Differential Covariation in Taste Responsiveness to Bitter Stimuli in Rats

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Abstract

Variation exists in the sensitivity of individual rodents and humans to different bitter tastants. An absence of uniform correlation in responsiveness to different bitter substances across individuals within a species suggests heterogeneity in the mechanisms underlying stimulus processing within this taste modality. Here, we examined taste responsiveness of individual rats to three bitter compounds (quinine hydrochloride, denatonium benzoate, and cycloheximide) in short-term lick tests to determine the magnitude of covariation among responses to these stimuli and infer commonalities in their receptor and neural mechanisms. Rats were tested with a given pair of bitter stimuli during three sessions comprising randomized trial blocks of six concentrations of each stimulus + deionized water. Psychophysical functions were generated for individual rats for respective stimulus pairs, and concentrations of each stimulus that produced equivalent lick suppression relative to water were correlated across animals. Behavioral taste responsiveness to quinine hydrochloride strongly covaried with responsiveness to denatonium benzoate ($r = +0.82$). Lick responsiveness to quinine was less robustly correlated with that to cycloheximide ($r = +0.44$), and denatonium and cycloheximide responses failed to correlate. These results imply substantial overlap in the bitter taste coding mechanisms for quinine and denatonium but some degree of independence in the mechanisms responsible for gustatory processing of cycloheximide. More generally, these data reinforce the notion that bitter taste processing is not a homogeneous event.

Key words: behavior, bitter taste, brief-access test, lick responses, psychophysics, rat

Introduction

Independent variation in sensitivity to different bitter-tasting substances among humans as well as individuals of other species has provided evidence that the perception of bitter taste is controlled by multiple biological mechanisms. For example, in humans individual sensitivity to the bitter compound 6-propylthiouracil does not reliably predict responsiveness to other bitter stimuli (Yokomukai et al., 1993; Delwiche et al., 2001). Several other bitter substances tend to cluster into two groups based on intercorrelations in individual sensitivities [i.e., quinine, caffeine, sucrose octaacetate (SOA), denatonium benzoate, and tetralone vs. urea, phenylalanine, tryptophan, and epicatechin; Yokomukai et al., 1993; Delwiche et al., 2001]. Similarly, behavioral avoidance of individual outbred mice to the bitter alkaloid quinine is correlated with avoidance to SOA (Boughter et al., 1992; Whitney and Harder, 1994), but aversion to phenylthiocarbamide (PTC) is dissociated from that to the former two compounds (Whitney and Harder, 1994). A lack of uniform covariation across individual organisms in responsiveness to various bitter stimuli suggests some complexity in the mechanisms responsible for bitter taste perception as variation (e.g., genetic polymorphism) in a single biological substrate would be expected to produce a consistent high degree of correlation in responsiveness among bitter substances.

Differences in gustatory coding mechanisms for certain classes of bitter substances are also supported by studies across species demonstrating perceptual distinction among various bitter compounds. Human psychophysical experiments examining cross-adaptation among several bitter stimuli have shown that adapting the tongue to quinine HCl reduces the perceived bitterness of some compounds (e.g., caffeine and SOA) but not others (e.g., urea and PTC; McBurney et al., 1972). In hamsters, conditioned taste aversions to quinine and denatonium cross-generalize with one another, suggesting perceptual similarity between these stimuli (Frank et al., 2004; see also Spector and Kopka, 2002), but neither compound generalizes to caffeine or SOA (Frank et al., 2004). Cross-habituation studies in nonmammalian species (Manduca sexta caterpillars) have further documented a segregation in taste-mediated aversive responses across bitter substances (Glendinning et al., 2002a).
Consistent with a diversity of mechanisms underlying the processing of bitter taste, a multigene family of ~30 putative G-protein–coupled bitter taste receptors (T2Rs) has been identified in mammals (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000; Fischer et al., 2005; Meyerhof, 2005, for review; Shi et al., 2003). It has been hypothesized that the significant number and sequence variation of these receptors reflect the need of the mammalian bitter taste system to recognize a multitude of structurally dissimilar bitter compounds (Adler et al., 2000; Matsunami et al., 2000). Even further heterogeneity in bitter taste processing is evident from recent studies showing extensive diversity in human bitter taste receptors, with as many as 151 different protein-coding haplotypes (Kim et al., 2005). Such diversity likely underlies the individual variation in bitter taste perception noted above.

The goal of the present study was to examine covariation in behavioral taste responsiveness among three structurally diverse bitter tastants (quinine hydrochloride, denatonium benzoate, and cycloheximide) in a sample of heterogeneous rats to assess the degree to which these stimuli may engage similar receptor and/or neural mechanisms. A brief-access taste exposure paradigm was used to generate individual psychophysical functions for respective pairs of stimuli, from which isoaversive concentrations were correlated across rats to determine covariation in gustatory responsiveness. The rationale underlying these experiments is that if two bitter stimuli activate similar receptor or neural mechanisms, then responsiveness to those stimuli should covary highly across individual animals, whereas if the mechanisms are heterogeneous they should not (e.g., see Delwiche et al., 2001).

**Materials and methods**

**Animals**

Seventy-two naive adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) with a mean body weight of 361.44 g (±4.64 SE) at the start of the experiment were used. Rats were randomly assigned to one of three stimulus pair conditions (n = 24/group). Animals were housed individually in standard tub cages (47 × 25.5 × 20.5 cm) in a vivarium that maintained a 12-h light/dark cycle and an ambient temperature of ~23°C. All training and testing occurred during the light phase of the cycle. Food and water were available *ad libitum* except for training and testing water restriction conditions noted below. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center.

**Apparatus**

Training and testing were conducted in a Davis MS-160 lickometer apparatus (DiLog Instruments, Tallahassee, FL). This device allows for automated within-session presentation of multiple stimulus solutions to an animal in the form of individual taste-sampling trials of short duration (e.g., 5 s) during which immediate lick responses are monitored (see Smith, 2001). Rats gained access to a stainless steel drinking spout on each trial through a small aperture in the front wall of a 30 × 15.4 × 15-cm testing chamber, with availability of the spout determined by the opening and closing of a motorized shutter. Delivery of a given stimulus solution was determined by the positioning of a motorized table/block apparatus just outside of the chamber that could accommodate up to 16 different stimulus tubes. Lick activity was detected via a high-frequency AC contact circuit, and all data collection (i.e., lick counts and latencies) as well as presentation and timing of all stimuli were controlled precisely via computer and associated software.

**Training**

Rats initially were given 2 days of training with water as the only available stimulus in order to familiarize them with the apparatus and to train them to lick the spout to receive fluid. During the training phase, an overnight water restriction schedule was in effect in order to motivate performance on the task, with rats receiving their sole daily fluid intake in the apparatus. On the first training day, subjects were given a 30-min period of continuous access to water through a single sipper tube that began when the animal took its first lick. On the second day of training, rats were allowed access to water during 40 5-s trials separated by 10-s intersession intervals to familiarize them with the brief-access trial procedure. All subjects successfully completed Day 1 training (with >1100 total licks) and Day 2 training (with >70% of trials sampled) except for one subject assigned to the quinine/denatonium test condition that failed to initiate training and was therefore eliminated from the experiment.

**Bitter stimulus testing**

Three bitter stimuli were used in these experiments: quinine hydrochloride, denatonium benzoate, and cycloheximide. Rats were tested during three consecutive daily sessions (Days 3–5) for lick responses to one of three different pairs of these bitter stimuli: (a) quinine/denatonium, (b) quinine/cycloheximide, or (c) denatonium/cycloheximide. A given animal was tested with only one stimulus pair, and testing was repeated across days on the assigned pair. Within each test session, rats were presented with six concentrations each of two of the following bitter stimuli (quinine and denatonium—0.01, 0.03, 0.1, 0.3, 1, and 3 mM; cycloheximide—0.1, 0.3, 1, 3, 10, and 30 μM) and a deionized water control during 5-s trials. Stimuli were presented randomly within blocks of 13 trials (four blocks total) such that animals were allowed to sample each stimulus concentration and the water control once/block and four times during a given test session (12 trial replications total at each concentration over the three test days). Each stimulus trial was preceded by a 2-s deionized water rinse trial that served to rinse the oral cavity and minimize sensory
adaptation effects. On opening of the shutter on each presentation, 30 s was allowed for trial initiation, and the trial duration began with the animal’s first lick on the sipper tube. If a rat failed to initiate sampling during the 30-s period, the shutter closed and the table was automatically repositioned for the next trial. All presentations were separated by 10-s interpresentation intervals during which the shutter remained closed. Test sessions were approximately 30–40 min in length. Brief-access testing has been used widely to measure taste responsiveness in rodents (Davis, 1973; Smith et al., 1992; Boughter et al., 2002; Glendinning et al., 2002b). The specific procedures reported here were based on those shown previously to produce reliable concentration-response avoidance functions to bitter stimuli in rats (St John et al., 1994; Spector and Kopka, 2002).

The water restriction conditions present during training remained in effect during the testing phase of the experiment, with the exception that prior to each test day rats were given 5 ml of supplemental water overnight in their home cages to facilitate maintenance of body weight. This fluid access schedule resulted in mean (±SE) body weight percentages across the three test days of 92% (±0.001), 91% (±0.002), and 91% (±0.002), respectively.

Taste stimuli

All solutions were prepared fresh prior to testing in deionized water using reagent grade chemicals when possible (Sigma-Aldrich, St Louis, MO) and were presented at room temperature. Quinine hydrochloride, denatonium benzoate, and cycloheximide concentrations were selected to encompass a full behavioral gustatory response range based on previous work (e.g., Spector and Kopka, 2002) and preliminary testing.

Data analysis

For each individual rat, the mean number of licks to each concentration of the two bitter stimuli tested and to the water control was calculated across all trials sampled over the three test days. Nonsampled trials (i.e., those with zero licks) were excluded from the data analysis; these trials constituted 20% of all trials in the data set (2161/10,920 stimulus trials total). Lick ratios for each stimulus concentration were then determined by dividing the mean number of licks to a stimulus by the mean number of licks to water, which served to standardize for individual differences in licking behavior that were nongustatory in nature. A lick ratio of 1.0 indicates equal responding to a given stimulus relative to water, with ratios approaching zero representing increased levels of lick suppression. For each rat, psychophysical curves for each bitter stimulus were generated by fitting the lick ratio data with a sigmoidal two-parameter logistic function

\[ f(x) = \frac{1}{1+(x/c)^b}, \]

where \( x \) represents stimulus concentration, \( b \) represents the slope, and \( c \) represents the stimulus concentration that evoked the half-maximal response (i.e., a 0.5 lick ratio value or 50% suppression of licking; see St John et al., 1994). The individual \( c \) parameter concentrations were converted to \( \log_{10} \) values to normalize the distribution of scores, and Pearson product–moment correlation coefficients \( r \) were then calculated for each of the three stimulus pairs using the \( \log c \) values to determine the degree of relationship in behavioral responsiveness between bitter stimuli. The concentration of each stimulus that produced 50% suppression of licking was chosen as the isointensity measure for the correlations because it fell within the dynamic range of the response curves (i.e., for assessment of individual variation). For each pair of stimuli, sigmoidal functions and associated \( c \) values were also generated on the mean lick ratio data across all subjects, and descriptive statistics were calculated on \( b \) and \( c \) values from the individual curves.

To determine if gustatory responding to a given bitter stimulus was influenced by the stimulus it was paired with during testing, separate one-way analyses of variance (ANOVAs) were run on the \( \log c \) parameter values for quinine, denatonium, and cycloheximide with paired stimulus (2) as a between-subjects factor.

Results

Correlations among taste responses to bitter stimuli

Sample individual psychophysical functions for four subjects tested with quinine HCl and denatonium benzoate (of 23 subjects total) along with concentrations evoking 50% lick suppression for each stimulus are shown in Figure 1 to illustrate the basis of the correlational analysis. Similar functions were derived for all rats on their tested stimulus pair (\( n = 23 \), quinine/denatonium; \( n = 23 \), quinine/cycloheximide; \( n = 24 \), denatonium/cycloheximide). Pearson \( r \) analysis on the \( \log c \) parameter values (i.e., 50% lick suppression concentrations) from the individual psychophysical curves revealed a strong and significant correlation between behavioral responsiveness to quinine and denatonium (\( r = +0.82, P = 0.000002 \); Figure 2A). In contrast, gustatory responsiveness to quinine was only moderately correlated with that to cycloheximide (\( r = +0.44, P = 0.04 \); Figure 2B), and individual responses to denatonium and cycloheximide failed to correlate significantly (\( x = 0.05, r = +0.34 \); Figure 2C). One subject assigned to the quinine/cycloheximide pair condition was excluded from the analysis because its 50% suppression value for quinine (0.0113 mM; \( \log_{10} \) value = −1.95) was a significant outlier (\( P < 0.05 \)) relative to all other subjects tested with this stimulus pair (Grubbs’ test). No other \( \log c \) values in the data set met criteria for an outlier according to this test.

The mean (±SE) of the individual \( b \) and \( c \) values for each stimulus by test pair condition is presented in Table 1. Overall, absolute \( c \) values for quinine were lower than those for
denatonium within the same millimolar concentration range, while cycloheximide was effective at evoking half-maximal response suppression at micromolar concentrations. The slopes (b values) of the individual curves for cycloheximide were steepest, followed by denatonium, with quinine producing the most gradual decline in responding across concentration.

Figure 1  Representative individual psychophysical functions for four rats (S1–S4) tested with quinine and denatonium. Lick ratio = mean licks to stimulus/mean licks to deionized water. Individual lick ratio data were fit with two-parameter logistic functions, and concentrations of each stimulus evoking 50% lick suppression relative to water were determined (c values; shown by drop lines). The log10 c values were correlated across rats to index the degree of behavioral covariation between the two stimuli.

Figure 2  Correlations across rats between log10 stimulus concentrations producing 50% response suppression for (A) quinine and denatonium, (B) quinine and cycloheximide, and (C) denatonium and cycloheximide. The Pearson correlation coefficient (r) for each stimulus pair is shown.
Effects of stimulus pairing

A one-way between-subjects paired stimulus (2) ANOVA on the log $c$ parameter values to quinine confirmed that 50% suppression values to this stimulus did not differ based on the stimulus it was paired with during testing [i.e., denatonium or cycloheximide; $F(1,44) = 1.15, P = 0.29$]. A similar ANOVA on half-maximal suppression values to denatonium also demonstrated no significant effect of stimulus pairing [$F(1,45) = 0.25, P = 0.62$]. The concentration of cycloheximide evoking 50% lick suppression, however, was lower when denatonium was the paired stimulus than when quinine was the alternative stimulus [$F(1,45) = 12.28, P < 0.01$; see Table 1]. This latter effect may be due in part to a somewhat reduced overall aversive potency of denatonium compared with quinine at identical concentrations, resulting in greater contrast when paired with cycloheximide and increased relative potency of cycloheximide to induce behavioral suppression (i.e., evidenced by lower $c$ values when paired with denatonium). This difference was unlikely to have influenced the correlational analysis given that the average log $c$ values for cycloheximide regardless of paired stimulus were well within the dynamic area of the response curves (i.e., $-0.11$ and 0.07 when paired with denatonium and quinine, respectively), and $r$ values for quinine/cycloheximide and denatonium/cycloheximide test pairs were highly similar.

Mean ($\pm$SE) lick ratio data and corresponding psychophysical functions for each pair of bitter stimuli are shown in Figure 3.

Discussion

The results of the present study demonstrate among heterogeneous rats that at equipotent stimulus concentrations behavioral taste responsiveness to quinine hydrochloride strongly covaries with responsiveness to denatonium benzoate, while lick responsiveness to these compounds is less robustly correlated with or dissociated from avoidance of cycloheximide. These data imply substantial overlap in the receptor and/or neural coding mechanisms underlying bitter taste perception of quinine and denatonium but some degree of independence in the mechanisms responsible for gustatory processing of cycloheximide. Importantly, these findings provide confirmatory evidence consistent with other species that bitter substances do not share uniform homogeneity in processing when assessed at the level of behavioral output (McBurney et al., 1972; Whitney and Harder, 1994; Delwiche et al., 2001; Glendinning et al., 2002a; Frank et al., 2004).

That individual taste responsiveness to quinine was a robust predictor of responsiveness to denatonium ($r = +0.82$) in the present investigation concurs with previous data that rats are unable to discriminate between these compounds (Spector and Kopka, 2002) and that conditioned taste aversions to these stimuli cross-generalize in hamsters (Frank et al., 2004). Individual ratings of bitterness for quinine and denatonium are also correlated in humans (Delwiche et al., 2001). Thus, evidence from several species supports commonality in the substrates that convey bitter taste information for these substances. What is the origin of the similarity in processing? Genetic studies in mice indicate that responding to quinine and denatonium is influenced by a common genetic locus (Soa; Whitney and Harder, 1994; Boughter and Whitney, 1998), a region which is associated with the Tas2r family of bitter taste receptor genes (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000). While denatonium is a known ligand for the murine receptor mT2R-8 and its human counterpart, hT2R-4 (Chandrashekar et al., 2000), a specific taste receptor for quinine has yet to be identified, and therefore similarity in the receptor mechanisms mediating initial recognition of these stimuli is unknown. The high correlation observed here in individual responsiveness to these compounds would suggest that they stimulate overlapping receptors such that polymorphisms influencing responses to one compound would concurrently affect the other. Alternatively, distinct receptors for these stimuli may exist for which levels of expression are influenced similarly by genetic variation at a given locus. Although calcium-imaging data indicate that single taste receptor cells respond selectively to these stimuli (Caicedo

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Table 1  Mean ($\pm$SE) individual curve parameters for each stimulus pair

<table>
<thead>
<tr>
<th>Test Pair</th>
<th>Quinine</th>
<th>Denatonium</th>
<th>Cycloheximide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$b$</td>
<td>$c$ (mM)</td>
<td>$b$</td>
</tr>
<tr>
<td>Quinine/denatonium $^a$</td>
<td>0.832</td>
<td>0.300</td>
<td>1.113</td>
</tr>
<tr>
<td></td>
<td>(±0.04)</td>
<td>(±0.03)</td>
<td>(±0.07)</td>
</tr>
<tr>
<td>Quinine/cycloheximide $^a$</td>
<td>0.956</td>
<td>0.271</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>(±0.06)</td>
<td>(±0.03)</td>
<td>(±0.07)</td>
</tr>
<tr>
<td>Denatonium/cycloheximide $^b$</td>
<td>—</td>
<td>—</td>
<td>1.332</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±0.09)</td>
</tr>
</tbody>
</table>

$b$ denotes slope and $c$ denotes stimulus concentration producing 50% response suppression. Mean ± SE $\log_{10} c$ values are shown in brackets.

$^a n = 23$.

$^b n = 24$.

$^c$ Significant difference in mean values within a column (across stimulus pair).
and Roper, 2001), both effectively activate the chorda tympani nerve in hamsters (Frank et al., 2004), and a correlation exists between responses to quinine, denatonium, and a subset of other bitter stimuli are influenced by the Soa gene locus, allelic variation at this locus is unrelated to avoidance of cycloheximide, caffeine, and thiamine (Boughter and Whitney, 1998). At the molecular level, a selective T2R receptor for cycloheximide has been characterized in mice (mT2R-5) and is known to be distinct from a second functional receptor binding denatonium (mT2R-8; Chandrashekar et al., 2000). Integrated recordings from the rat NST have shown that compared to quinine, denatonium, and several other bitter compounds, cycloheximide produces minimal taste-evoked activity in the NST via input from the seventh nerve (Lemon and Smith, 2005), even at concentrations 100-fold greater than that producing complete behavioral suppression in the current study. These data indicate that gustatory information for cycloheximide and certain other bitter substances (Lemon and Smith, 2005; see also Frank et al., 2004) must enter the brain via a different mechanism (e.g., ninth nerve input). Although no evidence exists regarding behavioral discrimination of cycloheximide from other bitter stimuli, the present results demonstrating a dissociation in individual responsiveness to this compound relative to denatonium and only a moderate correlation between responses to quinine and cycloheximide might predict potential discriminability of these stimuli by rats. The differential covariation in responsiveness observed among bitter compounds in the present study is consistent with similar findings of a dissociation in the stimulus processing of various sweeteners (i.e., sucrose and glucose vs. glycine; Eylam and Spector, 2004).

The methods used in the current study to assess correlations in individual responsiveness among different bitter substances offer a number of advantages. First, the stimuli chosen may be classified as relatively “pure” bitters (i.e., without significant side tastes such as bitter salts), avoiding complications in the measurement of bitter responsiveness by individual differences in sensitivity for other taste modalities with distinct transduction mechanisms (Herness and Gilbertson, 1999, for review). Second, the pair comparison procedure allowed for assessment of the relationship between two stimuli tested within the same session, equating the impact of motivational state and environmental variables on measurements of individual responsiveness to each compound. Finally, correlations in lick responsiveness were based on isoaversive concentrations of each stimulus determined from psychophysical functions obtained over multiple trial replications using a short-term taste-sensitive behavioral assay. While it is possible that olfactory cues from the bitter solutions may also have contributed to lick responses, average overall latencies to initiate licking on quinine, denatonium, and cycloheximide trials (3.81, 3.58, and 4.02 s, respectively) in the present study were very similar, suggesting little difference

![Figure 3 Mean concentration-response functions for each pair of bitter stimuli tested in separate groups of rats. (A) quinine and denatonium (n = 23), (B) quinine and cycloheximide (n = 23), and (C) denatonium and cycloheximide (n = 24). Lick ratio = mean licks to stimulus/mean licks to deionized water. Average (±SE) lick ratio data are shown. Drop lines indicate concentrations of each stimulus evoking 50% lick suppression (c values) determined from logistic functions fit to the group mean data.](image-url)
among these compounds in any olfactory-mediated response inhibition. It should further be noted that differential covariation in individual responsiveness among a subset of bitter stimuli does not affirm similarities or differences in perceptual discriminability of these stimuli per se (but rather individual differences in their aversive gustatory properties). The present findings contribute to a growing literature aimed at understanding commonalities and diversity in the mechanisms underlying stimulus processing within this taste modality.

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