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Influence of stimulus and oral adaptation temperature on gustatory responses in central taste-sensitive neurons

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Influence of stimulus and adaptation temperature on taste

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Abstract

The temperature of taste stimuli can modulate gustatory processing. Perceptual data indicate the adapted temperature of oral epithelia also influences gustation, albeit little is known about the neural basis of this effect. Here, we electrophysiologically recorded orosensory responses (spikes) to 25°C (cool) and 35°C (warm) solutions of sucrose (0.1 and 0.3 M), NaCl (0.004, 0.1, and 0.3 M), and water from taste-sensitive neurons in the nucleus of the solitary tract in mice under varied thermal adaptation of oral epithelia. Conditions included presentation of taste stimuli isothermal to adaptation temperatures of 25°C (constant-cooling) and 35°C (constant-warming), delivery of 25°C stimuli following 35°C adaptation (relative-cooling), and presentation of 35°C stimuli following 25°C adaptation (relative-warming). Responses to sucrose in sucrose-oriented cells (n = 15) were enhanced under the constant- and relative-warming conditions compared to constant-cooling, where contiguous cooling across adaptation and stimulus periods induced the lowest, and longest latency, responses to sucrose. Yet compared to constant-warming, cooling sucrose following warm adaptation (relative-cooling) only marginally reduced activity to 0.1 M sucrose, and did not alter responses to 0.3 M sucrose. Thus, warmth adaptation counteracted the attenuation in sucrose activity associated with stimulus cooling. Analysis of sodium-oriented (n = 25) neurons revealed adaptation to cool water and cooling taste solutions enhanced unit firing to 0.004 M (perithreshold) NaCl, whereas warmth adaptation and stimulus warming could facilitate activity to 0.3 M NaCl. The concentration-dependence of this thermal effect may reflect a dual effect of temperature on the sodium reception mechanism that drives sodium-oriented cells.

Keywords
taste  sucrose  sodium  temperature  adaptation
Introduction

The excitation of gustatory receptor cells by taste stimuli classified as sweet by humans involves molecular mechanisms with strong sensitivity to temperature. For instance, the cellular transduction of sucrose is mediated by inward current carried by the transient receptor potential melastatin 5 (TRPM5) ion channel (Perez et al. 2002; Zhang et al. 2003), which displays increased current passage with warming from 15°C to 35°C (Talavera et al. 2005). Warming taste solutions increases peripheral nerve activity to sucrose (Breza et al. 2006; Lu et al. 2012; Yamashita et al. 1970; Yamashita and Sato 1965), an effect that is lost following genetic deletion of TRPM5 (Talavera et al. 2005). Stimulus warming accordingly enhances taste responses to sucrose in oral sensory neurons in the rostral nucleus of the solitary tract (NTS; Wilson and Lemon 2013; 2014), which receives the central terminations of cranial nerve fibers carrying gustatory and oral somatosensory information. Furthermore, latency to respond to sucrose in NTS neurons and the slope of the sucrose concentration-response function are inversely related to stimulus temperature (Wilson and Lemon 2014), demonstrating progressive change in stimulus temperature can systematically modify the timing of gustatory activity to sucrose in the mammalian brain and how this activity changes with concentration.

Receptor mechanisms underlying salt taste transmission also show strong temperature dependence. The transduction of sodium salts involves passage of Na⁺ ions through epithelial sodium channels (ENaCs) expressed by taste receptor cells, as evidenced by reduction in neural activity to NaCl following pharmacological blockade of ENaC by the diuretic amiloride (e.g., Heck et al. 1984) and confirmed in genetic and molecular reports (Chandrashekar et al. 2010; Shigemura et al. 2008). Electrophysiological studies of ENaC in expression systems described a marked sensitivity of this channel to temperature, where, for instance, decreasing temperature (i.e., cooling) can increase amiloride-sensitive Na⁺ current and channel open probability (Askwith et al. 2001; Awayda et al. 2004; Chraibi and Horisberger 2003). Further, the transient receptor potential vanilloid 1 (TRPV1) ion channel, which is sensitive to capsaicin and
noxious heat stimulation (Caterina et al. 1997; Tominaga et al. 1998), may also contribute to the
oral transduction of sodium salts. For instance, recordings from peripheral nerves showed
ENaC-independent responses to lingual application of NaCl are facilitated when solutions are
heated to temperatures that approach or exceed threshold for noxious heat, an effect blocked
through oral application of antagonists of TRPV1 (Lyall et al. 2004). Thermal sensitivity of
receptors responsive to sodium agrees with other studies that showed change in stimulus
temperature can modify peripheral (Breza et al. 2006; Lundy and Contreras 1997; Nakamura
and Kurihara 1988; Ninomiya 1996; Ninomiya et al. 1996; Sato and Yamashita 1965; Yamashita
et al. 1970; Yamashita et al. 1964) and central (Wilson and Lemon 2013) gustatory activity to
NaCl. These data, along with those for sucrose, support the postulate that stimulus temperature
importantly guides the neural representation of sensory input in gustatory pathways.

Whereas psychophysical studies also support a role for stimulus temperature in the
generation of neural and perceptual signals for taste stimuli (see Green and Frankmann 1987;
Torregrossa et al. 2012), some of this work indicates that, under certain conditions, variation in
the temperature of oral epithelia can also modulate taste signals. For instance, thermal
stimulation of different regions of the tongue is sufficient to elicit sensory qualities common to
sweet, salty, sour, or bitter taste sensations in humans (Cruz and Green 2000; von Békésy
1964), implying lingual temperature may, by itself, activate or gate the actions of taste receptors.
Further, cooling taste solutions reduces the perceived intensity of sucrose in humans
(Bartoshuk et al. 1982; Calvino 1986; Green and Nachtigal 2012) albeit some studies reported
this effect was apparent only when both the tongue and solution were similarly cooled (to 20°C),
with stimulus cooling causing only slight reductions in sucrose intensity when the tongue was
thermally controlled and warmed (to 36°C; Green and Frankmann 1987). Tongue temperature
can approximate a constant, near core, “warm” value with the mouth closed (Green 1984;
Green and Gelhard 1987), yet certain behaviors may decrease oral temperature. Green (1986)
reported that, in humans, the temperature of the tongue is slightly reduced when the mouth is
closed for 30 s compared to 60 s, which suggests oral epithelia can lose heat to evaporative cooling when the mouth is opened. Further, sipping chilled water can rapidly decrease temperature inside the human mouth by several degrees Celsius (Pangborn et al. 1970), giving rise to the potential for heat loss to cooled ingesta by oral epithelia.

The present work used in vivo neurophysiological recordings in inbred mice to test the hypothesis that variation in the adapting temperature of oral epithelia modifies central activity for gustation. To do this, we recorded gustatory responses to temperature-adjusted solutions of sucrose and NaCl from single taste-sensitive neurons in the NTS of mice under oral adaptation to physiological warm and relatively cool temperatures. Results suggest orosensory activity to gustatory stimuli in medullary circuits for taste is influenced, in some cases, by interplay between oral adaptation and taste stimulus temperature.
Material and methods

Mice. Thirty adult male and female C57BL/6J (B6) mice (The Jackson Laboratory, stock #000664) weighing 18-35 g on the day of recording were used. All mice were housed in a vivarium that maintained a 12:12 h light:dark cycle and an ambient temperature of ~23°C. Food and water were available ad libitum.

Single-unit electrophysiology. Animals were prepared for electrophysiological recording in accordance with University of Oklahoma Institutional Animal Care and Use Committee and National Institutes of Health guidelines. Mice were acutely anesthetized using a mixture of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Atropine (24 µg/kg, i.p.) was administered to reduce bronchial secretions. Once anesthetized, mice were tracheotomized in order to facilitate breathing during flow of solutions into the mouth and for maintenance of gas anesthesia. Mice were secured in a stereotaxic instrument with ear bars (Model 930, David Kopf Instruments, Tujunga, CA) and the skull was leveled to position lambda and bregma on the same dorsal-ventral plane. The lower incisors were trimmed using rongeurs. A silk thread was passed caudal of these teeth and drawn lightly taut to deflect the mandible downward. The tongue was extended from the mouth by a small rostroventral suture. Anesthesia was maintained throughout recording sessions by 0.6-0.8% isoflurane in oxygen, which mice freely respired through their tracheostomy tube (Wilson and Lemon 2014). Body temperature was kept at 37°C by a heating pad. A portion of the occipital bone was removed and parts of the cerebellum were gently aspirated to allow dorsal access to the medulla. The rostral, taste-sensitive region of the NTS was targeted at 5.8-6.2 mm posterior to bregma, 1.2 mm lateral from the midline, and 500-800 µm below the surface of brain stem (Franklin and Paxinos 2008).

Trains of extracellular action potentials were recorded from taste-sensitive NTS neurons using conventional electrophysiological methods. Tungsten microelectrodes ($z = 2$ to 5 MΩ, FHC, Bowdoinham, ME) sampled unit electrophysiological activity, which was AC amplified (Grass...
P511, high-z probe), band-pass filtered (0.3 to 10 kHz), and monitored on an oscilloscope and loudspeaker. An electronic micropositioner (Model 2660, David Kopf Instruments, Tujunga, CA) advanced the electrode ventrally through the brain stem in 1 μm steps. Spikes generated by individual neurons were identified based on waveform consistency. For isolated thermo-gustatory neurons, spikes were digitally sampled at 25 kHz (1401 interface and Spike2 software, CED, Cambridge, UK) and time-stamped to the nearest 0.1 ms. All analyses of response data were performed offline.

**Taste Stimuli.** Stimulus delivery was accomplished using a custom apparatus, as described for mice (Wilson et al. 2012; Wilson and Lemon 2013; 2014). Solution flow rate was 0.9 ml/s at ambient temperature, albeit this rate could nominally increase with warming; this variance does not impact response quantification (Wilson and Lemon 2014). The taste delivery system was designed to stimulate broad regions of oral epithelia with solutions, including the rostral and caudal tongue, and the palate (Wilson et al. 2012).

All taste chemicals (Sigma, St. Louis, MO) were high purity and dissolved in deionized water. Multiple concentrations of sucrose and NaCl were tested at 25°C and 35°C on independent trials following oral application of a thermal adapting rinse of water adjusted to 25°C and 35°C, as below. Moreover, neurons were also tested with 25°C and 35°C water as stimuli following thermal adaptation to assess their temperature sensitivity characteristics in the absence of taste input. Stimulus solutions were stored in airtight glass bottles that were adjusted to either 25°C or 35°C in separate recirculating water baths. As referenced in this manuscript, 25°C (near room temperature) was considered “cool”, whereas 35°C (near physiological temperature) was “warm”. Human psychophysical studies have reported that oral stimulation with temperatures near 25°C are perceived as cool whereas the threshold for perception of warmth with increments in the temperature of oral tissue lies near 35°C (Green 1986; 1984).
Each taste trial was composed of pre-stimulus and stimulus periods. A trial began with
continuation of oral delivery of the adapting thermal water rinse, as below, for 5 s. At this point,
flow switched to the taste stimulus, which was delivered for 5 s. Switching between pre-stimulus
rinse and stimulus solutions was accomplished using a 3-way fluid valve that was controlled by
the data acquisition system. Flow switched back to the adapting rinse following completion of
taste delivery. The oral adapting rinse continued in between trials to facilitate thermal adaptation.
The inter-trial interval was 2–3 min and allowed cells to return to pre-stimulus activity levels.
During this interval, the stimulus delivery pathways and tubing were thoroughly rinsed with water
adjusted to the temperature of the adapting rinse. Mice did not ingest solutions as their open
mouth and anesthetized state prevented swallowing.

Once isolated, the gustatory tuning profile of each neuron was characterized through
initial measurement of responses, defined as below, to a set of 25°C “prototype” taste stimuli,
presented in random order. Prototype stimuli included 0.5 M sucrose (disaccharide), 0.1 M
sodium chloride (NaCl, sodium salt), 0.01 M citric acid (organic acid), and 0.01 M quinine-HCl
(alkaloid). These stimuli fall into taste categories described as, respectively, “sweet”, “salty”,
“sour”, and “bitter” by humans. The temperature of the adapting rinse during prototype testing
was fixed at 25°C; the adapting rinse temperature was controlled on all trials by a fluid-to-fluid

Next, cells were tested with multiple concentrations of NaCl (0.004, 0.1, and 0.3 M) and
sucrose (0.1 and 0.3 M), and also water, under varied adapting rinse and stimulus temperatures.
Trials for these stimuli involved 4 unique adaptation-stimulus temperature conditions (see Figure
2A): adaptation to 25°C followed by delivery of a 25°C stimulus (referred to herein as constant-
cooling); 35°C adapting rinse, 25°C stimulus (relative-cooling); 35°C adapting rinse, 35°C
stimulus (constant-warming); and 25°C adapting rinse, 35°C stimulus (relative-warming).
Constant-cooling, where both thermal adaptation and taste stimulation occurred at 25°C, served
as the baseline condition for relative-warming, where stimulus temperature increased following
adaptation to 25°C. Likewise, constant-warming, at 35°C, was the baseline condition for relative-cooling, where stimulus temperature decreased following 35°C adaptation. A fine thermocouple probe positioned inside the tip of the oral delivery tube continuously monitored rinse and stimulus temperatures at the moment of oral delivery. A digital thermometer (BAT-12, Physitemp Instruments, Inc., Clifton, NJ) coupled to the probe provided an analog voltage signal that co-varied with temperature and was sampled (at 1 kHz) alongside neural activity by the data acquisition system. Temperatures stated above are target values, albeit actual mean stimulus temperature, as measured during the last 3 s of stimulus delivery, marginally deviated from target by, on average, 1.3°C under the relative-warming (mean stimulus temperature = 33.7°C) and relative-cooling (mean stimulus temperature = 26.3°C) conditions. This deviation was probably caused by, respectively, heat loss to the cool and heat gain from the warm adapting rinse by the fluid control valve, which in turn imparted its thermal character to the stimulus as it flowed though.

Concentrations of NaCl covered a broad intensity range for B6 mice, from near detection threshold (0.004 M), as determined in NaCl avoidance tasks (Ishiwatari and Bachmanov 2012), to approximate indifference compared to water (0.1 M), to aversive (0.3 M; Bachmanov et al. 1998; Ninomiya et al. 1989). Sucrose concentrations were detectable by mice (Treesukosol and Spector 2012) and appetitive (e.g., Bachmanov et al. 2001).

The ordering of adaptation-stimulus temperature conditions for water, 0.1 and 0.3 M sucrose, and 0.004, 0.1, and 0.3 M NaCl was randomized for each cell. To begin, one of the two adapting rinse temperatures was randomly selected (e.g., 25°C) and water adjusted to this temperature by the heat exchanger was applied continuously to oral epithelia, except, of course, during periods of stimulus delivery. After ~5 min of adaptation, one of the two stimulus temperatures was chosen at random (e.g., 35°C) and water, sucrose, and NaCl stimuli adjusted to this temperature were tested as a group, followed by water, sucrose, and NaCl solutions adjusted to the remaining stimulus temperature (e.g., 25°C). Trials were randomized across
stimuli and concentrations within each temperature group. Following completion of this sequence, the temperature of the adapting rinse was changed to the remaining value (e.g., 35°C) and the mouth was bathed with this rinse for ~5 min, after which randomization and testing of 25°C and 35°C stimuli was repeated, as above.

Responses to temperature-varied solutions of 0.004, 0.1, and 0.3 M NaCl mixed with and without the competitive ENaC inhibitor amiloride (30 μM) were recorded from an additional group of neurons to explore receptor mechanisms mediating thermo-gustatory activity to NaCl. The ordering of adaptation-stimulus temperature conditions for amiloride testing was randomized, as was the ordering of stimulus concentration and amiloride treatment conditions. Trials that included amiloride began with flow of distilled water for 5 s, followed by delivery of the NaCl/amiloride mixture for 5 s; in some cases, we tested additional trials that began with flow of amiloride (30 μM) rather than water to inhibit ENaC prior to NaCl/amiloride delivery. On completion of each trial that involved amiloride, the tongue was rinsed with 20 ml of 0.1 M NaCl followed by distilled water. Trials were separated by more than 3 min to avoid carryover effects.

At the end of a daily recording session, a weak electrical current (100 μA/1.5 s) was passed through the recording electrode to make an electrolytic lesion of brain tissue at the recording site. For mice where multiple neurons were sampled, only one lesion was made at the location of the final cell. Anesthetized mice were then overdosed with 20% (w/v) urethane and perfused transcardially with isotonic saline followed by a mixture of 4% paraformaldehyde and 3% sucrose. Brains were removed and stored at least overnight in a mixture of 4% paraformaldehyde and 20% sucrose. Brain stems were cut by microtome into coronal sections (40 μm), mounted onto slides, and stained with thionin. Lesion sites were compared against an atlas of the mouse brain (Franklin and Paxinos 2008) to verify electrode placement.

**Data Analysis.** The strategy for data analysis was to classify cells into unit types and to explore the effect of temperature on gustatory activity to sucrose and NaCl in, respectively, neurons
oriented to sucrose (S-type units) or NaCl (N-type). Hierarchical cluster analysis was applied to a matrix of correlation distances among neurons computed from their responses to prototype stimuli to identify S- and N-type cells. For the purpose of cluster analysis, taste responses to prototype stimuli were calculated as the sum of spikes that arose during the 5 s stimulus presentation period minus the sum of spikes during the 5 s pre-stimulus period. Responses to each thermo-concentration of sucrose and NaCl were analyzed in detail for, respectively, S- and N-type units that showed significant activation to each input, as assessed through detection of latency to first spike on individual stimulus trials (Wilson and Lemon 2014). S- and N-type units that did not display significant latencies on, respectively, all sucrose or NaCl trials were discarded from further analysis. This measure aimed to avoid biasing statistical estimates of thermal effects on taste activity with data from cells that showed weak or null gustatory responses, albeit only a few recorded cells met discard criteria, as below. What is more, first spike latency was used to define the beginning of the thermo-gustatory response window on individual sucrose and NaCl trials so that factorial analysis of temperature effects on firing rates to these inputs, as below, reflected spiking activity during periods of significant responding. Finally, the effects of adapting and stimulus temperature on latency were also evaluated within each cell class.

On each trial, latency was defined as the time of the first spike since stimulus onset when the firing rate became significantly greater than the pre-stimulus rate. Only significant increases in discharge were assessed, as taste-sensitive NTS neurons in mice invariably show excitation when responding to sucrose or NaCl. Stimulus onset was defined as the 5 s mark of each trial, which corresponded to when solution flow switched from the adapting rinse to the stimulus. This mark preceded stimulus contact with the mouth and neural activation by a brief (< 1 s) delay during rinse-stimulus switchover, albeit trial structure was constant across all stimulus presentations. Stimulus onset was used as the zero point for expression of spike time stamps used to detect response latency. Latency was quantified by a binless algorithm (cf. Bair and
Koch 1996; Chase and Young 2007) adapted for analysis of NTS neurons (Wilson and Lemon 2014). Briefly, an iterative Poisson method estimated the probability that the firing rate at each sequential spike during taste delivery was due to lingering pre-stimulus drive, not taste input. When this probability became < 10^{-6}, the firing rate was considered unusually high relative to average pre-stimulus firing across all trials, which was indexed separately for 25°C and 35°C adaptation conditions. Thus, the time of the first peristimulus spike where this criterion was met was taken as response latency (see Figure 3A). Latency was left undefined if criterion was not met for spikes falling within 4 s of stimulus onset.

Due to repeated measures and deviation from normality, latency data were compared between temperature conditions using sign tests with Bonferroni-adjusted \( \alpha \) for multiple comparisons. A bootstrap resampling procedure estimated a confidence interval (CI) of median latency for each stimulus and thermal condition (cf. Wilson and Lemon 2014). To do this, latencies were resampled with replacement 1000 times, where the \( n \) of each resample was equivalent to the number of actual latencies. On each resample, a Studentized bootstrapped-\( t \) was computed as \( T^* = (m^* - m)/s^* \), where \( m^* \) was the median of the bootstrapped latencies, \( m \) was the median of the actual latency data, and \( s^* \) was the standard deviation of the medians of 100 nested bootstrapped resamples of the current bootstrap. A 68% CI, corresponding to approximately 1 standard deviation (SD), was computed using the distribution of \( T^* \) values. This CI was given by \( T_{0.16}^* \times S_{M^*} + m \) (lower bound) and \( T_{0.84}^* \times S_{M^*} + m \) (upper bound), where \( T_{0.16}^* \) and \( T_{0.84}^* \) were the respective 16th and 84th percentile \( T^* \) values, and \( S_{M^*} \) was the standard deviation of the distribution of medians for the 1000 bootstrap resamples of latency.

The time course of firing to individual concentrations of sucrose and NaCl was analyzed in, respectively, S- and N-type neurons that showed significant activation to each input across all 4 adaptation-stimulus temperature conditions. An initial statistical assessment revealed responses to sucrose and NaCl differed across these conditions, albeit these differences were
not the same at all concentrations (temperature condition × concentration interactions, two-way repeated measures ANOVAs, $P < 0.007$). Thus, neuronal firing rate, operationally defined as spikes per 500 ms epoch post latency, to each concentration of sucrose or NaCl was analyzed independently. To begin, spike trains were divided into half-second bins. A three-way repeated measures ANOVA using adapting temperature (2 levels: cool and warm), stimulus temperature (2 levels: cool and warm), and time bin ($n$ levels) as factors was applied to evaluate how these parameters influenced firing rates to sucrose and NaCl. Because stimulus onset was fixed at 5 s into each trial albeit latency to first spike could vary with stimulus and thermal condition, as reported below, the number of response bins captured during significant taste-induced firing was not equivalent across stimuli. However, 5 bins were used for all stimuli except 0.1 M sucrose, for which only 3 bins were analyzed due to a marked delay (i.e., > 3 s, yet stimulus was only 5 s long) in first spike latency to this input under constant-cooling in 3 S-type cells. Thus, analyses of temperature influence on firing rates to 0.004, 0.1, and 0.3 M NaCl and 0.3 M sucrose were based on 2.5 s of unit activity post latency, whereas firing rates to 0.1 M sucrose were evaluated using 1.5 s of activity.

Responses to temperature-varied mixtures of NaCl and amiloride were analyzed in an additional sample of N-type cells using repeated-measures ANOVAs. These analyses used NaCl concentration, amiloride treatment condition, stimulus temperature, and adaptation temperature as factors. To simplify analysis of amiloride effects, the total number of spikes that arose during the 3.5 s period that followed first spike latency quantified taste responses on compared trials. A longer response window than above was used because mixtures of 0.1 and 0.3 M NaCl and amiloride delivered after adaptation to cool or warm water induced an early, transient response prior to inhibition of sodium activity by amiloride.

The assumption of sphericity among differences between group means in each repeated-measures ANOVA was evaluated using Mauchly’s test. When the null hypothesis of sphericity was rejected, degrees of freedom ($df$) for $F$-values were corrected using the
Greenhouse-Geisser procedure. The corrected \( df \) are reported herein as real numbers, where applicable. Omnibus \( F \)-values were evaluated using \( \alpha = 0.05 \). Pairwise comparisons among group means that followed significant \( F \)-values were carried out using paired \( t \)-tests under Bonferroni-adjusted \( \alpha \).

All ANOVAs were executed in SPSS (IBM, Somers, NY), with pairwise comparisons performed in the R programming language (R Foundation for Statistical Computing, Vienna, Austria). All other analyses and the generation of data plots were carried out in MATLAB (The MathWorks, Natick, MA) using standard routines and custom code. Utilities from the statistics and bioinformatics modules for this platform were used.
Results

General Response Characteristics. Trains of action potentials were recorded from 49 NTS neurons in B6 mice. Thirty cells were sampled from female mice, and 19 cells were recorded from males. Each cell was tested with all stimuli and thermal conditions (28 trials) at least once, and a total of 1372 stimulus-response trials were analyzed. Recorded neurons showed low baseline spike discharge rates across all trials, albeit the mean pre-stimulus rate was higher ($F_{1,48} = 13.67, P = 0.001$) during adaptation to warm water (1.68 Hz ± 1.87 SD) compared to cool (1.04 Hz ± 1.14 SD). Sex did not influence responses to the prototype stimuli at 25°C (non-significant [n.s.] sex × stimulus interaction, two-way ANOVA, $P = 0.7$; n.s. main effect of sex, $P = 0.7$) and was not included as a factor in analyses. Cluster analysis identified 16 S-type and 26 N-type cells (Figures 1A, 1B, 2B, and 2C), with units in both groups composing 86% of sampled neurons. For these groups, trials that used water as a stimulus under relative-warming and relative-cooling were analyzed to assess sensitivity to temperature change. The number spikes per 5 s delivery of water during relative-cooling did not differ between S- and N-type cells (independent samples $t$-test, $P > 0.05$). On the other hand, stimulation with water during relative-warming evoked a larger number of spikes during the 5 s stimulus window in S-type (4.37 net spikes ± 3.86 SD) compared to N-type (-0.65 net spikes ± 2.46 SD) neurons (independent samples $t$-test corrected for unequal variances, $t_{22.6} = 4.7, P < 0.001$). Thus, S-type neurons showed heightened sensitivity to warming from 25°C in the absence of gustatory drive. Histological analysis revealed electrode positioning indeed targeted the NTS (Figure 1C).

Analysis of sucrose activity in S-type cells. Latency to first spike was estimated on each sucrose trial to quantify neuronal firing from the time point of significant activation, and to determine how change in adapting temperature affected lag to respond to sucrose. Latency to respond to 0.1 and 0.3 M sucrose was detected across all adaptation-stimulus temperature
conditions for all but one S-type neuron, which was excluded from further analysis. Figure 3A demonstrates measurement of latency in one of the 15 analyzed S-type cells. For this unit, contiguous warming between adaptation and stimulus epochs of taste trials (i.e., the constant-warming adaptation-stimulus temperature condition), and warming taste solutions following cool adaptation (relative-warming), appeared to reduce latencies for both 0.1 and 0.3 M sucrose compared to constant-cooling. This observation follows prior data from NTS cells that showed, under oral adaptation to ambient temperature water, warming sucrose solutions decreased response latency, whereas cooling substantially increased latency (Wilson and Lemon 2014). Yet for the unit in Figure 3A, cooling taste solutions following warm adaptation (relative-cooling) appeared to exert no effect on latencies for either 0.1 or 0.3 M sucrose when compared to constant-warming.

Analysis of latency data across all S-type neurons revealed a common trend. For 0.1 and 0.3 M sucrose, contiguous warming between the adaptation and stimulus phase of taste trials (constant-warming), and warming sucrose following cool adaptation (relative-warming), significantly decreased latency relative to constant-cooling (sign tests, $P < 0.001$, Figure 3B), which induced the longest latencies among significant responses to each sucrose concentration. However cooling sucrose solutions following warm adaptation (relative-cooling) produced latencies, for either concentration, that were not different from those measured under constant-warming (n.s. sign tests, $P > 0.05$), which decreased lag. Thus, warmth adaptation of the mouth counteracts the delay in response associated with cooling sucrose solutions.

The effects of adapting temperature, stimulus temperature, and time on neuronal firing rates to sucrose were evaluated separately for each concentration using three-way ANOVA. Cooling and warming solutions of 0.1 and 0.3 M sucrose caused a change in response to these stimuli that varied with adapting temperature (adapting temperature × stimulus temperature interactions, $F_{1,14} > 8$, $P < 0.05$), albeit these effects were constant across time for both concentrations (n.s. adapting temperature × stimulus temperature × time interactions, $P > 0.05$;
n.s. adapting temperature × time interactions, $P > 0.05$; n.s. stimulus temperature × time
interactions, $P > 0.05$). Contiguous warming between adaptation and stimulus epochs of taste
trials (constant-warming) increased firing rates to 0.1 and 0.3 M sucrose compared to the
constant-cooling condition (Bonferroni-adjusted pairwise comparisons, $P < 0.001$, Figure 4).
Thus, constant-cooling suppressed whereas constant-warming enhanced unit activity to sucrose.
Similarly, warming taste solutions following cool adaptation (relative-warming) also increased
firing rates to 0.1 and 0.3 M sucrose compared to constant-cooling (Bonferroni-adjusted
pairwise comparisons, $P \leq 0.001$). On the other hand, cooling taste solutions following warm
adaptation (relative-cooling) only moderately reduced unit firing to 0.1 M sucrose (Bonferroni-
corrected pairwise comparison, $P < 0.05$, Figure 4A) and did not affect activity to 0.3 M sucrose
(Bonferroni-adjusted pairwise comparison, $P = 0.39$, Figure 4B) compared with constant-
warming, which facilitated activity. Thus, warmth adaptation lessens or removes the attenuation
in response to sucrose normally induced by cooling. These data indicate oral adapting
temperature, in addition to stimulus temperature, can significantly influence neuronal sensitivity
to sucrose in medullary taste-sensitive neurons.

Analysis of NaCl activity in N-type cells. A separate analysis on temperature effects on firing
rate to NaCl was performed for each NaCl concentration, as described in the methods. Cells
were included in the analysis of data for one concentration only if they showed significant
response latencies to that concentration on each of the 4 adaptation-stimulus temperature trials.
Nine of the 26 N-type neurons that were recorded met this criterion for 0.004 M NaCl, whereas
25 N-type cells significantly responded to 0.1 and 0.3 M NaCl during each adaptation-stimulus
temperature condition. The analyses firing rates to 4, 100, and 300 mM NaCl that follow are
based on these respective subsets cells.

Although increasing solution concentration decreased latency to first spike to NaCl (sign
tests, $P < 0.001$), as described (Breza et al. 2010; Marowitz and Halpern 1977), latencies within
each concentration did not vary across adaptation-stimulus temperature conditions (Bonferroni-adjusted sign tests, \( P > 0.04 \), Figure 5). The lack of thermal influence on latency was distinct for NaCl compared to neural activity to sucrose, where lag to response was significantly modulated by temperature (Figure 3; cf. Wilson and Lemon 2014).

Firing rates to 0.004 M NaCl in N-type neurons were enhanced by cooling stimulus solutions, collapsed across adapting temperature and time (main effect of stimulus temperature, \( F_{1,8} = 8.3, P < 0.05 \), Figure 6A). There was no significant interaction between stimulus temperature and adapting temperature or time on firing rates to 0.004 M NaCl (\( P > 0.05 \)).

Adapting temperature affected firing rate to 0.004 M NaCl, albeit the effect was time-dependent (adapting temperature \( \times \) time interaction: \( F_{4,32} = 17.6, P < 0.001 \)): cool adaptation increased the firing rate to 0.004 M compared to warm adaptation during the first 1.5 sec of the stimulus response (Bonferroni-adjusted pairwise comparisons, \( P < 0.05 \), Figure 6B).

Firing rates to 0.1 M NaCl in N-type cells were unaffected by change in stimulus temperature (n.s. main effect, \( P = 0.3 \), Figure 6C; n.s. stimulus temperature \( \times \) time interaction, \( P = 0.17 \)), albeit spiking to this stimulus did vary with adapting temperature and time (adapting temperature \( \times \) time interaction, \( F_{1.8,43.9} = 8.1, P = 0.001 \)). Specifically, adaptation to warmth attenuated firing rates to 0.1 M NaCl compared to cool adaptation during the first second of the response (Bonferroni-adjusted pairwise comparisons, \( P < 0.01 \), Figure 6D). The interaction between adaptation and stimulus temperature on firing rates to 0.1 M NaCl was not significant (\( P > 0.05 \)).

Finally, firing rates to 0.3 M NaCl in N-type units were moderately elevated when stimulus solutions were warmed compared to cooled, collapsed across adaptation temperature conditions and time (main effect of stimulus temperature, \( F_{1.24} = 13.1, P = 0.001 \), Figure 6E).

There was no significant interaction between stimulus temperature and adaptation temperature or time on firing to 0.3 M NaCl (\( P > 0.05 \)). On the other hand, adaptation temperature modulated firing rates to 0.3 M NaCl in a manner that relied on time (adaptation temperature \( \times \) time
interaction, $F_{2,0.48.8} = 9.3, P < 0.001$). Warmth adaptation attenuated spiking to 0.3 M NaCl relative to cool adaptation during the first 0.5 s of the response, but enhanced firing to this input at 2.5 s post latency (Bonferroni-adjusted pairwise comparisons, $P < 0.05$, Figure 6F).

MIXTURES OF NaCl AND AMILORIDE WERE TESTED ON AN ADDITIONAL SAMPLE OF 10 N-TYPE CELLS TO EVALUATE THE POTENTIAL CONTRIBUTION OF AMILORIDE-SENSITIVE CHANNELS TO Thermo-gustatory activity to sodium. Amiloride eliminated significant firing to 0.004 M NaCl across adaptation-stimulus temperature conditions, as latencies were not detected on these trials (Table 1). On the other hand, latency to first spike was detected on all trials for 0.1 and 0.3 M NaCl with, and without, amiloride (Table 1). Factorial ANOVA applied to activity 0.1 and 0.3 M NaCl in this group of N-type neurons revealed the effect of stimulus temperature on neuronal firing varied with amiloride treatment (stimulus temperature × amiloride treatment interaction, $F_{1.9} = 8.58$, $P = 0.02$), but was independent of adaptation temperature (n.s. stimulus temperature × adaptation temperature interaction, $P = 0.11$) and concentration (n.s. stimulus temperature × concentration interaction, $P = 0.07$). Expectedly, application of amiloride substantially reduced responses to 0.1 and 0.3 M NaCl (main effect of amiloride, $F_{1.9} = 78.7$, $P < 0.001$). Although activity to these stimuli was enhanced when solutions were warmed compared to cooled (Bonferroni-adjusted pairwise comparisons, $P \leq 0.001$; Figure 7A), the difference in firing between warm and cool NaCl under control conditions (22.4 spikes difference ± 3.9 SD; Figure 7B) was reduced (dependent samples t-test, $t_9 = 3.0, P < 0.05$) in the presence of amiloride (9.4 spikes difference ± 2.0 SD).

Residual thermo-gustatory activity to NaCl on amiloride trials was due to an early, abbreviated response to NaCl (Figure 7C) likely contributed by transient competition between Na$^+$ and amiloride for receptor access, as oral adaptation to amiloride, as opposed to water, entirely abolished responses to NaCl (Figure 7D). Assuming a peripheral locus of effect, the above results suggest amiloride-sensitive channels wholly mediate temperature effects on gustatory activity to NaCl in N-type neurons.
An influence of adapting temperature on central gustatory activity to sucrose. Building on prior data that showed stimulus temperature can influence neural activity to sucrose (Breza et al. 2006; Lu et al. 2012; Talavera et al. 2005; Wilson and Lemon 2013; 2014; Yamashita et al. 1970; Yamashita and Sato 1965), the present work describes a novel effect of variation in oral adaptation temperature on neural sensitivity to this stimulus. Different adapting rinse temperatures were tested in some of the prior electrophysiological work on thermal influence on sucrose activity, albeit sucrose solutions were also adjusted to and tested at rinse temperature (Breza et al. 2006; Lundy and Contreras 1999; Talavera et al. 2005; Yamashita and Sato 1965). Thus, separating the effects of adapting from stimulus temperature was precluded in these studies, as both were presumably equal. By testing adapting rinse and sucrose solutions at multiple isothermal and anisothermal temperatures, here we show that change in adapting temperature can influence neural activity for sucrose taste. Cooling sucrose solutions to 25°C substantially decreased firing rates to 0.1 and 0.3 M sucrose in the NTS compared to warming, but only when the mouth was cool-adapted to 25°C (Figure 4). Following warm adaptation to 35°C, firing rates to both cooled and warmed solutions of sucrose were relatively enhanced, and only moderately different (0.1 M) or indistinguishable (0.3 M) from one another. Thus, warm adaptation largely counteracted the attenuation in magnitude, and also onset (Figure 3), of gustatory activity to sucrose imposed by cooling the stimulus solution.

Notwithstanding periods of taste delivery, the thermal adapting rinse was continuously applied to the mouth during data acquisition trials and inter-trial periods, which were around 2-3 min for both cool and warm adaptation conditions. The surface temperature of the tongue in rodents can reach a steady reduced or elevated value after less than 1 minute of adaptation to, respectively, chilled (e.g., 10°C) or heated (e.g., 40°C) water (Yamashita and Sato 1965). Thus, the present method of continuous thermal adaptation might have effectively changed the temperature of the oral epithelial surface. It is important to note that temperature measurements...
in the present work indexed solution temperature at the moment of oral delivery and cannot be interpreted as direct measurements of oral epithelia temperature. Nonetheless, a change in the thermal environment of taste receptor cells agrees with a potential mechanistic explanation of the present effects involving warmth potentiation of TRPM5-dependent receptor pathways for sucrose (Talavera et al. 2005). It is possible these pathways sensitized during extended stimulation with 35°C during warm adaptation, increasing the level of membrane depolarization in taste cells. This postulate agrees with our observation that pre-stimulus spiking activity in NTS neurons was higher under warm adaptation as opposed to cool. Heightened taste cell depolarization could, in turn, lead to enhanced firing to sucrose in downstream S-type neurons in the brain stem, even when sucrose was cooled to near-ambient temperature. Analogous sensitization effects of warmth adaptation were reported in psychophysical studies on sucrose taste perception. For instance, although cooling can reduce the intensity of sucrose perception (Bartoshuk et al. 1982; Calvino 1986; Green and Nachtigal 2012), humans reported only “slight and inconsistent” decrements in the sweetness of cooled (20°C) sucrose when the tongue was thermally controlled and warm-adapted to 36°C (Green and Frankmann 1987). These data, along with the present findings, indicate adaptation temperature importantly influences gustatory responses to sucrose, and that warming the mouth can, in some cases, facilitate activity to sucrose independently of stimulus temperature.

For humans and also mice, the temperature of oral epithelia would be expected to hold near-constant value when the mouth is closed (cf. Green 1984; Green and Gelhard 1987), and the 35°C adapting rinse used presently intended to target a normal closed-mouth temperature approximated for mammals (cf. Green 1986). On the other hand, the 25°C adapting rinse was at a temperature lower than closed-mouth, albeit oral epithelia may at times display decreased temperatures upon opening of the mouth and during the consumption of cooled ingesta (Green 1986; Pangborn et al. 1970), as discussed above. What is more, dipping the tongue into a 37°C solution of sucrose following adaptation to sucrose at 21°C counteracts the attenuation in
sweetness imposed by the cooled solution (Green and Nachtigal 2012). Such “re-warming” of the tongue was interpreted to explain in part the phenomenon where tasting sweet stimuli with the tongue outside the mouth, conducive to evaporative cooling of lingual epithelia, produces a reduced sensation of sweetness that quickly grows in magnitude once the tongue is retracted into the mouth, which would re-warm the tongue (Green and Nachtigal 2012). Thus, the temperature of oral epithelia can seemingly vary with ingestive behaviors and shape neural and perceptual responses to sucrose taste.

Warmth adaptation and stimulus warming decreased latency to fire to sucrose in S-type NTS neurons (Figure 3), similar to previous findings (Wilson and Lemon 2014). As above, a physiological mechanism supporting this effect may involve an influence of temperature on the TRPM5 component of taste receptors for sucrose. Current flow through TRPM5 at positive voltage appears to steepen with warming from ~25°C, and lower temperatures, to 35°C (Talavera et al. 2005). This suggests a depolarizing step in the membrane potential of taste receptor cells harboring TRPM5 would result in greater inward current flow at warm temperatures compared to cool, potentially leading to a more rapid cellular activation and signal transmission to follower neurons. Yet the contribution of TRPM5 to temperature effects on the timing of sucrose activity awaits investigation. It is noteworthy that the lack of thermal influence on latency to fire to NaCl in N-type units may reflect the disparate receptor process involved with transduction of this stimulus.

Although TRPM5 emerges as a likely and parsimonious molecular candidate for mediating thermal influence on gustatory activity to sucrose, this postulate is not without caveat. In addition to its role in sucrose taste, the warmth-sensitive (Talavera et al. 2005) TRPM5 ion channel also contributes critically to the transduction of select bitter-tasting stimuli, including the bitter prototype quinine (Damak et al. 2006; Zhang et al. 2003). This reliance of quinine taste on heat-sensitive TRPM5 predicts temperature should impact gustatory responses to quinine, along the vein of the marked influence of temperature on activity to sucrose if we assume
thermal action on this input arrives through TRPM5 (e.g., Lu et al. 2012; Talavera et al. 2005; Wilson and Lemon 2014). However, whereas change in stimulus temperature from cool (22°C) to warm (37°C) values can induce supralinear change in NTS unit activity to oral delivery of sucrose, such effect is not found with quinine - a stimulus that elicits taste responses with comparably low sensitivity to temperature (Wilson and Lemon 2013). The non-uniform effect of temperature across gustatory responses to sucrose and other stimuli whose transduction involves TRPM5 suggests other mechanisms may contribute to thermal modulation of sucrose activity. One possibility is that temperature may exert unique influence on the T1R-dependent receptor proteins involved with sweet taste transduction (Wilson and Lemon 2013), albeit this remains to be experimentally studied. Other potential mechanisms could include activation of central gustatory sensitization and habituation processes (cf. Di Lorenzo and Lemon 2000) that are engaged through extended oral stimulation with warm and cool adapting temperatures. Moreover, the NTS receives projections from the trigeminal (V) nerve (e.g., Blomquist and Antem 1965; Contreras et al. 1982; Corson et al. 2012; Hamilton and Norgren 1984; Marfurt and Rajchert 1991; Whitehead and Frank 1983), which conveys oral pain, touch, and temperature sensation. NTS neurons implicated for contributing to gustatory sensation are evidenced to receive synaptic input from mandibular processes of the trigeminal nerve (Boucher et al. 2003; Braud et al. 2012; Felizardo et al. 2009), suggesting these neurons may also combine information about gustation and oral somatosensation originating along anatomically and, presumably, functionally distinct afferent routes. It is possible that thermal stimulation of trigeminal pathways and the convergence of V input onto NTS neurons may have contributed, in part, to the present results, albeit this was not directly tested. The present data show adaptation temperature can markedly influence gustatory activity to sucrose in the brain stem, albeit additional studies are needed to delineate mechanisms that support the observed effects.
Temperature and central gustatory activity to sodium. The influence of adaptation and stimulus temperature on responses to NaCl in the present sample of N-type NTS neurons progressively varied with stimulus concentration. Firing rates to 0.004 M (perithreshold) NaCl were enhanced by stimulus cooling and also cool adaptation of oral epithelia, which markedly raised activity to 4 mM NaCl during an extended period of the taste response (Figure 6). Increasing NaCl concentration to 0.1 M induced responses that were relatively less susceptible to change with adaptation temperature and unaffected by cooling or warming of stimulus solutions. On the other hand, responses to 0.3 M NaCl were moderately enhanced by stimulus warming, with warmth adaptation also facilitating activity to this input during a late window of the taste response; across concentrations, this was the only instance where warmth adaptation enhanced unit firing to NaCl. Thus, warming could enhance unit firing to concentrated NaCl, whereas cooling facilitated neural responses to low-intensity solutions of NaCl. Considering perithreshold NaCl, the increased firing to this input under stimulus cooling and cool adaptation may suggest gustatory adaptation, or habituation, to weak intensities of NaCl decreases with decreasing temperature (cf. Figure 6A, 6B). This contrasts with the present and prior data on thermo-gustatory activity to sucrose, where firing to low (0.1 M, Figure 4A) and perithreshold (0.05 M, Wilson and Lemon 2014) intensities of sucrose is increased by warming, not cooling, possibly reflecting a decrease in the rate of gustatory adaptation to sucrose with rising temperature (cf. Green and Nachtigal 2012).

The gustatory signal for NaCl carried by “sodium-best” NTS neurons arises from taste receptor processes sensitive to blockade by the ENaC antagonist amiloride (Boughter et al. 1999; Scott and Giza 1990; Smith et al. 1996; St. John and Smith 2000). In confirmation, our experiments showed thermo-gustatory activity to 0.004, 0.1, and 0.3 M NaCl in N-class cells was inhibited or blocked when stimuli were mixed with amiloride, and abolished when such mixtures were tested following adaptation of oral epithelia to amiloride (Figure 7). Thus, assuming a peripheral locus of effect, enhancement or suppression of responses to NaCl in N-
class units due to cooling and warming, as reported here, would intuitively involve an influence of temperature on amiloride-sensitive channels. Along this line, Nakamura and Kurihara (1988) used whole-nerve recordings from the chorda tympani (CT) nerve, which supplies taste and oral somatic sensation to the anterior tongue, to reveal peripheral gustatory activity to NaCl is composed of two amiloride-sensitive components, each with a dependence on temperature and Na\(^+\) concentration that parallels the present effects of these parameters on central activity to NaCl. In their study, amiloride-sensitive CT responses to a reduced concentration (0.01 M) of NaCl peaked when solutions were cooled to around 10°C. On the other hand, amiloride-sensitive nerve activity to an elevated NaCl concentration (0.3 M) peaked at approximately 30°C. Amiloride-sensitive activity to an intermediate concentration (0.1 M) of NaCl showed, accordingly, two approximately equal peaks, one at around 10°C and another at 30°C. This result was interpreted as evidence for two amiloride-blockable taste receptor sites for NaCl (Nakamura and Kurihara 1988), one tuned to low concentrations of Na\(^+\) when cooled, and another maximally stimulated by high concentrations of Na\(^+\) when warmed. A similar “double effect” of temperature on amiloride-sensitive receptor processes for sodium was described in data showing amiloride-sensitive fibers of the CT nerve were divisible by whether they displayed heightened activation to NaCl tested at room temperature or cooled to around 12°C (Ninomiya 1996).

Biophysical studies have revealed a marked influence of temperature on amiloride-sensitive Na\(^+\) current mediated by ENaC. At physiological temperatures (e.g., 35°C), this current shows a rapid increase to “peak” amplitude that is followed by a fast relaxation to a decaying, or “rundown”, value (Chraibi and Horisberger 2002). The reduced current during rundown is thought to reflect a form of recurrent inhibition of Na\(^+\) current flow through ENaC induced by multiple potential mechanisms (cf. Chraibi and Horisberger 2002; Gilbertson and Zhang 1998). Recurrent inhibition of Na\(^+\) current also arises in taste receptor cells that show amiloride-sensitive responses to Na\(^+\) salts (Gilbertson and Zhang 1998). When cooling ENaC to around
25°C, amiloride-sensitive Na+ current retains its rapid onset-to-peak characteristic albeit the rate of decline after the peak slows, resulting in heightened rundown current compared to warm conditions (Chraibi and Horisberger 2002). Further reductions in temperature (e.g., 12°C) can largely remove the self-inhibitory effect of Na⁺ on ENaC, rendering similarities, or indifference, between the peak and rundown components of amiloride-sensitive responses (Chraibi and Horisberger 2003; 2002). Thus, cooling ENaC increases rundown current during stimulation with Na⁺, whereas warming tends to decrease this current.

Cooling-induced enhancement of responses to low concentrations (≤ 0.01 M) of NaCl in amiloride-sensitive peripheral (Nakamura and Kurihara 1988) and central (Figure 6) taste pathways agrees with the known effects of cooling on ENaC, where decrements in temperature can enhance the amplitude of rundown current and Na⁺ passage through this channel (Askwith et al. 2001; Awayda et al. 2004; Chraibi and Horisberger 2003; 2002). On the other hand, warmth enhancement of responses to elevated concentrations (0.3 M) of NaCl in amiloride-sensitive circuits, as shown previously (Nakamura and Kurihara 1988) and presently (Figure 6), seems at odds with ENaC, which displays reduced rundown and amiloride-blockable Na⁺ currents with warming above room temperature, as above. Yet some reports have shown that although cooling can indeed markedly raise the open probability of ENaC, warming can increase the conductance of this channel, indicative of a dual effect of temperature (Chraibi and Horisberger 2003). Although it is cumbersome to directly compare the influence of temperature on ENaC current in vitro with the in vivo performance of gustatory-sensitive neurons implicated to receive sodium taste input mediated by this channel, the available biophysical data suggest temperature can certainly exert complex effects on ENaC function. Such effects may contribute to the present, and previously observed (Nakamura and Kurihara 1988; Ninomiya 1996), dual action of temperature on gustatory activity to NaCl in amiloride-sensitive taste pathways.

It is important to consider that the amiloride-insensitive TPRV1 ion channel is also implicated for sodium transduction and responses in taste-sensitive nerves, and contributes
potentiated activity to high concentrations of Na\(^+\) paired with heat (Lyall et al. 2004). Such sensitivity might render TRPV1 as a candidate receptor contributing to warmth facilitation of gustatory activity to concentrated NaCl, as in Figure 6. However, the present (Figure 7) and prior data (Boughter et al. 1999; Scott and Giza 1990; Smith et al. 1996; St. John and Smith 2000) suggest Na\(^+\) input to N-class neurons arrives exclusively through ENaC.

A noteworthy study by Yamashita et al. (1970) evaluated how abrupt change in tastant temperature during gustatory stimulation would impact the continuation of taste responses recorded from single CT nerve fibers. In this work, a sudden drop in stimulus temperature from 30°C to 10°C augmented ongoing unit activity to a reduced concentration (0.03 M) of NaCl in select fibers, whereas a sudden increase in stimulus temperature from 10°C to 30°C inhibited responding to this stimulus. Also revealed were a small number of fibers where abrupt warming potentiated ongoing activity to elevated concentrations of NaCl, including 0.3 M as used presently. There are similarities between the results of this study and the present, albeit important procedural differences exist, including different adaptation conditions. Units were adapted to temperature-adjusted taste stimuli in Yamashita et al. (1970), which presumably promoted gustatory adaptation, as opposed to temperature-controlled water in the present effort. Along this line, Yamashita et al. (1970) showed ongoing activity by select CT fibers to 0.1 M NaCl could be inhibited by abrupt warming of the stimulus, albeit the present data revealed warming stimulus solutions did not affect, across adapting temperatures, responses to 0.1 M NaCl in N-type neurons (Figure 6C). Other single-fiber studies on temperature-taste interactions in the CT nerve that used thermal, rather than taste, adaptation showed no effect of temperature on responses to 0.1 M NaCl in sodium-best units (Breza et al. 2006; Lundy and Contreras 1999).

Although it is important to consider that Yamashita et al. (1970) classified units based on thermal rather than gustatory tuning, the above results taken together suggest the level of thermosensory and also chemosensory adaptation of sodium receptors may guide how temperature impacts gustatory activity to NaCl.
Final considerations

There are limited extant data on the frequency by which thermal sensitivity arises in taste-sensitive neurons in the mammalian NTS, with sometimes inconsistent findings reported across these studies. An electrophysiological description of the lamb NTS revealed approximately 30% of sensory neurons in this nucleus are sensitive to oral and epiglottal stimulation with temperature (Sweazey and Bradley 1989). Further work on rats showed that about 33% of taste-sensitive NTS neurons were sensitive to change in oral temperature, with cooling, but not warming, inducing excitation in firing in these cells (Travers and Norgren 1995). On the other hand, other electrophysiological investigations discovered a marked majority of gustatory-sensitive neurons in rodent NTS can respond with excitation to change in oral temperature, including cooling and also warming of oral epithelia (Ogawa et al. 1988; Wilson and Lemon 2013). Differences in findings across these studies may relate to methodological discrepancies. Along this line, the present, and recent (Wilson and Lemon 2013; 2014), data have further revealed an extended receptive range of taste-sensitive NTS neurons to particular combinations of gustatory stimulation and temperature. Temperature-induced change in firing to gustatory input in NTS neurons can markedly surpass responses by these units to oral thermal stimulation alone (Wilson and Lemon 2013; 2014). Thus, thermal sensitivity by taste-sensitive neurons may be effectively indexed, in some cases, only through tests that include pairings of temperature input with taste stimuli.

It is also important to consider that functional interactions between temperature and gustation partly show species dependence. Although the present and other data from rodents, as above, indicate temperature modulates taste responses to sugars and salts, a recent electrophysiological study in Manduca hornworms revealed peripheral taste responses to K⁺ salts and select sugars, including sucrose, showed no change with warming and cooling of receptors, albeit sensitivity to an aversive stimulus was found to rely on temperature (Afroz et al.
Considering other arthropods, *Drosophila* display neural and behavioral sensitivity to warmth mediated by a taste receptor protein (Ni et al. 2013) expressed in gustatory-sensitive neurons implicated in avoidance function (Thorne and Amrein 2008). Such cells may combine thermal and aversive gustatory input (Montell 2013). Temperature can modulate taste responses to aversive stimuli, such as acids and bitters, in mammalian neurons (Breza et al. 2006; Lundy and Contreras 1999; Yamashita and Sato 1965), albeit, as above, the effect of temperature on these responses can be markedly less pronounced compared to its influence on appetitive taste signals (Wilson and Lemon 2013). Different patterns of thermo-gustatory interaction across species may partly have an ecological basis (Afroz et al. 2013).

In closing, the present data continue to build on the literature indicating temperature is an underling and systematic parameter of the neural processing of taste in mammals. Given its omnipresent nature during taste experience and marked ability to modulate gustatory signals as shown here and in other studies, temperature should be considered in equal light to stimulus concentration and quality as a modifier of gustatory activity. Future studies that test extended concentration series of tastants across several adapting and stimulus temperatures may precisely describe how change in the balance between these temperatures modulates taste activity and perception. It is conceivable in humans that this balance may dynamically and rapidly shift during the consumption of flavorful foods and drinks served warmed and chilled.
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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions

J.L. performed experiments; J.L. and C.H.L. analyzed data; J.L. and C.H.L. interpreted results of experiments; J.L. and C.H.L. prepared figures; J.L. and C.H.L. edited and revised manuscript; J.L. and C.H.L. approved final version of manuscript; C.H.L. conception and design of research; J.L. drafted manuscript.


Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Perez CA, Shigemura N, Yoshida R, Mosinger B, Jr., Glendinning JI, Ninomiya Y, and Margolskee RF. Trpm5 null mice respond to bitter, sweet, and umami compounds. *Chem Senses* 31: 253-264, 2006.


Treesukosol Y, and Spector AC. Orosensory detection of sucrose, maltose, and glucose is severely impaired in mice lacking T1R2 or T1R3, but Polycose sensitivity remains relatively normal. *Am J Physiol Regul Integr Comp Physiol* 303: R218-235, 2012.


Figure Captions

**Figure 1.** Neural groups and verification of electrode placement. **A,** Dendrogram showing the outcome of cluster analysis applied to sort neurons into groups. Characters denoting each group refer to response profiles in panel **B.** **B,** Mean (± 1 standard error) responses by each neural group to 25°C solutions of 0.01 M citric acid (C), 0.1 M NaCl (N), 0.01 M quinine-HCl (Q), and 0.5 M sucrose (S). N-type, sodium-oriented cells; B-type, broadly responsive cells; H-type, acid/electrolyte-oriented cells; S-type, sucrose-oriented neurons. **C,** Coronal section (40 µm) through mouse brain stem (left) along with neuroanatomical sketch (right) highlighting the location of the nucleus of the solitary tract (NTS) relative to general landmarks (cf. Franklin and Paxinos 2008). Arrow indicates an electrolytic lesion made following unit recording. Abbreviations: SpVe, spinal vestibular nucleus; sp5, spinal trigeminal tract.

**Figure 2.** Examples of raw data. **A,** Family of traces showing real-time measurement of solution temperature, at the moment of oral delivery, against time on four separate trials where a cool (25°C) stimulus was delivered following cool adaptation of oral epithelia (constant-cooling; abbreviated C), a warm (35°C) stimulus was presented following warm adaptation (constant-warming; W), a cool stimulus was delivered following warm adaptation (relative-cooling; W→C); and a warm stimulus was given after cool adaptation (relative-warming; C→W). **B,** Electrophysiological sweeps showing responses by an S-type neuron to 0.1 and 0.3 M sucrose recorded across the four thermal conditions. The ordering of the stack of sweeps indicated for 0.1 M sucrose also applies to 0.3 M sucrose and data for NaCl in panel **C.** Upward and downward arrowheads indicate stimulus onset and offset, respectively. **C,** Electrophysiological sweeps showing responses by an N-type neuron to 0.004, 0.1, and 0.3 M NaCl recorded across the four thermal conditions.
**Figure 3.** Effects of adaptation and stimulus temperature on latency to first spike to sucrose in S-type neurons. A, Rastergrams from an example S-type cell depicting detection of latency to first spike on individual trials for 0.1 and 0.3 M sucrose across adaptation-stimulus temperature conditions. The blackened raster spike on each trial denotes latency, defined as the time of the first action potential during stimulus delivery when the firing rate of the cell became significantly greater than the pre-stimulus rate (see methods). Latency to first spike for 0.1 M sucrose on each adaptation-stimulus temperature condition was: constant-cooling (abbreviated C), 2.24 s; constant-warming (W), 1.59 s; relative-cooling (W→C), 1.68 s; relative-warming (C→W), 1.72 s. Latencies for 0.3 M sucrose were: C, 1.48 s; W, 0.99 s; W→C, 0.94 s; C→W, 1.06 s. B, Median latency (± 68% confidence limits) to first spike for 0.1 and 0.3 M sucrose for each adaptation-stimulus temperature condition, abbreviated as above (*, P < 0.0001). Confidence limits were approximated using a bootstrap resampling procedure (see methods).

**Figure 4.** Effects of adaptation and stimulus temperature on firing rate to sucrose in S-type neurons. Mean (± 1 standard error) firing rate (spikes/500 ms) to 0.1 M (panel A) and 0.3 M (panel B) sucrose during each adaptation-stimulus temperature condition (*, P < 0.05; ***, P < 0.001). Firing rates for 0.1 M sucrose reflect average spike discharge per half-second for 1.5 s post latency; firing rates for 0.3 M sucrose represent mean discharge per half-second for 2.5 s post latency. Thermal conditions were constant-cooling (abbreviated C), constant-warming (W), relative-cooling (W→C), and relative-warming (C→W). Error bars were normalized for between-neuron variability using the method of Cousineau (2005), albeit statistical comparisons were performed on uncorrected data.

**Figure 5.** Latency to first spike to NaCl in N-type neurons. Bars represent median latencies (± 68% confidence limits) for 0.004, 0.1, and 0.3 M NaCl across adaptation-stimulus temperature conditions. Conditions were constant-cooling (abbreviated C), constant-warming (W), relative-
cooling (W→C), and relative-warming (C→W). Confidence limits were approximated using a bootstrap resampling procedure (see methods).

**Figure 6.** Effects of stimulus temperature, adaptation temperature, and time on firing rates to NaCl in N-type neurons. Bar graphs in the left column show average responses (spike/500 ms ± 1 standard error) to 0.004 M (panel A), 0.1 M (panel C), and 0.3 M (panel E) NaCl at cool and warm stimulus temperatures (*, P < 0.05; ***, P < 0.001). Firing rates represent mean spike discharge per half-second for 2.5 s following latency to activation. Data are collapsed across time window and adaptation temperature to represent the main effect of stimulus temperature, which was significant for activity to 0.004 and 0.3 M NaCl (see results). Point and line graphs in right column depict the influence of adaptation temperature and time on average responses (spikes/500 ms ± 1 standard error) to 0.004 M (panel B), 0.1 M (panel D), and 0.3 M (panel F) NaCl, collapsed across stimulus temperature; a significant interaction between adapting temperature and time was found for unit firing to each concentration of NaCl (see results). Error bars were normalized for between-unit variability using the method of Cousineau (2005), albeit statistical comparisons were performed on uncorrected data.

**Figure 7.** The influence of amiloride on thermo-gustatory activity to NaCl in an additional sample of N-type neurons (n = 10). A, Average firing (spikes/3.5 s ± 1 standard error) to cool (25°C) and warm (35°C) NaCl mixed with (A+) and without (A-) 30 µM amiloride (***, P ≤ 0.001). Data are collapsed across concentration (0.1 and 0.3 M) and adaptation temperature to represent the interaction between amiloride condition and stimulus temperature on the magnitude of the NaCl response (see results). B, Mean difference (± 1 standard error) in firing between cool and warm NaCl in the A+ and A- conditions in panel A (*, P < 0.05). Error bars in panels B and A were normalized for between-neuron variability using the method of Cousineau (2005), albeit statistical comparisons were performed on uncorrected data. C, Mean time course
of firing to 0.004, 0.1 and 0.3 M NaCl across cells during the A+ (dashed line) and A- (solid line) conditions. Greyed areas surrounding each line denote ± 1 standard error. D, Raw electrophysiological traces showing activity by one N-class neuron to cool taste stimuli following oral adaptation to cool water (left column), to 0.1 and 0.3 M NaCl (cool) following adaptation to a cool amiloride solution (middle column), and the response of this cell to 0.3 M NaCl mixed with and following oral adaptation to amiloride (right column). Upward and downward arrowheads indicate stimulus onset and offset, respectively.
Table 1. Median latencies (s) to first spike for all thermo-concentration solutions of NaCl mixed with (A+) and without (A-) amiloride. Data are based on 10 N-type neurons. Parenthetical value gives the $n$ of cells that showed significant latency to first spike and were used to compute the median, otherwise $n = 10$. $\emptyset$, zero cells showed significant latency to respond to NaCl.

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A

![Graph showing the net spikes/5 s for neurons (n = 49).]

B

![Bar graph showing net spikes/5 s for different types of neurons.]

C

![Image of a brain section with labeled regions.]

**Neurons (n = 49)**

- N-type: 26 neurons
- B-type: 2 neurons
- H-type: 5 neurons
- S-type: 16 neurons

**Net spikes/5 s**

- N-type: 100 spikes
- B-type: 200 spikes
- H-type: 150 spikes
- S-type: 120 spikes
A 34
c
32
°C
24

-5  0  5  10

time post stimulus (s)

B

S-type neuron

W
W→C
C→W

0.1 M sucrose
0.3 M sucrose

C

N-type neuron

0.004 M NaCl
0.1 M NaCl
0.3 M NaCl

°C
A

0.1 M sucrose

stimulus onset

C

C→W

W→C

W

time (s)

-2 0 2 4

0.3 M sucrose

stimulus onset

C

C→W

W→C

W

time (s)

-2 0 2 4

B

[temperature condition]

-2 0 4 2

stimulus onset

A

B

[sucrose] (M)

0.1 M sucrose 0.3 M sucrose

latency (s)

0.1 0.5 1.0 1.5 2.0 2.5 3.0 3.5

* * * * * * *
A 0.1 M sucrose

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<td>W→C</td>
<td>15</td>
</tr>
<tr>
<td>W</td>
<td>17</td>
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</table>

B 0.3 M sucrose

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<th>temperature condition</th>
<th>spikes/500 ms</th>
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<tr>
<td>C→W</td>
<td>20</td>
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<tr>
<td>W→C</td>
<td>25</td>
</tr>
<tr>
<td>W</td>
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</table>
**Figure 1.**

(A) Spike/500 ms for 0.004 M NaCl with adaptation temperature.

(B) Graph showing the effect of 0.004 M NaCl on spike/500 ms over time post latency (s) for cool and warm temperatures.

(C) Spike/500 ms for 0.1 M NaCl with adaptation temperature.

(D) Graph showing the effect of 0.1 M NaCl on spike/500 ms over time post latency (s) for cool and warm temperatures.

(E) Spike/500 ms for 0.3 M NaCl with adaptation temperature.

(F) Graph showing the effect of 0.3 M NaCl on spike/500 ms over time post latency (s) for cool and warm temperatures.

*Significant difference compared to baseline.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>0.004 M NaCl</th>
<th>0.1 M NaCl</th>
<th>0.3 M NaCl</th>
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<td>Cool</td>
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<tr>
<td>Warm</td>
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<td></td>
</tr>
</tbody>
</table>
**A**

Cool NaCl vs. warm NaCl

**B**

Δ spikes

**C**

Dose response to NaCl

**D**

Adaptation to water and 30μM amiloride

- 0.01 M citric acid
- 0.1 M NaCl
- 0.01 M quinine
- 0.5 M sucrose
- 0.3 M NaCl

Stimulus + 30μM amiloride