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Making Time Count: Functional Evidence for Temporal Coding of Taste Sensation

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Abstract

Although the temporal characteristics of neural responses have been proposed as a mechanism for sensory neural coding, there has been little evidence thus far that this type of information is actually used by the nervous system. Here the authors show that patterned electrical pulses trains that mimic the response to the taste of quinine can produce a bitterlike sensation when delivered to the nucleus tractus solitarius of behaving rats. Following conditioned aversion training using either “quinine simulation” patterns of electrical stimulation or natural quinine (0.1 mM) as a conditioned stimulus, rats specifically generalized the aversion to 2 bitter tastants: quinine and urea. Randomization of the quinine simulation patterns resulted in generalization patterns that resembled those to a perithreshold concentration (0.01 mM) of quinine. These data provide strong evidence that the temporal pattern of brainstem activity may convey information about taste quality and underscore the functional significance of temporal coding.

Keywords

taste; temporal coding; nucleus of the solitary tract; conditioned taste aversion; electrical brain stimulation

The way in which sensory stimuli are encoded by neurons in the brain is one of the most basic questions in neuroscience. One mechanism that has received much attention in recent years is that of temporal coding, defined as the information contained in the temporal characteristics (e.g., spike timing, rate envelope, sequence of interspike intervals) of a stimulus-evoked spike train (see Hallock & Di Lorenzo, 2006; and Lestienne, 2001, for reviews). Most studies of temporal coding have shown that various temporal arrangements of spikes can convey information about a sensory stimulus but have not established that this information is actually used by the organism to generate a percept. However, a convincing demonstration of the functionality of temporal coding seems imperative for any assertion about the relevance of temporal coding in the nervous system. What kind of data would constitute such a demonstration?

One strategy that has been used is to correlate distinctive temporal patterns of sensory responses with behaviors indicative of specific sensory stimuli. For example, Glendinning, Davis, and Rai (2006) used this tack in a study of the *Manduca sexta* caterpillar. In that report, they showed that two stimuli that evoked responses in the primary taste cells differing only in whether the firing rate accelerated or decelerated over time were perceived as completely different. Furthermore, the pattern of generalization of habituation to a

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particular stimulus could be predicted solely on the basis of the temporal pattern of the evoked spike train.

Another strategy that has reaped positive results is based on the idea of driving the nervous system with electrical stimulation by replaying stimulus-specific temporal patterns of neural responses and examining the evoked behavior. In this context, the taste system is an attractive model for studying temporal coding because there are relatively few categories of similar tasting stimuli, called *qualities* (sweet, sour, salty, bitter, and possibly umami), and because the successful encoding of taste stimuli can often be gauged by both innate and learned behavioral reactivity. Taking advantage of these facts, Covey (1980) first recorded taste responses from the rat chorda tympani nerve, which innervates taste buds on the rostral 2/3 of the tongue. The temporal patterns of the evoked responses were then used to drive an electrical stimulator that delivered patterned electrical pulses to the chorda tympani of decerebrate rats. Remarkably, each of the temporal patterns of electrical stimulation associated with the various taste stimuli produced appropriate orofacial behaviors.

Building on the basic idea of Covey's work, we conducted a series of experiments on the nucleus tractus solitarius (NTS), the first synapse in the central gustatory pathway (Hamilton & Norgren, 1984). We began with a set of reasoned assumptions. In particular, we assumed that the temporal code for taste, if there is one, is contained in the temporal pattern of taste-evoked spike trains in individual cells, not in the average time course of response across cells. Although this may seem like an odd assumption given the plethora of evidence that population responses in other systems carry information, we hypothesized that averaging across many responses would obscure the information conveyed by spike timing in individual cells. In support of this argument, previous findings in the anesthetized rat showed that spike timing in individual cells does convey information about taste quality (Di Lorenzo & Victor, 2003, 2007; Roussin, Victor, Chen, & Di Lorenzo, 2008). It is also possible, perhaps likely, that the temporal characteristics of an individual neuron's response would share some common features with the temporal characteristics of responses in other neurons so that the response of an individual cell would contain and represent the essential information conveyed by spike timing in many cells. We also assumed that driving all the elements in a taste-related area (the NTS in our case) with this temporal pattern would stimulate the critical elements (admittedly along with many others) that can interpret the information; essentially, we assumed that there is some relationship, albeit undefined at this time, between a temporal code at the level of a single neuron's response and a temporal code at the level of the population response.

In our previous and present investigations, we delivered electrical pulse trains that were based on the NTS responses to natural tastes in intact behaving rats and tested the extent to which this stimulation was similar to a natural taste sensation (Di Lorenzo & Hecht, 1993; Di Lorenzo, Hallock, & Kennedy, 2003). We first showed that rats could learn to avoid lick-contingent NTS stimulation when it was paired with an intraperitoneal injection of LiCl in a conditioned aversion paradigm (Di Lorenzo & Hecht, 1993). In that study, a lick of water was followed by a 1-s electrical pulse train in which the temporal arrangement of pulses mimicked the sucrose response of an NTS neuron (called a *sucrose simulation* pattern). When the temporal pattern of electrical stimulation was changed from one that mimicked sucrose to one that mimicked quinine (called a *quinine simulation* pattern), rats avoided licking without any conditioned aversion training. Our most recent set of experiments supported and extended our previous findings (Di Lorenzo et al., 2003). In the first experiment, licking of water was suppressed when the lick-contingent pulse trains were based on two different single cell responses to quinine but not when the interpulse intervals contained in the pulse trains were randomly shuffled. In a second experiment, rats avoided lick-contingent electrical stimulation of the NTS that mimicked the temporal pattern of a

sucrose response following stimulation–illness pairings. It is important to note that this aversion generalized to natural sucrose but not to NaCl, HCl or quinine; extinction of the aversion to electrical stimulation also extinguished the aversion to sucrose.

Collectively, our experiments using electrical stimulation of the NTS have demonstrated that particular temporal patterns of electrical pulse trains (sucrose simulation pattern) could evoke a sweetlike sensation and that the precise temporal pattern of an electrical pulse train (quinine simulation pattern) was critical for evoking appropriate behavioral reactivity based on hedonic characteristics. However, the question of whether the temporal pattern per se of stimulation was critical for signaling taste quality remained open. That is, our previous work did not demonstrate unequivocally that the particular temporal pattern of pulses (and not, e.g., the frequency or length of the stimulation) was necessary to evoke sucroselike sensations; only that this particular pattern was sufficient. Furthermore, there were no data showing that the particular temporal order of pulses in the quinine simulation pattern evoked a bitterlike sensation; only that it was avoided as natural quinine would be. Whether the temporal sequence of pulses in the quinine simulation evoked a bitterlike taste sensation was not examined. Although the randomized quinine simulation pattern was not avoided in that experiment, it is still possible that the temporal pattern of pulses in the quinine simulation evoked sensations other than taste (e.g., tactile or thermal sensations; Ogawa, Hayama, & Yamashita, 1988; Ogawa, Imoto, & Hayama, 1984) and that the simulation pattern was avoided because of potentially unpleasant, but not tastelike, sensations rather than because it evoked a bitterlike taste sensation. The present series of experiments were designed to answer the question of whether the precise temporal order of pulses is both necessary and sufficient to evoke a tastelike sensation of an identifiable quality. Results showed that a conditioned aversion to a lick-contingent quinine simulation pattern of NTS stimulation, but not a conditioned aversion to its randomized counterpart, generalized specifically to bitter tastants.

General Method

Subjects

Fourteen male Sprague-Dawley rats (300–450 g) served as subjects for Experiment 1; 44 rats were used in Experiment 2. Rats were housed individually in plastic cages and maintained on a 12-hr light:dark schedule; lights on at 7:00 a.m. Rats had free access to food throughout the experiment, except for a half hour after each of the conditioned aversion acquisition sessions. Access to water was restricted according to the experimental paradigm described later. Rats were weighed daily to monitor their general health. All procedures were approved by the Binghamton University Institutional Animal Care and Use Committee and were in compliance with the National Institute of Health's Guide for the Use of Laboratory Animals.

Surgery

Rats were anesthetized with an intramuscular injection of a combination of ketamine (100 mg/kg) and xylazine (18 mg/kg) and mounted in a stereotaxic instrument. The skull above the NTS was then exposed. A stainless steel guide tube (24 gauge, thin wall) was positioned just above the taste-responsive portion of the NTS. One end of a stainless steel wire was soldered to the guide tube and the other end was crimped to a miniature Amphenol pin; this permitted the guide tube to serve as ground. The placement of the guide tube was determined by the presence of taste responses recorded from a tungsten microelectrode (18–20 M Ω ; FHC, Inc.) that was temporarily secured within the guide tube so that the tip protruded ~1.0 mm from the end. The guide tube was cemented in place when an electrophysiological response to NaCl (100 mM) bathed over the tongue was evident. The

tungsten microelectrode was then replaced with a bundle of three microwires used for electrical stimulation, as described later. Each stimulating electrode consisted of a 25- μ m diameter platinum-iridium wire, insulated except at its cross-section with formvar (California Fine Wire, Inc.). The entire assembly, along with several stainless steel screws anchored to the skull, was embedded in dental acrylic cement. Wound edges were treated with bacitracin ointment and closed with nylon suture. After surgery, rats were given an antibiotic, gentamycin (5 mg/kg sc) and an analgesic, Buprenex (0.05 mg/kg sc). Gentamycin was also administered the day after the surgery. Two weeks of recovery were permitted before behavioral testing. All rats regained or exceeded their presurgical weight by the end of the recovery period.

Apparatus

Experiments were conducted in a sound-insulated chamber. The test box was constructed of clear Plexiglas (10 cm $\times$ 20 cm $\times$ 12 cm) with a stainless steel floor. A slit on the top of the box allowed the cable from a computer to be attached to the plug on the rat's head. A graduated centrifuge tube fitted with a rubber stopper and stainless steel drinking tube was mounted so that the tip of the drinking tube was positioned 1.6 cm from a teardrop-shaped opening on one end of the box. The bottom of this opening was located 6.5 cm from the floor of the box and extended 4 cm vertically. At the widest part of the bottom, it measured 0.9 cm across; at the top, it measured 1.6 cm across. The number of licks was recorded through a low-current (1 μ A) lickometer circuit that utilized the drinking tube as one pole and the stainless steel floor for the other (Weijnen, 1989).

Screening

Before any experimental manipulations, rats were trained to lick water in the experimental chamber for 10 min/day and then screened for reactivity to the presentation of lick-contingent electrical stimulation. Previous work has shown that this sort of screening is a good predictor of the location of the stimulating electrodes in the taste-responsive portion of the NTS (Di Lorenzo et al., 2003). Screening also enabled the choice of the electrode to be used in subsequent portions of the experiment. In total, 122 rats were screened, but only 58 showed appropriate behavioral reactivity. The rats that failed the screening test most commonly showed no reaction to the stimulation even when the current was very high.

After recovery from surgery, rats were placed on a water deprivation regimen of 22 hr/day. One hour after each daily 10-min session, during which water was available in the experimental chamber, rats were given free access to water for 1 hr in their home cages. Daily experimental sessions were given for approximately 10 days until the number of licks per day was stable.

For screening, each animal was placed in the experimental chamber with water available as usual. However, on the screening day, licking resulted in the delivery of 1-s electrical pulse trains through one of the three implanted electrodes. Only complete 1-s pulse trains were given; licks that occurred during the stimulation train were counted but had no effect on the progress of the pulse train. The Q1 pattern (see Figure 1) was used for screening because previous work has shown that it is effective in producing avoidance (Di Lorenzo & Hecht, 1993; Di Lorenzo et al., 2003). Each electrode was tested separately for 1 min beginning with the first lick. Current levels were gradually increased up to a maximum of 200 μ A until the rat was observed to react to the stimulation. Reactivity consisted of behaviors such as gapes, paw pushing, head shakes, and tongue protrusions. These behaviors are typical signs of rejection (Grill & Norgren, 1978). Rats with positive signs of reactivity also avoided further licking. The electrode that was associated with the strongest signs of reactivity at the lowest current level was chosen for later stimulation during further experimental sessions.

Subsequent electrical stimulation was delivered through that electrode at the same current level determined by the screening procedure.

All behavioral procedures began 2 days after screening. Only water was given during the 10-min sessions on these days.

Electrical Pulse Trains

In constructing each pulse train, electrophysiological responses to quinine recorded in the NTS of anesthetized rats (Di Lorenzo & Monroe, 1995; Di Lorenzo & Victor, 2007) were used as templates. All of these units showed robust quinine responses. The units used for quinine simulation patterns, called Q1 and Q2, were the same as those used in previous work (Di Lorenzo et al., 2003). Both showed higher firing rates in response to HCl than to sucrose, NaCl, or quinine. That is, they were HCl best. The units chosen as templates for quinine simulation patterns called Q3 (quinine best), Q4 (NaCl best), and Q5 (NaCl best) were selected because of their vigorous responses to quinine, which are relatively rare in the anesthetized rat. Table 1 lists some basic information about the units that were used to construct the quinine simulation patterns. Each quinine response that served as a template for a quinine simulation pattern was recorded from a different rat. For each response, the time of occurrence of each spike within the first 1.0 s of response was recorded. These spike times were then used to drive an electrical stimulator (Grass S88X equipped with a SIU-BI optically isolated biphasic constant current unit, Astro-Med, Inc.). Biphasic pulses were 0.1 ms in length to minimize tip damage with repeated stimulation.

Randomized counterparts of the experimental temporal patterns were constructed to serve as controls for the temporal order of pulses. Interspike intervals were extracted from the experimental patterns and shuffled to create patterns with identical numbers of pulses but containing different temporal arrangements. Although each temporal pattern had a discrete beginning and end, rats were stimulated continuously during sustained bouts of licking. As such, we wanted to ensure that the randomized control patterns would not correlate with experimental patterns during any interval of sustained stimulation in which patterns would loop (e.g., the second half of a randomized pattern pulse train and the beginning half of the next pulse train correlating with the experimental pattern). As an example, a quinine pattern with 22 interspike intervals would have a randomized control counterpart that also had 22 interspike intervals. Thus, 22 staggered correlations were used to ensure that the randomized pattern was dissimilar to the experimental pattern at every point during a period of continuous stimulation.

Figure 1 shows the patterns of pulse trains for all experiments (Figure 1A), as well as the associated interpulse interval histograms (Figure 1B).

Histology

At the end of each experiment, we reconstructed recording sites using standard histological techniques. Briefly, rats were sacrificed with an overdose of sodium pentobarbital and perfused transcardially with isotonic saline and formol-saline. The brains were then removed and stored in formol-saline for several days. Serial frozen sections (40 μ m) through the NTS were mounted on gelatinized slides and stained with cresyl violet. To verify that repeated electrical stimulation did not degrade the tips of the wires and/or produce gliosis, electrolytic lesions were not produced before sacrifice.

Experiment 1A: Screening Quinine Simulation Patterns for Behavioral Avoidance

Procedure—Two days after screening, each of 8 rats was presented with one of five patterns of lick-contingent electrical pulse trains (Q1 to Q5) based on five different NTS

responses to quinine for the entire 10-min drinking session and the number of licks was recorded. The randomized counterparts of Q1 to Q5 are referred to as Q1r to Q5r. Q1, Q1r, Q2, and Q2r were identical to the quinine simulation patterns used in previous work (Di Lorenzo et al., 2003). All rats received the Q1 and Q1r simulation patterns. However, rats were tested with only two additional quinine simulation patterns and their randomized counterparts. When a quinine simulation pattern was tested, its randomized counterpart pattern was always also tested on a separate day. Days when water with electrical stimulation was presented were alternated with days when only water was presented.

Results—Results showed that rats avoided licking water only when licking produced any of three of the five quinine simulation patterns. When the sequence of interpulse intervals in these “effective” pulse trains was randomly shuffled, rats licked normally. Likewise when the two “ineffective” quinine simulation patterns or their randomized counterparts were presented, rats showed as many licks as they did without any electrical stimulation. The number of licks shown to Q1–Q3 were significantly lower (all p s < 0.2) than the number of licks shown to all other temporal patterns of stimulation. The number of licks shown to Q4 and Q5 were not significantly different than the number of licks shown to their randomized counterparts, Q4r and Q5r, respectively (p s > 1). A one-way analysis of variance (ANOVA) showed a main effect of stimulation pattern; $F(9, 32) = 5.15$, $p < 0.01$. We conducted post hoc pairwise tests using Fisher's least significant difference. Figure 2 shows the mean number of licks for each pattern of lick-contingent electrical stimulation.

Experiment 1B: Creating and Testing a Composite Quinine Simulation Pattern

Procedure—Our next step was to create and test a pattern of electrical stimulation that captured the common features of the effective quinine simulation patterns. This composite pattern and its randomized counterpart are shown in Figure 1.

The composite quinine simulation pattern of electrical stimulation was constructed on the basis of the assumption that the temporal pattern of pulses in each or all of the effective quinine simulation patterns (Q1–Q3) identified in Experiment 1A conveyed critical information used by the rat to produce rejection. Because the number and temporal arrangement of pulses in these three quinine simulation patterns were obviously different, we hypothesized that each pattern contained both essential temporal sequences of pulses (sequences that were informative to the rat), as well as nonessential temporal sequences of pulses (sequences that were basically “noise”). Our goal in composing a composite temporal pattern of pulses was to identify and combine these essential sequences into a single temporal pattern of electrical pulses. To do this, we derived a “consensus sequence” by first aligning the three quinine simulation patterns to each other and identifying pulses (and their timing) that were common to at least two of the three patterns. Because each pattern began with a different temporal sequence of pulses, it was not obvious where the informative portion of the 1-s simulation pattern began. For example, it was possible that the first four or five pulses in Q1 were irrelevant noise but only the first two pulses in Q2 were noise and the informative temporal sequence(s) might begin at the first pulse in Q3. To maximize our chances of incorporating the critically informative temporal sequences as part of our consensus pattern, we first identified a distinctive temporal “feature” (e.g., some interpulse interval or short sequence of interpulse intervals) that all three of the effective quinine simulation patterns shared. For the quinine simulation patterns Q1–Q3, a pair of pulses separated by 8–9 ms occurred within the initial 108 ms of all three responses. The three quinine simulation responses were then realigned so that each response began with that pair of pulses. The composite pattern was constructed by including only those pulses that occurred in at least two of the quinine simulation patterns within 10 ms of each other.

Six rats that had no experience with any quinine simulation pattern were used in this experiment. Two days after screening, each rat was presented with either the lick-contingent composite pattern of electrical stimulation or its randomized control pattern (3 rats for each pattern) for their 10-min drinking session, and the number of licks were recorded. Two days after that session, rats received whichever pattern they had not received earlier during their drinking session. Three rats were tested with the composite pattern first, and the other 3 rats were tested with the randomized composite pattern first.

Results—The results of tests using lick-contingent presentation of the composite pattern (shown in Figure 3) confirmed that rats avoided licking water when the composite quinine pattern of electrical stimulation was presented but not when the randomized composite quinine pattern was presented, Student's paired t test, $t(5) = 4.24$, $p < 0.01$.

Experiment 2: Generalization of an Aversion to the Quinine Simulation Patterns of Electrical Stimulation to Natural Tastants

Procedure—Forty-four rats were used in this experiment; 26 of these were implanted with electrodes for electrical stimulation of the NTS. Surgical preparation, initial behavioral training, and screening were identical to those in Experiment 1.

Eight groups of rats were used in this experiment. Rats in the composite group ($n = 8$) were trained to avoid a lick-contingent 1.0-s train of electrical pulses that was derived from Experiment 1. Rats in the randomized composite group ($n = 5$) were trained to avoid a randomized pattern of electrical stimulation that contained the same number and complement of interpulse intervals as the composite pattern. Rats in the Q1 group ($n = 7$) were trained to avoid a lick-contingent 1.0-s train of electrical pulses that was demonstrated to produce avoidance in previous experiments (Di Lorenzo & Hecht, 1993; Di Lorenzo et al., 2003). Rats in the Q1r group ($n = 6$) were trained to avoid a randomized pattern of electrical stimulation that contained the same number and distribution of interpulse intervals as the Q1 pattern. However, the order of presentation was randomized. Three groups of unoperated rats were also included: the 0.1-mM ($n = 6$) and 0.01-mM ($n = 6$) quinine groups were trained to avoid licking 0.1- or 0.01-mM quinine, respectively, and an unoperated naïve control group ($n = 6$) was trained to lick water and then tested with all the natural tastants as in the generalization tests described later. The concentration of 0.01-mM quinine was chosen because it is just about at the recognition threshold for rats (Koh & Teitelbaum, 1961; Shaber, Brent, & Rumsey, 1970; St. John & Spector, 1996; Thaw & Smith, 1994). The higher concentration of quinine (0.1 mM) is a log step above the lower concentration and is clearly above threshold but not so high as to produce complete avoidance without training (Markison, St. John, & Spector, 1999). A final group, the histological control group ($n = 9$), was derived following histological analyses of electrode placements. Rats in this group had electrodes with tips that were at least 150 μ m from the border of the NTS. Data from rats in the histological control group were analyzed separately from other experimental and control groups.

On the first day of conditioned aversion training, either lick-contingent NTS stimulation (composite, randomized composite groups) or a quinine solution (0.1-mM quinine and 0.01-mM quinine groups) was presented for the 10-min drinking session, and the number of licks was recorded. Immediately after the training session, all rats in these groups were given an injection of LiCl (0.3 M ip, 1% body weight). We used this dosage of LiCl to ensure the rapid acquisition of an aversion (Nachman & Ashe, 1973; Shumake, Sterner, Gaddis, & Crane, 1982). Training sessions were given every other day; water without stimulation was presented on the intervening days.

On the day that the number of licks with electrical stimulation fell below 150, a series of 1-min exposures to five tastants and water was presented. These stimuli were available in the following order: quinine (.025 mM), NaCl (25 mM), HCl (2.5 mM), urea (0.1 M), sucrose (125 mM), and plain water. For quinine, NaCl, HCl, and sucrose, the concentrations were those used previously (Di Lorenzo et al., 2003) and were intentionally weak. The concentrations of the bitter substances, quinine and urea, were intentionally weak to ensure that the rats were willing to drink them but strong enough so that they could be identified as bitter. Little is known about the relationship between concentration and ingestion for urea in rats. In the present experiment, we hypothesized that, like quinine, the concentration of urea that evokes an electrophysiological response in the NTS would be much higher than the concentration that would be behaviorally acceptable. Thus, the concentration of urea was 1 log unit below the concentration used for electrophysiological studies of taste responses in the NTS (e.g., Roussin et al., 2007). Each 1-min exposure was begun when the rat took the first lick. This ensured that the rat sampled every tastant presented. Naïve control rats were trained to lick in the experimental chamber for 10 days and then tested with the natural tastants as in the generalization test for conditioned rats. For all electrical stimulation groups, the conditioned aversion was extinguished by repeated presentation of the lick-contingent electrical stimulation without subsequent LiCl injections every other day. For the 0.01-mM and 0.1-mM quinine groups, quinine at the same concentration used for conditioned aversion training was presented without subsequent LiCl injections every other day. For all groups, water without stimulation was presented on the intervening days. The day after the number of licks with electrical stimulation (or number of licks with the conditioned concentration of quinine as appropriate) were within 90% of pretraining rates, the natural tastants were presented a second time as described earlier. We conducted two-way ANOVAs with stimulus and group as factors and licks as the dependent variable to compare the patterns of generalization and extinction across groups. An alpha level of 0.05 was used for these analyses.

To assess generalization of conditioned aversions to particular taste stimuli, we calculated a lick suppression ratio as follows: $\text{Suppression ratio} = (1 - [\text{number of licks for a taste stimulus during the generalization test} / \text{mean licks for the same stimulus in the naïve control group}]) \times 100$. The mean number of licks in the naïve control group was used as a benchmark, as none of the rats in the electrical stimulation groups were conditioned with a natural stimulus. This measure is similar to that used by Frank, Formaker, and Hettinger (2003). A value of 100 indicated total lick suppression, implying complete generalization of a conditioned aversion; a value of 0 indicated no suppression and no generalization. We then analyzed suppression ratios with a one-sample Student's *t* test to determine whether they were different from zero (no suppression). Considering the large number of comparisons to which this test was applied ($6 [\text{groups}] \times 5 [\text{taste stimuli}]$), an alpha level of 0.01 was used as a criterion for significance to minimize Type I error.

Results—All groups of rats that were conditioned to avoid either patterned electrical stimulation or natural tastants learned the aversion. Groups that were conditioned to avoid electrical stimulation took more acquisition trials to learn the aversion. The median number of trials to meet criterion were as follows: For composite, $Mdn = 6$; for randomized composite, $Mdn = 5$; for Q1 and Q1r, $Mdn = 4$; for 0.01- and 0.1-mM quinine, $Mdn = 3$. Figure 4 shows the mean proportion of licks shown for each conditioned stimulus (either lick-contingent electrical stimulation or natural quinine) plus or minus the standard error of the mean for all groups before any conditioning. A one-way ANOVA showed that there were no significant differences among groups in this measure, $F(4, 23) = 1.86, p > .15$.

Our first hypothesis was that the generalization patterns across tastants shown by the composite and Q1 groups would not differ. Statistical analyses supported this hypothesis; a

two-way ANOVA showed a significant effect of stimulus, $F(4, 40) = 5.87, p < .001$; but no significant effect of group, $F(1, 40) = 0.72, p = .40$; and no significant Stimulus $\times$ Group interaction, $F(4, 40) = 0.19, p = .94$. On the basis of this result, subjects in these groups were pooled in subsequent analyses. Similarly, we hypothesized that the randomized composite and Q1r groups would not differ on the basis of the idea that randomization would obscure the quality-specific information conveyed by the temporal patterns of the composite and Q1 pulse patterns. Statistical analyses also supported this contention; a two-way ANOVA showed a significant effect of stimulus, $F(4, 25) = 22.46, p < .001$; but no significant effect of group, $F(1, 25) = 3.45, p = .08$; and no significant Group $\times$ Stimulus interaction, $F(4, 25) = 2.67, p = .06$. Subjects in the randomized composite and Q1r groups were therefore pooled in subsequent analyses.

Next, the pooled quinine simulation group was compared with the pooled randomized group. A two-way ANOVA showed a significant effect of stimulus, $F(4, 75) = 16.53, p < .001$; and group, $F(1, 75) = 10.81, p = .002$; but no significant Group $\times$ Stimulus interaction, $F(4, 75) = 1.78, p = .14$. Inspection of the pattern of generalization shown by these groups (see Figure 5) revealed the basis for the lack of a significant Group $\times$ Stimulus interaction: Licking for quinine and urea was suppressed for all groups, and licking for sucrose was near normal. However, in the randomized quinine simulation group, licking for NaCl (Student's t test, $p < .05$), HCl (Student's t test, $p < .05$), and urea (Student's t test, $p < .01$) was suppressed, compared with the quinine simulation group (see Figure 6). In effect, the quinine simulation group specifically generalized a conditioned aversion to quinine and urea, the two bitter tastants, whereas the randomized quinine group generalized a conditioned aversion to all tastants except sucrose.

We then compared these results to patterns of generalization when natural quinine served as a conditioned stimulus. Results confirmed that the pooled quinine simulation group did not differ from the 0.1-mM quinine group; a two-way ANOVA showed a significant effect of stimulus, $F(4, 70) = 9.17, p < .001$; but no significant effect of group, $F(1, 70) = 2.50, p = .12$; or any significant Group $\times$ Stimulus interaction, $F(4, 70) = 0.66, p = .62$. In addition, the pooled randomized quinine simulation group did not differ from the 0.01-mM quinine group; a two-way ANOVA showed a significant effect of stimulus, $F(4, 55) = 22.90, p < .001$; but no significant effect of group, $F(1, 55) = 0.06, p = .81$; or any significant Group $\times$ Stimulus interaction, $F(4, 55) = 0.54, p = .71$.

We also compared the histological control group to the naïve control group with a two-way ANOVA and found a significant main effect of group, $F(1, 70) = 5.20, p = .03$; but no significant effect of stimulus, $F(4, 70) = 1.92, p = .12$; or any Group $\times$ Stimulus interaction, $F(4, 70) = 1.093, p = .37$. Fewer licks overall in the histological control group, compared with the naïve control groups, accounted for the group effect (see Figure 7).

For extinction, our hypothesis was that none of the groups would differ from each other or from the naïve control group. However, a two-way ANOVA revealed a significant main effect of both group, $F(7, 175) = 3.90, p = .001$; and stimulus, $F(4, 175) = 12.763, p < .001$; but no Group $\times$ Stimulus interaction, $F(28, 175) = 0.91, p = .61$. Post hoc comparisons using the Bonferroni correction showed that the 0.01-mM quinine group differed from the composite ($p = .02$), randomized composite ($p = .026$), and naïve control ($p = .001$) groups, but no other group differences were significant (see Figures 4 and 5). These differences were most likely due to a failure to fully recover from an aversion to urea (see Figure 6). This result is not unexpected, given the fact that quinine, not urea, was presented in extinction trials.

To determine which stimuli produced lick suppression after conditioned aversion training, we calculated a lick suppression ratio, as described earlier. Results showed that the pattern of generalization of avoidance (the particular complement of tastants that were avoided) was different for quinine simulation and randomized simulation groups (comparison of mean suppression ratios in the pooled quinine simulation group vs. the pooled randomized simulation group), $\chi^2(4) = 663.8$, $p < .01$. Suppression ratios for each of five stimuli in each of the four electrical stimulation groups were analyzed with one-sample Student's *t* tests to determine whether they were significantly different from zero (no suppression); an alpha level of 0.01 was imposed to determine significance, given the large number of comparisons. Results showed that the composite and Q1 groups avoided quinine ($ps < .008$) and urea ($ps < .002$) but not NaCl ($ps > .07$), HCl ($ps > .04$), or sucrose ($ps > .02$). In contrast, the randomized composite group avoided all tastants (all $ps < .009$) except sucrose ($p > .29$). The Q1r group was similar to the randomized composite group, except that it did not significantly avoid NaCl ($p = .016$). Figure 8 shows the average suppression ratios for each stimulus for each group.

Finally, we examined the number of licks that were shown during baseline (before any conditioned aversion training), on days between conditioning sessions, and on days between extinction sessions for all groups (composite, randomized composite, Q1, Q1r, 0.01-mM quinine, 0.1-mM quinine). Two-way ANOVA showed a significant effect of both group, $F(5, 71) = 5.10$, $p < .001$; and time, $F(2, 71) = 19.61$, $p < .001$; but no significant Group $\times$ Time interaction, $F(10, 71) = 1.25$, $p = .28$. Bonferroni post hoc pairwise comparisons showed that the 0.1-mM quinine group licked more water on water-only days than any other group ($ps < .03$). No other group differences were found. (In addition, the number of licks shown during baseline $>$ extinction $>$ acquisition periods, $ps < .03$.)

Histological Results—The locations of the tips of the stimulating electrodes for all implanted rats are shown in Figure 9. It can be seen that the electrodes were placed in or near the rostral NTS for all experimental groups. Electrode placements were scattered across both the medial-lateral and rostro-caudal extent of the rostral NTS. A few placements were below the NTS in the reticular formation but were within $\sim 200 \mu\text{m}$ of the border of the NTS. Four electrode placements in the histological control group were either lateral or ventral to the NTS (see Figure 8D). In 3 rats in this group (not shown), the electrode tips were ventral to the NTS, but the guide cannula had essentially produced a lesion of the NTS. In the remaining 2 rats (not shown), the electrode tips were located $\sim 1 \text{ mm}$ caudal to the taste-responsive portion of the NTS and $\sim 0.5 \text{ mm}$ ventral.

Discussion

Results of these experiments demonstrate that information contained solely in the temporal pattern of activity can evoke a taste perception with an identifiable quality. These data speak directly to the functionality of temporal coding in the nervous system by demonstrating that a specific perception can be generated by microstimulation and that the identity of this percept is dependent on the temporal sequence of pulses in the train of stimulation. These findings may have broad implications for the design of sensory prostheses and brain-machine interfaces. They also provide support for the taste system as a model for investigation of temporal coding.

Collectively, data from Experiments 1 and 2 showed that the temporal patterns of taste-evoked spike trains in single cells in the NTS convey enough information to evoke a specific and predictable taste sensation along with stimulus-appropriate behavioral reactivity. In particular, rats in Experiment 1 avoided lick-contingent pulse trains that shared the same temporal pattern as that of spike trains evoked by quinine recorded from single cells in the

NTS. Furthermore, rats also avoided a composite pulse train that combined the shared temporal features of effective quinine-evoked spike trains, suggesting that there may be a “universal” temporal code for quinine in the NTS. In support of this notion, the results of Experiment 2 showed that the shared temporal characteristics of responses to quinine evoked a bitter sensation, as evidenced by the specific generalization of composite and Q1 aversions to bitter taste stimuli. The pattern of generalization to these temporal sequences of NTS stimulation was indistinguishable from the generalization pattern after a conditioned aversion to 0.1 mM quinine. Conversely, the pattern of generalization of a conditioned aversion to the randomized composite or Q1r sequences of electrical stimulation was distinctly different from that produced by the composite and Q1 temporal sequences. This underscores the assertion that the temporal sequence of pulses per se is both necessary and sufficient for evoking a bitter sensation. The pattern of generalization across tastants after a conditioned aversion to the randomized composite or Q1r pulse trains (i.e., avoidance of all natural tastants except sucrose) was identical to the pattern of generalization after a conditioned aversion to a perithreshold concentration of quinine (0.01 mM), suggesting that the randomized composite and Q1r pulse trains and the very low concentration of quinine evoked taste sensations that were difficult to identify. The fact that sucrose was singularly not avoided may derive from its unique ability to drive overconsumption.

Data from the present experiments are a natural extension of previous findings with similar techniques (Di Lorenzo & Hecht, 1993; Di Lorenzo et al., 2003). That is, previous work demonstrated that a temporal sequence of pulses based on a single cell's response to quinine produced avoidance but that its randomized counterpart did not. This suggested that the temporal pattern of NTS stimulation was important for evoking behavior that was appropriate to the sensation that it was intended to evoke (i.e., bitterness). However, those data did not show that the rat was experiencing bitterness; only that it was avoiding the lick-contingent stimulation as though it were bitter. Here we showed that these quinine-based temporal patterns of pulse trains actually evoked bitter sensations to the extent that both quinine simulation pulse trains and natural bitter tastants were treated identically in generalization tests of conditioned aversions. The demonstration that conditioned aversions to quinine simulation patterns of electrical stimulation generalized not only to quinine but also to urea, another bitter substance, suggests that the quinine simulation patterns evoked a sensation of a specific taste quality, not just a specific stimulus. These results buttress previous data showing that a conditioned aversion to a sucrose simulation pattern (constructed similarly to the quinine simulation patterns but using a response to sucrose instead) generalized to natural sucrose but not to NaCl, HCl, or quinine. Together, these data strongly suggest that the temporal pattern of NTS stimulation conveys quality-specific information.

Our data also extend observations that spike timing of taste responses in the NTS can convey information about taste quality (Di Lorenzo & Victor, 2003, 2007), especially when taste stimuli evoke similar taste qualities (Roussin et al., 2008). Present data imply that spike timing cannot only be used to discriminate among tastants of different qualities but can actually convey identifying features of a tastant.

Comparison of Quinine Simulation and Composite Quinine Simulation Patterns

Data from Experiment 1A showed that not every quinine simulation pattern evoked rejection when paired with licking water. In particular, Q1–Q3 produced avoidance but Q4 and Q5 did not. These results beg the question of what distinguished the effective quinine simulation patterns (Q1–Q3) from the ineffective patterns (Q4–Q5). Examination of Table 1 shows that all quinine simulation patterns were based on NTS cells that responded well to quinine and were relatively broadly tuned across tastants (Uncertainty measures close to 1.0). However, Q1–Q3 were based on quinine responses in NTS cells that responded best to HCl or quinine,

whereas the other, ineffective quinine simulation patterns were based on cells that responded best to NaCl. Because HCl and quinine are sometimes confused (Nowlis, Frank, & Pfaffmann, 1980), it is possible that HCl- and quinine-best cells are specialized to identify quinine, thereby making their quinine responses more informative about the presence of quinine than quinine responses in NaCl-best cells. Conversely, NaCl-best cells may be specialized to convey information only about NaCl so that their quinine responses are less informative, at least with respect to their temporal pattern of response. Related to this point are results showing that NaCl-best cells are specifically affected by experimental manipulations designed to affect NaCl perception, such as Na⁺ deprivation (Jacobs, Mark, & Scott, 1988) or application of amiloride, a Na⁺ channel blocker (St. John & Smith, 2000). Whether the best stimulus of an NTS cell was the critical distinguishing feature that accounted for differences in the effectiveness of the various quinine simulation patterns remains to be determined. However, the observation that only a subset of quinine simulation patterns were effective suggests that not every cell's response to quinine contains information in its temporal pattern that is relevant to provoking avoidance. Results from studies in anesthetized rats showing that temporal coding is used to distinguish among taste qualities by about half of the cells in the NTS (Di Lorenzo & Victor, 2003, 2007) are consistent with these observations. Moreover, there may be other mechanisms, such as cooperative firing among ensembles of cells, that may be important for a more complete message to be conveyed. This mechanism is evident in recordings from the gustatory neocortex (Katz, Simon, & Nicolelis, 2002). In that case, the "ineffective" quinine simulation patterns may not have contained quite enough information on their own to trigger an avoidance response.

In Experiment 1B, the composite quinine simulation pattern, constructed from the common temporal features of the three effective quinine simulation patterns, was also effective in producing avoidance. In Experiment 2, this pattern was also shown to evoke a bitter sensation. The implications of these results are twofold. First, they suggest that individual quinine responses such as those used in Experiment 1A are "noisy" in that they contain spikes with timing that may not be informative. Second, these results imply that there may be a common temporal pattern that is associated with quinine (or bitterness) in which each individual response only partially participates. The composite pattern may have served as a closer approximation of this ideal temporal pattern of activity than any of the three individual quinine responses. Presumably, an even better approximation may be gleaned from more exemplars of effective quinine responses.

It is reasonable to question what aspects of these seemingly idiosyncratic temporal patterns convey the information needed to make the appropriate behavioral choices. Examination of Figure 1A provides little help in this regard, because there is seemingly no discernible pattern to the pulse trains that can distinguish one from another. Examination of Figure 1B shows that the interpulse interval distributions for the effective quinine simulation patterns, Q1-Q3 and the composite pattern, all show the majority of interpulse intervals between 8 and 32 ms. This implies that pulse frequencies between 31 and 125 Hz were most effective. However, the fact that the randomized counterparts to each of these quinine simulation patterns produced very different behavioral consequences than the original patterns implies that the relevant information is carried in the order of interpulse intervals and/or the timing of the pulses, presumably with respect to the beginning of a lick. In this regard, quantitative analyses of the temporal characteristics of NTS taste responses in anesthetized rats suggests that spike timing within a response, not the sequence of interspike intervals, conveys a significant amount of information about taste quality (Di Lorenzo & Victor, 2003, 2007; Roussin et al., 2008).

Differences Between Quinine Simulation Patterns and Natural Quinine Groups

Natural quinine and quinine simulation patterns of electrical stimulation may have evoked similar taste sensations but with different intensities. Specifically, the fact that the electrical stimulation was unilateral, relatively brief, anatomically limited, and without any olfactory or postingestional cues suggests that it probably evoked a weak sensation. This could account for differences between the electrical stimulation and the natural tastant groups in the number of acquisition trials (fewer for natural quinine) and the intensity and specificity of the aversion to real quinine. In addition, the electrical pulse trains may have evoked sensations in addition to bitterness. In this regard, there are cells in the taste-responsive portion of the NTS that respond well to tactile and thermal stimulation (Ogawa et al., 1984, 1988). Thus, electrical stimulation of all cells in the vicinity of the electrodes in the Q1 and composite groups may have produced more pronounced tactile and thermal sensations than one might expect natural quinine to produce. On the other hand, the randomized electrical patterns would also be expected to drive tactile and thermal responses; however, rats that were trained to avoid these patterns did not show specific aversions to bitter tastants.

It is possible, although unlikely, that the order of stimulus presentation in the generalization tests affected the pattern of cross-generalization. As described in General Method, tastants in the generalization tests were always presented in the same order. A weak quinine solution was presented first when the rat was most motivated to drink and before NaCl to avoid the potential bitter-suppressing effects of NaCl–quinine mixtures that have been noted in the literature (Keast, Canty, & Breslin, 2004). In general, the effects of potential mixture interactions (derived from the idea that rats retained the taste of one tastant when they first licked the next) on the cross-generalization results have been discussed in detail (Di Lorenzo et al., 2003). Briefly, two compelling observations argue strongly that order effects cannot account for the present results: First, rats in control groups drank all tastants with the same avidity; second, rats in those groups that cross-generalized to bitter tastants recovered their licking after extinction of the conditioned aversion.

Necessity for Histological Precision

It is interesting to note that rats in which the electrodes were far from the NTS did not generalize conditioned aversions to bitter tastants. In fact, although rats in the histological control group learned to avoid lick-contingent patterned electrical stimulation, none of them generalized that aversion to any of the natural tastants. Even so, they did not lick the natural tastants to the same degree as the naïve control group. This suggests that some generalized suppression of licking, perhaps due to enhanced neophobia or a mild place aversion, was produced by the conditioned aversion training. Recovery of licking after extinction supports this contention.

A Functional Role for Temporal Coding in the NTS

It is not unreasonable to ask the question of how a temporal sequence of pulses that presumably drives a relatively large area of the NTS can produce predictable behaviors and taste sensations. Both previous (Di Lorenzo et al., 2003; Di Lorenzo & Hecht, 1993) and present data point to the idea that the temporal sequence of spikes in the response from a single cell contains all the necessary information for these effects. However, as discussed earlier, responses from different cells may express an approximation of the ideal temporal pattern, but the collective activity of numerous cells may be necessary for a perfect behavioral response and specific percept. On the other hand, it has been shown that the activity of a single cortical cell can suffice as a guide to behavior (Houweling & Brecht, 2008).

The observation that the temporal pattern of spikes in the response of a single cell can convey such a rich trove of information suggests that the neural code for taste in the NTS is quite sparse. This idea would certainly be consistent with the fact that the gustatory portion of the NTS contains a relatively small number of cells that are available for conveying information. In spite of the paucity of neural coding elements in the NTS, these cells are charged with multiplexing information about taste quality, intensity (concentration), and hedonic value (pleasure or disgust). Present results provide functional evidence that support previous studies showing that temporal patterns of response in individual cells can convey information about taste quality. Whether the temporal characteristics of NTS taste responses can also convey information about concentration and hedonic value is under investigation.

Electrical microstimulation has been used extensively in recent times to evoke sensations in other sensory systems (reviewed in Tehovnik, Tolia, Sultan, Slocum, & Logothetis, 2006). For example, microstimulation of the visual (Murphy & Maunsell, 2007) and somatosensory (Leal-Campanario, Delgado-García, & Gruart, 2006) cortices, or thalamus (Pezaris & Reid, 2007), has been shown to produce sensations that can be used as conditioned stimuli in various learning paradigms. Results such as these show that this sort of artificial stimulation can produce an identifiable sensation but not one that compares with what a natural stimulus might invoke. In studies of the rodent olfactory bulb, a distributed arrangement of stimulation sites has been used to add to the discriminability of different spatial patterns of microstimulation (Mouly & Holley, 1986; Mouly, Vigouroux, & Holley, 1985), but again, no claim was made that the stimulation mimicked a natural odor. Here we show that using the temporal patterns of response that occur naturally as templates for microstimulation enables a better definition of the sensory characteristics that are evoked. This sort of paradigm may well inform future development of brain-machine interfaces and sensory prostheses.

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Figure 1.

(A) Temporal patterns of electrical stimulation. Graph showing the temporal arrangement of electrical pulses in all quinine simulation patterns (Q1–Q5), the composite pattern (C) and their randomized controls (Q1r–Q5r and Cr). (B) Interpulse interval (IPI) histograms for each of the six patterns and their randomized control patterns. For each quinine simulation pattern, the histogram for the randomized counterpart was identical by definition.

Figure 2.

Number of licks for all quinine simulation patterns in Experiment 1A. Mean number of licks ($\pm SEM$) for water baseline (no electrical stimulation) and for all lick-contingent quinine simulation patterns (Q1–Q5) and their randomized counterparts (Q1r–Q5r) in 10-min testing sessions. All rats drank plain water.

Figure 3.

Number of licks for the composite quinine simulation pattern in Experiment 1B. Mean number of licks ($\pm SEM$) for water baseline (no electrical stimulation) and for the lick-contingent composite quinine simulation pattern and its randomized counterpart, randomized composite. All rats drank plain water.

Figure 4.

Proportion of licks (mean $\pm$ SEM) show the conditioned stimulus, either electrical stimulation or natural quinine, for all groups. C = composite; Cr = randomized composite; 0.01 Q = 0.01 mM quinine; 0.1 Q, 0.1 mM quinine.

Figure 5.

Number of licks for generalization and extinction tests after conditioned aversion training using either quinine simulation patterns of NTS stimulation or 0.1-mM quinine as conditioned stimuli in Experiment 2. Mean number of licks ($\pm SEM$) for each of five tastants presented for 1 min, timed from the first lick, for the composite, Q1, and 0.1-mM quinine groups. Generalization tests (left) were given on the day after rats reached criterion for a conditioned aversion; extinction tests were given on the day after rats regained 90% of their baseline intake during lick-contingent electrical stimulation (only water presented) or 0.1 mM quinine depending on the group.

Figure 6. Number of licks for generalization and extinction tests following conditioned aversion training using either randomized quinine simulation patterns of NTS stimulation or 0.01 mM quinine as conditioned stimuli in Experiment 2. Details are as in Figure 4.

Figure 7.

Number of licks for taste stimuli tested in the Naïve Control groups and for generalization and extinction tests following conditioned aversion training in the Histological Control group in Experiment 2. Rats in the Naïve Control group were unoperated and tested after being trained to lick water in the experimental chamber. Rats in the Histological Control group consisted of rats from all experimental groups with electrode placement outside of the NTS. All rats in this group learned a conditioned aversion but did not generalize the aversion to any of the taste stimuli tested.

Figure 8.

Suppression ratios for generalization tests. Mean suppression ratio $\pm$ SEM for the pooled quinine simulation (Composite and Q1) and randomized quinine simulation (Randomized Composite and Q1r) are shown as filled and gray columns. Square symbols indicate the mean suppression ratio for the 0.1 mM quinine group and round symbols represent the mean suppression ratios for the 0.01 mM quinine group. Abbreviations for taste stimuli are: Q, quinine; N, NaCl; H, HCl; U, urea; S, sucrose.

Figure 9.

Results of histological analyses for Experiments 1 and 2. Numbers in center of figure between drawings of brainstem sections refer to the distance in mm caudal to bregma. Line at lower left of each panel indicates 1 mm. A. Location of electrode tips for all rats in Experiment 1. B.-D. Location of electrode tips for all rats in Experiment 2 with the exception of placements in 5 animals in the Histological Control group. See text for details.

Table 1
Basic Information About NTS Units Used to Construct the Quinine Simulation Patterns (Q1–Q5) for Experiment 1

Simulation pattern	Mean firing rate in first 2 s (sps)					No. of pulses in quinine simulation		
	Baseline (sps)	Sucrose	NaCl	HCl	Quinine	Uncertainty	1s	200 ms
Q1	3.1	2.5	15.7	27.9	24.9	0.86	46	9
Q2	2.9	9.3	14.2	63.6	45.1	0.83	69	6
Q3	2.6	8.3	4.6	9.1	11.3	0.97	41	13
Q4	1.9	1.2	23.9	16.6	19.3	0.84	49	19
Q5	6.3	5.5	63.5	18.5	33.0	0.81	40	11

Note. Baseline refers to firing rate (in spikes per second) without any stimulus on the tongue. Response magnitude in the first 2 s is the average firing rate (in spikes per second) minus the baseline firing rate. Uncertainty refers to a measure of breadth of tuning (Smith & Travers, 1979) for which a value of 1.0 implies equal responses to all taste stimuli and a value of 0 indicates responsivity to a single taste stimulus. Numbers of pulses in the entire 1-s pulse train as well as in the first 200 ms are also shown. NTS = nucleus tractus solitarius.