taken to minimize ischemia to the sensilla. We also completely sealed the oral cavity with Parafilm to prevent ingestion of the stimulating solution.

Once the taste sensilla had been secured, we positioned one glass micropipette (diameter of tip approximately 25 μm) directly above, and another (with same tip diameter) directly below, the lateral sensillum. The upper micropipette was connected to a programmable infusion pump (Harvard Apparatus, model 70-2002, PHD 2000 series), which delivered drips of caffeine or control solution to the tip of the sensillum according to a precisely controlled schedule (see below for details). The lower micropipette was connected to a vacuum, which drew away virtually all the caffeine or control solution after it had dripped over the sensillum.

Experimental protocol

On day 1, we recorded neural responses of the bitter-sensitive taste cell (to 5 mmol l^{-1} caffeine, 50 mmol l^{-1} salicin, 0.1 mmol l^{-1} aristolochic acid) and the inositol-sensitive taste cell (to 10 mmol l^{-1} *myo*-inositol) within a lateral sensillum (henceforth, the test sensillum) using the protocol described above. Then, we secured the caterpillar in the dripping device, and began dripping either the control or caffeine solution (see below) directly onto the lateral sensillum. At the end of the 23 h exposure period, which was sufficient to produce the desensitization phenomenon (see below), we removed the caterpillar from the vial and repeated the neural recording procedure conducted on day 1.

The control solution was White's basal salt mixture (Sigma), an aqueous medium used for plant tissue culture. We selected this salt mixture because, in contrast to other solutions (e.g. 100 mmol l^{-1} KCl or distilled H_2O), it did not, on its own, reduce the responsiveness of the bitter- and inositol-sensitive taste cells taste cells during preliminary experiments. The caffeine solution was identical to the control solution in all respects except that it contained 5 mmol l^{-1} caffeine.

Over the 23 h exposure period, we dripped the control or caffeine solution intermittently onto a single lateral sensillum. Each drip cycle lasted 40 min and consisted of 10 min of continuous dripping followed by 30 min of no dripping. This protocol was designed to reflect the pattern of

ad libitum feeding by *M. sexta* (see, for example, Reynolds et al., 1986). The drip cycle was repeated 35 times throughout the 23 h exposure period, resulting in a total of 5.8 h of dripping.

Some caterpillars had to be rejected from the experimental results for any one of three reasons. First, the position of the test sensillum occasionally shifted during the 23 h exposure period so that it was no longer directly underneath the dripping micropipette. To ensure that each test sensillum received drips according to our fixed schedule, we checked the preparation 3–4 times during the initial 12 h of the exposure period and once at the end of the exposure period. If the test sensillum was not located directly beneath the dripping micropipette during all these checks, then the caterpillar was rejected. Second, in a small number of cases, we could not obtain a normal neural response to inositol at the end of the exposure period (i.e. the magnitude of the neural response to inositol on day 1 was less than 60 % of that obtained on day 2). Because preliminary experiments indicated that chronic dripping of the control or caffeine solution did not alter the responsiveness of the inositol-sensitive taste cell, we assumed that any caterpillar with a subnormal response to inositol after the exposure period had been damaged in some way during the experiment. Third, in a few cases, the vacuum micropipette did not draw the dripped solution away from the caterpillar correctly, leaving its body wet. We only used caterpillars that were dry on day 2 to reduce the possibility of caffeine being absorbed through the cuticle and producing unintended systemic effects.

Data analysis

To determine whether the caffeine or control solution changed the firing rate of a particular taste cell, we compared the firing rate of the cell before and after the exposure period using the Wilcoxon matched-pairs signed-rank test. We made these comparisons separately for each taste cell and drip solution ($P \leqslant 0.05$).

Does desensitization of one bitter-sensitive taste cell transfer to the contralateral taste cell?

In this final experiment (experiment 5), we asked whether desensitizing one of the bitter-sensitive taste cells with caffeine (using the dripping procedure described in the previous experiment) would also desensitize the bitter-sensitive taste cell in the contralateral lateral sensillum. If not, then this would indicate that desensitization is induced by a localized effect of chronic caffeine exposure on the bitter-sensitive taste cell.

We used virtually the same experimental procedures as described in experiment 4, but with the critical difference that we monitored sensitivity changes in taste cells within both the left and right lateral sensilla. To this end, we recorded the responses of the bitter-sensitive taste cells to 5 mmol l^{-1} caffeine and of the inositol-sensitive taste cells to 10 mmol l^{-1} inositol, both before and after dripping the caffeine solution (see previous experiment) onto one of the lateral sensilla for 23 h. The unstimulated (i.e. non-dripped-upon) sensillum served as a within-animal control, so it was not necessary to include an additional control group (i.e. caterpillars that were chronically stimulated by the control solution).

Because of the complex nature of this experiment, we used three criteria for rejecting caterpillars, in addition to those used in experiment 4. First, the bitter- and inositol-sensitive taste cells of the caterpillar (in the left and right lateral sensilla) had to exhibit a moderately vigorous response (i.e. $\geqslant$50 Hz) to their respective stimuli on day 1 of the experiment, thus ensuring that all taste cells were healthy and responsive. Second, the inositol-sensitive taste cell in the left and right lateral sensilla had to exhibit a moderately vigorous response to 10 mmol l^{-1} inositol on day 2 (i.e. $\geqslant$60 % of the day 1 response); this served as a positive control, ensuring that any reduction in responsiveness of the bitter-sensitive taste cells was not due to any unintended damage to the caterpillar during the dripping procedure. Third, the bitter-sensitive taste cell in the stimulated sensillum had to be desensitized (i.e. fire at a rate $\leqslant$60 % of its day 1 response); this was a necessary precondition for determining whether the desensitization phenomenon transferred to the contralateral, unstimulated bitter-sensitive taste cell.

Data analysis

To determine whether the dripping protocol desensitized the bitter- or inositol-sensitive taste cells in the unstimulated lateral sensillum, we compared the responsiveness of each taste cell both before and after the dripping protocol, using the Wilcoxon matched-pairs signed-rank test ($P \leqslant 0.05$). We made similar comparisons for the bitter- or inositol-sensitive taste cells in the stimulated sensillum.

Results

Does dietary exposure to any bitter compound desensitize the bitter-sensitive taste cell?

The responsiveness of the bitter-sensitive taste cell to 5 mmol l^{-1} caffeine was significantly lower in caterpillars exposed to the caffeine diet than in those exposed to the control diet (Fig. 1A). In contrast, dietary exposure to the salicin or aristolochic acid diets did not significantly alter the responsiveness of the bitter-sensitive taste cell to 50 mmol l^{-1} salicin or 0.1 mmol l^{-1} aristolochic acid, respectively (Fig. 1B,C). Thus, only the caffeine diet induced sensitivity changes in the bitter-sensitive taste cell.

That the caterpillars actually ingested all three bitter diets is demonstrated by the fact that they all gained weight (albeit to varying degrees) over the 48 h exposure period. The percentage increase in mass (median ± median absolute deviation) over the 2-day exposure period was 219±9 % for the salicin diet, 200±5 % for the caffeine diet and 165±5 % (N=11–14 per diet) for the aristolochic acid diet. Analogous figures for caterpillars on the corresponding control diets, in respective order, were 316±8 %, 302±9 % and 319±10 % (N=11–14 per diet). These

latter values reveal that the caterpillars gained more weight on the palatable control diets.

Is the caffeine-induced desensitization localized to the bitter-sensitive taste cell?

Two days of dietary exposure to the control diet failed to induce significant changes in responsiveness of any of the taste cells to their respective sapid solutions (Fig. 2A–D). In contrast, 2 days of dietary exposure to the caffeine diet significantly reduced the responsiveness of the bitter-sensitive taste cell to caffeine, but did not reduce the responsiveness of the inositol-, sugar- or salt-sensitive taste cells to their respective sapid solutions (Fig. 2E–H). Thus, dietary exposure to the caffeine diet specifically desensitized the bitter-sensitive taste cell.

What mechanism(s) underlie the caffeine-induced desensitization phenomenon?

Two days of dietary exposure to the control diet was correlated with a small but significant increase in responsiveness of the bitter-sensitive taste cell to many of the caffeine and salicin concentrations (Fig. 3A,B). We did not observe a similar increase in responsiveness of the same taste cell to any of the aristolochic acid concentrations (Fig. 3C). In contrast, 2 days of dietary exposure to the caffeine diet significantly decreased the responsiveness of the bitter-

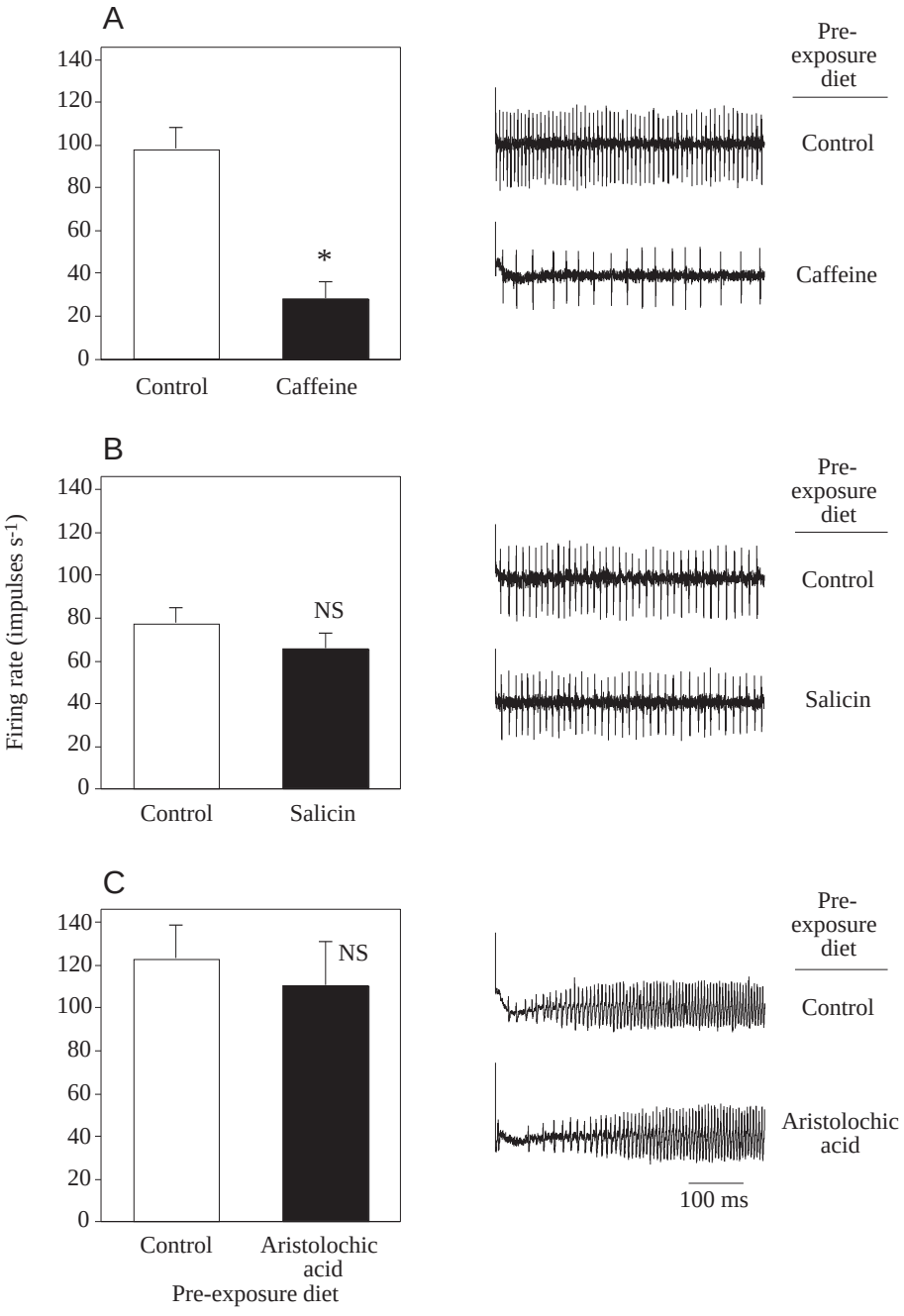

Fig. 1. Sensory response of the bitter-sensitive taste cell in the lateral sensillum (A) to 5 mmol l^{-1} caffeine after dietary exposure to the control or caffeine diet (5 mmol kg^{-1} diet; fresh mass), (B) to 50 mmol l^{-1} salicin after dietary exposure to the control or salicin diet (157 mmol kg^{-1} diet) or (C) to 0.1 mmol l^{-1} aristolochic acid after dietary exposure to the control or aristolochic acid diet (0.4 mmol kg^{-1} diet). The dietary exposure period lasted for 48 h. For the neural recordings, we dissolved the bitter compounds in 100 mmol l^{-1} KCl. Each column indicates the median (+ median absolute deviation) response of 11–14 bitter-sensitive taste cells (each from a different caterpillar). To the right, we provide typical neural responses from caterpillars in each of the treatment groups. Medians within in each panel were compared using the Mann–Whitney *U*-test (*$P \leq 0.05$; NS, $P > 0.05$; two-tailed).

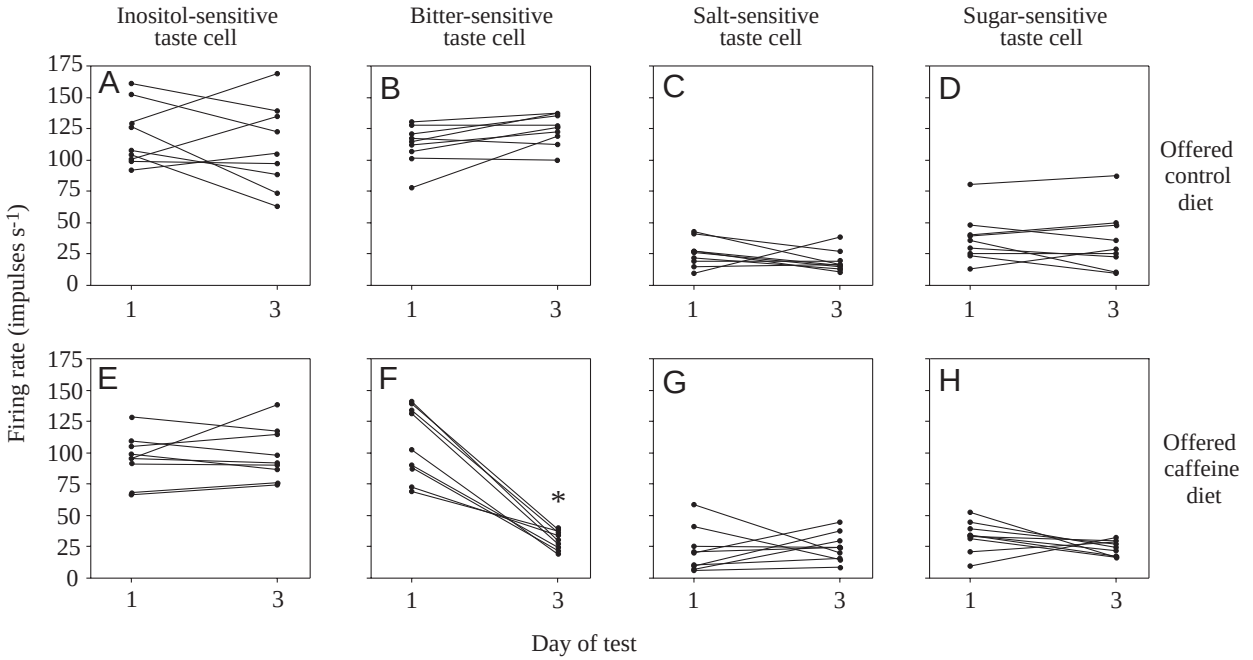

Fig. 2. Sensory response of the four different classes of taste cell within the lateral sensillum to specific sapid stimuli, both before (i.e. on day 1) and after (i.e. on day 3) dietary exposure to the control (A–D) or caffeine (E–H) diet. We stimulated the inositol-sensitive taste cell with 10 mmol l^{-1} inositol (A,E), the bitter-sensitive taste cell with 5 mmol l^{-1} caffeine (B,F), the salt-sensitive taste cell with 100 mmol l^{-1} KCl (C,G) and the sugar-sensitive taste cell with 200 mmol l^{-1} sucrose (D,H). Each line (in a given panel) joins the sensory response of a specific taste cell on day 1 and day 3 of the test. We tested for a diet-induced change in responsiveness in each taste cell (N=9 per panel) using the Wilcoxon matched-pairs signed-rank test (*$P \leq 0.05$; two-tailed).

sensitive taste cell to all caffeine and salicin concentrations (Fig. 3D,E). The concentration–response (C/R) curves for both compounds on day 3 were nearly flat. There were no significant exposure-induced changes in responsiveness of the bitter-sensitive taste cell to aristolochic acid (Fig. 3F).

These results demonstrate that dietary exposure to caffeine does not simply shift the C/R curve for caffeine to the right or reduce the slope of the C/R curve at 50 % of the maximal firing rate. Rather, it reduces the maximal response of the bitter-sensitive taste cell to all soluble concentrations of caffeine by approximately 70 %. The results also show that the caffeine-induced desensitization phenomenon generalized to salicin, but not to aristolochic acid.

Can caffeine induce desensitization through gustatory stimulation alone?

Chronic dripping of the control solution onto the lateral sensillum did not alter the responsiveness of the bitter- or inositol-sensitive taste cells to each of their respective sapid solutions (Fig. 4A–D). However, chronic dripping of the caffeine solution significantly reduced the responsiveness of the bitter-sensitive taste cell to caffeine and salicin, but not to aristolochic acid (Fig. 4E–G). The caffeine solution did not alter the responsiveness of the inositol-sensitive taste cell to inositol (Fig. 4H). Taken together, these results indicate that direct stimulation of the bitter-sensitive taste cell with caffeine (in the absence of ingestion) is a sufficient condition for inducing the desensitization phenomenon described in earlier experiments.

Does desensitization of one bitter-sensitive taste cell transfer to the contralateral taste cell?

Chronic dripping of the caffeine solution significantly reduced the responsiveness of the bitter-sensitive taste cell in the stimulated sensillum, but there was no evidence that the desensitization phenomenon transferred to the bitter-sensitive taste cell in the contralateral (i.e. unstimulated) sensillum (Fig. 5A,B). The caffeine treatment failed to alter the sensitivity of the inositol-sensitive taste cell in either sensillum (Fig. 5C,D).

Discussion

Our results demonstrate that chronic exposure to a suprathreshold concentration of caffeine selectively desensitizes the bitter-sensitive taste cell within the lateral sensillum. They also indicate that the desensitization phenomenon is localized to the transduction pathway that is activated by caffeine and salicin. This latter conclusion is based on the observation that chronic exposure to the caffeine diet virtually eliminated the response to caffeine and salicin, but had no measurable impact on the response to aristolochic acid, which activates a different transduction pathway within the same taste cell (Glendinning and Hills, 1997). We discovered recently that the desensitization phenomenon generalizes to other structurally related compounds. For example, chronic exposure to the caffeine diet desensitizes the bitter-sensitive taste cell to theophylline (a methylxanthine)

and helicin (a phenolic glycoside) (S. Ensslen and J. I. Glendinning, unpublished data).

The finding that the desensitization phenomenon can be localized to a single transduction pathway within a single taste cell is novel. It is important because it reveals that exposure-induced desensitization does not necessarily render a taste cell 'blind' to all its ligands. Such selective desensitization may be particularly adaptive in the case of bitter (or otherwise noxious) compounds, which often differ greatly in toxicity (Bernays and Chapman, 1987; Glendinning, 1994). For example, because aristolochic acid is substantially more toxic than caffeine (J. I. Glendinning, unpublished data), generalization of the desensitization phenomenon to aristolochic acid would increase the chances of *M. sexta* unwittingly ingesting a toxic dose of this compound. Generalization of this phenomenon to salicin, however, would not have such dire consequences because it is much less toxic than caffeine.

It has been suggested that single taste cells within the maxillary sensilla of locusts respond to two classes of nutrient (amino acids and sugars), and that high levels of one class of nutrient in the hemolymph (e.g. amino acid) selectively desensitize that taste cell to amino acids, but this idea has been difficult to substantiate owing to an inability to distinguish among the 6–10 taste cells within each sensillum (Simpson and Simpson, 1992). In the caterpillar *Spodoptera littoralis*, such nutrient-specific modulation of responsiveness to amino acids or sugars is mediated by different taste cells (Simmonds et al., 1992).

It is likely that chemosensory cells in other species (both vertebrate and invertebrate) undergo transduction-pathway-specific desensitization. For instance, there is evidence from *Caenorhabditis elegans* that chronic exposure to one odorant desensitizes identified olfactory cells to only a subset of their ligands (Colbert and Bargmann, 1995). In addition, *Paramecium caudatum* appear to possess at least two transduction pathways for bitter stimuli, and exposure-induced desensitization of one of these pathways does not impair the responsiveness of the other (Oami, 1998). Finally, there is evidence that some taste (Bernhardt et al., 1996) and olfactory (Dione and Dubin, 1994)

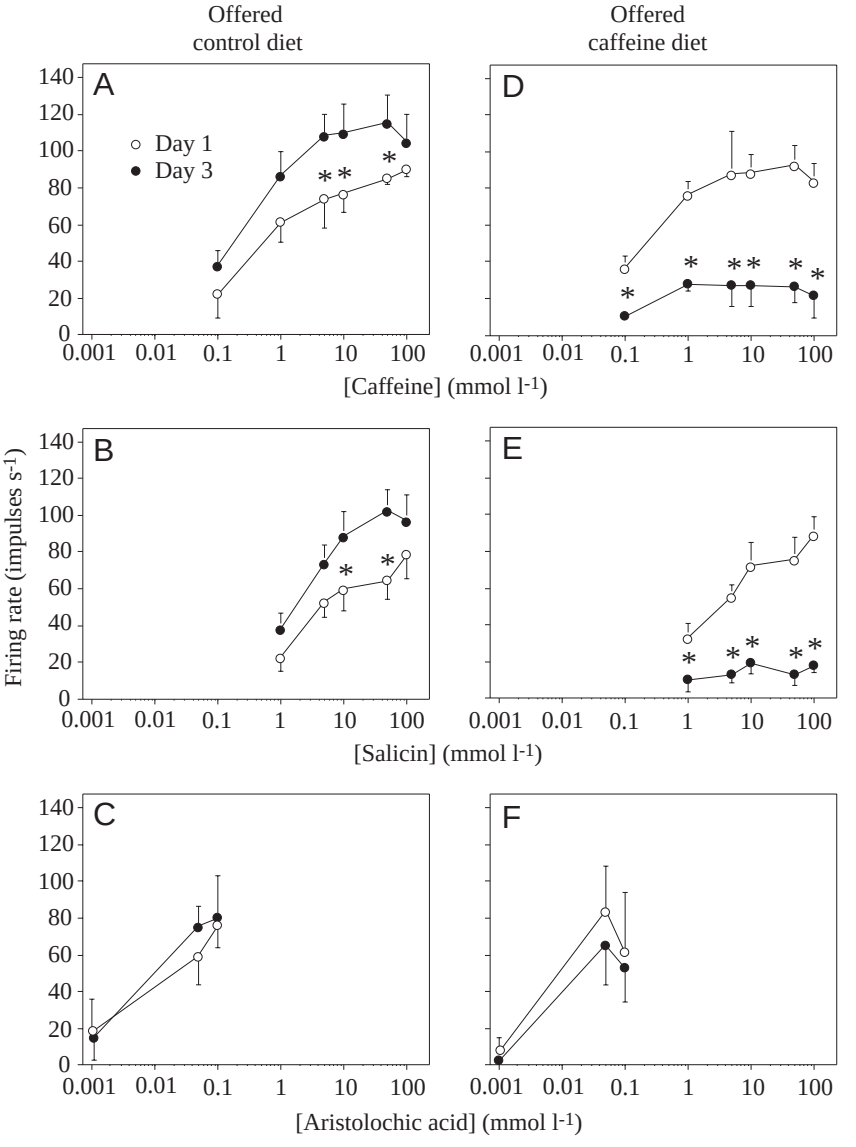

Fig. 3. Sensory response of the bitter-sensitive taste cell in the lateral sensillum to an ascending series of caffeine, salicin and aristolochic acid concentrations both before (day 1) and after (day 3) dietary exposure to the control (A–C) or caffeine (D–F) diet. The dietary exposure period occurred between days 1 and 3 of the experiment. Individual taste cells were stimulated with all concentrations of all three bitter compounds on both days. Points are median (± median absolute deviation) responses of 15 bitter-sensitive taste cells (each from a different caterpillar). We tested for diet-induced changes in responsiveness to each bitter compound using the Wilcoxon matched-pairs signed-rank test, separately for each compound and concentration (*$P \leqslant 0.05/6$ for caffeine, *$P \leqslant 0.05/5$ for salicin and *$P \leqslant 0.05/3$ for aristolochic acid; two-tailed).

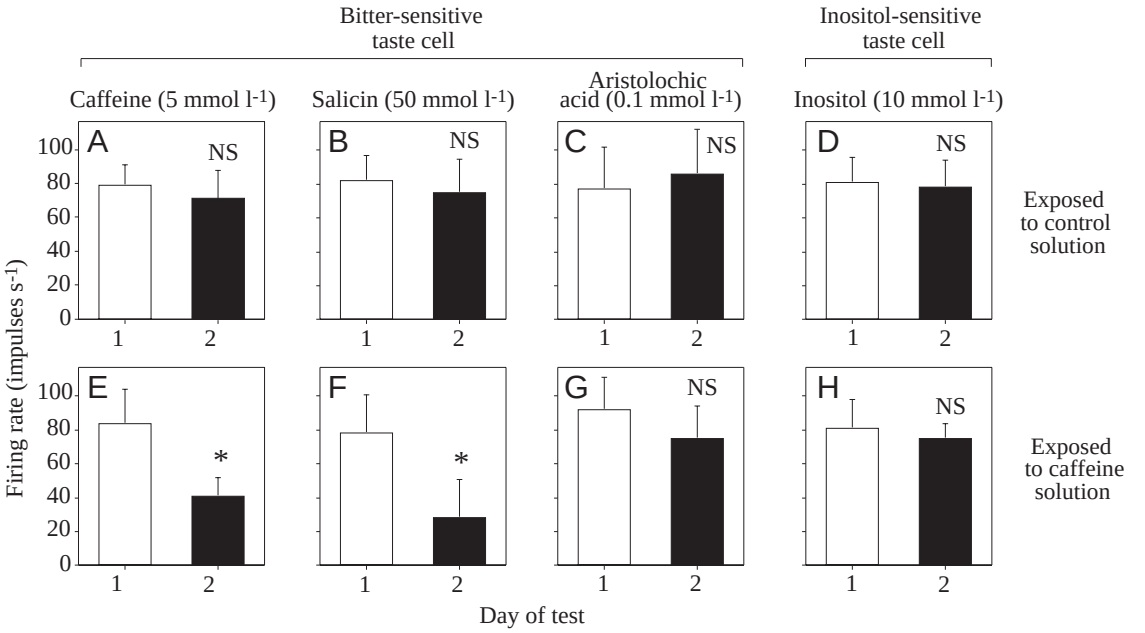

Fig. 4. Sensory response of the bitter-sensitive taste cell to three bitter compounds (5 mmol l^{-1} caffeine, 50 mmol l^{-1} salicin and 0.1 mmol l^{-1} aristolochic acid) and of the inositol-sensitive taste cell to 10 mmol l^{-1} inositol, both before and after treatment with the control (A–C) or caffeine (D–F) solution. We dripped the control or caffeine solution directly onto the lateral sensillum for 23 h, between days 1 and 2 of the experiment. Columns indicate median (+ median absolute deviation) responses of 15 lateral sensilla (each from a different caterpillar). We compared medians in each panel using the Wilcoxon matched-pairs signed-rank test (*$P \leqslant 0.05$; NS, $P > 0.05$; two-tailed).

cells in vertebrates and invertebrates contain two transduction pathways, which are activated by different ligands. However, it is not known whether either of these latter transduction pathways can be selectively desensitized by a chronic exposure regime. Progress in this area is slow owing to an inability to make repeated recordings from identified chemoreceptors in most model systems.

Why did salicin and aristolochic acid fail to desensitize the bitter-sensitive taste cell?

On the basis of previous work with *M. sexta* (Schoonhoven, 1969, 1976, 1978), we expected that dietary exposure to caffeine would desensitize the bitter-sensitive taste cell to caffeine and that dietary exposure to salicin would desensitize the same taste cell to salicin. However, we found that only the caffeine diet produced the expected effect. This discrepancy cannot be attributed to differences in the age of the caterpillars or in the composition of the artificial diet because these two variables did not differ between our

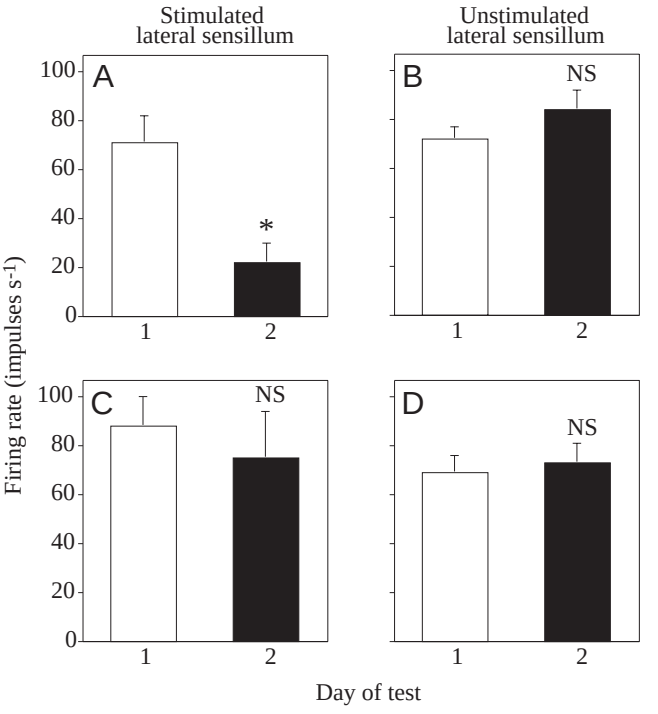

Fig. 5. Test to determine whether desensitization of the bitter-sensitive taste cell in one lateral sensillum transfers to the analogous taste cell in the contralateral sensillum. To induce desensitization, we dripped the caffeine solution directly onto one lateral sensillum (i.e. the stimulated sensillum) for 23 h during days 1 and 2 of the experiment. We did not drip anything onto the contralateral (i.e. unstimulated) sensillum during the same time period. We show the sensory responses of the bitter-sensitive taste cell within the stimulated and unstimulated sensilla to 5 mmol l^{-1} caffeine both before and after treatment with the caffeine solution (A,B). We also show the sensory responses of the inositol-sensitive taste cell within the same stimulated and unstimulated sensilla to 10 mmol l^{-1} inositol (as a positive control) both before and after treatment with the caffeine solution (C,D). Columns indicate median (± median absolute deviation) responses of seven bilateral pairs of lateral sensilla (each from a different caterpillar). Medians in each panel were compared using the Wilcoxon matched-pairs signed-rank test (*$P \leqslant 0.05$; NS, $P > 0.05$; one-tailed).

experiments. However, there were three notable differences between our experiments. First, Schoonhoven (1969, 1976, 1978) exposed caterpillars to the salicin diet for 2.5 or more days, while we did so for only 2 days. To address this discrepancy, we exposed six caterpillars to the salicin diet for 2.5 days, but still failed to observe any desensitization (J. I. Glendinning, unpublished data). Second, Schoonhoven (1969, 1976, 1978) used a lower concentration of salicin in the exposure diet than we did (10 *versus* 157 mmol kg^{-1} diet). We selected the higher salicin concentration because it was the minimum concentration required to inhibit feeding robustly in our brief-access biting assay (Glendinning et al., 1999). Finally, Schoonhoven (1969, 1976, 1978) conducted his studies more than two decades ago using a genetically distinct population of caterpillars. Accordingly, there may have been genetic differences between our two populations of *M. sexta* that affected their respective responses to chronic salicin exposure. That small mutations can dramatically alter the ability of chemosensory cells to undergo exposure-induced sensitivity changes has been demonstrated convincingly in *C. elegans* (Colbert and Bargmann, 1995).

Even though we cannot explain why exposure to the salicin and aristolochic acid diets did not desensitize the bitter-sensitive taste cell, we can eliminate two possible explanations. If one assumes that the desensitization phenomenon is induced by sustained vigorous stimulation of the bitter-sensitive taste cell, then one might hypothesize that only the caffeine diet produced a sufficient level of sustained stimulation. We attempted to avoid this possibility by using concentrations of caffeine, salicin and aristolochic acid in the exposure diets that are approximately iso-stimulatory to the bitter-sensitive taste cell (Glendinning et al., 1999). A second possibility is that the caffeine-activated transduction pathway was desensitized more readily than the aristolochic-acid-activated pathway. However, according to this hypothesis, the salicin diet should have induced desensitization as effectively as the caffeine diet. Clearly, this was not the case.

How did caffeine induce desensitization?

We can envision four ways that chronic exposure to dietary caffeine induced desensitization. One mechanism involves caffeine producing a systemic effect (on the central nervous system or the lateral sensillum *via* the hemolymph) after it has been ingested. Even though the results of experiments 4 and 5 do not enable us to reject this possibility, they nevertheless establish that caffeine ingestion and absorption are not necessary for inducing desensitization. The only way that caffeine could have acted systemically during experiments 4 and 5 was by diffusing from the sinus of the lateral sensillum into the blood of the caterpillar. If caffeine did enter the blood *via* this route, the amounts would have been vanishingly small and would have been diluted rapidly by the circulatory system of the caterpillar.

A second mechanism for inducing desensitization incorporates the fact that, during exposure to the caffeine diet, the bitter-sensitive taste cell of the caterpillar would have provided a persistent stream of sensory input to the central nervous system (CNS). The CNS could have responded to this input by specifically inhibiting the caffeine-activated transduction pathway within the bitter-sensitive taste cell, either through centrifugal neural feedback or through hormonal feedback to the taste cell (see discussion in Simpson and Simpson, 1992). We consider this explanation unlikely, however, because it assumes that the CNS of the caterpillar can discriminate between the neural responses of the bitter-sensitive taste cell to caffeine, aristolochic acid and salicin. While it is conceivable that the CNS could discriminate between the neural responses to caffeine and aristolochic acid, given that each compound elicits a markedly different temporal pattern of spiking and maximal firing rate, it seems inconceivable that the CNS could do so for the neural responses to caffeine and salicin, given that each compound elicits a strikingly similar temporal pattern of spiking and maximal firing rate (e.g. see Fig. 1, and Glendinning and Hills, 1997; Glendinning et al., 1999).

A third possibility is that the desensitization phenomenon is an example of sensory adaptation, stemming from the persisting presence of elevated levels of caffeine in the fluids bathing the taste cells. We consider this explanation implausible, however, for two reasons. First, it would not explain the compound-specificity of the desensitization phenomenon. For instance, it is difficult to explain why caffeine, but not salicin or aristolochic acid, would have persisted in the fluids bathing the taste cells. Second, we have found that the desensitization phenomenon takes at least 24 h to offset once the caterpillar has been transferred to a control diet (J. I. Glendinning and M. E. Eisenberg, unpublished data). It seems unlikely that elevated levels of caffeine would persist in the fluids bathing the taste cells over such a long period.

The final, and most likely, mechanism for inducing desensitization involves caffeine acting on signaling mechanisms within the bitter-sensitive taste cell. During dietary exposure to caffeine, the caffeine could have accumulated to high levels within the bitter-sensitive taste cell and specifically disrupted the caffeine-activated transduction pathway. This hypothesis would explain (i) why dietary exposure to salicin failed to desensitize the bitter-sensitive taste cell to salicin in experiment 1, (ii) why the desensitization phenomenon generalized to salicin but not to aristolochic acid in experiment 2, and (iii) why desensitization of the bitter-sensitive taste cell in one sensillum, through the dripping protocol in experiment 5, did not transfer to the analogous taste cell in the contralateral sensillum. The plausibility of this explanation rests on the observation that caffeine can inhibit the activity of phosphodiesterase enzymes in the nervous system of *M. sexta* (Nathanson, 1984); these enzymes play a central role in regulating cyclic-AMP- and cyclic-GMP-mediated second-messenger systems. Although virtually nothing is known about the nature of the caffeine-activated transduction pathway within the bitter-sensitive taste cell, there is evidence that cyclic-AMP- and cyclic-GMP-mediated second-messenger systems are involved in both sweet taste

transduction in other species of insect (Amakawa et al., 1990) and bitter taste transduction in several species of mammal (e.g. Kolesnikov and Margolskee, 1995; Rosenzweig et al., 1999).

In conclusion, we have shown that chronic dietary exposure to caffeine can selectively desensitize a single transduction pathway within a single taste cell. The remarkable specificity of this phenomenon is further revealed by the fact that caffeine exposure did not alter the responsiveness of the sugar-, salt- or inositol-sensitive taste cell within the same sensillum. To understand how caffeine exerts such specific effects on the bitter-sensitive taste cell, more studies are needed to determine the nature of the transduction pathways within this cell. To understand the functional significance of the desensitization phenomenon, further studies are needed to determine how it influences the taste-mediated behavioral responses of the caterpillar to diets containing caffeine, salicin or aristolochic acid.

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