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Orosensory Responsiveness to and Preference for Hydroxide-Containing Salts in Mice

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Abstract

Historically, taste researchers have considered the possibility that the gustatory system detects basic compounds, such as those containing the hydroxide ion, but evidence for an "alkaline taste" has not been strong. We found that, in 48 h, 2-bottle preference tests, C3HeB/FeJ (C3) mice showed a preference for $Ca(OH)_2$, whereas SWR/J (SW) mice showed avoidance. Strain differences were also apparent to NaOH but not $CaCl_2$. Follow-up studies showed that the strain difference for $Ca(OH)_2$ was stable over time (Experiment 2) but that C3 and SW mice did not differ in their responses to $Ca(OH)_2$ or NaOH in brief-access tests, where both mice avoided high concentrations of these compounds (Experiment 3). In order to assess the perceived quality of $Ca(OH)_2$, mice were tested in 2 taste aversion generalization experiments (Experiments 4 and 5). Aversions to $Ca(OH)_2$ generalized to NaOH but not $CaCl_2$ in both strains, suggesting that the generalization was based on the hydroxide ion. Both strains also generalized aversions to quinine, suggesting the possibility that the hydroxide ion has a bitter taste quality to these mice, despite the preference shown by C3 mice to middle concentrations in long-term tests.

Key words: astringency, basic, calcium, mice, pH, taste

Introduction

Sapid stimuli can be classified into a number of qualities, and although there has been debate about the distinctness and even number of these qualities (Bartoshuk 1978; Erickson 2000), recent experimental evidence suggests the existence of 5: sweet, salty, sour, bitter, and umami (e.g., Ishimaru 2009). Notably, each sense is linked to a key ingestive process: Sweet signals the presence of a food rich in easily digestible calories, umami signals the presence of a protein source, saltiness the presence of vital micronutrients and minerals, and bitter signals potential toxins (Glendinning et al. 2000). Sour taste, which increases as the pH of the detected acid decreases, signals increasingly dangerous pH levels that can cause tissue damage (Beidler 1971). That our gustatory sense warns us of deviations from neutral pH in 1 direction presents a curious asymmetry—deviations in the direction of higher pH may not be detected by the gustatory sense. It may be that other orosensory signals, perhaps arising from the trigeminal system, warn of fluids or foodstuffs containing bases (e.g., Bryant and Silver 2000).

High-pH compounds such as NaOH certainly evoke sensations when applied to the tongue. In an early review by Parker (1922), alkaline taste was explained as a mixture of "several tastes and touch," including a "sweetish" taste at low concentrations; olfaction was also implicated. Kloehn and Brogden (1948) attempted to determine whether the gustatory sense contributed to the sensation of NaOH by comparing detection thresholds on taste bud-rich and taste bud-poor regions of the tongue in humans and tentatively concluded that gustation contributed (but did not compare these thresholds with those of nonalkaline sodium-containing compounds). Liljestrand and Zotterman (1956) used a cat taste nerve preparation to demonstrate that NaOH (pH 11.7–12.2) evokes a response in both the whole nerve and individual chorda tympani fibers, especially those classified as responding best to salt, quinine, or water. Although an "alkaline-best" cell type was not found, the authors still reason that alkaline stimuli might be discriminated from other qualities based upon the pattern of neural activity. Other investigators have demonstrated that animals can behaviorally differentiate basic compounds from water, including rejection at high pH levels (Bell 1963; Goatcher and Church 1970).

In the current experiment, we measured behavioral responses to hydroxide-containing compounds NaOH and

$Ca(OH)_2$ in 2 common inbred strains of mice, SWR/J (SW) and C3HeB/FeJ (C3). These particular strains have been well characterized with respect to sweet and bitter taste and intake, and differ in sensitivity to many stimuli of these qualities (e.g., Lush 1989; Capeless and Whitney 1995; Boughter and Whitney 1998; Boughter et al. 2002; Nelson et al. 2003; St. John and Boughter 2004; St. John et al. 2005). Using behavioral tests, including 2-bottle intake and brief-access tests, we sought to measure and define the ingestive (intake) and orosensory response (brief-access, conditioned taste aversion, CTA) to these compounds. Somewhat surprisingly, SW and C3 mice differed substantially in preference for $Ca(OH)_2$ and NaOH, although brief-access tests indicated that this strain variation was not likely due to orosensory factors. Finally, we utilized a CTA procedure in order to assess whether or not alkaline stimuli possessed a unique taste, or cross-generalized to 1 of the established taste qualities.

Experiment 1: 2-bottle preference tests

Materials and methods

Subjects

Nine adult C3HeB/FeJ (C3: 4 males, 5 females; initial body weights 22–32 g) and 9 adult SWR/J (SW: 5 males, 4 females; initial body weights 18–24 g) were tested in experiments to determine preference for $Ca(OH)_2$, $CaCl_2$, and NaOH as part of a larger study that included 5 inbred strains. Because C3 and SW mice exhibited dramatic differences in preference behavior, the current manuscript focuses on these 2 strains. After acclimation to the animal colony, mice were placed in individual shoebox cages in a colony room where lighting (12:12 h light:dark cycle), temperature, and humidity were automatically controlled. Food (Harlan Teklad Rodent Diet 7012) was available throughout the experiment. Water was available during nontesting phases in a single bottle.

Procedure

Mice were tested for their intake of water versus a test compound in 3 12-day blocks. The first block was $Ca(OH)_2$ preference testing, the second $CaCl_2$ preference testing, and the final block NaOH preference testing. Within each block, mice were offered an ascending concentration series in 48-h sessions.

Two 50-mL Pyrex centrifuge tubes were fitted with silicone stoppers through which was placed a stainless-steel, leakproof sipper tube. Silicone (rather than rubber) stoppers were chosen because, to the authors and lab personnel, they do not contaminate the solutions with a detectable taste or smell. Distilled water was always placed in 1 of the tubes. The other was filled with distilled water, 0.3, 1, 3, or 10 mM concentration of the test stimulus. Position (left or right) of the test stimulus was randomized on the first day of testing for a given concentration. Prior to the second day of testing, bottles were weighed to the nearest tenth of a g, topped off, and replaced on the cage with the left–right positions of the stimuli reversed. Following these 10 days of testing, a replication trial was given with the 10 mM concentration for the final 2 days to assess stability of the response at this concentration.

A block always consisted of 12 consecutive days. Mice were always given time off between blocks (1–9 weeks) during which they had access to a single bottle of tap water.

Analysis

Intake of stimuli was first expressed as a preference ratio (intake of test stimulus over total intake) for each 24-h test and the 2 preference ratios were averaged to assess solution preference over 48 h. Preference ratios of 0.5 (equal intake of the test solution and water) indicated neutrality. The data were analyzed using 3 Strain × Concentration analyses of variance (ANOVAs), 1 for each stimulus, with Strain as a between-subjects factor and Concentration as a within-subjects factor. Concentrations included were 0, 0.3, 1, 3, and 10 mM; the second presentation of 10 mM was analyzed separately. Degrees of freedom for the ANOVAs differ slightly because mice were removed from individual analyses in the case of rare missing observations.

In order to determine whether a preference ratio at a particular concentration differed from neutrality, a 1-sample *t*-test assessed significant departures from a preference ratio of 0.5. Significantly lower means were considered evidence of avoidance; significantly higher means evidence of preference. The statistical rejection criterion (i.e., alpha) was set at 0.05, and Bonferroni corrections for multiple *t*-tests were used as appropriate.

Results

Over the concentration range tested, strain differences in the response to some stimuli were readily apparent, especially at higher concentrations of $Ca(OH)_2$ and NaOH (Figure 1). For $Ca(OH)_2$, the ANOVA indicated a significant effect of Strain ($F(1,14) = 8.575$, $P = 0.011$) and a Strain × Concentration interaction ($F(4,56) = 6.505$, $P = 0.00023$). The C3 mice significantly deviated from neutrality at the 3 mM concentration ($t(8) = 8.015$, $P = 0.00026$) and the replication trial of 10 mM ($t(8) = 4.834$, $P = 0.0078$). The SW mice significantly differed from neutrality at 10 mM ($t(6) = -4.036$, $P = 0.0068$; replication trial: $t(6) = -8.655$, $P = 0.00013$). Strikingly, when differences from neutrality were found, C3 mice preferred $Ca(OH)_2$ and SW mice avoided it.

In contrast, the ANOVA found no main effect of Strain or a Strain × Concentration interaction in the behavior of C3 and SW mice toward $CaCl_2$. There was, however, a main effect of Concentration ($F(4,60) = 2.632$, $P = 0.043$). The C3 mice did significantly differ from neutrality at 10 mM ($t(7) = 3.663$, $P = 0.048$; replication trial: $t(7) = 4.486$, $P = 0.0028$).

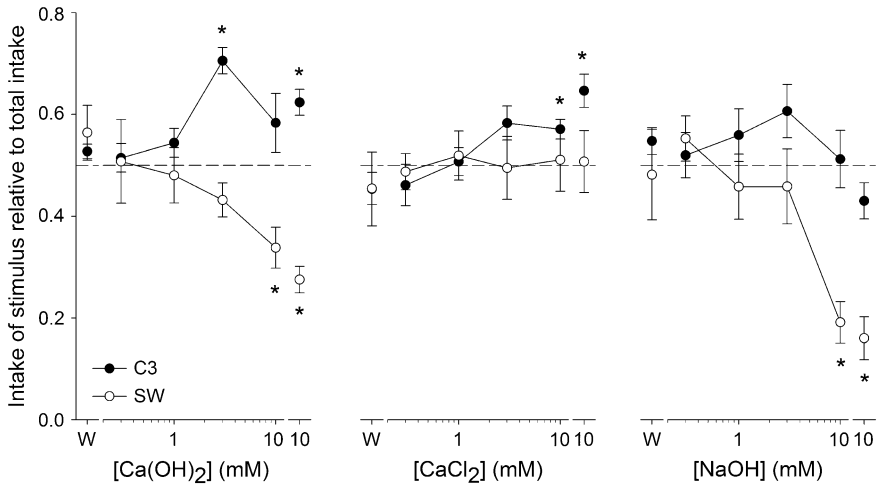

Figure 1 Two-bottle preference test results for $CaOH_2$, $CaCl_2$, and NaOH for C3 (C3HeB/FeJ) and SW (SWR/J) mice. Concentrations were 0.03–10 mM in near half-logarithmic steps. The first data point represents water in both bottles and the last data point a replication trial of 10 mM. The dependent measure is mean (±standard error) preference ratio (intake of the test solution over total fluid intake in 48 h). Asterisks indicate a significant difference from neutrality (1-sample *t*-test, Bonferroni correction, $P < 0.0083$).

For NaOH, the ANOVA indicated a main effect of Strain ($F(1,14) = 4.849$, $P = 0.045$), Concentration ($F(4,56) = 4.570$, $P = 0.0029$), and a Strain × Concentration interaction ($F(4,56) = 3.214$, $P = 0.019$). The *t*-tests did not reveal differences from neutrality in the C3 mice, but SW mice significantly avoided 10 mM ($t(8) = -7.524$, $P = 0.00041$; replication: $t(8) = -8.023$, $P = 0.00026$).

Experiment 2: stability of $Ca(OH)_2$ preference

In Experiment 1, C3 and SW mice differed in their response to $Ca(OH)_2$, particularly at higher concentrations. Because preference results are often susceptible to effects of test order (Harder et al. 1989), we determined the stability of the strain difference at this particular concentration by testing naïve C3 and SW mice at a concentration (3 mM) eliciting divergent preference behavior for an extended 12-day period (6 consecutive 48-h tests).

Materials and methods

Subjects

Ten C3 (5 males, 5 females) and 10 SW (5 males, 5 females) mice purchased from Jackson Labs were housed in conditions similar to Experiment 1.

Procedure

Similar preference testing procedures as detailed for Experiment 1 were used to test naïve C3 and SW mice with 3 mM $CaOH_2$ over 12 consecutive days, with the exception that 25-mL graduated cylinders were used and volumes consumed were read visually to the nearest 0.1 mL. Fresh solution was presented in the stimulus bottle every 48 h, and the position of stimulus and water bottles was switched every 24 h.

Analysis

Preference data were analyzed using a Strain × Time Period repeated measures ANOVA. Strain deviations from 0.5 were assessed with 1-sample *t*-tests, as in Experiment 1.

Results

Strain differences in preference for 3 mM $Ca(OH)_2$ were readily apparent, and the magnitude of the difference was stable over the 12-day period (Figure 2). Both strains increased their preference for $Ca(OH)_2$ over the 12-day period, although this appeared to level off by the fourth 48-h test. There was a main effect of Strain ($F(1,20) = 26.966$, $P = 0.000044$) and of Test Period ($F(5,100) = 6.044$, $P = 0.000063$) but no Strain × Test Period interaction.

The C3 mice showed a significantly greater preference ratio than 0.5 on Tests 3–6 (P values < 0.05), although only the difference on Test 5 was significantly different when the more conservative Bonferroni-adjusted P value was applied ($t(9) = 4.35$, 0.0019). The SW mice showed a significantly lower preference ratio than 0.5 on Tests 1–3 (P values < 0.0042) but not on Tests 4–6 (P values > 0.05).

Experiment 3: brief-access testing

The results of Experiment 1 indicated that, among other strain differences, C3 and SW mice behaved quite differently to the high concentrations of the hydroxide-containing compounds. In particular, C3 mice showed a preference for 10 mM $Ca(OH)_2$ (and, to a lesser extent, NaOH), whereas

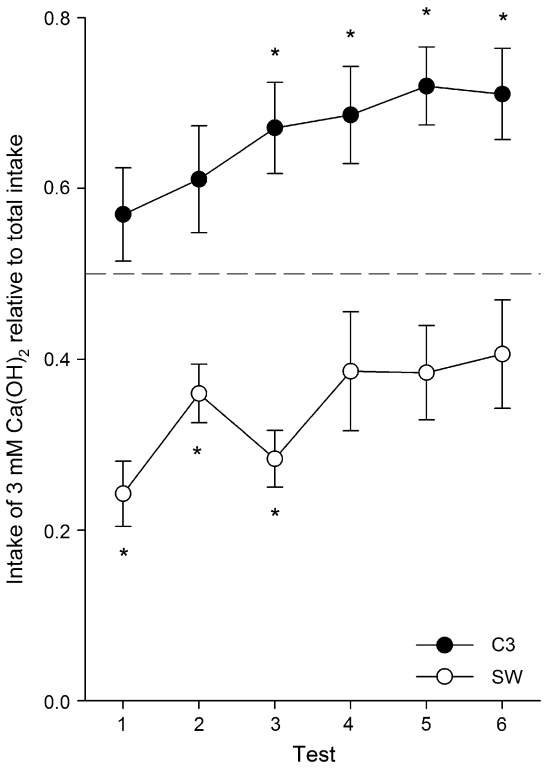

Figure 2 Mean (±standard error) preference for 3 mM $CaOH_2$ (intake of $CaOH_2$ over total fluid intake) in 6 consecutive 48-h 2-bottle preference tests ($CaOH_2$ vs. distilled water) for C3 and SW mice. Asterisks indicate a significant difference from neutrality (1-sample *t*-test, Bonferroni correction, $P < 0.0083$).

SW mice showed an aversion to hydroxide-containing compounds.

Because preference tests measure behavior over a 24-h period, orosensory differences between the strains are only 1 possibility in explaining the strain difference. We therefore next used a brief-access licking paradigm to better assess the origin of the strain differences (Boughter et al. 2002; Glendinning et al. 2002; St. John and Spector 2008).

In addition to testing the 3 compounds from Experiment 1, we also tested quinine. Quinine is a stimulus that SW mice are known to strongly avoid and C3 mice weakly avoid (St. John and Boughter 2004). Use of this stimulus therefore provided an "anchor" in interpreting results to the previously untested compounds. In order to determine whether avoidance of these compounds was linked to bitter taste receptors, we also examined the responses of 6 C3.SW congenic bitter taster mice. These mice contain a ~3- to 4-Mb segment of distal Chr 6 donated from the SW strain and transposed to a C3 genomic background via a program of serial backcrossing (Boughter and Whitney 1995; Bachmanov et al. 2001). The mice are therefore maintained as homozygotes for this segment, which includes the bitter-tasting *Tas2r*-cluster haplotype.

Materials and methods

Subjects

Twenty-eight mice of 2 inbred and 1 congenic strain served as subjects. Mice were bred from 2 males and 2 females of each inbred strain purchased from Jackson Labs. Subjects were 10 C3 (5 males, 5 females), 12 SW (8 males, 4 females), and 6 C3.SW (3 males, 3 females) adult mice. Approximately 1 week prior to the beginning of the experiment, mice were transferred from group housing (littermates by sex) into individual shoebox cages in a colony room where lighting (12:12 h light:dark cycle), temperature, and humidity were automatically controlled. Food was available throughout the experiment. Water was available except where noted below.

Apparatus

Brief-access testing was conducted in an automated, multistimulus lickometer known as the Davis Rig (MS-160, DiLog Instruments, Tallahassee, FL). The Davis Rig (Smith 2001; St. John and Boughter 2004) consists of an animal chamber with 3 Plexiglas walls and a fourth wall made of stainless steel. This front wall contains an oval window through which a mouse can access taste solutions. Access to taste solutions is controlled by a computer-operated shutter. Opening of the shutter signals fluid availability; at the end of a taste trial, the shutter swings closed. Outside the animal chamber, up to 16 stimulus bottles are held on a motorized tray (in this experiment, the maximum number of stimuli offered was 6) and can be driven into position opposite the stimulus-access window for the ensuing trial. Stimulus bottles were glass with stainless-steel sipper tubes.

The control program for the Davis Rig permits control of several parameters for the session such as session length (in seconds or number of trials), trial length, intertrial interval, and sequence of tube presentation. Another parameter is the amount of time the shutter will remain open to allow the mouse to initiate a trial. This "clock" begins when the shutter opens, but the "trial" does not begin until the mouse makes its first lick. In our experiments, it was possible for the shutter to open and later close without the mouse initiating a lick. This was not counted as a trial because the mouse did not come into contact with the fluid. These nontrials generally occurred late in a session when the mouse was fully rehydrated.

Procedure

General notes. The experiment lasted 3 weeks. To motivate stimulus sampling, water was removed from the home cage late in the afternoon on Monday of each week (no behavioral sessions were conducted on Monday). On Tuesday–Friday, mice received all of their daily fluid from the behavioral sessions in the Davis Rig (see below), with the exception of a 1-mL supplement 1–4 h after behavioral testing concluded for the day. Body weight was monitored daily to verify that

all mice tolerated this restricted water schedule well. Immediately after the session on Friday, unlimited water was returned to the home cage until the following Monday.

The first week consisted of training, during which mice learned to lick in the Davis Rig to obtain fluid (distilled water). During the second week, the acceptability of quinine hydrochloride, NaOH, $Ca(OH)_2$, and $CaCl_2$ was measured, 1 stimulus per day (the order of stimuli was partially counterbalanced across animals). During the third week, mice received a second session with each of the 4 stimuli. Replication trials were conducted in reverse order of the previous week in an effort to minimize order effects (mice generally are more motivated by thirst as the week goes on.)

Training. During the first 2 training sessions, mice had access to a single bottle of distilled water. Sessions ended after 30 min or 15 min from the mouse's first lick (whichever came first). In the rare event that a mouse did not initiate at least 50 licks during these sessions, the mouse was given a second session later in the day. During the final 2 days of training, distilled water was placed in 6 stimulus tubes. During a maximum 30-min session, the mouse could initiate up to 24 5-s trials. The shutter remained open for up to 60 s waiting for the mouse to initiate its first lick. After shutter closure, a 10-s intertrial interval elapsed before a new bottle was positioned. The bottles were presented in randomized blocks of 6, ensuring that each bottle was offered once every 6 trials and that the order of presentations within a block was unpredictable.

Testing. Testing sessions resembled the final training session except that only 1 of the 6 stimulus tubes contained distilled water. The remaining tubes contained NaOH or $CaCl_2$ (1, 3, 10, 30, and 100 mM), $Ca(OH)_2$ (0.1, 0.3, 1, 3, and 10 mM), or quinine hydrochloride (hereafter: quinine; 0.03, 0.1, 0.3, 1, and 3 mM). Concentrations were chosen from pilot work or previously published work (St. John and Boughter 2004) to span the dynamic range of behavior (i.e., from maximal licks to a few licks in a 5-s trial). Stimuli were presented 1 per day so that each mouse received each stimulus array once per week. Roughly equal numbers of mice received the stimuli in the following 3 orders: 1) NaOH, $CaCl_2$, $Ca(OH)_2$, and quinine; 2) $CaCl_2$, $Ca(OH)_2$, quinine, and NaOH; and 3) $Ca(OH)_2$, quinine, NaOH, and $CaCl_2$. The second week was identical to the first except that the order of stimuli was reversed (e.g., a mouse receiving Order A in the first week of testing received quinine, $Ca(OH)_2$, $CaCl_2$, and NaOH during the second week).

Results

In contrast to the preference test results, strain differences were not particularly evident in the brief-access studies (Figure 3). Separate 2-way (Strain × Concentration) ANOVAs were conducted for each stimulus. There was a main effect of Concentration in each case, reflecting the fact that, for all 4 stimuli, mice tended to reduce their licking of the higher concentrations. The similarity of C3 and SW responses to $Ca(OH)_2$ and NaOH may appear surprising given the results of Experiment 1 and 2. However, brief-access tests in water-restricted mice are not sensitive to detecting preferences, because mice tend to lick at their maximal rates to neutral stimuli like water (St. John and Spector 2008). Thus, if C3 mice prefer some stimuli at lower concentrations (up to 10 mM, the highest concentration used in Experiment 1), these tests would not be revealing.

Strain differences were only apparent for quinine (Strain: $F(2,25) = 12.68$, $P < 0.0005$; Strain × Concentration: $F(8,100) = 1.46$, n.s.). Consistent with previous work (St. John and Boughter 2004), SW and C3.SW mice were similar to one another and avoided quinine to a greater degree than did C3 mice. Somewhat surprisingly, C3 and SW mice responded similar to each other and less similar to C3.SW mice in responses to $CaCl_2$ and the lower concentrations of NaOH (cf., Figure 3), although this trend was not statistically significant.

Experiment 4: generalization of CTA

In Experiments 1 and 2, C3 mice preferred $Ca(OH)_2$ relative to water, whereas SW mice did not. In Experiment 3, both strains avoided the high concentrations of hydroxide salts. In an effort to determine the similarity of these compounds to known aversive and preferred taste stimuli, we conducted taste aversion generalization experiments. Mice were conditioned (by LiCl injection) to avoid $Ca(OH)_2$. We assessed generalization of the aversion to taste stimuli representative of sweet, sour, salty, and bitter taste qualities as well as to other hydroxide-containing compounds.

Materials and methods

Subjects

Twenty-four mice of 2 inbred strains served as subjects (Jackson Labs). Subjects were 13 C3 (7 males, 6 females) and 11 SW (7 males, 4 females) adult mice. Approximately 1 week prior to the beginning of the experiment, mice were transferred from group housing (littermates by sex) into individual shoebox cages in a colony room where lighting (12:12 h light:dark cycle), temperature, and humidity were automatically controlled. Food was available throughout the experiment. Water was available except where noted below.

Procedure

Mice were tested over 23 days, which consisted of "Davis Rig Training" (Days 1–5), "Taste Aversion Conditioning 1" (Days 6–14), "Generalization Testing 1" (Days 15–16), "Taste Aversion Conditioning 2" (Days 17–21), and "Generalization Testing 2" (Days 22–23).

Davis Rig training. Similar to Experiment 3, water was removed on Monday, and the mice were trained in the Davis

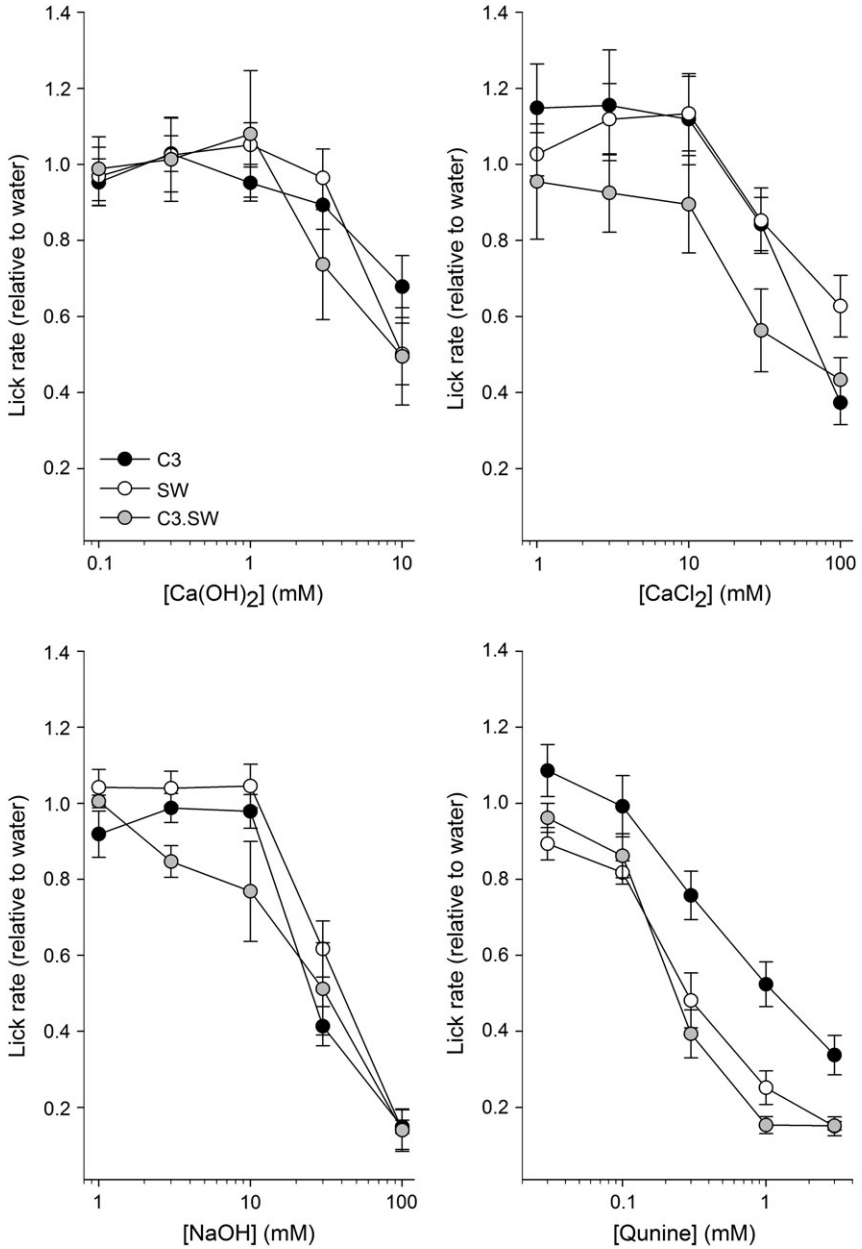

Figure 3 Mean (±standard error) lick rate (relative to water) of $CaOH_2$, $CaCl_2$, NaOH, and quinine in 5-s brief-access taste trials for C3 (filled circles), SW (open circles), and C3. SW congenic (gray circles) mice.

Rig over the next 4 days. The first 2 sessions consisted of access to a single tube of distilled water in a single, 20-min trial. In the rare case a mouse did not initiate at least 50 licks during this session, a second session was provided later in the day. The second 2 sessions consisted of 24, 5-s trials during which 6 tubes were filled with distilled water. These sessions were identical to the corresponding training sessions in Experiment 2 except that the shutter remained open for 300 s rather than 60 s waiting for trial initiation.

Taste aversion conditioning 1. Over days 6–14, mice received fluid twice daily. These sessions were conducted in the colony room (not in the Davis Rig). Fluid was offered in 50-mL Pyrex centrifuge tubes fitted with silicone stoppers pierced with stainless-steel sipper tubes. Fluid was measured (to the nearest 0.1 g) before and after each fluid access period.

Fluid was available for 15 min in the morning and for 45 min in the afternoon (precisely 5 h after the beginning of the morning session). Food was temporarily removed from the home cage during the morning session only. Fluid was always distilled water during the afternoon sessions, and was distilled water during the morning sessions of days 6–8, 10–11, and 13–14.

On days 9 and 12, mice received 10 mM $Ca(OH)_2$ (the conditioned stimulus). If a mouse did not consume at least 2 mL of

the conditioned stimulus, 1 mL was infused into the oral cavity by syringe. Following the morning fluid access period, 5 SW mice (referred to as SW+) and 6 C3 mice (C3+) were injected intraperitoneally with 3 mEq of 0.15 M LiCl. The remaining mice (SW- and C3-) were injected with 3 mEq 0.15 M NaCl.

Generalization testing 1. Mice were placed in the Davis Rig for 30-min sessions on 2 consecutive days. Sessions consisted of up to 24 5-s trials. Six bottles were filled with compounds from either the "standard panel" (distilled water, 10 mM $Ca(OH)_2$, 0.3 M sucrose, 0.15 M NaCl, 10 mM citric acid, and 3 mM quinine) or the "chloride–hydroxide panel" (distilled water, 10 mM $Ca(OH)_2$, 10 mM $CaCl_2$, 10 mM NaOH, 10 mM NaCl, and 30 mM $Ca(OH)_2$). Half of the mice were exposed to each panel; a single mouse received the same stimuli over these 2 sessions.

Taste aversion conditioning 2. Because Generalization Testing 1 sessions represented potential extinction trials, mice were given another conditioning experience with $Ca(OH)_2$ prior to further generalization testing. Over Days 17–21, the mice again received access to fluid twice daily as in Taste Aversion Conditioning 1. On Day 19, the morning fluid was 10 mM $Ca(OH)_2$. Mice that did not drink at least 2 mL during this session had 1 mL infused orally via a syringe. The morning fluid access was followed by a single intraperitoneal injection of 3 MEq of 0.15 M LiCl (C3+ and SW+ mice) or 3 MEq of 0.15 mM NaCl (C3- and SW- mice).

Generalization testing 2. Over the final 2 days, mice that had received the standard panel in Generalization Testing 1 received the chloride–hydroxide panel during Generalization Testing 2 and vice versa. Thus, all mice received both generalization tests, but order of the tests was counterbalanced.

Analysis

Because many test stimuli were unconditionally aversive (e.g., citric acid and quinine), we calculated an "aversion index" (St. John and Hallagan 2005) by dividing the average number of licks (relative to water) to a test stimulus for each mouse in the SW+ and C3+ by the group average number of licks to that test stimulus in the appropriate taste aversion control group (i.e., SW- and C3-). The aversion index ranges from near 0 to around 1, with 1 indicating that the mouse in the conditioning group licked a stimulus as much as a mouse in the taste aversion control group (i.e., no aversion). An aversion was considered statistically significant if the aversion index differed from 1.0 (1-sample *t*-test). Alpha level was set at 0.01 to correct for multiple comparisons using Bonferroni's method (assuming 5 interdependent means per test panel). One mouse in the SW+ group who showed an unusually high lick count to the conditioned stimulus during all conditioning phases was removed from the analysis.

Results

Mice were successfully conditioned to avoid $Ca(OH)_2$ (Figure 4). C3 and SW mice reduced their intake of the conditioned stimulus relative to water intake and controls by the second presentation and conditioning trial (i.e., day 12). The aversion was also evident during the second conditioning phase (i.e., day 19) in between the 2 generalization tests.

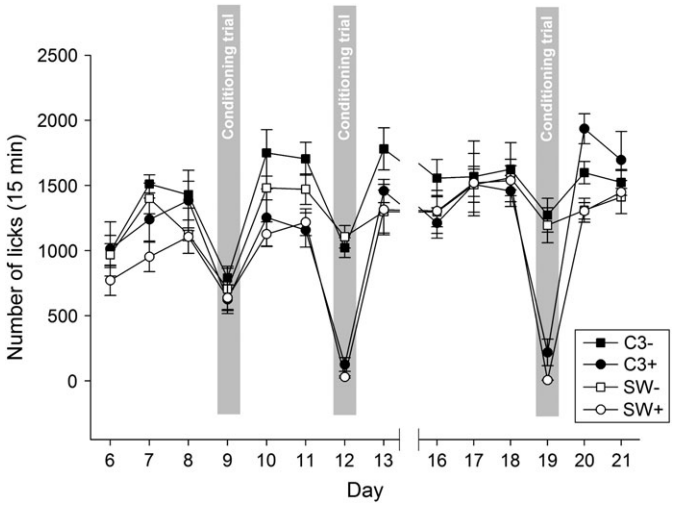

Figure 4 Mean (±standard error) 15-min intake (number of licks) of fluid for C3 (filled symbols) and SW (open symbols) mice during daily sessions. The fluid offered was water except on days 9, 12, and 19 (Conditioning trials), when the fluid was 10 mM $CaOH_2$. C3+ and SW+ mice received LiCl injections following $CaOH_2$ sessions; C3- and SW-received saline injections. No sessions occurred on days 14–15 and 22–23 because mice were being tested in brief-access tests (see Figure 5).

For the Standard Panel (Figure 5A), C3+ and SW+ mice showed a strong avoidance of the conditioned stimulus, 10 mM $Ca(OH)_2$. Both strains also avoided quinine, but because SW mice strongly avoided quinine even in the control group (cf., Figure 3), only the C3 aversion index was significantly less than 1.0. SW mice also significantly generalized their aversion to citric acid, whereas C3 mice did not.

For the Chloride–Hydroxide Panel (Figure 5B), both strains again demonstrated a strong aversion to the conditioned stimulus, both at 10 and 30 mM. Neither strain generalized the $Ca(OH)_2$ aversion to isomolar $CaCl_2$, whereas both strains generalized the aversion to isomolar NaOH.

Experiment 5: further taste aversion generalization studies

Interpretation of the results of Experiment 4 was rendered somewhat problematic because of the aversive concentration of quinine tested. Experiment 5 was essentially similar to Experiment 4 with the following modifications: 1) The test array was focused on $Ca(OH)_2$, quinine, and citric acid; 2) a low and high concentration of each was tested; and 3) aversions were conditioned to either $Ca(OH)_2$ or quinine, as the existence of cross-generalizations provides more compelling evidence of qualitative similarity than unidirectional generalization (Spector and Grill 1988).

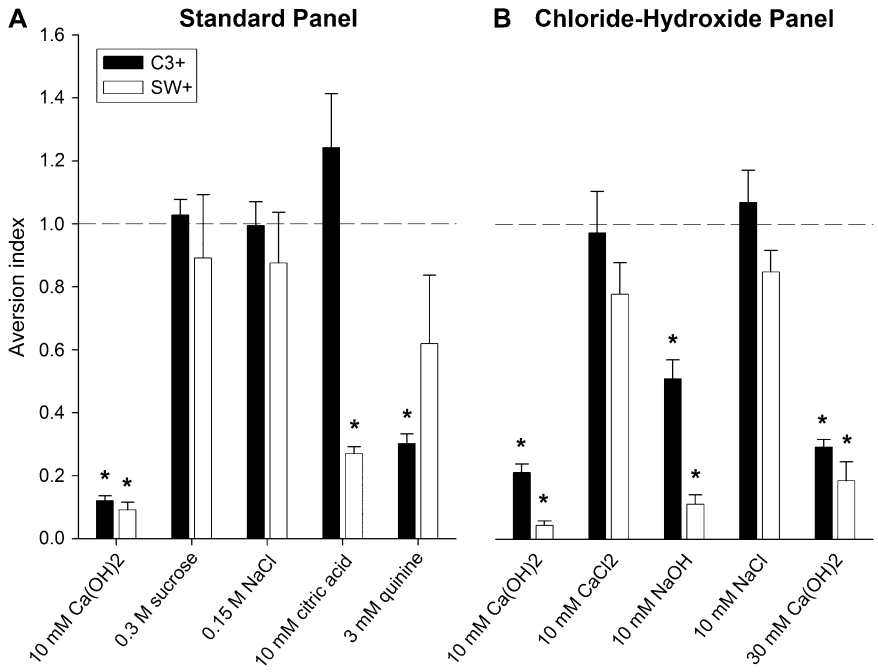

Figure 5 The Aversion Index (±standard error: standardized lick rate to a tastant over average lick rate to the tastant among control mice; see text for further details) as a function of stimulus for C3 (filled bars) and SW (open bars) mice given taste aversions to 10 mM $Ca(OH)_2$. Stimuli were delivered during 2 generalization tests (the Standard Panel, **A**, and the Chloride–Hydroxide Panel, **B**). Asterisks indicate a significant difference from 1.0 (1-sample *t*-tests, Bonferroni correction for multiple comparisons). In general, both C3 and SW mice generalized aversions to $Ca(OH)_2$ and NaOH but not NaCl or $CaCl_2$.

Materials and methods

Subjects

Eighteen mice of the C3 and 18 of the SW strain served as subjects. One SW mouse escaped from its cage during the experiment leaving a final $n = 17$. Approximately 1 week prior to the beginning of the experiment, mice were transferred from group housing (littermates by sex) into individual shoebox cages in a colony room where lighting (12:12 h light:dark cycle), temperature, and humidity were automatically controlled. Food was available throughout the experiment. Water was available except where noted below.

Procedure

Training, conditioning, and testing of mice were identical to Experiment 3 with the following exceptions:

Conditioning. Some of the mice received 10 mM $Ca(OH)_2$ as the conditioned stimulus and some received 1 mM quinine. As before, some mice received a LiCl injection and some received a NaCl (control) injection. These manipulations created 8 groups, with group sizes in parentheses: C3/Q+ (6), C3/Q- (3), C3/OH+ (6), C3/OH- (3), SW/Q+ (6), SW/Q-(2), SW/OH+ (6), and SW/OH- (3). For analysis purposes, the Q- and OH- groups were combined into a single control group for each strain (the C3/CON, $n = 6$; SW/CON, $n = 5$).

Testing. A single, 2-day test was given with 8 test stimuli: $Ca(OH)_2$ (1 and 10 mM), quinine (0.1 and 1 mM), and citric acid (1 and 10 mM). The remaining 2 bottles were filled with distilled water (and thus distilled water was presented twice in each block of 8 trials).

Results

There was evidence for cross-generalization of taste aversions between quinine and $Ca(OH)_2$ for both C3 and SW mice (Figure 6), although overall, the aversions conditioned in this experiment were weaker than in Experiment 4. Mice given an aversion to 10 mM $Ca(OH)_2$ showed a significant aversion to the conditioned stimulus (Figure 6A; assessed in the same way as in Experiment 4). C3 mice but not SW mice generalized this aversion to a lower concentration of $Ca(OH)_2$ (though the comparison for SW mice at 1 mM $Ca(OH)_2$ just missed the statistical rejection criterion of $\alpha = 0.00833$; $t(4) = 2.43$; $P = 0.036$). The aversion in both strains generalized to 1 mM quinine but not citric acid (Figure 6A).

Aversions conditioned to quinine were somewhat weaker; in fact, only SW mice showed a significant aversion to the conditioned stimulus of 1 mM quinine (Figure 6B). (The aversion index for C3 mice was significantly less than 1.0 at the $\alpha = 0.05$ level but not at the corrected $\alpha = 0.00833$ level). The significant aversion in SW mice cross-generalized to the higher concentration of $Ca(OH)_2$ but not to citric acid.

Discussion

Compounds that are low in pH are perceived as sour and elicit physiological reflexes (such as salivation) and, at low

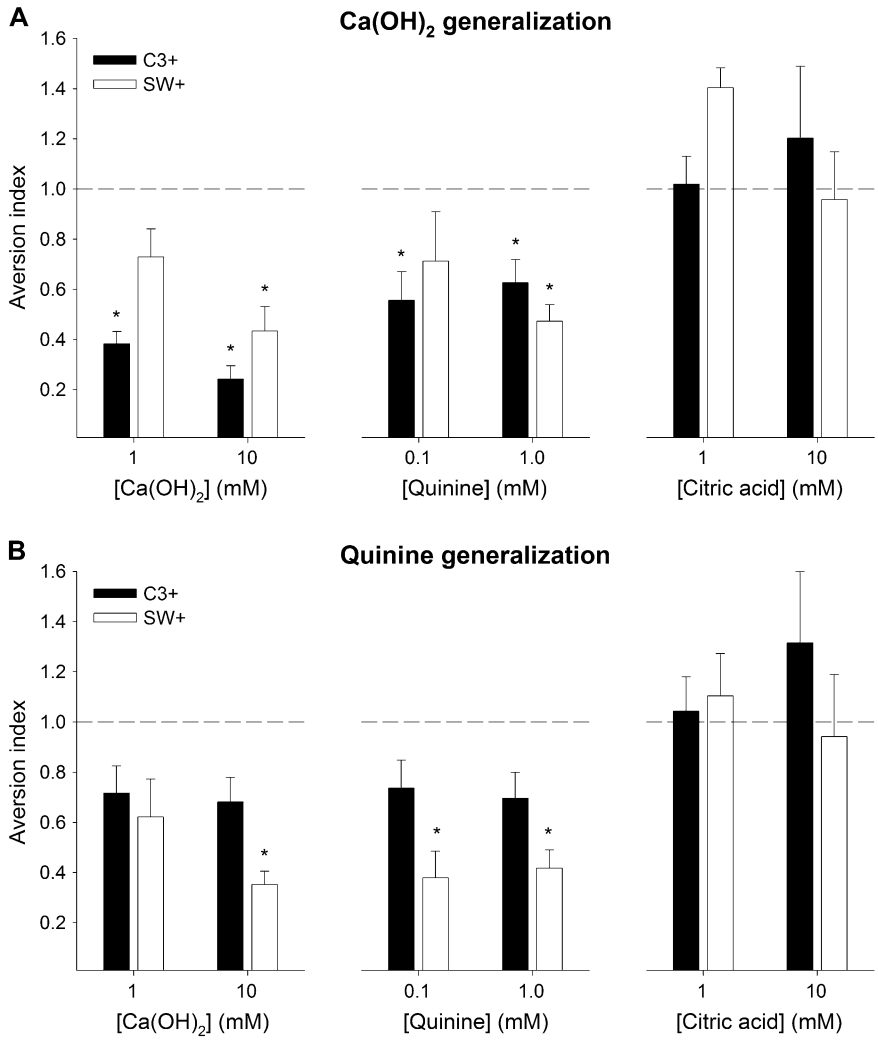

Figure 6 The Aversion Index (±standard error) as a function of stimulus for C3 (filled bars) and SW (open bars) mice given aversions to $Ca(OH)_2$ **(A)** or quinine **(B)**. Asterisks indicate a significant difference from 1.0 (1-sample *t*-tests, Bonferroni correction for multiple comparisons).

enough pH, behavioral rejection (Beatty and Cragg 1935; Harriman 1980; Norris et al. 1984; Brining et al. 1991). Organisms also may reject compounds with a high enough pH (Goatcher and Church 1970), but the sensory mechanisms underlying this rejection response have been less rigorously examined. Interestingly, we found that even among mice, responses to hydroxide-containing compounds vary. SW mice, for example, rejected hydroxide-containing compounds in 2-bottle preference tests, whereas C3 mice preferred these compounds, at least at some concentrations. The several experiments presented here further documented these behavioral differences.

In our first experiment, the SW mice displayed an expected rejection of increasing concentrations of $Ca(OH)_2$ and NaOH. These same mice, however, responded neutrally to $CaCl_2$, suggesting that, at least over the concentration range tested (0.3–10 mM), the hydroxide ion was more critical than the cation in mediating this behavioral avoidance. In contrast, C3 mice showed a preference for $Ca(OH)_2$, particularly at 3 and 10 mM, concentrations that SW mice rejected. C3 mice did not reject $CaCl_2$ or NaOH over the concentration range tested, and generally behaved similarly to all 3 compounds. Thus, it is less clear whether the preference in C3 mice is driven by the hydroxide ion or the cation presented.

Preference in 2-bottle tests can be driven by orosensory and/or postingestive factors. In a second experiment, 3 mM $Ca(OH)_2$ preference tests were conducted over 12 consecutive days. From the very first 2-day test, the strain difference between SW and C3 mice was evident. C3 mice showed a nonsignificant preference for 3 mM $Ca(OH)_2$ and SW mice showed a statistically significant rejection. Over days, both strains showed increased relative intake of $Ca(OH)_2$, resulting in a statistically-significant preference in C3 mice by the fourth 2-day preference test. Two trends were clear (and statistically supported by ANOVA): C3 and SW differed in their preference for 3 mM $Ca(OH)_2$, and both strains increased their

preference with time, suggesting the development of tolerance or learned safety.

Because our first experiment presented ascending series, it is possible that tolerance effects were involved in the concentration–response functions obtained in that experiment. The 3 mM $Ca(OH)_2$ concentration would have been the third concentration encountered by the mice in Experiment 1. Indeed, there is a striking correspondence in the preference results for 3 mM $Ca(OH)_2$ in Experiment 1 and for Test 3 from experiment 2 (cf., Figures 1 and 2). Although tolerance to an aversive taste is likely contributing to the behavioral responses in these experiments, it is important to note that the magnitude of the C3 and SW strain difference remained constant over the 12-day test (cf., Figure 2), a conclusion supported statistically by the significant Strain and Test Period factors but the lack of a significant Strain × Test Period interaction.

The complex nature of preference tests led us to examine C3 and SW responses to hydroxide-containing compounds in a brief-access paradigm that minimizes or eliminates the role of postingestive feedback (St. John and Boughter 2004; St. John and Spector 2008). In this test, strain differences in licking hydroxide-containing compounds were not apparent (cf., Figure 3). The possibility that the brief-access test is not sensitive to detect strain differences is unsupported by the data: These same mice did display conspicuous differences in sensitivity to quinine, replicating earlier work (Boughter et al. 2002; St. John and Boughter 2004). The tension between Experiment 3 and Experiments 1 and 2 might be explained by C3 and SW mice having similar orosensation for hydroxide-containing compounds, with differences in the 48-h preference tests being due to nonorosensory factors.

However, resolving the disparity between the 2 studies is complicated by the fact that brief-access tests in water-restricted animals (such as that used in Experiment 3) are not sensitive to detecting preferred stimuli. Because mice lick at their maximal rate to water (usually considered a neutral stimulus), the mice cannot lick faster to a preferred stimulus. Indeed, C3 mice preferred 3 mM $Ca(OH)_2$, for example, in 2-bottle tests (cf., Figures 1 and 2) but licked this concentration at the same rate as water in the brief-access test, with a taste-licking/water-licking ratio near 1.0 (cf., Figure 3). In the brief-access test, on the other hand, these mice did reduce licking 10 mM $Ca(OH)_2$, a concentration one might expect maximal licking based on the preference test results.

What can be determined from the brief-access tests is that both C3 and SW mice do respond to the orosensory qualities of hydroxide-containing compounds, at least at concentrations above 10–30 mM. Both SW and C3 mice avoided hydroxide-containing compounds as concentration increased above these intensities. This response pattern is consistent with the notion that these concentrations have an aversive taste, although it is also possible that behavioral rejection is driven by aversive trigeminal or olfactory cues.

We next assessed the quality of the orosensory experience of hydroxide-containing compounds to the C3 and SW mice using CTA methodology (Tapper and Halpern 1968; Nowlis et al. 1980; St. John et al. 2005). Our first attempt, in Experiment 4, was to condition a taste aversion to $Ca(OH)_2$ and examine the generalization of that aversion to a host of other compounds. It is important to note that despite its name, a "taste aversion" conditioned with the methods used in this experiment can be used to assess the similarity of various test compounds to $Ca(OH)_2$, but cannot indicate whether such similarity is based on taste, olfaction, or mouth feel. Some compounds in our generalization array are likely to elicit olfactory or trigeminal cues (e.g., citric acid and sucrose), whereas others may be less likely to do so (e.g., NaCl and quinine).

Both C3 and SW mice developed strong aversions to $Ca(OH)_2$ and generalized these aversions to a subset of the test stimuli. Both strains strongly avoided quinine, although whether such avoidance in the SW mice represented the generalization of the taste aversion was difficult to assess because of a floor effect (sham-aversion SW mice also rejected quinine strongly). Thus, although C3 mice showed a preference for $Ca(OH)_2$ in the 48-h 2-bottle preference tests of Experiments 1 and 2, these mice avoided 10 mM $Ca(OH)_2$ in the brief-access test of Experiment 3 and generalized a $Ca(OH)_2$ aversion to quinine in Experiment 4. These results provide converging evidence that $Ca(OH)_2$ has an aversive orosensory component to both C3 and SW mice despite the interesting difference which emerges over a 48-h test.

Also significantly, both SW and C3 mice generalized the $Ca(OH)_2$ aversion to NaOH but not $CaCl_2$. A number of recent studies have examined the calcium appetites of rats and mice (Tordoff 2001, 2008; McCaughey et al. 2005), demonstrating that this cation can be recognized by rodents to guide calcium intake to address a physiological mineral deficiency. Our emphasis on the hydroxide anion in this report is justified by the results of the taste aversion generalization in both strains in Experiment 4. The fact that the $Ca(OH)_2$ aversion did not generalize to $CaCl_2$ does not mean that the calcium ion is unimportant. However, these results do suggest that, at the concentrations used, $Ca(OH)_2$ is more similar to NaOH than to $CaCl_2$. Whatever the sensation evoked by licking $Ca(OH)_2$, the hydroxide ion appears to overshadow the contribution of calcium at these concentrations.

A weakness of Experiment 4 (the difficulty in assessing generalization to quinine in SW mice) prompted the final experiment, in which mice were given aversions to either $Ca(OH)_2$ or quinine. Although the aversions generated in Experiment 5 were unexpectedly weak (compare the magnitude of aversion with the conditioned stimulus in Figures 5 and 6), there was evidence that SW mice given an aversion to quinine cross-generalized this aversion to $Ca(OH)_2$. We also reassessed whether a $Ca(OH)_2$ aversion would generalize to citric acid, because Experiment 4 found a significant generalization to this compound in SW mice. In Experiment 5, there was absolutely no evidence of generalization between $Ca(OH)_2$ and citric acid for either C3 or SW mice. The explanation of this discrepancy awaits further experimentation.

It is common for strains to differ in preference and intake of aversive or bitter-tasting stimuli; indeed, a large-magnitude difference between SW and C3 mice can be found for the bitter acetylated sugar sucrose octaacetate, with SW mice showing aversion, and C3 mice displaying complete neutrality to a large concentration range (Boughter and Whitney 1998). However, in the current study, C3 mice actually displayed a preference for alkaline compounds at certain concentrations that SW mice avoided. Although this stark behavioral difference was not due to variation in orosensory sensitivity to the aversive aspects of $Ca(OH)_2$ and NaOH, it is possible that the preference seen by C3 mice is due to some other property of the stimulus. Interestingly, although humans often describe sodium hydroxide as bitter or aversive, it is also noted to have a sweet-tasting component (reviewed in Parker 1922; Liljestrand and Zotterman 1956). However, C3HeB/FeJ mice possess a lower level of avidity to sweet-tasting compounds such as sucrose or saccharin than do SWR/J mice (Lush 1989; Capeless and Whitney 1995; Reed et al. 2004), precluding a simple link between alkaline preference and the potential sweet taste of this compound. More likely, the preference is due to postingestive factors. Intriguingly, Glendinning (1993) showed that *Peromyscus* mice are capable of developing preferences for lower concentrations of normally avoided "deterrent" compounds such as QHCl or tannic acid. This is the so-called "Schweppes" effect, named for the preference of many humans for bitter-flavored tonic water. It is also worthwhile to point out that rats and mice will voluntarily ingest kaolin or clay following poisoning (Mitchell et al. 1976, 1977; Takeda et al. 1993; Yamamoto et al. 2002), and clay soils often possess a high pH. This "geophagia" is suggested to be a protective response.

In summary, C3 and SW mouse strains vary in preference for $Ca(OH)_2$ and NaOH, not only in degree but also in valence, in long-term preference tests. Strong differences occurred between C3 mice (which preferred these compounds at 3 mM) and SW mice (which avoided them). These strain differences to 3 mM $Ca(OH)_2$ were evident on the first 48-h exposure to $Ca(OH)_2$ and persisted over a 12-day period, with the C3 preference for $Ca(OH)_2$ increasing over the 12 days and the SW avoidance of $Ca(OH)_2$ decreasing toward neutrality at the same rate. The preference for $Ca(OH)_2$ in C3 mice does not appear to be orosensory in nature because C3 and SW mice equally avoided this compound (at comparable concentrations) in a brief-access licking test, and both generalized $Ca(OH)_2$ to quinine in a taste aversion generalization test. Finally, the hydroxide ion is salient in the orosensory evaluation of $Ca(OH)_2$ given that aversions to this compound generalized to another hydroxide-containing compound (NaOH) but not another calcium-containing compound ($CaCl_2$).

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