The Journal of Experimental Biology 215, 1412-1421

doi:10.1242/jeb.059832

RESEARCH ARTICLE

Not all sugars are created equal: some mask aversive tastes better than others in an herbivorous insect

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Accepted 26 December 2011

SUMMARY

***Manduca sexta* caterpillars are unusual because they exhibit strong peripheral gustatory responses to sugars, but nevertheless fail to show immediate appetitive responses to them. We hypothesized that the primary function of the peripheral gustatory response to sugars is to mask the taste of noxious compounds, which abound in host plants of *M. sexta*. We compared 10 s biting responses to water with those to mixtures of a noxious compound [caffeine (Caf) or aristolochic acid (AA)] and various combinations of sugars [i.e. sucrose (Suc), glucose (Glu), inositol (Ino), Suc+Glu, Suc+Ino or Glu+Ino]. The biting assays indicated that the aversive taste of AA was completely masked by Suc+Ino, and partially masked by Suc+Glu, Glu+Ino and Suc, whereas that of Caf was completely masked by Suc+Ino and Suc+Glu, and partially masked by Glu+Ino, Suc and Ino. To examine the contribution of the peripheral taste system to the masking phenomenon, we recorded responses of the maxillary gustatory sensilla to each stimulus mixture. The sugars differed greatly in their capacity to suppress peripheral gustatory responses to AA and Caf: Suc+Ino and Suc+Glu produced the greatest suppression, and Glu and Ino the least. Further, the extent to which each sugar stimulus suppressed the peripheral gustatory responses to AA reliably predicted the extent to which it masked the taste of AA in biting assays; no such predictive relationship was observed for the sugar/Caf mixtures. We conclude that some, but not all, sugars act on both peripheral and central elements of the gustatory system to mask the taste of noxious compounds.**

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/8/1412/DC1

Key words: taste, mixture suppression, herbivore, insect, sugar, bitter.

INTRODUCTION

Most insects show immediate appetitive responses to sugars. For instance, the probability that butterflies, bees and flies will extend their proboscis in response to taste stimulation increases with sugar concentration (Dethier, 1976; Schoonhoven and van Loon, 2002; Omura and Honda, 2003; de Brito Sanchez et al., 2005; Dahanukar et al., 2007; Gordon and Scott, 2009; Zhang et al., 2010). Likewise, the rate at which lepidopteran caterpillars either bite from disks (Ma, 1972) or swallow solutions (Sasaki and Asaoka, 2006) increases with sugar concentration. *Manduca sexta* caterpillars (Sphingidae; Lepidoptera) are unusual because they exhibit a strong peripheral taste response to sugars, but nevertheless fail to show immediate appetitive responses to them – e.g. they bite at the same rate from sugar-treated disks as from water-treated disks (Glendinning et al., 2007). Here, we examined the functional significance of the peripheral gustatory response to sugars in *M. sexta*.

The solanaceous host plants of *M. sexta* are laden with noxious compounds (Harborne, 1986; Pomilio, 2008). We hypothesized that the primary function of the peripheral gustatory response to sugars is to mask the aversive taste of these noxious compounds. It is important to note that ingestion of noxious (and potentially toxic) compounds in solanaceous foliage does not typically pose a serious mortality risk to *M. sexta* caterpillars because they possess an array of mechanisms for coping with toxic compounds. These include: (1) a constitutive insensitivity to some solanaceous poisons (Morris, 1984; Murray et al., 1994); (2) an ability to induce cytochrome P450 detoxification enzymes in response to ingestion of solanaceous compounds (Snyder and Glendinning, 1996; Stevens et al., 2000); (3) post-oral chemosensory mechanisms that permit caterpillars to sense ingested poisons rapidly (Glendinning, 1996), and subsequently regulate their intake in proportion to the extent of P450 enzyme induction (Snyder and Glendinning, 1996); and (4) multidrug transporters that pump alkaloids from the central nervous system to the hemolymph, and then from the hemolymph into the lumen of the malpighian tubules for excretion (Murray et al., 1994; Gaertner et al., 1998).

Manduca sexta caterpillars have eight bilateral pairs of taste sensilla associated with their mouthparts. Each of these sensilla contains three to four gustatory receptor neurons (GRNs), the axons of which project directly to the central nervous system (Schoonhoven and van Loon, 2002). There is a sugar-sensitive GRN in both the lateral and medial styloconic sensilla, which responds to glucose (Glu) and inositol (Ino), and a second sugar-sensitive GRN in the lateral (but not the medial) styloconic sensilla, which responds to sucrose (Suc) (Table 1). Glu, Ino and Suc are all present in the solanaceous foliage that *M. sexta* caterpillars ingest (Nelson and Bernays, 1998). There is also a bitter-sensitive GRN in both the lateral and medial styloconic sensilla, which responds to a variety of noxious compounds, including caffeine (Caf) and aristolochic acid (AA) (Table 1). Although Caf and AA do not occur in solanaceous foliage, they can rapidly inhibit biting in *M. sexta* caterpillars by activating the bitter-sensitive GRNs in the lateral and medial styloconic sensilla (Glendinning et al., 1999).

Table 1. Chemical stimuli that elicit excitatory responses in gustatory receptor neurons (GRNs) within the lateral styloconic, medial styloconic, epipharyngeal and maxillary palp sensilla of *Manduca sexta*

Sensillum	GRN	Compounds that elicit excitatory responses
Lateral styloconic (*N*=1 bilateral pair)	1	Aristolochic acid, caffeine, salicin, theophylline
	2	Glucose, inositol
	3	Sucrose
	4	NaCl, KCl
Medial styloconic (*N*=1 bilateral pair)	1	Aristolochic acid, *Canna* extract
	2	Glucose, inositol
	3	NaCl, KCl
	4	–
Epipharyngeal (*N*=1 bilateral pair)	1	Aristolochic acid, caffeine, salicin, *Canna* extract
	2	Oxalic acid, NaCl, KCl
	3	Oxalic acid
Maxillary palp (*N*=5 bilateral pairs)	1	*Grindelia* extract
	2	NaCl, KCl
	3	–
	4	–

There are four GRNs in each of the lateral and medial styloconic sensilla, three GRNs in each of the epipharyngeal sensilla, and four GRNs in each of the taste sensilla on the maxillary palp. These molecular receptive ranges are derived from prior studies (Schoonhoven and Dethier, 1966; Schoonhoven, 1972; de Boer et al., 1977; Glendinning et al., 1998; Glendinning et al., 2002; Glendinning et al., 2007).

Most models of taste processing (e.g. labeled-line coding) treat each GRN as an independent processing unit (i.e. 'silo'), which relays its responses to the central gustatory system with high fidelity (de Brito Sanchez and Giurfa, 2011). Although this perspective may be relevant to single-component taste stimuli (e.g. 0.3 mol l^{-1} Suc), it is not relevant to complex mixtures of taste stimuli (e.g. what is typically present in foods). This is because inhibitory interactions occur between GRNs within the same sensillum. For instance, when two taste stimuli (e.g. S_1 and S_2) that activate different GRNs (e.g. GRN_1 and GRN_2) are presented in a mixture, insect taste sensilla usually exhibit mixture suppression (i.e. generate fewer action potentials than would be predicted based on the sum of the action potentials elicited by each stimulus alone). Two different types of mixture suppression have been reported. In one type (reciprocal inhibition), S_1 stimulates GRN_1 and S_2 stimulates GRN_2, but when S_1 and S_2 are presented together, the excitatory responses of both GRN_1 and GRN_2 are diminished (Ishikawa, 1963; White et al., 1990; Shields and Mitchell, 1995a; Bernays and Chapman, 2001; Glendinning et al., 2007; Wright et al., 2010). In the other type of mixture suppression (non-reciprocal inhibition), S_1 stimulates GRN_1 and S_2 fails to stimulate any GRN; nevertheless, when S_1 and S_2 are presented together, the excitatory response of GRN_1 is diminished (Dethier and Bowdan, 1989; Bernays and Chapman, 2000; Bernays and Chapman, 2001; de Brito Sanchez et al., 2005).

Previously, we reported that the aversive taste of Caf could be masked by the presence of Ino, but not Glu, in *M. sexta* caterpillars (Glendinning et al., 2000). Here, we re-examined this prior work based on studies indicating that noxious compounds are differentially sensitive to peripheral mixture suppression (Hiroi et al., 2004), and that binary mixtures of sugars can produce more mixture suppression than single sugars (Shields and Mitchell, 1995b). We tested three sugars (Suc, Glu and Ino) and two noxious compounds (Caf and AA). In Experiment 1, we examined the extent to which the sugars masked the aversive taste of Caf and AA. In Experiment 2, we asked whether any of the observed instances of taste masking could be explained (at least in part) by peripheral mixture suppression.

MATERIALS AND METHODS

Subjects and rearing conditions

We maintained a colony of tobacco hornworms [*Manduca sexta* (Linnaeus 1763); Sphingidae] in our laboratory. This colony was derived from eggs that were donated by the *Manduca* rearing facility at the University of Arizona, Tucson, AZ, USA. The caterpillars were reared on a wheat-germ-based artificial diet (Bell and Joachim, 1976), and were maintained in an environmental chamber with a 16 h:8 h light:dark cycle at 25°C. The experiments were conducted during the first or second day of the fifth larval growth stage (instar). All caterpillars were naive to the taste stimuli prior to testing. To control for differences between caterpillars from different egg batches, individuals from each batch were interspersed randomly across treatment levels, according to a blind procedure. We provide sample sizes in the figure legends.

Experimental solutions

We used (1) 200 mmol l^{-1} Suc and Glu because it is the lowest concentration of each sugar that elicits a maximal excitatory response in both the lateral and medial styloconic sensilla; (2) 1 mmol l^{-1} Ino because it elicits response similar in magnitude to 200 mmol l^{-1} Suc in the lateral styloconic sensillum; and (3) 0.1 mmol l^{-1} AA (sodium salt) and 5 mmol l^{-1} Caf because they elicit robust aversive responses in *M. sexta* (Glendinning et al., 2006). We purchased all chemicals from Sigma-Aldrich (St Louis, MO, USA).

During the behavioral and electrophysiological tests, we presented each caterpillar with one of two test series. For the AA test series, we included a negative control stimulus (water), a positive control stimulus (AA alone), AA plus one of three sugars (Suc, Glu and Ino), and AA plus binary mixtures of the three sugars (Suc+Glu, Suc+Ino or Glu+Ino). The Caf test series was the same in all respects, except that we used Caf instead of AA. For the electrophysiological tests, we also tested Suc+Glu, Suc+Ino and Glu+Ino.

For the biting assay, the chemical stimuli were dissolved in deionized water. For the electrophysiological recordings, they were dissolved in an electrolyte solution (i.e. 100 mmol l^{-1} KCl) and presented at room temperature (i.e. 22–24°C).

Experiment 1 – short-term biting assay

We measured the number of bites taken from the experimental disk during the initial 10 s of the meal. This disk consisted of a 4.25 cm diameter glass-fiber disk (Whatman GF/A) treated with 400 µl of each test solution. We ran the behavioral test immediately after applying the test solution so as to minimize evaporative water loss. By focusing

on the initial 10 s of biting, we minimized the contribution of any post-ingestive effects of the test solutions. To avoid experiential effects, we ran each caterpillar through a single biting assay.

Short-term biting assay

The assay consisted of four stages. (1) We placed a caterpillar in the 'food-deprivation arena', which consisted of a clean (inverted) Petri dish covered by a clear plastic cylinder (7.5 cm in diameter, 10 cm tall). We fasted the caterpillar in this arena for 30 min to standardize its 'hunger' state. (2) Then, we transferred the caterpillar to the 'test arena', which was identical to the food-deprivation arena in all respects except that it contained a piece of cork in the center, containing a disk pre-moistened with 400 µl experimental solution. The observer was kept blind to the identity of the test solution. (3) Next, we positioned the caterpillar on the edge of the glass-fiber disk so that its legs and prolegs grasped the edge. (4) Finally, we recorded the number of bites taken from the disk over the first 10 s of feeding.

To ensure that the observer could record the number of bites accurately and without bias, we positioned the test arena on a turntable-like device, which permitted us to rotate the turntable and keep the caterpillar's mandibles clearly visible as it fed.

Data analysis

To compare the number of bites taken from each experimental solution, we used a two-way factorial ANOVA. One factor was test series (AA or Caf test series) and the second factor was test solution (water, noxious compound alone, or noxious compound plus sugars). We used a *post hoc* Tukey's test to determine the relative number of bites taken from each test solution, separately for the AA or Caf test series. We inferred that an experimental disk (e.g. a disk treated with 5 mmol l^{-1} Caf) was less acceptable than the control disk (i.e. a disk treated with water alone) if the caterpillars took significantly fewer bites from the experimental disk. For each factorial ANOVA, we confirmed that each dependent measure met the normality assumption (Kolmogorov–Smirnov test), and that there was homogeneity of variance across treatment levels (Levene's test). In all comparisons, we set the alpha level at 0.05.

Experiment 2 – electrophysiological responses

We focused on the lateral and medial styloconic sensilla, each of which responds to sugars and noxious plant compounds (see Table 1 for the molecular receptive range of the each GRN). Our goal was to determine how the presence of the sugars influenced peripheral taste responses to Caf or AA.

Recording technique

For each caterpillar, we recorded neural responses of a lateral and medial styloconic sensilla to the AA or Caf test series, using a previously described tip recording technique (Glendinning et al., 2007). Neural recordings were pre-amplified (10×) with a TasteProbe (Syntech, Hilversum, The Netherlands), filtered (100–1200 Hz), digitized (IDAC-4, Syntech) and analyzed with Autospike software (Syntech).

Data analysis

We tallied the total number of action potentials during each successive 100 ms bin across the initial 1000 ms of each recording. Because many of the recordings contained responses from multiple GRNs with temporally variable spike amplitudes, it was impossible to assign spikes based solely on amplitude and shape. As a result, we incorporated the distinct temporal pattern of firing that each stimulus elicited into our spike assignments.

We used repeated-measures ANOVAs to ascertain whether the instantaneous firing rate changed systematically with time. We confirmed equality of variance across treatment levels of a repeated-measures factor with Mauchly's sphericity test. We compared the total number of spikes that the stimulus elicited over 1000 ms with that elicited by the KCl solution, using a paired *t*-test. We inferred that the test solution elicited a detectable response when total number of spikes differed significantly from than that elicited by the electrolyte solution. In comparisons involving multiple *t*-tests, we used the Bonferroni correction.

To test for mixture suppression, we first compared the number of the spikes elicited by the stimulus mixture (i.e. mixed condition) with the total number of spikes elicited by each component of the stimulus mixture separately (i.e. unmixed condition). We made no attempt to discriminate between spikes from different GRNs; we simply counted the total number of spikes that occurred during the initial 1000 ms of response. Second, we compared the magnitude of mixture suppression across the different stimulus mixtures with a repeated-measures ANOVA and a Tukey-type *post hoc* test (corrected for a within-subject design). We calculated percent mixture suppression as follows: 100×[1–(number of spikes during the mixed condition/number of spikes during the unmixed condition)]. We confirmed equality of variance across treatment levels of the repeated-measures factor with Mauchly's sphericity test. Third, we used mixed-model ANOVA and unpaired *t*-tests to compare % mixture suppression in the AA test series with that in the Caf test series. Finally, we conducted a fine-grained analysis of mixture suppression. To this end, we examined changes in instantaneous firing rates and spike amplitude over time.

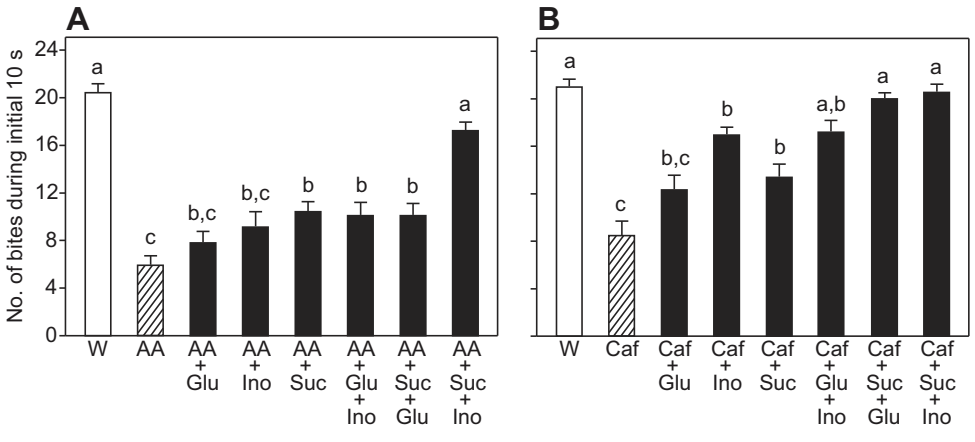

Fig. 1. Number of bites taken by *Manduca sexta* caterpillars during the initial 10 s of feeding on disks containing solutions in both the (A) aristolochic acid (AA) and (B) caffeine (Caf) test series. We compared the number of bites for each solution using a *post hoc* Tukey's test; different letters (a,b,c) above the bars indicate means that differ significantly from one another (P<0.05). Data are means ± s.e.m., and are based on biting responses of 18–21 caterpillars per test solution. Each caterpillar was tested only once. AA, 0.1 mmol l^{-1} aristolochic acid; Caf, 5 mmol l^{-1} caffeine; Glu, 200 mmol l^{-1} glucose; Ino, 1 mmol l^{-1} inositol; Suc, 200 mmol l^{-1} sucrose; W, water.

We also compared the total number of spikes (over the initial 1000 ms of response) elicited by each binary (or ternary) mixture with that elicited by each of its component stimuli, using a repeated-measures ANOVA and a *post hoc* Tukey's test. We used these analyses to assign spikes to individual cells (whenever possible), and to draw inferences into the nature of the mixture suppression (e.g. relative contribution of reciprocal *versus* non-reciprocal inhibition).

RESULTS

Experiment 1 – short-term biting assay

The caterpillars exhibited nearly continuous biting activity during the trials with water alone (producing approximately 20 bites), but highly disrupted (or inhibited) biting activity during the trials with AA alone or Caf alone (producing ≤8 bites; Fig. 1). The addition of sugars to the AA (or Caf) solution inhibited biting to varying degrees. There was a significant main effect of test solution series ($F_{1,311}$=86.2, P≤0.05), revealing that the caterpillars generally took fewer bites from stimulus mixtures in the AA test series than in the Caf test series. There was also a significant main effect of stimulus mixture ($F_{7,311}$=46.4, P≤0.05) and a significant interaction of test solution series × stimulus mixture ($F_{7,311}$=4.3, P≤0.05).

For the AA test series, the relative acceptability was: water=AA+Suc+Ino>AA+Suc+Glu=AA+Glu+Ino=AA+Suc>AA alone (Fig. 1, left panel). The number of bites taken for AA+Glu and AA+Ino could not be distinguished from that taken for AA alone. AA+Suc+Ino was the only solution that was as acceptable as water. Although AA+Suc+Glu, AA+Glu+Ino and AA+Suc were more acceptable than AA alone, they were significantly less acceptable than water.

For the Caf test series, the relative acceptability was: water = Caf+Suc+Ino = Caf+Suc+Glu > Caf+Suc = Caf+Ino > Caf alone (Fig. 1, right panel). The number of bites taken for Caf+Glu could not be distinguished from that taken for Caf alone. Caf+Suc+Ino and Caf+Suc+Glu were the only solutions that were as acceptable as water. Even though Caf+Glu+Ino, Caf+Suc and Caf+Ino were more acceptable than Caf alone, they were still less acceptable than water.

Experiment 2 – electrophysiological responses

The neural response to AA in both sensilla was unique in two respects: both spike amplitudes and instantaneous firing rates increased over time (Fig. 2). In contrast, the spike amplitudes elicited by Caf and the sugars either remained constant or decreased over time in both sensilla. The firing rates generated by KCl did not change systematically over time in either sensillum.

In Fig. 3, we compare total spikes elicited by each plant chemical with that elicited by KCl alone (the reference stimulus). In the lateral styloconic sensillum, the total number of spikes elicited by Caf, AA, Suc, Ino and Glu were all significantly greater than that elicited by KCl alone. In the medial styloconic sensillum, the total number of spikes elicited by AA, Ino and Glu (but not Caf and Suc) was significantly greater than that elicited by KCl alone.

Mixture suppression: coarse-grained analysis

All sugar solutions, except Ino, inhibited the response of the lateral and medial styloconic sensilla to both AA and Caf (Fig. 4A). The magnitude of mixture suppression, however, varied greatly across the test solutions (Fig. 4B). For solutions containing AA, the magnitude of mixture suppression was greatest for AA+Suc+Ino in the lateral styloconic sensillum, and for AA+Suc+Ino and AA+Suc+Glu in the medial styloconic sensillum. For solutions containing Caf, the magnitude of mixture suppression was greatest for Caf+Suc+Ino and Caf+Suc+Glu in the lateral styloconic sensillum. There were no significant differences in magnitude of mixture suppression across the stimulus mixtures in the medial styloconic sensillum. Finally, the magnitude of mixture suppression in the lateral styloconic sensillum was significantly greater for the AA test series than for the Caf test series (main effect of noxious compound: $F_{1,78}$=12.3, P≤0.05); no such difference was observed in the medial styloconic sensillum (main effect of noxious compound: $F_{1,78}$=0.2, P>0.05; Fig. 4C).

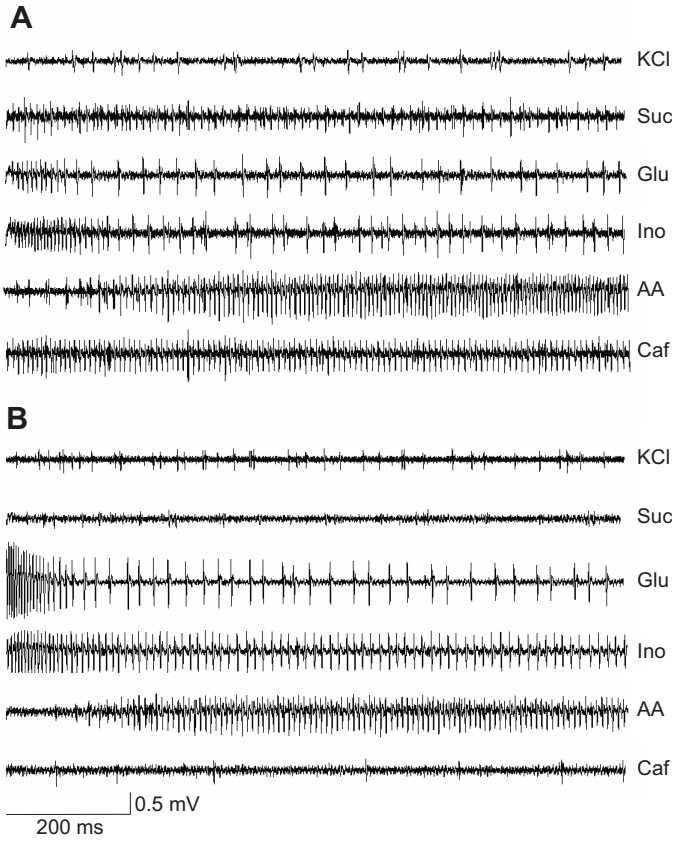

Fig. 2. Typical neural recordings from (A) a lateral and (B) a medial styloconic sensillum of *M. sexta* to six solutions: KCl, Suc, Glu, Ino, AA and Caf (note that the latter five solutions were dissolved in the 0.1 mol l^{-1} KCl solution). See Fig. 1 for a key to the abbreviations. Each recording illustrates the initial 1000 ms of response.

Mixture suppression also occurred in test solutions containing sugars alone (i.e. Suc+Ino, Suc+Glu and Ino+Glu; Fig. 5). The magnitude of mixture suppression was greatest for Glu+Suc in the lateral styloconic sensillum, and for Ino+Suc in the medial styloconic sensillum.

Mixture suppression: fine-grained analysis

Binary mixtures of AA+Suc and Caf+Suc

The presence of Suc altered the peripheral response to AA and Caf in different ways (Fig. 6, supplementary material Fig. S1). The instantaneous firing rate for AA+Suc increased significantly with time in both sensilla ($F_{9,69}$>6.1, P≤0.05), and resembled the response to AA alone (Fig. 6A, top trace). However, the total number of spikes elicited over the initial 1000 ms was significantly less than that elicited by AA alone (supplementary material Fig. S1A). These observations indicate that Suc partially inhibited the response to AA in both sensilla.

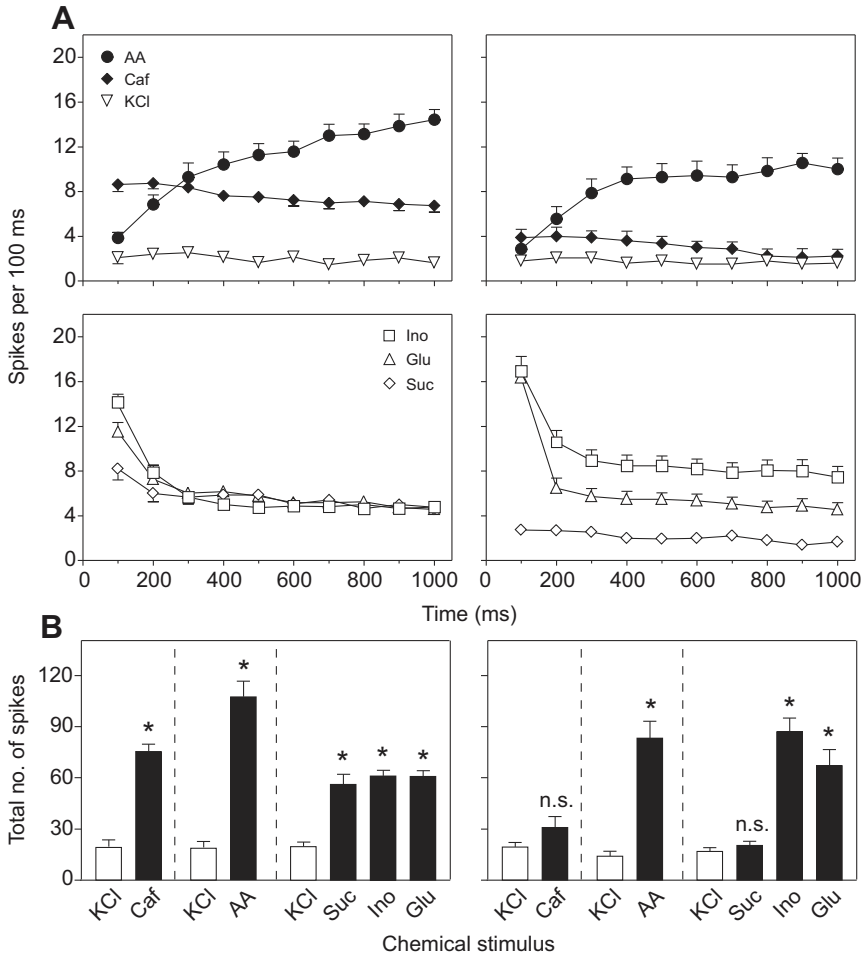

Fig. 3. Neural responses of the lateral (left column) and medial (right column) styloconic sensilla of *M. sexta* to different test solutions. (A) Instantaneous firing rates (i.e. spikes per 100 ms) across the initial 1000 ms of stimulation with AA, Caf and KCl (top row), and Glu, Suc and Ino (bottom row). (B) Comparison of the total number of spikes elicited by each test solution with that elicited by KCl alone, using *post hoc* Tukey's tests. Asterisks indicate means that differ significantly from the corresponding control KCl solution (*P<0.05; n.s., P>0.05). We distinguish separate statistical comparisons within each panel with a vertical stippled line. See Fig. 1 for a key to the abbreviations. Data are means ± s.e.m., and are based on responses of seven to 15 medial or lateral sensilla, each from a different caterpillar.

Caf+Suc elicited an instantaneous firing rate that decreased significantly with time in both sensilla ($F_{9,79}$>3.1, P≤0.05). However, because of the weak responses of the medial styloconic sensilla to Caf, Suc and Caf+Suc (supplementary material Fig. S1B), it is difficult to draw any inferences about mixture interactions. In contrast, there were two distinctive features of the response of the lateral styloconic sensilla to Caf+Suc: it contained significantly more total spikes than did the response to Caf or Suc alone, and multiple GRNs fired out of phase with one another (Fig. 6B). This indicates that Suc produced only limited inhibition of the response to Caf.

Binary mixtures of AA+Glu and Caf+Glu

The instantaneous firing rate elicited by AA+Glu decreased over time in both sensilla ($F_{9,69}$>3.8, P≤0.05; supplementary material Fig. S2A). The high firing rates during the initial 200 ms were virtually identical in intensity and duration to those elicited by Glu alone. Further, the firing rates during the final 800 ms were more intense than those elicited by Glu alone, implicating a contribution of AA.

Caf+Glu elicited an instantaneous firing rate that decreased over time in both sensilla ($F_{9,79}$>12.7, P≤0.05), and resembled the temporal pattern of firing elicited by Glu alone (supplementary material Fig. S2B). The total number of spikes generated by Caf+Glu was significantly greater than that generated by Glu alone in the lateral but not the medial styloconic sensillum (supplementary material Fig. S2B).

Binary mixtures of AA+Ino and Caf+Ino

The instantaneous firing rate elicited by AA+Ino changed significantly over time in both sensilla ($F_{9,69}$>6.7, P≤0.05; supplementary material Fig. S3A). In the lateral styloconic sensillum, the temporal pattern of firing appears to reflect a composite of the response to both AA and Ino. For example, in Fig. 6A (second trace from top), the initial 200 ms resembles the phasic response to Ino, and the final 700 ms resembles the increasing firing rate to AA. Although the instantaneous firing rate in the medial styloconic sensillum followed a typical phasic–tonic pattern, it also appears to contain a mixture of the responses to both AA and Ino.

The neural responses to Caf+Ino in both sensilla resembled a composite of that to Caf and Ino alone (supplementary material Fig. S3B). There are elements of both the phasic response to Ino and the tonic response to Caf (Fig. 6B).

Ternary mixtures of AA+Ino+Glu and Caf+Ino+Glu

In response to AA+Ino+Glu, the instantaneous firing rate decreased over time in the medial ($F_{9,69}$=10.6, P≤0.05) but not in the lateral styloconic sensilla ($F_{9,69}$=0.7, P>0.05; supplementary material Fig. S4A, left panels). Further, the total number of spikes elicited by AA+Ino+Glu was significantly greater than that elicited by AA alone in the medial but not the lateral styloconic sensillum (supplementary material Fig. S4A, right panels). These findings indicate that the binary mixture of Ino+Glu largely eliminated the AA response in the lateral styloconic sensillum, but failed to do so in the medial styloconic sensillum.

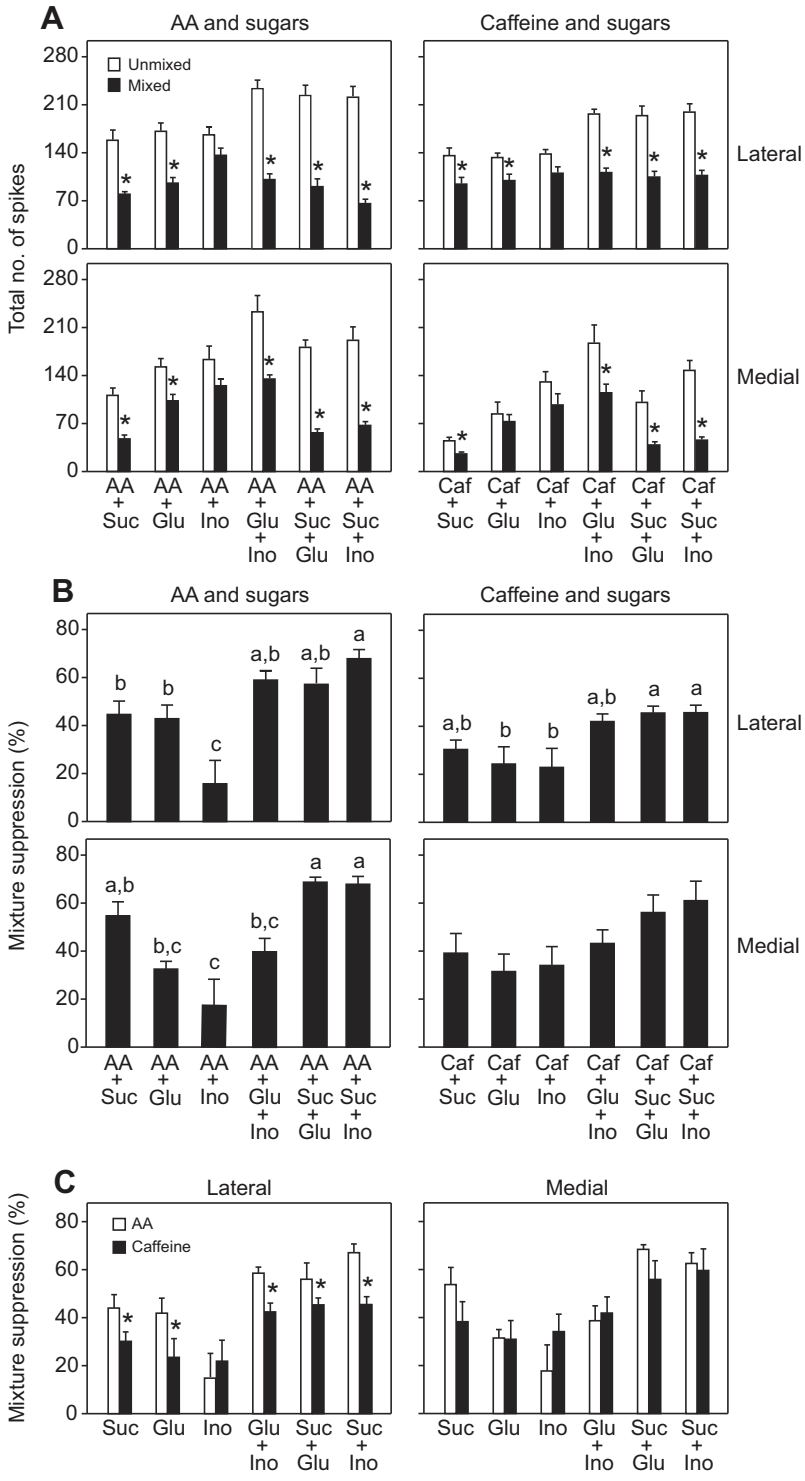

Fig. 4. Evidence for mixture suppression in the lateral and medial styloconic sensilla of *M. sexta* during stimulation with the mixtures of AA, Caf, Glu, Ino and Suc. (A) Effect of mixing sugars and AA (or Caf) on the total number of spikes generated during the initial 1000 ms of response in the lateral and medial sensilla. In each panel, we show the total number of the spikes elicited by a stimulus mixture (i.e. mixed) and that elicited by each of the components of the stimulus mixture (i.e. unmixed). We used paired *t*-tests to compare the total number of spikes elicited by each mixed *versus* unmixed (**P*<0.008) stimulus configuration. (B) Magnitude of mixture suppression in the different stimulus mixtures in the lateral and medial sensilla. We used *post hoc* Tukey's tests to compare percent mixture suppression across the test solutions, separately for each panel. Different letters above each bar (a,b,c) indicate means that differ significantly from one another (*P*<0.05). (C) Comparison of mixture suppression in sugar solutions treated with AA or Caf. We used unpaired *t*-tests to compare percent mixture suppression in a sugar solution (e.g. sucrose) containing AA with that of the same sugar solution containing Caf (**P*<0.008), separately for each sensillum. See Fig. 1 for a key to the abbreviations. Data are means ± s.e.m., and are based on responses of seven to 15 medial or lateral sensilla, each from a different caterpillar.

Caf+Ino+Glu elicited an instantaneous firing rate that decreased significantly over time in both sensilla ($F_{9,79}$>25.3, P≤0.05; supplementary material Fig. S4B, left panels). These decelerating firing rates presumably stem from strong phasic responses to Glu and Ino.

Ternary mixtures of AA+Suc+Glu and Caf+Suc+Glu

The instantaneous firing rate elicited by AA+Suc+Glu changed slightly but significantly over time in both sensilla ($F_{9,69}$>2.3, P≤0.05; supplementary material Fig. S5A). These temporal changes included a small phasic response (during the initial 300 ms) followed by an increasing instantaneous firing rate over the remaining 700 ms. The small phasic response to AA+Suc+Glu probably reflects the inhibitory effect of Suc on the initial phasic response to Glu, which is apparent particularly in the medial sensillum (supplementary material Fig. S6).

Caf+Suc+Glu elicited an instantaneous firing that decreased significantly over time in both sensilla ($F_{9,79}$>7.0, P≤0.05;

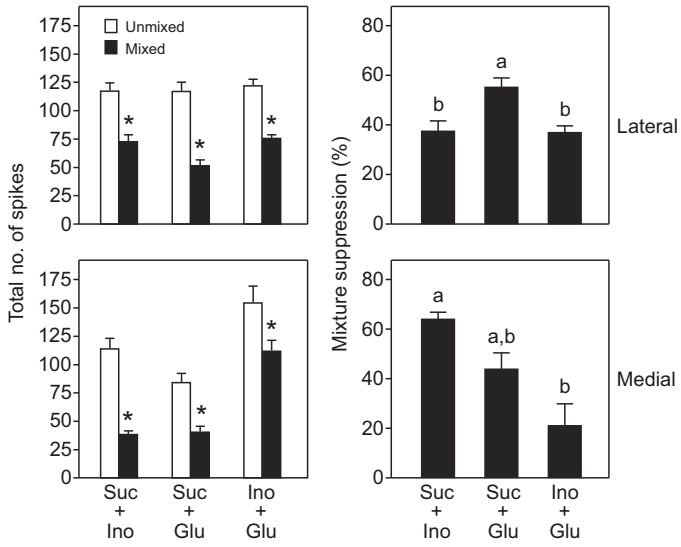

Fig. 5. Evidence for mixture suppression in the lateral and medial styloconic sensilla of *M. sexta* during stimulation with mixtures of sugars (Ino, Glu and Suc). Left column: effect of mixing the sugars on the total number of spikes generated during the initial 1000 ms of response within the lateral and medial sensilla. In each panel, we show the total number of the spikes elicited by a stimulus mixture (i.e. mixed) and that elicited by each of the components of the stimulus mixture (i.e. unmixed). We used paired *t*-tests to compare the total number of spikes elicited by each mixed *versus* unmixed (*P<0.008) stimulus configuration. Right column: magnitude of mixture suppression in the different stimulus mixtures. We used the *post hoc* Tukey's test to compare percent mixture suppression across the stimulus mixtures, separately for each sensillum. Different letters above each bar (a,b,c) indicate means that differ significantly from one another (P<0.05). See Fig. 1 for a key to the abbreviations. Data are means ± s.e.m., and are based on responses of seven to eight medial or lateral sensilla, each from a different caterpillar.

supplementary material Fig. S5B). In the lateral styloconic sensillum, Caf appears to have contributed to the vigorous response to Caf+Suc+Glu. This is based on the observation that Suc+Glu generated the same number of total spikes as Suc or Glu alone (supplementary material Fig. S6), whereas Caf+Suc+Glu generated nearly three times more spikes than did Suc+Glu (supplementary material Fig. S5B). This suggests that Suc+Glu did not effectively inhibit the response of the lateral styloconic sensillum to Caf.

Ternary mixtures of AA+Suc+Ino and Caf+Suc+Ino

The instantaneous firing rate elicited by AA+Ino+Suc did not change significantly over time in either sensilla ($F_{9,69}$<1.4, P>0.05; supplementary material Fig. S7A). Further, the total number of spikes generated by AA+Ino+Suc was significantly less than that generated by AA alone in the lateral sensillum. These results indicate that the response to AA was completely inhibited in the ternary mixture of AA+Ino+Suc.

Caf+Ino+Suc elicited an instantaneous firing that decreased significantly over time in both sensilla ($F_{9,79}$>2.1, P≤0.05; supplementary material Fig. S7B). In the lateral styloconic sensillum, Caf likely contributed to the vigorous response to Caf+Ino+Suc. This is based on the observation that Ino+Suc generated the same number of total spikes as did Ino or Suc alone (supplementary material Fig. S8), whereas Caf+Ino+Suc generated nearly two times more spikes than did Ino+Suc (supplementary material Fig. S7B). This suggests that Ino+Suc did not effectively inhibit the response of the lateral styloconic sensillum to Caf.

DISCUSSION

Some, but not all, sugars masked the aversive taste of AA and Caf

In many species of insect, sugars cause immediate stimulation of feeding. Sugars do not have this effect in *M. sexta*, despite causing strong peripheral taste responses (Glendinning et al., 2007). To explain this conundrum, we hypothesized that the primary function of the peripheral taste response to sugars is to mask the taste of aversive compounds. We found strong support for this hypothesis in our short-term biting assay. Some, but not all, of the sugars masked the taste of AA and Caf. The most effective masking agent was Suc+Ino; it completely masked the taste of both AA and Caf. Although Suc+Glu completely masked the taste of Caf, it only partially masked that of AA. Suc was the only single-component sugar stimulus that partially masked the taste of both AA and Caf. Glu, in contrast, was the only single-component sugar stimulus that failed to even partially mask the taste of AA or Caf.

Mixture suppression in the peripheral taste system

To assess the contribution of peripheral mixture suppression to the behavioral results, we examined how the medial and lateral styloconic sensilla responded to binary and ternary mixtures of the sugars and noxious compounds. We observed extensive mixture suppression, both between sugars and noxious compounds and between the different sugars (Figs 4, 6).

The extent of mixture suppression differed greatly across the binary and ternary mixtures. At one extreme was Ino. Despite eliciting a vigorous response in the lateral and medial styloconic sensilla, Ino did not produce any mixture suppression when mixed with AA or Caf. Indeed, the neural responses to AA+Ino or Caf+Ino appeared to reflect a composite of the response to each stimulus component alone (supplementary material Fig. S3). Given that Ino stimulates a different GRN than both AA and Caf (Table 1), it appears that each GRN was able to respond to its own ligand without inhibiting the response of the neighboring GRNs. At the other extreme was Ino+Suc, which produced the most extensive mixture suppression. Based on the fact that the neural responses to AA+Ino+Suc in both sensilla lacked any of the features typical of the AA response (i.e. spike amplitudes and instantaneous firing rates that increase over time; supplementary material Fig. S6), it appears that Ino+Suc strongly inhibited the response of the AA-sensitive GRN in both the lateral and medial styloconic sensillum.

It is notable that the magnitude of mixture suppression was, in most cases, significantly greater for solutions containing AA than for those containing Caf in both the medial and lateral styloconic sensillum (Fig. 4C). This finding is even more remarkable given that Caf and AA are thought to stimulate the same GRN in the lateral styloconic sensillum (and perhaps in the medial styloconic sensillum as well) (Glendinning and Hills, 1997). One explanation for this result stems from the observation that Caf and AA stimulate different signaling pathways within the same bitter-sensitive GRN, and that these pathways generate distinct temporal patterns of spiking (Glendinning and Hills, 1997). The peripheral taste response to AA has a relatively long latency of activation (i.e. >200 ms; Figs 2, 3), which could make it more vulnerable to inhibitory effects of neighboring sugar-sensitive GRNs, which reach their maximal firing rates more quickly (i.e. <100 ms; e.g. Fig. 2). This explanation could be tested by evaluating mixture suppression with noxious

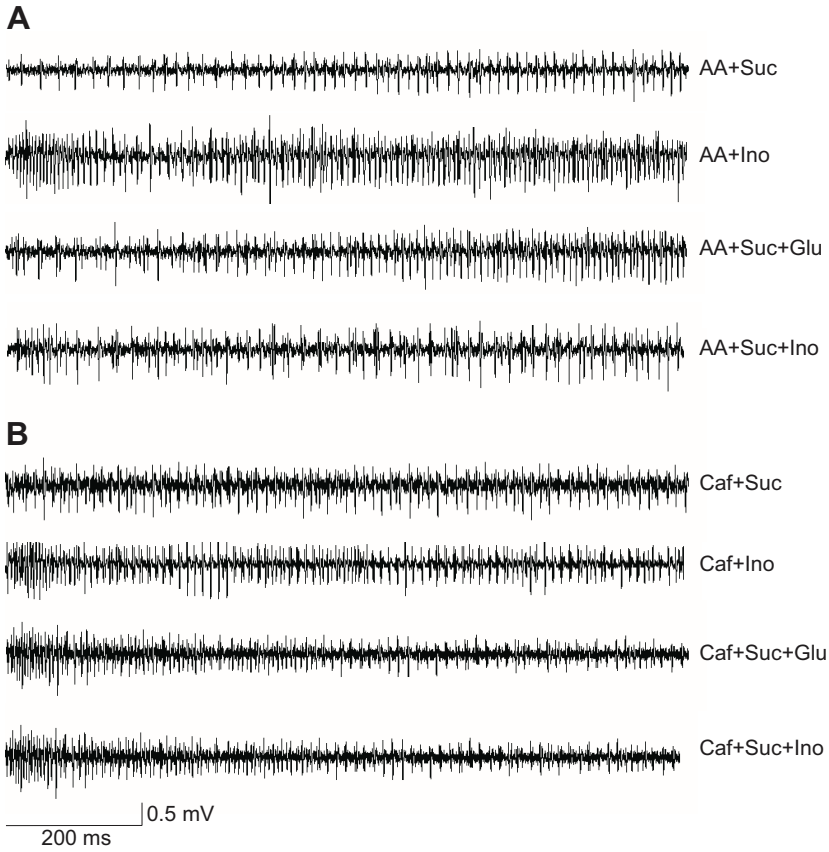

Fig. 6. Typical responses of the lateral styloconic sensillum of *M. sexta* to solutions containing (A) AA plus Suc, Ino, Suc+Glu or Suc+Ino, or (B) Caf plus Suc, Ino, Suc+Glu or Suc+Ino. See Fig. 1 for a key to the abbreviations. All chemicals were dissolved in the 0.1 mmol^{-1} KCl solvent. Each trace illustrates the initial 1000 ms of response.

compounds that produce temporal patterns of spiking that vary in latency of activation. Accordingly, mixture suppression should increase with the latency of activation.

There was no obvious relationship between the firing rate elicited by a sugar-sensitive GRN and the magnitude of mixture suppression it caused. For instance, when Suc was presented alone, it elicited a feeble excitatory response in the medial styloconic sensillum, but when presented in a binary mixture (i.e. AA+Suc, Suc+Ino or Suc+Glu), it produced robust mixture suppression (i.e. >40%). In contrast, when Ino was presented alone, it elicited a vigorous excitatory response in the medial styloconic sensillum, but when presented in a binary mixture (i.e. AA+Ino, AA+Glu and Ino+Glu), it produced modest mixture suppression (i.e. <40%).

Our findings indicate that mixture suppression in the lateral styloconic sensillum was mediated largely by reciprocal inhibition. This is because when each of the plant chemicals was presented individually, it elicited a strong excitatory response in a single GRN. However, when the same chemicals were presented in binary or ternary mixtures, they caused varying degrees of inhibition in multiple GRNs (Fig. 3). The only exceptions to this pattern were the binary mixtures of AA+Ino and Caf+Ino, which failed to produce any mixture suppression. Although little is known about the mechanistic basis of mixture suppression in insect taste sensilla, the most parsimonious explanation is that activation of one GRN stimulates paracrine release into the sensillar lumen, which modulates the response of neighboring GRNs. There is widespread evidence for these types of modulatory signaling networks in mammalian taste buds (Herness and Zhao, 2009).

In the medial styloconic sensillum, mixture suppression was mediated by reciprocal and non-reciprocal inhibition. For instance, AA, Glu and Ino each elicited a strong excitatory response in a single GRN when presented individually, but caused varying degrees of reciprocal inhibition in multiple GRNs when presented in binary or ternary mixtures. In contrast, Suc produced a feeble excitatory response when presented alone (Fig. 3), but caused non-reciprocal inhibition of the excitatory responses of GRNs to the other plant chemicals (Figs 4–6). One possible explanation for this latter case of non-reciprocal inhibition is that Suc directly antagonized the signaling pathways for AA, Glu and Ino (see Talavera et al., 2008).

Peripheral mixture suppression was also observed between sugars (Fig. 5). This finding is notable because it indicates that some of the mixture suppression produced by the ternary mixtures (Fig. 4) was due to inhibitory interactions among the sugars. It is important to note, however, that the existence of peripheral mixture suppression between sugars is tangential to the question of whether sugars inhibited the peripheral gustatory response to AA and Caf. Indeed, we were able to establish specific inhibitory effects of the sugars on responses of the bitter-sensitive GRNs.

Did peripheral mixture suppression mask the aversive taste of AA and Caf?

Before discussing the neural basis of the biting responses, it is necessary to highlight four caveats about our experimental approach. First, we did not record from the epipharyngeal sensilla because they do not generate excitatory responses to sugars (de Boer et al., 1977; Glendinning et al., 2000). Although prior reports indicate that the excitatory response of epipharyngeal sensilla to Caf is suppressed by Glu but not Ino (Glendinning et al., 2000), we are not certain how this class of sensillum contributed to our behavioral results. Second, because little is known about the actual sugar concentrations that *M. sexta* caterpillars encounter while ingesting solanaceous foliage, we selected concentrations that elicit maximal responses in the sugar-sensitive GRNs (Glendinning et al., 2007). We reasoned

that these high concentrations would provide the clearest evidence of peripheral mixture suppression. Third, we used Caf and AA as model noxious plant compounds because they strongly activate the peripheral taste system of *M. sexta* caterpillars. Additional studies are needed to determine whether there are noxious compounds in solanaceous foliage that activate the same GRNs as Caf and AA, and how sugars influence taste responses to these compounds. Fourth, because consumption of host-plant foliage can alter the responses of the peripheral taste system of *M. sexta* (del Campo et al., 2001; Glendinning et al., 2009), it is possible that the nature of the mixture suppression differs between caterpillars reared on artificial diet and those reared on host-plant tissue. Notwithstanding these caveats, we discuss below the extent to which peripheral mixture suppression contributed to the masking phenomenon.

For the AA test series, the extent to which each sugar masked the taste of AA increased with the magnitude of peripheral mixture suppression. For instance, the one sugar stimulus that completely masked the aversive taste of AA (Suc+Ino) also strongly inhibited the peripheral taste of both sensilla to AA. The three sugar stimuli that partially masked the aversive taste of AA (Suc, Glu+Ino and Suc+Glu) also partially blocked the response of both sensilla to AA. Finally, the two sugar stimuli that failed to mask the aversive taste of AA (Glu and Ino) also provided the weakest suppression of the peripheral response to AA. The strong correlation between the acceptability of the AA-containing solutions and the magnitude of peripheral taste suppression points to a causal connection between these two phenomena.

For the Caf test series, there was no clear relationship between taste acceptability of each stimulus mixture and magnitude of peripheral taste suppression. For instance, even though Suc+Glu and Suc+Ino completely masked the aversive taste of Caf, they only partially suppressed the peripheral response to Caf. Likewise, even though Ino partially masked the aversive taste of Caf, it failed to produce any significant suppression of the peripheral taste response to Caf. These findings, together with those of an earlier report (Glendinning et al., 2000), indicate that the ability of the sugars to mask the aversive taste of Caf cannot be explained by peripheral taste suppression alone. We propose, instead, that sugars masked the taste of Caf by activating inhibitory mechanisms in both the peripheral and central taste systems. A similar explanation has been proposed to explain how sugars mask the aversive taste of quinine in mammals (Kroeze and Bartoshuk, 1985; Formaker and Frank, 1996).

Perspectives and significance

This study, together with prior reports (Glendinning et al., 2000; Glendinning et al., 2007), indicates that sugars can modulate immediate appetitive responses of *M. sexta* caterpillars, but only when they are presented together with noxious plant compounds. It is important to emphasize that this result does not preclude a role of sugars in the stimulation of feeding. For instance, some sugar solutions (e.g. Suc+Ino) can increase meal length in *M. sexta* caterpillars (Glendinning et al., 2007). However, it took more than 6 min of feeding before the effect of Suc+Ino on meal length manifested itself. This indicates that the stimulation of feeding was mediated primarily by a post-oral mechanism (Burke and Waddell, 2011; Dus et al., 2011; Fujita and Tanimura, 2011).

Although all sugars modulated taste responses to AA and Caf, Suc had the greatest impact. For instance, the only binary sugar mixtures that completely masked the taste of AA and/or Caf contained Suc (i.e. Suc+Ino and Suc+Glu). Likewise, the only single-component sugar that partially blocked the taste of AA was Suc. For the peripheral taste recordings, the sugar stimuli that produced the greatest mixture suppression almost always contained Suc. This finding is remarkable because *M. sexta* possesses only one bilateral pair of GRNs that responds to Suc, and two bilateral pairs of taste sensilla that respond to Glu and Ino (Table 1). If the ability of sugars to mask the taste of an aversive compound was determined simply by the relative amount of taste input from sugar-sensitive GRNs, then one would expect that mixtures of Glu and Ino would be more effective than Suc (Figs 3, 5). The fact that we observed the opposite result indicates that the taste system of *M. sexta* caterpillars pays particular attention to input from the bilateral pair of sucrose-sensitive GRNs. More information about the composition of host-plant foliage is needed to explain the functional significance of Suc (or input from the sucrose-sensitive GRN) to the feeding behavior of *M. sexta* caterpillars.

FUNDING

Funding was provided by the Howard Hughes Medical Institute to Barnard College. Deposited in PMC for release after 6 months.

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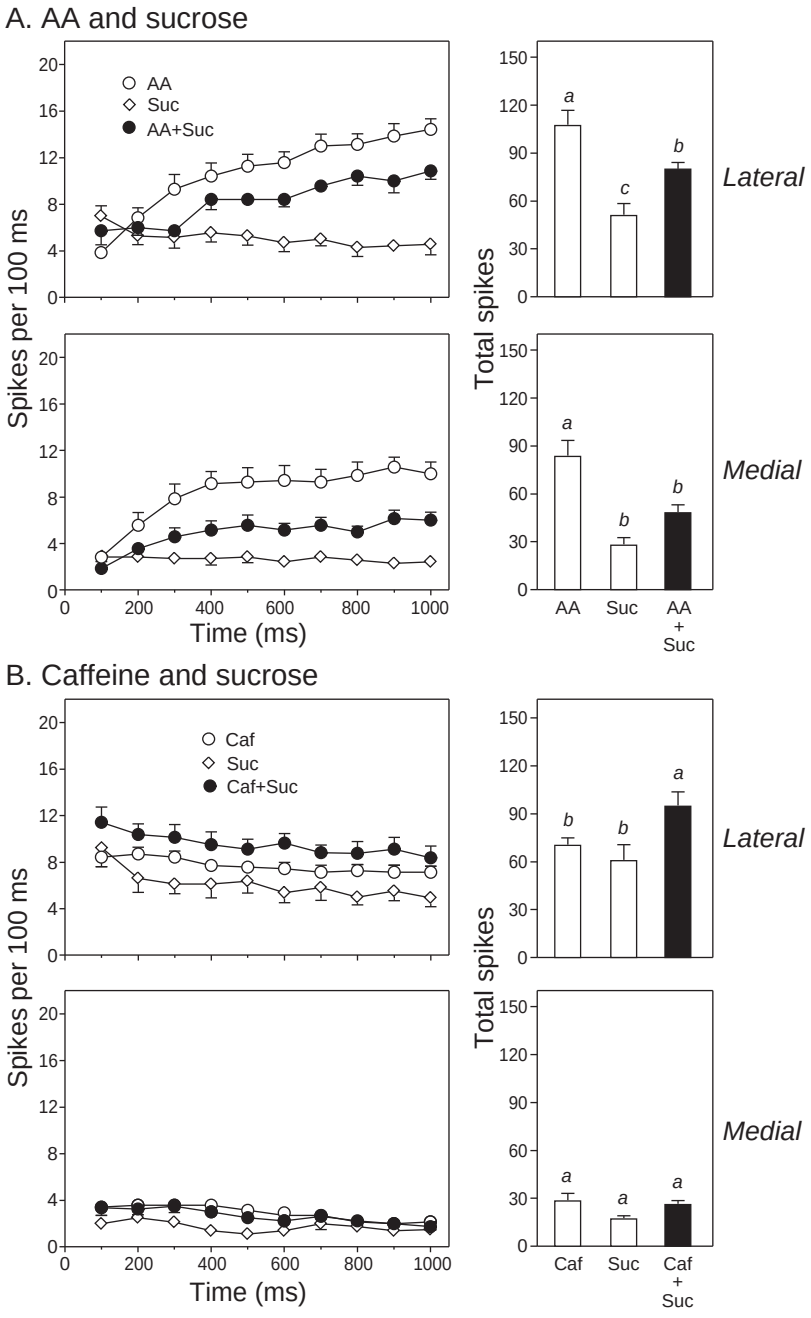

Figure S1. Neural responses of the lateral and medial styloconic sensilla to (A) Suc, AA and the binary mixture of both, or (B) Suc, Caf and the binary mixture of both. *Left row of panels:* We show instantaneous firing rates (i.e., spikes per 100 ms) of GRNs in response to each stimulus configuration during the initial 1000 ms of stimulation. *Right row of panels:* We compare the total number of spikes that were elicited by each test solution, using Tukey post-hoc tests. Different letters (i.e., a, b, c) above bars indicate means that differ significantly from one another ($P < 0.05$). See Figure 1 for a key to the abbreviations for each chemical stimulus. Data are means ± s.e.m., and are based on responses of 7-8 medial or lateral sensilla, each from a different caterpillar.

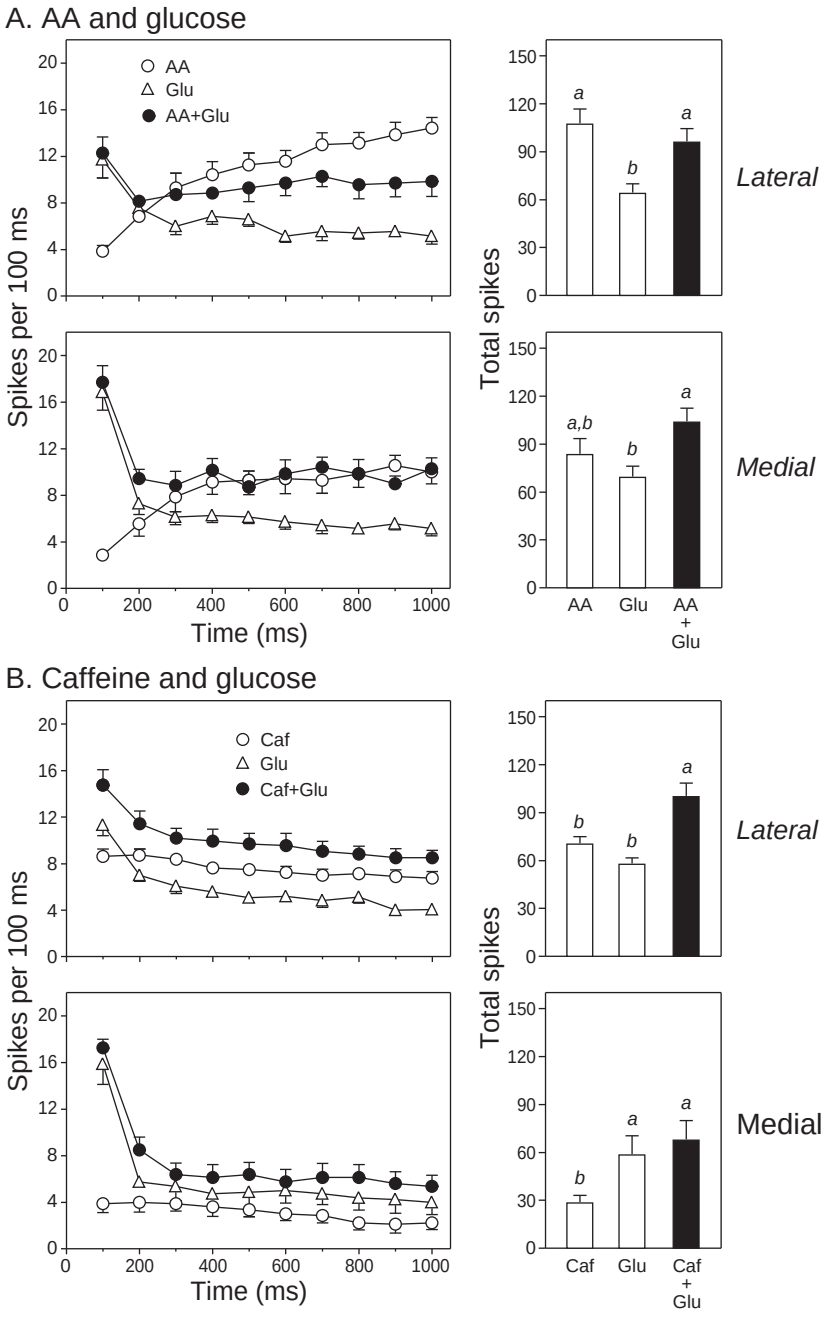

Figure S2. Neural responses of the lateral and medial styloconic sensilla to (A) Glu, AA and the binary mixture of both, or (B) Glu, Caf and the binary mixture of both. *Left row of panels:* We show instantaneous firing rates (i.e., spikes per 100 ms) of GRNs in response to each stimulus configuration during the initial 1000 ms of stimulation. *Right row of panels:* We compare the total number of spikes that were elicited by each test solution, using Tukey post-hoc tests. Different letters (i.e., a, b, c) above bars indicate means that differ significantly from one another ($P < 0.05$). See Figure 1 for a key to the abbreviations for each chemical stimulus. Data are means ± s.e.m., and are based on responses of 7-8 medial or lateral sensilla, each from a different caterpillar.

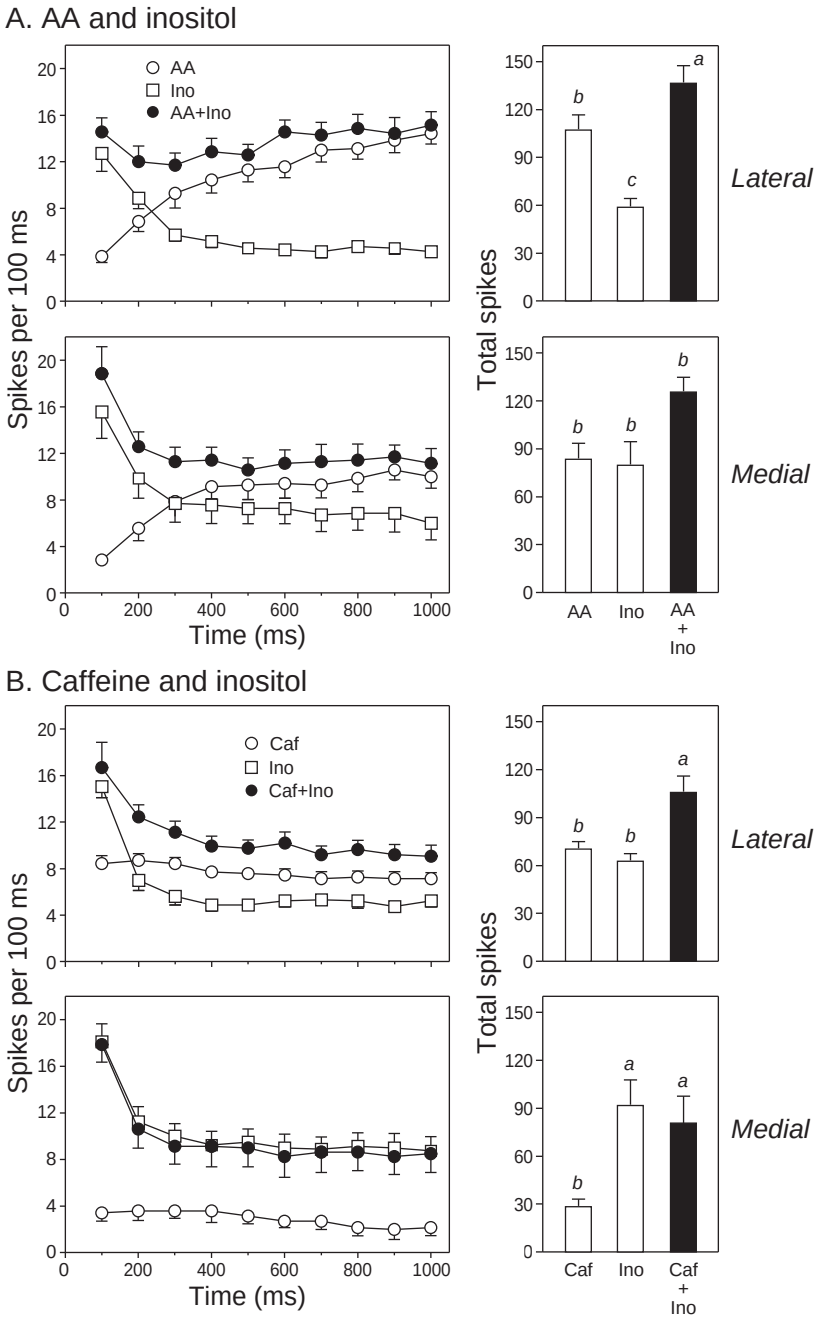

Figure S3. Neural responses of the lateral and medial styloconic sensilla to (A) Ino, AA and the binary mixture of both, or (B) Ino, Caf and the binary mixture of both. *Left row of panels:* We show instantaneous firing rates (i.e., spikes per 100 ms) of GRNs in response to each stimulus configuration during the initial 1000 ms of stimulation. *Right row of panels:* We compare the total number of spikes that were elicited, using Tukey post-hoc tests. Different letters (i.e., a, b, c) above bars indicate means that differ significantly from one another ($P < 0.05$). See Figure 1 for a key to the abbreviations for each chemical stimulus. Data are means ± s.e.m., and are based on responses of 7-8 medial or lateral sensilla, each from a different caterpillar.

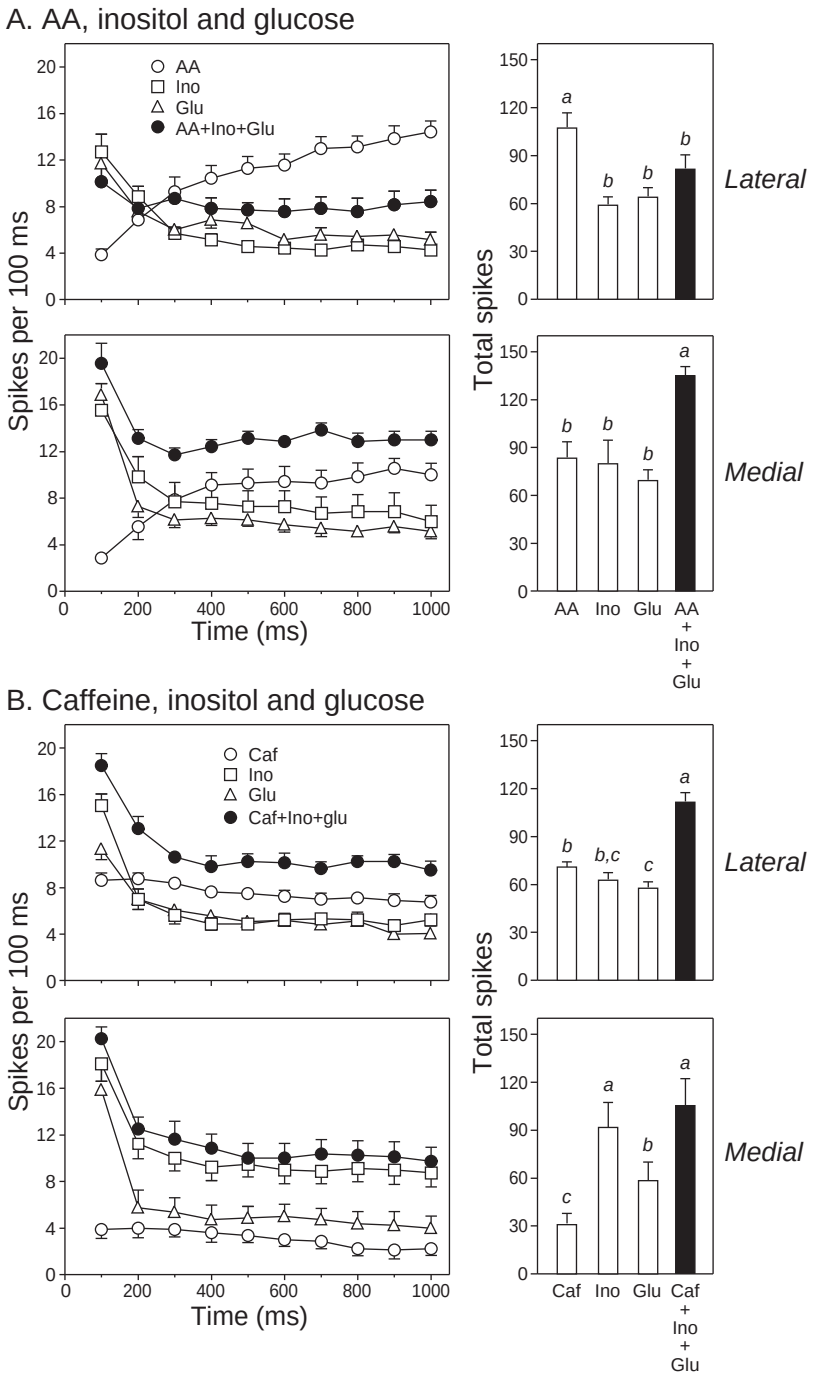

Figure S4. Neural responses of the lateral and medial styloconic sensilla to (A) Ino, Glu, AA and the ternary mixture of all three, or (B) Ino, Glu, Caf and the ternary mixture of all three. *Left row of panels:* We show instantaneous firing rates (i.e., spikes per 100 ms) of GRNs in response to each stimulus configuration during the initial 1000 ms of stimulation. *Right row of panels:* We compare the total number of spikes that were elicited, using Tukey post-hoc tests. Different letters (i.e., a, b, c) above bars indicate means that differ significantly from one another ($P < 0.05$). See Figure 1 for a key to the abbreviations for each chemical stimulus. Data are means ± s.e.m., and are based on responses of 7-8 medial or lateral sensilla, each from a different caterpillar.

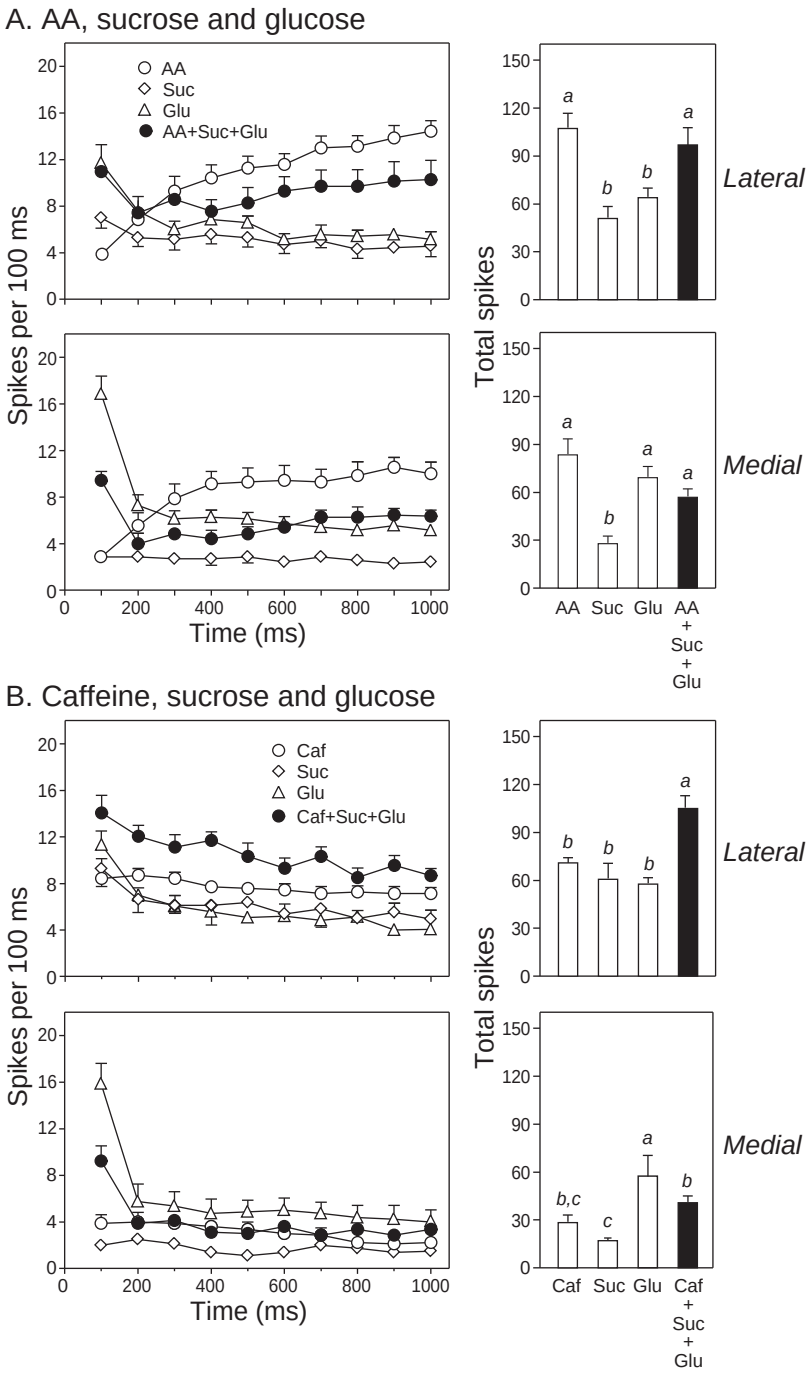

Figure S5. Neural responses of the lateral and medial styloconic sensilla to (A) Suc, Glu, AA and the ternary mixture of all three, or (B) Suc, Glu, Caf and the ternary mixture of all three. *Left row of panels:* We show instantaneous firing rates (i.e., spikes per 100 ms) of GRNs in response to each stimulus configuration during the initial 1000 ms of stimulation. *Right row of panels:* We compare the total number of spikes that were elicited, using Tukey post-hoc tests. Different letters (i.e., a, b, c) above bars indicate means that differ significantly from one another ($P < 0.05$). See Figure 1 for a key to the abbreviations for each chemical stimulus. Data are means ± s.e.m., and are based on responses of 7-8 medial or lateral sensilla, each from a different caterpillar.

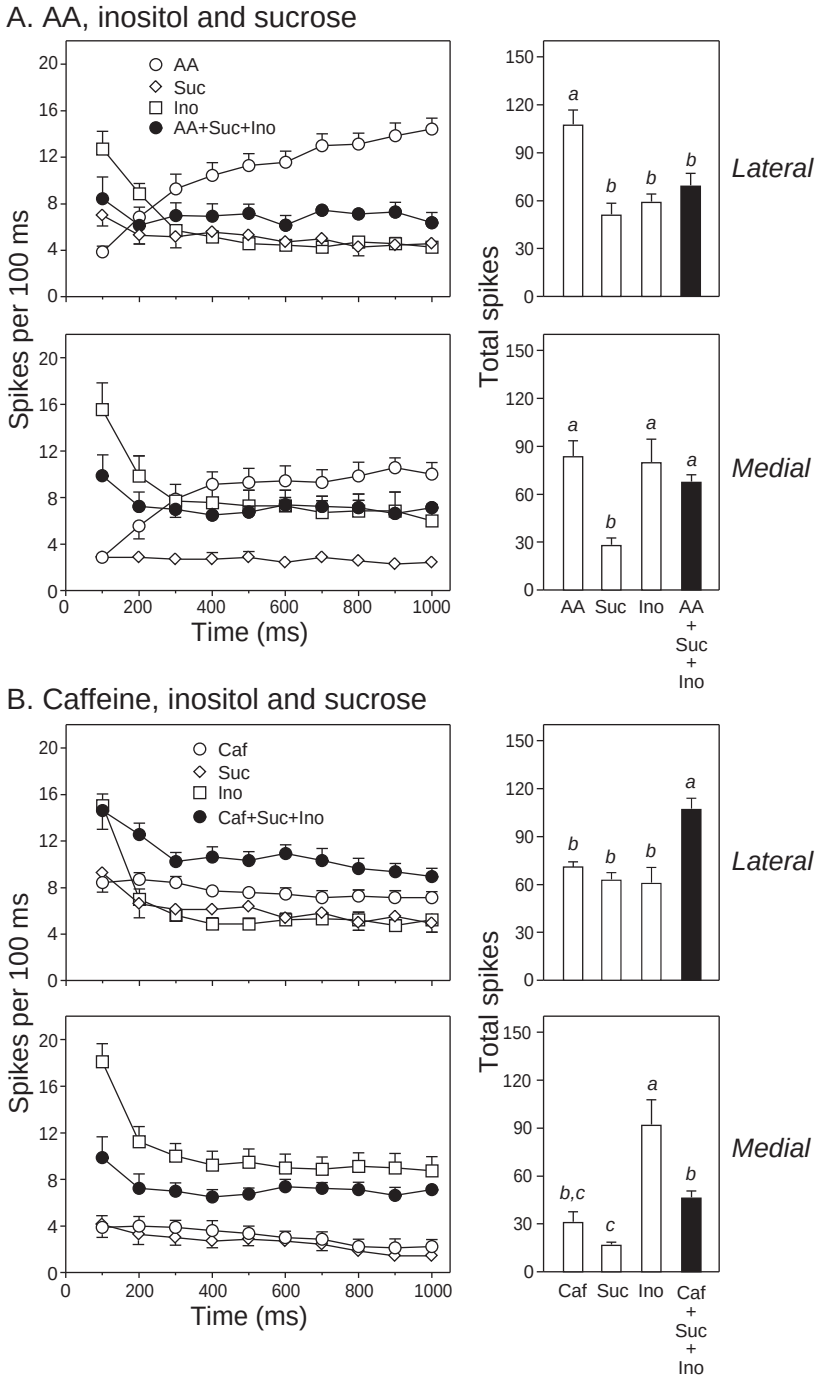

Figure S6. Neural responses of the lateral and medial styloconic sensilla to (A) Suc, Ino, AA and the ternary mixture of all three, or (B) Suc, Ino, Caf and the ternary mixture of all three. *Left row of panels:* We show instantaneous firing rates (i.e., spikes per 100 ms) of GRNs in response to each stimulus configuration during the initial 1000 ms of stimulation. *Right row of panels:* We compare the total number of spikes that were elicited, using Tukey post-hoc tests. Different letters (i.e., a, b, c) above bars indicate means that differ significantly from one another ($P < 0.05$). See Figure 1 for a key to the abbreviations for each chemical stimulus. Data are means ± s.e.m., and are based on responses of 7-8 medial or lateral sensilla, each from a different caterpillar.

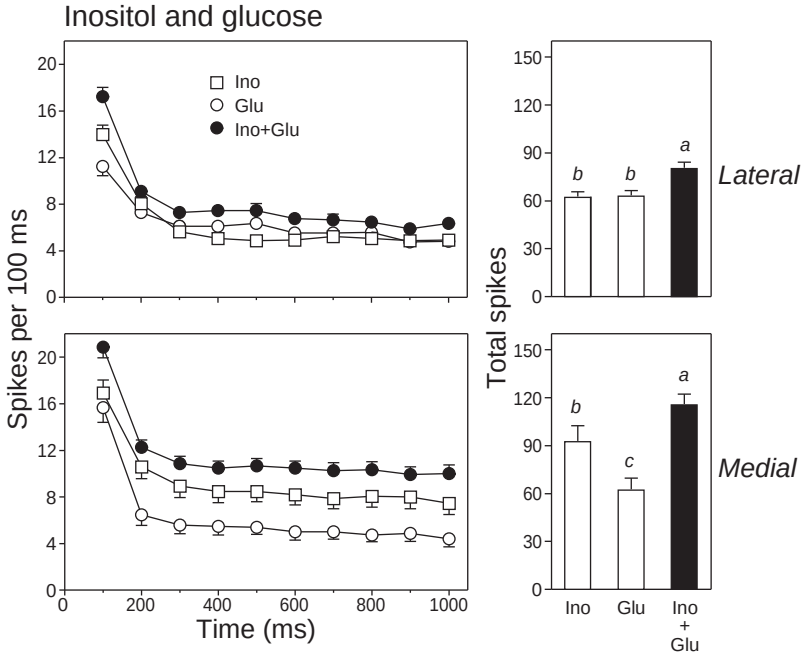

Figure S7. Neural responses of the lateral and medial styloconic sensilla to Ino, Glu and the binary mixture of both. *Left panels:* We show instantaneous firing rates (i.e., spikes per 100 ms) of GRNs and total number of spikes elicited by the three chemical stimuli over 1000 ms. *Right panels:* We compare total number of spikes with Tukey post-hoc tests; different letters (i.e., a, b, c) above bars indicate means that differ significantly from each other ($P < 0.05$). See Figure 1 for a key to the abbreviations for each chemical stimulus. Data are means ± s.e.m., and are based on responses of 7-8 medial or lateral sensilla, each from a different caterpillar.

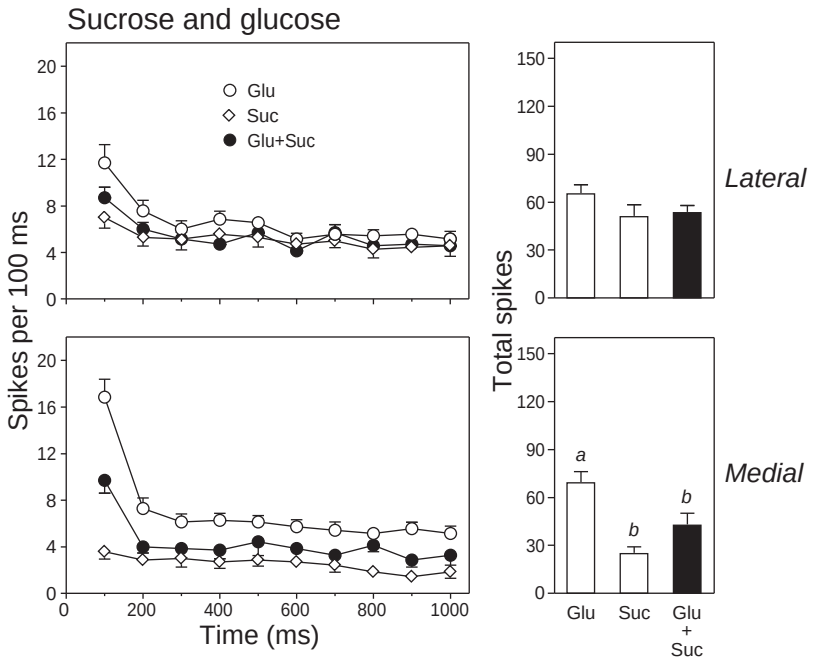

Figure S8. Neural responses of the lateral and medial styloconic sensilla to Suc, Glu and the binary mixture of both. *Left panels:* We show instantaneous firing rates (i.e., spikes per 100 ms) of GRNs and total number of spikes elicited by the three chemical stimuli over 1000 ms. *Right panels:* We compare total number of spikes with Tukey post-hoc tests; different letters (i.e., a, b, c) above bars indicate means that differ significantly from each other ($P < 0.05$). See Figure 1 for a key to the abbreviations for each chemical stimulus. Data are means ± s.e.m., and are based on responses of 7-8 medial or lateral sensilla, each from a different caterpillar.

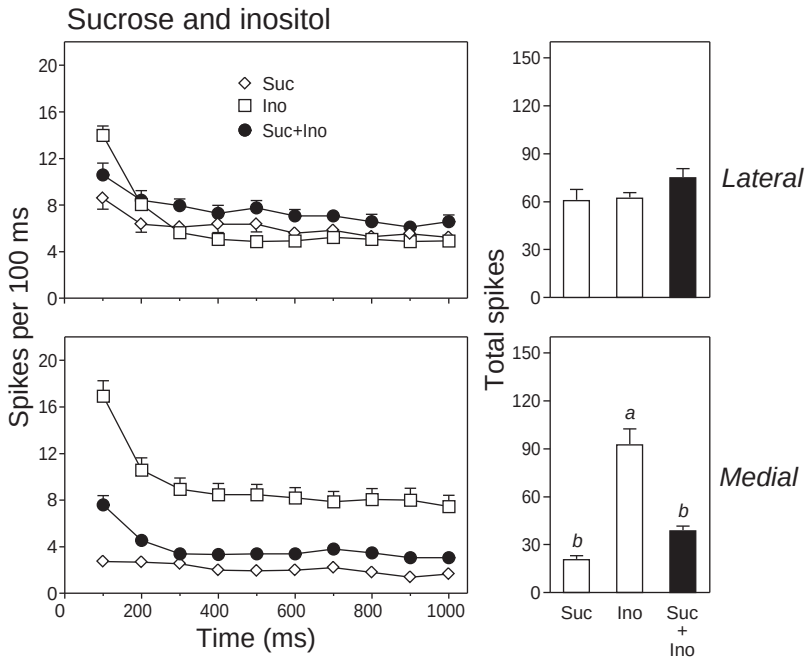

Figure S9. Neural responses of the lateral and medial styloconic sensilla to Suc, Ino and the binary mixture of both. *Left panels:* We show instantaneous firing rates (i.e., spikes per 100 ms) of GRNs and total number of spikes elicited by the three chemical stimuli over 1000 ms. *Right panels:* We compare total number of spikes with Tukey post-hoc tests; different letters (i.e., a, b, c) above bars indicate means that differ significantly from each other ($P < 0.05$). See Figure 1 for a key to the abbreviations for each chemical stimulus. Data are means ± s.e.m., and are based on responses of 7-8 medial or lateral sensilla, each from a different caterpillar.