Contribution of the TRPV1 channel to salt taste quality in mice as assessed by conditioned taste aversion generalization and chorda tympani nerve responses

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Smith KR, Treesukosol Y, Paedae AB, Contreras RJ, Spector AC. Contribution of the TRPV1 channel to salt taste quality in mice as assessed by conditioned taste aversion generalization and chorda tympani nerve responses. Am J Physiol Regul Integr Comp Physiol 303: R1195–R1205, 2012. First published October 10, 2012; doi:10.1152/ajpregu.00154.2012.—In rodents, at least two transduction mechanisms are involved in salt taste: 1) the sodium-selective epithelial sodium channel, blocked by topical amiloride administration, and 2) one or more amiloride-insensitive cation-nonsensitive pathways. Whereas electrophysiological evidence from the chorda tympani nerve (CT) has implicated the transient receptor potential vanilloid-1 (TRPV1) channel as a major component of amiloride-insensitive salt taste transduction, behavioral results have provided only equivocal support. Using a brief-access taste test, we examined generalization profiles of water-deprived C57BL/6J (WT) and TRPV1 knockout (KO) mice conditioned (via LiCl injection) to avoid 100 μM amiloride-prepared 0.25 M NaCl and tested with 0.25 M NaCl, sodium gluconate, KCl, NH4Cl, 6.625 mM citric acid, 0.15 mM quinine, and 0.5 M sucrose. Both LiCl-injected WT and TRPV1 KO groups learned to avoid NaCl+amiloride relative to controls, but their generalization profiles did not differ; LiCl-injected mice avoided the nonsodium salts and quinine suggesting that a TRPV1-independent pathway contributes to the taste quality of the amiloride-insensitive portion of the NaCl signal. Repeating the experiment but doubling all stimulus concentrations revealed a difference in generalization profiles between genotypes. While both LiCl-injected groups avoided the nonsodium salts and quinine, only WT mice avoided the sodium salts and citric acid. CT responses to these stimuli and a concentration series of NaCl and KCl with and without amiloride did not differ between genotypes. Thus, in our study, TRPV1 did not appear to contribute to sodium salt perception based on gustatory signals, at least in the CT, but may have contributed to the oral somatosensory features of sodium.

The sodium ion is essential for life and critical for numerous physiological processes. As such, mammals must have strict regulatory control of their sodium balance (45). One means of this regulation is through the gustatory system, which has become a major research focus for investigating salt and its sensory transduction mechanisms and associated neural pathways. Two salt taste transduction pathways have been proposed, at least in rodents, translating the chemical salt stimulus to a neural signal. One pathway is selective for sodium (and lithium) and is dependent on the apically located epithelial sodium channel (ENaC) serving as the receptor (e.g., 3, 7–9, 11, 16, 24–26, 30, 37, 39). The other pathway is cation nonselective, but the identity of its transduction mechanism is unknown. It has been suggested that the receptor is a variant of the transient receptor potential vanilloid receptor-1 (TRPV1t) (33, 34).

The evidence supporting the contribution of the ENaC to sodium taste transduction in the rodent is compelling. Electrophysiological assessments demonstrate that oral treatment of amiloride, a drug known to block ENaC and shown to be tasteless in rodents (15, 35), reduces the whole nerve sodium chloride (NaCl) response in the chorda tympani (CT), a branch of the facial nerve that innervates taste buds in the anterior tongue (e.g., 3, 9, 10, 19, 26, 38). Paralleling the electrophysiological behavior, studies demonstrate that an amiloride blockade of ENaC reduces taste sensitivity to sodium but not to KCl (12–14, 20, 21, 49) and alters the perceived taste quality of sodium, making it more similar to nonsodium salts (14, 27, 48). Amiloride, along with its analog benzamil, however, does not completely eliminate the CT response to NaCl, indicating that sodium also engages an additional taste transduction pathway(s).

TRPV1 is a nonselective cation channel activated by capsaicin, heat, and acids that is found in various neuronal tissues including the dorsal root ganglia, trigeminal ganglia, nodose ganglia, hypothalamus, cerebral cortex, and in some non-neuronal tissues (see Ref. 41). A variant of TRPV1 (TRPV1t) is reportedly expressed in taste buds (31, 33), but other studies have not found explicit expression in taste receptor cells (28, 29). The response of the CT to Na+, K+, and NH4+ has been reported to be enhanced by TRPV1 agonists such as resiniferatoxin (RTX) and capsaicin (33, 34) and suppressed by antagonists of the channel such as SB366791 (33, 34, 51), iodo-resiniferotoxin (I-RTX) (1, 52), and capsazepine (33), but the effectiveness of antagonism varies across studies (6). In some experiments with rodents, blocking both lingual ENaC and TRPV1, has been shown to eliminate the CT response to NaCl (33, 34). Importantly, whereas oral treatment with benzamil alone reduced the tonic CT response to NaCl in C57BL/6J (B6) mice, it entirely eliminated the response in mice homozygous null for the Trpv1 gene (B6.129X1-Trpv1<sup>IntLux/IntLux</sup>) (TRPV1 KO) (33, 49). Additionally, TRPV1 KO mice have reduced tonic responses to high concentrations of KCl. Based on these collective electrophysiological data, especially the results from TRPV1 KO mice, it has been postulated that the TRPV1 channel may be a major component of amiloride-insensitive salt taste transduction (33, 34).

Although behavioral tests of this hypothesis are scarce, the findings from such experiments raise questions as to what functional role the TRPV1t channel actually plays in salt taste. Both Ruiz et al. (44) and Treesukosol et al. (49) found that
TRPV1 KO and WT mice had similar behaviorally assessed NaCl detection thresholds. Treesukosol et al. (49) reported that amiloride shifted the psychometric detectability function for NaCl to the right by similar degrees in both TRPV1 KO and WT mice. In contrast, Ruiz et al. (44) found that although treatment of NaCl with amiloride increased detection thresholds in WT mice, it surprisingly had no effect on NaCl thresholds in TRPV1 KO mice. Ruiz et al. (44) also observed that TRPV1 KO mice displayed greater preference for NaCl and KCl compared with WT mice at some concentrations, including, in the case of NaCl, hypertonic concentrations that are normally avoided in a 24-h two-bottle intake test (2, 12). Intake tests, however, can be influenced by postigestive factors (see Ref. 47). Therefore, whether the TRPV1 KO mouse is phenotypically different from WT mice in taste-related behavioral responses to salts has yet to be fully resolved. Although there is evidence that the normal responsiveness of the CT nerve to salts including NaCl depends in part on TRPV1, this may not be the case for other gustatory nerves innervating regions other than the anterior tongue, which may account for the failure to find differences in behaviorally assessed taste sensitivity to NaCl and KCl between the KO and WT mice. Although the removal of TRPV1 does not appear to affect taste detection thresholds to NaCl and KCl and, in at least one study, amiloride treatment had a similar effect on NaCl detectability between TRPV1 knockout (KO) and wild-type (WT) mice, it remains possible that TRPV1 deletion changes the perceived quality of NaCl without affecting its detectability. Therefore, we employed a conditioned taste aversion (CTA) generalization paradigm, modeled after Hill et al. (27), to assess the contribution of the amiloride-insensitive salt taste transduction pathway to the taste quality of NaCl in TRPV1 KO and WT mice. In this paradigm, a CTA was first established by pairing ingestion of NaCl mixed with amiloride, a novel tastant assigned as the conditioned stimulus (CS), with visceral malaise experimentally induced by injection of LiCl. Upon successful acquisition of the CTA, as indicated by significant decreases in the ingestion of the CS on subsequent presentations, we offered several different salt and nonsalt stimuli in a brief-access test to determine the degree to which the CTA to the CS generalized to other taste compounds. This provided a response profile reflective of the qualitative nature of the CS. In our experiments, we prepared NaCl with 100 μM amiloride to serve as the CS to condition an aversion specifically to the amiloride-insensitive component of NaCl taste. Accordingly, we sought to determine whether the perceived qualitative nature of the amiloride-insensitive portion of the NaCl taste signal between TRPV1 KO and WT mice, as represented by the taste generalization profiles, is similar or different. At the conclusion of the behavioral tests, we conducted electrophysiological recordings of the CT responses to a range of stimuli in a subset of behaviorally tested animals as well as additional mice. Stimuli included those used in the behavioral test in addition to various concentrations of NaCl and KCl prepared with and without amiloride.

MATERIALS AND METHODS

Experiment 1

Subjects. Thirty-eight (N = 9–10/group) naïve male B6.129X1-Trpv1tm1Hdl/J (TRPV1 KO) and C57BL/6J (WT) mice (Jackson Lab-
were presented with 5-s trials (starting with first lick) separated by 5-lick water rinses during which the sample sphere was extended in front of the slot for lick access then retracted for the wash cycle. After a ~6-s intertrial interval, the sample sphere was again extended in front of the access slot for the start of another trial. Mice could initiate as many trials as possible during each session.

After 2 days of rehydration following gustometer training, mice were introduced to a water restriction schedule consisting of home-cage presentations of fluid (water or CS depending on the schedule) for 15 min in the morning staggered across animals (8:00–10:15 AM) and presented with water for 30 min in the afternoon beginning ~5 h after start of the morning session. The mice were given water in the morning for the first 4 days and total intake was measured. Using the data collected from the gustometer training and the first 4 days of home-cage water intake, we subdivided TRPV1 KO and WT mice into two groups defined by the unconditioned stimulus (US; NaCl or LiCl) in the conditioned taste aversion protocol. Injection groups were established such that body weight, trials taken and total licks in the gustometer, and total 15-min morning water intake were not significantly different between groups. The following morning, after having access to the CS for 15 min, each mouse was given an intraperitoneal injection of either 0.15 M NaCl or LiCl at a dose of 3.0 mg/kg. Each CS presentation, and therefore injection, was separated by 2 days of water. This cycle of one CS day followed by two water days for morning fluid presentations was repeated three more times. On the fifth and last conditioning trial, water bottles were returned to the home cages for 48 h.

Next, the animals were again placed on the 23.5-h deprivation schedule for the last 3 days of the behavioral experiment. Before the brief-access taste test with the stimulus array, the mice were given 2 days of further gustometer training using 5-s water trials preceded by water rinses (5 licks or 5-s access after initial lick, whichever came first). On the test day, the panel of taste stimuli was delivered in individual trials, each of which was preceded by a water rinse. The first stimulus following the initial 5-lick rinse trial for each session was sucrose, a tantant that is normally preferred, to promote stimulus sampling. With the exception of the first trial, the stimulus trials (including the water stimulus) were presented in randomized blocks of eight, and the mice could initiate as many trials as possible during the session.

Data analysis. The total intake for each mouse for each CS presentation was analyzed using a three-way (genotype × trial × injection) analysis of variance (ANOVA) to assess the acquisition of an aversion to the CS. A series of t-tests were then conducted on the last CS presentation testing for any genotype differences by 1) comparing the CS intake between the two saline-injected groups, and 2) comparing the CS intake of the two LiCl-injected groups. The CS intake of the saline-injected group was compared with that of the LiCl-injected group for each genotype to confirm that an aversion was conditioned.

To assess CS generalization, a Taste/Water Lick Ratio was derived based on the responses of each mouse to each tantant during the generalization test using the following equation:

\[
\text{Taste/Water Lick Ratio} = \frac{\text{Mean Licks to Taste Stimulus}}{\text{Mean Licks to Water}}
\]

where, relative to water, a value of 1.0 represents equal licking of the tantant, a value >1.0 represents greater licking of the tantant, and a value <1.0 represents lesser licking of the tantant. A three-way ANOVA (genotype × stimulus × injection) was performed on the taste-to-water lick ratios. When there were no main or interactive effects involving the genotype factor, a two-way ANOVA comparing lick ratios of all saline-injected animals to LiCl-injected animals was conducted. Also, individual t-tests were performed for each tantant in the stimulus array panel between each of the injection groups collapsed across genotype. We present both the unadjusted and conservative Bonferroni-adjusted P values. Two animals were excluded from this analysis since their brief-access data could not be used due to technical malfunctions that occurred during their gustometer session. This reduced the sample size to N = 8 in the LiCl-injected WT group and N = 9 in LiCl-injected TRPV1 KO group. An α level of 0.05 was adopted in all analyses to indicate statistical significance.

Experiment 2

Subjects. Thirty-six (N = 9/group) male naïve B6.129X1-Trpv1<tm1Jul1/J (TRPV1 KO) and C57BL/6J (WT) mice (Jackson Laboratory) weighing between 15.5 and 20.6 g at the onset of the experiment served as subjects and were housed and handled in the same conditions as in experiment 1.

Stimulus solutions. The concentrations of both the CS and test stimuli were doubled such that 0.5 M NaCl mixed with 100 μM amiloride served as the CS, and 0.5 M KCl, 0.5 M NaHCO3, 0.5 M NaCl, 0.5 M NaGlu, 13.25 mM citric acid, 0.3 M quinine hydrochloride, and 1.0 M sucrose served as the test stimuli.

Procedure. Training and conditioning paradigms followed the same procedure as in experiment 1 with the exception that three conditioning trials were implemented rather than five because intake of the CS reached very low levels in the LiCl-injected groups by the third trial.

Data analysis. Like experiment 1, the total intake for each mouse for each CS presentation was analyzed using a three-way (genotype × trial × injection) ANOVA. A main effect of genotype was revealed so the analyses were separated into two two-way ANOVAs (injection × trial) for each genotype with injection as the between-subjects factor and trial as the within-subjects factor. As in experiment 1, a series of t-tests were then conducted on the last CS presentation for each group comparing the CS intake between the two saline-injected groups and another comparing the CS intake of the two LiCl-injected groups. The CS intake of the saline-injected group was compared with that of the LiCl-injected group for each genotype to confirm that an aversion was conditioned.

For the CS generalization analyses, taste-to-water lick ratios were calculated for each stimulus. A three-way ANOVA (genotype × injection × stimulus) was conducted on the taste-to-water lick ratios. When there was a significant effect of genotype, two separate two-way ANOVAs (injection × stimulus) were conducted, one for WT and the other for TRPV1 KO mice. When significant main effects and interactions were found, individual t-tests were conducted for each taste-to-water lick ratio. We present both the unadjusted and conservative Bonferroni-adjusted P values. An α level of 0.05 was adopted in all analyses to indicate statistical significance.

Experiment 3

Subjects. Twenty-two adult male C57BL/6J (WT) and 23 adult male B6.129X1-Trpv1<tm1Jul1/J (TRPV1 KO) mice (Jackson Laboratory), including a subset of the mice used in the behavioral task, served as subjects. Of these mice, 12 WT and 9 TRPV1 KO recordings met the criterion posed for an acceptable preparation (see Data analysis below). Subjects weighed between 23 and 40 g at the onset of the experiment and were housed and handled in the same conditions as in the behavioral experiments.

Surgical procedure. Mice were anesthetized with ketamine (30 mg/kg) followed by an injection of urethane (1.2 g/kg). Supplemental urethane injections were given until no reflex was elicited upon foot pinch. Mice were tracheotomized with PE-200 tubing and secured in a nontraumatic head holder (model 926B Mouse Nose/Tooth Bar Assembly David Kopf Instruments, Tujunga, CA) angled to facilitate right CT transection via a mandibular approach. The tongue was extended and secured to the grounding table by a suture. The nerve assembly was introduced to a water restriction schedule consisting of home-cage presentations of fluid (water or CS depending on the schedule) for 5h morning fluid presentations was repeated three more times. On the fifth and last conditioning trial, water bottles were returned to the home cages for 48 h.
electrode (negative polarity) was attached to the skin overlying the cranium with a stainless steel alligator clip. Body temperature was maintained at 37°C.

**Stimuli and stimulus delivery.** Solutions were presented to the tongue at a constant flow rate (50 μl/s) and temperature (35°C ± 0.3°C) by an air-pressurized 32-channel commercial fluid-delivery system and heated perfusion cube (OcraFlow II; ALA Scientific Instruments, Farmingdale, NY), respectively. The time constant was set at 200 ms, and the flow rate, temperature, and rinse composition were essentially identical to the recent Breza et al. (5) geniculate ganglion study. All solutions and pharmacological reagents were reagent grade and purchased from VWR International or Sigma Aldrich. Artificial saliva (0.015 M NaCl, 0.022 M KCl, 0.003 M CaCl₂, and 0.0006 M MgCl₂; pH = 5.8 ± 0.2) served as the rinse solution and solvent for all stimuli. We tested CT nerve responses to 10-s applications of 0.5 M NH₄Cl, 1.0 M sucrose, 13.25 mM citric acid, 0.3 and 20 mM quinine, 0.5 M NaGlu, and 100 μM amiloride-treated and untreated NaCl and KCl at a range of concentrations (0.1 M, 0.3 M, 0.5 M, and 1.0 M).

**Nerve recordings.** The average baseline activity for the 10 s immediately preceding each chemical stimulus presentation was subtracted from the integrated response resulting from the 10-s stimulus to calculate the area under the curve (AUC). Whole nerve responses to the various stimuli were normalized to responses to 1.0 M sucrose presented periodically throughout the nerve recording. This served as indicators of nerve integrity and recording stability. Neural activity was differentially amplified (AC X 10,000; A-M Systems, Sequim, WA, band-pass 300–5,000 Hz), observed with an oscilloscope, digitized with waveform hardware and software (Spike 2; Cambridge Electronic Design, Cambridge, UK), and stored on a computer for off-line analysis.

**Data analysis.** The standard stimulus was 1.0 M sucrose, and any CT responses for which the preceding and subsequent sucrose responses deviated more than 15% were discarded from analysis. If multiple presentations of the stimulus series were performed on one animal, we used the series in which sucrose responses deviated the least for analysis. A two-way ANOVA (genotype × standard) was conducted to determine any significant differences between the sucrose standards throughout the duration of the preparation. Next, a three-way ANOVA (genotype × amiloride × concentration) comparing the effect of amiloride across genotypes was conducted for both NaCl and KCl responses. When main effects and interactions were found, individual t-tests were performed with unadjusted and Bonferroni-adjusted P values for each concentration. Next, a two-way ANOVA (genotype × stimulus) was conducted on the stimulus array, including concentrations used in the brief-access test, comparing the responses of TRPV1 KO and WT mice.

**RESULTS**

**Experiment 1**

**Conditioned taste aversion acquisition.** The conditioning of an aversion to 0.25 M NaCl prepared with 100 μM amiloride was achieved in both WT and TRPV1 KO mice (Fig. 1). A three-way ANOVA measuring the effect of injection on conditioning trials revealed no main effect or interaction of genotype on total CS intake across conditioning trials but did reveal a main effect of injection [F(1,32) = 75.861, P < 0.001], a main effect of conditioning trial [F(4,128) = 60.484, P < 0.001], and a trial × injection interaction [F(4,128) = 23.690, P < 0.001]. No significant differences were found for intake on the last conditioning trial between saline-injected TRPV1 KO and WT or between LiCl-injected TRPV1 KO and WT mice indicating that both genotypes treated the CS similarly in both injection groups. Finally, analysis of the final conditioning trial in both animals revealed significant differences in CS intake between LiCl- and NaCl-injected mice for both genotypes [TRPV1 KO: t(16) = −7.37, P < 0.001; WT: t(16) = −6.08, P < 0.001], confirming that an aversion was acquired in TRPV1 KO and WT mice.

**Generalization test.** All mice initiated at least one trial per stimulus in the brief-access test. No statistical difference was observed between groups for the number of stimulus trials initiated on the test day: NaCl-injected KO mice averaged 50.2 stimulus trials, LiCl-injected KO mice averaged 53.1 stimulus trials, NaCl-injected WT mice averaged 48.1 stimulus trials, and the LiCl-injected WT mice averaged 50 stimulus trials per session. Because no main effect [F(1,32) = 0.24, P = 0.878] or interactions (genotype × injection [F(1,32) = 0.32, P = 0.732]; genotype × stimulus [F(6,192) = 0.953, P = 0.459]; genotype × injection × stimulus [F(6,192) = 0.298, P = 0.937]) were observed for genotype in a three-way ANOVA, we collapsed the WT and TRPV1 KO subgroups and conducted a two-way ANOVA to compare the effect of injection on licking responses to the stimuli in the test array. This revealed main effects of injection [F(1,34) = 10.098, P = 0.003] and stimulus [F(6,204) = 59.587, P < 0.001] and a significant stimulus × injection interaction [F(6,204) = 11.918, P < 0.001]. Significant differences between injection groups for KCl, NH₄Cl, and for quinine were found regardless of whether a Bonferroni-adjustment was applied (Table 1; Fig. 2), suggesting that the CS had a quality similar to nonsodium salts and quinine regardless of genotype. There were no significant differences in licking to sucrose, citric acid, and the sodium salts between injection groups. Additionally, because sucrose was presented as the first stimulus of the test session...
for all mice, we also conducted a three-way ANOVA in which the response of each animal to the first sucrose presentation was omitted from the analysis. This did not change the significance of the statistical outcomes.

Experiment 2

Conditioned taste aversion acquisition. The conditioning of an aversion to 0.5 M NaCl treated with 100 μM amiloride was achieved in both WT and TRPV1 KO mice (Fig. 3). Fewer conditioning trials were necessary to condition an aversion in this experiment, likely due to the increased salience of the stimulus. The three-way ANOVA measuring the effect of injection on intake during conditioning trials revealed main effects of injection [F(1,32) = 14.695, P = 0.001] and trial [F(2,64) = 189.821, P < 0.001] and both a significant genotype × injection interaction [F(1,32) = 7.674, P = 0.009] and an injection × trial interaction [F(2,64) = 15.809, P < 0.001]. A two-way ANOVA performed on CS intake across conditioning trials revealed main effects of injection [F(1,16) = 19.028, P < 0.001] and both a significant genotype × injection interaction [F(1,16) = 19.028, P < 0.001] and an injection × trial interaction [F(2,32) = 9.754, P < 0.001]. A similar analysis in KO mice indicated a main effect of trial [F(2,32) = 94.517, P < 0.001] and an injection × trial interaction [F(2,32) = 7.319, P = 0.002]. A large decrease in intake from the first CS presentation to the second presentation was observed in saline-injected animals likely attributable to the hypertonicity of the CS. Indeed by the third conditioning trial there was a clear and significant difference in CS intake between the LiCl-injected mice and the saline-injected mice for both genotypes [TRPV1 KO mice: t(16) = −3.302, P = 0.013; WT mice: t(16) = −7.59, P < 0.001, Bonferroni adjusted], but there was no significant difference between saline-injected TRPV1 KO and WT or between LiCl-injected TRPV1 KO and WT mice regardless of whether a Bonferroni adjustment was applied or not. These data indicate an aversion to the CS was successfully conditioned.

Generalization test. All mice initiated at least one trial per stimulus in the brief-access test. No statistical differences were observed between groups for the number of stimulus trials initiated on the test day: NaCl-injected KO mice averaged 52.7 stimulus trials, LiCl-injected KO mice averaged 50 stimulus trials, NaCl-injected WT mice averaged 49.2 stimulus trials, and the LiCl-injected WT mice averaged 49 stimulus trials per session. A three-way ANOVA comparing the effect of injection between genotypes on the responses to the stimuli in the array indicated main effects of injection [F(1,32) = 18.788, P < 0.001] and an injection × trial interaction [F(2,32) = 94.517, P < 0.001] and a significant genotype × injection interaction [F(1,32) = 9.754, P < 0.001] and an injection × trial interaction [F(2,32) = 7.674, P = 0.009] and an injection × trial interaction [F(2,64) = 15.809, P < 0.001].

Table 1. t-Values comparing Taste/Water Lick Ratio lick ratio per stimulus between injection groups, collapsed across animals

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>t Value</th>
<th>P Value</th>
<th>Bonferroni-Adjusted Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M NaCl</td>
<td>34</td>
<td>t = −1.469</td>
<td>P = 0.150</td>
<td>adjusted P = 1.000</td>
</tr>
<tr>
<td>0.25 M NaGlu</td>
<td>34</td>
<td>t = 0.526</td>
<td>P = 0.602</td>
<td>adjusted P = 1.000</td>
</tr>
<tr>
<td>0.25 M KCl</td>
<td>34</td>
<td>t = −5.880</td>
<td>P &lt; 0.001</td>
<td>adjusted P &lt; 0.001</td>
</tr>
<tr>
<td>0.25 mM Quinine</td>
<td>34</td>
<td>t = −4.282</td>
<td>P &lt; 0.001</td>
<td>adjusted P &lt; 0.001</td>
</tr>
<tr>
<td>0.25 M NaGlu</td>
<td>34</td>
<td>t = −1.620</td>
<td>P = 0.109</td>
<td>adjusted P = 0.761</td>
</tr>
<tr>
<td>0.25 mM Quinine</td>
<td>34</td>
<td>t = −3.309</td>
<td>P &lt; 0.002</td>
<td>adjusted P = 0.016</td>
</tr>
<tr>
<td>0.5 M Sucrose</td>
<td>34</td>
<td>t = 0.692</td>
<td>P = 0.494</td>
<td>adjusted P = 1.000</td>
</tr>
</tbody>
</table>

df, Degrees of freedom. Statistically significant P values are in bold.

Table 2. t-Values comparing Taste/Water Lick Ratios between NaCl- and LiCl-injection groups for each instant in WT mice

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>t Value</th>
<th>P Value</th>
<th>Bonferroni-Adjusted Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M NaCl</td>
<td>16</td>
<td>t = −3.302</td>
<td>P &lt; 0.005</td>
<td>adjusted P = 0.031</td>
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<tr>
<td>0.5 M NaGlu</td>
<td>16</td>
<td>t = −3.163</td>
<td>P = 0.006</td>
<td>adjusted P = 0.042</td>
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<tr>
<td>0.5 M KCl</td>
<td>16</td>
<td>t = −4.201</td>
<td>P &lt; 0.001</td>
<td>adjusted P = 0.005</td>
</tr>
<tr>
<td>0.5 M NH4Cl</td>
<td>16</td>
<td>t = −4.646</td>
<td>P &lt; 0.001</td>
<td>adjusted P = 0.002</td>
</tr>
<tr>
<td>13.25 mM Citric acid</td>
<td>16</td>
<td>t = −3.816</td>
<td>P &lt; 0.002</td>
<td>adjusted P = 0.011</td>
</tr>
<tr>
<td>0.3 mM Quinine</td>
<td>16</td>
<td>t = −2.288</td>
<td>P = 0.036</td>
<td>adjusted P = 0.253</td>
</tr>
<tr>
<td>1.0 M Sucrose</td>
<td>16</td>
<td>t = −1.458</td>
<td>P = 0.164</td>
<td>adjusted P = 1.000</td>
</tr>
</tbody>
</table>

Statistically significant P values are in bold.
Table 3. t Values comparing Taste/Water Lick Ratios between NaCl- and LiCl-injection groups for each tastant in KO mice

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>KO: t Value</th>
<th>P Value</th>
<th>Bonferroni-Adjusted Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M NaCl</td>
<td>16</td>
<td>2.045</td>
<td>0.058</td>
<td>adjusted P = 0.404</td>
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<tr>
<td>0.5 M NaGlu</td>
<td>16</td>
<td>0.369</td>
<td>0.717</td>
<td>adjusted P = 1.000</td>
</tr>
<tr>
<td>0.5 M KCl</td>
<td>16</td>
<td>-2.302</td>
<td>0.035</td>
<td>adjusted P = 0.246</td>
</tr>
<tr>
<td>0.5 M NH4Cl</td>
<td>16</td>
<td>-3.069</td>
<td>0.0008</td>
<td>adjusted P = 0.051</td>
</tr>
<tr>
<td>13.25 mM Citric acid</td>
<td>16</td>
<td>-0.932</td>
<td>0.365</td>
<td>adjusted P = 1.000</td>
</tr>
<tr>
<td>0.3 mM Quinine</td>
<td>16</td>
<td>-4.146</td>
<td>0.001</td>
<td>adjusted P = 0.005</td>
</tr>
<tr>
<td>1.0 M Sucrose</td>
<td>16</td>
<td>0.360</td>
<td>0.723</td>
<td>adjusted P = 1.000</td>
</tr>
</tbody>
</table>

Statistically significant P values are in bold.

P < 0.001] and stimulus [F(6,192) = 177.546, P < 0.001] as well as both a significant genotype × injection interaction [F(1,32) = 6.387, P = 0.017] and a significant genotype × injection × stimulus interaction [F(6,192) = 3.699, P = 0.002]. Because significant effects involving genotype were found, the analysis was further broken down into two two-way ANOVAs, one for the Taste/Water Lick Ratios for WT mice and the other for TRPV1 KO mice. The two-way ANOVA performed on Taste/Water Lick Ratios for WT mice revealed main effects of injection [F(1,16) = 20.842, P < 0.001] and stimulus [F(6,96) = 98.205, P < 0.001], but no injection × stimulus interaction. The two-way ANOVA conducted on Taste/Water Lick Ratios for TRPV1 KO mice revealed a main effect of stimulus [F(6,96) = 82.134, P < 0.001] and a significant stimulus × injection interaction [F(6,96) = 3.502, P = 0.004]. LiCl-injected WT mice avoided all salt and acid stimuli, whereas LiCl-injected KO mice avoided only the nonsodium salts and quinine. This difference between LiCl-injected and NaCl-injected KO mice was marginal for NH4Cl and KCl as the statistical values did not survive the conservative Bonferroni adjustment (Table 2 and 3; Fig. 4).

Doubling the concentration of all of the taste stimuli changed the generalization profile for WT mice but had no effect on TRPV1 KO mice responses relative to that observed in experiment 1. The most salient difference between the profiles for WT and TRPV1 KO mice resides in the fact that the WT mice avoided the sodium salts as well as citric acid, whereas the TRPV1 KO mice did not. Additionally, as in experiment 1, we conducted a three-way ANOVA in which the first sucrose presentation was omitted from the analysis. Although a stimulus × injection interaction emerged, the new analysis had little impact on the results because a genotype × stimulus × injection interaction remained.

**Experiment 3**

All stimuli were normalized to sucrose, and those stimuli presented between sucrose standards deviating more than 15% were discarded from analysis (see Figs. 6, 7, and 8 for examples of WT and TRPV1 KO CT response traces). A two-way ANOVA comparing all sucrose standards for significant differences across genotypes revealed no main effect of genotype or sucrose presentation, indicating that all sucrose standards were relatively similar throughout the preparations (Fig. 5). A
three-way ANOVA comparing the effect of amiloride between genotypes across NaCl concentrations revealed main effects of amiloride \[F(1,19) = 137.838, P < 0.001\] and concentration \[F(3,57) = 133.626, P < 0.001\] and an amiloride \times concentration interaction \[F(3,57) = 40.687, P < 0.001\] but no main effect or interactions involving genotype (Fig. 6). Amiloride significantly reduced the CT response to NaCl at each concentration \[0.1 \text{ M}: t (20) = 5.813, P < 0.001; 0.3 \text{ M}: t (20) = 11.911, P < 0.001; 0.5 \text{ M}: t (20) = 11.485, P < 0.001; 1.0 \text{ M}: t (20) = 10.620, P < 0.001 (Bonferroni adjusted)\].

Three-way ANOVA comparing the effect of amiloride across genotypes on KCl also failed to reveal any genotype effects but found main effects of amiloride \[F(1,19) = 23.696, P < 0.001\] and concentration \[F(3,57) = 123.496, P < 0.001\] and an amiloride \times concentration interaction \[F(3,57) = 12.752, P < 0.001\] (Fig. 7). Amiloride significantly reduced the CT response to KCl at the two highest concentrations \[0.5 \text{ M}: t (20) = 5.379, P < 0.001; 1.0 \text{ M}: t (20) = 5.474, P < 0.001 (Bonferroni adjusted)\], and the difference in responses observed at 0.3 M concentration just missed the statistical rejection criterion \[t (20) = 2.060, P = 0.053\]. Nonetheless, the extent of this suppression was weak and suggests that at the high concentrations a small amount of K\(^+\) can penetrate through ENaCs. The two-way ANOVA conducted on responses of WT and TRPV1 KO mice to the array of solutions including stimulus concentrations used in the behavioral study failed to identify a main effect or interaction of genotype but did reveal a main effect of stimulus \[F(6,114) = 97.010, P < 0.001\] (Fig. 8).

**DISCUSSION**

The purpose of this study was to determine whether the qualitative nature of the amiloride-insensitive component of NaCl taste would be altered in mice lacking the TRPV1 channel, especially considering prior reports that the CT of TRPV1 KO mice appears to completely lack an amiloride-
insensitive response to NaCl placed on the anterior tongue (33, 34, 49). Here, we adopted the CTA generalization paradigm to pursue this question in which we conditioned an aversion to NaCl mixed with amiloride and then tested the conditioned mice for their generalization to various sodium and nonsodium salts as well as to other taste stimuli.

The generalization profiles of the TRPV1 KO mice mirrored those of WT mice to the stimulus array when 0.25 M NaCl mixed with amiloride served as the CS. Both LiCl-injected groups generalized the aversion to nonsodium salts and quinine, suggesting that the CS was qualitatively similar between genotypes. This profile of conditioned avoidance for WT mice was expected and is consistent with electrophysiological recordings from acid-generalist CT nerve fibers and neurons in the rat geniculate ganglion (where the soma of the CT nerve fibers reside), which respond best to salts, acids, and alkaloids (4, 5, 17, 32, 37). Additionally, our data correspond well with behavioral studies demonstrating that, when mixed with amiloride, NaCl adopts a nonsodium salt taste quality (27) and cannot be distinguished from other nonsodium salts in rodents (14, 21, 48). Furthermore, these data complement other behavioral studies demonstrating the similarity of NaCl detection thresholds in TRPV1 KO and WT mice (44, 49). The parallel generalization profiles between the two genotypes challenges the necessity of the TRPV1 ion channel in salt taste and is supported by our electrophysiological findings discussed below.

As seen with the generalization profiles generated from the animals conditioned to avoid the lower CS concentration, TRPV1 KO and WT mice generalized the aversion of 0.5 M NaCl mixed with amiloride to the nonsodium salts (KCl and NH₄Cl) and quinine, although avoidance of quinine in WT mice and avoidance of KCl and NH₄Cl in TRPV1 KO mice did not survive Bonferroni correction. Using an amiloride blockade of ENaC to isolate the amiloride-insensitive component involved in salt taste during the conditioning phase of the experiment, we prevented the stimulation of sodium-specialist neurons. This, therefore, changed the taste perception of NaCl,
which may explain why neither TRPV1 KO nor WT mice avoided the sodium salts when not prepared with amiloride during the test, at least at the lower CS concentration. When the concentration of the CS was doubled along with the stimuli in the test array, however, differences in the generalization profiles between the genotypes emerged. The conditioned (i.e., lithium paired) WT mice avoided the sodium salts during the test, whereas the conditioned TRPV1 KO mice showed no such aversion. This generalization to the sodium salts observed at the higher stimulus array concentrations in WT mice complements data from a similar study in which rats conditioned to avoid a 0.5 M NaCl solution mixed with amiloride subsequently avoided 0.5 M NaCl (with no amiloride) (27). The presence of an aversion to sodium salts in WT mice but not in TRPV1 KO mice may implicate the involvement of TRPV1 in the oral perception of 0.5 M NaCl when it is mixed with amiloride. This suggests that at higher NaCl concentrations, regardless of an amiloride blockade, nongustatory fibers are being activated through the mediation of TRPV1 channels. Arai et al. (1) demonstrated that fibers in the glossopharyngeal nerve, a mixed gustatory and somatosensory nerve, may be activated in a manner mediated by TRPV1 in the presence of high but not low or moderate concentrations of NaCl, KCl, NH₄Cl, and acetic acid. This study showed that the potent TRPV1 antagonist I-RTX had no effect on CT responses but significantly suppressed responses of the glossopharyngeal nerve to high concentrations of acids and salts. This evidence implicates the contribution of TRPV1 to the afferent neural signal in the presence of higher NaCl concentrations. Combined with evidence suggesting that hypertonic concentrations of NaCl, quinine, and acids elicit responses from the lingual nerve (42, 46, 53), TRPV1 activation may explain why WT but not TRPV1 KO mice formed an aversion to the sodium salts. Experiments conducted in humans have shown that higher concentrations of NaCl (i.e., 0.5 M) elicit irritation and such sensations can be attenuated by desensitizing the tongue with capsaicin (22, 23). This suggests that oral somatosensory sensations stimulated by NaCl might be mediated by TRPV1.

Just as the possibility that oral somatosensory signals were involved in the perceived quality of the taste stimuli, we cannot entirely rule out that olfaction could have played a role as well, especially at high concentrations of NaCl and sucrose (36, 43). If olfaction is contributing to the perceived quality of the high concentration of NaCl and NaGlu, however, then it would have to be mediated in part by TRPV1 as indicated by the pronounced difference between the WT and TRPV1 KO mice in their response to these stimuli.

Both WT and TRPV1 KO mice conditioned to avoid 0.25 M NaCl mixed with amiloride did not generalize the aversion to citric acid in the brief-access test. This was surprising given that acid-generalist fibers and ganglion cells are highly responsive to alkaloids, salts, and acids (4, 5, 17, 18, 32). In a similar behavioral study, Hill et al. (27) likewise found that Sprague-Dawley rats conditioned to avoid amiloride-treated NaCl avoided the nonsodium salts but not citric acid. When we doubled the concentrations of the CS and the stimulus array in the brief-access test, however, WT mice avoided citric acid but the TRPV1 KO mice showed no such aversion. The aversion to citric acid observed in experiment 2 is consistent with behavioral and electrophysiological data demonstrating that 1) citric acid may share perceptual characteristics with quinine (40, 50) and 2) some neurons and ganglion cells in the rodent respond best to acids and quinine, respectively (4, 5, 17, 18, 32). Alternatively, it may be that the higher concentration of citric acid is stimulating somatosensory fibers where TRPV1 is expressed in a manner similar to that observed by Arai et al. (1). These are not mutually exclusive possibilities.

Finally, we measured CT responses in WT and TRPV1 KO mice, including a subset of the subjects used in the brief-access test, to the stimulus array and also a concentration series of NaCl and KCl solutions prepared with and without amiloride. Our results are consistent with previous findings in the literature demonstrating the suppressive effect of amiloride on the CT response to NaCl (e.g., 3, 9, 19, 24, 38) but conflict with data from other CT recordings of TRPV1 KO mice in the

Fig. 8. Example CT trace (R.M.S. = root mean square) of WT (A) and TRPV1 KO (B) and CT response (means ± SE) (C) of WT mice (black) and TRPV1 KO mice (grey) to an array of stimuli representative of the canonical tastants.

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literature (33, 34). Subtle differences in the CT preparation such as the temperature at which the solution was presented on the tongue, use of amiloride rather than benzamil to block ENaC, duration of lingual presentation of the taste solution, and use of a vacuum chamber for stimulus presentation (33, 34) may account for the disparity observed in WT and TRPV1 KO recordings here and in other literature. Nevertheless, the similarity observed in CT responses in TRPV1 KO and WT mice in our study may explain the ability of TRPV1 KO mice to acquire a CTA to either 0.25 M or 0.5 M NaCl mixed with amiloride and also may explain the lack of a difference observed in the generalization profiles between the two genotypes at the lower CS concentration. Additionally, the similarity in CT responses of TRPV1 KO and WT mice supports the view that other TRPV1-independent amiloride-insensitive salt taste transduction pathways exist. The emergence of a phenotypic difference in the behavioral responses of WT and TRPV1 KO at the higher sodium concentrations, which in humans is known to cause irritation (22, 23), suggests that these concentrations are potentially stimulating nongustatory afferents such as in the glossopharyngeal nerve (1). Given the outcome of our experiment, the trigeminal nerve, which provides somatosensory innervation to the anterior tongue, should be electrophysiologically examined in TRPV1 KO and WT mice to investigate the contribution of TRPV1-dependent signals in this nerve in response to high concentrations of salts.

**Perspectives and Significance**

Salt taste depends on at least two transduction mechanisms, the well-documented ENaC that is selective for sodium and blocked by amiloride, and the other(s), an unknown amiloride-insensitive mechanism that has been an unsolved riddle for many years. It has been proposed (33, 34) that the amiloride-insensitive taste transduction component may involve an apically located TRPV1 channel broadly responsive to several cations including sodium, but this mechanism has not been supported. Breza and Contreras (6) showed that TRPV1 agonists, SB-366791 and cetylpyridinium chloride, were without effect on CT nerve or single-cell responses to NaCl. The present study found that the CT nerve responses to NaCl with and without amiloride were the same in WT and TRPV1 KO mice. Furthermore, it is apparent that the ablation of TRPV1 does not lead to a clear taste-related behavioral phenotype in mice, at least with respect to low to mid-range concentrations of salt stimuli (44, 49). This means that there is a TRPV1-independent amiloride-insensitive salt transduction pathway(s) that is sufficient to maintain relatively normal salt taste perception even when oral ENaCs are blocked. At the same time, our behavioral data do implicate a role for TRPV1 in mediating oral perception of NaCl and perhaps citric acid at high concentrations in mice, likely through lingual somatosensory processes. Such a possibility highlights the importance of controlling for such nontaste signals in experiments designed to assess the behavioral consequences of manipulations of the gustatory system or at least considering such signals in the interpretation of outcomes.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

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