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Diverse Bitter Stimuli Elicit Highly Similar Patterns of Fos-like Immunoreactivity in the Nucleus of the Solitary Tract

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Abstract

Previous studies have demonstrated that oral stimulation with quinine elicits Fos-like immunoreactivity in the first-order gustatory nucleus, the NST, with a different topographic distribution than sucrose or citric acid. However, it is unknown whether the quinine pattern is unique to this alkaloid or common across bitter stimuli with different chemical structures. Indeed, recent physiological experiments suggest that taste receptor cells and primary afferent neurons may exhibit selectivity for various bitter tastants. The present investigation compared the distribution of FLI in NST following stimulation with three bitter chemicals: QHCl, denatonium and propylthiouracil, stimuli that evoked Ca^{2+} currents in almost entirely different sets of receptor cells. The results demonstrate that the quinine pattern is not idiosyncratic but instead generalizes to the other two tastants. Although it remains possible that intermingled but different NST neurons are activated by these stimuli, these data suggest that a specialized region in the NST is preferentially involved in processing a common aspect of bitter tastants. In contrast to citric acid, quinine, denatonium and propylthiouracil all elicited vigorous oromotor rejection responses, consistent with our earlier hypothesis that the medial third of the NST may be an afferent trigger zone for oromotor rejection.

Key words: chemotopy, gape, gustatory, parabrachial, rejection, taste

Introduction

Previous studies in our laboratory have demonstrated that oral stimulation with QHCl elicits Fos-like immunoreactivity (FLI) in the first-order gustatory processing center, the nucleus of the solitary tract (NST) that is distributed in a distinct topographic pattern compared to the FLI observed after sucrose or citric acid (Harrer and Travers, 1996; Travers, 2002). The quinine pattern is stable across a log step of concentrations (Travers, 2002), with FLI concentrated medially, compared to the more lateral, even distributions produced by both the sweet and sour tastants.

Our knowledge of peripheral transduction of bitter stimuli has advanced markedly in recent years. Molecular studies have demonstrated a large family of G-protein coupled receptors, the T2Rs, which comprise putative bitter receptors (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000). The molecular results suggest that each family member has a restricted stimulus range (Chandrashekar *et al.*, 2000; Bufe *et al.*, 2002) but that multiple mRNAs for T2Rs are co-expressed (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000), such that a common set of taste receptor cells detect bitter substances with diverse chemical structures (Chandrashekar *et al.*, 2000; Bufe *et al.*, 2002). On the other hand, calcium imaging suggests that a given taste receptor cell responds to a limited number of bitter stimuli (often only one; Caicedo and Roper, 2001) and neurophysiological recordings from the chorda tympani and glossopharyngeal nerves also imply some specificity (Dahl *et al.*, 1997).

Psychophysical studies in humans are equivocal as to whether there are different bitter taste qualities (Delwiche *et al.*, 2001; Lindsey (2001) and rodent psychophysical studies have not shown an ability to discriminate between denatonium and quinine (Spector and Kopka, 2002), two bitter substances with different chemical structures. These results could imply that regardless of whether different bitter receptors activate the same or different receptor cells, the signals ultimately converge upstream. Because we have shown that quinine reproducibly activates a particular NST region, it seems reasonable to determine whether other bitter tasting compounds activate the same area. Further, because this medial NST zone has been implicated in bitter-evoked reflexes (King *et al.*, 1999), it is important to establish whether different bitter-tasting compounds elicit the same

oral reflex behaviors and FLI distributions. Studies with Fos immunohistochemistry cannot, of course, resolve which individual central neurons are activated by different stimuli. However, in light of the possibility of differential peripheral processing, the unique topography of QHCl-elicited FLI in rNST raises the question of whether this pattern is idiosyncratic or common to other bitter tastants. In the current experiment, we compared the NST FLI distributions and oromotor responses elicited by one sour stimulus, citric acid, with those elicited by three chemically diverse bitter stimuli: an alkyloid (QHCl), an amine (denatonium) and a pyrimidine (propylthiouracil).

Materials and methods

Animals, surgery and behavioral testing

Twenty-nine adult, male, Sprague–Dawley rats weighing 289–440 g at the beginning of the experiment served as subjects. Rats were implanted with intraoral cannulae for the delivery of taste solutions using techniques similar to those originally described by Grill and Norgren (1978). Animals recovered for 3–5 days after surgery. For 6–7 days before the test day, animals were placed in the testing chamber for adaptation to stimulation procedures. During adaptation, water was delivered continuously for 30 min. The total volume delivered was ~8ml. On test days, animals were randomly assigned to five stimulation conditions: 30 mM citric acid (n = 6), 2.7 mM denatonium benzoate (denatonium, n = 6), 10 mM propylthiouracil (n = 6), 3 mM quinine hydrochloride (quinine, n = 6), or distilled water (water, n = 5). An additional water-stimulated rat was discarded from analysis because he became anorexic due to poor dental occlusion following the cannulae implants. An unstimulated group was not included in this study, because our previous experiments have shown that water elicits small, sometimes significant, increases in FLI in NST compared to unstimulated animals (Harrer and Travers, 1996; King *et al.*, 1999; Travers and Hu, 2000; Travers, 2002) and thus is the most appropriate control for any of the non-gustatory consequences of the testing session. During test sessions, animals were videotaped for subsequent analysis of oromotor behaviors.

Tissue processing and immunohistochemistry

After fluid delivery, animals were left undisturbed for 45 min and then perfused with a mixture of 1.3% acrolein and 4% paraformaldehyde. Brains were usually post-fixed (≤1.25 h) and all were soaked overnight in a mixture of 20% sucrose and phosphate-buffered saline (PBS). On the second day, brains were cut with a sliding microtome into 30 µm sections, divided into four series and stored in cryoprotectant (Hoffman *et al.*, 1992) at –20°C for further processing. Sections 120 µm apart were processed with standard DAB/ABC techniques for identifying neurons expressing FLI, as described previously (Harrer and Travers, 1996; Travers and Hu, 2000; Travers, 2002). After removing the tissue from cryoprotectant, tissues were rinsed with PBS, sequentially treated with 1% sodium borohydride, then with 5% H_2O_2. Prior to primary antibody incubation, tissues were put in blocking solution, 10% normal sheep serum diluted in PBS, for 1 h. Tissues were then incubated in the primary antibody (AB5; Oncogene Research Products, San Diego, CA) at a 1:25K dilution in a solution of 0.4% Triton X-100 in phosphate buffer (PB) for ~72 h at 4°C. Subsequently, tissue was placed in goat anti-rabbit antibody [diluted 1:600 in phosphate buffer (PB) with 0.4% Triton X-100] for 90 min and then in an avidin–biotin mixture (Elite kit; Vector Laboratories, Burlingame, CA) diluted in PB–0.1% bovine serum albumin for another 90 min period. The final chromagen reaction began with incubation in 0.05% DAB (3,3′-diaminobenzidine-HCl) with 0.02% nickel ammonium sulfate followed by the final oxidation stage, achieved by adding H_2O_2 to a final concentration of 0.003%. Most of the oxidation reactions were controlled at 25°C for 2.5 min. Reacted sections were mounted on chrome-alum subbed slides, dehydrated through ascending alcohols, cleared with HEMO-DE (Fisher Scientific, Hanover Park, IL) and coverslipped. Another series of adjacent sections was stained with cresyl violet to reveal cytoarchitecture.

Immunohistochemical data analysis and photomicroscopy

FLI neurons in the NST were plotted for six sections from each brain, chosen from the intermediate and rostral NST as defined by Herbert *et al.* (1990). One section was chosen from the intermediate NST at the rostral pole of the area postrema (Ip), a level of the NST that primarily receives visceral afferent input from the Xth cranial nerve. The remaining five sections analyzed were NST levels that receive oral afferent input: one section from the rostral region of the intermediate NST (Ir) and four equally spaced sections from the rostral NST—R1–R4; see Travers (2002) for a horizontal schematic for these levels. At levels Ir and R1, gustatory input is primarily from the IXth cranial nerve, at R4 from the VIIth and at R3 from both nerves (Hamilton and Norgren, 1984). NST borders were drawn with the aid of darkfield and if necessary, adjacent cresyl violet stained sections. Subsequently, the NST at each level was divided into 'subfields', as originally described by King *et al.* (1999), which essentially splits the nucleus into mediolateral thirds and dorsal and ventral halves (Figure 3, lower right panel). FLI neurons were plotted with a computer-based camera lucida system (Neurolucida; Microbrightfield Inc., Williston, VT). Plotting was done by an investigator unaware of the stimulus condition. The sequence of analysis was arranged so that animals stimulated with the same compounds or immuno-processed simultaneously were not analyzed as a group.

Statistical analyses were performed using Systat statistical software (Point Richmond, CA). To ascertain whether gustatory stimulation was more effective in eliciting FLI

than water, separate analyses were conducted for the single visceral NST level and the oral NST, summed across all five levels, using one-way ANOVAs and post-hoc LSD tests. A series of more detailed ANOVAs were subsequently performed for the oral NST to determine whether topographic differences existed between stimuli. After performing a two-way ANOVA with stimulus as one factor and either subfield or anteroposterior level as a second, repeated measures factor, separate one-way ANOVAs for each subfield (summed across levels) and each level (summed across subfields) were performed with post-hoc LSD tests. In addition to ANOVAs, differences in the topographic distribution of FLI evoked by pairs of stimuli were quantified using Pearson's *r*. Photographs were taken with a digital camera (DXM 1200; Nikon) and files were subsequently imported into Canvas (Deneba), adjusted for brightness, contrast and sharpness and then labeled.

Behavioral analysis

The number of gapes that occurred during the first 2 min of stimulation were counted by reviewing the videotapes in slow motion or frame-by-frame. The criteria for a gape were set as initially described by Grill and Norgren (1978), including a triangular-shaped mouth opening, exposed internal oral labia and a longer duration of the entire mouth movement (compared to a lick). The onset of stimulation was defined as the time of the first mouth movement, which typically occurred just a few seconds after starting the pump. However, in some cases the first mouth movement was missed due to a slight delay in beginning videotaping and so the count was performed simultaneous with the beginning of taping. In one case (citric acid stimulation), counting was delayed until min 5, due to unclear taping during the first 5 min. Counts were adjusted for the amount of time the mouth could be clearly viewed and expressed as gapes/sec. Data were analyzed with a one-way ANOVA and post-hoc LSD tests.

For all ANOVAs (both behavioral and anatomical), statistical significance was defined as $P \leq 0.05$. Significance levels are rounded up to the nearest single digit; levels < 0.0001 are rounded up to that level.

Results

Fos-like immunoreactivity in NST

Overall efficacy

The mean numbers of FLI neurons at the one visceral level of NST analyzed, the rostral pole of the area postrema, are depicted in Figure 1 (left side). An ANOVA indicated a tendency for a differential production of FLI as a function of stimulus condition, but this just missed statistical significance ($P = 0.056$). Figure 1 (right side) also shows the mean numbers of FLI neurons in the oral NST summed over the five levels counted. ANOVA revealed a highly significant

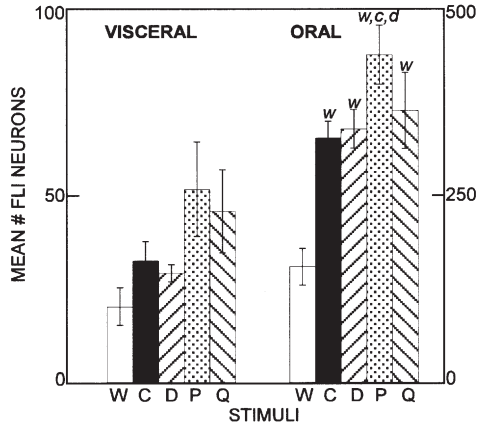

Figure 1 Mean number of FLI neurons at the one visceral level (Ip) of the NST analyzed (left) or summed across the five oral levels of the NST (Ir, R1, R2, R3, R4) analyzed (right). Letter in italics above each bar indicate stimuli associated with a significantly smaller number of FLI neurons than the one associated with the bar. Stimulus abbreviations: W, water; C, citric acid; D, denatonium; P, propylthiouracil; Q, quinine.

effect of stimulus ($P < 0.0001$). Each taste stimulus elicited at least twice as much FLI as water (all Ps < 0.002). In contrast, the overall numbers of FLI-labeled neurons observed after stimulation with the various tastants were roughly similar although propylthiouracil was slightly (1.3×) more effective than citric acid and denatonium (both Ps < 0.05).

Topographic distribution

Coronal plane: differences between subfields. Figure 2 presents photomicrographs of FLI at an NST level between R1 and R2 and captures the major topographic differences in Fos expression observed between stimuli. Water was associated with sparse FLI distributed widely along the mediolateral axis. Citric acid stimulation evoked FLI in a greater number of neurons, but they were distributed in a pattern similar to water. The three bitter tastants also produced more FLI neurons relative to water. The FLI distributions for the three bitter stimuli were distinct compared to both water and citric acid but they closely resembled each other. Denatonium, quinine and propthiouracil all elicited FLI preferentially distributed in the medial third of the nucleus. We also noted that, despite the similar number of immunoreactive neurons, the intensity of the FLI staining following citric acid tended to be lighter than that observed after stimulation with the bitter tastants.

Figure 3A,B quantifies the topographic pattern of FLI in the coronal plane, divided into the six subfields and summed across the five oral levels analyzed. Figure 3A emphasizes the pattern of FLI distribution across subfields for a given stimulus; Figure 3B replots the data so that FLI within a given subfield can be compared across stimuli. Figure 3A shows a medial shift in peak FLI expression for the three bitter stimuli compared to water and citric acid and high-

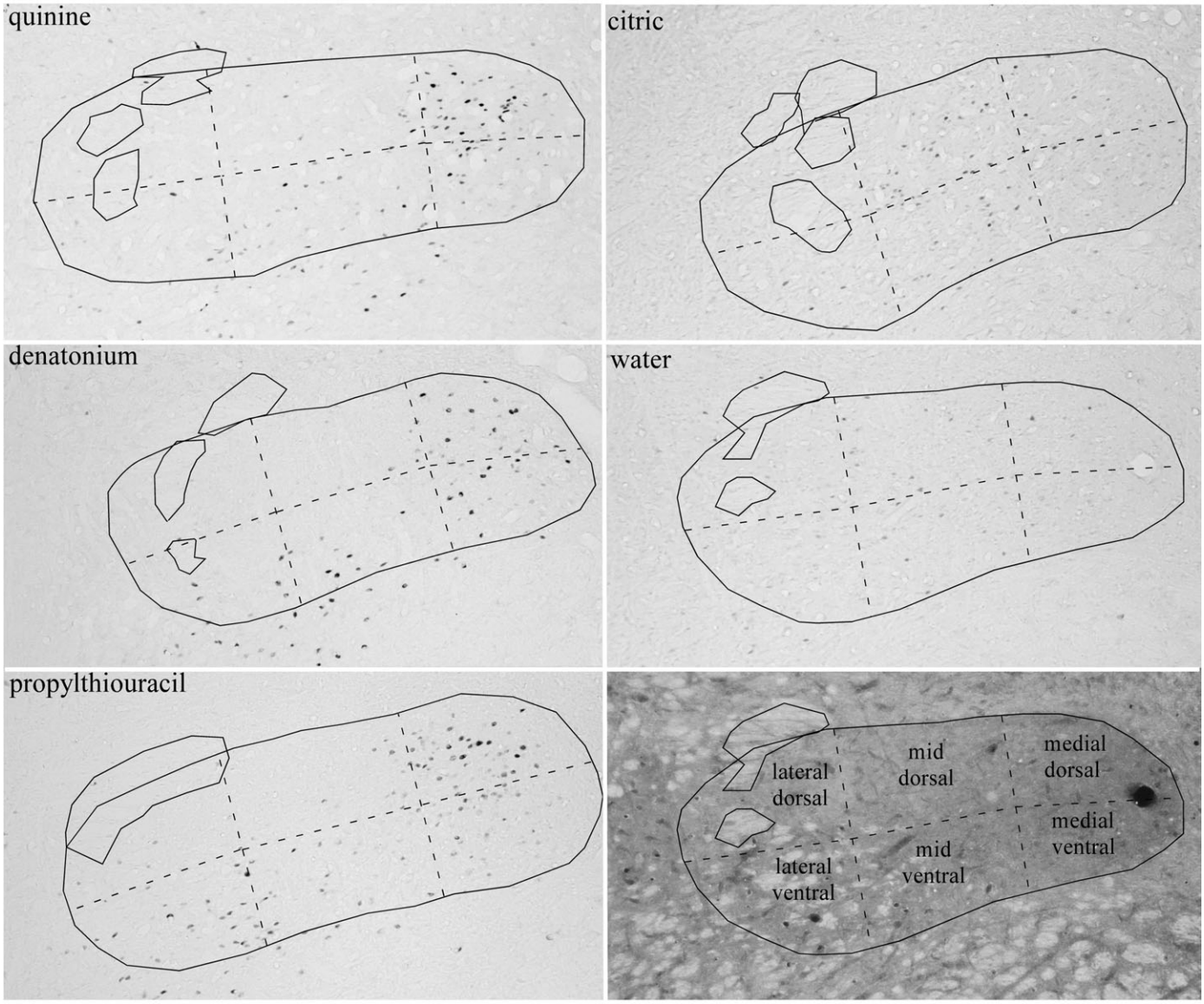

Figure 2 Photomicrographs of coronal sections through the rNST at a level just rostral to where the NST moves lateral to the IVth ventricle (between levels R1 and R2 in the current study). After stimulation with all three bitter stimuli, FLI was maximal in the medial subfields (left panels). In contrast, after citric acid, FLI was maximal in the middle-dorsal subfield (right upper panel). Water stimulation was associated with weak Fos staining in a pattern resembling citric acid (right middle panel). The lower-right panel shows a darkfield photomicrograph of the same NST section as shown in the water panel, depicting the borders of the nucleus and the position of the six subfields. Scale bar = 250 μm.

lights the similarity of the across-subfield patterns evoked by the different bitter chemicals. An ANOVA with stimulus and subfield as factors yielded main effects for both factors, as well as an interaction between them (stimulus, $F = 9.93$, df = 4, 24, $P < 0.0001$; subfield, $F = 56.38$, df = 5, 120, $P < 0.0001$; stimulus × subfield, $F = 12.58$, df = 20, 120, $P < 0.001$). Subsequent ANOVAs and post-hoc tests clarified the subfields associated with differential FLI expression; these differences are best viewed in Figure 3B. Separate ANOVAS for each subfield yielded significant effects for stimulus (all Ps < 0.05), with at least one tastant more efficacious than water in each subfield. In fact, in the lateral- and middle-ventral subfields and in the medial-dorsal subfield, each gustatory stimulus elicited significant increases in FLI compared to water (all Ps < 0.05). However, in the lateral- ($P < 0.05$) and middle-dorsal ($P < 0.0001$) subfields, only citric acid produced more FLI neurons than water. Conversely, in the medial-ventral subfield, only the bitter stimuli were effective (all Ps < 0.005).

There were also differences between taste stimuli in certain subfields. In both the lateral- and middle-dorsal subfields, citric acid evoked FLI in more neurons than any of the three bitter stimuli (all Ps < 0.05). Conversely, all three bitter stimuli produced more FLI than citric acid in the dorsal-

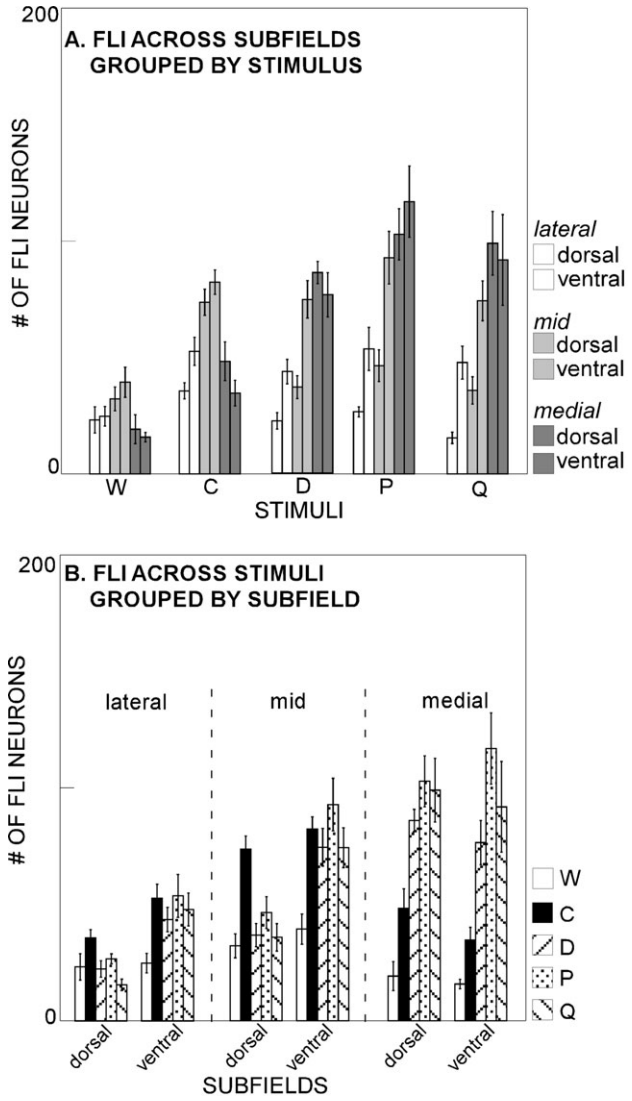

Figure 3 Mean (±SEM) number of FLI neurons elicited in each subfield. (A) Bars are grouped by stimulus and emphasize patterns across subfields. Successively darker shades of grey code subfields into lateral, middle and medial thirds; within each third, subfields are divided into dorsal and ventral halves ordered lateral to medial. Note that FLI peaks in the middle subfields for water and citric acid, but in the medial subfields for all three bitter stimuli. (B) Bars are grouped by subfield and emphasize differences between stimuli for a given subfield. Most notably, citric acid elicited significantly more FLI than the bitter stimuli in the lateral dorsal and middle dorsal subfields whereas the converse was true for the medial dorsal and ventral subfields. Stimulus abbreviations: W, water; C, citric acid; D, denatonium; P, propylthiouracil; Q, quinine.

and ventral-medial subfields (all Ps < 0.05). In contrast, differences between the bitter tastants were smaller and merely appeared to reflect differences in overall efficacy. Propylthiouracil was nominally the most effective bitter stimulus in each subfield, but was statistically different from quinine only in the lateral-dorsal subfield ($P < 0.05$) but denatonium only in the medial-ventral subfield ($P < 0.05$).

Topographic differences were further analyzed by calculating across-subfield correlations between each pair of

Table 1 Correlations (Pearson's r) for FLI Patterns across six subfields

	W	C	D	P
W				
C	0.93			
D	–0.16	0.08		
P	–0.25	–0.03	0.97	
Q	–0.28	–0.02	0.99	0.98

Abbreviations: W, water; C, citric acid; D, denatonium; P, propylthiouracil; Q, quinine.

stimuli (Table 11). The correlations strongly support the impression gained from inspecting the photomicrographs in Figure 2 and mean subfield plots in Figure 3A. Citric acid and water produced highly similar patterns of FLI distribution across subfields ($r = 0.93$), that were distinct from the patterns produced by the bitter stimuli (rs ≤ 0.08). In contrast, the FLI patterns associated with the three bitter stimuli were very similar to one another (rs ≥ 0.97).

Horizontal plane: differences across levels. In agreement with our previous studies (Harrer and Travers, 1996; Travers, 2002), differential FLI expression as a function of stimulus was not nearly as apparent along the rostro-caudal axis. An ANOVA with stimulus and anteroposterior level as factors revealed main effects for stimulus ($F = 9.93$, df = 4, 24, $P < 0.0004$) and level ($F = 68.91$, df = 4, 96, $P < 0.0001$). For each stimulus, there was a dramatic decrease in the number of FLI neurons at the most rostral NST level analyzed. However, this merely reflects the fact that the nucleus becomes markedly smaller as it approaches the rostral pole. A rostral decrease in FLI was not apparent if the number of Fos neurons were replotted as density (not shown). In addition to the main effects of stimulus and level, there was an interaction between these two variables ($F = 2.74$, df = 16, 96, $P < 0.005$) but post-hoc tests revealed only minor differences according to stimulus. Indeed, at every level, each tastant tested evoked significant increases in the number of FLI neurons compared to water (all Ps < 0.05). The sole hint of a stimulus topography along the anteroposterior axis was that propylthiouracil evoked significantly more FLI than citric acid or denatonium only at the two most caudal levels (level IR: propylthiouracil > citric and denatonium, Ps < 0.05; level R1: propylthiouracil > citric, $P < 0.05$). However, propylthiouracil was nominally the most effective stimulus at each level. The lack of a topography along the anteroposteror axis is emphasized by the high across-level correlations between stimuli; all were ≥0.82.

Although there was not a stimulus topography along the anteroposterior axis, *per se*, FLI distribution changed in a more subtle way along this axis. The relative effectiveness for a given stimulus across subfields was essentially the same at different levels but the patterns were blunted at more rostral locations. Accordingly, across-subfield correlations

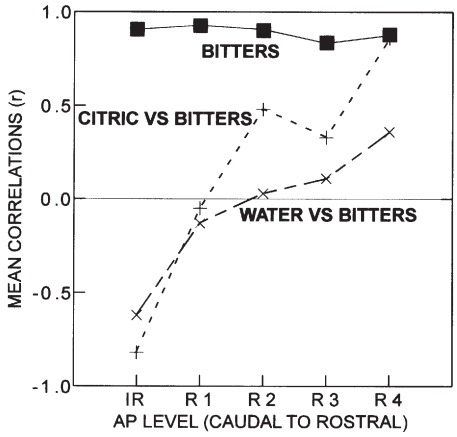

Figure 4 Across-subfield correlations for the distribution of FLI as a function of anteroposterior level. The across-subfield pattern for bitter stimuli is most distinct caudally and becomes progressively more similar to both citric acid and water at more rostral levels.

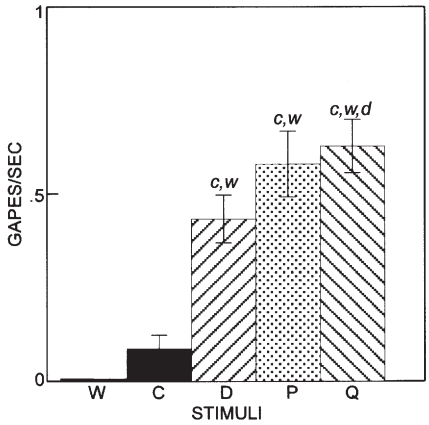

Figure 5 Mean number of gapes/s during the first 2 min of stimulation elicited by each of the five stimuli. The letters in italics above each bar indicate the stimuli that elicited a significantly smaller number of gapes than the one associated with the bar. Stimulus abbreviations: W, water; C, citric acid; D, denatonium; P, propylthiouracil; Q, quinine.

calculated at each anteroposterior level revealed a systematic increase in the correlations between water or citric acid versus the bitter stimuli, from the most caudal to the most rostral level analyzed (Figure 4), suggesting that the FLI pattern produced by bitter stimuli became less distinct rostrally. However, the high similarity in the patterns elicited by the different bitter stimuli was maintained throughout the longitudinal extent of the oral NST.

Behavioral analysis

Figure 5 shows the mean number of gapes/second for each of the five stimulus conditions. An ANOVA for stimulus condition was highly significant ($F = 24.9$, df = 4, 24, $P < 0.0001$). Water produced virtually no gapes and citric acid a few ($X = 0.1$/s), but the three bitter stimuli elicited 5–7 times as many gapes as citric acid. Post-hoc tests confirmed that each bitter stimulus elicited more gapes than either citric acid (all Ps < 0.0005) or water (all Ps < 0.0001). The bitter stimuli were much more similar in their ability to cause gapes. Nevertheless, denatonium was somewhat less effective than the other two stimuli, eliciting only 69% as many gapes as quinine ($P < 0.05$) and 75% as many as propylthiouracil, but the latter difference only approached significance ($P = 0.07$) Although not quantified, it was clear that citric acid was associated with more licking and very little passive rejection relative to the bitter stimuli. Thus, following citric acid stimulation, for 3/6 rats, the floor was clean and dry at the end of the test session; for the remaining two of the three rats in which the condition of the floor was noted, the only fluid present appeared to be a small amount of urine. These results confirm and extend our earlier findings with a higher concentration of citric acid. Even with a concentration one half log-step higher, rats gape minimally, show little passive rejection and instead exhibit many ingestive responses (Travers, 2002). In contrast, for each of the 18 rats stimulated with bitter stimuli, by the end of the test session, the floor was very wet and cloudy, apparently with rejected fluid.

Discussion

Functional significance of 'bitter topography'

The results of the present study demonstrate that three different stimuli that humans describe as bitter and which are avoided and rejected by rats, elicit highly similar topographic patterns of FLI in the rat NST which are different from the pattern elicited by a sour stimulus, citric acid. However, the NST chemotopy observed in this and previous studies is not a strict segregation by quality. Instead, the bitter stimuli elicit the most distinctive distribution, which contrasts with both sucrose (Harrer and Travers, 1996) and citric acid (present study; Travers, 2002), which result in FLI distributions that are similar to one another. In addition, bitter-activated neurons show some overlap with cells activated by the other qualities (Harrer and Travers, 1996; Travers and Hu, 2000). Despite this overlap, however, the distributions are strikingly preferential. In the present study, citric acid elicited FLI in twice as many neurons in the dorsal half of the middle third of NST than any of the three bitter stimuli and all three bitter stimuli elicited FLI in twice as many neurons in the medial third of NST, compared to citric acid. The current experiment further showed that, on a behavioral level, denatonium and propylthiouracil were similar to quinine because they elicited vigorous bouts of gaping but different than citric acid, which elicited minimal gaping. These data support our previous hypothesis (King *et al.*, 1999; Travers, 2002) that a circumscribed medial NST region plays a preferential role in triggering the oral rejection response. The specialization appears most pronounced in the more caudal rNST, where the quinine-activated neurons are most segregated and numerous. An association

between the bitter FLI neurons with oral rejection was originally prompted by the finding that IXth nerve section, which dramatically reduces the number of quinine-elicited gapes (Travers *et al.*, 1987), likewise reduces the number of neurons expressing Fos after quinine stimulation and obliterates their distinctive topography (King *et al.*, 1999). In contrast, quinine-evoked Fos is minimally disrupted by VIIth nerve section (King *et al.*, 1999) or decerebration (Travers *et al.*, 1999), manipulations that spare oral rejection. Furthermore, our previous studies showed that quinine, over a log step of concentration, differentially activated the medial third of NST in comparison to either sucrose or citric acid, two stimuli that share a lack of potency in eliciting gapes but which themselves have distinctive taste qualities ('sweet' and 'sour') and motivational properties (Harrer and Travers, 1996; Travers and Hu, 2000; Travers, 2002). Thus, Fos chemotopy does not appear correlated with quality or motivational properties *per se*, but with a specific function of bitterness, plausibly reflex rejection.

Aside from their preferential expression of bitter-elicited FLI, any unique properties of neurons in the medial third of NST are largely unknown. This area roughly corresponds to the medial half of a morphologically distinct NST subnucleus, the rostral central, a site of dense termination of primary afferent fibers (Travers and Norgren, 1983; Whitehead, 1988; Halsell *et al.*, 1996; DiNardo and Travers, 1997; Travers *et al.*, 1997, 2000), suggesting that Fos expression may include neurons directly activated by peripheral input. It was interesting, that despite producing FLI in similar numbers of neurons, bitter tastants appeared to elicit more intense immunostaining than citric acid, similar to our previous observations comparing quinine and sucrose (Harrer and Travers, 1996). This prompts the speculation that a component of the bitter circuitry in NST differentially engages neurotransmitter systems, e.g. NMDA receptors, which are preferentially involved in upregulating Fos (Sheng and Greenberg, 1990; Ferguson *et al.*, 2003) and are distributed, albeit not confined to this region of the nucleus (King, 2003). It is also notable that double-labeling data suggest that the majority of NST neurons expressing FLI after quinine are interneurons, instead of having direct projections to the PBN or subjacent reticular formation, the region implicated in coordinating gustatory-modulated rejection and ingestion (Travers and Norgren, 1983; DiNardo and Travers, 1997; Travers *et al.*, 1997, 2000). This suggests that even a relatively simple oromotor response may require multi-stage processing within NST, underscoring the complex nature of the first-order gustatory nucleus. The observation of a systematic topographic organization should aid in designing studies to unravel the mechanisms by which specific functions are accomplished in this nucleus.

Bitter processing

Despite the fact that denatonium, propylthiouracil and quinine evoke Ca2+ currents in separate sets of receptor cells (Caicedo and Roper, 2001), they activate neurons in a common region of the NST. It remains possible that topographic differences between bitter tastants would be apparent at higher-level gustatory relays. However, we qualitatively analyzed the FLI distribution in the parabrachial nucleus but observed no obvious differences for the three bitter stimuli in this region either. Instead, FLI label was robust in both the 'waist' area and further rostrally in the external lateral and medial subnuclei (data not shown), in a pattern apparently identical to that described by other laboratories for quinine (Yamamoto and Sawa, 2000; King *et al.*, 2003).

Although the presence of a topographic distinction would have provided evidence for central discrimination, their lack does not lead to the opposite conclusion. Inhibited cells are unlikely to express Fos and even excited neurons vary in their potential for expressing this immediate-early gene (Hunt *et al.*, 1987; Bullitt, 1990) and thus not all of the neurons affected by bitter tastants are likely to be revealed with this technique. Even if they were, a differential topography is certainly not a requirement for discrimination. Instead, it is possible that the activated cells in the medial NST are comprised of populations of neurons preferentially or even specifically activated by one or another bitter stimulus. Thus, the question of between-bitter neural discrimination needs to be addressed with single-unit recording studies. Such investigations are few, but two are particularly relevant. Somewhat surprisingly, responses of single fibers in the chorda tympani and glossopharyngeal nerves yielded across-neuron patterns for diverse bitter stimuli that were no more similar to each other than to non-bitter tastants, implying great discriminabilty (Dahl *et al.*, 1997). However, except for quinine, bitter chemicals elicited weak responses, raising the concern that the apparent differences between bitter stimuli could be representative of the variability between multiple trials, rather than stable differences between stimuli (Di Lorenzo and Victor, 2003). Central neurophysiological data are also provocative but not entirely conclusive. In the monkey insular cortex, stimuli with a dominant bitter component evoked responses that were more similar to each other than those elicited by other qualities but as in the periphery, responses to different bitter tastants were not identical (Scott *et al.*, 1999). On the other hand, the bitter tastants eliciting the most distinct cortical patterns were those with 'side-tastes'; for example, NH_4Cl and urea were rather poorly correlated with quinine, but these bitter chemicals also taste salty and/or sour.

Although further neurophysiological studies are needed, the most definitive evidence for between-bitter discrimination would come from psychophysical or behavioral investigations, since these capacities represent the ultimate output of neural processing. Delwiche *et al.* (2001) demonstrated

that individuals vary in their intensive ratings of a battery of bitter stimuli, supporting the idea of multiple neural mechanisms (e.g. different receptors) but not necessarily a discriminative capacity based on quality (and the authors make no such claim). In fact, an abstract reported that humans have great difficulty discriminating among several chemically diverse bitter compounds when concentrations are adjusted appropriately (Lindsey, 2001). Further, it was recently demonstrated that without intensity cues, rats cannot discriminate between quinine and denatonium (Spector and Kopka, 2002). Although these two stimuli represent only a minority of the many existing bitter compounds, it is significant that they appeared to taste identical to rats and evoked highly similar FLI patterns in NST (present study) despite the fact that they elicited calcium currents in almost completely separate sets of taste receptor cells (Caicedo and Roper, 2001) and are apparently transduced by different T2R receptors (Chandrashekar *et al.*, 2000). On the other hand, conditioned taste aversion generalization gradients in hamster suggested perceptual differences between ionic (quinine, denatonium and $MGSO_4$) versus non-ionic (caffeine and sucrose octaacetate) bitter stimuli (Frank *et al.*, 2004), but at the same time raised the possibility that the differences may have been non-gustatory. Thus, although there are provocative hints for between-bitter discrimination, contrary data also exist and a definitive conclusion is not yet possible.

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