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Oral Amiloride Treatment Decreases Taste Sensitivity to Sodium Salts in C57BL/6J and DBA/2J Mice

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Abstract

Sodium taste transduction is thought to occur via an amiloride-sensitive, sodium-selective pathway and an amiloride-insensitive, cation nonselective, anion-dependent pathway(s). It has been shown by others that amiloride, an epithelial sodium channel (ENaC) blocker, significantly reduces the chorda tympani nerve response to lingually applied NaCl in C57BL/6 (B6) mice but not in DBA/2 (D2) mice, suggesting that the latter strain might not possess functional ENaCs in taste receptor cells. We psychophysically measured and compared taste detection thresholds of NaCl and sodium gluconate (NaGlu) prepared with and without 100 μM amiloride in these two strains (eight/strain). Mice were trained and tested in a two-response operant signal detection procedure conducted in a gustometer. Surprisingly, no strain effect was found for the detection thresholds of both salts (~0.05–0.06 M). Moreover, these thresholds were increased by almost an order of magnitude by amiloride adulteration of the solutions. This marked effect of amiloride on sodium detection thresholds suggests that ENaCs are necessary for normal sensitivity to sodium salts in both strains. In addition, because NaGlu is thought to stimulate primarily the amiloride-sensitive pathway, especially at low concentrations, the similarity of NaCl and NaGlu thresholds ($r > 0.81$ both strains) suggests that ENaCs are also sufficient to support the detection of sodium in weak solutions by B6 and D2 mice.

Key words: animal psychophysics, epithelial sodium channels, gustatory system, inbred mice, sodium chloride

Introduction

In rodents, sodium taste transduction appears to occur through at least two transduction pathways. One pathway is completely suppressed by oral treatment with the epithelial sodium channel (ENaC) blocker amiloride and the other is unaffected by this drug. The amiloride-sensitive component is thought to reflect the action of a transcellular transduction pathway that involves the relatively selective entry of Na^+ (and Li^+) through ENaCs in the apical membrane of a subset of taste receptor cells (Heck *et al.*, 1984; Brand *et al.*, 1985; DeSimone and Ferrell, 1985; Avenet and Lindemann, 1988; Formaker and Hill, 1988; Ninomiya and Funakoshi, 1988; Elliott and Simon, 1990; Ye *et al.*, 1993). The second, amiloride-insensitive, pathway is thought to reflect the electroneutral diffusion of cations and small anions through tight junctions between taste receptor cells after which the ions contact submucosal receptor sites (Avenet and Lindemann, 1988; Formaker and Hill, 1988; Elliott and Simon, 1990; Hettinger and Frank, 1990; Ye *et al.*, 1991, 1993; Doolin and Gilbertson, 1996). In addition, there is evidence for the presence of nonselective ion channels located on the apical membranes of some taste receptor cells (Gilbertson and Zhang, 1998; DeSimone *et al.*, 2001). Because amiloride treatment drops the chorda tympani (CT) nerve response to sodium salts with large organic anions, such as sodium acetate or sodium gluconate, to seemingly negligible values, these salts are thought to be transduced predominantly through the sodium selective transcellular pathway (Formaker and Hill, 1988; Elliott and Simon, 1990; Ye *et al.*, 1991, 1993, 1994; Simon, 1992).

From a taste coding perspective, it is worth noting that, in some rodents, amiloride treatment suppresses sodium salt responses in relatively narrowly tuned sodium responsive afferent fibers in the peripheral gustatory system, but has little effect on salt responses in nerve fibers that are broadly tuned (Ninomiya and Funakoshi, 1988; Hettinger and Frank, 1990; Ninomiya, 1998; Lundy and Contreras, 1999). In rats, normal sodium taste detection and recognition is dependent on the amiloride-sensitive transcellular sodium transduction pathway. Stimulus adulteration with amiloride reduces the sodium taste sensitivity of rats (Geran and Spector, 2000a,b; Kopka and Spector, 2001) and appears to change the taste quality of NaCl, making it more similar to

that of nonsodium chloride salts (Bernstein and Hennessy, 1987; Hill *et al.*, 1990; Spector *et al.*, 1996; Geran and Spector, 2001; Kopka *et al.*, 2000).

Interestingly, amiloride treatment does not universally suppress CT responses to NaCl in all strains of mice. Oral treatment with amiloride reduces the CT response to NaCl in C57BL/6 (B6) mice, but has no significant effect in the DBA/2 (D2), 129/J (129) and BALB/c (BALB) strains (Gannon and Contreras, 1995; Ninomiya *et al.*, 1989), even though the nerve in all four strains responds well to this salt. Also, in BALB mice, NaCl responses are affected by amiloride in significantly less taste receptor cells compared with B6 mice (Miyamoto *et al*., 1999). Thus, on the basis of these electrophysiological findings, B6 mice appear to possess an amiloride-sensitive sodium transduction pathway in the taste receptor cells of the anterior tongue (innervated by the CT), whereas it appears that the latter three strains may not have, or at least have a significantly lower number of, amiloride-sensitive taste receptor cells.

In previous work, we have shown that amiloride adulteration of NaCl solutions significantly raises the NaCl detection threshold by about an order of magnitude in B6 mice (Eylam and Spector, 2002). This finding links the amiloride-sensitive transcellular sodium transduction pathway to NaCl taste sensitivity in this strain. Assuming that in these mouse strains the CT nerve responds best to NaCl, as is the case in rats, the electrophysiological findings mentioned above lead to the prediction that amiloride should *not* alter taste sensitivity to NaCl in D2 mice. Moreover, these findings, along with the demonstration that the CT of B6 mice is more responsive to NaCl than is the CT of D2 mice (Frank and Blizard, 1999), also suggests that the D2 strain would be less sensitive to NaCl as assessed behaviorally. Although this question was previously addressed using two-bottle intake tests [e.g. (Lush, 1989; Ninomiya *et al.*, 1989; Kotlus and Blizard, 1998; Bachmanov *et al.*, 2002)], these studies report somewhat conflicting results and as noted in our prior work with B6 mice, the two-bottle intake test is not an optimal assay for discerning differences in NaCl sensitivity at low concentrations. Nonetheless, predictions about perception based on neurobiological observations of peripheral processes must be confirmed behaviorally. Accordingly, we used a two-response operant conditioning procedure to measure the effect of amiloride on NaCl detection thresholds in B6 and D2 mice.

In Sprague–Dawley rats, sodium gluconate (NaGlu) and NaCl detection thresholds are virtually the same (Geran and Spector, 2000b). Moreover, when the ENaC blocker is mixed in the solutions, thresholds for NaGlu are shifted by an even greater margin compared with NaCl, as would be expected based on the electrophysiological evidence, suggesting that NaGlu is transduced predominantly, if not exclusively, through the amiloride-sensitive transcellular sodium transduction pathway (Geran and Spector, 2000b). This implicates the amiloride-sensitive sodium taste transduction pathway as not only necessary, but also sufficient for normal taste sensitivity to low concentrations of sodium salts in rats. Therefore, we additionally determined thresholds for sodium gluconate, with and without amiloride treatment, to examine whether salt anion size would influence sensitivity in either strain.

Materials and methods

Subjects

Eight C57BL/6J (B6) and 8 DBA/2J (D2) naive adult (7 weeks ± 2 days old) male mice (Jackson Laboratories, Bar Harbor, ME), with mean body masses of 23.3 ± 0.23 g and 19.3 ± 0.58 g, respectively, on arrival, served as subjects. The mice were housed individually in polycarbonate shoebox cages in a colony room where the temperature, humidity and lighting (12 h light/12 h dark) were controlled automatically. Subjects had free access to pellets of laboratory chow (LabDiet 5001, PMI Nutrition International Inc., Brentwood, MO) and distilled water. One week after arrival, the mice were put on a restricted water-access schedule. Fluid was available only during the training or testing session on Monday to Friday; home-cage water bottles were replaced after the last session on Friday and removed on Sunday. While on the water-restriction schedule, mice that dropped below 85% of their body mass based on *ad libitum* drinking weight received 1 ml supplemental water after the end of the testing session. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Taste stimuli

All taste solutions were prepared daily with reagent grade chemicals, and presented at room temperature. The NaCl and NaGlu (Fisher Scientific, Atlanta, GA) concentrations used for testing were 0.0125, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6 and 0.8 M prepared with distilled water. During the amiloride phase of the experiment, 100 μM amiloride hydrochloride (Sigma Chemical Co., St Louis, MO) was prepared with distilled water at least 1 h prior to testing in a glass flask covered with aluminum foil to prevent photodegradation. A 100 μM amiloride concentration was selected because (i) it or lower concentrations have been commonly used in rodent electrophysiology including studies involving B6 and D2 mice (DeSimone and Ferrell, 1985; Ninomiya and Funakoshi, 1988; Ninomiya *et al.*, 1989; Ye *et al*., 1993; Miyamoto *et al*., 1999); (ii) rats appear to treat this concentration as tasteless (Markison and Spector, 1995); and (iii) we have previously demonstrated that this concentration significantly shifts NaCl detection thresholds in B6 mice (Eylam and Spector, 2002). The amiloride solution was used in place of distilled water in preparation of all other solutions used in this phase, including water reinforcers.

Procedure

The procedure and apparatus were described in detail by Eylam and Spector (Eylam and Spector, 2002). Briefly, animals were trained and tested in a specially designed testing apparatus referred to as a gustometer [modified from Spector *et al.* (Spector *et al.*, 1990)]. The test cage was enclosed in a sound-attenuating chamber (BRS/LVE, Laurel, MD) and white noise was presented to minimize extraneous auditory cues. All fluid deliveries were computer-controlled. The mice had access to a centrally positioned sample spout through a slot in the side wall of the test chamber. The initial lick filled the shaft of the sample spout and subsequent licks deposited ~1.6 μl into the fluid column. Reinforcement fluid was delivered from two stationary horizontally oriented 'reinforcement' spouts located on each side of this access slot. Contact with the correct reinforcement spout during the choice phase (see below) resulted in the delivery of water (~1.6 μl/lick).

Trial structure

The trial structure was described in detail by Eylam and Spector (Eylam and Spector, 2002). The mice were tested in daily 25 min sessions during which they were allowed to complete as many trials as possible. Each trial began with the sample phase. To initiate a trial, the mouse had to lick the spout two times within 250 ms to insure that the mouse was engaged in active licking when the stimulus was presented. Once a trial was initiated, the fluid stimulus was presented through the sample spout and the mouse was allowed up to five licks or 2 s spout access (whichever came first) before the sample spout was rotated away from the animal's reach. Following the sample phase, the mouse had 10 s (limited hold) to lick one of the two reinforcement spouts; this was referred to as the choice phase. The reinforcement phase began as soon as contact was made with one of the reinforcement spouts. If a correct choice was made, the mouse could receive up to 15 licks of the water reinforcer in a 30 s period. If an incorrect choice was made or no response was initiated within the allocated time, the mouse received a 30 s time-out during which no fluid was available. When 15 licks were taken, 30 s had passed, or when a time-out was completed, the sample spout was rotated over the funnel, rinsed with distilled water and dried with pressurized air, and then rotated back into position in front of the slot. This intertrial interval lasted ~6 s. Some of these parameters varied during training as described below.

Training

Mice were trained to respond to the presentation of NaCl by licking one reinforcement spout and to respond to the presentation of water by licking the other reinforcement spout (side counterbalanced between mice within strains). The training schedule can be seen in Table 1.

NaCl training structure. First, we trained the mice to lick the different spouts for fluid delivery in the gustometer (Spout training, Table 1) by presenting the animals with only one spout each day while covering (reinforcement spouts) or retracting (sample spout) the others. Water was the fluid delivered on all 3 days of this phase of training and it was available from the spout *ad libitum* throughout the session. Following these 3 days, we trained the mice to lick from a specific reinforcement spout in response to the presentation of either water or 0.6 M NaCl (delivered through the sample spout) by providing access only to the corresponding reinforcement spout while the other reinforcement spout was covered (Side training, Table 1). The sample solution (water or NaCl) and the matching reinforcement spout were

Table 1 NaCl training and testing schedule

Phase	Sessions	Stimuli	Limited hold[a] (s)	Time out (s)	Presentation schedule
Spout training	3	dH_2O	none	none	constant[b]
Side training	6	0.6 M NaCl or dH_2O	180	none	constant
Alternation	30	0.6 M NaCl and dH_2O	15	30	criterion[c] (6→1)
Detection training I	5	0.2–0.6 M NaCl and dH_2O	10	30	randomized blocks[d]
Detection training II	23	0.025–0.8 M NaCl and dH_2O	10	30	randomized blocks
Pre-amiloride NaCl testing (PRE-AMIL)	20	0.0125–0.8 M NaCl and dH_2O	10	30	randomized blocks
Amiloride testing (AMIL)	25	0.1–0.8 M NaCl and 100 μM amiloride	10	30	randomized blocks
Post-amiloride NaCl testing (POST-AMIL)	15	0.0125–0.8 M NaCl and dH_2O	10	30	randomized blocks

[a]*Limited hold* refers to the amount of time the mouse was given to make a response.

[b]*Constant* presentation schedule refers to presentation of the same stimulus throughout the entire session (no randomization).

[c]A stimulus is presented repeatedly until a certain number of correct responses are made (not necessarily successive). This criterion number of responses was decreased from six to four, to two and finally to one. Mice were moved from one criterion to the next when their performance reached 75% correct responses. The mice progressed individually, but the next phase did not start until all mice reached a criterion of 1.

[d]Solutions were presented in a semirandom fashion; a new randomized block was not presented until all solutions were presented once in the previous block.

alternated between days. In this phase, mice were allowed up to 180 s to respond after sampling (limited hold) and no time-out contingency was in effect. The alternation phase followed in which both NaCl and water were presented and both reinforcement spouts were available for response. During this phase of training, the limited hold was shortened to 15 s and a criterion number of correct responses (non-consecutively) were required for a change in the sample stimulus (from water to NaCl and vice versa). The criterion, which started at six correct responses, was gradually reduced across sessions according to the performance of each individual animal (at least 75% overall correct performance) until all mice reached a criterion of 1 (Alternation, Table 1). The time-out was introduced in this phase as a punishment for incorrect responses. Once performance was adequate (>80% correct responses in most cases), mice were trained to discriminate 0.6 M NaCl from water presented in randomized blocks (Detection training I, Table 1) and the limited hold was shortened to 10 s. After 1 week, two lower NaCl concentrations were added (0.2, 0.4 M) and the mice were trained for 3 additional weeks (Detection training II, Table 1).

NaGlu training structure. Five weeks after the completion of the NaCl detection experiment, the animals were retrained to discriminate sodium gluconate (NaGlu) from water. During the interim period between the two experiments these mice were further tested for their NaCl sensitivity under a different paradigm, but this is not the topic of this report. The mice were 'trained' with 0.6 M NaGlu and distilled water presented in randomized blocks (Detection training I, Table 2). After four sessions, two lower NaGlu concentrations were added (0.2, 0.4 M) and the mice were trained for 1 additional week (Detection training II, Table 2).

Testing

Detection testing (NaCl PRE-AMIL). Mice were tested with a range of NaCl concentrations (0.0125–0.8 M NaCl) for 4 weeks. During each session, half of the reservoirs were filled with different concentrations of NaCl and the other half, as well as the two reservoirs connected to the reinforcement spouts, were filled with distilled water. To maintain and assess stimulus control, the same concentrations were presented every Monday (0.025, 0.05, 0.1, 0.2, 0.4 M NaCl; referred to as the 'standard array'), while on Tuesday to Friday this range was varied weekly according to the overall performance of the group. This 'alternate array' always included one or two clearly detectable concentrations to maintain and measure stimulus control. Stimuli were delivered in randomized blocks of 10 so that the probability of a NaCl stimulus presentation was 0.5.

NaCl detection in the presence of amiloride (NaCl AMIL). In this phase, the NaCl taste threshold was reassessed in the presence of amiloride. During this phase all NaCl solutions were prepared with amiloride hydrochloride (100 μM) as the solvent instead of distilled water. Amiloride was also placed in the water stimulus and reinforcer fluid to help maintain its pharmacological effect on epithelial sodium channels. A similar range of NaCl concentrations was planned for this phase of the experiment as for the previous phase, but the mice clearly had difficulty performing the task. In order to prevent loss of stimulus control, the number of concentrations used per session was reduced and only the high end of the concentration range was utilized until performance reached adequate levels. The standard array was not used here and the mice were tested for 5 weeks with 8 NaCl concentrations ranging from 0.025 to 0.8 M.

Post-amiloride detection testing (NaCl POST-AMIL). A second determination of NaCl threshold was conducted after the performance-disrupting amiloride manipulation to test the reliability of the procedure. The schedule of daily stimulus presentation was similar to that described for the NaCl PRE-AMIL phase.

NaGlu testing. The testing schedule for NaCl testing was repeated here (Table 2); first, the mice were tested for their NaGlu detection threshold (NaGlu PRE-AMIL) for 5 weeks, followed by a redetermination of the threshold in the presence of amiloride (NaGlu AMIL) for 3 weeks, and

Table 2 NaGlu training and testing schedule

Phase	Sessions	Stimuli	Limited hold[a] (s)	Time out (s)	Presentation schedule
Detection training I	4	0.6 M NaGlu and dH_2O	10	30	randomized blocks[b]
Detection training II	5	0.2–0.6 M NaGlu and dH_2O	10	30	randomized blocks
Pre-amiloride NaGlu testing (PRE-AMIL)	24	0.0125–0.8 M NaGlu and dH_2O	10	30	randomized blocks
Amiloride testing (AMIL)	15	0.025–0.8 M NaGlu and 100 μM amiloride	10	30	randomized blocks
Post-amiloride NaGlu testing (POST-AMIL)	20	0.0125–0.8 M NaGlu and dH_2O	10	30	randomized blocks

[a]*Limited hold* refers to the amount of time the mouse was given to make a response.

[b]Solutions were presented in a semirandom fashion; a new randomized block was not presented until all solutions were presented once in the previous block.

lastly, a post-amiloride threshold measurement (NaGlu POST-AMIL) for 4 additional weeks.

Water control test. This test was conducted at the end of the experiment. All reservoirs were filled with distilled water with half of the reservoirs arbitrarily assigned to the left and half assigned to the right reinforcement spout. Mice were tested for two consecutive days to examine whether there were any extraneous cues guiding responses other than the chemical nature of the stimulus.

Data analysis

Both the NaCl and NaGlu data were corrected for false alarm (FA) rate using the following formula:

$$\text{corrected hit rate} = P(\text{hit})_c = \frac{P(\text{hit}) - P(\text{FA})}{1 - P(\text{FA})} \times 100 \quad (1)$$

where P(hit) is the proportion of correct responses on NaCl trials (responses on the salt side) and P(FA) is the proportion of incorrect responses on water trials (responses on the salt side). Only trials with a response were included in the analysis. Sigmoidal three-parameter logistic curves were fit to the corrected data using the following formula:

$$f(x) = a/(1+(10^{\log_{10}(x)-c)*b})) \quad (2)$$

where x is the NaCl concentration, a is the maximum asymptote of performance, b is the slope, and c is the $\log_{10}$ NaCl concentration at half-asymptotic performance. The latter parameter from the curve fit, c, was defined as the detection threshold.

A three-way analysis of variance (ANOVA; Phase × Strain × Concentration) was used for the common concentrations of the three phases (four concentrations) to see if there were any main effects or interactions. If significant effects were found, further analyses were performed. A two-way ANOVA (Phase × Concentration) was used in comparison of the corrected hit rates across the three phases of each experiment (PRE-AMIL, AMIL, POST-AMIL). Since the two lowest concentrations were not tested during the AMIL phase, they were not included in this analysis. Also, the parameters of the logistic functions were compared across the three phases using a one-way ANOVA (Phase). *Post hoc* paired comparisons were made between the curve parameters of the PRE-AMIL and POST-AMIL phases to test the reliability of this task as well as to ensure that the amiloride manipulation did not have lasting carry-over effects on sensitivity.

In order to test for adaptation effects from prior NaCl presentations, especially presentations of high NaCl concentration followed by low ones, we identified and separately analyzed NaCl trials after water reinforcement, which served as a functional water rinse. Since no significant difference was found between all trials and trials following water reinforcement only (see results), this analysis was not repeated for the NaGlu trials.

The data from NaCl POST-AMIL were compared to that of the NaGlu PRE-AMIL using ANOVA, and a Pearson product–moment correlation procedure was used to test the relationship between the threshold values of the two salts. Finally, the normal approximation of the binomial distribution (one-tailed test) was used to determine any deviation of performance from chance on water control test sessions. The conventional P-value 0.05 was considered significant in all statistical tests.

Results

Sodium chloride (NaCl) detection threshold

Figure 1 displays the mean corrected hit rate results for the two strains of mice. As can be seen from the relatively high asymptotic performance achieved (85.5 ± 5.7 for B6 mice and 95.3 ± 3.2 for D2 mice during the PRE-AMIL phase), both the B6 and the D2 mice were successfully trained in this paradigm.

A three-way ANOVA (Phase × Strain × Concentration) of the corrected hit rate for NaCl indicated no main effect of strain [$F(1,14) = 1.2$; $P = 0.3$]. However, there was a concentration main effect [$F(3,42) = 76.3$; $P < 0.01$], a phase main effect [$F(2,28) = 202.9$; $P < 0.01$], as well as a Phase × Strain interaction [$F(2,28) = 5.7$; $P < 0.01$]. A comparison between strains for each phase separately demonstrates no strain effect in the NaCl PRE-AMIL and the NaCl POST-AMIL phases [all $F(1,14) \leq 0.04$; all $P > 0.8$]. During the NaCl AMIL phase, however, there was a significant strain effect [$F(1,14) = 5.0$; $P = 0.04$]; the D2 mice appeared to be more disrupted by the amiloride treatment.

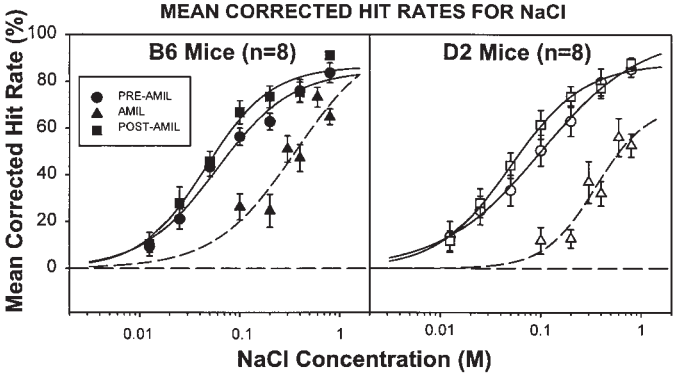

Figure 1 Mean (± SE) performance (corrected hit rates) as a function of NaCl concentration for pre-amiloride detection testing (PRE-AMIL; circles), amiloride testing (AMIL; triangles), and post-amiloride detection testing (POST-AMIL; squares) phases of the eight C57BL/6J (B6) mice (left panel, closed symbols) and eight DBA/2J (D2) mice (right panel, open symbols). The curves were fit to the corrected hit rate data by using the logistic function described in the text. Amiloride shifted the performance curve to the right, demonstrating an increase in thresholds to NaCl in both mouse strains.

The reduction in the corrected hit rate in the presence of amiloride was reversed when the blocker was removed, as indicated by the absence of a significant difference between the NaCl PRE-AMIL and NaCl POST-AMIL phases for either strain when the two strains were analyzed separately [all $F(1,7) < 3.0$, all $P > 0.1$]. There was only a main effect of concentration [all $F(6,42) > 119.7$; all $P < 0.01$] and the interaction was not significant [all $F(6,42) < 1.0$; all $P > 0.4$]. The addition of amiloride shifted the curve to the right in both strains and a Phase × Concentration ANOVA revealed a significant phase effect when amiloride was included in the analysis [all $F(2,14) > 60.2$; all $P < 0.01$]. It is important to note that only four of the seven concentrations tested were common to all three phases of the experiment and, therefore, only partial data could be compared using this statistical test. Nonetheless, amiloride caused a clear reduction in sensitivity in both strains (Figure 1). As expected, there was a dose-dependent change in the corrected hit rate and therefore a concentration main effect was confirmed [$F(3,21) > 28.6$; $P < 0.01$]. Also, there was a significant Phase × Concentration interaction [$F(6,42) > 3.1$; $P < 0.02$].

The curve fit to the mean of corrected hit rates as well as the curve fit for individual concentration-response data accounted for the variance well, especially for the NaCl PRE-AMIL and NaCl POST-AMIL phases (B6 mean $r^2 = 0.96$ and 0.93, respectively; D2 mean $r^2 = 0.91$ and 0.88, respectively). The curve fit to the mean of corrected hit rates of the NaCl AMIL phase also accounted for the variance well (B6 $r^2 = 0.83$; D2 $r^2 = 0.88$). However, the curve fit to individual concentration-response data of this phase was not as good (B6 mean $r^2 = 0.69$; D2 mean $r^2 = 0.65$ of the five mice whose data could be fit with a curve). The poor fits reflect the apparent 'confusion' of the animals in this task and demonstrate the marked effect of amiloride on taste-related behavior to NaCl.

Surprisingly, the two mouse strains performed similarly and had comparable thresholds for NaCl in this task (Table 3); a two-way ANOVA (Phase × Strain) including all three phases for each of the three curve parameters across animals showed no strain effect or interaction. There was also no phase effect for the asymptote (a) or slope (b) parameters (Table 3). The analysis of asymptotes has to be regarded with caution because in some cases the asymptotes were clearly extrapolated. The threshold, however, did significantly differ across phases [$F(2,22) = 41.4$; $P < 0.01$] but there was no interaction with strain. Oral treatment with amiloride caused an increase in the mean individual NaCl threshold by a little over 0.6 $\log_{10}$ units in both strains, with the caveat that no curve could be fit to the data from the NaCl AMIL phase of three of the eight D2 mice. Nonetheless, the performance of all the mice was clearly impaired by the amiloride adulteration of the NaCl solutions. On average, the threshold was consistent between the NaCl PRE- and NaCl POST-AMIL phases (Table 3, Figure 2).

In the water control test no animal responded significantly different from chance (50%; all P-values >0.05), with a performance average of 49.9 ± 1.6% for the B6 mice and 50.0 ± 1.3% for the D2 mice, confirming that the mice were not guided by any extraneous cues, but rather responded on the basis of the chemical nature of the stimulus.

Sodium gluconate (NaGlu) detection threshold

There was no difference between the two strains in their responses during the three phases of the experiment (Figure 3). A three-way ANOVA of Phase × Strain × Concentration of the corrected hit rate data revealed no main effect of strain [$F(1,11) = 0.5$; $P = 0.5$], and no Strain × Concentration [$F(5,55) = 0.7$; $P = 0.6$] or Strain × Phase interactions [$F(2,22) = 0.4$; $P = 0.6$]. As was the case for NaCl, amiloride shifted the mean curve of both strains to the right. There was a main effect of phase [$F(2,22) = 120.6$; $P < 0.01$], and concentration [$F(5,55) = 135.2$; $P < 0.01$], as well as a Phase × Concentration interaction [$F(10,110) = 10.1$; $P < 0.01$].

In addition, as was the case for NaCl, amiloride significantly increased the NaGlu threshold in both mouse strains (Table 3, Figure 4). A Phase × Strain ANOVA of

Table 3 Mean (± SE) individual curve parameters across phases

	Threshold ($\log_{10}M$)		Asymptote (%)		Slope	
Phase	B6 mice	D2 mice	B6 mice	D2 mice	B6 mice	D2 mice
NaCl PRE-AMIL	–1.242 (±0.1)[a]	–1.008 (±0.1)[a]	85.49 (±5.7)[a]	95.33 (±3.2)[a]	–1.815 (±0.5)[a]	–1.130 (±0.1)[a]
NaCl AMIL	–0.602 (±0.1)[a]	–0.386 (±0.1)[b]	87.42 (±5.9)[a]	81.60 (±13.2)[b]	–1.641 (±0.3)[a]	–1.728 (±0.4)[b]
NaCl POST-AMIL	–1.325 (±0.1)[a]	–1.307 (±0.1)[a]	88.03 (±3.1)[a]	87.02 (±3.6)[a]	–1.550 (±0.2)[a]	–1.867 (±0.6)[a]
NaGlu PRE-AMIL	–1.276 (±0.1)[a]	–1.279 (±0.1)[c]	85.52 (±3.1)[a]	87.93 (±4.0)[c]	–1.146 (±0.1)[a]	–1.208 (±0.1)[c]
NaGlu AMIL	–0.117 (±0.1)[d]	–0.053 (±0.2)[e]	100.00 (±0.0)[d]	100.00 (±0.0)[e]	–5.221 (±3.3)[d]	–3.958 (±2.2)[e]
NaGlu POST-AMIL	–0.957 (±0.1)[c]	–0.906 (±0.1)[f]	92.22 (±2.5)[c]	83.32 (±5.1)[f]	–1.449 (±0.1)[c]	–2.070 (±0.3)[f]

[a]$n = 8$ (all mice), [b]$n = 5$, [c]$n = 7$, [d]$n = 4$, [e]$n = 2$, [f]$n = 6$.

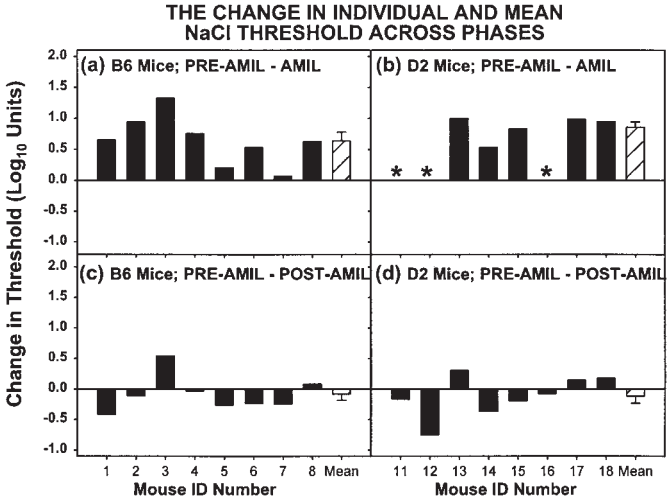

Figure 2 The changes in individual threshold (solid bars) as well as the mean (± SE) of these thresholds (hatched bars) across NaCl testing phases. Bars going up represent an increase in threshold and a decrease in sensitivity while bars going down represent the opposite. **(a)** The change in NaCl thresholds from PRE-AMIL to AMIL for B6 mice and **(b)** for D2 mice, **(c)** the change in NaCl threshold from PRE-AMIL to POST-AMIL for B6 mice and **(d)** for D2 mice. The asterisks (*) represent mice the data of which could not be fit by an individual curve during the AMIL phase resulting in no threshold calculated for this phase and therefore no difference between phases measured.

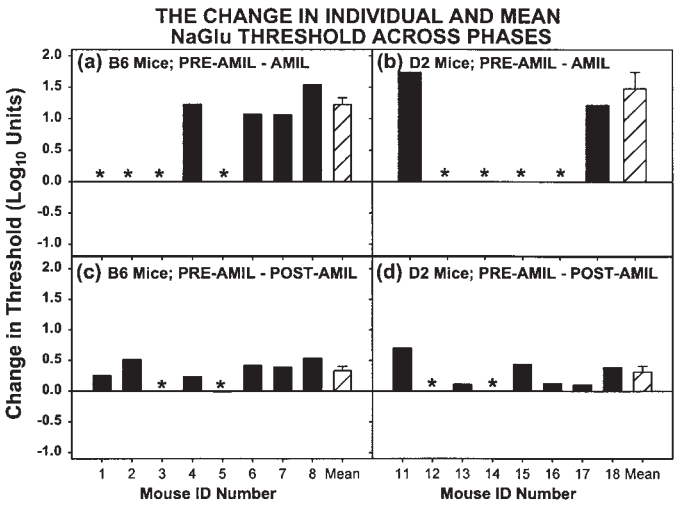

Figure 4 The changes in individual threshold (solid bars) as well as the mean (± SE) of these thresholds (hatched bars) across NaGlu testing phases. Bars going up represent an increase in threshold and a decrease in sensitivity while bars going down represent the opposite. **(a)** The change in NaGlu thresholds from PRE-AMIL to AMIL for B6 mice and **(b)** for D2 mice, **(c)** the change in NaGlu threshold from PRE-AMIL to POST-AMIL for B6 mice and **(d)** for D2 mice. The asterisks (*) represent mice the data of which could not be fit by an individual curve resulting in the lack of threshold calculated for this phase and therefore no difference between phases measured.

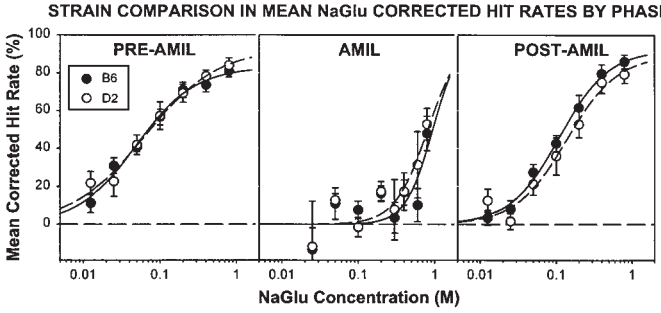

Figure 3 A strain comparison between the mean (± SE) performance (corrected hit rates) to NaGlu and the curves fit to these mean data during the PRE-AMIL phase (left panel), AMIL phase (middle panel) and POST-AMIL phase (right panel) of the eight B6 mice (closed symbols, solid lines) and the eight D2 mice (open symbols, hatched lines). The curves were fit to the corrected hit rate data by using the logistic function described in the text. No strain difference was found in either phase.

the curve fit parameters for the three phases of testing revealed a significant main effect of phase for the threshold [$F(2,8) = 106.4$; $P < 0.01$] but no strain effect [$F(1,4) = 0.7$; $P = 0.4$] or interaction [$F(2,8) = 1.3$; $P = 0.3$]. There were no significant main effects or interactions in either the asymptote (a) or the slope (b) [all $F(2,8)<1.4$; all $P > 0.3$]. Although curves could not be fit in most individual mouse cases for data from the NaGlu AMIL phase, the data clearly indicate that amiloride treatment severely disrupted the performance in all mice. Because the sensitivity of all mice was severely impaired by the addition of this ENaC blocker, the NaGlu AMIL phase had to be cut short to circumvent the possible loss of stimulus control.

Unlike with NaCl, there seems to have been a carry-over effect from the AMIL phase to the POST-AMIL phase, and two mice (one from each strain) lost stimulus control completely, selectively responding on one reinforcement spout during the NaGlu POST-AMIL phase regardless of the stimulus presented to them. This was supported by the outcomes of a three-way ANOVA (Phase × Strain × Concentration) of the concentration–response data from NaGlu PRE-AMIL and NaGlu POST-AMIL, indicating a significant effect of phase [$F(1,11) = 35.0$; $P < 0.01$] and a significant Phase × Concentration interaction [$F(6,66) = 5.1$; $P < 0.01$] as well as a Strain × Concentration interaction [$F(6,66) = 2.4$; $P = 0.04$]. This carry-over effect occurred in both strains as indicated by a lack of a significant Phase × Strain interaction [$F(1,11) = 1.1$; $P = 0.3$] or a Phase × Strain × Concentration interaction [$F(6,66) = 0.3$; $P = 0.9$].

Once again, the water control test did not identify any mouse performing at levels above chance (all $P > 0.05$), with a performance average of 47.6 ± 3% for the B6 mice and 49.9 ± 1.4% for the D2 mice, confirming that during the prior phases animals were guided by the orosensory characteristics of the stimuli and not extraneous cues.

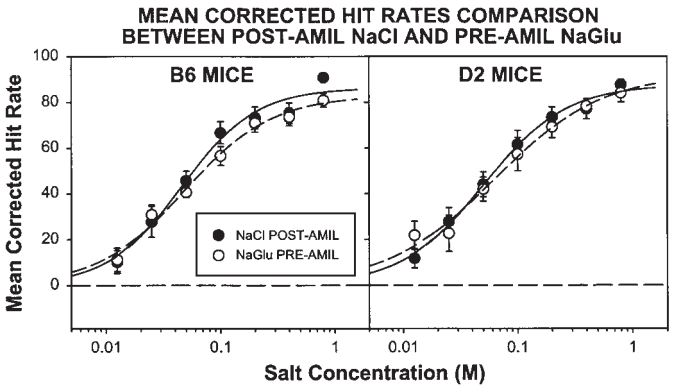

Figure 5 A comparison between mean (± SE) performance (corrected hit rates) and the curves fit to these mean data during NaCl post-amiloride testing (POST-AMIL; solid circles, solid lines) and NaGlu pre-amiloride testing (PRE-AMIL; open circles, hatched lines) for eight B6 mice (left panel) and for eight D2 mice (right panel). No significant difference was found between the two phases in either strain.

A comparison between NaCl and NaGlu detection thresholds

There was no difference in either the overall mean corrected hit rate data or the threshold in both strains when NaCl was replaced with NaGlu (Figure 5). The results for the two salts were remarkably similar even though they were separated by a few months and the stimulus was changed. A three-way ANOVA of Salt × Strain × Concentration of the corrected hit rate data for the NaCl POST-AMIL and NaGlu PRE-AMIL phases indicated only a significant main effect of concentration [$F(6,78) = 189.4$; $P < 0.01$], with no other main effects [all $F(1,13) \leq 2.2$; all $P > 0.1$] or interactions (all $P > 0.13$). In addition, no significant main effects or interactions were found in a two-way Phase × Strain ANOVA for the curve parameters between these two phases of testing [all $F(1,13) \leq 3.6$; all $P > 0.08$]. Moreover, a significant correlation was found between the NaCl threshold measurement (NaCl POST-AMIL) and the threshold measurement of NaGlu (NaGlu PRE-AMIL) of individual animals in both strains (both $r > 0.8$; both $P < 0.02$; Pearson correlation).

Oral treatment with amiloride had a larger effect on detection performance when added to NaGlu than when it was added to NaCl. This was confirmed by a three-way ANOVA (Salt × Strain × Concentration) conducted on the corrected hit rates observed during the AMIL phase which indicated a significant main effect of salt [$F(1,13) = 38.1$; $P < 0.01$] and a significant Salt × Concentration interaction [$F(5,65) = 9.0$, $P < 0.01$]. There was also a significant main effect of concentration [$F(5,65) = 28.1, P < 0.01$], but no main or interaction effects involving strain (all $P > 0.05$). The results of this analysis are in contrast to the three-way ANOVA (Salt × Strain × Concentration) conducted on the corrected hit rates observed during the PRE-AMIL phase, which only indicated a significant main effect of concentration [$F(6,78) = 213.4$, $P < 0.01$] with no other significant main or interaction effects.

Discussion

In rats, normal sensitivity to NaCl is dependent on the amiloride-sensitive transcellular sodium taste transduction pathway. This transduction mechanism was shown behaviorally to be both necessary and sufficient for detection of weak sodium concentrations (Geran and Spector, 2000a,b; Kopka and Spector, 2001). These behavioral studies and others (Bernstein and Hennessy, 1987; Hill *et al.*, 1990; McCutcheon, 1991; Contreras and Studley, 1994; Markison and Spector, 1995; Spector *et al.*, 1996; Roitman and Bernstein, 1999; Brot *et al.*, 2000) have complemented neurophysiological examinations of the effect of oral amiloride treatment on neural responses to taste stimuli in the same species (Brand *et al.*, 1985; DeSimone and Ferrell, 1985; Formaker and Hill, 1988; Eliot and Simon, 1990; Scott and Giza, 1990; Simon, 1992; Ye *et al.*, 1993; Doolin and Gilbertson, 1996; Gilbertston and Zhang, 1998; Kitada *et al.*, 1998; Sollars and Hill, 1998; Lundy and Contreras, 1999; St John and Smith, 2000).

In mice, the amiloride-sensitive sodium taste transduction pathway has been primarily studied electrophysiologically. These studies have demonstrated that CT responses to lingually applied NaCl (Ninomiya *et al.*, 1989; Gannon and Contreras, 1995) are significantly suppressed by oral treatment with amiloride in B6 mice, as is the case in rats. In striking contrast, amiloride treatment is without effect on CT responses to NaCl in D2 mice (Ninomiya *et al.*, 1989), implying that this strain lacks functional ENaCs, at least in the apical membranes of the taste receptor cells of the anterior tongue. Based on these studies, along with the demonstration of a strain difference in CT responsiveness to NaCl (Frank and Blizard, 1999), we hypothesized that the D2 mice would be less sensitive to sodium than B6 mice. However, we found no strain difference between the sodium detection thresholds of B6 and D2 mice; both strains had NaCl and NaGlu thresholds of ~0.05–0.06 M. Thus, our unexpected results demonstrate that the D2 mice are as sensitive to sodium as are the B6 mice.

Not only were the sodium detection thresholds similar between the B6 and D2 mice, but detection performance in both strains was also severely impaired by amiloride adulteration of the stimuli. In fact, when 100 μM amiloride served as the solvent, performance became so disrupted in some of the mice that detection thresholds could not be derived. For those mice in which sensitivity could be assessed under the amiloride condition, NaCl detection thresholds were raised by 0.64 $\log_{10}$ units in B6 mice and 0.86 $\log_{10}$ units in D2 mice. The amiloride-induced rightward shifts in the detectability functions for sodium gluconate were larger still, ~1.2 $\log_{10}$ units in both strains. These findings provide clear evidence that amiloride affects

sodium taste detection in not only B6 mice, as we have previously shown (Eylam and Spector, 2002), but also in D2 mice, a strain for which the neurophysiology literature has suggested otherwise. These results also strongly imply, but do not prove, that functional ENaCs are present in both strains.

Although we cannot explain the apparent disparity between the electrophysiologically and behaviorally assessed effects of amiloride on taste responses to NaCl without further experiments, we can offer a few hypotheses. First, the amiloride-sensitive taste receptor cells of D2 mice may be distributed in receptor fields innervated by gustatory nerves other than the CT. A possible candidate for this alternate neural pathway is the greater superficial petrosal (GSP) branch of the facial nerve, which innervates palatal taste buds. In rats, the GSP is responsive to palatal application of NaCl and amiloride treatment is very effective at suppressing these responses (Sollars and Hill, 1998).

Secondly, we used the DBA/2J substrain, whereas the electrophysiological work was conducted in DBA/2CrSlc mice (Ninomiya *et al*., 1989). We cannot entirely refute the possibility that a substrain difference is at the root of the disparity between our behavioral data and the electrophysiological effects regarding amiloride sensitivity. However, the high degree of genetic relationship between the two substrains makes this explanation less parsimonious then the others. Nevertheless, this possibility remains to be resolved by an explicit test comparing the two substrains.

Thirdly, we cannot dismiss the possibility that the efficacy of the amiloride treatment in D2 mice may have been induced by our training and testing conditions. One procedural component that may have contributed to the unexpected effectiveness of amiloride in D2 mice is the water-restriction schedule. The effect of hydrational state on NaCl taste sensitivity is not yet clear, but endocrine factors have been implicated in the modulation of taste receptor cell responsivity. Aldosterone, a hormone associated with hydromineral balance, when administered in rats, has been reported to increase: (i) the apical expression of the beta and gamma ENaC subunits in taste receptor cells, (ii) the number of amiloride-sensitive taste cells, (iii) the magnitude of amiloride-sensitive Na^+ currents in a subset of taste receptor cells, and (iv) the percentage of suppression of the CT response to lingually applied NaCl during amiloride treatment (Herness, 1992; Lin *et al.*, 1999). In addition, vasopressin, a hormone released in response to extracellular hyperosmolality, has been implicated at modulating the properties of amiloride-sensitive ion channels of frog and hamster taste receptor cells (Okada *et al.*, 1991; Gilbertson *et al.*, 1993). Water restriction was used in our experiment as a means to generate potent motivation for stimulus sampling and responding in the operant conditioning task. We avoided a caloric restriction schedule because the chemical composition of a food reinforcer could potentially interfere with taste receptor processes. In light of these issues, it would be instructive to compare the effects of food and water deprivation on threshold measurements in this task. Moreover, it would be useful to assess the effects of food and water restriction schedules on taste-evoked neural responses in these mouse strains.

Another possible contributor to the amiloride sensitivity in D2 mice in our paradigm may have been the repeated exposure to sodium during the course of the experiment. Bachmanov *et al.* (Bachmanov *et al.*, 1999) reported that B6 mice pre-exposed to several days of two-bottle intake tests with NaCl displayed enhanced amiloride suppression of CT responses to weak NaCl concentrations. The magnitude of this effect was not remarkable, however, and it remains to be seen whether similar effects would occur in D2 mice. It would be instructive to test the CT response to NaCl with and without lingual amiloride treatment in D2 mice that have been trained and tested for many weeks in our signal detection procedure to examine whether any of the factors listed above are capable of inducing some sensitivity to the ENaC blocker in the anterior tongue taste receptors.

Lastly, it is possible that amiloride itself is not tasteless to D2 mice. In rats, a 100 μM concentration of amiloride has been shown to be an ineffective conditioned stimulus in taste aversion conditioning experiments, strongly suggesting that amiloride is basically tasteless to these animals (Hill *et al.*, 1990; Markison and Spector, 1995). However, such experiments have yet to be conducted in mice. If amiloride has a taste to these mice, their compromised performance in the presence of this drug may have been due to perceptual masking rather than interference with sodium taste transduction. To our knowledge there is no mention in the literature of lingual amiloride application alone generating responses in gustatory nerves of mice but this remains to be comprehensively assessed in all taste nerves across relevant strains. We are currently conducting conditioned taste aversion studies using amiloride as a conditioned stimulus in the B6 and D2 mouse strains to explicitly test the possibility that the ENaC blocker has a perceptible taste to these particular rodents.

Some of the mice from both strains were able to detect, albeit poorly, high concentrations of NaGlu in the presence of this ENaC blocker. It is unclear what pathway is utilized by NaGlu in this case since oral treatment of amiloride is thought to block ENaCs and activation of the amiloride-insensitive sodium transduction pathway(s) is primarily precluded by the gluconate anion, at least it is in rats. It is possible, however, that some degree of taste sensitivity to NaGlu is maintained as a result of incomplete inactivation of one of these pathways. At high concentrations, some sodium may be able to pass through ENaCs despite amiloride blockade. In hamsters, the degree of suppression of CT responses to lingually applied NaCl caused by amiloride appears to depend on the relative concentrations of both the drug and the salt (Hettinger and Frank, 1990). Likewise, at high NaGlu concentrations, some sodium may penetrate

through tight junctions in taste buds to reach basolateral receptor sites or leak through an amiloride-insensitive non-selective cation channel recently proposed to be positioned in the apical membrane of some taste receptor cells (DeSimone *et al.*, 2002). Alternatively, NaGlu might stimulate trigeminal or olfactory receptors once its concentration reaches a certain level. Lastly, despite our adulteration of all solutions with amiloride to maintain constant bathing of the tongue with this ENaC blocker, a temporal delay before channel blockade may play a role in the residual sensitivity to high NaGlu concentrations. Regardless of the mechanism, it is noteworthy that rats are also able to detect higher concentrations of NaGlu (Geran and Spector, 2000b). In rats, however, amiloride completely eliminates the enhanced licking responses to 0.3 M concentrations of NaGlu, NaCl and sodium acetate normally observed when animals are acutely depleted of sodium by natriuretic treatment (Geran and Spector, 2001). This finding weakens the hypothesis that at this concentration the sodium is able to pass through amiloride blocked ENaCs. Thus, the basis for the residual sensitivity to high concentrations of NaGlu under conditions of amiloride blockade observed in rats and mice remains to be completely understood.

Interestingly, taste detection thresholds derived for NaGlu in both mouse strains were similar to and highly correlated with those derived for NaCl. Apparently, normal detection of sodium salts is independent of anion size in B6 and D2 mice. As mentioned previously, sodium gluconate is thought to be transduced primarily through the sodium selective, amiloride-sensitive transcellular pathway because lingual treatment with this ENaC blocker virtually eliminates the CT nerve response to sodium salts with organic anions in rats (Formaker and Hill, 1988; Elliott and Simon, 1990; Ye *et al.*, 1991, 1993, 1994; Simon, 1992) and the glossopharyngeal nerve responds very poorly to sodium gluconate even at concentrations as high as 2.0 M (Kitada *et al*., 1998). To the extent that such electrophysiological findings can be generalized to the strains of mice used in our behavioral study, it would appear that the amiloride-sensitive transcellular sodium taste transduction pathway is both necessary and sufficient for the normal detection of low concentrations of sodium salts regardless of the anion in B6 and D2 mice, as has been previously demonstrated in rats (Geran and Spector, 2000b).

These results, coupled with our prior findings comparing NaCl concentration-dependent performance in a conditioned signal detection task and a two-bottle intake test in B6 mice, highlight the complexity of taste-related behavior and the need to view the analysis of function from different angles. In our prior work, B6 mice were relatively indifferent to low concentrations of NaCl compared with water as measured in a 24 h two-bottle intake test (Eylam and Spector, 2002). These animals did not begin to avoid NaCl solutions until the concentration reached hypertonic values. Moreover, amiloride had only modest effects, at best, on NaCl avoidance, a result consistent with the behavior of F-344 rats (Chappell *et al.*, 1998). Apparently, the amiloride-sensitive transduction pathway is not necessary for the expression of NaCl avoidance behavior to be maintained. Consequently, the two-bottle preference test would not have been an optimal behavioral assay to examine potential strain differences in NaCl sensitivity at low concentrations or its potential disruption by amiloride. In contrast, the conditioned signal detection task used here clearly indicated that amiloride treatment had robust effects on NaCl sensitivity in both B6 and D2 mice, in spite of the fact that the CT response to lingually applied NaCl is unaffected by ENaC blockade in receptor cells in the latter strain. Although, as mentioned above, the disparity between the electrophysiological and psychophysical results regarding strain differences in the effect of amiloride treatment on peripheral taste processes remains to be explained, such findings underscore the need to apply and link various approaches toward understanding taste function.

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