A psychophysical and electrophysiological analysis of salt taste in Trpv1 null mice

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A psychophysical and electrophysiological analysis of salt taste in Trpv1 null mice. Am J Physiol Regul Integr Comp Physiol 292: R1799–R1809, 2007. First published January 18, 2007; doi:10.1152/ajpregu.00587.2006.—Current evidence suggests salt taste transduction involves at least two mechanisms, one that is amiloride sensitive and appears to use apically located epithelial sodium channels relatively selective for Na⁺ and a second that is amiloride insensitive and uses a variant of the transient receptor potential vanilloid receptor 1 (TRPV1) that serves as a nonspecific cation channel. To provide a functional context for these findings, we trained Trpv1 knockout (KO) and wild-type (WT) C57BL/6J mice (n = 9 or 10/group) in a two-response operant discrimination procedure and measured detection thresholds to NaCl and KCl with and without amiloride. The KO and WT mice had similar detection thresholds for NaCl and KCl. Amiloride shifted the NaCl sensitivity curve to the same degree in both groups and had virtually no effect on KCl thresholds. In addition, a more detailed analysis of chorda tympani nerve (CT) responses to NaCl, with and without benzamil (Bz, an amiloride analog) treatment revealed that the tonic portion of the CT response of KO mice to NaCl + Bz was absent, but both KO and WT mice displayed some degree of a phasic response to NaCl with and without Bz. Because these transients constitute the entire CT response to NaCl + Bz in Trpv1 KO mice, it is possible that these signals are sufficient to maintain normal NaCl detectability in the behavioral task used here. Additionally, there may be other amiloride-insensitive salt transduction mechanisms in taste receptor fields other than the anterior tongue that maintain normal salt detection performance in the KO mice.

vanilloid receptors; taste ion channels; chorda tympani nerve; C57BL/6 mice; gustatory system

CURRENT EVIDENCE SUGGESTS salt taste transduction involves at least two distinct mechanisms in rodent models (1, 2, 5, 6, 9, 11, 16–18, 21, 29, 38). One appears to use apically located epithelial sodium channels (ENaCs) relatively selective for sodium and lithium ions and is inhibited by the ENaC blocker amiloride. The second is thought to be amiloride insensitive and involves nonselective cation channels that serve as receptors for a variety of salts, including Na⁺, K⁺, NH₄⁺ and Ca²⁺. Studies have suggested large organic anions inhibit the ability of these cations to stimulate this nonselective pathway (6, 9, 39, 40). It has been proposed that these nonselective cation channels are distributed submucosally along the basolateral membrane of taste receptor cells and that cations coupled with small anions are capable of electroneutrally diffusing through the tight junctions between the cells (6, 38, 39).

However, recent evidence suggests that an amiloride-insensitive channel may exist on the apical membrane. With the application of 5 μM benzamil (Bz), an amiloride analog, the Bz-insensitive component of the chorda tympani response to NaCl has been shown to be modulated by cetylpyridinium chloride (CPC) and two transient receptor potential vanilloid receptor (TRPV1) agonists, resiniferatoxin, and capsaicin. The stimulating effect of these agonists on the Bz-insensitive NaCl chorda tympani response is reversed by the application of TRPV1 antagonists, capsazepine and SB-366791 (24). This nonselective cation pathway has been identified by CPC (4) and vanilloid modulation (24) as a variant of the TRPV1 channel and appears to be expressed in some taste receptor cells. Direct measurement of the unilateral apical Na⁺ flux in polarized taste receptor cells and of the voltage sensitivity of the chorda tympani (CT) response in the presence of TRPV1 agonists indicate that the conductance mediating the nonspecific salt responses is present in the taste cell apical membrane (24).

Ultimately, it is important to link these biophysical findings regarding salt taste transduction to behavior and, by inference, perception. The application of amiloride in animal psychophysical experiments, for example, has revealed much about the amiloride-sensitive component of salt taste transduction due partly to ideal features of amiloride, such as its rapid and reversible effects and the fortuitous fact that it appears to be tasteless to rats (26) and mice (8). Specific antagonists to investigate the role of the amiloride-insensitive component have not been available until the recent discovery of CPC (4) and vanilloid modulation (24) of CT responses to NaCl as described above. Although CPC has rapid and reversible effects, psychophysical testing in rats suggests, unlike amiloride, it has a taste (36), complicating its utility as a tool for investigating the amiloride-insensitive pathway in a functional context. Similarly, the vanilloids complicate psychophysical testing because of their pungency.

Electrophysiological recordings show that Trpv1 knockout (KO) mice demonstrate no amiloride-insensitive CT responses to NaCl and display attenuated responses to KCl as assessed by the quasi-steady-state (i.e., tonic) portion of the signal. These electrophysiological findings suggest that a large component of the amiloride-insensitive salt taste transduction pathway depends on the products of the Trpv1 gene (24). On the basis of these findings, it would be expected that Trpv1 null mice would display more severe impairments in the detection of NaCl under conditions of amiloride blockade compared with the
performance of wild-type (WT) mice. Here, we tested this hypothesis by psychophysically assessing the effects of amiloride treatment on NaCl and KCl taste sensitivity in Trpv1 KO and WT mice. In addition, a more detailed analysis of electrophysiological responses to NaCl and KCl, with and without treatment with benzamid (an amiloride analog) and amiloride, recorded from the chorda tympani nerves of Trpv1 KO and WT mice, was conducted with an emphasis on both the phasic and tonic components of the signal.

MATERIALS AND METHODS

Behavioral

Subjects. Ten male wild-type C57BL/6J (B6) mice and 10 male homozygous Trpv1 KO B6.129S4-Trpv1tm1kol mice (Jackson Laboratory, Bar Harbor, ME) with mean body masses of 21.38 ± 0.49 g and 20.66 ± 0.77 g, respectively, upon arrival, served as subjects. All mice were experimentally naive at the start of the experiments.

The mice were housed individually in polycarbonate shoebox cages in a colony room where the temperature, humidity, and lighting (12:12-h light-dark) were controlled automatically. Mice were given free access to pellets of laboratory chow (LabDiet 5001; PMI Nutrition International, Brentwood, MO) and water (Elix 10; Millipore, Billerica, MA).

After 7 days of habitation to the laboratory environment, the mice were placed on a restricted water-access schedule in which fluid was available only during the training and testing sessions on Monday through Friday. Testing and training took place during the lights-on phase. On weekends, the mice received ad libitum water and food; water bottles were replaced on the home cages of the mice after their testing session on Friday and removed again on Sunday, no more than 23 h before testing. While on the water-restriction schedule, mice that dropped below 85% of their free-drinking weight received 1 ml supplemental water 1 h after the end of the testing session.

After training, one mouse was eliminated from the study because of apparent illness, and its data were not included in the analysis. All procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Taste stimuli. All taste solutions were prepared daily with purified water (Elix 10; Millipore, Billerica, MA) and reagent-grade chemicals and presented at room temperature. Test stimuli consisted of seven concentrations of NaCl (0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 M; Fisher Scientific, Atlanta, GA) and KCl (0.03, 0.06, 0.125, 0.25, 0.5, 0.6, 1.0 M; Fisher Scientific). During the amiloride phase of the study, 100 mM amiloride hydrochloride (Sigma Chemical, St. Louis, MO) was prepared at least 1 h before testing with purified water in a glass flask covered with aluminum foil to prevent photodegradation. This amiloride solution was used in place of purified water in preparation of all solutions in this phase, including reinforcers.

Apparatus. Mice were trained and tested in a specially designed computer-controlled testing apparatus referred to as a gustometer (35). Animals were placed in a testing cage, enclosed in a sound-attenuating chamber. A speaker (8 Ohm 2 W 3A05Z8, Quam Chicago, IL) and a ventilation fan in the chamber collectively produced a background broadband masking noise of ~84 dB sound pressure level, which served to minimize extraneous auditory cues. All taste stimuli and reinforcement fluids were placed in up to 12 pressurized reservoirs outside the chamber. The solutions in these reservoirs were rotated daily. Computer-controlled solenoid valves regulated fluid delivery from these reservoirs and were calibrated to deliver a controlled amount of fluid into the drinking spout. By extending its tongue, the mouse had access to the centrally positioned sample spout through a slot located in the side wall of the testing cage. The initial lick caused the sample spout to be filled with the appropriate taste stimulus and each subsequent lick delivered ~2 µl of fluid into the fluid column. Reinforcement fluid was delivered from two stationary horizontally oriented “reinforcement” spouts located on each side of the access slot. Contact with the correct reinforcement spout during the decision phase resulted in the delivery of ~2 µl/lick of reinforcement fluid (water, or 100 µM amiloride hydrochloride during the amiloride phase).

Trial structure. The mice were tested in daily 25-min sessions, during which each mouse was allowed to complete as many trials as possible. The mouse had to lick the dry spout two times within 250 ms to initiate a trial, which ensured the mouse was engaged in active licking. The trial began with the “sample phase.” The fluid stimulus was presented through the sample spout for 2 s or up to 5 licks (whichever came first), after which the sample spout was rotated away from the reach of the mouse. During the “decision phase,” the mouse had 5 s (limited hold) to lick one of the two reinforcement spouts. 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 reinforcement fluid in a 4-s period. If an incorrect choice was made or no response was initiated within the allocated time, the mouse received a 30-s timeout, during which no fluid was presented. When 15 licks were taken or 4 s had passed, the sample spout was rotated over a funnel, rinsed with purified water, dried with pressurized air, and rotated back into position in front of the slot. This intertrial interval lasted ~6 s.

Training. “Spout training” consisted of training the mice to lick the different fluid delivery spouts in the gustometer by presenting the mice with only one spout for 30 min each day, while making the other spouts inaccessible (see Table 1). Water was delivered on all 3 days of this training phase and was available ad libitum throughout the session.

“Side training” involved training the mice to associate one of the reinforcement spouts with the presentation of either water or 0.8 M NaCl delivered through the sample spout. The sample spout and one of the reinforcement spouts were available, while the other reinforcement spout was retracted and its access slot covered. The sample solution (water or 0.8 M NaCl) and the matching reinforcement spout were alternated between days.

“Alternation” involved presenting one stimulus repeatedly until the correct response was made a predetermined number of times. The other stimulus was then presented until an equivalent number of correct responses was made. This alternation continued over eight sessions, with the criterion number decreasing every second day. During the first two sessions, the stimulus alternated after 6 correct responses, after 4 correct responses for the third and fourth sessions, after 2 correct responses for the fifth and sixth sessions, and after 1 correct response for the seventh and eighth sessions. The reinforcement period was 30 s (or 20 licks) during this phase of training.

Table 1. Training schedule

<table>
<thead>
<tr>
<th>Days</th>
<th>Phase</th>
<th>Timeout, s</th>
<th>Limited Hold, s</th>
<th>Stimuli</th>
<th>Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Spout training</td>
<td>None</td>
<td>None</td>
<td>Water</td>
<td>Constant</td>
</tr>
<tr>
<td>6</td>
<td>Side training</td>
<td>None</td>
<td>180</td>
<td>0.8 M NaCl or water</td>
<td>Constant</td>
</tr>
<tr>
<td>8</td>
<td>Alternation</td>
<td>10</td>
<td>15</td>
<td>0.8 M NaCl and water</td>
<td>Alternated after x correct responses</td>
</tr>
<tr>
<td>32</td>
<td>Discrimination training I</td>
<td>20</td>
<td>15</td>
<td>0.8 M NaCl and water</td>
<td>Semirandom</td>
</tr>
<tr>
<td>11</td>
<td>Discrimination training II</td>
<td>30</td>
<td>10</td>
<td>0.2, 0.4, 0.8 M NaCl and water</td>
<td>Semirandom</td>
</tr>
</tbody>
</table>
“Discrimination training” involved the presentation of taste stimuli in randomized blocks, with the number of stimuli gradually increasing from two to six, the limited hold decreasing from 15 s to 10 s, the timeout increasing from 20 s to 30 s, and the testing session decreasing from 30 min to 25 min. All mice were trained to discriminate water from 0.8, 0.4, and 0.2 M NaCl.

Sodium chloride threshold testing. This testing period required 29 sessions. Each day, 10 fluid reservoirs contained taste solutions to be delivered via the sample spout. Five of the ten reservoirs contained 0.2, 0.4, or 0.8 M, and the other five reservoirs contained purified water. Stimuli were delivered in randomized blocks of 10, so that each animal had a 1 in 2 probability of receiving a salt stimulus on any given trial. At the end of each day, the reservoirs and stimulus delivery lines were rinsed thoroughly, and their stimulus assignments were rotated to reduce the possibility that a mouse could associate an extraneous cue with a particular solenoid or reservoir. For the first week, mice were only given 0.025, 0.05, 0.1, 0.2, or 0.8 M NaCl, referred to as the “standard array.” Starting on the second week of discrimination training, every Monday, the mice were presented with concentrations from the standard array and from Tuesday through Friday, they were presented with 0.0125, 0.025, 0.05, 0.1, 0.4 M NaCl referred to as the “alternate array.” The purpose of the standard array presentations was to maintain and measure stimulus control of behavior. Similarly, the higher concentrations in the alternate array were included for the same purpose.

Amiloride testing. After NaCl sensitivity was assessed, a second test consisting of 10 testing sessions was performed using the standard array concentration series of NaCl but with all stimuli dissolved in 100 μM amiloride hydrochloride instead of purified water. Although the amiloride analog Bz was used in the electrophysiological studies, we chose to use amiloride because it appears to be tasteless to rats (26) and mice (8), and Bz has yet to be tested for this possibility. Control sessions without amiloride were interposed between the amiloride sessions to maintain and measure stimulus control of the behavior.

KCl threshold testing. After sensitivity to NaCl was assessed in the presence of amiloride, we trained mice to respond to a KCl presentation by reinforcing correct responses to the same reinforcement spout that had previously been associated with NaCl delivery. Mice were tested with 0.03, 0.06, 0.125, 0.25, 0.5, 0.6, and 1.0 M KCl across 25 sessions. This was followed by a 6-wk break in testing, during which the mice received ad libitum water and food in their home cages. After the break, KCl threshold testing was run over another period of 25 sessions. KCl threshold was determined in the same manner as NaCl. Sessions with 0.03, 0.06, 0.125, 0.25, and 0.6 M KCl dissolved in 100 μM amiloride hydrochloride were included over 11 sessions, and control sessions without amiloride were interposed between the amiloride days to determine whether the drug had any effect on KCl sensitivity in mice.

Water testing. Two water control sessions were conducted, in which all reservoirs were filled with purified water. Five reservoirs were assigned to the “water” reinforcement spout and five to the “salt” reinforcement spout. This was done to exclude the possibility that extraneous cues contributed to responses during threshold testing.

Data analysis. Detection threshold was defined as the stimulus concentration corresponding to one-half the maximum asymptote of performance. Performance scores were derived from corrected hit rates expressed by the following equation:

\[ P(\text{hit}) = \frac{P(\text{hit}) - P(\text{FA})}{1.0 - P(\text{FA})} \times 100 \]  

where \( P(\text{hit}) \) is equal to the corrected hit rate, \( P(\text{hit}) \) is equal to the proportion of salt (NaCl or KCl) trials that resulted in a response on the salt reinforcement spout, and \( P(\text{FA}) \) is equal to \( P(\text{false alarm}) \), which is equal to the proportion of water trials that resulted in a response on the salt reinforcement spout. Trials for which the mouse did not respond were not included in the calculation of this score.

The following logistic function was used to fit a curve to the corrected hit rates for each mouse:

\[ f(x) = \frac{a}{1 + 10^{\frac{c - x}{s}}} \]  

where \( a \) equals maximum asymptote of performance, \( b \) equals slope, \( c \) equals log_{10} stimulus concentration corresponding to one-half the maximum asymptote of performance, and \( x \) equals stimulus concentration in log_{10} units.

The parameters of the logistic functions fit to the corrected hit rate data were compared across the two phases for NaCl and KCl (with and without amiloride), using a two-way ANOVA: phase × genotype. Paired t-tests were performed to test for differences in individual parameters across phases of the experiment.

On water control test sessions, the normal approximation of the binomial distribution (one-tailed test) was used to determine any positive deviation of performance from chance. The \( P \) value 0.05 was considered significant in all statistical tests.

Genotyping. To confirm the genotypes, tail tissue from a sample of Trpv1 KO mice (\( n = 8 \)) and WT mice (\( n = 7 \)) were taken after behavioral testing and sent to Charles River Genetic Testing Services (Troy, NY) for PCR analysis.

DNA was extracted, and PCR was performed in a final reaction mixture volume of 25 μl, including 1 μl of the isolated DNA and a Trpv1 primer mixture (1 μl) of Trpv1-F: 5’- CCT GCT CAA CAT GCT GCT TG -3’, Trpv1-R: 5’-TCC TCA TGC ACT TCA GGA AA-3’ and Neo3193: 5’-CGA TAG TAG TGA GAC GT TGA CTT CC-3’. PCR conditions were as follows: a preheating step for 2 min at 94°C followed by 40 cycles of 45 s at 94°C, 45 s at 60°C, and 1 min 30 s at 72°C and an autoextension step of 5 min at 72°C. The PCR products were separated using electrophoresis on a 2% agarose gel.

Electrophysiology

Subjects. We recorded the CT response in WT B6 and homozygous Trpv1 KO B6.129S4-Trpv1tm1jul mice (Jackson Laboratory, Bar Harbor, ME). Mice with body mass 30–40 g were housed three per polycarbonate cage in a vivarium where the temperature, humidity, and lighting (12:12-h light-dark cycle) were controlled automatically. Mice were given free access to pellets of LM-485 mouse/rat sterilized diet 7012 (Harlan Teklad, Madison, WI) and water. All procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Procedure. Mice were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), and supplemental pentobarbital (10 mg/kg) was administered as necessary to maintain surgical anesthesia. The animal’s corneal and pedal withdrawal reflexes and breathing rate were used to monitor the depth of anesthesia. We used a tuft of cotton to stimulate the corneal reflex and forceps to pinch a toe to stimulate the pedal withdrawal reflex. Eye blinking or pedal withdrawal, and an acute increase in breathing rate were assumed to be evidence of an inadequate level of anesthesia. In that case, a supplemental dose of pentobarbital (at one-quarter of the original dose) was administered. Body temperature was maintained at 36–37°C with a circulating water heating pad.

The left chorda tympani nerve was exposed laterally as it exited the tympanic bulla and placed onto a 32-gauge platinum-iridium wire electrode. An indifferent electrode was placed in contact with exposed muscle tissue in the vicinity of the CT nerve. Neural responses were differentially amplified with an optically coupled isolation amplifier. For display, responses were filtered using a band-pass filter with cutoff frequencies 40 Hz to 3 kHz and fed to an oscilloscope. Responses were then full-wave rectified and integrated with a time constant of 1 s. Integrated neural responses were recorded on a chart.
The lingual surface was stimulated with NaCl at 0.01 M, 0.02 M, 0.05 M, 0.1 M, 0.3 M, 0.5 M, and 1.0 M and was rinsed with 10 mM KCl, which approximates the potassium levels in saliva, between NaCl stimulations. Bz (5 μM; Sigma, St. Louis, MO) was used to block Na\(^+\) ion entry via the apical epithelial Na\(^+\) channels (23). To demonstrate their functional equivalence, Bz (5 μM) and amiloride (100 μM) were each shown to block the entire CT response to NaCl in Trpv1 KO mice (see Fig. 9A). We also used KCl + 5 μM Bz as a stimulus for both WT and KO mice. The KCl concentrations used were 0.01, 0.05, 0.1, 0.3, and 0.5 M. Between KCl + Bz stimulations, we used a water rinse. Control stimuli consisting of 300 mM NaCl and 300 mM NH₄Cl, applied at the beginning and at the end of the experiment, were used to assess preparation stability. Only preparations with steady unstimulated activity were used for data collection, and these typically had tonic responses to 300 mM NH₄Cl and 300 mM NaCl at the end of an experiment that differed by no more than 2–5% from those initially recorded. The data were digitized and analyzed off line. The typical response duration was 1 min, but in a few cases, the stimulus was allowed to remain on the tongue longer. The numerical value of a tonic-integrated CT response was obtained during the NaCl stimulation period was typically at least 1 min (23). The change in the area under the integrated chorda tympani response curve for a 30-s time interval measured from the end of a typical stimulation period. The duration of a stimulation period was typically at least 1 min (23). The change in the area under the integrated chorda tympani response curves to the NaCl stimuli under different conditions was normalized to the tonic response over a similar 30-s interval observed in each animal to 300 mM NH₄Cl. This ratio of areas was averaged across the number of animals in each group (n) and expressed as the means ± SE. The numerical value of a phasic integrated CT response for a given stimulus was obtained as the peak CT response relative to the mean tonic response level for that stimulus. For each animal, each peak above tonic value was normalized to the mean tonic value of the response to 300 mM NH₄Cl. These normalized phasic responses to a given stimulus concentration were then averaged across the number of animals in each group (n) and expressed as the means ± SE.

RESULTS

Sodium Chloride Threshold Testing

All mice initiated at least 15 trials for a given concentration during the NaCl threshold phase of testing and usually many more (up to 158). To avoid losing stimulus control, a lower number of trials was obtained for weaker concentrations. Mice initiated between 13 and 64 trials for a given concentration during the NaCl\(^+\) amiloride threshold testing phase. Logistic functions were fit to performance data of each mouse for each test condition. These curves represented the data points accurately, as evidenced by the mean R\(^2\) value (Table 2).

A two-way ANOVA (phase × genotype; see Table 2) revealed that the curve parameter representing NaCl threshold (c) significantly increased when amiloride was used (P < 0.01). Amiloride adulteration of the stimuli increased the NaCl detection threshold by a mean of 0.7 log\(_{10}\) units. There was no significant main effect of genotype and no significant phase × genotype interaction. Thus, NaCl thresholds were similar for the two groups, regardless of amiloride treatment.

No main effects of either genotype, or phase, on asymptotic performance (i.e., curve parameter a) were indicated, nor was there a significant interaction. Similarly, the slope of the NaCl detectability function (i.e., curve parameter b) did not significantly differ between genotypes, and there was no main effect
of phase and no interaction. These statistics confirm what is obvious in Figs. 1 and 2; both groups of mice performed similarly during NaCl threshold testing, and amiloride had comparable effects on NaCl detection for both genotypes.

**KCl Threshold Testing**

All mice initiated at least 13 trials for a given concentration during the KCl threshold testing phase of testing and usually many more (up to 164). A lower number of trials were presented for weaker concentrations to avoid losing stimulus control of behavior. Mice initiated between 13 and 34 trials for a given concentration during the KCl + amiloride threshold testing phase. With the exception of a few mice, logistic functions accurately accounted for the corrected hit rate data of each mouse for the various threshold determination phases, as evidenced by the mean $R^2$ value (Table 2). *Mouse 5* was not included in the statistical tests examining a possible amiloride-induced shift in threshold because a curve could not be fit to its data collected during the amiloride phase of testing. The data for this mouse were included in all of the calculations and statistical analyses that did not involve amiloride curve parameters. Although the data clearly showed a concentration-dependent increase to KCl, logistic functions could not accurately account for the corrected hit data for *mouse 20* during the latter 25 sessions of KCl testing, and instead, the corrected hit rate values for two of the mid-range concentrations were replaced by their average to fit a curve to the data and obtain parameter values.

When the first 25 sessions of KCl testing were compared with the KCl + amiloride testing phase, a two-way ANOVA (phase × genotype; Table 2) of the $c$ parameter, defined as threshold, for WT and KO mice across the two phases revealed no main effect of genotype and no main effect of phase but did reveal a significant phase × genotype interaction ($P = 0.008$). It is clear from Figs. 3 to 5 that the magnitude of this interaction was modest at best. In fact, when the thresholds measured during the KCl + amiloride testing phase were compared with the second, more recent, 25 sessions of KCl...
testing in a two-way ANOVA, the significant phase × genotype interaction disappeared \((P = 0.074)\), and there were also no main effects of either genotype or phase. Thus, when comparing data from the KCl + amiloride threshold testing phase with KCl threshold testing data obtained during the 5 wk immediately before, there were no reliable genotype differences, and KCl detection threshold did not significantly change with amiloride adulteration.

A two-way ANOVA (phase × genotype; Table 2) of asymptotic performance \((a\) parameter) for WT and KO mice across the two phases (KCl and KCl + amiloride) revealed a main effect of genotype when comparing KCl + amiloride testing with data from the first 25 sessions of KCl testing \((P = 0.034)\). Although the mean value for asymptote was slightly higher for KO mice than WT mice for both KCl testing (KO: 94.904, WT: 90.611) and KCl + amiloride testing (KO: 97.308, WT: 87.430), when we conducted \(t\)-tests to evaluate the phases separately, no significant differences were revealed between KO and WT mice for either KCl testing, \(t(9) = 1.023, P = 0.322,\) or KCl + amiloride testing, \(t(9) = 1.974, P = 0.066\). Also, when KCl + amiloride testing was compared with the second, more recent, 25 sessions of KCl testing, a two-way ANOVA did not reveal a main effect of genotype. A two-way ANOVA did not reveal a significant main effect of phase on asymptote when KCl + amiloride was compared with the first 25 sessions of KCl testing. However, a main effect of phase was found when KCl + amiloride was compared with the latter 25 sessions of KCl testing \((P = 0.033)\), but this disparity in the outcomes of the two analyses appeared to be driven by a single outlier \((\text{mouse 3})\) that had an asymptotic performance value of 36.7 for KCl testing and 100.0 for KCl + amiloride testing. When this animal was not included in the analysis, the significant main effect of phase on asymptote disappeared \((P = 0.069)\). Regardless of whether the asymptotes from the KCl + amiloride phase were compared with those from the first or the second 25 sessions of KCl testing, a two-way ANOVA did not reveal significant interactions. Thus, on the whole, there was no strong statistical support for genotype or amiloride affecting asymptotic performance in the KCl detection task.

A two-way ANOVA (phase × genotype: Table 2) of the slope of the detectability function \(\text{i.e.,} \ b\) parameter for WT and KO mice across the two phases (KCl and KCl + amiloride) did not reveal a significant main effect for genotype when comparing the first 25 sessions of KCl testing or the second 25 sessions of KCl testing, did not reveal a significant main effect of phase when comparing the first 25 session of KCl testing or the second 25 sessions of KCl testing and did not reveal a significant interaction effect when comparing the first 25 sessions of KCl testing or the second 25 sessions of KCl testing. Thus these statistics indicate that the slope of the curves describing data for both the WT and KO mice were not significantly different and were not significantly changed with the addition of amiloride.

**Water Testing**

During the water control test, no mouse responded significantly above chance \(50\%;\) all \(P\) values \(>0.05\), with performance for the B6 wild-type mice averaging \(47.8 ± 0.02\%\) and for the KO mice averaging \(47.8 ± 0.02\%\). Thus, there is no evidence to conclude that any mouse could perform the task in the absence of chemical cues (Fig. 6).

**Genotyping**

PCR confirmed genotypes for the WT and Trpv1 KO mice with a band obtained at \(\sim 980\) bp for the WT mice and a band obtained at \(\sim 600\) bp for the Trpv1 KO mice.

**Electrophysiology: Chorda Tympani Responses**

Figure 7A shows an integrated CT response in a WT mouse to \(1.0 \text{ M NaCl}\) and \(1.0 \text{ M NaCl} + \text{Bz}\). Note that in the presence of Bz, both the phasic maximum response and the quasi-steady state response (tonic response) were reduced. These reduced responses represent the amiloride-insensitive part of the NaCl response.

Figure 7B shows the integrated tonic CT response to NaCl and NaCl + Bz across concentrations in WT mice. In Fig. 7B
Accordingly, the data in Fig. 7B show phasic responses also depend on solution flow rate parameters as a function of concentration. However, unlike tonic responses, the phasic response to NaCl is also a saturating function of concentration:

\[ R = \frac{R_m c}{K_m + c} \]  

(3)

Here, \( R \) is the normalized response, \( R_m \) is its maximum value, \( K_m \) is the NaCl concentration at which \( R \) has one-half its maximum value, and \( c \) is the NaCl concentration. For the response to NaCl alone, \( R_m = 1.32 \pm 0.05 \text{ M} \), and \( K_m = 0.11 \pm 0.02 \text{ M} \). For the response to NaCl + Bz, \( R_m = 0.26 \pm 0.02 \text{ M} \), and \( K_m = 0.035 \pm 0.01 \text{ M} \). These parameters are consistent with those found previously in the rat (4).

Figure 7C shows the normalized phasic response to NaCl and NaCl + Bz across concentrations in WT mice. Like the tonic response, the phasic response to NaCl + Bz is also a saturating function of concentration. However, unlike tonic responses, phasic responses also depend on solution flow rate parameters (22). Accordingly, the data in Fig. 7C were fit to a modified saturating function of concentration:

\[ R = R_o + \frac{R_m c}{K_m + c} \]  

(4)

Here \( R \) is the normalized phasic response, \( R_o \) is a concentration-independent phasic response, \( R_m \) is the maximum response relative to \( R_o \), \( K_m \) is the NaCl concentration at which \( R \) relative to \( R_o \) has one-half its maximum value, and \( c \) is the NaCl concentration. For phasic responses to NaCl alone in WT mice: \( R_o = 0.53 \pm 0.38 \text{ M} \), \( R_m = 1.33 \pm 1.01 \text{ M} \), and \( K_m = 0.15 \pm 0.17 \text{ M} \). Thus, for the phasic responses to NaCl the mean \( R_m \) and \( K_m \) values are comparable to their corresponding tonic response values, but the phasic response values are established with much less precision. For phasic responses to NaCl + Bz, the basic model was poor (correlation coefficient was 0.17). We, therefore, elected to display the data without a fitted curve. The data points in Fig. 7C were connected by dotted line segments. It is clear, however, that phasic responses to NaCl + Bz, like the corresponding tonic responses to NaCl + Bz, are nearly concentration independent. Also at 0.5 M and 1.0 M NaCl, the phasic responses with Bz are significantly less than the corresponding phasic responses to NaCl alone.

Fig. 8A, shows an integrated tonic CT response in a Trpv1 KO mouse to 1.0 M NaCl and 1.0 M NaCl + Bz. In contrast to the wild-type controls, the tonic response to 1.0 M NaCl + Bz is at or near 0 level. However, a distinct phasic response remains (see Fig. 8A). The tonic response to NaCl + Bz was at or near 0 for all NaCl concentrations (see Fig. 8B).

Figure 8B shows the integrated tonic CT response to NaCl and NaCl + Bz across concentrations in Trpv1 KO mice. In Fig. 8B, the data points for the response to NaCl were fit to Eq. 3. The parameters are \( K_m = 1.47 \pm 0.08 \text{ M} \), \( R_m = 0.17 \pm 0.03 \text{ M} \), and the correlation coefficient was 0.99. For the tonic response to NaCl + Bz, all data points were at or near zero. Accordingly, the model represented by Eq. 3 was notably less precise, so we present the data points merely connected by dotted line segments. The virtual absence of a tonic integrated CT response to NaCl + Bz at all NaCl concentrations compared with the large residual responses to NaCl + Bz seen in Fig. 7 is consistent with the hypothesis that the amiloride-insensitive response to NaCl depends on a TRPV1 gene product.

Figure 8C shows the normalized phasic response to NaCl and NaCl + Bz across concentrations in Trpv1 KO mice. Similar to WT mice, the phasic response to NaCl in Trpv1 KO mice was a saturating function of concentration that could be modeled using Eq. 4. For phasic responses to NaCl alone in Trpv1 KO mice: \( R_o = 0.65 \pm 0.26 \text{ M} \), \( R_m = 1.34 \pm 0.84 \text{ M} \), and \( K_m = 0.20 \pm 0.18 \text{ M} \). As was the case for WT mice, the phasic responses to NaCl for Trpv1 KO mice have mean \( R_m \) and \( K_m \) values that are comparable to their corresponding tonic response values, but as noted above, the phasic response values...
are established with less precision. Similar to WT mice, phasic responses to NaCl + Bz in Trpv1 KO mice were greater than zero but nearly concentration independent. In Fig. 8C they are displayed as data points connected by dotted-line segments.

We also studied the phasic and tonic CT responses to KCl + Bz. Fig. 9B shows the tonic responses to KCl + Bz plotted as a function of KCl concentration for both WT and KO mice. Below 0.1 M KCl, the tonic responses were not significantly different from baseline. However, at 0.3 and 0.5 M KCl, WT tonic responses exceeded KO responses significantly (P < 0.05, n = 3). This is consistent with results in rats that show that part of the tonic CT response to KCl is vanilloid sensitive (24). Similar to phasic responses to NaCl + Bz, the phasic CT responses to KCl + Bz were nearly concentration independent and did not differ significantly between WT and KO mice (data not shown).

**DISCUSSION**

On the basis of the electrophysiological analysis of tonic responses of the CT to NaCl applied to the anterior tongue, it appears as though mice lacking the Trpv1 gene do not possess an amiloride-insensitive NaCl taste transduction pathway (24). Therefore, it was expected that relative to WT mice, oral application of amiloride would much more severely disrupt, if not eliminate, the ability of Trpv1 KO mice to detect NaCl. In contrast to this prediction, mice lacking the Trpv1 gene did not display any major differences in gustatory function relative to their WT controls for any of the compounds tested in the behavioral task used in the present study. Moreover, not only were detection thresholds for NaCl similar for both the KO and WT mice, but sensitivity was impaired by amiloride treatment to the same degree in both groups. Both groups also had similar detection thresholds for KCl, and amiloride had no effect on KCl thresholds. Thus, contrary to predictions based on at least the tonic CT responses to NaCl + benzamil, the behavioral findings of the current study suggest that the TRPV1 variant is present in KO mice.

**Fig. 8.** A: CT response of KO mouse to 1.0 M NaCl (N) followed by a 10 mM KCl rinse (R). This was followed by the response to 1.0 M NaCl + 5 μM benzamil (N + Bz). Note the tonic (steady-state response) response is entirely eliminated in the presence of Bz. The phasic (peak response above tonic) response is reduced by Bz. B: normalized tonic CT response to NaCl (●) and NaCl + Bz (○) over the dynamic range of NaCl concentrations. C: normalized phasic CT response to NaCl (●) and NaCl + Bz (○) over the dynamic range of NaCl concentrations.

**Fig. 9.** A: CT response in KO mouse to 100 mM NaCl (N) followed by 100 mM NaCl + 5 μM benzamil (N + Bz) or 100 mM NaCl + 100 μM amiloride (N + Am). The rinse (R) was 10 mM KCl. B: normalized tonic CT response to KCl + 5 μM benzamil in WT mice (●) and in KO mice (○) over the dynamic range of KCl concentrations.
not necessary for the normal taste detection of NaCl or KCl with or without 100 μM amiloride.

To assess salt taste function, the behavioral task we used in the current study assessed the ability of the mice to detect the presence or absence of the salt taste stimuli and did not address issues of taste quality. We cannot rule out the possibility that other types of taste-guided responses to NaCl in other behavioral tasks may be impaired in the KO mice.

Amiloride treatment raised the NaCl detection threshold by ~0.7 log₁₀ units. The magnitude of this amiloride-induced change in sensitivity to NaCl is comparable to the ~0.8 log₁₀ unit shift observed in a previous study in B6 and D2 mice (7) and the ~0.9 log₁₀ unit shift observed in Sprague-Dawley rats (15). This demonstrates consistency not only across genotypes of mice but also across rodent species (i.e., rats) and across different studies as well.

In rats, the detection threshold for NaCl with 100 μM amiloride is similar to the detection threshold for KCl (14, 15). On the basis of these studies, we would have expected the KCl detection thresholds in B6 and Trpv1 KO mice to be similar with those seen for NaCl when it is adulterated with amiloride. However, for the mice in this study, the detection threshold for NaCl with 100 μM amiloride was ~0.3 log₁₀ units higher than that for KCl, suggesting that KCl and NaCl with amiloride differentially stimulate amiloride-insensitive pathways. This is consistent with the only partial inhibition of KCl responsiveness observed in the CT nerve of rats with the application of CPC compared with the complete inhibition of the amiloride-insensitive portion of the NaCl response by CPC application (4).

Recently, Ruiz et al. (32) performed long-term two-bottle preference tests in Trpv1 KO and C57BL/6J WT mice and also measured detection thresholds to NaCl with and without amiloride using an operant shock-avoidance task. The two-bottle preference tests showed that, contrary to their predictions, the Trpv1 KO mice preferred NaCl and KCl compared with water more than did the WT mice. Furthermore, with the addition of 100 μM amiloride, Trpv1 KO mice still preferred NaCl, even though amiloride-sensitive channels were blocked. In the same study, NaCl detection thresholds measured in the shock-avoidance task did not differ between WT and KO mice, consistent with the findings of the present report. The disparity in the outcomes between the two-bottle preference tests and the operant taste detection tasks remains to be understood, but there are at least two possibilities. First, it is possible that although the Trpv1 gene ablation does not affect performance in tasks that merely require the animal to detect the presence or absence of salt stimuli but has more profound effects on suprathreshold taste function involving either hedonic evaluation or qualitative identification of the stimulus. Second, it is possible that the two genotypes differ in the postigestive stimulation associated with the salts. Expression of the gene product of Trpv1 has been found in the gut and has been implicated in gastrointestinal function (13, 19). Importantly, behavior displayed in long-term two-bottle preference tests is known to be influenced by postigestive events (27, 31, 33, 34, 37). Thus, any differences between WT and KO mice in the postigestive processing of salt stimuli may contribute to the differences between these mice observed in the intake tests. The operant taste detection task used here, as well as that in the Ruiz et al. (32) study, involved the presentation of small volumes of taste stimuli and the measurement of immediate responses and thus minimize the involvement of postigestive factors in discrimination performance. It is important to stress, however, that the two hypotheses posited to explain the differential effect of the gene manipulation on the results from the two types of tasks are not mutually exclusive.

There were a couple of differences between the results of the shock avoidance experiment conducted by Ruiz et al. (32) and those presented here that are worthy of note. First, the detection thresholds for NaCl were lower by more than an order of magnitude in the former study. This is not particularly surprising given that taste detection thresholds are known to be influenced by the nature of the behavioral task and are dependent on the way performance is quantified, as well as the definition of the limen (34). Although both the task used here and the one employed by Ruiz et al. (32) were based on operant conditioning, there were several parametric differences in the methods that could serve as the basis for the lack of correspondence in the thresholds. Nevertheless, precisely because thresholds vary across procedures, it is best to compare the psychophysical functions generated across conditions within an experiment. In this sense, the lack of an effect of Trpv1 deletion on the psychometric functions for NaCl is consistent between the two studies. In our study, however, amiloride shifted the psychometric functions for NaCl detection by a similar amount in both WT and KO mice, whereas in the Ruiz et al. (32) experiment, although amiloride blunted the sensitivity of WT mice to NaCl, it had no effect on the NaCl detection thresholds measured in KO mice. The basis of this disparity remains to be determined, but it is interpretively vexing because these discrepant outcomes have different ramifications regarding the contribution of amiloride-sensitive channels to NaCl detection by Trpv1 KO mice compared with WT controls.

Similar to Sprague-Dawley rats, B6 and WT mice display both an amiloride-sensitive and an amiloride-insensitive CT response to NaCl (24, 30). The normalized maximum value of the tonic amiloride-insensitive CT response to NaCl + Bz, Rₘ, is Rₘ = 0.26 ± 0.02. This maximum value is achieved at relatively low NaCl concentrations given that Kₘ is, in this case, 0.035 ± 0.01 M. In contrast, Trpv1 KO mice have a normalized maximum value of the tonic amiloride-insensitive CT response to NaCl + Bz at, or just above, baseline (Fig. 8B), even at high NaCl concentrations. The small deviation from 0 that is observed in some individual preparations is seen at NaCl concentrations above 0.1 M. This probably reflects partial escape from complete Bz blockade of epithelial Na⁺ channels rather than a residual conductance through the Trpv1 variant salt taste receptor (Trpv1t), which, in any case, is presumably absent in Trpv1 KO mice. Thus Trpv1 KO mice show essentially no tonic response to NaCl in the presence of benzamil or equivalently in the presence of amiloride (see Fig. 9A), whereas WT display a significant tonic response to NaCl + Bz at all concentrations of NaCl tested.

Given the potent effects that deleting the Trpv1 gene has on CT responses to NaCl and KCl, why were we and Ruiz et al. (32) unable to see this manifest in the behavioral salt detection performance in the KO mice? There are a number of possible hypotheses addressing the apparent discrepancy between the electrophysiology and the behavioral findings of the current study. Although major differences were found in the tonic response of the CT to NaCl + Bz, as seen in Figs. 7A and 8A,
the nerves from both WT and Trpv1 KO mice displayed a phasic response to NaCl with and without benzamil. The phasic response to NaCl alone is, of course, influenced by Na⁺ flux through ENaC. Both WT and Trpv1 KO mice showed concentration-dependent phasic responses to NaCl that increased in accordance with a saturation function. Phasic responses to NaCl + Bz for both WT and Trpv1 KO mice were diminished (especially at higher NaCl concentrations) compared with phasic responses to NaCl alone but, in Trpv1 KO mice, unlike the tonic responses, phasic responses to NaCl + Bz were not eliminated completely. Because these transients constitute the entire CT response to NaCl + Bz in Trpv1 KO mice (cf. Fig. 8), it is possible that these signals are sufficient to maintain normal detectability of the presence of NaCl in the behavioral task used here. Moreover, it would appear that these transients represent a separate salt detection mechanism. If so, this would be the second example of a class of taste stimuli for which transduction mechanisms for phasic and tonic parts differ. It has been suggested that the tonic and phasic neural responses to acids derive from separate transduction mechanisms (3, 25).

The electrophysiological recordings that demonstrate that Trpv1 KO mice display essentially no tonic response to NaCl with benzamil adulteration was limited to the CT nerve. It remains to be seen how other taste nerves such as the glossopharyngeal (GL) and the greater superficial petrosal (GSP) would respond to NaCl with and without benzamil adulteration. In rats, the ENaC blocker amiloride has been shown to partially inhibit the response to sodium salts in not only the CT but also in the GSP, suggesting that sodium transduction in these receptor fields of these nerves involves both amiloride-sensitive and amiloride-insensitive components. Amiloride treatment does not appear to significantly inhibit sodium responsiveness of the GL nerve in rats (10, 20) or mice (28). Nevertheless, the possibility that NaCl engages amiloride-insensitive transduction mechanisms in taste receptor cells of the posterior tongue and palate, other than those associated with a TRPV1 variant, and that this may be the basis of the normal salt sensitivity displayed by the Trpv1 KO mice in this study, cannot be dismissed.

Indeed, this is reminiscent of the normal NaCl detectability and its disruption by amiloride seen in inbred mice that do not display an amiloride-sensitive component of the CT response to this salt (12, 30). Eylam and Spector (7) hypothesized that ENaCs distributed in receptor fields other than the anterior tongue were helping to maintain normal performance in these strains. Apparently, a direct correspondence between the peripheral electrophysiology of a single gustatory nerve and taste-guided behavior cannot always be expected because signals in other taste nerves may be sufficient to maintain performance depending on the behavioral task.

The use of inbred and genetically altered strains of mice is rapidly becoming a primary tool for exploration of the neurobiological basis of gustatory function. It is through behavior that inferences can be made about perception. Since behavior is the output of the system, variability at any one of the stages of taste signal processing along the gustatory neuraxis can affect the outcome of phenotype assessment. The lack of concordance between the electrophysiological and behavioral findings reported here provide a salient example of this reality.

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