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Original Article

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Analysis of the rat chorda tympani nerve response to "super salty" sodium carbonate

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In behavioral experiments, rats perceive sodium carbonate (Na_2CO_3) as super salty. In fact, when the dissociated Na^+ ions are accounted for, rats perceive Na_2CO_3 as 5× saltier than equinormal concentrations of NaCl. The chorda tympani nerve (CT) responds to salts through at least two receptor mechanisms and is a model system for understanding how salt taste is transmitted to the brain. Here, we recorded CT nerve activity to a broad range of NaCl (3–300 mM) and Na_2CO_3 (3–300 mN) to investigate why Na_2CO_3 tastes so salty to rats. Benzamil, a specific epithelial sodium channel (ENaC) antagonist, was used to determine the relative contribution of apical ENaCs in Na_2CO_3 transduction. The benzamil-insensitive component of CT nerve responses was enhanced by increasing the adapted tongue temperature from 23°C to 30°C. Na_2CO_3 solutions are alkaline, so we compared neural responses (with and without benzamil) to 100 mM NaCl alone (6.2 pH) and at a pH (11.2 pH) that matched 100 mN Na_2CO_3. As expected, NaCl responses increased progressively with increasing concentration and temperature. Responses to 3 mN Na_2CO_3 were greater than 3 mM NaCl with and without benzamil, but the shape of the first log-fold range of was relatively flat. Adjusting the pH of NaCl to 11.2 abolished the thermal enhancement of 100 mN NaCl through the benzamil-insensitive pathway. Rinsing Na_2CO_3 off the tongue resulted in robust aftertaste that was concentration dependent, thermally sensitive, and benzamil-insensitive. Responses to alkaline NaCl did not recapitulate Na_2CO_3 responses or aftertaste, suggesting multiple transduction mechanisms for the cations ($2Na^+$) and anion (CO_3^{-2}).

Key words: salt taste, chorda tympani, ENaC, temperature, pH, aftertaste

Sodium (Na^+) is an essential nutrient, but consuming it in excess is associated with increased risk of hypertension and stroke (Gleiberman 1973; Page 1976). This increased health risk is more prevalent in industrialized nations, where the majority of sodium is consumed via processed foods (James et al. 1987; CDC 2020). While sodium serves as an essential element in the preservation and safe storage of packaged food items, it has come with a price, with billions of dollars per year spent on salt-related disease. The gustatory system is responsible for detecting sodium concentration, but preservatives with large organic anions attenuate neural-firing rates of sodium-responsive neurons (Beidler 1954; Elliott and Simon 1990; Ye et al. 1991, 1993; Rehnberg et al. 1993; Breza and Contreras 2012b) and reduce the perceived intensity of sodium taste in rats (Geran and Spector 2000) and humans (van der Klaauw and Smith 1995). As the quest for the discovery of a salt taste substitute ensues, so does an impetus for an understanding of the cellular mechanisms underlying salt taste.

Researchers interested in studying mechanisms of salt taste most-commonly employ sodium chloride (NaCl) as the prototypical salty stimulus. A variety of other salty substances are routinely consumed, and one, sodium carbonate (Na_2CO_3), while rarely studied in research laboratories, has been found to have peculiar properties. Writings as far back as ancient Egypt describe the differences in the taste of salts such as natron, a salt rich in Na_2CO_3, which was gathered at dried lake beds and used an essential component in the mummification of human remains (Kurlansky 2003). Using inventive operant procedures designed to assess perceived intensity, Morrison (1969, 1972) demonstrated that rats treated 0.05 M Na_2CO_3 similarly to 0.8 M NaCl, and thirsty rats found Na_2CO_3 to be about tenfold more aversive than NaCl (Morrison 1972).

The possibility that Na_2CO_3 has primarily a salty taste quality to rats was supported by the work of Morrison and Young (1972) who demonstrated that rats will consume 0.03 M Na_2CO_3 as avidly as 0.3 M NaCl when sodium deplete. Morrison and Young in fact used these stimuli to investigate whether consumption during sodium appetite was governed by sodium absorption or perceived saltiness. Since 0.3 M NaCl contains 5 times as much sodium ion as 0.03 M Na_2CO_3—whereas Morrison's earlier work demonstrated they were perceived as equally intense—Morrison and Young concluded that the strong salty taste of Na_2CO_3 provoked consumption. Perhaps more convincingly, recent work replicated Morrison and Young's observations in furosemide-injected sodium-deplete rats across a broad concentration range (St. John et al. 2017). Because both the NaCl and Na_2CO_3 concentration–response functions in that study were an inverted-U across 10-fold different concentration ranges, St. John et al. argued that both NaCl and Na_2CO_3 were primarily salty to rats.

An understanding of the mechanism(s) underlying this "super saltiness" could reveal new ways to manipulate the

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intensity of salt taste. The majority of sodium taste is carried by the chorda tympani (CT) nerve (St John et al. 1995, 1997), a branch of the facial nerve, which innervates taste buds in the anterior 2/3rds of the tongue. As such, the CT has served as model system for understanding of salt taste transduction for decades. Much of our understanding of the CT neuron types and their role in behavior have been studied using the Sprague-Dawley rat as the model system. Here, we recorded from the CT nerve in anesthetized Sprague-Dawley rats, to investigate how Na_2CO_3 is sensed by the gustatory system. For consistency with whole nerve and single-unit recordings from rat CT neurons, we used benzamil to dissect sodium responses carried through Na^+ selective neurons, from nonselective salt-generalist neurons (Breza et al. 2010; Breza and Contreras 2012a, 2012b). In the gustatory system, benzamil is more selective for sodium salts compared to amiloride (Lundy and Contreras 1997), and specifically inhibits Na^+ selective neurons in the CT (Breza et al. 2010; Breza and Contreras 2012a, 2012b). Generalist neurons in the non-selective pathway were further studied by investigating the effects of elevated temperature (Ogawa et al. 1968; Lundy and Contreras 1999; Lyall et al. 2004; Breza et al. 2006) on salt-taste responses with and without benzamil.

Based on recent behavioral experiments (St John et al. 2017), we hypothesized that responses to Na_2CO_3 would be greater than those to NaCl at lower concentrations, since rats treated equinormal concentrations (same concentration of dissociated Na^+ ions) of Na_2CO_3 as five-fold saltier compared to NaCl. We also hypothesized that blocking apical epithelial sodium channels (ENaCs) with benzamil would attenuate a portion of the response, based on amiloride's suppression of Na_2CO_3 over a range of behaviorally relevant concentrations (St John et al. 2017), but expected to see differences in the shape of CT responses given that Na_2CO_3 is perceived as "super salty" to rats (Morrison 1972; St John et al. 2017).

Materials and methods

All procedures were performed in accordance with, and approved by, the Institutional Animal Care and Use Committee at Eastern Michigan University.

Animals and surgery

Adult male (n = 11) Sprague-Dawley rats (Harlan—now Envigo) were housed in plastic cages in a temperature-controlled (23°C) colony room on a 12:12-h light–dark cycle with lights on at 0700. Food (Purina, 5001) and tap water were provided ad libitum. Rats weighed between 365–450 g.

Rats were anesthetized with urethane (1.5 g/kg body wt), tracheostomized, and secured in a custom-made brass head holder (J.M. Breza), which could be rotated for the nerve dissection. The whole CT nerve was exposed by a mandibular approach, transected proximally, and desheathed for recording. Each rat's tongue was gently extended and held in place by a suture attached to its ventral surface. Body temperature was maintained at 37°C with a custom-made (Paul Hendrick; FSU) heating pad and temperature controller.

Neurophysiology

The CT nerve was cut near its entrance into the tympanic bulla and draped over a platinum wire hook (positive polarity), and the entire cavity was then filled with high-quality paraffin oil (VWR) to isolate the signal from ground and maintain nerve integrity. An indifferent electrode (negative polarity) was attached to the skin overlying the cranium with a stainless-steel wound clip. Neural activity was differentially amplified (alternating current X10,000; A-M Systems, Sequim, WA, bandpass 300–5,000 Hz), observed with an oscilloscope, digitized with waveform hardware and software (Spike 2; Cambridge Electronic Design, Cambridge, England), integrated using the root mean square (RMS) method (250 ms time constant), and stored on a computer for offline analysis.

Stimulus delivery and stimulation protocols

Solutions were presented to the tongue at a constant flow rate (100 µl/s) and temperature (23°C and 30°C ± 0.3°C for the present study) by an air-pressurized, 16-channel commercial, fluid-delivery system and heated perfusion cube (OctaFlow; ALA Scientific Instruments, Farmingdale, NY). We chose to first deliver solutions to the tongue at 23°C as this is the room temperature at Rollins College where behavioral experiments with Na_2CO_3 were conducted. All 11 rats were tested with stimuli at 23°C. To enhance the benzamil-insensitive pathway of salt taste, we presented the temperature of the rinse and taste solutions again at 30°C in 6 out of the 11 rats. The presentation of each stimulus was signaled to Spike 2 by square waveforms produced by the fluid-delivery system. All solutions and pharmacological reagents were reagent grade and purchased from Fischer Scientific or Sigma Aldrich. For consistent with behavioral experiments, deionized H_2O (18 MΩ/cm^2) served as the rinse solution and solvent for all stimuli. Although we have used artificial saliva as the rinse and solvent for taste solutions in several our whole nerve experiments in rats (Breza and Contreras 2012a, 2012b), Na_2CO_3 precipitated the $CaCl_2$ out of solution and therefore it could not be used. We tested CT nerve responses to 5-s applications of 0.3M NH_4Cl, NaCl (3–300 mM), Na_2CO_3 (1.5–150 mM or 3–300 mN), and 100 mM NaCl with a pH of 11.2 to match 100 mN Na_2CO_3. The initial pH of 100 mM NaCl was 6.2. To adjust the pH of NaCl to 11.2, we used a minute amount of 2M NaOH, pulled into a glass tube (A-M Systems, inner diameter 0.4 mm, #625000) by capillary action. The pH of the stirring solution was monitored and the tube was inserted into the solution and withdrawn until the pH reached 11.2. If the solution pH went over 11.2, it was discarded—HCl was never used to adjust the pH down. Because Na_2CO_3 is a disodium salt, we will refer to solution concentrations of Na_2CO_3 in normality, so that the reader understands concentration in terms of the number of dissociated Na^+ ions. Prior to taste application, the tongue adapted (60 s) to H_2O or H_2O + 5 µM benzamil to block apical Na^+ channels as described previously (Breza and Contreras 2012a). There was an uninterrupted flow throughout the course of the experiment, so that the experimental environment on the epithelium remained controlled.

Data analysis

For consistency with our previous studies using similar experimental conditions (Breza and Contreras 2012a, 2012b), we analyzed the area under the curve (AUC) for the 5-s integrated-nerve response and subtracted that from the 5-s period of baseline activity immediately prior to each stimulus (area of the 5-s response-area of baseline). Normalized responses were calculated for each stimulus by

dividing each response by the average 23°C 300 mM NH_4Cl (responses to 300 mM NH_4Cl at the beginning and end of the protocol) response. Because we noticed that nerve responses to Na_2CO_3 continued to remain elevated during the H_2O rinse, we calculated 5 s AUC measurements for up to 30 s (6 bins) during the rinse period for each taste stimulus for comparison. We refer to this post-stimulus rinse time as "aftertaste." Consistent responses (criteria ± 10%) to 300 mM NH_4Cl (23°C and 30°C) at the beginning and end of each temperature protocol were indicators of nerve integrity and recording stability.

At the start of this experiment, we chose to use a 250 ms time constant to give us the best opportunity to detect differences in the phasic component of the response, such as peak responses, latency to peak and slope, as they are the earliest sensory component during active licking, and reliable features for concentration-dependent responses (Whiddon et al. 2018). Consistent with our previous reports, we found peak responses increased with increasing concentration, which can be clearly seen in Fig. 1. However, because there were no substantial differences in the peak, latency to peak, or slope of responses between NaCl and Na_2CO_3 solutions, we omitted average data from the current report, as the added statistics did not improve the overall quality of the report.

We also analyzed tonic responses (4.5 s after stimulus onset), for consistency with our previous whole-nerve studies using nearly identical stimulus parameters (Whiddon et al. 2018). We observed interesting differences in tonic responses to NaCl and Na_2CO_3 across the concentration range, such as a dose-dependent inhibition of tonic responses from 3 to 30 mN Na_2CO_3 (which can clearly be seen in Fig. 1 and were significant). We have, however, limited data presentation to 100 mM/mN concentration of the salts because without proper pH controls, these data are not easily interpreted. 100 mM NaCl is a midrange-hypotonic concentration and the standard stimulus for many whole nerve and single unit studies, as both salt taste pathways (amiloride/benzamil-sensitive and amiloride/benzamil-insensitive) are active at this concentration (Ninomiya and Funakoshi 1988; Rehnberg et al. 1993; Lyall et al. 2001, 2002; Breza et al. 2010; Breza and Contreras 2012a, 2012b) and it is not hyperosmotic. Na_2CO_3 is alkaline and we therefore compared 100 mN NaCl responses alone (pH 6.2) and at a pH that matched 100 mN Na_2CO_3 (pH 11.2) at 23°C and 30°C, with and without benzamil treatment. Matching the pH of NaCl across the Na_2CO_3 concentration range was beyond the scope of the present investigation but worth exploring in the future.

Two-way repeated-measures (RM) ANOVAs were used to compare 5 s AUC responses to NaCl and Na_2CO_3 across the concentration range (with and without benzamil), differences in response to NaCl and Na_2CO_3 at 23°C and 30°C, differences in AUC and tonic responses to NaCl alone, alkaline pH, effects of temperature on Na_2CO_3 at 23°C and 30°C, and the first 30s (six 5 s bins) of aftertaste (Statistica; StatSoft, Tulsa, OK). One-way RM ANOVAs were used to compare AUC and tonic responses to NaCl alone, alkaline pH Na_2CO_3 alone and with benzamil at 23°C or 30°C. Specific comparisons within ANOVAs were accomplished via contrast coefficients. A paired *T* test was used to indicate whether 5 s AUC and tonic responses to NH_4Cl were statistically different from each other at 23°C and 30°C (supplemental). Contrast tests were used to understand main effects and interactions. Cohen's *d* method $\left[d = (\bar{x}_1 - \bar{x}_2)/SD_{pooled}\right]$ was also used to indicate the magnitude of the effect for pairwise comparisons. By convention, effect sizes (d) ≤ 0.2 were considered small, whereas 0.5 were considered medium, and ≥ 0.8 were considered large (Cohen 1992). Criteria for statistically significant effects were set at $P < 0.05$. For transparency exact F, and P values are reported unless values were less than 1×10^{-6}. To correct for multiple comparisons within each ANOVA, we applied the Benjamini–Hochberg (B–H) linear set up procedure (Benjamini and Hochberg 1995), for consistency with our whole nerve and single unit experiments (Baumer-Harrison et al. 2020). B–H critical value = $p < (i/m)Q$, where i is the rank, m is the total number of comparisons, and Q is the false discovery rate. For the procedure, we used a false discovery rate of 5% as it is analogous to the alpha criterion. All graphic data are presented as means ± standard error of the mean (SEM).

Results

Figure 1A shows integrated CT nerve responses to a concentration series of NaCl and Na_2CO_3 at 23°C and 30°C. Overall, the response patterns we observed with Na_2CO_3 on nerve activity were complex. Figure 1B shows integrated CT nerve responses to 3 mM NaCl and 3 mN Na_2CO_3 and how measurements of taste and aftertaste were calculated. As shown, responses to 3 mM NaCl were entirely phasic, whereas 3 mN Na_2CO_3 showed robust activity throughout the 5-s stimulation period, and a robust aftertaste during the rinse post Na_2CO_3 application.

Average AUC responses to NaCl and Na_2CO_3 at 23°C and 30°C are shown in Fig. 2. When NaCl and Na_2CO_3 were compared at 23°C, there was a main effect of Concentration ($F(4,40) = 211.33$, $P < 1 \times 10^{-6}$) and a Salt × Concentration interaction ($F(4,40) = 4.50$, $P = 0.004$). Both salts increased nerve activity with increasing concentration. The Concentration × Salt interaction appears to reflect the differences in the shape of concentration dependent responses between NaCl and Na_2CO_3. Contrast tests at 23°C showed that AUC responses to 3 mN Na_2CO_3 were significantly greater (B–H, $P < 0.01$) than those to 3 mM NaCl (AUC: $P = 4 \times 10^{-4}$, $d = 1.3$). Responses to 3 mN Na_2CO_3 were 1.9× greater than those to NaCl.

When NaCl and Na_2CO_3 were compared at 30°C, there was a main effect of Concentration ($F(4,20) = 98.16$, $P < 1 \times 10^{-6}$) and a significant Salt × Concentration interaction ($F(4,20) = 5.24$, $P = 0.005$). Contrast tests showed that responses to 3 mN Na_2CO_3 were 2.3× greater (B–H $P < 0.02$) than responses to 3 mM NaCl ($P = 4 \times 10^{-4}$, $d = 2.1$). Responses to Na_2CO_3 were similar to NaCl at 10–100 mM/mN, but significantly less at 300 mM/mN ($P = 0.002$, $d = 2.0$;). As expected, NaCl responses increased from 23°C to 30°C (Temperature: $F(1,5) = 24.81$, $P = 0.004$; Concentration: $F(4,20) = 419.14$, $P < 1 \times 10^{-6}$; Temperature × Concentration: $F(4,20 = 66.71$, $P < 1 \times 10^{--}$). Surprisingly, while there were main effects of Na_2CO_3 concentration ($F(4,20) = 34.04$, $P < 1 \times 10^{-6}$), there were neither significant main effects of Temperature nor a Temperature × Concentration interaction for Na_2CO_3. Therefore, unlike NaCl, responses to Na_2CO_3 were not significantly modulated by temperature. NH_4Cl is transduced primarily through the benzamil-insensitive pathway (Lundy and Contreras 1997) and NH_4Cl increases

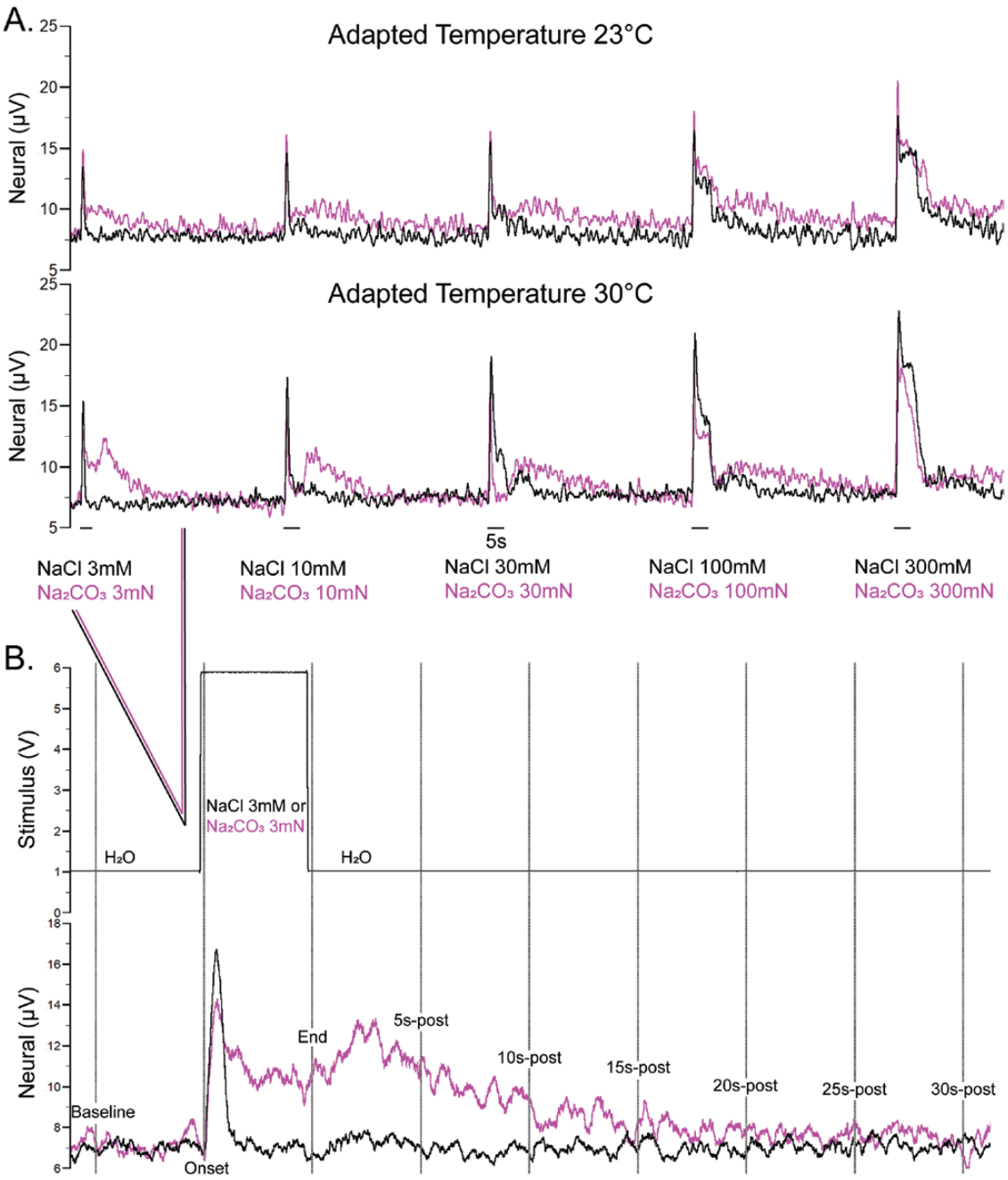

Fig. 1. *Temperature modulates NaCl and Na_2CO_3 responses and Na_2CO_3 aftertaste.* Example integrated CT nerve recording to NaCl and Na_2CO_3 across 2 log-fold concentration range (A). Responses to NaCl (black) and Na_2CO_3 (magenta) are superimposed for easy comparison. Responses to NaCl follow a predictable pattern; neural activity increases with concentration and warmer solutions (23–30°C), and neural activity quickly returned to baseline during the rinse period. In contrast, Na_2CO_3 responses across the concentration range were not significantly impacted by temperature, but the onset of H_2O rinse produced a robust aftertaste, which was modulated by temperature and concentration and persisted long after the taste stimulus was rinsed off the tongue. Line under each stimulus indicates the start and stop of the 5-s stimulus presentation, as determined by square waves (not shown in A for space). Responses to 30°C 3 mN Na_2CO_3 and 3 mM NaCl from A are shown in B on a smaller scale to show differences in the shape of responses and how responses were quantified. 3 mM NaCl responses are entirely phasic, whereas 3 mN Na_2CO_3 have phasic and tonic components, and water produces a robust aftertaste that persisted for several times the length of taste stimulation. Square wave (stimulus) indicates the switch from H_2O to NaCl or Na_2CO_3. Vertical lines are labeled to indicate measurement of 5-s baseline to stimulus onset, 5-s stimulation from onset to end (AUC), and aftertaste, which was quantified in 5-bins for the first 30s of the rinse period.

with warmer temperatures. Therefore, as a control experiment, we compared NH_4Cl responses at 23°C and 30°C. As expected, responses to NH_4Cl were significantly increased (by 51%) from 23°C to 30°C (see Supplementary Data S1).

Effects of benzamil on NaCl and Na_2CO_3

As expected, the addition of 5 μM benzamil to the rinse solution significantly attenuated NaCl responses (Fig. 2) across the concentration range at 23°C (Benzamil: $F(1,10) = 18.09$, $P =$

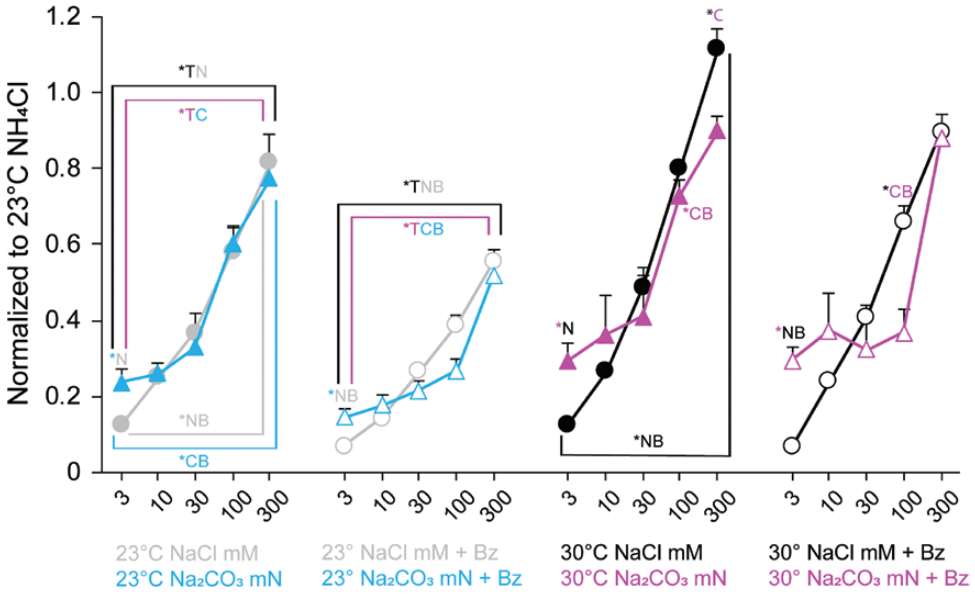

Fig. 2. *Differences in concentration responses profiles to NaCl and Na_2CO_3 at 23°C and 30°C.* Average AUC responses to NaCl (circles) and Na_2CO_3 (triangles) (closed symbols) and with benzamil (open symbols). *, letters, and brackets are color coordinated (23°C NaCl is gray; 23°C Na_2CO_3 is blue; 30°C NaCl is black; 30°C Na_2CO_3 is magenta) to reflect significant differences. * indicates B–H adjusted significant differences. N is NaCl; C is Na_2CO_3; NB is NaCl + benzamil; CB is Na_2CO_3 + benzamil; TN is differences in NaCl from 23°C to 30°C; TC is differences in Na_2CO_3 from 23°C to 30°C; TNB is differences in NaCl + benzamil from 23°C to 30°C; TCB is differences in Na_2CO_3 + benzamil from 23°C to 30°C. Color coordination with * and letters helps to clarify specific differences.

0.002; Concentration: $F(4,40) = 189.18$, $P < 1 \times 10^{-6}$; Benzamil × Concentration: $F(4,40) = 13.06$, $P = 1 \times 10^{-6}$) and 30°C (Benzamil: $F(1,5) = 47.47$, $P = 0.001$; Concentration: $F(4,20) = 500.23$, $P < 1 \times 10^{-6}$; Benzamil × Concentration: $F(4,20) = 14.57$, $P = 1 \times 10^{-6}$). As expected, and consistent with previous studies with amiloride (Lundy and Contreras 1997), the percent suppression of NaCl responses with benzamil was greater at 23°C than at 30°C (Temperature: $F(1,5) = 41.06$, $P = 0.001$; Concentration: $F(4,20) = 355.05$, $P < 1 \times 10^{-6}$; Temperature × Concentration: $F(4,20) = 84.56$, $P < 1 \times 10^{-6}$).

Benzamil significantly attenuated Na_2CO_3 responses at 23°C (Benzamil: $F(1,10) = 42.24$, $P = 7 \times 10^{-5}$; Concentration: $F(4,40) = 132.45$, $P < 1 \times 10^{-6}$; Benzamil × Concentration: $F(4,40) = 38.14$, $P < 1 \times 10^{-6}$] and 30°C (Benzamil: $F(1,5) = 10.74$, $P = 0.022$; Concentration: $F(4,20) = 22.39$, $P < 1 \times 10^{-6}$; Benzamil × Concentration: $F(4,20) = 19.36$, $P = 1 \times 10^{-6}$). Although there was a main effect and interaction of Benzamil on Na_2CO_3 responses at 30°C, contrast tests of the interaction showed that benzamil significantly inhibited (50% reduction) responses at 100 mN only ($P = 5 \times 10^{-4}$, $d = 2.8$, B–H $P < 0.01$). Interactions appear to reflect differences in the shape of concentration dependent responses—steeper dose–response function for Na_2CO_3 without Benzamil and a response plateau at 3–100 mN for Na_2CO_3 with benzamil. Benzamil suppressed tonic responses tonic responses to 100 mN Na_2CO_3 by 56% at 23°C and by 50% at 30°C (see Fig. 3, pH control below).

Consistent with numerous studies, benzamil-insensitive nerve responses to NaCl increased from 23°C to 30°C (Temperature: $F(1,5) = 41.06$, $P = 0.001$; Concentration: $F(4,20) = 355.05$, $P < 1 \times 10^{-6}$; Temperature × Concentration $F(4,20) = 84.56$, $P < 1 \times 10^{-6}$). Benzamil-insensitive responses to Na_2CO_3 also increased from 23°C to 30°C (Temperature: $F(1,5) = 25.54$, $P = 0.004$; Concentration: $F(4,20) = 43.80$, $P < 1 \times 10^{-6}$; Temperature × Concentration: $F(4,20) = 9.53$; $P = 2 \times 10^{-4}$). Benzamil-insensitive responses to NaCl vs Na_2CO_3 were significantly different at 23°C (Concentration: $F(4,40) = 275.74$, $P < 1 \times 10^{-6}$; Salt × Concentration: $F(4,40) = 9.74$, $P < 1 \times 10^{-6}$), and 30°C (Concentration: $F(4,20) = 172.26$, $P < 1 \times 10^{-6}$; Salt × Concentration: $F(4,20) = 16.40$, $P = 4 \times 10^{-6}$). Salt × Concentration interactions appear to reflect the differences in the shape of concentration-dependent responses between NaCl and Na_2CO_3. At 23°C, benzamil-insensitive (B–H $P < 0.03$) responses to 3 mN Na_2CO_3 were significantly greater (2.1×) than those to 3 mM NaCl ($P = 0.003$, $d = 1.4$). Responses to NaCl, however, were significantly greater than those to Na_2CO_3 at 30 mM/mN ($P = 0.024$, $d = 0.7$) and 100 mM/mN ($P = 0.002$, $d = 1.3$). Thus, while benzamil insensitive responses to 23°C Na_2CO_3 were greater than those to NaCl at the lowest concentration, midrange concentrations were steeper for NaCl than they were for Na_2CO_3, but equal at the highest concentration. Contrast tests (B–H $P < 0.02$) at 30°C showed that benzamil-insensitive responses to Na_2CO_3 at 3 mN were significantly greater (4.5×) than those to 3 mM NaCl ($P = 4 \times 10^{-4}$, $d = 3.2$) but similar at 10, 30, and 300 mm/mN. Interestingly, benzamil-insensitive NaCl responses were significantly greater (1.8×) than Na_2CO_3 responses at 100 mM/mN ($P = 0.014$, $d = 2.2$).

pH effects

Because 100 mM NaCl and 100 mN Na_2CO_3 responses differed in terms of their temperature and benzamil sensitivity, we then examined whether these differences were due to changes of pH between NaCl and Na_2CO_3. To analyze the effects of pH on Na^+ responses through benzamil-sensitive and benzamil insensitive pathways, we compared AUC and tonic responses of (with and without benzamil) 100 mM NaCl (pH 6.2), 100 mN Na_2CO_3 (pH 11.2), and NaCl pH 11.2. Figure 3 shows raw responses to 30°C NaCl (6.2), NaCl pH 11.2, and Na_2CO_3 (11.2), alone (3A) and with benzamil

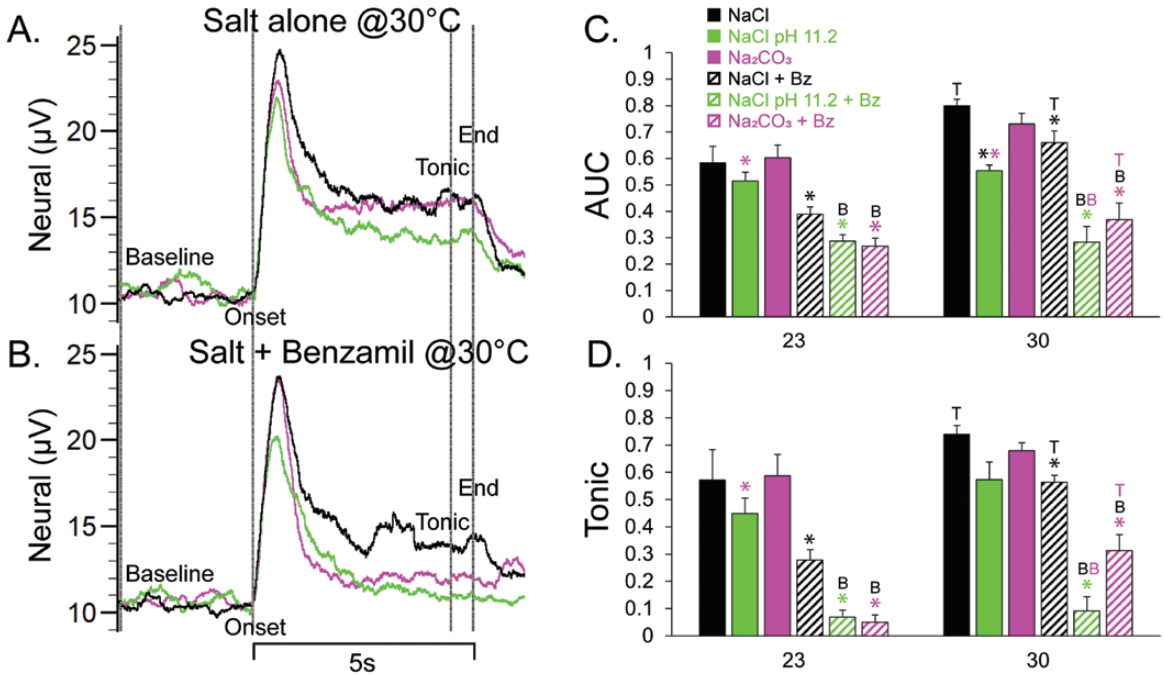

Fig. 3. *Alkaline pH differentially modulates benzamil-insensitive 100 mm NaCl and 100 mN Na_2CO_3 responses at 23°C and 30°C.* Example raw responses to 30°C NaCl (black), Na_2CO_3 (magenta), and NaCl pH 11.2 (green) alone (A) and with benzamil (B). Vertical lines are labeled to indicate measurement of 5-s baseline to stimulus onset, 5-s stimulation from onset to end (AUC), and tonic responses measured 4.5 s after stimulus onset. Average 5-s AUC responses are shown in C, whereas average tonic responses are shown in D. Colors schemes are the same as raw data, and labeled in the key (C). * and letters are color coordinated to clarify specific differences. * indicates B-H adjusted significant differences. T is differences from 23°C to 30°C. B stands for benzamil.

(3B), and average AUC (3C) and tonic (3D) responses to these stimuli presented at 23°C and 30°C. There were significant main effects of Stimulus for AUC (23°C: $F(5,50) = 26.03$, $P < 1 \times 10^{-6}$; 30°C: $F(5,25) = 28.98$, $P < 1 \times 10^{-6}$) and tonic (23°C: $F(5,50) = 20.38$, $P < 1 \times 10^{-6}$; 30°C: $F(5,25) = 26.48$, $P < 1 \times 10^{-6}$) responses. Contrast tests for AUC (B–H $P < 0.033$ for 23°C and $P < 0.039$ for 30°C) and tonic responses (B–H $P < 0.033$ for 23°C and 30°C) showed that benzamil significantly attenuated AUC and tonic responses to NaCl at 23°C (AUC: $P = 0.002$, $d = 1.2$; tonic $P = 0.006$, $d = 3.5$) and 30°C (AUC: $P = 0.01$, $d = 1.6$; tonic: $P = 0.01$, $d = 2.5$). Similarly, benzamil significantly attenuated AUC and tonic responses to Na_2CO_3 at 23°C (AUC: $P = 2 \times 10^{-5}$, $d = 2.5$; tonic $P = 1 \times 10^{-5}$, $d = 2.8$) and 30°C (AUC: $P = 5 \times 10^{-4}$, $d = 2.8$; tonic: $P = 0.004$, $d = 3.2$).

Overall, benzamil had the greatest suppression on tonic responses compared to AUC. This is primarily because the phasic component of the response lasted ~1.65 s (33% of the 5-s response) and peak responses to NaCl and Na_2CO_3 were not significantly attenuated by benzamil (average peak data not presented). In fact, tonic responses to NaCl were significantly suppressed by ~52% at 23°C and by 24% at 30°C, whereas Na_2CO_3 responses were suppressed by 92% at 23°C and by 54% at 30°C. This is convincing evidence that pretreating the tongue with benzamil is sufficient for blocking Na^+ responses throughout the 5-s duration of stimulation, consistent with our previous studies of whole nerve and single unit recordings (Breza and Contreras 2012a). It is also consistent with previous data that amiloride and benzamil are more effective at cooler temperatures where the amiloride/benzamil-insensitive component is less pronounced (Lundy and Contreras 1997).

At 23°C, AUC and tonic responses to NaCl were not significantly different than NaCl pH 11.2, but at 30°C, AUC responses to NaCl pH 11.2 were significantly less (31%) than those to NaCl ($P = 4 \times 10^{-4}$, $d = 4.4$), which can be seen in raw (Fig. 3A) and average (3C). Tonic responses to NaCl vs NaCl pH 11.2 were not significantly different at 23°C or 30°C (22% and 23% decrease, respectively), but it is worth noting that the effect size was large at 30°C ($P = 0.122$, $d = 1.3$). However, despite being matched for pH responses to Na_2CO_3 were significantly greater than NaCl pH 11.2 at 23°C (AUC $P = 0.004$, $d = 0.7$; tonic $P = 0.006$, $d = 0.6$) and at 30°C (AUC $P = 4 \times 10^{-4}$, $d = 2.2$). Interestingly, AUC and tonic responses of benzamil-insensitive NaCl responses were further reduced when the pH of NaCl was increased to 11.2 at 23°C (AUC $P = 0.005$, $d = 1.2$; tonic $P = 0.003$, $d = 1.9$) and 30°C (AUC $P = 0.002$, $d = 2.9$; tonic 4×10^{-4}, $d = 4.8$), suggesting that the benzamil-insensitive component was inhibited by an alkaline pH. In fact, the benzamil-insensitive tonic response to NaCl was essentially eliminated at a pH of 11.2. Although benzamil-insensitive responses to NaCl pH 11.2 and Na_2CO_3 were not significantly different at 23°C, benzamil-insensitive AUC and tonic responses to 30°C Na_2CO_3 were significantly greater than those to NaCl pH 11.2 (AUC $P = 0.047$, $d = 0.6$; tonic $P = 0.017$, $d = 1.6$). The increased temperature enhances the benzamil-insensitive pathway, and suggests that alkaline NaCl is not equivalent to Na_2CO_3 through the benzamil-insensitive pathway.

When the effect of pH on NaCl responses at 23°C and 30°C were compared to Na_2CO_3, there were significant main effects of Temperature on AUC ($F(1,5) = 21.21$, $P = 0.006$) and tonic ($F(1,5) = 17.85$, $P = 0.008$) responses, significant main effects of Stimulus on AUC ($F(5,25) = 36.33$, $P < 1 \times 10^{-6}$) and

tonic ($F(5,25) = 29.65$, $P < 1 \times 10^{-6}$) responses, and significant Temperature × Stimulus interactions (AUC: $F(5,25) = 9.25$, $P = 4 \times 10^{-5}$; Tonic: $F(5,25) = 7.17$, $P = 3 \times 10^{-4}$). After the B–H adjustment, $P < 0.025$ was considered significant for contrast tests of AUC and tonic responses. As expected, AUC and tonic responses to NaCl significantly increased from 23°C to 30°C (AUC: $P = 0.002$, $d = 3.5$; tonic: $P = 0.005$, $d = 2.8$). An unexpected finding was that increasing the pH of NaCl to 11.2 eliminated this temperature effect on AUC and tonic responses, as NaCl pH 11.2 at 23°C and 30°C were not significantly different. This result is consistent with responses to 100 mN Na_2CO_3 (11.2 pH), which also were not significantly different at 23°C and 30°C.

These results suggested that the benzamil-insensitive component of NaCl, which is known to increase with increasing stimulus temperature, was eliminated by increasing the pH of NaCl to 11.2. To test, this directly, we examined whether the temperature effect through the benzamil-insensitive pathway was eliminated at an alkaline pH. As predicted, the addition of benzamil revealed that the temperature effect on NaCl responses through the benzamil-insensitive pathway was eliminated when the pH of NaCl was increased to 11.2. While AUC and tonic responses to NaCl + benzamil significantly increased from 23 to 30°C (AUC: $P = 0.001$, $d = 4.2$; Tonic: $P = 0.002$, $d = 4.9$), responses to NaCl + benzamil pH 11.2 were not significantly different at 23 and 30°C. In contrast, benzamil-insensitive AUC and tonic responses to Na_2CO_3 significantly increased from 23 and 30°C (AUC $P = 0.020$, $d = 1.1$; tonic $P = 9 \times 10^{-4}$, $d = 2.2$). Thus, although the temperature effects on benzamil-insensitive NaCl responses were eliminated with an alkaline pH, the same was not true for Na_2CO_3 implying that perhaps additional mechanisms are in play.

Aftertaste of NaCl and Na_2CO_3

Averages of aftertaste to NaCl and Na_2CO_3 can be seen in raw data Fig. 1 and analyzed data Figs 4 and 5. Aftertaste is clearly demonstrated as a response that remains elevated during the rinse period. As shown in Figs 1, 4, and 5, nerve activity for NaCl decreased quickly to baseline during the rinse, whereas nerve activity following Na_2CO_3 stimulation remained elevated. As a rule, the weaker the NaCl response, the faster nerve activity returned to baseline during the aftertaste rinse period. Aftertaste to NaCl was not significantly affected by benzamil or adapted temperature. Cooler temperatures and benzamil reduce the magnitude of the NaCl response and so neural activity is quicker to return to baseline. In contrast, the post-rinse period of Na_2CO_3 was marked by a robust aftertaste, that was modulated by concentration and temperature. After 15–20 s of rinsing, responses to NaCl were at or near baseline levels, but the magnitude of Na_2CO_3 aftertaste was still present and typically ranged from 4–36× greater than NaCl.

Neural activity remained elevated after 3 mN Na_2CO_3 application at 23°C and 30°C, producing a robust aftertaste. 3 mN Na_2CO_3 aftertaste was significantly greater than to NaCl aftertaste at 23°C (Salt: $F(1,10) = 65.00$, $P = 1 \times 10^{-5}$; Time: $F(5,50) = 22.26$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,50) = 14.48$, $P < 1 \times 10^{-6}$; 5 s post–25 s post, B–H $P < 0.042$, $d = 0.8$–2.3) and 30°C (Salt: $F(1,5) = 25.26$, $P = 0.004$; Time: $F(5,25) = 24.05$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,25) = 10.42$, $P = 2 \times 10^{-5}$; 5 s post–25 s post, B–H $P < 0.042$, $d = 2.7$–3.7). In the presence of benzamil, aftertaste to 3 mN Na_2CO_3 remained significantly elevated relative to aftertaste of 3 mM NaCl at 23°C (Salt: $F(1,10) = 15.25$, $P = 0.003$; Time: $F(5,50) = 60.46$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,50) = 33.62$, $P < 1 \times 10^{-6}$; 5 s post–20s post, B–H $P < 0.033$, $d = 1.2$–3.1) and 30°C (Salt: $F(1,5) = 26.15$, $P = 0.004$; Time: $F(5,25) = 32.39$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,25)= 28.84$, $P < 1 \times 10^{-6}$; 5 s post–25 s post, B–H $P < 0.042$, $d = 1.9$–3.5). Aftertaste to 3 mN Na_2CO_3 with benzamil was significantly greater at 30°C than at 23°C (Temperature: $F(1,5) = 36.48$, $P = 0.002$; Time: $F(5,25) = 47.01$, $P < 1 \times 10^{-6}$; Temperature × Time: $F(5,25) = 10.99$, $P = 1 \times 10^{-5}$) at each timepoint post-stimulation (5 s post–30 s post, B–H $P < 0.05$, $d = 0.6$–2.1).

10 mN Na_2CO_3 produced robust aftertaste, as neural activity during the H_2O rinse remained significantly elevated over NaCl at each timepoint post stimulation at 23°C (Salt: $F(1,10) = 30.86$, $P = 2 \times 10^{-4}$; Time: $F(5,50) = 60.05$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,50) = 12.54$, $P < 1 \times 10^{-6}$; 5 s post–30 s post, B–H $P < 0.05$, $d = 1.0$–2.4) and 30°C (Salt: $F(1,5) = 78.51$, $P = 3 \times 10^{-4}$; Time: $F(5,25) = 25.25$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,25) = 8.57$, $P = 8 \times 10^{-5}$; 5 s post–30 s post, B–H $P < 0.05$, $d = 2.3$–5.6). Benzamil had no main effect on attenuating aftertaste at either 23°C or 30°C. Aftertaste to 10 mN Na_2CO_3 increased significantly from 23°C to 30°C (Temperature: $F(1,5) = 29.46$, $P = 0.003$; Time: $F(5,25) = 26.98$, $P < 1 \times 10^{-6}$; Temperature × Time: $F(5,25) = 6.06$, $P = 8 \times 10^{-4}$; 5 s post–30 s post, B–H $P < 0.05$, $d = 2.3$–5.6) and was still robust with benzamil (Temperature: $F(1,5) = 33.97$, $P = 0.002$; Time: $F(5,25) = 47.18$, $P < 1 \times 10^{-6}$; Temperature × Time: $F(5,25) = 21.05$, $P < 1 \times 10^{-6}$; 5 s post–30 s post, B–H $P < 0.05$, $d = 1.6$–2.7). At 23°C, aftertaste to 10 mN Na_2CO_3 at was significantly greater than aftertaste to 10 mM NaCl alone (Salt: $F(1,10) = 30.86$, $P = 2 \times 10^{-4}$; Time: $F(5,50) = 60.05$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,50) = 12.54$, $P < 1 \times 10^{-6}$; 5 s post–30 s post, B–H $P < 0.05$, $d = 1.0$–2.4) and with benzamil (Salt: $F(1,10) = F(1,10) = 38.75$, $P = 1 \times 10^{-4}$; Time: $F(5,50) = 27.19$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,50) = 17.86$, $P < 1 \times 10^{-6}$; 5 s post–30 s post, B–H $P < 0.05$, $d = 1.1$–3.6). At 30°C, aftertaste to 10 mN Na_2CO_3 at was also significantly greater than aftertaste to 10 mM NaCl alone (Salt: $F(1,5) = 78.51$, $P = 3 \times 10^{-4}$; Time: $F(5,25) = 25.25$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,25) = 8.57$, $P = 8 \times 10^{-5}$; 5 s post–30 s post, B–H $P < 0.05$, $d = 2.3$–5.6) and with benzamil (Salt: $F(1,5 = 66.32$, $P = 5 \times 10^{-4}$; Time: $F(5,25) = 44.39$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,25) = 19.05$, $P < 1 \times 10^{-6}$; 5 s post–30 s post, B–H $P < 0.05$, $d = 1.8$–4.7].

At 23°C and 30°C, aftertaste to 30 mN Na_2CO_3 was robust and benzamil had no main effect on aftertaste nerve activity. Temperature, however, had a profound effect on the magnitude of aftertaste to 30 mN Na_2CO_3, and responses significantly increased (Temperature: $F(1,5) = 24.40$, $P = 0.004$; Time: $F(5,25) = 16.75$, $P < 1 \times 10^{-6}$; 10 s post–30 s post, B–H $P < 0.042$, $d = 0.8$–1.5). The temperature effects were still robust in the presence of benzamil (Temperature: $F(1,5) = 56.79$, $P = 7 \times 10^{-4}$; Time: $F(5,25) = 23.68$, $P < 1 \times 10^{-6}$; Temperature × Time: $F(5,25) = 8.78$, $P = 7 \times 10^{-5}$; 5 s post–30 s post, B–H $P < 0.05$, $d = 1.5$–2.2) indicating the mechanism(s) behind this were benzamil-insensitive. At 23°C, aftertaste to 30 mN Na_2CO_3 was significantly greater than aftertaste to 30 mM NaCl alone (Salt: $F(1,10) = 41.56$, $P = 7 \times 10^{-5}$; Time: $F(5,50) = 37.74$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,50) = 11.67$, $P < 1 \times 10^{-6}$; 10 s post–30 s post, B–H $P < 0.042$, $d = 1.1$–2.0) and with benzamil (Salt: $F(1,10) = 12.63$, $P = 0.005$;

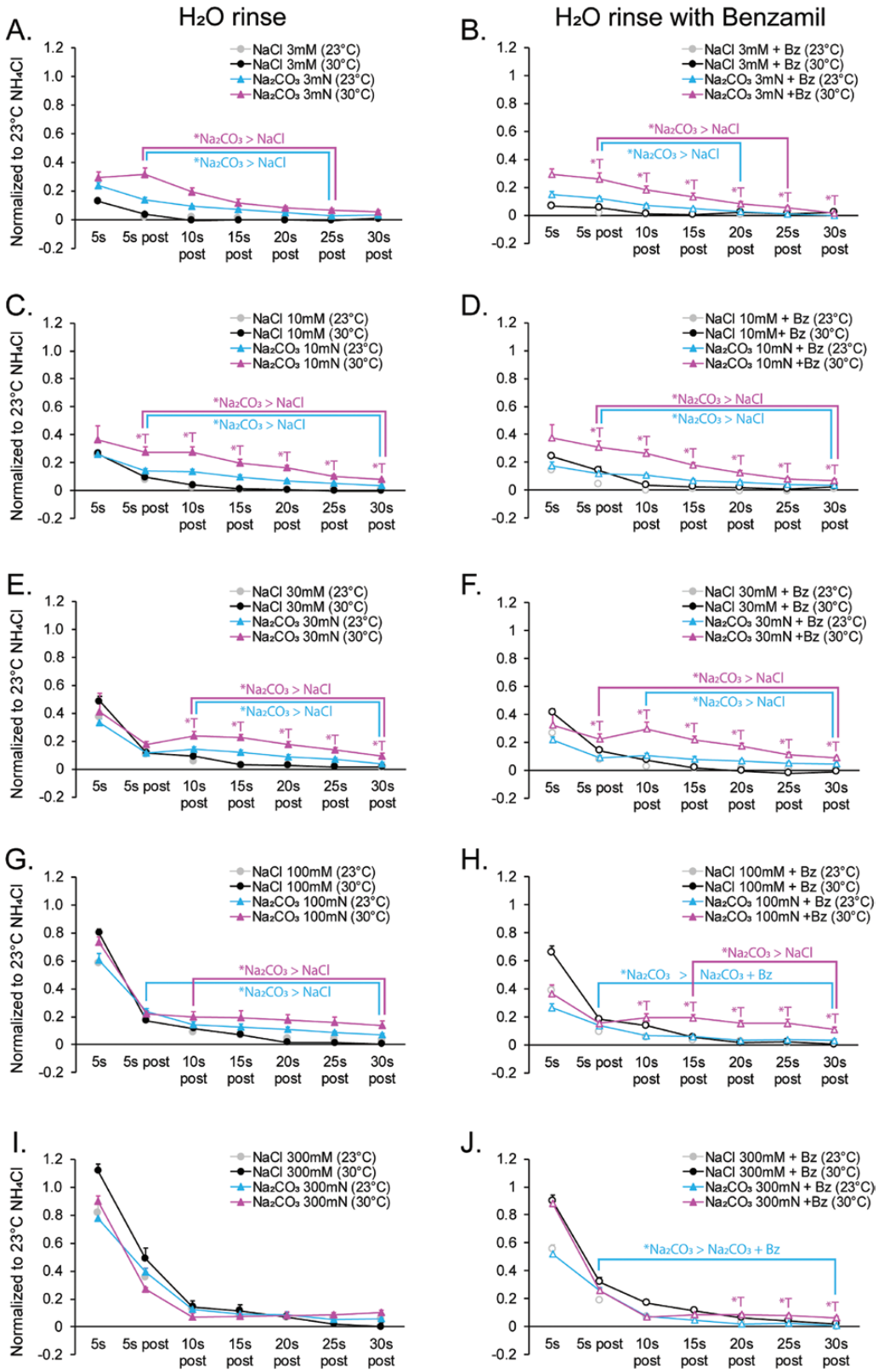

Fig. 4. *Na_2CO_3 evokes a robust aftertaste to H_2O rinse that is modulated by temperature and concentration.* Average responses to NaCl (circles), Na_2CO_3 (triangles) alone (closed symbols) and with benzamil (open symbols). *, letters, words, and brackets are color coordinated (23°C NaCl is gray; 23°C Na_2CO_3 is blue; 30°C NaCl is black; 30°C Na_2CO_3 is magenta) to reflect significant differences. Responses to NaCl were compared to Na_2CO_3 across the concentration range alone (A, C, E, G, and I) and with benzamil (B, D, F, H, and J). * indicates B-H adjusted significant differences. T indicates significant differences from 23°C to 30°C for Na_2CO_3 Bz stands for benzamil.

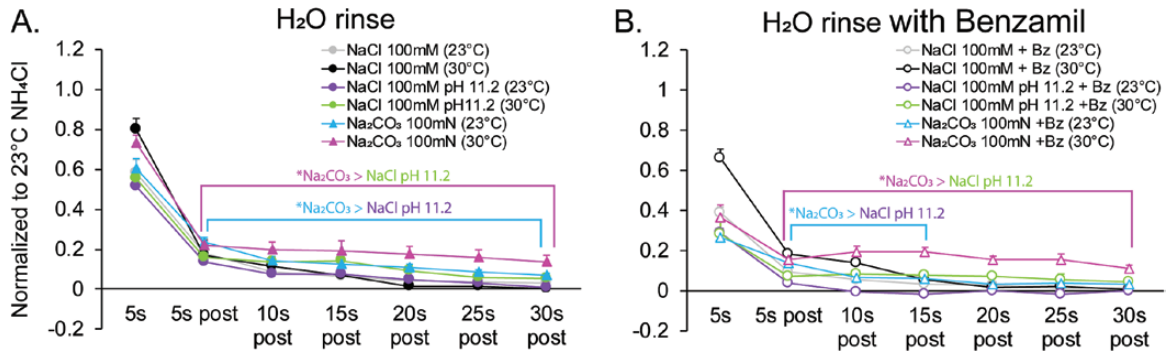

Fig. 5. *Alkaline NaCl does not recapitulate Na_2CO_3 aftertaste.* Average AUC responses to NaCl (squares), Na_2CO_3 (triangles) alone (A; closed symbols) and with benzamil (B; open symbols). Symbols, words, and brackets are color coordinated to reflect significant differences. 23°C NaCl is gray; 30°C NaCl is black; 23°C NaCl pH 11.2 is purple; 30°C NaCl pH 11.2 is green; 23°C Na_2CO_3 is blue; 30°C Na_2CO_3 is magenta. * indicates B–H adjusted significant differences. The temperature effect on benzamil-insensitive Na_2CO_3 responses are not indicated on the figure, as they have already been accounted for in Fig. 2. Bz stands for benzamil.

Time: $F(5,50) = 19.60$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,50) = 8.86$, $P = 5 \times 10^{-6}$; 10 s post–30 s post, B–H < 0.042, d = 1.2–2.0). At 30°C, aftertaste to 30 mN Na_2CO_3 was also significantly greater than aftertaste to 30 mM NaCl (Salt: $F(1,5) = 35.32$, $P = 0.002$; Time: $F(5,25) = 22.16$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,25) = 9.41$, $P = 4 \times 10^{-5}$; 10 s post–30 s post, B–H $P < 0.042$, d = 2.0–3.8) and this effect was remained in the presence of benzamil (Temperature: $F(1,5) = 73.77$, $P = 4 \times 10^{-4}$; Time: $F(5,25) = 28.84$, $P < 1 \times 10^{-6}$; Temperature × Time: $F(5,25) = 18.51$, $P < 1 \times 10^{-6}$; 5 s post–30 s post, B–H $P < 0.05$, d = 1.0–4.6).

100 mN Na_2CO_3 aftertaste was significantly attenuated by benzamil at 23°C (Benzamil: $F(1,10) = 27.92$, $P = 4 \times 10^{-4}$; Time: $F(5,50) = 41.36$, $P < 1 \times 10^{-6}$; Benzamil × Time: $F(5,50) = 4.55$, $P = 0.002$; 5 s post–30 s post, B–H $P < 0.05$, d = 1.0–1.7) but not 30°C. Aftertaste to 100 mN Na_2CO_3 alone was not significantly different at 23°C and 30°C. In the presence of benzamil, however, aftertaste to 100 mN Na_2CO_3 increased from 23°C to 30°C (Temperature: $F(1,5) = 64.06$, $P = 5 \times 10^{-4}$; Time: $F(5,25) = 18.55$, $P < 1 \times 10^{-6}$; Temperature × Time: $F(5,25) = 6.23$, $P = 7 \times 10^{-4}$; 10 s post–30 s post, B–H $P < 0.042$, d = 2.3–3.1). At 23°C, 100 mN Na_2CO_3 aftertaste was significantly greater than 100 mM NaCl aftertaste during the H_2O rinse (Salt: $F(1,10) = 29.56$, $P = 3 \times 10^{-4}$, Time: $F(5,50) = 52.24$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,25) = 19.05$, $P < 1 \times 10^{-6}$; 5 s post–30 s post, B–H $P < 0.05$, d = 1.0–1.4), but in the presence of benzamil, aftertaste to 100 mN Na_2CO_3 and 100 mM NaCl were not significantly different. At 30°C, however, aftertaste to 100 mN Na_2CO_3 was significantly greater than aftertaste to 100 mM NaCl (Salt: $F(1,5) = 18.83$, $P = 0.007$; Time: $F(5,25) = 26.96$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,25) = 16.47$, $P < 1 \times 10^{-6}$; 10 s post–30 s post, B–H $P < 0.042$, d = 1.8–3.6), and this effect remained in the presence of benzamil (Salt: $F(1,5) = 22.14$, $P = 0.005$; Time: $F(5,25) = 52.33$, $P < 1 \times 10^{-6}$; Salt × Time: $F(5,25) = 18.89$, $P < 1 \times 10^{-6}$; 15 s post–30 s post, B–H $P < 0.033$, d = 3.0–3.6).

At 23°C, 300 mN Na_2CO_3 aftertaste was significantly less in the presence of benzamil (Benzamil: $F(1,10) = 14.28$, $P = 0.004$; Time: $F(5,50) = 156.71$, $P < 1 \times 10^{-6}$; Benzamil × Time: $F(5,50) = 13.94$, $P < 1 \times 10^{-6}$; 5 s post–30 s post; B–H $P < 0.05$, d = 0.9–1.5), but at 30°C, benzamil had no significant effect on 300 mN Na_2CO_3 aftertaste. Aftertaste to 300 mN Na_2CO_3 did not increase from 23°C and 30°C. In the presence of benzamil, however, there was a borderline significant main effect of Temperature ($F(1,5) = 6.57$, $P = 0.050$) a significant main effect of Time ($F(5,25) = 76.78$, $P < 1 \times 10^{-6}$), and a significant Temperature × Time interaction ($F(5,25) = 10.30$, $P = 2 \times 10^{-5}$). Contrast tests of the interaction showed that aftertaste to 300 mN Na_2CO_3 + benzamil at 30°C initially reached near baseline values at 10 s post-stimulation, and then significantly increased relative to aftertaste at 23°C at 20–30 s post-stimulation (B–H $P < 0.025$, d = 1.8–3.0). This appeared to reflect a weak but bonafide aftertaste, as neural activity rebounded during the rinse period—in contrast to hypertonic NH_4Cl (supplemental data S1) and NaCl (Fig. 4), where responses decayed over time. Compared to low and midrange concentrations of Na_2CO_3, the phenomenon of Na_2CO_3 aftertaste was essentially eliminated at the hypertonic concentration. In fact, aftertaste to 300 mN Na_2CO_3 and 300 mM NaCl were not significantly different with or without the benzamil rinse at 23°C and 30°C.

Effects of stimulus pH on aftertaste

Adjusting the pH of 100 mM NaCl to 11.2 (to match 100 mN Na_2CO_3) had no significant effect on NaCl aftertaste at either 23°C or 30°C with or without benzamil. Despite being matched for pH, aftertaste to 100 mN Na_2CO_3 was greater than 100 mM NaCl pH 11.2 at 23°C (Salt $F(1,10) = 18.55$, $P = 0.002$; Time $F(5,50) = 96.23$, $P < 1 \times 10^{-6}$; 5 s post–30 s post, B–H $P < 0.05$, d = 1.0–1.8) and 30°C (Salt $F(1,5) = 33.34$, $P = 0.002$; Time $F(5,25) = 9.47$, $P < 4 \times 10^{-5}$; Salt × Time $F(5,25) = 4.72$, $P = 0.004$; 15 s post–30 s post, B–H $P < 0.033$, d = 2.3–4.4). In the presence of benzamil, aftertaste to 100 mN Na_2CO_3 was significantly greater than 100 mM NaCl pH 11.2 during the first 15 s of aftertaste at 23°C (Salt $F(1,10) = 12.71$, $P = 0.005$; Time $F(5,50) = 30.47$, $P < 1 \times 10^{-6}$; Salt × Time = $F(5,50) = 3.34$, $P = 0.011$; B–H $P < 0.025$, d = 1.7–2.0), whereas each of the timepoints were significantly different at 30°C (Salt $F(1,5) = 25.15$, $P = 0.004$; Time $F(5,25) = 5.03$, $P = 0.003$; Salt × Time $F(5,25) = 2.93$, $P = 0.032$; 5 s post–30 s post, B–H $P < 0.05$, d = 5.3–9.0). As described above, and in Fig. 5, the benzamil-insensitive portion of 100 mN Na_2CO_3 increased from 23°C to 30°C. Thus, aftertaste of Na_2CO_3 was modulated by temperature and primarily benzamil-insensitive, but adjusting the pH of NaCl could not recapitulate these effects.

Discussion

To our knowledge, this is the first comprehensive examination of neural responses to Na_2CO_3 in the gustatory system. We used the Sprague-Dawley rat, as it is a model organism for salt taste physiology and behavior. Historically, the vast majority of physiological studies of the taste system have used the rat CT nerve as the model system. Rat whole nerve (Heck et al. 1984; Lundy and Contreras 1997) and single unit (Boudreau et al. 1983; Ninomiya and Funakoshi 1988; Lundy and Contreras 1999; Breza et al. 2006) studies of CT neurons provided the foundation for our understanding of salt-taste pathways.

Together with behavioral studies, it was shown that the CT nerve is critical for normal salt detection (Spector et al. 1990; St. John et al., 1995, 1997; Geran and Spector 2007), salt discrimination (Spector and Grill 1992; Blonde et al. 2010), and Na^+ appetite (Roitman and Bernstein 1999). These behavioral studies supported physiological data that the CT nerve transduces salts through Na^+ selective ENaCs (amiloride/benzamil-sensitive) and nonselective (amiloride/benzamil-insensitive) salt-generalist pathways. Here, we recorded from the CT nerve in anesthetized rats to gain a better understanding of how Na_2CO_3 is sensed by the gustatory system through benzamil-sensitive and benzamil-insensitive pathways. The nonselective pathway was further studied by investigating the effects of elevated temperature on salt-taste responses with and without benzamil HCl. Lastly, 100 mM NaCl, a midrange concentration that has been used extensively in physiological studies, was studied at a neutral (6.2) and alkaline (11.2) pH to determine whether the "super saltiness" of Na_2CO_3 was due, in part, to its alkalinity.

Based on behavioral experiments in rats (St John et al. 2017), which showed that rats perceived the Na_2CO_3 as 10× saltier than NaCl when matched for concentration (5× when dissociated Na^+ ions are accounted for), we hypothesized that CT nerve responses to equinormal Na_2CO_3 would be 5-fold greater than those to NaCl. Specifically, St John et al. (2017) showed behavioral evidence that Na_2CO_3 activated both salt-sensing pathways more than NaCl over a wide range of concentrations, so we reasoned that these effects would be reflected in CT nerve responses. The overall shape of CT nerve responses to Na_2CO_3 during stimulation and rinse periods were quite different than those to NaCl and NH_4Cl. For NaCl, responses increased progressively with increasing concentration and the upward trend was steep. In contrast, responses to Na_2CO_3 were relatively flat from 3–30 mN and increased sharply at 100 and 300 mN. Although not reported for space, we found that while the peak responses to Na_2CO_3 increased with increasing concentration, tonic responses decreased from 3–30 mN and then increased abruptly at 100 and 300 mN (Fig. 1A). This was the main reason for the flat concentration response profile for 3–30 mN Na_2CO_3, because the phasic component (includes the peak—latency to peak was 500–660 ms) lasted ~1.65 s, which was 33% of the 5 s response. Despite having the same number of dissociated Na^+ ions, Na_2CO_3 responses at the lowest concentration (3 mN) were ~2× greater than those to 3 mM NaCl. When the benzamil-insensitive component was enhanced at 30°C, responses to 3 mN Na_2CO_3 were 4.5× greater than those to 3 mM NaCl, which is consistent with the behavior (St. John et al. 2017). Responses to higher concentrations of Na_2CO_3, however, were not significantly greater than those to NaCl, but each concentration of Na_2CO_3, particularly hypotonic concentrations, evoked robust aftertaste during a water rinse (see below).

NaCl responses were suppressed more by benzamil at 23°C than at 30°C, which is consistent with a previous CT nerve study in Sprague-Dawley rats with amiloride (Lundy and Contreras 1997). A hallmark of the amiloride/benzamil-insensitive pathway is that responses to NaCl and NH_4Cl increase with increasing temperature (Lundy and Contreras 1997; Breza et al. 2006; Lu et al. 2016), which results in a stronger influence of the amiloride/benzamil-insensitive pathway at warmer temperatures. The responses we observed with NaCl and NH_4Cl (supplemental) at 23°C and 30°C are consistent with those in the aforementioned literature, and therefore stood as a good reference for comparing responses to 23°C and 30°C Na_2CO_3.

Like NaCl, Na_2CO_3 responses at 23°C consisted of benzamil-sensitive and benzamil-insensitive components, which is consistent with brief access tests at the same temperature (St John et al. 2017). Although each concentration of Na_2CO_3 was significantly inhibited by benzamil, responses to 3mN Na_2CO_3 had benzamil-insensitive components, and neural activity was present throughout stimulation. In contrast, 3 mM NaCl consisted of only a phasic component and evoked no tonic nerve activity (Fig. 1B). For space, mean tonic activity to 3mN was not presented, but can clearly be seen in Fig. 1.

Benzamil reduced tonic responses to 100 mM NaCl by 52%, and 100 mN Na_2CO_3 by 92%. This implies that at 23°C, tonic responses to 100 mN Na_2CO_3 were almost entirely benzamil sensitive. To test whether the differences in NaCl and Na_2CO_3 responses were due to the alkaline pH of Na_2CO_3 solutions alone, we tested NaCl at the same alkaline pH as Na_2CO_3 with and without benzamil. We chose to make these comparisons at 100 mM/mN, as this is a standard stimulus used in in vivo physiology experiments and both salt pathways respond well to Na^+ salts at this concentration (Rehnberg et al. 1993; Breza and Contreras 2012b). Interestingly, we found there was a synergistic effect of an alkaline pH on the benzamil-insensitive pathway. At 23°C, benzamil reduced AUC NaCl responses by 33% and alkaline AUC NaCl responses by 51%. Benzamil reduced tonic NaCl responses by 52% and tonic responses to alkaline NaCl by 88%. To examine how the alkaline pH was decreasing the benzamil-insensitive pathway, we then compared NaCl + benzamil to NaCl + benzamil pH 11.2, and observed that the alkaline pH decreased the benzamil-insensitive tonic response by an additional 75%. The suppression of NaCl by benzamil at an alkaline pH was synergistic, as the sum of the suppression of benzamil and alkaline pH was not equal to the parts (AUC sum = 45% vs actual 51%; tonic sum 72% vs actual 88%). The combination of benzamil and pH 11.2 reduced tonic NaCl responses by 88%, which is highly consistent with how benzamil reduced tonic Na_2CO_3 responses by 92%, and strongly suggests that the alkaline pH was responsible for attenuating the benzamil-insensitive component. This is probably one of the most important findings of these experiments, because other than decreasing the temperature and substituting Cl^- for large anions such as acetate or gluconate (Ye et al. 1991, 1993; Rehnberg et al. 1993; Breza and Contreras 2012b), nobody has found a reliable way to decrease the amiloride/benzamil-insensitive component.

Because the NaCl responses through amiloride/benzamil-insensitive component are enhanced by warm temperatures (Lundy and Contreras 1999; Lyall et al. 2004; Breza et al. 2006; Lu et al. 2016), we repeated the same alkaline experiment at 30°C to determine how the benzamil insensitive component operated under these favorable conditions. At 30°C, increasing the pH of NaCl to 11.2 significantly decreased AUC NaCl responses by 31% (Fig. 3) and tonic responses by 23%. Peak responses to alkaline pH were also significantly suppressed by 32%, and this is clearly shown in raw trace in Fig. 3 (average data not presented). We then tested how the alkaline pH directly affected the benzamil-insensitive component and discovered that increasing the pH of NaCl + benzamil to 11.2 decreased AUC responses by an additional 57%, peak responses by 35% (average data not presented for space but seen in Fig. 3), and tonic responses by an additional 84%.

A fascinating finding is that the total reduction of the tonic NaCl response vs NaCl + benzamil pH 11.2 was 88% at both 23°C and 30°C, but when the benzamil-insensitive portion of the response was enhanced at 30°C, the ratio of the reduction in the benzamil-insensitive component was greater (75% @ 23°C vs 84% @ 30°C). This is consistent with the literature in that warmer temperatures increase the ratio of amiloride/benzamil-insensitivity of the chorda tympani nerve (Lundy and Contreras 1997) and supports the notion that alkaline pH is inhibiting the benzamil-insensitive component of NaCl. For Na_2CO_3, however, benzamil reduced tonic responses at 23°C by 92%, and by 52% at 30°C. This rebound of the benzamil-insensitive component of Na_2CO_3 at 30°C suggests that perhaps while the alkaline pH is inhibiting the Na^+ entry through benzamil-insensitive channels, the dissolved CO_3^{-2} anion was exerting its own effects.

Na_2CO_3 is a rather complicated stimulus. Two Na^+ cations dissociate from CO_3^{-2} and the dissociated CO_3^{-2} anion binds up available H^+ ions in solution to produce carbonic acid (H_2CO_3), which makes the solution pH alkaline since the anion remains protonated. Little is known about the effects of extracellular alkalinity on salt responses in taste cells, but the mechanisms of H_2CO_3 on TBCs are fairly understood. Dissolving CO_2 in water will convert to H_2CO_3, which is a powerful taste stimulus—it decreases intracellular pH, increases CT nerve activity in rats and mice, and these responses are attenuated by carbonic anhydrase blockers (Lyall et al. 2001; Chandrashekar et al. 2009). The dissociated Na^+ ions will also stimulate both salt-taste pathways. Type III taste bud cells express PKD2L1, carbonic anhydrase 4 (Chandrashekar et al. 2009), and respond to salts through an amiloride-insensitive pathway (Oka et al. 2013; Lewandowski et al. 2016). Genetic ablation of PKD2L1 TBCs eliminates the taste of carbonation (Chandrashekar et al. 2009) and silencing their synaptic communication with afferents (Oka et al. 2013) attenuates amiloride-insensitive salt taste.

Elevating the temperature enhanced the benzamil-insensitive response to Na_2CO_3, which is consistent with how temperature modulates responses to salts, acids, and quinine through amiloride/benzamil-insensitive acid generalist neurons in the geniculate ganglion (Lundy and Contreras 1999; Breza et al. 2006). This suggests that the thermal enhancement of the benzamil-insensitive component to Na_2CO_3 is transduced through Type III cells—most likely the H_2CO_3 component as the alkalinity inhibited the benzamil-insensitive NaCl response and its thermal sensitivity. Although several studies have shown that amiloride/benzamil-insensitive responses to salts and acids increase with increasing temperature, the mechanisms for this are completely unknown. While it has been suggested that the receptor for the amiloride/benzamil-insensitive component is a variant of TRPV1, we (Breza and Contreras 2012a) and others (Ruiz et al. 2006; Smith et al. 2012) were not able to reproduce those findings.

Lyall et al. (2002) showed that benzamil-sensitive 100mM NaCl responses in the rat CT nerve were inhibited at an acidic pH (2.0) but enhanced at an alkaline pH (10.3). The pH of Na_2CO_3 solutions in the present study ranged from 10.6 to 11.3, whereas 100 mM NaCl was at 11.2, so we cannot compare our data directly to Lyall et al. (2002). Although the methods for stimulus delivery and quantifying tonic CT nerve activity in the present study were much different from those in Lyall et al. (2002), we do feel that there are similarities in our findings. Specifically, although the magnitude of the tonic CT nerve response to NaCl pH 6.2 and NaCl pH 11.2 were not statistically different at 23°C or 30°C, the response to NaCl pH 11.2 was almost entirely benzamil sensitive. This implies that the benzamil-sensitive portion was enhanced, consistent with Lyall et al. (2002).

Intriguingly, we observed that the CT nerve showed robust activity during the water rinse after Na_2CO_3 application. We thereby deemed this response "aftertaste," and it was concentration and temperature dependent. Benzamil in the rinse allowed for us to determine whether the origin of aftertaste to NaCl and Na_2CO_3 was benzamil sensitive or benzamil insensitive. Elevating the temperature in the presence of benzamil allowed us to enhance and isolate the benzamil-insensitive component. Adjusting the pH of NaCl to match Na_2CO_3 allowed for us to examine whether aftertaste to Na_2CO_3 was due to its alkaline pH.

Aftertaste to Na_2CO_3 was primarily benzamil insensitive across the concentration range, with the only benzamil sensitivity being at 100 and 300 mN at 23°C. This was further validated by demonstrating that this effect was substantially enhanced with increasing the solution temperature from 23°C to 30°C demonstrating the signal is carried through amiloride/benzamil-insensitive acid generalist neurons of the CT (Ogawa et al. 1968; Lundy and Contreras 1999; Lyall et al. 2004; Breza et al. 2006). The shape of aftertaste to Na_2CO_3 over the concentration range was a somewhat inverted u-shaped function, peaking at 30 mN and decreasing thereafter—it was essentially eliminated at 300 mN. Aftertaste to Na_2CO_3 remained significantly greater (4–36× after 15–20 s of rinsing) than NaCl at 3–100 mN/mM, which might explain why rats perceive Na_2CO_3 carbonate as saltier than NaCl (St. John et al. 2017). Although the pH of Na_2CO_3 is alkaline, adjusting the pH of 100 mM NaCl to match 100 mN Na_2CO_3 did not recapitulate aftertaste responses to Na_2CO_3 through the benzamil-insensitive pathway. Interestingly, the aftertaste we saw in response to removal of Na_2CO_3 was eerily similar to those by Zocchi et al. (2017), where removal of bicarbonate (HCO_3^-) resulted in a "water response," which was dependent on the expression of carbonic anhydrase 4 expression in PKD2L1 cells. Because the CO_3^{-2} anion can be converted to HCO_3^- or H_2CO_3, and carbonic anhydrase can quickly remove the proton at the surface of the membrane, it is possible that the aftertaste we reported here are similar to those by Zocchi et al. (2017), where removal of HCO_3^- from the tongue with water resulted in a "water response." Exactly how that

works has not been borne out, but our data with Na_2CO_3 are consistent with Zocchi et al. (2017), where removal of the Na_2CO_3, but not alkaline NaCl, resulted in aftertaste when switched to H_2O.

We are unsure what sensation this aftertaste signal sends to the brain, but if it is carried through Type III PKD2L1 cells, as shown by Zocchi et al. (2017), then it could be perceived as sour (Huang et al. 2006; Chandrashekar et al. 2009) or salty through an amiloride/benzamil-insensitive pathway (Lewandowski et al. 2016). Brief access tests strongly suggest that rats perceive Na_2CO_3 as salty, and that it is transduced through both amiloride-sensitive and amiloride-insensitive pathways (St John et al. 2017), which was supported here, in part, using a neurophysiological approach. In a previous report, using spike train analyses, we showed that amiloride-sensitive NaCl specialist neurons were more responsive to qualitative differences in the stimulus set and less sensitive to intensity differences, while acid generalist neurons were more responsive to intensity differences (Wu et al. 2013). This might explain differences in perceived salt taste intensity to NaCl and Na_2CO_3 in rats. Anecdotally, we perceived 100 mN Na_2CO_3 aftertaste as a mixture of salty/carbonic like taste, and it persisted for long after only a small amount was introduced to the mouth. The sensation was not pleasant, and although Na_2CO_3 might not serve as the "supersalty" salt substitute we hoped for, a mechanistic explanation of these phenomena might allow for specific targets for industry to enhance salt taste, which would have a favorable impact on human health.

Supplementary Data

Supplementary material is available at *Chemical Senses* online.

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Conflict of interest

None declared.

Data availability

Data will be shared on a reasonable request to the corresponding author.

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