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# Behavioral and neural responses to gustatory stimuli delivered non-contingently through intra-oral cannulas

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**Abstract**

The act of eating requires a decision by an animal to place food in its mouth. The reasons to eat are varied and include hunger as well as the food's expected reward value. Previous studies of tastant processing in the rat primary gustatory cortex (GC) have used either anesthetized or awake behaving preparations that yield somewhat different results. Here we have developed a new preparation in which we explore the influences of intra-oral and non-contingent tastant delivery on rats' behavior and on their GC neural responses. We recorded single-unit activity in the rat GC during two sequences of tastant deliveries, PRE and POST, which were separated by a waiting period. Six tastants ranging in hedonic value from sucrose to quinine were delivered in the first two protocols called 4TW and L-S. In the third one, the App L-S protocol, only hedonically positive tastants were used. In the 4TW protocol, tastants were delivered in blocks whereas in the two L-S protocols tastants were randomly interleaved.

In the 4TW and L-S protocols the probability of ingesting tastants in the PRE sequence decreased exponentially with the trial number. Moreover, in both protocols this decrease was greater in the POST than in the PRE sequence likely because the subjects learned that unpleasant tastants were to be delivered. In the App L-S protocol the decrease in ingestion was markedly slower than in the other protocols, thus supporting the hypothesis that the decrease in appetitive behavior arises from the non-contingent intra-oral delivery of hedonically negative tastants like quinine.

Although neuronal responses in the three protocols displayed similar variability levels, significant differences existed between the protocols in the way the variability was partitioned between chemosensory and non-chemosensory neurons. While in the 4TW and L-S protocols the former population displayed more changes than the latter, in the App L-S protocol variability was homogeneously distributed between the two populations. We posit that these tuning changes arise, at least in part, from compounds released upon ingestion, and also from differences in areas of the oral cavity that are bathed as the animals ingest or reject the tastants.


*Keywords:* Taste; Gustation; Chemical senses; Gustatory cortex; Insular cortex; Feeding

## 1. Introduction

The gustatory system participates in the maintenance of energetic and nutritional homeostasis by processing the chemical, thermal, and textural attributes of food [1–6]. This system normally functions in the context of ingestive behavior, and chemosensory responses occur only after one is sufficiently

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motivated to place food in one's mouth. The degree to which animals can exert control over events to which they are exposed is well known to exert a strong impact on their behavior and physiological functioning [7]. The act of eating is an ideal model in which to investigate the influence of motivation on animal behavior and neural activity. This is because food delivery can be manipulated in such a way that an animal can learn to control – or to be unable to control – what is placed in its mouth.

In previous studies we have investigated the neural responses obtained in the rat gustatory cortex (GC) to both hedonically positive (e.g. sucrose) and negative (e.g. quinine) tastants that were randomly, but contingently, delivered into the mouth via intra-oral cannulae (IOC) [8,9]. In these experiments, tastants were delivered only after water-deprived and head-restrained animals were sufficiently motivated to press a bar to receive both hedonically positive and negative tastants. Responses in the GC were investigated because it is a primary cortical area involved in the processing of gustatory information [4,10–12].

Here we have developed a new preparation in which we explore the influences of intra-oral and non-contingent tastant delivery on rat behavior and GC neural responses. In addition, these animals were not water-deprived, were not trained to press a bar to receive a tastant, and tastants were delivered via an IOC. Consequently, these animals could not control either when or which of the tastants would be delivered. In this study our goal was to investigate the changes in behavioral responses and the associated GC firing patterns when these subjects were unable to control when and which tastant was to be delivered.

Tastants were administered according to one of the three stimulus-delivery protocols, which differed in the degree of stimulus predictability and hedonic valence. We hypothesized that these disparities would differentially influence the time course of subjects' willingness to ingest the tastants. In each protocol, a 20-minute waiting period separated two identical sequences of tastant deliveries. In the 4TW protocol, individual tastants were delivered in blocks of four trials, while stimuli were randomly interleaved in the L-S (Latin-Square) protocol. Therefore, only in the 4TW protocol, but not in the L-S protocol, was the animal able to predict what tastant would be delivered after the onset of each block. In the Appetitive L-S protocol, hedonically positive stimuli were randomly delivered. In the first sequence of the 4TW and L-S protocols the probability of ingesting tastants decreased exponentially with the number of completed trials and, in the second sequence, the exponential decrease was accentuated. In contrast, the exponential decay was markedly slower for both sequences of the Appetitive L-S protocol. These results suggest that differences in palatability between tastants are lessened throughout the course of the experiment mainly as a consequence of the expectation of the non-contingent intra-oral delivery of hedonically negative tastants. The behavioral changes in the first two protocols coincided with extensive inter-sequence variability in chemosensory responses, while chemosensory responses obtained under the Appetitive L-S protocol were more stable.

## 2. Materials and methods

### 2.1. Animal care

Eleven Long–Evans rats weighing between 250 and 350 g were used in the present study. Animals were obtained from Harlan Bioproducts for Science Inc. (Indianapolis, IN) and housed in a common colony. Rats had ad libitum access to food and water at all times and were maintained on a fixed 12 h/12 h light/dark cycle. Experiments were performed at approximately the same time of day. All procedures conformed to standards established by the National Institutes of Health and were approved by the Duke University Institutional Animal Care and Use Committee.

### 2.2. Surgical procedures

We followed procedures detailed previously [8]. Briefly, animals were anesthetized using 5% halothane followed by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Adequate anesthesia levels were maintained throughout surgery with additional intraperitoneal injections of pentobarbital (0.07 cc as needed). Anesthetized animals were secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, Ca.) using atraumatic ear bars. After exposing the skull, stainless-steel screws were inserted to stabilize the electrodes and also to serve as electrical grounds. Craniotomies were performed bilaterally and electrodes were then lowered into the gustatory cortex (GC) and were cemented to the skull with dental acrylic resin. In addition, a stainless-steel screw used to restrain the rat's head was cemented above the occipital bone.

Recording electrodes consisted of groups of 16 microwires (California Fine Wire Corp., Grover Beach, CA) arranged either in bundles (25 μm diameter tungsten or 15 μm diameter nichrome wires) or in a 4-by-4 square lattice (35 μm tungsten wires with a 250 μm spacing). Bundles of microwires were attached to a custom-made microdrive that permitted them to be vertically displaced up to 2 mm from their initial positions [8,13]. Moveable electrodes were lowered in increments of approximately 100 μm the day prior to each experiment in order to allow the neural tissue to recover and to guarantee the sampling of distinct neurons. Relative to bregma, the centers of the electrodes were at AP +1.3 mm, ML +5.2 mm, and DV ~ −5 mm and their tips were spread across the GC [14].

### 2.3. Intra-oral cannulae (IOC)

Each rat was implanted with an intra-oral cannula that consisted of a PE-100 tube that was pulled through the masseter muscle and exited dorsally where it was secured to the skull with dental acrylic resin [15]. The IOC provided access to the intra-oral cavity through an opening lateral to the first maxillary molar.

### 2.4. Histology

Following completion of the experiments, rats were given an intraperitoneal injection of Nembutal (150 mg/kg) and

subsequently perfused transcardially with 120 cc of PBS followed by 120 cc of paraformaldehyde (4%). Brains were removed and stored in a sucrose/paraform solution (sucrose 30% w/v) for at least 24 h of postfixation. The tissue was sectioned through GC in 50 μm coronal slices and counter-stained with cresyl violet to visualize cell bodies. The staining revealed electrode tracks and their location in GC (see Fig. 2).

### 2.5. Video recording

The rats were restrained inside a custom-made restraint apparatus [8] that, in turn, was placed into a sound-attenuating box (Med Associates Inc., St. Albans, Vermont). The subjects were habituated to restraint for 3–5 days prior to the first recording session. Subjects were kept in the restraint chamber throughout the entire experiment, which lasted approximately 90 min. For offline behavior analysis, experiments were videotaped with a Panasonic B332 digital video camera that was synchronized to the data acquisition hardware by means of a digital timer (For-A, Company Limited, Japan).

### 2.6. Stimulus delivery

Tastants, at room temperature, were delivered through either a single PE-10 tube or custom-made bundles of six polyimide microtubes (Medsource Technologies, Trenton, Georgia) that were inserted into the IOC and interfaced to a nitrogen-pressurized, computer-controlled, solenoid valve system [18]. The opening of the valves was used to obtain time stamps of tastant delivery. Each tastant or water-rinse trial consisted of the delivery of 50 μl (80 μl for water-rinse trials) of the appropriate solution that in each rat was injected into the same side of the mouth.

In accordance with our earlier work [8,16], we used the following taste stimuli: citric acid (0.02 M), NaCl (0.1 M), sucrose (0.1 M), quinine HCl (0.001 M), nicotine (0.01 M), and water (separate from its use as a rinse between tastants). These tastants were chosen to represent, respectively, sour, salty, sweet, bitter, and "water" taste qualities. In a separate set of control experiments to test the effects of hedonics on chemical tuning, citric acid, quinine and nicotine were omitted as tastants. Preference tests have indicated that, at the concentrations tested, NaCl and sucrose are palatable [29], while citric acid, quinine HCl and nicotine are not [28,30].

### 2.7. Experimental protocols

Three tastant delivery protocols, designated as 4TW, Latin-Square (L-S), and Appetitive Latin-Square (App L-S) were tested. These protocols differ in tastant identity, delivery order and timing, as well as in the number of tastant and water rinse trials used.

All protocols consisted of two sequences of tastant deliveries, denoted as PRE and POST, which were separated by an inter-sequence interval lasting approximately 20 min. Tastants were delivered in two sequences so that a sufficient number of trials could be obtained in order to characterize neural responses in each sequence separately (see below) so that neural stability could be assessed across the two sequences.

For the 4TW and L-S protocols, six tastants were tested and eight trials per tastant were delivered, totaling 48 tastant trials in each sequence. In the App L-S protocol three tastants were used: NaCl, sucrose and water. The total number of tastant trials was the same as in the other two protocols. Consequently, in each sequence sixteen trials per tastant were administered.

An experiment therefore was composed of a total of sixteen trials for each of the six tastants (32 for the Appetitive L-S protocol), corresponding to a total of 4.8 ml of sapid fluid. The volumes of water used in water rinse trials were 1.92 ml for the 4TW protocol and 4.8 ml for the L-S and App L-S protocols.

### 2.8. Delivery protocols

#### 2.8.1. 4TW protocol

In the 4TW protocol, tastants were delivered in blocks of four trials each (abbreviated as 4T), and blocks were separated by a water rinse (denoted as W). The inter-trial interval for all trials, including water rinse trials, was 30 s. In each sequence, two blocks of each tastant were delivered, totaling eight trials. The first block was either sucrose or NaCl to ensure that the first four trials were hedonically positive, thus maximizing the number of appetitive trials. In the following five blocks, the remaining tastants were delivered in random order. The second group of six blocks repeated the same order of the first group of six blocks, and completed the PRE sequence. Tastant delivery order in the POST sequence was the same as that for the PRE sequence.

#### 2.8.2. Latin-Square protocol

Tastants were delivered pseudorandomly following a Latin-Square (L-S) scheme, with a water rinse trial interleaved between every tastant trial. Water rinse trials were delivered at 10 and 20 s after the previous and before the following tastant trials, respectively. Again, the first trial (T1) was either sucrose or NaCl.

#### 2.8.3. Appetitive Latin-Square protocol

Tastants were delivered as in the Latin-Square protocol, but only NaCl, sucrose and water were tested. Consequently in each sequence sixteen trials per tastant were delivered. Therefore subjects in this protocol were given the same number of tastant trials as in the two other protocols.

### 2.9. Electrophysiology

Action potentials having a greater than 3:1 signal to noise ratio were isolated using band-pass filters (0.3–6 kHz) and digitized at 40 kHz using a parallel processor (Plexon, Dallas, TX, USA). All spike waveforms were digitized and saved for offline analysis with the program "Offline Sorter" (Plexon, Dallas, TX, USA). A group of waveforms was only classified as a single neuron if it produced discrete clusters of exemplars in a space made up of the first three principal components of the original waveform space, and if its inter-spike interval plot showed a recognizable refractory period followed by a sloping increase to a maximum at times >1.2 ms [8].

### 2.10. Data analysis

Data analysis was performed using custom programs and built-in functions in Matlab (The MathWorks, Inc.), R (R Development Core Team) and SigmaPlot (Systat Software, Inc.).

### 2.11. Behavioral analysis

Animal behaviors under the 4TW, L-S, and App L-S protocols were characterized by analyzing off-line the video records of eleven, thirteen, and five experiments obtained from four, six, and two rats, respectively. Some of the subjects were tested under multiple protocols across different days.

All trials were classified into appetitive or aversive groups according to the type of oro-facial movements elicited by tastant administration. In appetitive trials, subjects ingested the delivered solutions and displayed lateral tongue protrusions, licking or paw licking whereas in aversive trials animals refused to ingest, displaying gapes and/or drools [17].

### 2.12. Electrophysiological data analysis

For each neuron the number of spikes that occurred in the 2.5 s immediately preceding (background activity) and following (evoked activity) each stimulus delivery was initially counted [8,9]. Single-trial spike counts were then grouped according to the tastant delivered, the sequence in which they occurred and the stimulus-delivery protocol employed, thus yielding sets of eight spike counts. Each count within the set was then modeled as a Poisson process in the statistical program "R" (www.R-project.org). Because the Poisson rates ($\lambda$) and the experimentally observed average spike counts (or firing rates, obtained by dividing spike counts by 2.5 s) are numerically identical, they are used interchangeably throughout the paper.

The Poisson firing rates for each cell were identified by the following four descriptors, which can take the levels denoted in parentheses: Peri-stimulus Time, which designates the background or stimulus-evoked firing rate ('background' or 'evoked', respectively); Tastant, which indicates the tastant delivered ('*Na*', '*C*', '*Ni*', '*Q*', '*S*' or '*W*'); Sequence, which corresponds to the tastant delivery sequence ('PRE' or 'POST'); and Delivery protocol, which designates the delivery protocol employed ('4TW', 'L-S', or 'App L-S'). Henceforth, firing rates for a given cell are denoted by R(PeriStim, Tast, Seq, Prot) (Hz), where PeriStim, Tast, Seq, and Prot (protocol type) are abbreviations of the names of the different descriptors.

To determine whether GC neuronal spiking activity was affected by the delivery of a given tastant (Tast) throughout a particular sequence (Seq) in an experiment run under a certain delivery protocol (Prot), the Poisson estimate of the background firing rate, *R*(Back, Tast, Seq, Prot), was compared to the estimated evoked firing rate, *R*(Evok, Tast, Seq, Prot) through a Poisson generalized linear model (GLM) [19]. All neurons were modeled together such that

$$\ln(\lambda) = \beta_0 + X\beta,$$

where the columns of the matrix $X$ refer to the covariates to be modeled, which included background vs. evoked firing rate, protocol, sequence, tastant, and neuron number. Subsets of relevant higher order interactions were also included on the basis of their contributions to the model. The parameter vector $\beta$ is the weighting or relative contribution of each covariate to the fit of the data, and the vector $\beta_0$ is the intercept. Because multiple variables were examined, the GLM required that tastant deliveries were repeated a large number of times in order to ensure that a sufficient number of spike trains could be modeled without overfitting the data [19,20]. Because a large number of trials were needed, we presented two sequences of tastants, each with eight deliveries of tastants. Chi-square and normal tests based on the asymptotic normality of the estimates of the models were used to compare firing rates. Multiple comparisons were adjusted for family-wise type-I error using the Bonferroni method.

In order to assess the goodness of fit of the Poisson-based GLM, we generated predictive coverage estimates for our cell-level model. For each cell in the sample an approximate 90% predictive interval was calculated using the mean estimated by the model. Then, the proportion of observations from that cell falling inside the interval was obtained. Models that adequately fit the data should exhibit coverage probabilities close to 90% [19,20].[2]

The difference in firing rates between evoked and background responses is referred to as a PeriStimDelta and is denoted by PeriStimDelta (Tast, Seq, Prot). For example, the magnitude of the response elicited by sodium chloride during the POST sequence for a particular cell recorded under the 4TW delivery protocol would be denoted by PeriStimDelta (Na, POST, 4TW). If the differences between the log ratios of the background and evoked activities as given by the GLM were significant, (i.e. if any PeriStimDelta (Tast, Seq, Prot) significantly differed from zero), then the response for a given neuron under those conditions (tastant, sequence and delivery protocol, respectively) was considered significant. Positive and negative Deltas corresponded to excitatory and inhibitory responses, respectively. Similarly, if the difference between response magnitudes of PeriStimDelta (Tast, PRE, Prot) and PeriStimDelta (Tast, POST, Prot) (denoted by InterSeqDelta (Tast, Prot)) was significant, then this indicated that the response magnitudes to a particular tastant changed between sequences.

A chemosensory neuron was defined as one exhibiting responses of different magnitudes to distinct tastants. For those cells that exhibited significant responses of the same direction to all tastants, we next determined whether any tastant elicited responses that were significantly different from the others, by

[2] Note, however, that since we do not account for the uncertainty in the estimates of the firing rates, this is a lower bound for the real coverage probability: the true predictive interval is wider than and contains the one presented. This means that observed probabilities slightly under 90% are reasonable. Inactive cells during any of the experiments were excluded from the analysis because of the large uncertainty associated with the estimates. For most of the cells, the coverage probability is at least 75%.

comparing their 95% confidence intervals given by the GLM. All cells that displayed significantly different responses (PeriStimDelta) to the tastants were classified as chemosensory. Any cell whose responses did not differ between tastants was classified as mechanosensory [18].

For each protocol, the statistical significance of the *Z*-scores associated with each of the Deltas (PeriStimDelta(Tast, PRE, Prot), PeriStimDelta(Tast, POST, Prot), and InterSeqDelta(Tast, Prot)) for each cell was determined by comparison to a critical *Z*-score. For the 4TW and L-S stimulus -delivery protocols, $C=6\times N$ (6 tastants, $N=85$ and $N=57$ cells in the 4TW and L-S protocols, respectively) comparisons were performed simultaneously. This yielded critical *Z*-scores of 3.9 and 3.8 for this test at a family-wise type-I error rate of 0.05 (Bonferroni correction method) for the 4TW and L-S protocols, respectively. For the App L-S protocol, $C=3\times N$ (3 tastants, $N=44$ cells) and the associated *Z*-score is 3.6 for the same error rate. Results are displayed graphically in Figs. 4, 6, and 8.

### 2.13. Contribution of changes in background- versus evoked-firing rates to response changes

Response magnitudes, PeriStimDelta (Tast, Seq, Prot), are defined as the difference between the evoked and the immediately preceding background activity. Therefore, inter-sequence alterations in both evoked and background activity may lead to changes in response magnitudes for a given neuron and tastant, InterSeqDelta (Tast, Prot). Thus, it is necessary to characterize the contribution of these two types of alterations in the observed inter-sequence response magnitude changes.

We therefore analyzed the data collected under the 4TW and L-S protocols by running linear correlations between the *Z*-scores associated with InterSeqDelta (Tast, Prot) and both EvokDelta (Tast, Prot) and BackDelta (Tast, Prot), where

$$\begin{aligned}\text{EvokDelta}(\text{Tast},\text{Prot}) &= R(\text{Evok},\text{Tast},\text{POST},\text{Prot}) - R(\text{Evok},\text{Tast},\text{PRE},\text{Prot}),\\ \text{BackDelta}(\text{Tast},\text{Prot}) &= R(\text{Back},\text{Tast},\text{POST},\text{Prot}) - R(\text{Back},\text{Tast},\text{PRE},\text{Prot})\end{aligned}$$

and where $R(\ldots.)$ is the firing rate under the different conditions.

This yielded the correlation coefficients $R^2$(Evok) and $R^2$(-Back), respectively, as well as their associated *p*-values, *p* (Evok) and *p*(Back). Low *p*-values indicate significant correlations, meaning that changes in the corresponding firing rates – either background or evoked – played a significant role in the observed inter-sequence response magnitude changes. The magnitude of such correlations is given by $R^2$.

Analysis of the data (Figs. 3A and 4A and additional data not shown) revealed that the *p*-values associated with the correlation of the overall InterSeqDelta (Tast, Prot) with EvokDelta (Tast, Prot) (4TW: $p(\text{Evok})<1.99\times10^{-34}$, L-S: $p(\text{Evok})<1.55\times10^{-10}$), are consistently smaller than those associated with BackDelta(Tast, Prot) (4TW: $p(\text{Back})<9.88\times10^{-19}$, L-S: $p(\text{Back})<4.8\times10^{-3}$). Additionally, the $R^2$ values between the overall InterSeqDelta(Tast, Prot) and EvokDelta(Tast, Prot) were higher (4TW: $R^2(\text{Evok})=0.51$; L-S: $R^2(\text{Evok})=0.33$) than those for BackDelta(Tast, Prot) (4TW: $R^2(\text{Back})=0.38$; L-S: $R^2(\text{Back})=0.15$). From these analyses we conclude that inter-sequence changes in the responses arise primarily from changes in evoked firing rates.

### 2.14. Behavioral analysis

The time course of behavioral responses in each stimulus-delivery protocol was analyzed by first calculating the experiment-wide probability of observing an appetitive response in each trial (averaging data from 11, 8, and 5 experiments for the 4TW, L-S, and App L-S protocols, respectively).

Probabilities for each separate sequence (Seq) in each protocol (Prot) were then empirically fitted (SigmaPlot, Systat Software) with an exponential function of the form

$$P_{\text{Prot,Seq}}(T) = K_{\text{Prot,Seq}} + a_{\text{Prot,Seq}}\cdot e^{\frac{-(T-1)}{\tau_{\text{Prot,Seq}}}},$$

where $P_{\text{Prot,Seq}}(T)$ is the experiment-wide probability of observing an appetitive response in trial number *T*, and, $K_{\text{Prot,Seq}}$, $a_{\text{Prot,Seq}}$ and $\tau_{\text{Prot,Seq}}$ are the three parameters describing the exponential fit to the data.

To fit the experimentally observed probability of an appetitive trial occurring in the first trial of a sequence, we enforced $P_{\text{Prot,Seq}}(1)=K_{\text{Prot,Seq}}+a_{\text{Prot,Seq}}$. The parameter $\tau_{\text{Prot,Seq}}$ therefore describes the apparent time constant with which subjects progressively rejected the delivered stimuli. At the end of the sequence (comprising 48 trials), the probability of the occurrence of an appetitive trial approximates $K_{\text{Prot,Seq}}$: that is $P_{\text{Prot,Seq}}(48)\approx K_{\text{Prot,Seq}}$.

## 3. Results

### 3.1. Marked changes in subjects' behavior during the 4TW and L-S protocols

To quantify the ingestive behavior of non-deprived animals after tastants were delivered non-contingently through an IOC, we analyzed the video records of four rats run under the 4TW, L-S and App L-S protocols. Depending on the type of oro-facial movements displayed by the rat upon stimulus delivery each trial was categorized as appetitive or aversive [17].

The results, summarized in Fig. 1A (4TW), Fig. 1B (L-S), and Fig. 1C (App L-S) display the probability (P) with which each trial elicited appetitive responses in each of the PRE and POST sequences. In both protocols and sequences, subjects mainly responded with appetitive behaviors at the beginning of each sequence. Specifically, they were equally likely to display appetitive responses to stimulus delivery in the first trial (T1) of either sequence (mean probability $P(\text{T1})=0.95$, $\chi^2(1)=0.114$, $p>0.74$). This demonstrates that they were about equally receptive at the start of each session. These data also confirm that the subjects accepted tastants again at the beginning of the POST sequence regardless of previous avoidance behaviors during the PRE period. As the experiment progressed, however, the subjects began to reject the delivered tastants. The first rejected trial in each sequence was

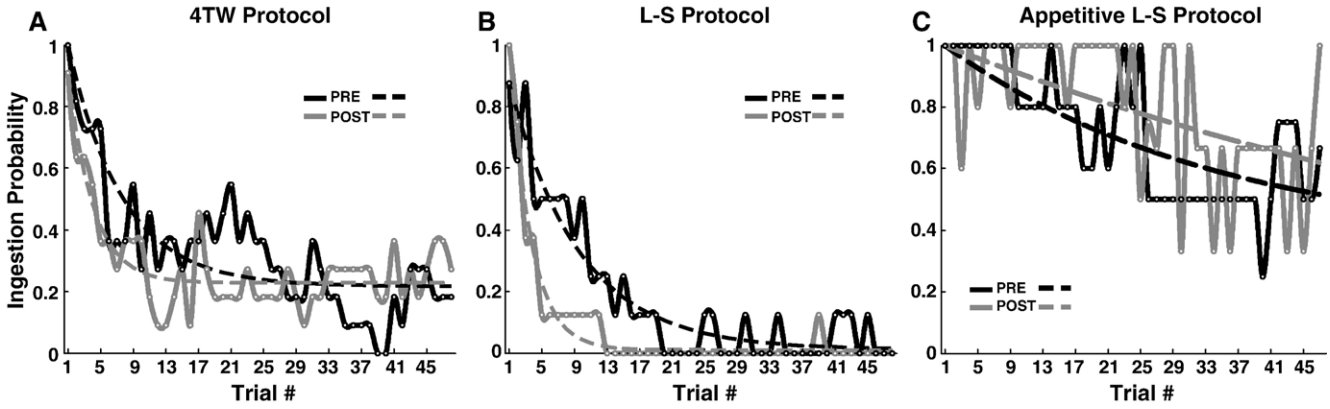


Fig. 1. Changes in stimulus-triggered responses throughout the experiment. The experiment-wide probability of eliciting appetitive responses in each trial (solid lines) was plotted for both PRE and POST sequences (grey and black, respectively). For each sequence, a decaying exponential was fitted to the experimental data and was overlaid on the experimental data (dashed lines, same color scheme). Exponential parameters are presented in figure insets and their $R^2$ values and 95% confidence intervals are given below. A. 4TW protocol (average of 11 experiments). Exponential fit: $R^2_{PRE}=0.91$, $R^2_{POST}=0.94$; parameter for 95% confidence intervals: $\tau_{PRE}$ [5.6, 8.3], $\tau_{POST}$ [2.6, 3.7]; $K_{PRE}$ [0.19, 0.24], $K_{POST}$ [0.21, 0.24]; $a_{PRE}$ [0.7, 0.9], $a_{POST}$ [0.6, 0.8]. B. L-S protocol (average of 8 experiments). Exponential fit: $R^2_{PRE}=0.93$, $R^2_{POST}=0.96$; parameter 95% confidence intervals: $\tau_{PRE}$ [7.7, 9.7], $\tau_{POST}$ [2.5, 2.8]; $K_{PRE}$ [−0.01, 0.03], $K_{POST}$ [0.003, 0.02]; $a_{PRE}$ [0.8, 0.91], $a_{POST}$ [0.95, 1.03]. C. Appetitive L-S protocol (average of 5 experiments). Exponential fit: $R^2_{PRE}=0.97$, $R^2_{POST}=0.95$; parameter for 95% confidence intervals: $\tau_{PRE}$ [19, 169], $\tau_{POST}$ [20, ∞]; $K_{PRE}$ [0.07, 0.63], $K_{POST}$ [0, 3.1]; $a_{PRE}$ [0.006, 0.05], $a_{POST}$ [0, 4.0].

usually classified as a "gape", characterized by subjects' active attempt to expel the tastant solution. Subsequent rejected trials were "drools", wherein subjects left their mouths open and passively let the delivered solution drip out of their mouths.

The two delivery protocols resulted in different time courses of the subjects' probability of ingesting the stimuli throughout the experiment. The behavioral time courses were quantified by fitting subject-wide average data from each separate sequence with a decaying exponential function, yielding the following regression curves that are shown in Fig. 1. For the 4TW protocol (Fig. 1A) the fit is,

$$P_{4TW,PRE}(T) = 0.22 + 0.78 \cdot e^{\frac{-(T-1)}{6.65}}, \quad (1)$$

$$P_{4TW,POST}(T) = 0.23 + 0.68 \cdot e^{\frac{-(T-1)}{3.06}} \ldots\ldots$$

and for the L-S protocol (Fig. 1B) the fit is,

$$P_{L-S,PRE}(T) = 0.01 + 0.86 \cdot e^{\frac{-(T-1)}{8.62}}, \quad (2)$$

$$P_{L-S,POST}(T) = 0.01 + 0.99 \cdot e^{\frac{-(T-1)}{2.62}} \ldots\ldots$$

The protocols are similar in that the probability of displaying an appetitive response declined faster in the POST than in the PRE sequence, as indicated by the smaller POST relative to PRE time constants $\tau$ ($\tau_{4TW,POST} < \tau_{4TW,PRE}$ and $\tau_{L\text{-}S,POST} < \tau_{L\text{-}S,PRE}$). This occurred, as noted, even though the initial trial (T1) acceptance probability was approximately the same in the PRE (4TW: 100%, L-S: 87%) and POST (4TW: 92%, L-S: 100%) sequences. We also found that the time constants were not statistically different between the protocols (overlapping confidence intervals between $\tau_{4TW,PRE/POST}$ and $\tau_{L\text{-}S,PRE/POST}$). In addition, we also found that the number of appetitive trials did not significantly vary between sequences in the 4TW protocol (PRE: 16.4±13.1, POST: 13.3±13.5; $\chi^2(1)=2.27$, $p>0.13$), whereas a significant inter-sequence decrease was observed in the L-S protocol (PRE: 8.4±5.5; POST: 3.6±3.6; $\chi^2(1)=15.04$, $p<1.05\times10^{-4}$). One important difference between the protocols was that the animals still ingested the stimuli in 22–23% of the trials at the end of the 4TW sequences. In contrast, at the end of either of the L-S sequences the animals rejected all of the stimulus trials regardless of whether they contained hedonically positive or negative tastants.

We next investigated possible causes of the observed decrease in ingestion by grouping data from both experiments and correlating, for each sequence, the trial number corresponding to the first aversive trial with that corresponding to the first delivery of each of the different tastants. In the PRE sequence, we found that only quinine significantly correlated with the onset of aversive responses (quinine: $p<0.0006$, $R^2=0.73$; all other tastants: $p>0.23$, $R^2<0.3$). In the POST sequence, however, no tastant yielded statistically significant correlations although the initial delivery of quinine again most strongly correlated with the onset of aversive responses (quinine: $p>0.054$, $R^2=0.46$; all other tastants: $p>0.19$, $R^2<0.32$).

To further establish the influence of aversive tastants on the decrease of appetitive behavior, we conducted 5 additional experiments on two rats using a modified version of the L-S protocol. In this protocol citric acid, quinine, and nicotine were not used. For this reason this protocol was given the name "Appetitive L-S". Fitting the subject-wide average behavioral data with the same type of decaying exponentials (Fig. 1C) yielded the following,

$$P_{App\ L-S,PRE}(T) = 0.35 + 0.65 \cdot e^{\frac{-(T-1)}{33.9}}, \quad (3)$$

$$P_{App\ L-S,POST}(T) = e^{\frac{-(T-1)}{96.1}} \ldots\ldots\ldots$$

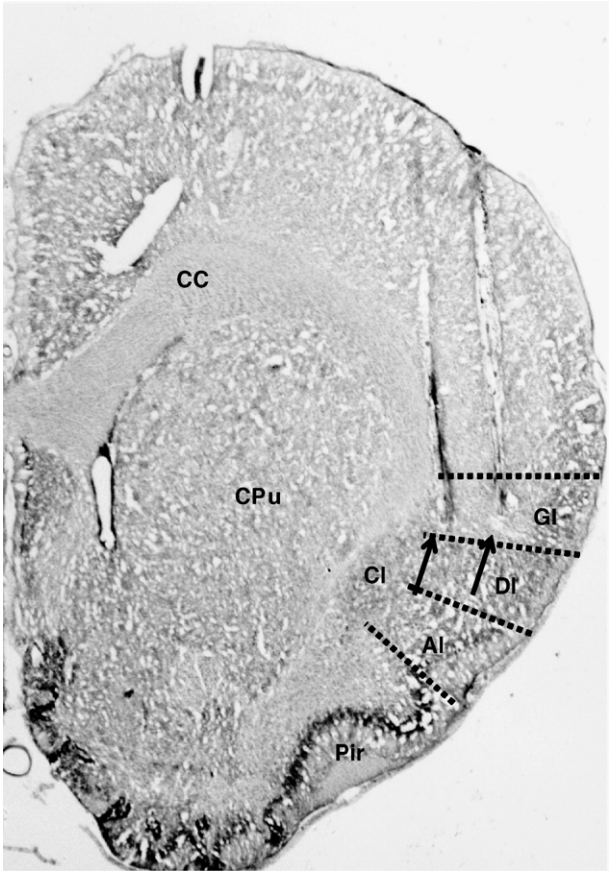


Fig. 2. Localization of electrodes in rat gustatory cortex. This 50 μm thick coronal section of a rat cortex is stained with cresyl violet. The paths of the electrodes are clearly visible in the tissue, and the locations of the electrode tips are indicated with arrows. This electrode array is seen to terminate in the granular insular cortex. The transitional zones between the granular, dysgranular, and agranular cortices are demarcated with dashed lines. Abbreviations are as follows: AI, agranular insular cortex; CC, corpus callosum; Cl, claustrum; CPu, caudate putamen; DI, dysgranular insular cortex; GI, granular insular cortex; Pir, piriform cortex.

which were superimposed onto the corresponding experimental data. It is evident that the decrease in appetitive behavior observed under the Appetitive L-S protocol is markedly slower than either of the 4TW or L-S protocols in both PRE and POST sequences ($\tau$*AppL-S,PRE/POST* >> $\tau$ *L-S, PRE/POST and* $\tau$*AppL-S,PRE/POST* >> $\tau$ *4TW, PRE/POST*, respectively). We additionally note that, in contrast to the 4TW and L-S protocols, no significant ( $p > 0.05$) inter-sequence difference in the decay rate of ingestion probability was observed in this protocol ($\tau_{\text{App L-S,PRE}} \approx \tau_{\text{App L-S,POST}}$).

### 3.2. Electrophysiological studies

Electrode placement in the GC has been verified for all implanted rats. Fig. 2 shows an example where the positions of the electrode tips are clearly visible in the GC. In general, the electrodes spanned layers II–III to VI and were distributed across the granular to the agranular gustatory cortex.

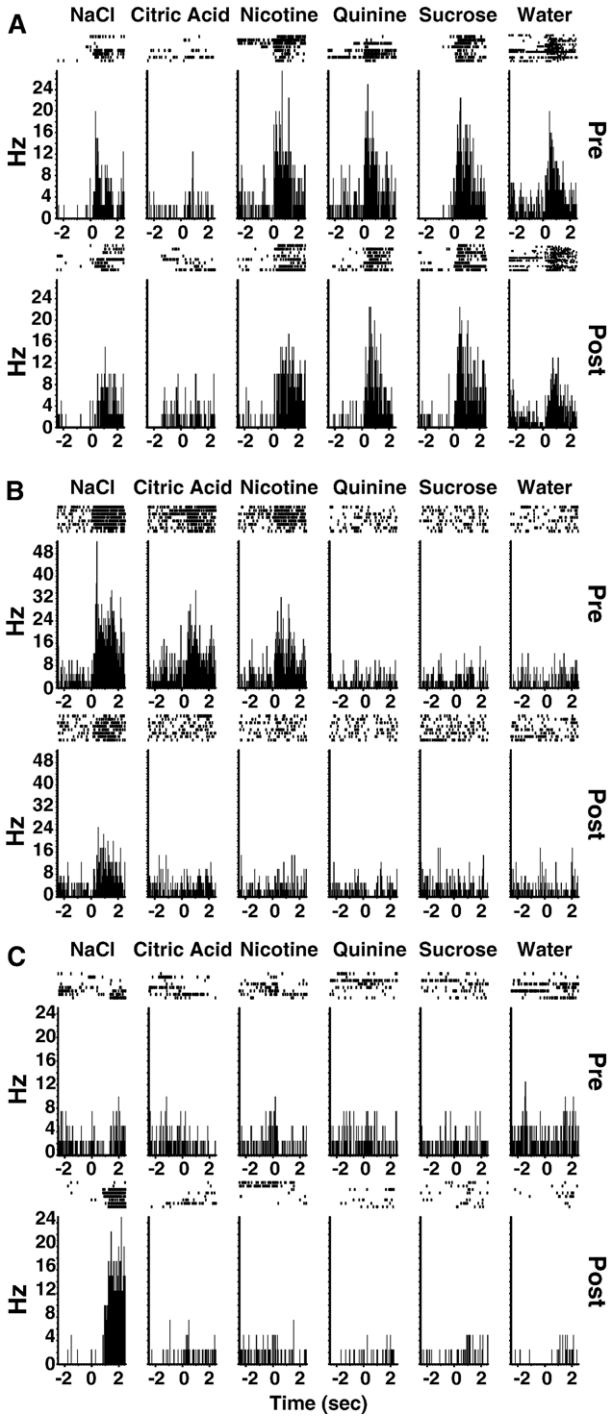


Fig. 3. Representative examples of GC single neuron response variability. Presented is the peri-stimulus histogram activity of three different GC neurons that exemplify the distinct neuronal activity patterns observed throughout the two sequences (PRE and POST). For each neuron, responses to the six tastants in each sequence are displayed in the raster plots (above: vertical ticks indicate peri-stimulus spiking times and each row represents one trial) and in peri-stimulus time histograms (below: PSTH, instantaneous trial-wide average firing rate). A. Stability of response: All tastants except citric acid (C) evoked responses in the PRE and POST sequences. B. Selective inhibition: In the PRE sequence, Na (NaCl), C (citric acid) and Ni (nicotine), evoked responses whereas in the POST sequence only Na evoked a response. C. Unmasking of a response. In this example none of the tastants evoked a response in the PRE sequence whereas Na evoked a response in the POST sequence.

### 3.3. Single-cell analysis of gustatory cortical single-unit responses

We next investigated how the behavioral changes shown in Fig. 1 obtained during each of the three delivery protocols are reflected in the activity of GC neurons. Fig. 3 shows the raster plots and PSTHs of three tastant responsive neurons that were chosen to illustrate the range of the changes in chemical tuning that were observed between the PRE and POST sequences. Fig. 3A depicts an example of a neuronal response to several tastants that remained unchanged between the PRE and POST sequences. The neuronal responses displayed in Fig. 3B exhibited tastant-selective chemical tuning changes. During the PRE sequence, NaCl, citric acid, and nicotine all evoked responses from this neuron, while in the POST sequence only NaCl remained an effective stimulus. Note that between the sequences the baseline activity did not significantly change (unpaired t-test, $p>0.05$). Fig. 3C illustrates a neuronal response exhibiting the selective unmasking of an excitatory response to NaCl in the POST sequence.

The results for all the neuronal responses obtained for the 4TW, L-S, and App L-S protocols are shown in Figs. 4, 6, and 8, respectively. In each figure, trial-wide average responses in the PRE and POST sequences, PeriStimDelta(Tast, PRE, Prot) and PeriStimDelta(Tast, POST, Prot), are plotted in the left and middle panels, respectively. Average inter-sequence response magnitude changes, given by InterSeqDelta(Tast, Prot), are plotted in the right panel. Each row across the three panels represents the same neuron recorded throughout the experiment, columns refer to each of the six taste stimuli tested, and firing rate values are color-coded. Cells have been numbered in ascending order from the top to the bottom of the panels. Statistically significant responses or response changes, corresponding to *Z*-scores with an absolute value greater than the critical Z-score, have been marked with a (×) sign.

### 3.4. 4TW protocol

The activity of 85 neurons, displaying an average firing rate of 6.2±8.1 Hz, was recorded in fourteen experiments obtained from three rats tested under the 4TW delivery protocol (Fig. 4). We found that 42.3% (36/85) of the neurons responded at least to one tastant in the PRE sequence, of which all were classified as chemosensory. That is, no mechanosensory neurons were detected.

In Fig. 5A we show the percentage of neurons that, in the PRE and POST sequences, were excited (20% and 24%, respectively), inhibited (20% and 16%, respectively) or showed composite responses (2% and 1%, respectively). Fig. 5B shows the proportion of cells for each tastant that exhibited excitatory or inhibitory responses in the PRE and POST sequences.

No tastant exhibited significantly more excitatory or inhibitory responses than any other (PRE excitatory responses: $\chi^2(5)=2.9$, $p>0.72$; PRE inhibitory responses: $\chi^2(5)=3.9$, $p>0.56$; POST excitatory responses: $\chi^2(5)=10.6$, $p>0.06$;

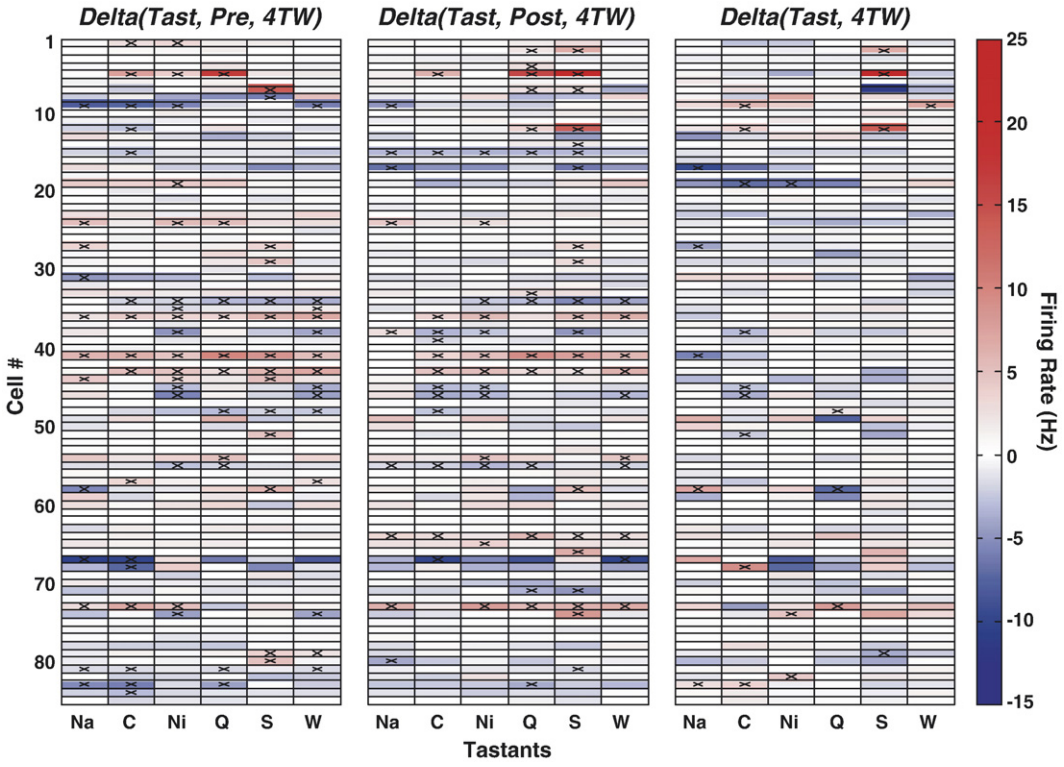


Fig. 4. Variability of the chemical tuning profiles in the 4TW protocol. The chemical tuning profiles of 85 GC neurons assessed in the PRE and POST sequences are presented in the left and middle panels, respectively. The inter-sequence differences in responses are presented in the rightmost panel. Each row represents the same neuron across sequences and columns correspond to the different tastants tested. Firing rates have been color-coded. Significant responses (left and middle) and inter-sequence response changes (right) are indicated by an x.

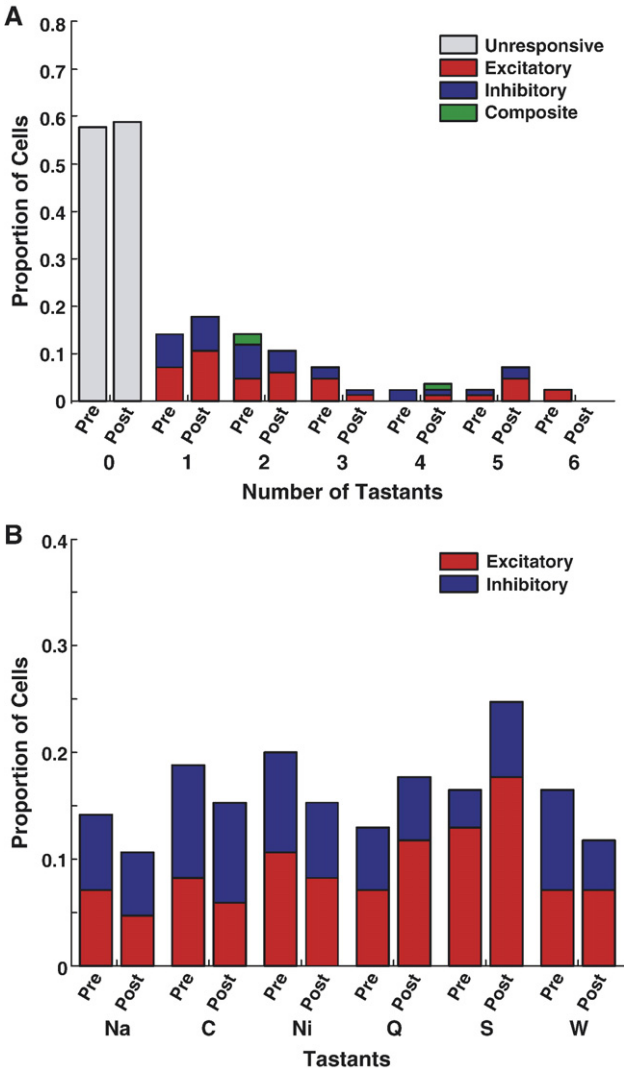


Fig. 5. A. Histograms of the proportion of cells responding to different numbers of tastants in each sequence. Cells displaying both excitatory and inhibitory responses to the tested tastants were labeled as composite responders. B. Histograms of the proportion of cells responding to the different tastants in each sequence. Color scheme: white (in A) and grey (in B) — unresponsive, red — excitatory, blue — inhibitory, green — composite responses (in B only) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

POST inhibitory responses: $\chi^2(5)=1.6$, $p>0.89$). We note that in the POST sequence, sucrose elicited more excitatory responses than the other tastants.

Of the total neuronal population, 23.5% (20) showed significant inter-sequence chemical tuning changes, of which 75% (15) and 25% (5) changed responses to one and two tastants, respectively. Across the entire 4TW population, the average number of tuning changes per neuron is 0.29. Considering only those 20 neurons that exhibited chemical tuning changes, the average number of changes per neuron is 1.25.

The possibility that the neural representation of certain tastants is more labile than others, i.e. more prone to inter-sequence response changes, would suggest the existence of tastant-specific physiological processes involving the GC. We therefore calculated the distribution of significant response changes across the six different tastants to assess differences in inter-sequence chemical tuning variability between tastants. This distribution was not statistically different from a homogeneous distribution ($\chi^2(5)=8.84$, $p>0.12$). We nevertheless note that the tastant that showed the most chemical tuning changes was citric acid, followed by NaCl and sucrose, displaying 36%, 20% and 16% of the total number of changes, respectively.

### 3.5. L-S protocol

We similarly analyzed the activity of 57 single units recorded from eight experiments run with four rats under the L-S delivery protocol (Fig. 6). The cell-wide average firing rate was observed to be 4.5±4.9 Hz.

In this protocol 52.6% (30/57) of neurons were found to be chemosensory, and as with the 4TW protocol, no mechanosensory neurons were detected. In Fig. 7A we show the percentage of neurons in the PRE and POST sequences that were excited (28% and 25%, respectively), inhibited (14% and 16%, respectively) and demonstrated composite responses (10% and 4%, respectively). Fig. 7B presents the proportion of cells that exhibited excitatory or inhibitory responses for the various tastants in the PRE and POST sequences. Whereas inhibitory responses were homogeneously distributed across tastants in both sequences (PRE inhibitory responses: $\chi^2(5)=1.6$, $p>0.9$; POST inhibitory responses: $\chi^2(5)=2.3$, $p>0.8$), sucrose elicited significantly more excitatory responses than the other tastants in both sequences (PRE excitatory responses: $\chi^2(5)=28.7$, $p<2.6\times10^{-5}$; POST excitatory responses: $\chi^2(5)=22.0$, $p<5.2\times10^{-4}$).

Of these 57 neurons, 17 (30%) showed significant inter-sequence changes in responses, of which 71% (12), 17.6% (3), and 11.8% (2) displayed changes to one, two and three tastants, respectively. The average number of changes for all cells in this protocol is therefore 0.42, and the average number for cells that exhibited changes is 1.41.

As above, we explored the possibility that the neural representation of certain tastants is more labile than others. As in the case of the 4TW protocol, no differences in response variability between tastants were detected ($\chi^2(5)=9.5$, $p>0.09$). In this protocol, the tastants whose responses were the most variable were sucrose, with 37.5% of the total number of changes, followed by citric acid, nicotine and water, all three displaying 16.7% of the total number of changes.

### 3.6. Appetitive L-S protocol

To determine whether the behavioral change from acceptance to rejection of tastants precipitated a corresponding change in the chemical tuning of the neural responses, we repeated the L-S design but tested only NaCl, sucrose, and water, all of which are hedonically positive. We obtained 44 single units from five experiments conducted with two rats (Fig. 8), exhibiting a cell-wide average firing rate of 12.8±10 Hz.

In this protocol, 54% (24) of cells were found to respond to tastants in the PRE sequence. Of these, 71% (17) were found to be chemosensory whereas 29% (7) were classified as

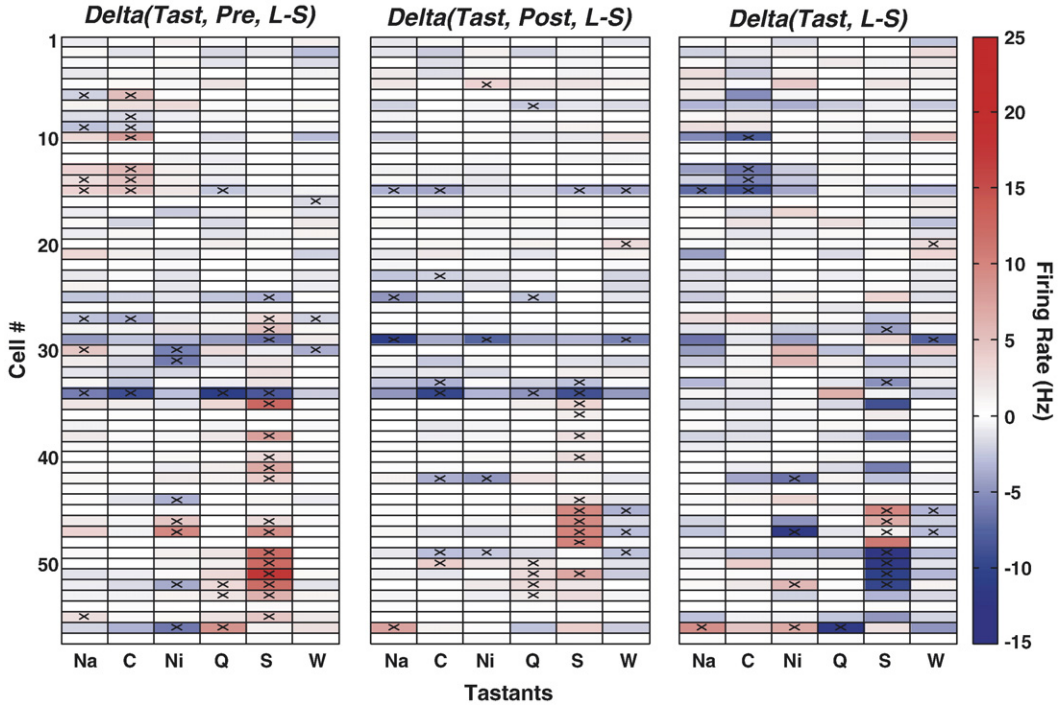


Fig. 6. Variability of the chemical tuning profiles in the L-S protocol. The chemical tuning profiles of 57 GC neurons assessed in PRE and POST sequences are presented (left and middle panels, respectively) as well as inter-sequence response changes (right panel). The color scheme used is identical to that of Fig. 4.

mechanosensory because there were no statistical differences between tastants in the magnitude of their evoked responses.

In Fig. 9A we show the percentage of cells in the PRE and POST sequences that were excited (36% and 39%, respectively) and inhibited (18% and 20%, respectively). No cells exhibiting composite responses were found in either sequence. Fig. 9B depicts the proportion of cells that exhibited excitatory or inhibitory responses for the various tastants in the PRE and POST sequences. In this protocol, both inhibitory (PRE inhibitory responses: $\chi^2(2)=2.92$, $p>0.23$; POST inhibitory responses: $\chi^2(2)=1.86$, $p>0.39$) and excitatory (PRE excitatory responses: $\chi^2(2)=1.12$, $p>0.57$; POST excitatory responses: $\chi^2(2)=0.053$, $p>0.97$) responses were homogeneously distributed across tastants in both sequences.

Of the total neuronal pool, 27% (12) showed significant inter-sequence changes in responses, of which 67% (8) and 33% (4) displayed changes to one and two tastants, respectively. The average number of inter-sequence chemical tuning changes for all cells in this protocol is 0.36, and the average number for cells that exhibited changes is 1.3.

Again, no tastant was found to exhibit responses that were significantly more variable than any other ($\chi^2(2)=0.88$, $p>0.64$). Nevertheless, responses to water were most variable, followed by those to sucrose and NaCl (44%, 31% and 25% of the total number of changes).

### *3.7. Comparison of the neuronal variability across the three protocols*

We found no difference between the three protocols in the overall levels of variability ($\chi^2(2)=0.72$, $p>0.70$), with 23.5%, 29.8% and 27.3% of the neuronal samples exhibiting at least one inter-sequence chemical tuning change in the 4TW, L-S and Appetitive L-S protocols, respectively.

To further explore whether substantive differences existed between the types of changes seen in each of the protocols we separated the neurons into chemosensory and non-chemosensory classes (the latter of which included both non-responsive and mechanosensory units [18]) according to their chemical responsiveness to stimuli in the PRE sequence. Their contributions to the total neural variability were then computed and compared. No statistically significant difference in the size of these two populations was detected (for all experiments, $\chi^2(1)<2.3$, $p>0.13$) in any of the protocols: in the 4TW, L-S and App L-S protocols, 42% (36/85), 53% (30/57) and 38.6% (17/44) of all neurons, respectively, were chemosensory. Furthermore, no difference between protocols in the proportion of chemosensory versus non-chemosensory neurons was detected ($\chi^2(2)=2.3$, $p>0.32$).

We note that despite the observed neuronal variability, the overall GC physiological properties, namely the total numbers of excitatory, inhibitory and non-significant responses remained constant throughout the experiment in all protocols (4TW: $\chi^2(2)=0.4$, $p>0.82$; L-S: $\chi^2(2)=2.8$, $p>0.25$l; Appetitive L-S: $\chi^2(2)=0.41$, $p>0.81$).

However, significant differences between protocols were found in the relative variability exhibited by the non-chemosensory versus chemosensory populations ($\chi^2(2)=8.38$, $p<0.015$). In the 4TW and L-S protocols, chemosensory neurons were more variable than non-chemosensory neurons (4TW: $\chi^2(1)=19.5$, $p<1.02\times10^{-5}$; L-S: $\chi^2(1)=8.6$, $p<0.004$). The proportions of chemosensory and non-chemosensory neurons that exhibited

chemical tuning variability in the 4TW protocol were 47.2% (17/36) and 6.1% (3/49), respectively, and, in the L-S protocol, 46.7% (14/30) and 11.1% (3/27), respectively. In contrast, in the Appetitive L-S protocol no significant difference in variability between the chemosensory and non-chemosensory populations was found ($\chi^2(1)=0.1, p>0.76$), with 29.17% (7/24) and 25% (5/20) of the former and latter populations, respectively, exhibiting chemical tuning changes.

In summary, we found that although the neuronal responses in the three protocols displayed similar overall levels of variability, there exist significant differences between them in the way the variability was partitioned between chemosensory and non-chemosensory neurons. Whereas in the 4TW and L-S protocols the former population displayed more changes than the latter, the variability was homogeneously distributed between the two in the Appetitive L-S protocol.

## 4. Discussion

Using three distinct stimulus-delivery protocols we recorded behavioral and GC neuronal responses to tastants in awake but restrained rats that were non-contingently delivered directly into their intra-oral cavities. Six tastants, half of which were hedonically positive and the other half hedonically negative, were tested on two of the protocols, denoted L-S and 4TW. These differed in the order of tastant delivery and in the amount of water rinse trials used. In the third protocol, called Appetitive L-S, only the three hedonically positive tastants were used.

Two sources contributed to the changes in ingestion over the course of the test sessions. In the case of the App L-S protocol, subjects gradually decreased their ingestion rates during both sequences, although the subjects were still willing to consume the stimuli on more than half of the trials. Such a decrease in response rates might be due to increasing satiety or simple disengagement from the task [22]. Behavioral studies in the L-S and 4TW protocols indicate that the delivery of hedonically negative tastants, such as quinine, additionally and significantly potentiated the decrease of appetitive behavior observed during the experiment. Furthermore, we suggest that the greater uncertainty in the occurrence of a negatively hedonic stimulus in the case of the L-S protocol also led to an accelerated decrease in ingestive drive.

GC tuning responses showed considerable variability between the two consecutive and identical sequences of tastant deliveries, regardless of the protocol type or the probability with which tastants were accepted and ingested. That similar overall levels of variability were observed in the three protocols, despite the fact that the percentages of tastant rejection markedly differed among them, suggest that several factors contribute to the variability in chemosensory tuning.

### *4.1. Responses to tastants in animals unable to control the delivery of tastants*

We have previously explored two protocols in which food or water deprived rats controlled when they were going to obtain food. In the first, they were able to move to a sipper tube where they could freely lick for a tastant [18] and in the second they were restrained and had to press a bar to receive fluid, but did not know which particular fluid would be delivered [8]. In two aspects the stimulus-delivery protocols that we employed in awake and restrained rats contrast with those used in previous studies of GC activity. In those studies food- or water-deprived rats controlled the time of stimulus delivery by bar pressing or freely licking [8,9,14,21–23]. In the present study the subjects were neither water- nor food-deprived and the stimulus delivery was not contingent upon the subjects' behavior. The present results pertain to a novel behavioral phenomenon which describes, for the first time, in an awake, restrained, passively stimulated (by IOC) and potentially stressed animal model, dynamic GC responses to tastants throughout marked behavioral changes.

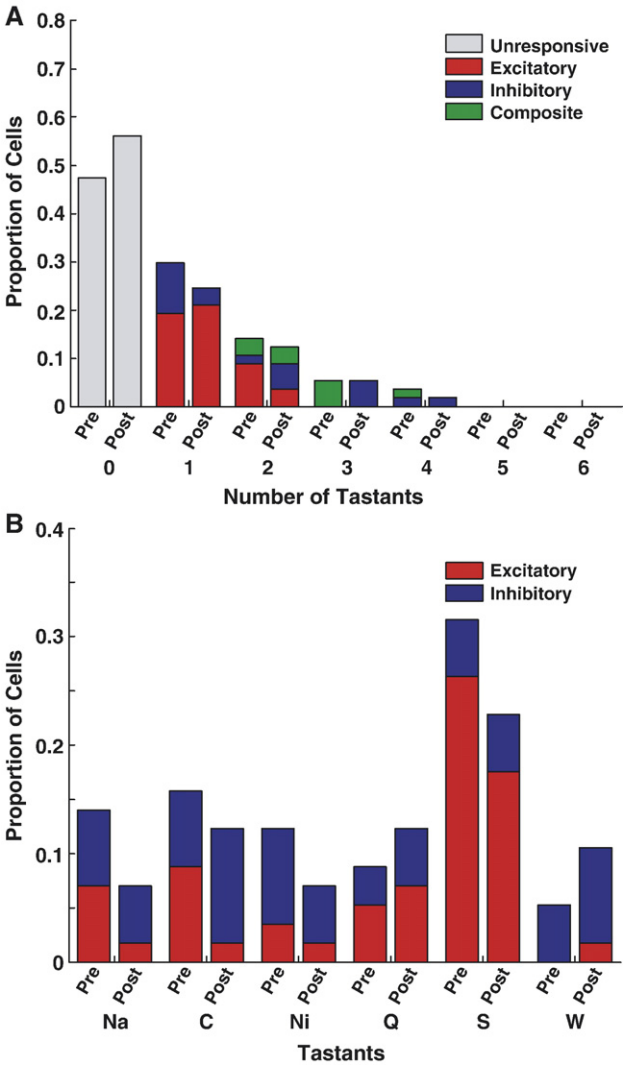


Fig. 7. A. Histograms of the proportion of cells responding to different numbers of tastants for both sequences. B. Histograms of the number of cells responding to the different tastants for both sequences. The color scheme used is identical to that of Fig. 6.

### *4.2. Changes in behavior throughout the experiment*

We found both similarities and differences between the 4TW and L-S protocols. In both these protocols the subjects exhibited

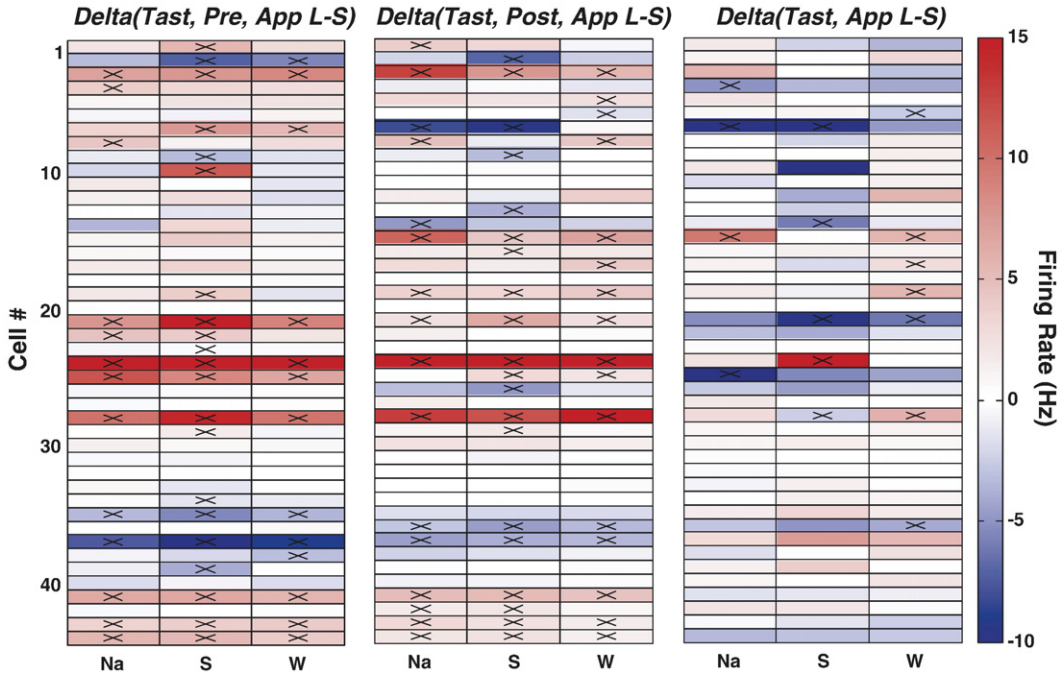


Fig. 8. Variability of the chemical tuning profiles in the Appetitive L-S protocol. The chemical tuning profiles of 44 GC neurons assessed in the PRE and POST sequences are presented (left and middle panels, respectively) as well as the inter-sequence response changes (right panel). The color scheme used is identical to that of Fig. 4.

qualitatively similar behavioral time courses, which were characterized by decaying exponentials with a constant term. Also, in both protocols following the 20-minute inter-sequence resting period, subjects essentially recovered from the PRE period and initially began to accept tastants again after the start of the POST period (Fig. 1A and B). Although this suggests that the subjects were initially similarly motivated to ingest the positively hedonic stimuli at the onset of both PRE and POST sequences, we found that, for both these protocols, the probability of observing appetitive trials decayed faster in the POST versus PRE sequence.

One rationale for this behavior is associated with the delivery of quinine which correlates with the appearance of rejective responses and thus could progressively degrade the overall motivation to ingest stimuli. We posit that during the PRE sequence subjects learned to anticipate receiving hedonically negative tastants, particularly quinine. (In brief access tests we found that quinine is more aversive than nicotine [28]). In the POST sequence, subjects therefore resorted to more quickly rejecting all stimuli as a strategy to avoid the incoming hedonically negative tastants. Such an explanation is consistent with the observation that the decay in appetitive behavior in the Appetitive L-S protocol, where hedonically negative tastants were not delivered, was both much slower and equivalent between the PRE and POST sequences (Fig. 1C), which is contrary to what was found in the 4TW and L-S protocols.

One important difference between the 4TW and L-S protocols was that, in the 4TW protocol, the animals still ingested the stimuli in 22–23% of the trials at the end of either the PRE or POST sequence. In contrast, at the end of either of the L-S sequences the animals rejected all of the stimulus trials regardless of whether they contained hedonically positive or negative tastants. We again suggest this to be in part due to a higher level of uncertainty about stimulus identity in the L-S versus the 4TW protocol.

### *4.3. What are the possible origins of the observed changes in chemical tuning?*

Previously, we found that GC responses were quite stable over a period of several hours when the subjects were permitted to freely lick to receive tastants [16]. Hence, it is unlikely that the response profiles of gustatory cortical neurons are always "noisy" and inherently variable. With respect to the modeling of the responses, the glm automatically corrects for potential differences in baseline firing rates that might occur across the sequences, so only genuine tuning changes were identified. Additional analysis confirmed that the chemical tuning changes were directly due to actual changes in evoked responses and were not the results of firing rate changes relative to a noisy baseline.

It is certain that some of the chemical tuning changes arise from the differences across sequences as the animals changed ingestion strategies for the PRE and POST sequences. That is, the observed inter-sequence behavioral differences provide a basis for some of the neuronal variability as different behavioral patterns will translate into distinct afferent activity patterns generated at the periphery which could lead to different chemical tuning patterns. Additionally, disengagement from a taste task has been shown to affect the tuning of GC neurons [22]. A comparison of the variability observed under the three protocols, however, suggests that more central mechanisms should also be considered. In fact, in the Appetitive L-S protocol no inter-sequence behavioral differences were detected (Fig. 1C). It

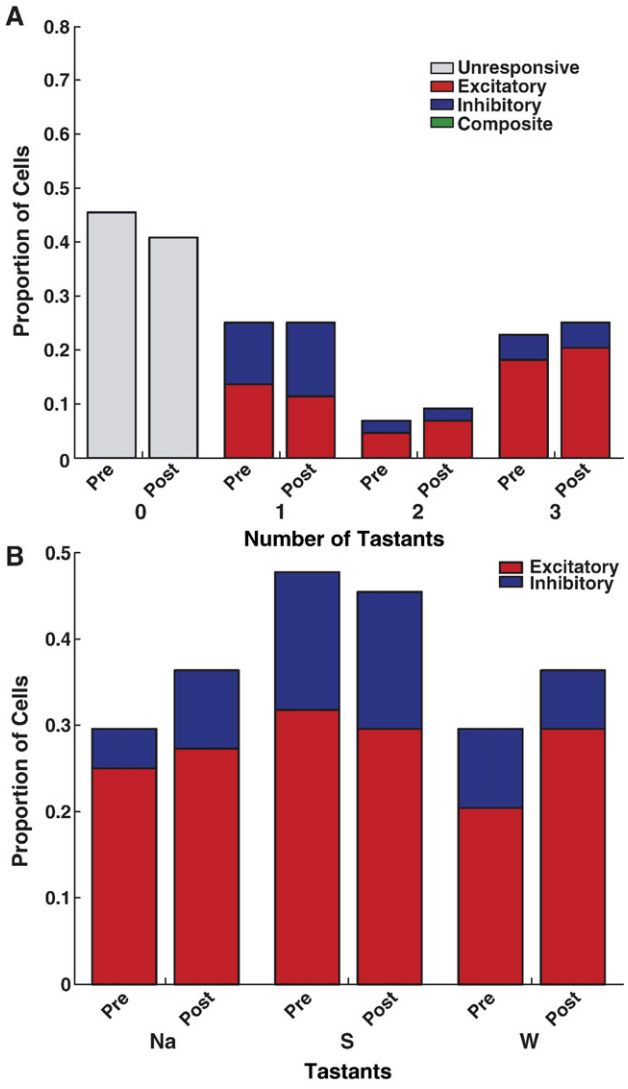


Fig. 9. A. Histograms of the proportion of cells responding to different numbers of tastants for both sequences. B. Histograms of the number of cells responding to the different tastants for both sequences. The color scheme is identical to that of Fig. 6.

follows that subjects' oral cavities were stimulated in a more consistent pattern than in the other two protocols. Nevertheless, this protocol still exhibited as high a level of overall neuronal variability as the 4TW and L-S protocols, with approximately 30% of neurons displaying changes in their chemical tuning properties. Since all three protocols exhibited the same extent of variability, some of the chemical tuning changes must have a common origin. That is, in all cases, the animals ingested tastants and water and for this reason we suggest that some of the tuning changes observed in all three protocols arise from evolving differences in the subjects' internals states over the course of each test session, in part due to processes involving feeding (sucrose, salt) and satiety. In this regard it has been shown that chemicals such as insulin [24,31], opiates [25], glucose [26,31] or glucagon [27] that are released throughout the feeding cycle may differentially affect taste responses throughout the gustatory axis.

Interestingly, we discovered that in those protocols that showed significant inter-sequence behavioral differences (4TW and L-S), chemosensory neurons exhibited much higher levels of variability than non-chemosensory neurons (chemosensory: approximately 50%; non-chemosensory: approximately 10%). In contrast, in the Appetitive L-S protocol, both neuronal populations displayed similar variability levels (approximately 25%). Given that larger alterations in ingestive probability (as seen in the 4TW and L-S protocols) corresponded to the higher variability levels of chemosensory neurons, we suggest that this pool of neurons strongly participates in, or is influenced by, the central processes involved in the dynamic representation of internal variables associated with feeding.

In summary, we have characterized for the first time, the behavioral and gustatory cortical time courses in an awake, restrained, non-contingently stimulated (by IOC) and potentially stressed animal model. We found that despite differences in behavioral responses in the three protocols tested, we observed similar and extensive inter-sequence variability in chemosensory GC responses. We attribute these chemical tuning changes to differences in areas of the oral cavity that are subjected to the tastants as the animals ingest or reject the tastants, and to changes in tuning properties that occur as a consequence of more central processes resulting from changes in the state of the animal [22] and to compounds that are released upon ingestion that can affect gustatory responses [24–27,31].

## Acknowledgements

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