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Amiloride is an Ineffective Conditioned Stimulus in Taste Aversion Learning in C57BL/6J and DBA/2J Mice

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Abstract

The epithelial sodium channel (ENaC) blocker amiloride has been shown to increase the behaviorally measured NaCl detection threshold in mice. In this study, a conditioned taste aversion (CTA) paradigm was used to examine whether 100 μM amiloride has a perceptible taste that could contribute to this observed decrease in behavioral responsiveness. Eighty-four C57BL/6J (B6) and 64 DBA/2J (D2) mice were divided into eight groups (n = 8–12 per group), in which half received an injection of 0.15 M LiCl (2 mEq/kg) and the other half an equivalent saline injection, in three conditioning trials. The four conditioned stimuli were 100 μM amiloride hydrochloride, water, 0.1 and 0.3 M NaCl. Neither strain demonstrated acquisition of a CTA to amiloride in a brief-access (BA) taste test (5 s trials in the gustometer). Although 0.3 M NaCl is inherently aversive, its pairing with LiCl led to significantly further decreases in licking during the BA test on salt trials in both strains. The D2 strain clearly avoided 0.1 M NaCl, whereas avoidance of this stimulus was more equivocal in B6 mice. The inefficacy of amiloride to serve as a conditioned stimulus in taste aversion learning involving three LiCl pairings suggests that the effects of this ENaC blocker on taste-related behavioral responses to NaCl are likely due to its pharmacological interference with sodium taste transduction.

Key words: conditioned taste aversion, epithelial sodium channels, mouse strains, sodium transduction, taste psychophysics

Introduction

In rodents, sodium taste transduction appears to occur through at least two transduction pathways; one pathway is sensitive to the oral treatment of the epithelial sodium channel (ENaC) blocker amiloride and the other(s) is unaffected by this drug. The amiloride-sensitive (AS) pathway is thought to involve the selective entry of Na^+ (and Li^+) through ENaCs in the apical membrane of a subset of taste receptor cells (e.g. 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), while the amiloride-insensitive (AI) pathway(s) is less selective for cations but is dependent on anion size (e.g. Formaker and Hill, 1988; Elliott and Simon, 1990; Hettinger and Frank, 1990; Ye *et al.*, 1991, 1993, 1994; Simon, 1992; Doolin and Gilbertson, 1996; DeSimone *et al.*, 2001). In rats, when amiloride is applied to the lingual epithelium, the suppression of chorda tympani (CT) nerve responsiveness to sodium salts is marked (e.g. Heck *et al.*, 1984; Brand *et al.*, 1985; DeSimone and Ferrell, 1985; Ninomiya and Funakoshi, 1988; Formaker and Hill, 1988; Elliott and Simon, 1990; Ye *et al.*, 1991, 1993).

Interestingly, the CT responses to NaCl of some inbred mouse strains, such as DBA/2 (D2), 129/J and BALB/c, are not suppressed by lingual application of amiloride (Ninomiya *et al.*, 1989; Gannon and Contreras, 1995). Although amiloride blocks sodium currents in some anterior tongue taste receptor cells (Miyamoto *et al.*, 1999), the CT response to NaCl is unaffected by the drug (Ninomiya *et al.*, 1989). In contrast, the CT responses to this salt are markedly suppressed by amiloride treatment in the C57BL/6 (B6) strain, as they are in the rat (Ninomiya *et al.*, 1989; Gannon and Contreras, 1995). These electrophysiological findings suggest that, unlike the B6 mouse strain, the D2, 129 and BALB/c strains have a significantly lower number of functional ENaCs in the taste receptor cells of the anterior tongue.

Based on these findings, we previously hypothesized that mice unaffected by the ENaC blocker (e.g. D2 mice), as assessed by CT electrophysiology, would demonstrate a reduced sensitivity to NaCl and that amiloride would have little or no effect on the behavioral detection threshold. In contrast to these predictions, we found that not only did B6 and D2 mice have similar behavioral sensitivity to NaCl, but

detection thresholds in both strains were also significantly raised by stimulus adulteration with 100 μM amiloride (Eylam and Spector, 2002, 2003). Although the amiloride-induced shift in sensitivity to NaCl in B6 mice is consistent with the documented effect of the ENaC blocker on CT responses to this salt, the behavioral results are somewhat difficult to reconcile with the electrophysiology in the D2 strain. It is possible that AS taste receptor cells are located in other oral fields in the D2 mice, and thus, the ENaC blocker might still exert its effect behaviorally but the effect of amiloride may not be apparent from CT recordings.

Alternatively, amiloride may not be tasteless to these mice and the apparent confusion of the animals in the signal detection task in the presence of amiloride may have been due to perceptual masking rather than interference with sodium taste transduction. In other words, these mice might be able to perceive an additional taste cue from the amiloride, such as the bitter sensation described in humans (Smith and Ossebaard, 1995; Halpern, 1998), and this may disrupt performance in taste-related behavioral tests. Although a 100 μM concentration of amiloride appears to be tasteless to rats based on its ineffectiveness to serve as a conditioned stimulus (CS) in taste aversion learning (Hill *et al.*, 1990; Markison and Spector, 1995), this has not yet been tested in mice. We are unaware of any electrophysiological evidence that gustatory nerves in mice are responsive to lingual application of amiloride alone, but all gustatory nerves have not been comprehensively examined.

Accordingly, we used a conditioned taste aversion procedure similar to the one used by Markison and Spector (1995) to examine the possibility that amiloride has a detectable taste to B6 and D2 mice. Because it has been shown that mice from both strains can acquire an aversion to NaCl (Risinger and Cunningham, 2000; Risinger and Boyce, 2002), we used this taste stimulus as a positive control condition to confirm the competence of the strains in the experimental paradigm.

Methods

Subjects

Eighty-four male naïve adult C57BL/6J (B6) and 64 male naïve adult DBA/2J (D2) mice from the Jackson Laboratory (Bar Harbor, ME) were used as subjects. Upon arrival, the mice were 7–8 weeks old; the mean body mass (± SEM) for the B6 mice was 26.6 ± 0.19 g and for the D2 mice was 23.15 ± 0.28 g. Mice were individually housed in shoebox cages in a colony room kept at a controlled temperature with an automatic lighting cycle (12 h light:12 h dark). All mice were handled and tested during the light phase. The mice received free access to pellets of laboratory chow (LabDiet 5001, PMI Nutrition International Inc., Brentwood, MO) and distilled water. At least 10 days after arrival, they were put on a restricted water access schedule in which fluid was only available during the training or testing sessions (see below). Body weight was monitored daily. All mice received 1 ml of supplemental distilled water after their corresponding session every other day during gustometer training. All procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Solutions

The conditioned stimuli were 100 μM amiloride hydrochloride (Sigma Chemical Co., St Louis, MO), 0.1 M NaCl, 0.3 M NaCl (Fisher Scientific, Atlanta, GA) and distilled water. The amiloride was prepared at least one h before use and was kept in a flask wrapped with aluminum foil to prevent photodegradation. We chose a high concentration of amiloride that has been used very effectively in electrophysiological studies of rodent salt taste transduction. Indeed, the majority of electrophysiological and behavioral research with mice has involved concentrations at this value or lower (e.g. Ninomiya *et al.*, 1989; Miyamoto *et al.*, 1999; Eylam and Spector, 2002, 2003; Yasumatsu *et al.*, 2003). The unconditioned stimuli were 0.15 M LiCl and 0.15 M NaCl. All solutions were prepared fresh daily with distilled water.

Apparatus

The one-bottle intake tests were conducted in the home cages. Fluids were presented in 25 ml graduated pipettes, modified in a similar fashion as described previously (Bachmanov *et al.*, 1996; Eylam and Spector, 2002). These modified pipettes were introduced to each cage by inserting the sipper-tube end between the metal rods of the cage lid and securing them with a cable clip to the shelf above, designed to reduce spillage.

In the brief-access paradigm the mice were trained and tested in a specialized computer-controlled gustometer, modified from the original rat apparatus (Spector *et al.*, 1990) as described previously (Eylam and Spector, 2002, 2003). Briefly, the animals were placed in a testing cage surrounded by an outer sound-attenuating chamber to minimize external cues. Fluids were held in up to six reservoirs, and were delivered through a single drinking-spout situated just behind a small opening in the side wall of the testing chamber. Licks were monitored with a contact circuit (<50 nA). When the mouse licked the drinking spout twice within a 250 ms period, a designated solenoid valve opened, which filled the shaft of the drinking spout and then further deposited ~1.6 μl of fluid upon each lick. After each trial, the drinking spout rotated out of the reach of the mouse, was rinsed with distilled water, and dried with pressurized air (~6 s intertrial interval).

Procedure

Gustometer training

The mice were trained under a restricted water access schedule. The water bottles were removed from the home cages the day before the start of the gustometer training

Table 1 Timeline of the training and testing schedule

	Gustometer training phase[a,b]					Rehyd.		Conditioning phase[a,c,d]											Rehydration			Gustometer testing[a,b]	
	Stationary spout		5 s trials					Water				CS ↓ US	Water		CS ↓ US	Water		CS ↓ US				Water alone	Water + CS
Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23

[a]Water bottles removed the day before the start of this phase.
[b]30 min session.
[c]Tested in the home cage.
[d]15 min sessions. There was 1 h of water rehydration 5 h after the morning session every day.

phase and were replaced for 2 days before the conditioning phase commenced. The mice were first trained for 2 days in the gustometer with a stationary drinking spout and distilled water (Table 1). The drinking spout was positioned in front of the access slot. The mice were allowed to take as many licks as possible within a 30 min session. Next, the mice were trained for 3 days in the gustometer with the brief-access paradigm, whereby access to distilled water through the drinking spout was available in 5 s trials from three reservoirs (Table 1). The 5 s trial period was initiated when the mouse made two licks in 250 ms, after which the fluid stimulus was delivered. The animals were permitted to lick as many times as possible within each trial before the spout rotated out of the animal's reach and was cleaned. The mice were allowed to complete as many trials as possible within the 30 min session. Two gustometers were used and thus two animals were tested every ~30 min throughout each day of gustometer training and testing.

Conditioning

Water bottles were removed the day before the start of the conditioning phase. During this phase, each mouse received water from an intake tube in its home cage for 15 min in the morning at the same time each day. All manipulations began at 09.00 h, but the start of the trial for each animal was staggered by 5 min to allow for time to take measurements and perform injections. Exactly 5 h after the start of the morning session, the mice were given access to distilled water for 1 h to allow rehydration. After 4 days of one-bottle water testing, each strain was equally divided into eight groups according to the CS (amiloride, water, 0.1 and 0.3 M NaCl) and US (LiCl and NaCl) they would receive. Mice were assigned to groups according to their body weight, mean intake in the first 4 days on intake testing (water intake), mean licks/trial and mean number of trials during the 3 days of BA training. There were no significant differences between injection groups in both strains regarding these assignment parameters.

Once the animals were assigned to their treatment groups, three conditioning trials followed in which the mice were presented with the appropriate CS for 15 min in the morning, immediately followed by an i.p. injection (2.0 mEq/kg body wt) of either 0.15 M LiCl or 0.15 M NaCl (Table 1). The purpose of the LiCl injection was to induce visceral malaise. Fluid intake was measured to the nearest 0.1 ml throughout conditioning. Each conditioning trial was separated by 2 days of the restricted water-access schedule. After the third conditioning trial, water bottles were replaced on the home cages for 2 days.

Brief-access testing

Water bottles were removed the day before the brief-access phase of testing began. On the first day of testing, the mice were presented with 5 s water trials from three reservoirs in the gustometer as described above for brief-access training. This water testing was intended to reacquaint the mice to the task in the gustometer and to increase their motivation for licking on the following testing day. On the second day of testing, the mice were presented with 5 s trials of their specific CS as well as water in the gustometer. The fluids were presented from three of the six reservoirs used in the experiment. The presentation of the solutions (i.e. CS and water stimulus) was randomized (without replacement) in repeated blocks with a water rinse trial in between each stimulus trial.

Data analysis

For each conditioning trial, the intake of each mouse in the experimental groups was divided by the mean intake of their matched control group to derive a CS-intake ratio. A ratio of 1.0 signifies equal intake between the experimental and control animals, while a ratio less than or greater than 1.0 signifies respectively decreased or increased intake by the experimental animals in comparison with their matched controls. A logarithmic conversion was applied to these data so that increases from a ratio of 1.0 would be statistically symmetrical with decreases.

In the brief-access paradigm, a CS-lick ratio was calculated for each mouse representing the mean number of CS licks per trial divided by the mean number of water stimulus licks per trial (water rinse trials were not included in the analysis). In the case of the mice that received water as a CS, trials from one reservoir were designated as the CS and the trials from another reservoir were designated as the water

comparison. The mean CS-lick ratios for the experimental and the control group for each CS were statistically compared. In addition, these CS-lick ratio scores were tested for significant departures from 1.0. A CS-lick ratio of 1.0 signifies that licking during CS trials was equal to licking during water trials. These comparisons were conducted with both the raw and the $\log_{10}$ transformed values.

Finally, we calculated a conditioned change ratio by dividing the CS-lick ratios of each mouse in the LiCl-injected experimental group by the mean CS-lick ratio for the NaCl-injected control group for each CS. This score scales the CS-lick suppression (standardized to water licks) for each LiCl-injected mouse relative to the mean performance of the relevant control group. Thus, a conditioned change ratio score of 1.0 signifies that a LiCl-injected experimental mouse showed the same degree of lick suppression (or enhancement) relative to water that the relevant control group displayed on average. The $\log_{10}$-transformed values were used in the statistical tests.

These data were analyzed with analyses of variance (ANOVAs) and *t*-tests. The strains were analyzed separately and the statistical rejection criterion was set at the conventional value of 0.05.

Results

B6 mice

Amiloride

The B6 mice injected with LiCl did not display conditioned decreases in the intake of amiloride during the one-bottle conditioning trials (Table 2, Figure 1). A one-way ANOVA of CS intake in the LiCl-injected mice revealed a significant main effect of trial [$F(2,22) = 7.95$, $P = 0.003$], but this was due to an increase in intake rather than a conditioned decrease over the three trials (Table 2). A one-way ANOVA conducted for the $\log_{10}$ of the CS-intake ratio based on the mice receiving amiloride indicated that there was no significant effect of conditioning trials. Moreover, the $\log_{10}$ of the CS-intake ratio did not differ from 0 on any of the

Table 2 CS intake across conditioning trials

Group	B6 mice			D2 mice		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Amil/LiCl	1.04 ± 0.10	1.21 ± 0.10	1.29 ± 0.09	1.69 ± 0.09	1.54 ± 0.11	1.16 ± 0.12
Amil/Sal	0.99 ± 0.11	1.27 ± 0.06	1.37 ± 0.06	1.45 ± 0.09	1.55 ± 0.09	1.46 ± 0.13
H_2O/LiCl	1.12 ± 0.07	1.25 ± 0.06	1.27 ± 0.05	1.56 ± 0.10	1.36 ± 0.11	1.45 ± 0.17
H_2O/Sal	1.05 ± 0.05	1.02 ± 0.06	1.15 ± 0.10	1.42 ± 0.10	1.60 ± 0.13	1.39 ± 0.13
0.1 M NaCl/LiCl	1.97 ± 0.09	1.80 ± 0.15	1.76 ± 0.14	1.99 ± 0.18	0.67 ± 0.19	0.54 ± 0.13
0.1 M NaCl/Sal	1.74 ± 0.10	1.89 ± 0.08	1.81 ± 0.09	1.97 ± 0.13	2.01 ± 0.17	1.81 ± 0.17
0.3 M NaCl/LiCl	1.96 ± 0.10	0.54 ± 0.16	0.55 ± 0.19	1.79 ± 0.24	0.29 ± 0.08	0.14 ± 0.03
0.3 M NaCl/Sal	1.75 ± 0.10	1.14 ± 0.16	1.32 ± 0.15	1.19 ± 0.18	0.52 ± 0.10	1.12 ± 0.17

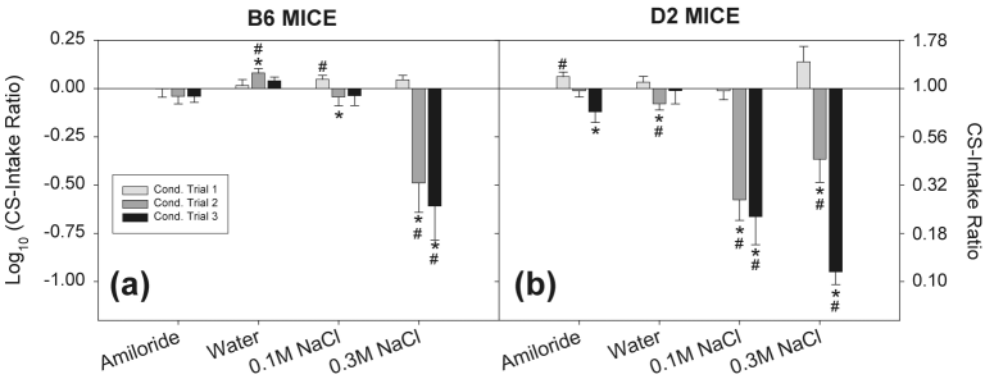

Figure 1 Mean (± SEM) $\log_{10}$ values (left axis) of the CS-intake ratio (right axis) for each conditioned stimulus (CS) for B6 mice **(a)** and D2 mice **(b)** on the three conditioning trials. The light gray bars represent the baseline intake on the first conditioning trial and the dark gray and the black bars represent the intake during the second and the third conditioning trials, respectively. An asterisk (*) denotes a significant difference from baseline intake while a pound sign (#) denotes a significant difference from a value of zero (equal to a ratio value of 1.0). The $\log_{10}$ of the ratio was calculated to make increases statistically symmetrical with decreases from a ratio of 1.0.

conditioning trials, indicating that the CS intake in the LiCl- and NaCl-injected mice was comparable.

In the brief-access test, there was no evidence of a conditioned taste aversion when amiloride was the CS. The CS-lick ratio for either the LiCl- and NaCl-injected mice was not significantly different from a value of 1.0 regardless of whether a $\log_{10}$ transform was used in the analysis. The water licks upon which the CS-lick ratios are based did not significantly differ between the LiCl- and NaCl-injected mice (Table 3). Similarly, the $\log_{10}$ of the conditioned change ratio for the amiloride CS (Figure 3) was not significantly different from 0, indicating that the LiCl- and NaCl-injected CS-lick ratios were statistically comparable.

Water

As was the case for amiloride, there was no evidence of conditioned decreases in intake across trials on any measure when water was the CS (Figure 1, Table 2). Likewise, no conditioned suppression of CS licking was observed during the brief-access test in LiCl-injected mice receiving water as the CS (Figures 2 and 3).

Table 3 Brief-access water stimulus licks

Group	B6 mice	D2 mice
Amil/LiCl	31.10 ± 2.27	26.40 ± 3.45
Amil/Sal	30.19 ± 1.71	27.53 ± 2.71
H_2O/LiCl	29.86 ± 1.99	32.92 ± 1.83
H_2O/Sal	26.75 ± 1.83	28.73 ± 1.97
0.1 M NaCl/LiCl	29.49 ± 2.06	28.56 ± 1.34
0.1 M NaCl/Sal	23.55 ± 2.21	31.71 ± 3.67
0.3 M NaCl/LiCl	35.00 ± 1.95	34.26 ± 4.27
0.3 M NaCl/Sal	32.57 ± 1.92	24.98 ± 3.80

NaCl

The LiCl-injected group receiving 0.1 M NaCl as the CS did not demonstrate convincing evidence of conditioned decreases in one-bottle intake across sessions (Table 2). The slight decrease in CS intake across trials was not significant. Although a one-way ANOVA conducted for the $\log_{10}$ CS-intake ratio from the mice receiving 0.1 M NaCl indicated a significant effect of conditioning trial [$F(2,22) = 3.99$, $P = 0.03$], paired t-tests revealed a significant difference only between the first and second trial [$t(11) = 2.57$, $P = 0.03$] but not between the first and third trial (Figure 1).

In the brief-access test, the CS-lick ratio for 0.1 M NaCl was >1.0 for the NaCl-injected B6 mice, whereas the CS-lick ratio for this taste stimulus was <1.0 for the LiCl-injected mice (Figure 2). Although neither of those differences were significant, the CS-lick ratio significantly differed between the LiCl- and NaCl-injected mice [$t(20) = 2.71$, $P = 0.014$]. Interestingly, the NaCl-injected mice licked water at significantly lower rates than the LiCl-injected mice [$t(20) = 2.11$, $P = 0.048$], perhaps because these control mice were licking the 0.1 M NaCl CS more than the experimental mice. These statistical outcomes occurred regardless of whether a $\log_{10}$ transform of the data was employed. The $\log_{10}$ of the conditioned change ratio (Figure 3) revealed a significant decrease from the value of 1.0 for the 0.1 M NaCl [$t(11) = 4.76$, $P = 0.001$], indicating that the CS-lick ratio of the experimental group was low relative to that in the control mice.

Although the conditioned aversion to 0.1 M NaCl was equivocal in B6 mice, there was clear evidence of conditioning when 0.3 M NaCl was the CS. Even though there was a significant decrease in CS intake in both the LiCl- and NaCl-injected mice over trials [both $F(2,14) > 6.02$, both $P < 0.015$, Table 2], the $\log_{10}$ CS-intake ratios were significantly below 0 on conditioning trials 2 and 3 [both $t(7) > 3.20$, $P \leq 0.015$], indicating that the CS intake in the

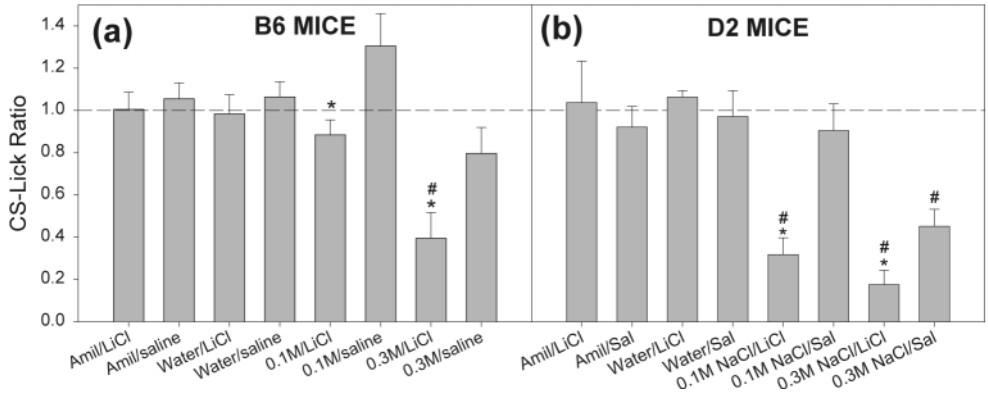

Figure 2 Mean CS-lick ratio (± SEM) during brief-access testing in the gustometer (mean CS licks/trial divided by mean water stimulus licks/trial) for each group for the B6 mice **(a)** and the D2 mice **(b)**. The reference line drawn at the ratio value of 1.0 designates CS licks/trial equal to the water licks/trial. A mean CS-lick ratio that significantly differed from a value of 1.0 is denoted with the pound sign (#). The CS-lick ratios of the LiCl-injected groups that were significantly different from the ratios of their matched control group were indicated with an asterisk (*).

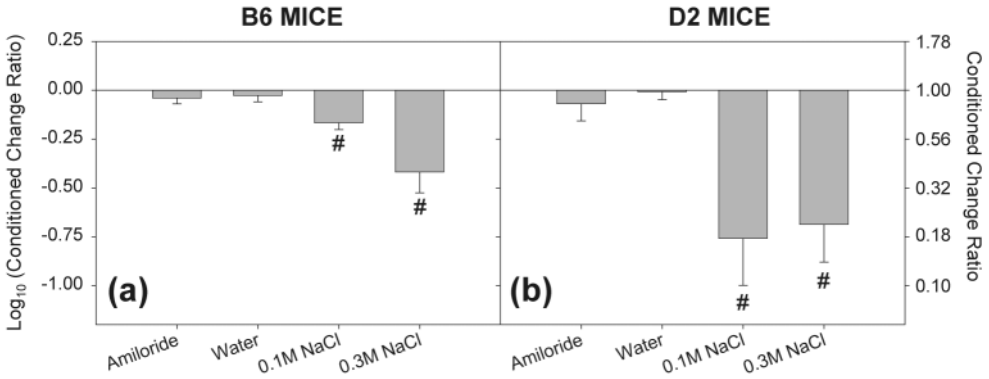

Figure 3 Mean (± SEM) $\log_{10}$ values (left axis) of the conditioned change ratio (right axis) for each conditioned stimulus for B6 mice **(a)** and D2 mice **(b)**. A pound sign (#) denotes a significant difference from a value of zero (equal to a ratio of 1.0). The $\log_{10}$ of the ratio was calculated to make increases statistically symmetrical with decreases from a ratio of 1.0.

experimental group was low relative to that in the control mice (Figure 1). Also, an ANOVA of those values indicated a significant effect of trial [$F(2,14) = 11.19$, $P = 0.001$] and subsequent *t*-tests indicated that both the second and third trials were significantly lower than the first [both $t(7) > 3.42$, $P \leq 0.011$].

The CS-lick ratio (Figure 2) for 0.3 M NaCl in the brief-access test for the LiCl-injected mice was significantly lower than 1.0 [$t(7) = 5.84$, $P = 0.001$] and also significantly different from the ratio for the NaCl-injected mice [$t(14) = 3.72$, $P = 0.002$]. The water licks upon which the CS-lick ratios are based did not significantly differ between the LiCl- and NaCl-injected mice. These statistical outcomes were unaffected by a $\log_{10}$ transform of the data. As might be expected, the $\log_{10}$ transform of the conditioned change ratio (Figure 3) for the 0.3 M CS was significantly different than 0 [$t(7) = 3.95$, $P = 0.006$].

D2 mice

Amiloride

Unlike the B6 strain, the D2 mice injected with LiCl did display modest decreases in the intake of amiloride across trials [$F(2,14) = 6.96$; $P = 0.008$], but this change relative to the NaCl-injected controls was not compelling (Table 2). A one-way ANOVA (conditioning trial) conducted for the $\log_{10}$ CS-intake ratio based on the mice receiving amiloride indicated a significant effect of conditioning trial [$F(2,14) = 5.86$, $P = 0.01$]. Paired *t*-tests revealed a significant difference between the $\log_{10}$ CS-intake ratio on the third trial in comparison to the first [$t(7) = 2.62$, $P = 0.03$] but not the second trial (Figure 1). Importantly, however, the $\log_{10}$ CS-intake ratio did not significantly differ from a value of 0 on both the second and third conditioning trials, indicating that LiCl-injected mice receiving amiloride as the CS were not different from their matched controls on those trials.

In the brief-access test, there was absolutely no evidence of a conditioned taste aversion when amiloride was the CS. The CS-lick ratio for neither the LiCl- nor NaCl-injected mice was significantly different from a value of 1.0 regardless of whether a $\log_{10}$ transform was used in the analysis (Figure 2). The water licks upon which the CS-lick ratios are based did not significantly differ between the LiCl- and NaCl-injected mice either (Table 3). The same was true for the $\log_{10}$ of the conditioned change ratio for the amiloride CS (Figure 3), which was not significantly different from 0, indicating that the LiCl- and NaCl-injected CS-lick ratios were statistically comparable.

Water

There was no evidence of conditioned decreases in intake across trials on any measure when water was the CS (Figure 1, Table 2). Likewise, no conditioned suppression of CS licking was observed during the brief-access test in LiCl-injected mice receiving water as the CS (Figures 2 and 3).

NaCl

Unlike the B6 mice, the D2 mice displayed clear evidence of conditioned aversion when 0.1 M NaCl was the CS (Figure 1). The intake of 0.1 M NaCl in LiCl-injected mice (Table 2) significantly decreased over trials [$F(2,14) = 43.33$, $P < 0.001$]. A one-way ANOVA conducted for the $\log_{10}$ CS-intake ratio based on the mice receiving 0.1 M NaCl revealed a significant effect of conditioning trial [$F(2,14) = 20.07$, $P < 0.001$]. The CS-intake ratio was significantly lower than 0 on both conditioning trials 2 and 3 [both $t(7) > 4.57$, both $P \leq 0.003$], indicating that the CS intake of the experimental group was low relative to that in the control mice after the baseline trial (i.e. Trial 1).

In the brief-access test, the CS-lick ratio for 0.1 M NaCl was significantly lower than 1.0 for the LiCl-injected D2 mice [$t(7) = 8.68$, $P < 0.001$], whereas the CS-lick ratio for this taste stimulus was not statistically different from 1.0 for the NaCl-injected mice (Figure 2). Accordingly, the CS-lick ratio differed between the LiCl- and NaCl injected mice [$t(14) = 4.61$, $P < 0.001$]. The water licks upon which the CS-

lick ratios are based did not significantly differ between the LiCl- and NaCl-injected mice (Table 3). These statistical outcomes occurred regardless of whether a $\log_{10}$ transform of the data was employed. As expected, the $\log_{10}$ transform of the conditioned change ratio (Figure 3) revealed a significant decrease from a ratio of 1.0 for the groups receiving 0.1 M NaCl [$t(7) = 3.17$, $P = 0.02$], indicating that the CS-lick ratio of the experimental group was low relative to that in the control mice.

There was clear evidence of conditioning when 0.3 M NaCl was the CS. The LiCl-injected mice, displayed a continual decrease in intake across conditioning trials [$F(2,14 = 31.43$, $P < 0.001$); Table 2]. A one-way ANOVA of the $\log_{10}$ CS-intake ratio revealed a significant effect of trial [$F(2,14) = 28.63$, $P < 0.001$] and the $\log_{10}$ CS-intake ratios on conditioning trials 2 and 3 were significantly below 0 [both $t(7) > 3.05$, both $P < 0.02$], indicating that the CS intake in experimental group was low relative to that in the control mice after the baseline trial (i.e. Trial 1).

In the brief-access test, the CS-lick ratios for mice receiving 0.3 M NaCl as the CS were significantly lower than 1 regardless of the US [both $t(7) > 5.1$, both $P < 0.002$]. Importantly, however, the CS-lick ratio of the LiCl-injected mice was significantly different from the ratio for the NaCl-injected mice [$t(14) = 2.55$, $P = 0.02$]. The water licks upon which the CS-lick ratios are based did not significantly differ between the LiCl- and NaCl-injected mice (Table 3). These statistical outcomes were unaffected by a $\log_{10}$ transform of the data. As would be expected on the above profile of effects, the $\log_{10}$ of the conditioned change ratio (Figure 3) was significantly different from 0 for mice receiving 0.3 M NaCl as the CS [$t(7) = 3.57$, $P = 0.009$].

Discussion

In the conditioned taste aversion paradigm used here, neither B6 nor D2 mice appeared to develop a clear avoidance response to 100 μM amiloride suggesting that, at this concentration, this compound is tasteless to them. In the LiCl-injected B6 mice, if anything, the amount of amiloride consumed actually increased over the three conditioning trials and their response in the brief-access paradigm was similar to that of their matched controls injected with saline and that of the mice receiving water. Although the LiCl-injected D2 mice did demonstrate a modest drop in amiloride intake across conditioning trials, this was not as robust as the avoidance observed by the mice receiving NaCl as the CS in this strain, nor was their intake of the CS on the second and third conditioning trials appreciably lower than the average control value.

The failure for any amiloride avoidance to be expressed in the brief-access test draws into question whether the change observed in the one-bottle test in LiCl-injected D2 mice is attributable to an inherent taste of the compound. Perhaps a weak olfactory cue was generated by amiloride. The apparatus used in the brief-access taste test was designed to minimize olfactory cues and maybe that is why no conditioning was evident in that context. Alternatively, the blockade of ENaCs in the oral cavity may have altered oral sensations arising from the adaptation state associated with saliva. In other words, although amiloride itself could be tasteless, it could alter the taste of saliva over the course of a one-bottle test allowing amiloride and water sessions to be differentiated. In contrast, in the brief-access test it would be difficult for the animal to differentiate whether water or amiloride was the stimulus during any given trial based on saliva taste due to the short duration of each trial. Whatever the reason for the slight decline in amiloride intake across the one-bottle intake tests in the LiCl-injected D2 mice, there was clearly no evidence that amiloride was treated differently from water in either group in the brief-access taste test.

Overall, our results with B6 and D2 mice are in concordance with the failure of amiloride to serve as an effective CS in taste aversion learning in rats (Markison and Spector, 1995). The inefficacy of amiloride cannot be attributed to an ability of these mice to perform competently in our taste aversion procedure. Mice from both strains were able to form clear aversions to 0.3 M NaCl when it was paired with LiCl. The lithium-injected D2 mice also clearly acquired an aversion to 0.1 M NaCl, reducing both their one-bottle intake as well as their licking of the CS in the brief-access test. The B6 mice, however, showed only weak conditioning, at best, to this stimulus. Although the LiCl-injected B6 mice were statistically distinguishable from their NaCl-injected counterparts in their responsiveness to the 0.1 M NaCl in the brief-access taste test, this was largely because the control animals displayed slightly increased licking of the CS compared with water and the experimental group displayed slightly decreased licking. The difference in the effectiveness of 0.1 M NaCl to serve as a CS in B6 and D2 mice was unexpected because NaCl detection thresholds are similar between the two strains (Eylam and Spector, 2002, 2003). One possible explanation is that the US was stronger for the D2 mice compared with the B6 mice as has been suggested by others (Ingram, 1982; Risinger and Cunningham, 2000).

Because these are negative results we cannot conclusively dismiss the possibility that 100 μM amiloride does have some type of perceptible taste to the mice tested here. For example, if more conditioning trials had been included or if a stronger US dose had been used, perhaps an aversion would have become apparent. Nevertheless, that no amiloride aversion was evident in the brief-access test after three conditioning trials in the context of aversions being conditioned to the 0.3 M concentration of NaCl in both strains and to the 0.1 M concentration in the D2 mice suggests that if amiloride has a detectable taste, it is weak at best. It also is conceivable that a higher concentration of amiloride would have been a more effective CS. We chose this concentration, however, because it was used in all our prior behavioral studies and has been demonstrated to induce a significant reduction in the sodium sensitivity of

both mouse strains as well as rats (e.g. Markison and Spector, 1995; Spector *et al.*, 1996; Geran and Spector, 2000a,b; Kopka and Spector, 2001; Geran *et al.*, 2002; Eylam and Spector, 2002, 2003). Thus, these results suggest that the amiloride-induced decrease in behaviorally assessed taste sensitivity to NaCl in the B6 and D2 strains is attributable to the action of the drug on ENaCs in receptor cells rather than to the addition of a masking taste sensation (Eylam and Spector, 2002, 2003). Moreover, our findings pave the way for further use of 100 μM concentrations (and lower) of amiloride in behavioral tests exploiting its pharmacological effects on ENaCs without concern that the drug has a detectable taste itself.

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