Strain Differences in the Neural, Behavioral and Molecular Correlates of Sweet and Salty Taste in Naïve, Ethanol- and Sucrose-exposed P and NP Rats

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ABSTRACT

Strain differences between naïve, sucrose- and ethanol-exposed P and NP rats were investigated in their consumption of ethanol, sucrose and NaCl; chorda tympani (CT) nerve responses to sweet and salty stimuli and gene expression in the anterior tongue of T1R3 and TRPV1/1TRPV1t. Preference for 5% ethanol and 10% sucrose, CT responses to sweet stimuli and T1R3 expression were greater in naïve P rats than NP rats. The enhancement of the CT response to 0.5 M sucrose in the presence of varying ethanol concentrations (0.5%-40%) in naïve P rats was higher and shifted to lower ethanol concentrations than NP rats. Chronic ingestion of 5% sucrose or 5% ethanol decreased T1R3 mRNA in NP and P rats. Naïve P rats also demonstrated bigger CT responses to NaCl+benzamil and greater TRPV1/1TRPV1t expression. TRPV1t agonists produced biphasic effects on NaCl+benzamil CT responses, enhancing the response at low concentrations and inhibiting it at high concentrations. The concentration of a TRPV1/1TRPV1t agonist (Malliard Reacted Peptides conjugated with glalacturonic acid) that produced a maximum enhancement in the NaCl+benzamil CT response induced a decrease in NaCl intake and preference in P rats. In naïve P rats and NP rats exposed to 5% ethanol in a no choice paradigm, the biphasic TRPV1t agonist versus NaCl+benzamil CT response profiles were higher and shifted to lower agonist concentrations than naïve NP rats. TRPV1/1TRPV1t mRNA expression increased in NP rats but not in P rats exposed to 5% ethanol in a no choice paradigm. We conclude that P and NP rats differ in T1R3 and TRPV1/1TRPV1t expression, neural and behavioral responses to sweet and salty stimuli and to chronic sucrose and ethanol exposure.

Key Words: Salt taste, sweet taste, chorda tympani, RTX, benzamil
INTRODUCTION

Ethanol is a gustatory stimulus and elicits responses in chorda tympani (CT) and glossopharyngeal taste nerves and in neurons of the nucleus of the solitary tract (Danilova and Hellekant 2000; Hellekant et al. 1997; Lemon et al. 2004; Lyall et al. 2005a,b; Sako and Yamamoto 1999; Brasser et al. 2010). In conditioned taste aversion studies in inbred mice, C57BL/6J mice generalized taste aversions from sucrose and quinine solutions to 10% ethanol and, reciprocally, aversions to 10% ethanol were generalized to each of these solutions presented separately. Only conditioned aversions to quinine generalized to ethanol in the DBA/2J mice but an aversion conditioned to ethanol did not generalize reciprocally to quinine. Thus, considering these two gustatory qualities, 10% ethanol tastes both sweet and bitter to C57BL/6J mice, but only bitter to DBA/2J (Blizard 2007). In contrast in outbred rats sucrose and quinine aversions did generalize to various alcohol concentrations but ethanol did not generalize to sucrose and quinine but only to mixtures of these compounds (Lawrence and Kiefer 1987; Kiefer and Mahadevan 1993; Kiefer and Lawrence, 1998; Blizard 2007). These studies suggest that in some inbred strains, ethanol has both a sweet taste quality as well as a bitter taste quality and, is thus, expected to interact with both sweet and bitter taste transduction pathways.

In mixtures, ethanol acutely enhances sweet (Hellekant et al. 1997; Sako and Yamamoto 1999) and salty responses (Lyall et al. 2005a,b) and suppresses sour and bitter responses in the CT nerve (Hellekant et al. 1997; Sako and Yamamoto 1999). Ethanol produces changes in taste nerve responses to salty, sour, sweet and bitter stimuli by interacting with quality specific taste receptors or with intracellular signaling.
effectors in taste receptor cells (TRCs) (Lyall et al. 2005a,b). The sweet taste receptor serves as a receptor for both sucrose and ethanol. Both the taste of sucrose and ethanol are represented similarly in gustatory regions of the central nervous system. Gurmarin, a sweet receptor blocker, administered orally specifically inhibited both ethanol and sucrose responses (Lemon et al. 2004; Brasser et al. 2010). In mixtures, ethanol acutely modulates NaCl CT responses by specifically interacting with a putative TRPV1t-dependent Bz-insensitive salt taste receptor (Lyall et al. 2005a,b). The alcohol preferring (P) and alcohol nonpreferring (NP) rats (Bice and Kiefer, 1990) and alcohol-naïve high (HAD) and low (LAD) alcohol drinking rats demonstrated similar taste reactivity responses to a range of alcohol concentrations. However, differences between taste reactivity between two strains became apparent only after rats were chronically exposed to ethanol (Kiefer et al. 1995).

A phenotypic linkage has been demonstrated between ethanol intake and several taste qualities. Differences in sensitivity to or preference for salty taste have been reported in subjects with a paternal history of alcohol-dependence relative to control subjects with no paternal history (Scinska et al. 2001). A positive association exists between ethanol intake and sweet taste (Stewart et al. 1994; Woods et al. 2003) involving the gene for T1R3 (Bachmanov et al. 2001, 2002; Blednov et al. 2007, 2008; Blizard, 2007; Inoue et al. 2004; Lu et al. 2005; Nelson et al. 2001; Brasser et al. 2010), a G-protein coupled receptor (GPCR) that combines with another GPCR (T1R2) to function as the broadly tuned, heterodimeric, sweet-taste receptor, T1R2+T1R3 (Li et al. 2002; Zhao et al. 2003). Alcohol-dependence and use also show significant association with the T2R38 gene, a marker for 6-n-propylthiouracil bitterness and with hT2R16, a
gene encoding a taste receptor for the bitter-tasting β-glucopyranosides (Duffy et al. 2004; Hinrichs et al. 2006).

Accordingly, we hypothesize that neural, behavioral and molecular correlates of sweet and salty taste quality can be modulated by acute and chronic ethanol exposure and by genetically inducing changes in taste preferences. To test this hypothesis, studies were performed on selectively bred P and NP rats. P rats were chosen because they voluntarily consume ethanol and develop tolerance and dependence through free-choice drinking (Files et al. 1993; Heyman 2000). In P rats, high oral alcohol preference appears to be positively associated with consumption of sweet-tasting solutions and negatively associated with intake of salty solutions (Stewart et al. 1994). We investigated taste behavior differences between P and NP rats by standard two-bottle 24 hr preference tests for ethanol, sucrose and NaCl, in their CT responses to sweet-tasting and salty stimuli and in their expression levels of T1R3 and TRPV1/TRPV1t mRNA in the anterior lingual epithelium containing fungiform taste papillae before and after chronic oral exposure to ethanol or sucrose. Our results suggest that in P rats, the increase in TRPV1t and T1R3 mRNA expression in the anterior tongue correlates with the increased neural and behavioral responsiveness to salty and sweet-tasting stimuli in the absence and presence of ethanol.
MATERIALS AND METHODS

Animals were housed in the Virginia Commonwealth University animal facility in accordance with institutional guidelines. All animal protocols were approved by the Institutional Animal Care and Use Committee. Six week old 75 alcohol-preferring (P) and 71 alcohol-nonpreferring (NP) female rats (~150 gm) were obtained from Indiana University School of Medicine, Indianapolis, IN. In addition to P and NP rats, 9 Sprague-Dawley (SD) rats (150–200 gm) were obtained from Charles River Laboratories International, Inc. Wilmington, MA. We also obtained 12 wildtype (WT; C57BL/6J) and 3 homozygous TRPV1 knockout (KO) mice (B6. 129S4-Trpv1tmjul) (30-40 gm) from The Jackson Laboratory, Bar Harbor, ME. Upon arrival, P and NP rats were placed in individual plastic cages and given ad libitum access to water and rat chow and kept in quarantine in the VCU animal facility for 3 weeks. The animals were housed in rooms maintained at 22-26°C and 30-70% humidity on 12 hr day and night cycle. At the end of 3 weeks the P and NP rats were moved along with SD rats, WT and KO mice to sterile rooms for the duration of the experiment. The animals were divided into 12 groups and used as described in Table 1.

CT taste nerve recordings

The female rats were anesthetized by intraperitoneal injection of pentobarbital (60 mg/Kg body weight) and supplemental pentobarbital (20 mg/Kg body weight) was administered as necessary to maintain surgical anesthesia. The animal’s corneal reflex and toe-pinched reflex were used to monitor the depth of surgical anesthesia. Body temperatures were maintained at 37°C with a Deltaphase Isothermal PAD (Model 39
The left CT taste nerve was exposed laterally as it exited the tympanic bulla and placed onto a 32G platinum/iridium wire electrode. Stimulus solutions maintained at room temperature were injected into a Lucite chamber (3 ml; 1 ml/s) affixed by vacuum to a 28 mm² patch of anterior dorsal lingual surface. The CT responses were recorded under zero lingual current-clamp and analyzed as described previously (Katsumata et al. 2008; Lyall et al. 2009a, 2010). During surgery P and NP rats in particular demonstrated a propensity to bleed. However, following surgery bleeding stopped within 10-15 minutes. The wound around the isolated CT nerve was then cleaned with a cotton swab dipped in saline to remove clotted blood. The wound cavity was filled with mineral oil before commencing with recording. We were able to record stable and reproducible CT responses from P and NP rats for extended periods (Supplementary Fig. 1).

CT responses were also monitored in WT and TRPV1 KO mice. Mice (30-40 gm) were anesthetized by intraperitoneal injection of pentobarbital (30 mg/Kg body weight) and supplemental pentobarbital (10 mg/Kg body weight) was administered as necessary to maintain surgical anesthesia. The rest of the procedure was the same as in rats. At the end of each experiment animals were humanely killed by an intraperitoneal overdose of pentobarbital (approximately 195 mg/Kg body weight for rats and 150 mg/Kg weight for mice).

The composition of the various stimulating solutions and the list of drugs and their concentrations used in the CT experiments are given in Table 2. The anterior lingual surface was stimulated with the rinse solution (R; pH 6.4) and then with a salt solution (N; pH 6.4) with or without the TRPV1 agonists: ethanol (ETOH; 0-40%),
resiniferatoxin (RTX; 0-10x10^-6 M) and Maillard Reacted Peptides (MRPs) conjugated with galcturonic acid (GalA-MRPs; 0-1.5%). In previous studies, the TRPV1t agonist-induced increase in the Bz-insensitive NaCl CT response varied with pH. The relationship between pH and the magnitude of the CT response was bell shaped. The maximum increase in the CT response was observed around pH 6.4 (Lyall et al. 2004). Benzamil (Bz; 5x10^-6M) was used to block Na^+ entry via the apical epithelial Na^+ channels (ENaC) and N-(3-methoxyphenyl)-4-chlorocinnamide (SB-366791) was used to block TRPV1t activity. In some studies, we topically applied U73122, a non-specific blocker of phospholipase Cs (PLCs) and its inactive analogue, U73343. BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-(acetoxyethyl) ester) was used to chelate intracellular Ca^{2+} (Table 2).

Typically, stimulus solutions remained on the tongue for 1-2 min. Control stimuli consisting of 0.3 M NH_4Cl and 0.3 M NaCl (Table 2) applied at the beginning and at the end of experiment were used to assess preparation stability (Fig. 4B). The preparation was considered stable only if the difference between the magnitude of the control stimuli at the beginning and at the end of the experiment was less than 10% (Lyall et al. 2009a,b, 2010). The following stimulus series were used in the CT experiments:

\[
\begin{align*}
R & \rightarrow 0.3 \text{M } \text{NH}_4\text{Cl} \rightarrow R \rightarrow 0.3 \text{M } \text{NaCl} \rightarrow R \rightarrow (N+Bz) \rightarrow R \rightarrow (N+Bz+\text{TRPV1t agonist}) \rightarrow R
\end{align*}
\]

The \( R \rightarrow (N+Bz+\text{TRPV1t agonist}) \rightarrow R \) step was repeated for each agonist concentration (Table 2). At the end of the RTX concentration series, the control stimuli were again applied (\( R \rightarrow 0.3 \text{ M } \text{NH}_4\text{Cl} \rightarrow R \rightarrow 0.3 \text{ M } \text{NaCl} \rightarrow R \)). At the end of experiment
the rats were humanely killed by an intraperitoneal overdose of pentobarbital (approximately 195 mg/Kg body weight).

**Data and statistical analysis**

The data were digitized and analyzed off line. In CT experiments tonic (steady state) part of the NaCl neural responses were quantified. To quantify the tonic part of a response the area under the response versus time curve was taken over the final 30s of the response. To normalize, this area was divided by the area under the 0.3 M NH₄Cl response curve over the final 30s of the tonic response period. The normalized data were reported as mean±SEM of the number of animals (n). To display a comparison of the relative magnitudes of the responses of NP and P rats to given taste stimuli, the data were normalized by dividing the response to each taste stimulus by the mean tonic response of the rat to 0.3 M NH₄Cl. Student’s t-test was employed to analyze the differences between sets of data. Since we are comparing the normalized CT responses before and after TRPV1t modulators in the same CT preparation, a paired t-test was used to evaluate statistical significance. When comparing normalized data between P and NP rats the unpaired ‘t’ test was used. In experiments in which repeated measurements to sequential drug applications were obtained, the data were analyzed using two-way ANOVAs with Bonferroni post-hoc tests (Oliveira-Maia et al. 2009).

For clarity the points on the graphs of the mean normalized tonic responses vs. the logarithm of the agonist concentration were connected respectively by smooth curves. The curves were generated using a fitting function that models the characteristic biphasic property of the agonist concentration versus the magnitude of the CT response. The biphasic property has been observed with every agonist of amiloride-
and Bz-insensitive NaCl CT response thus far examined (Katsumata et al. 2008; Lyall et al. 2004, 2005b, 2007, 2009a, 2010). The fitting function used was:

\[ R = \frac{r + bh(x)}{1 + h(x) + j(x)} \]  

(1)

where

\[ h(x) = 10^{n(x-a)} \]  

(2)

and

\[ j(x) = 10^{n(x-a)+m(x-d)} \]  

(3)

Here \( R \) is the response, \( x \) is the logarithm of the agonist concentration expressed in moles/liter and \( a, b, d, m, n, \) and \( r \) are parameters chosen by least squares criteria (Katsumata et al. 2008; Lyall et al. 2009a, 2010). For responses plotted against increasing ethanol concentration we used the fitting function:

\[ R = r + \frac{a c^n}{c^n + k^n} \]  

(4)

Here \( R \) is the response, \( c \) is ethanol concentration, and \( r, a, k, \) and \( n \) are parameters chosen by least squares criteria.

**Chronic ethanol and sucrose exposure**

P and NP rats were initially maintained on free access to food and water. The sucrose-fading paradigm (Samson 1986) was used to induce ethanol intake in rats (Table 1). Rats were given free access in their cages to 5% sucrose solution as reinforcement. Then, every third day the rats were switched to a sucrose-ethanol mixture containing: 3% sucrose/2% ethanol, 1% sucrose/4% ethanol, and finally to 0
sucrose/5% ethanol. The 5% ethanol regimen was maintained for 2-3 weeks (Table 1).

In a test choice paradigm group, 4 P and 4 NP rats always had two bottles in their cages, one bottle contained H₂O and the other the test solution (either a varying sucrose/ethanol mixture or ethanol alone; Group 5). In a control choice paradigm group (Group 6), another set of 4 P and 4 NP rats always had 2 bottles, one bottle contained H₂O and the other a test solution (either varying sucrose concentration or H₂O). In Group 7 additional 3 P and 3 NP rats were given 2 bottles, one containing H₂O and the other 10% sucrose. In a no choice paradigm test group, additional 14 P and 4 NP rats were given one bottle containing the test solution (either a varying sucrose/ethanol mixture or ethanol alone; Group 1). In the control no choice paradigm group (Group 2), additional 25 P and 28 NP rats were given free access in their cages to 5% sucrose solution and then every third day were switched to 3% and 1% sucrose and finally to H₂O. Control rats were then maintained for 2-3 weeks on H₂O. In Group 3, 4 P and 14 NP rats were given one bottle containing 5% sucrose and in Group 4, 5 P and 3 NP rats were given one bottle containing 10% sucrose. P and NP rats were used for CT nerve recordings or were sacrificed and their tissues were collected for gene expression and protein analysis.

Behavioral Studies

Behavioral studies were performed in naïve female P and NP rats (150-200 gm) using standard two bottle/24 hr tests. In Group 8 (Table 1), 13 P and 8 NP female rats were given a choice between two bottles, one containing H₂O and the other a test solution in the following order H₂O, 0.15M NaCl, 0.15M NaCl+5x10⁻⁶ M Bz or 0.15M
NaCl+5x10^{-6} M Bz+0.25\% GalA-MRPs (Tordoff and Bachmanov 2003). When Bz was used with the salt solution, Bz was also added to the second bottle containing H2O. For each 24 hr period the volume of H2O versus the volume of the test solution consumed by each rat was measured. The volumes consumed for each solution were converted to g of fluid consumed by taking into account the density of each solution. The preference ratio for a taste stimulus was calculated as the gm of the test solution consumed/24 hr/gm body weight divided by the total fluid intake (gm of H2O/24 hr/gm body weight + gm of the test solution/24 hr/gm body weight). The bottles containing H2O or the test solution were switched from left to right every day. The same rat was tested for fluid consumption for each solution for 4 days from Monday to Friday of each week. From Friday to next Monday the animals were maintained on two bottles containing H2O. Before the start of the experiment rats were given two bottles with H2O for 2 weeks. The experiment was started when rats were accustomed to drinking equally from two bottles. At this time point the preference ratios for two bottles containing water in 13 P rats (0.49±0.03 and 0.50±0.03) and 8 NP rats (0.46±0.03 and 0.49±0.03) were not statistically different.

In some P and NP rats in Groups 1, 2, 5 and 6, we monitored intake of 5% sucrose during the start of the sucrose-fading protocol (0-3 days; Table 1). At the end of the sucrose-fading protocol, we also monitored their intake of 5% ethanol (ETOH (10-13 days; Table 1). In 5 naïve P and 3 naïve NP rats (Group 4; Table 1), fluid consumption was measured when rats were given a single bottle containing 10% sucrose over a period of 4 days. In Group 7, fluid consumption was measured in 3 P and 3 NP rats given a choice between two bottles, one containing H2O and the other 10% sucrose.
over a period of 4 days. In a separate experiment, we also tested the effect of Bz on H2O intake in 3 female SD rats using standard two bottle/24 hr test (Group 10; Table 1). The rats were given a choice between two bottles, one containing H2O and the other H2O or H2O+5x10^{-6} M Bz. The data were analyzed using one-sample t-tests against 0.5, a reference value meaning indifference of the test solution with respect to the control solution (Oliveira-Maia et al., 2009).

**Western blots for TRPV1/TRPV1t and T1R3**

Some P and NP rats in Groups 2 (Table 1) were sacrificed and their tissues were collected for protein expression studies. The isolated anterior epithelia containing fungiform taste buds or intestinal mucosal cells were pooled from 4 P or 4 NP rats maintained on H2O (Group 2; Table 1). Rats were anesthetized by exposing them to an inhalation anesthetic, isoflurane (1.5 ml) in a desiccator. When the rats were fully unconscious, a midline incision was made in the chest wall and the aorta severed. The tongue and small intestine were then rapidly removed and stored in ice-cold Ringer’s solution (Table 2). The lingual epithelium was isolated by collagenase treatment as described earlier (Lyall et al. 2004). The small intestine was cut open, washed with ice-cold Ringer’s solution (Table 2) and the mucosa was scrapped gently using a glass slide. Protein from pooled tissues was isolated in RIPA Lysis buffer (Pierce) supplemented with the Complete Mini protease inhibitor pill (Roche). Protein was measured using the Pierce BCA kit and 40 μg protein samples were loaded into each well of a Criterion 10% polyacrylamide gel (BioRad), separated by PAGE and transferred to PVDF. The resulting blots were blocked in Odyssey Blocking Buffer (LI-
COR Biosciences) and then incubated in $\alpha$-T1R3 (sc-22459; polyclonal; dilution 1-1000; Santa Cruz), $\alpha$-TRPV1 antibody (C-15, sc-12503; polyclonal; 1-1000 dilution; Santa Cruz), $\beta$-actin (A-5441; dilution 1-5000; Sigma) or GAPDH (sc-32233; 1-1000; Santa Cruz). After washing, the blots were incubated in IRDye donkey $\alpha$-goat secondary antibody (LI-COR Biosciences), washed and rinsed, and then scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences). The resulting bands were analyzed using AIDA v.3.52 image analysis software (Raytest).

Quantitative (q)-RT-PCR for TRPV1/TRPV1t and T1R3

RNA was isolated from lingual epithelium containing fungiform taste buds from a separate group of 5 P and 5 NP rats in Groups 1, 2 and 3 (Table 1) using RNEasy (Qiagen) and cDNA was synthesized using M-MLV (Invitrogen) from 5 $\mu$g RNA in a 50 $\mu$l reaction. Primers for qRT-PCR were designed using Micro SEQ ® ID Analysis Software V2.0 and made by Integrated DNA Technologies (IDT, Inc, Coralville, IA) with rat actin used as an internal control. Expression of T1R3 and TRPV1 were analyzed using the TaqMan PCR Master Mix Reagent Kit on the ABI Prism 7300 system. Each sample was tested in triplicate. Cycle threshold ($C_T$) values were obtained graphically for T1R3 and TRPV1/TRPV1t and $\beta$-actin. The difference in $C_T$ values between $\beta$-actin and T1R3 or TRPV1/TRPV1t was represented as $\Delta C_T$ values. Because the tissue samples were obtained from different animals, there is no means to justify which positive sample is compared with which control sample, Therefore, we did not use the $2^{-\Delta \Delta CT}$ method to calculate the data (Schmittgen and Livak, 2008). The mean±SEM for each group of P and NP rats was calculated as individual data points using:
\[ 2^{-\Delta CT} \left[ 2^{-(CT_{\text{test}} - CT_{\beta-actin})} \right] \]  

An unpaired Student’s t-test was used to determine whether the difference in the mean±SEM values of \(2^{-\Delta CT}\) between two groups was significant (Schmittgen and Livak, 2008). The relative fold change in gene expression was calculated as the ratio between mean \(2^{-\Delta CT}\) value of the test group divided by the mean \(2^{-\Delta CT}\) value of the control group. The efficiencies of the amplification for qRT-PCR assays for TRPV1 and T1R3 were 100.2% and 102.0%, respectively.

**Primers for qRT-PCR**

**Rat TRPV1**

Sense  
5’- GACATGCTTCTCGTGAACCCTTG-3’  nucleotides 1312-1334

Anti-sense  
5’ –CCACAGGCCGATAGTAGGCAGC-3’  nucleotides 1452-1431

**Rat T1R3**

Sense  
5’-CCAGGTGCCAGTCTCCCA GTGC-3’  nucleotides 1491-1512

Anti-sense  
5’-GTAGCTCCCTGCCTTGCAGTCCAC-3’  nucleotides 1602-1579

**RT-PCR for TRPM5, \(\alpha\)-gustducin and TRPV1/TRPV1t**

The lingual epithelium was isolated from additional 5 WT mice (Group 12; Table 1) by collagenase treatment (Lyall et al. 2004). The anterior part of the tongue was used to isolate fungiform taste buds (FF TBs) and the posterior part of the tongue was used to isolate circumvallate taste buds (CV TBs) as described before (Lyall et al. 2004). We also harvested the non-gustatory lingual epithelium (NG Epi) without taste buds. RT-PCR was performed to detect the presence of TRPM5, \(\alpha\)-gustducin and TRPV1/TRPV1t...
in isolated mouse FF TBs, CV TBs and NG Epi. All primers for RT-PCR were made by IDT.

*Mouse TRPV1* (Wang et al. 2004)

<table>
<thead>
<tr>
<th>Sense</th>
<th>5'-CGGTTTATGTTCTACCTCGTGTTCTTGGTTTGG-3'</th>
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<tbody>
<tr>
<td>Anti-sense</td>
<td>5'-GCTCTCTTGTGACATCTTGGACAGTCTCGCC-3'</td>
</tr>
</tbody>
</table>

*Mouse α-gustducin* (DeFazio et al. 2006)

<table>
<thead>
<tr>
<th>Sense</th>
<th>5'-GCAACCACCTCCATTGTCTT-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-sense:</td>
<td>5'-AGAAGAGCCCACAGTCTTTGAG-3'</td>
</tr>
</tbody>
</table>

*Mouse TRPM5* (DeFazio et al. 2006)

<table>
<thead>
<tr>
<th>Sense</th>
<th>5'-GTCTGGAATCACAGGCCAAC-3'</th>
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<tbody>
<tr>
<td>Anti-sense:</td>
<td>5'-GTTGATGTGCCCCAAAAC-3'</td>
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**Quantification of fungiform papillae in P and NP rat tongues**

The lingual epithelium from 3 P and 3 NP rats (Group 9; Table 1) was isolated by collagenase treatment as described earlier (Lyall et al. 2004). The lingual epithelium was placed in Ringer's solution (Table 2), cut open and placed on a black rubber stopper with the basolateral membrane facing up and stretched with small stainless steel pins. The fungiform papillae were visualized and counted using a JENA stereomicroscope at 6.3X2.5 magnification.
RESULTS

Ethanol, sucrose and NaCl consumption and preference ratios in P and NP rats

In two-bottle 24 hr preference tests, NP rats (Group 5; Table 1) consumed significantly less 5% ethanol (Fig. 1A; filled bar) relative to H2O (open bar). This indicates that NP rats find ethanol aversive (Fig. 1A; crosshatched bar; **p<0.001; one sample t test). Consistent with previous studies (Kiiianmaa et al. 1991), P rats (Group 5; Table 1) consumed a significantly greater amount of 5% ethanol relative to H2O and, thus, demonstrated an innate preference for ethanol (Fig. 1A; hatched bar; p <0.002; one sample t test). Both naïve P and NP rats (Group 7; Table 1) also consumed a significantly greater amount of 10% sucrose than H2O (Fig. 1A). This indicates that like many species of rodents, NP (**p <0.001) and P (***p <0.0001; one sample t test) rats also demonstrate a preference for sweet-tasting stimuli (Boughter and Bachmanov 2007). P rats consumed a greater amount of sucrose relative to NP rats (Fig. 1A; filled bar; p = 0.0005; unpaired), and, thus, demonstrate a greater preference for sucrose relative to NP rats (Fig. 1A; hatched bar; p = 0.025; unpaired).

When given a single bottle containing 5% ethanol (Group 1; Table 1), NP rats consumed a greater amount of ethanol (Fig. 1B) relative to NP rats given a choice between H2O and ethanol (Fig. 1A; p = 0.0001; unpaired). P rats (Group 1; Table 1) still consumed significantly more ethanol than NP rats from a single bottle containing 5% ethanol (Fig. 1B; p = 0.009). However, their consumption of ethanol was the same as in P rats given a choice between H2O and ethanol (Fig. 1A; p>0.05; unpaired). In a no choice paradigm, P rats (Group 4; Table 1) still consumed more 10% sucrose solution than NP rats (Fig. 1B; p = 0.047), however, their consumption of sucrose was not
different from P rats given a choice between H$_2$O and sucrose (Fig. 1A; p>0.05). In a no choice paradigm, P and NP rats consumed a greater amount of 5% sucrose (Group 2; Table 1) than 10% sucrose solutions (Group 4; Table 1). P rats also consumed a significantly greater amount of 5% sucrose relative to NP rats (Fig. 1B; p = 0.004).

Taken together, the results show that genetically induced alcohol preference in P rats is associated with increased preference for sweet (sucrose) taste stimuli.

In two-bottle 24 hr preference tests, both naïve P and NP rats (Group 8; Table 1) consumed a significantly greater amount of 0.15 M NaCl (Fig. 1C; filled bars) relative to H$_2$O (open bars) with preference values significantly greater than 0.5 (*p <0.002 and **p <0.001, respectively; one-sample t-test). This is consistent with previous observations that rats prefer hypotonic and isotonic NaCl solutions to water (Fregly 1956). No difference was observed in NaCl preference between P and NP rats (p >0.05). P and NP rats also consumed a significantly greater amount of 0.15M NaCl+5x10$^{-6}$ M Bz (Fig. 1C; solid bars) than H$_2$O+Bz (Fig. 1C; open bars) with preference values significantly greater than 0.5 (*'p <0.03 and **'p <0.01, respectively; one-sample t-test). In sodium-replete rats, amiloride (an ENaC blocker) did not alter NaCl lick-rate (Brot et al. 2000). This suggests that sodium-replete rats retain preference for NaCl in the presence of amiloride. It is likely that in our long-term behavioral studies, rats maintained on Bz in two-bottle/24 hr preference tests become sodium depleted. In sodium depleted animals one would predict an increased NaCl intake to maintain sodium homeostasis. In rats made ‘sodium depleted’ by using the diuretic, furosemide, in short term licking tests, adding amiloride to the NaCl solutions reduced lick rates to ~20-25% of baseline (Brot et al. 2000). These results suggest that even under sodium depleted condition, inhibiting
ENaC activity by amiloride or Bz decreases sodium intake. However, Bz did not alter NaCl intake or preference in P and NP rats (p >0.05) suggesting that under the experimental conditions used in our studies, P and NP rats were not sodium depleted. Alternately, it is also possible that the effects of Bz on NaCl intake or preference are more complex. Post-absorptive effects of Bz may cause sodium depletion and sodium appetite, but effects of Bz on taste bud cells suppressed increased preference for NaCl.

Unlike amiloride, rats react to Bz indifferently. In 3 SD rats (Group 10; Table 1) given a choice between H₂O and H₂O+5x10^-6 M Bz, the preference ratio for H₂O+Bz was 0.55±0.05 (mean±SEM), a value not different from 0.5 (p>0.05). This indicates that at the concentration used in our experiments, Bz is not an aversive stimulus (i.e. bitter) to rats, and thus offers a significant advantage over amiloride in taste behavioral studies. These results suggest that both Bz-sensitive (ENaC-dependent) and Bz-insensitive (putative TRPV1t-dependent) salt taste receptors in the peripheral taste receptive fields contribute to NaCl preference in P and NP rats.

We hypothesize that modulating the activity of the putative TRPV1t-dependent Bz-insensitive salt taste receptor will produce differential effects on salt taste behavior in P and NP rats. To test this hypothesis, we investigated the effect of 0.25% GalA-MRPs on NaCl and H₂O consumption in P and NP rats (Group 8; Table 1). Consistent with our previous observations in SD rats and WT mice (Katsumata et al. 2008), 0.25% GalA-MRPs produced a maximum enhancement in the Bz-insensitive NaCl CT response in NP rats (Supplementary Fig. 1). Addition of 0.25% GalA-MRPs to NaCl+Bz solutions produced a significant decrease in NaCl intake (Fig. 1C; filled bars; p = 0.0043) and NaCl preference in P rats (Fig. 1C; crosshatched bars; p = 0.0015; unpaired; N = 13). In
contrast, in NP rats, GalA-MRPs produced a small but significant decrease in NaCl intake ($p = 0.047$) without a change in NaCl preference ($p > 0.05$). These results suggest that in P rats, further up-regulating a putative TRPV1t-dependent salt taste receptor activity by GalA-MRPs decreases NaCl intake and preference. When added to the rinse solution, GalA-MRPs (0.05%-1%) do not elicit a CT response [Katsumata et al. 2008]. Similarly, in human sensory evaluation, MRPs were also reported to have no taste of their own [Katsumata et al. 2008]. Thus, it is unlikely that the effect of GalA-MRPs on NaCl intake and preference can be related to its aversive taste. Furthermore, if rats react to GalA-MRPs as an aversive stimulus, it should affect NaCl intake and preference equally in both P and NP rats.

T1R3 and TRPV1/TRPV1t expression in the anterior lingual epithelium containing fungiform taste buds and in intestinal mucosal cells in P and NP rats

In preliminary studies (Supplementary Figs. 2A, 2B and 2C), in cDNA made from C57BL/6J WT mice (Group 12; Table 1) or rat (Oliveira-Maia et al. 2009) fungiform taste buds, circumvallate taste buds and non-gustatory lingual epithelium devoid of taste buds, TRPM5 and $\alpha$-gustducin were detected in taste bud cells and were not detected in the non-gustatory lingual epithelium or the CT nerve (Supplementary Figs. 2A and 2B). These results are consistent with the notion that the molecular correlates involved in sweet, bitter and umami taste are expressed only in a subset of taste cells (Zhao et al. 2003). TRPV1 message was detected in fungiform and circumvallate taste bud cells and in testis but not in the non-gustatory lingual epithelium (Supplementary Fig. 2C). Therefore, we used the anterior lingual epithelium rather than isolated taste buds to
quantitate the expression of T1R3 and TRPV1/TRPV1t mRNA and protein using qRT-PCR and Western blots in P and NP rats.

Using qRT-PCR from cDNA made from lingual epithelium containing fungiform taste papillae, T1R3 mRNA, normalized to the mRNA for β-actin, was significantly increased in 5 P rats (Table 3; p = 0.028; unpaired) relative to 5 NP rats maintained on H₂O (Group 2; Table 1). Western blots of T1R3 with bands for two reference genes, β-actin and GAPDH from protein samples pooled from isolated lingual epithelia containing fungiform taste papillae from 4 P or 4 NP (Group 2; Table 1) rats are shown in Fig. 2A. The density of the T1R3 bands was computed relative to β-actin or GAPDH bands (Fig. 2B). T1R3 protein was increased in lingual epithelium containing fungiform taste papillae in P rats relative to NP rats (Figs. 2A and 2B). Thus, there is a positive association across P and NP rat strains with increased sucrose preference and levels of T1R3 mRNA and protein in fungiform taste bud cells.

Using qRT-PCR from cDNA made from lingual epithelium containing fungiform taste papillae, TRPV1/TRPV1t mRNA normalized to the mRNA for β-actin was significantly increased in P rats (Table 3; p = 0.034; unpaired) relative to NP rats maintained on H₂O (Group 2; Table 1). Western blots of TRPV1/TRPV1t with bands for two reference genes, β-actin and GAPDH from protein samples pooled from isolated lingual epithelia containing fungiform taste papillae from 4 P or 4 NP rats (Group 2; Table 1) are shown in Fig. 2A. The density of the TRPV1/TRPV1t bands was computed relative to β-actin or GAPDH bands (Fig. 2B). Consistent with the qRT-PCR data, the results support the hypothesis that P rats maintained on H₂O have a higher expression of TRPV1/TRPV1t protein relative to NP rats maintained on H₂O (Group 2; Table 1).
interesting to note that in P rats maintained on H₂O both T1R3 and TRPV1/TRPV1t mRNA (Supplementary Table 1; p = 0.0076 and 0.022, respectively; n = 5; unpaired) and protein (Supplementary Fig. 3; n = 3) levels in intestinal mucosal cells were also increased relative to NP rats maintained on H₂O.

The observed differences in T1R3 and TRPV1t could be due to up or down regulation of gene transcription/translation or due to the higher density of fungiform papillae in P rats relative to NP rats. Accordingly, we counted the number of fungiform papillae in isolated lingual epithelia from naïve P and NP rats. In 3 P and 3 NP rats (Group 9; Table 1) the number of fungiform papillae in the tongue varied between 170 and 175 (172.7±2.2 papillae per tongue; M±SEM) and between 172 and 182 (177.5±2.2 papillae per tongue; M±SEM), respectively, and were not significantly different between the two genotypes (p>0.05). These values are within the range of fungiform papillae (175-201) found per male Sprague-Dawley rat tongue (187±2.9; M±SEM; n = 10) (Miller and Preslar 1974). These results indicate that the observed differences in T1R3 and TRPV1t expression in P and NP rats are independent of the number of fungiform taste papillae per rat tongue in the two genotypes.

**NaCl CT responses in P and NP rats**

We further investigated the strain differences in the CT responses to salty and sweet-tasting stimuli in P and NP rats in the absence and presence of specific modulators of a putative TRPV1t-dependent salt taste receptor and T1R2+T1R3 sweet taste receptor, respectively. CT responses were measured in P and NP rats maintained on H₂O (Group 2; Table 1) while their tongues were stimulated with a rinse solution (R).
and then with NaCl or NaCl+Bz (Table 2). P rats spontaneously elicited a significantly bigger NaCl CT response (Fig. 3A; filled bar) relative to NP rats (Fig. 3A; open bar; "p = 0.0133; unpaired; n = 3). The increase in the NaCl CT response was specifically due to the increase in the Bz-insensitive TRPV1t-dependent NaCl CT component (’p = 0.0355; unpaired). No statistical difference was observed in the Bz-sensitive ENaC-dependent NaCl CT response between P and NP rats.

To investigate the differences between TRPV1t activity in P and NP rats (Group 2; Table 1), we generated agonist concentration versus the magnitude of the Bz-insensitive NaCl CT response relations in P and NP rats using three TRPV1t agonists: RTX, ethanol (ETOH) and GalA-MRPs (Lyall et al. 2004, 2005a,b, 2007; Katsumata et al. 2008).

Acute effects of RTX on Bz-insensitive NaCl CT responses in P and NP rats

Consistent with previous studies (Lyall et al. 2004), in the rinse solution (R; Table 2), RTX elicited no CT response above baseline between 0.1x10^-6 M and 10x10^-6 M (data not shown). In NP rats, RTX produced a biphasic effect on the Bz-insensitive NaCl CT response (Fig. 3B; ○). Between 0.25x10^-6 M and 1x10^-6 M, RTX enhanced the CT response. RTX produced a maximum increase in the Bz-insensitive NaCl CT response at 1x10^-6 M and inhibited the CT response at 3x10^-6 and 10x10^-6 M. In P rats, the Bz-insensitive NaCl CT response in the absence and presence of RTX was greater relative to NP rats. In P rats, RTX produced a maximum enhancement in the CT response at 0.75x10^-6 M and at 1x10^-6, 3x10^-6 and 10x10^-6 M RTX inhibited the CT response (Fig. 3B; ●).

Acute effects of ethanol on Bz-insensitive NaCl CT responses
CT responses were monitored in P and NP rats (Group 2; Table 1) to NaCl+Bz alone and in mixtures containing 0-40% ethanol (ETOH). The corresponding rinse solutions were R or R+ETOH (0-40%), respectively. Unlike RTX, stimulating the tongue with rinse solution, R containing 10-40% ethanol elicited CT responses in P and NP rats that were not significantly different between the two genotypes (Fig. 3C). In mixtures containing NaCl+ethanol, the tonic CT responses were measured relative to the tonic CT responses to the same concentrations of R+ethanol. In NP rats, ethanol enhanced the Bz-insensitive NaCl CT response starting at around 20% and the response increased with 30% and 40% ethanol (Fig. 3D; ○). In P rats, ethanol induced an increase in the Bz-insensitive NaCl CT response starting at 10% and the response further increased with increasing ethanol concentration (Fig. 3D; ●).

Acute effects of GalA-MRPs on Bz-insensitive NaCl CT responses in P and NP rats

Consistent with previous studies (Katsumata et al. 2008), GalA-MRPs produced biphasic effects on NaCl+Bz CT responses in both P and NP rats (Group 2; Table 1) (Supplementary Fig. 1). In P rats, the GalA-MRPs concentration versus the magnitude of the Bz-insensitive NaCl response curve was higher and was shifted to the left on the concentration axis.

Effect of chronic ethanol and sucrose treatment on the Bz-insensitive NaCl CT responses in P and NP rats

NP rats given a choice between H_2O and 5% ethanol (Group 5; Table 1) did not show any changes in the RTX versus the magnitude of the Bz-insensitive NaCl CT response relations relative to NP rats maintained on H_2O (Group 6; Table 1). Similarly, P rats given 5% ethanol in a choice (Group 5; Table 1) or no choice paradigm (Group 1;
Table 1), did not show any changes in the RTX versus the magnitude of the Bz-insensitive NaCl CT response relations relative to P rats maintained on H2O (Groups 2 and 6; Table 1). Therefore, the data in P rats from choice and no choice treatments were combined and are plotted in Fig. 4A (▲) versus the data obtained in P rats maintained on H2O (Fig. 4A; ●). However, in the case of NP rats given 5% ethanol in the no choice paradigm (Group 1; Table 1), the RTX dose response curve was shifted upwards and to the left on the RTX concentration axis (Fig. 4A; ∇) and was not different from the RTX dose response relation in P rats maintained on H2O (Fig. 4A; ●). When given a choice between water and ethanol, NP rats responded to ethanol as an aversive stimulus. Thus, their ethanol consumption per day was negligible (Fig. 1A). In contrast, when given 5% ethanol in the no choice paradigm, NP rats consumed a significantly greater amount of ethanol per day (Fig. 1B) relative to NP rats given a choice between water and ethanol (Fig. 1A). These results suggest that changes in RTX concentration versus the NaCl+Bz CT response relationship are associated with increased ingestion of ethanol in NP rats in a no choice paradigm relative to NP rats given a choice between water and ethanol.

Since in some inbred strains, ethanol has a sweet taste quality, we further tested if chronic sucrose exposure will also modulate changes in TRPV1/TRPV1t expression and function in the anterior taste receptive field. The NP rats maintained chronically on 5% sucrose in a no choice paradigm (Group 3; Table 1) demonstrated a similar relationship between varying RTX concentration and the magnitude of the Bz-insensitive NaCl CT response (Supplementary Fig. 4A; ▲) as NP rats maintained on H2O (Supplementary Fig. 4A; ○). The maximum increase in the Bz-insensitive NaCl CT
response in both cases was observed at $1 \times 10^{-6}$ M RTX. Similarly, in P rats exposed to 5% sucrose in a no choice paradigm (Group 3; Table 1), the varying RTX concentration versus the Bz-insensitive NaCl CT profile (Supplementary Fig. 4B; ▲) was same as in P rats maintained on H$_2$O (Supplementary Fig. 4B; ○). It is important to note that the maximum increase in the Bz-insensitive NaCl CT response in both cases was observed at $0.75 \times 10^{-6}$ M RTX. These results suggest that in NP rats, the relationship between varying RTX concentration and the magnitude of the NaCl+Bz CT response shown in Fig. 4A (∇ versus ○) is specifically altered by chronic ethanol ingestion in a no choice paradigm and is not affected by chronic sucrose ingestion. We have previously shown that RTX specifically modulates the Bz-insensitive NaCl CT responses. RTX did not alter CT responses to sweet, bitter and umami stimuli between $0.1 \times 10^{-6}$ and $10 \times 10^{-6}$ M (Lyall et al. 2004). Taken together, these results suggest that TRPV1t activity is modulated by ethanol but not by sucrose.

**Effect of SB-366791 on Bz-insensitive NaCl CT responses**

In NP rats given chronic 5% ethanol in a no choice paradigm (Group 1; Table 1), the Bz-insensitive NaCl CT response was inhibited in the presence of $1 \times 10^{-6}$ M SB-366791 (Fig. 4B), a specific TRPV1/TRPV1t blocker (Gunthorpe et al. 2004; Lyall et al. 2004). In P rats maintained on H$_2$O (Group 2; Table 1), SB-366791 (Fig. 4C; grey bars) inhibited the Bz-insensitive NaCl CT response and its subsequent enhancement in the presence of TRPV1t agonists RTX ($0.75 \times 10^{-6}$ M) or ethanol (40%) relative to control (Fig. 4C; open bars).

To directly demonstrate that RTX, ethanol and GaIA-MRPs produce their effects on the NaCl+Bz CT responses by acting on TRPV1/TRPV1t, further studies were
performed on WT and TRPV1 KO mice (Group 12; Table 1). In WT mice GalA-MRPs (0.25%), RTX (1x10^{-6} M) and ethanol (ETOH; 30%) enhanced the CT response to NaCl+Bz (Supplementary Fig. 5; open bars). Consistent with previous studies (Lyall et al. 2004, 2005a,b, 2007, 2009a, 2010; Katsumata et al. 2008), TRPV1 KO mice elicited no spontaneous CT response to NaCl+Bz above the rinse baseline and no increase in the NaCl+Bz CT response was observed in the presence of GalA-MRPs, RTX or ethanol above the baseline rinse level (Supplementary Fig. 5; filled bars). Taken together, the above results provide direct evidence that RTX, ethanol and GalA-MRPs produce their effects on the Bz-insensitive NaCl CT responses by interacting with TRPV1t. RTX, capsaicin and H^+ modulate TRPV1 activity by binding to different sites on the protein molecule (Petrocellis and Marzo 2005).

**Effect of chronic ethanol and sucrose treatment on the TRPV1/TRPV1t mRNA levels in P and NP rats**

As shown in Table 4, in 5 NP rats chronically exposed to 5% sucrose for 2 weeks in a no choice paradigm (Group 3; Table 1) the TRPV1/TRPV1t mRNA level was not different from its corresponding value in 5 NP rats maintained on H_2O (Group 2; Table 2). These results suggest that chronic sucrose exposure does not affect TRPV1/TRPV1t mRNA levels in NP rats. In contrast, 3 NP rats chronically exposed to 5% ethanol in a no choice paradigm demonstrated a significant increase in TRPV1/TRPV1t mRNA level relative to 4 NP rats maintained on H_2O (Table 4). In 5 P rats chronically exposed to 5% ethanol in a no choice paradigm (Group 1; Table 1), the ratio (mean 2^{ΔCT} P rat_{ETOH}/mean 2^{ΔCT} P rat_{H2O}) of TRPV1/TRPV1t mRNA relative to 5 P rats maintained on H_2O (Group 2; Table 1) was 0.97 (data not shown). These results suggest that the
effect of chronic ethanol exposure in a no choice paradigm in NP rats is related to an
increase in TRPV1t mRNA in the taste receptive field in the anterior tongue.

CT responses to sweet-tasting stimuli in P and NP rats

P rats maintained on H₂O (Group 2; Table 1) (P rats+H₂O; Fig. 5A; n = 3)
spontaneously elicited a bigger CT response to 0.5 M sucrose (p = 0.0013; unpaired)
and 0.008 M SC45647 (p = 0.0289; unpaired) relative to NP rats (NP+H₂O; Fig. 5A; n =
3; Group 2; Table 1). In mixtures containing a fixed concentration of sucrose (0.5 M)
and varying ethanol concentration (0-40%), ethanol enhanced the CT response to
sucrose in both NP and P rats (Fig. 5B). In P rats, ethanol increased the CT response to
sucrose, starting at around 10%. The response further increased at 20% and 30%. At
40% ETOH the CT response was less than its maximum value (Fig. 5B; ●). In NP rats,
in the absence of ethanol, the sucrose CT response was lower than in P rats. Ethanol
enhanced the CT response of sucrose starting around 20% and thereafter the response
increased with increasing ethanol concentration to 30% and 40% (Fig. 5B; ○).

Effect of chronic exposure to sucrose and ethanol on CT responses to sweet stimuli and
T1R3 mRNA levels in P and NP rats

Chronic exposure to 5% sucrose in NP rats (Group 3; Table 1) in a no choice
paradigm produced a 27.6% decrease in T1R3 mRNA relative to NP rats maintained on
H₂O (Table 5). However, the magnitude of the CT response to 500 mM sucrose in NP
rats chronically exposed to 5% sucrose was not different from NP rats maintained on
H₂O (Table 5). In contrast, chronic exposure to 5% ethanol in P rats (Group 1; Table 1)
in a no choice paradigm decreased T1R3 levels by 49% relative to P and NP rats
maintained on H$_2$O (Group 2; Table 1). In addition, the magnitude of the CT response to 500 mM sucrose in P rats chronically exposed to 5% ethanol was significantly decreased ($p = 0.0007$; unpaired) from P rats maintained on H$_2$O (Table 5). These results suggest that chronic sucrose or ethanol exposure can alter neural responses to sweet stimuli by modulating T1R3 mRNA levels in Type II TRCs.

**Effect of U73122 on CT responses to sweet-tasting stimuli in the absence and presence of ethanol**

In Type II TRCs, the enzyme PLC$\beta_2$ is an essential downstream intracellular signaling effector that is involved in the transduction of sweet, bitter and umami taste (Chandrashekar et al. 2006). To test, if alcohol increases CT responses to sweet taste stimuli by also targeting the sweet taste transduction mechanism involving PLC$\beta_2$, we topically applied the non-specific PLC blocker, U73122, to the tongue at 150x10$^{-6}$ M (Table 2). CT responses in SD rats (Group 11; Table 1) were recorded while the tongue was stimulated with 0.008M SC45647 in the presence and absence of 30% ethanol, before and after U73122 exposure. U73122 inhibited the tonic CT response to SC45647 to near baseline and also inhibited the alcohol-induced increase in SC45647 CT response (Supplementary Fig. 6A). U73122 inhibited the tonic CT response to 0.5 M sucrose to near baseline (Lyall et al. 2010). In addition, U73122 also inhibited the ethanol-induced increase in the CT response to sucrose (data not shown). The inactive analogue U73343 had no effect on the sweet taste responses in the absence (Lyall et al. 2010) or presence of ethanol (data not shown). These results suggest that ethanol modulates sweet taste responses by targeting (T1R2+T1R3)-PLC$\beta_2$-TRPM5 sensing pathways for sweet taste transduction (Chandrashekar et al. 2006).
Effect of chelating TRC \([Ca^{2+}]\), on the CT response to sweet stimuli in the absence and presence of ethanol.

To test, if alcohol also increases CT responses to sweet stimuli by modulating TRC \([Ca^{2+}]\), levels, we loaded TRCs in vivo with BAPTA-AM. Rat (Group 11; Table 1) CT responses in SD rats were recorded in the presence of 0.5 M sucrose solutions containing 0 or 30% ethanol before and after topical lingual application of \(33 \times 10^{-3}\) M BAPTA-AM (Table 2). Chelating TRC \([Ca^{2+}]\), did not affect the magnitude of the CT response to sucrose (Supplementary Fig. 6B) relative to control. However, it inhibited the ethanol-induced increase in the CT response to sucrose observed under control conditions. This suggests that the synergistic effect of ethanol on the CT response to sweet taste stimuli is \(Ca^{2+}\)-dependent.
DISCUSSION

The results presented in this paper support the hypothesis that in P rats, genetically induced alcohol-preference is associated with: (i) increased preference for sweet-taste stimuli; (ii) increased expression of T1R3 and TRPV1/TRPV1t mRNA and protein in the fungiform taste receptive field; (iii) spontaneously bigger CT responses to sweet and salty taste stimuli and (iv) increased sensitivity of the CT responses to mixtures containing ethanol plus sweet or salty taste stimuli. Below we will discuss the physiological significance of increased alcohol preference and its potential role in sweet and salty taste during acute and chronic ethanol consumption.

Alcohol preference and salt taste

The Bz-insensitive component of the NaCl CT response is spontaneously enhanced in P rats relative to NP rats maintained on H2O (Figs. 3A and 3B). Several lines of evidence suggest that the Na\(^+\) entry via a putative non-specific cation channel, TRPV1t contributes to the Bz-insensitive NaCl CT response: (i) In P and NP rats (Figs. 3B, 3D, 4A and Supplementary Fig. 1), SD rats and WT mice, TRPV1t agonists (e.g. RTX, ethanol and GalA-MRPs) modulate the Bz-insensitive NaCl CT responses in a biphasic manner; (ii) A specific TRPV1/TRPV1t blocker, SB-366791, inhibited the spontaneous NaCl+Bz CT response (Fig. 4B) and the subsequent increase in the NaCl+Bz CT response in the presence of TRPV1t agonists (Fig. 4C); and (iii) TRPV1t KO mice demonstrate no spontaneous CT response to NaCl+Bz and no increase in the NaCl+Bz CT response above baseline in the presence of NaCl+Bz+TRPV1t agonists (Supplementary Fig. 5) (Lyall et al. 2004, 2005a,b, 2007, 2009a, 2010; Katsumata et al.
Thus, our studies have identified the P rat as the first animal model in which a putative TRPV1t-dependent salt taste receptor activity is spontaneously enhanced.

Our qRT-PCR data (Table 3) and Western blot data (Fig. 2) suggest that the increased activity and sensitivity of the Bz-insensitive NaCl CT responses to TRPV1t agonists in P rats (Figs. 3B, 3D and Supplementary Fig. 1) is associated with increased expression of TRPV1/TRPV1t mRNA and protein in the fungiform taste receptive field. The increase in TRPV1t activity in P rats cannot be explained simply by differences in the regulation of TRPV1t by intracellular effectors in P and NP rats. We have previously shown that increasing the phosphorylation state of TRPV1t by activating PKC or inhibiting its dephosphorylation by blocking the phosphatase, calcineurin, enhanced channel activity without a leftward shift in RTX concentration versus the magnitude of the NaCl+Bz CT response relationship (Lyall et al. 2009a). Similarly, relieving the channel from PIP2 inhibition increased TRPV1t activity without a shift in the RTX concentration versus the magnitude of the NaCl+Bz CT response relationship (Lyall et al. 2010).

While P rats demonstrated a spontaneous increase in TRPV1t-dependent NaCl CT responses, NP rats, surprisingly, displayed the same relationship between RTX concentration and the magnitude of the Bz-insensitive NaCl CT response as P rats when given chronic 5% ethanol in a no choice paradigm (Fig. 4A). In a no choice paradigm (Fig. 1B), NP rats consumed more ethanol relative to NP rats given a choice between H2O and ethanol (Fig. 1A). The increase in ethanol consumption was associated with an increase in the expression of TRPV1/TRPV1t mRNA in the fungiform taste receptive field (Table 4). These results suggest that a shift in the agonist...
concentration versus the magnitude of the Bz-insensitive NaCl CT response relationship is associated with increased expression of the TRPV1t channel protein in the fungiform taste receptive field. However, at present it is not clear, if the induction of TRPV1t in NP rats is also related to the amount of ethanol consumed and the length of time of chronic ethanol consumption. It is also presently not known if the NP rat regains its phenotypically smaller and right-shifted CT response to NaCl+Bz+RTX after it is allowed to choose water again.

It is important to note that similar to P and NP rats (Bice and Kiefer, 1990), naïve high alcohol drinking (HAD) and low alcohol drinking (LAD) rats demonstrated similar taste reactivity responses to a range of alcohol concentrations. Differences between taste reactivity between two strains became apparent only after rats had continuous access to alcohol during which time HAD rats consumed significantly more alcohol than LAD rats (Kiefer et al. 1995). Following alcohol exposure, HAD rats displayed a general increase in ingestive responses and a decrease in aversive responses relative to LAD rats. These results suggest that experience with ethanol produces an increase in ingestive and a decrease in aversive responses. In the no choice paradigm, P and NP rats were forced to consume ethanol over a period of 2-3 weeks. The alcohol consumption of P rats remained the same as in the choice paradigm. However, NP rats consumed a significantly greater amount of ethanol relative to rats in a choice paradigm (Figs. 1A and 1B). Thus it is likely that the long-term experience with ethanol induces changes in gene expression of TRPV1t in the fungiform taste receptive field and in the TRPV1t-dependent NaCl CT response profile.
Conditioned taste aversion studies suggest that in some inbred strains ethanol has both a sweet taste quality as well as a bitter taste quality (Blizard 2007). At low concentrations, some humans perceive ethanol as sweet and at higher concentrations it is perceived as bitter (Scinska et al. 2000). However, besides its taste, additional factors contribute to ethanol preference and oral avoidance. Recent studies suggest that TRPV1 KO mice display elevated preference and consumption of ethanol and reduced oral avoidance responses to ethanol regardless of concentration, insensitivity to capsaicin, and little to no difference in sweet or bitter taste responses relative to WT mice (Blednov and Harris 2007; Ellingson et al. 2009). TRPV1 KO mice not only lack TRPV1 in the trigeminal nerve endings but also do not display Bz-insensitive NaCl CT responses, suggesting that they also do not have a functional TRPV1t, a TRPV1 variant cation channel in their fungiform taste receptive field. (Lyall et al. 2004). These data suggest that elevated alcohol preference in TRPV1 KO mice is not related to the absence of a putative TRPV1t-dependent salt taste receptor per se but is rather related to the absence of trigeminal irritation caused by ethanol. Thus, while increased alcohol preference in P rats is associated with increased TRPV1/TRPV1t expression in the fungiform taste receptive field, the elevated preference for ethanol in TRPV1 KO mice is independent of a putative TRPV1t-dependent salt taste receptor but is associated with decreased trigeminal irritation induced by ethanol.

Our results further suggest that chronic ethanol usage and changes in the diet can have important consequences on salt taste receptor expression, function and changes in NaCl preference in animal models. Ethanol drinking and the conditions under which ethanol is consumed has been shown to differentially affect protein

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expression levels in the nucleus accumbens and amygdala (Bell et al. 2006). Chronic oral ethanol consumption increased the expression of TRPV1/TRPV1t mRNA (Table 4) in NP rats, increased neural responses to salty stimuli and increased their sensitivity to TRPV1t agonists (Fig. 4A). In a previous study (Stewart et al. 1994), in P rats, high oral alcohol preference appeared to be negatively associated with the intake of salty solutions varying in concentration between 0.004 and 0.55 M. In the study by Stewart et al. (1994) the NaCl solutions were presented continuously with water and food always available, and the concentrations of NaCl were doubled every 48 hr. However, in our studies we only used one NaCl concentration. There was no significant difference between NaCl preference between P and NP rats when presented with isotonic NaCl solutions (Fig. 1C). Since the relationship between NaCl concentration and the NaCl preference is a bell shaped curve, we also expect that at NaCl concentrations above 0.15 M, the NaCl consumption and the NaCl preference will decrease in both P and NP rats. In P rats, further increasing TRPV1t activity with GalA-MRPs induced a significant decrease in NaCl intake and NaCl preference (Fig. 1C). We have previously shown that in C57BL/6J mice (Rhyu et al. 2009), NaCl preference for 0.1 M NaCl+10x10^{-6} M amiloride is modulated by the addition of MRPs in a biphasic manner. At the concentration (0.25%) at which MRPs were shown to maximally enhance the Bz-insensitive NaCl CT response (Supplementary Fig. 1), MRPs decreased NaCl intake and preference. Alternately, at the concentration at which MRPs inhibited the CT response (0.5%), MRPs increased NaCl preference. These studies support our hypothesis that modulating TRPV1t activity by the addition of MRPs in the diet can modulate NaCl taste. However, additional behavioral studies need to be performed over
a wide range of NaCl concentrations in P and NP rats to determine if NaCl preference
curves are significantly different between P and NP rats in the absence and presence of
MRPs.

During stimulation of the tongue with salt solutions containing agonists, the
contribution of TRPV1t to the total NaCl CT response can be increased by 100% or
more (DeSimone and Lyall 2006; Lyall et al. 2009a, 2010). In humans, salt taste is
predominantly amiloride-insensitive and may contribute as much as 80% to human salt
taste perception (Ossebaard and Smith 1995; Feldman et al. 2003). Recent studies
suggest that the activation of TRPV1t, especially with non-pungent agonists, such as,
MRPs, produced increased salt taste sensitivity in humans (Katsumata et al. 2008) and
in animal models (Rhyu et al. 2009). Human subjects demonstrate significant increase
in salt taste perception when NaCl was presented in mixtures containing low
concentrations of MRPs. At high MRPs concentrations, the same human subjects
reported a significant decrease in salt taste perception (Katsumata et al. 2008). This
suggests that TRPV1t may also play a role in human salt taste perception.

Alcohol preference and sweet taste

In our studies, genetically induced alcohol preference in P rats was associated
with increased T1R3 mRNA (Table 3) and protein expression in the fungiform taste
receptive field (Fig. 2) for the detection of sweet taste stimuli and also in the gut
enteroendocrine cells (Supplementary Table 1 and Supplementary Fig. 3) that regulate
nutrient-responsive secretion of gut hormones (Kokrashvili et al. 2009a,b). Chronic
sucrose exposure in NP rats and chronic ethanol exposure in P rats resulted in a
decrease in T1R3 mRNA (Table 5). These results suggest that changes in the sweet
taste receptor can be induced by chronic ethanol usage and/or diets containing high
sugar.

In P rats the neural responses to sweet taste stimuli demonstrated a significantly
greater sensitivity to ethanol relative to NP rats (Fig. 5B). This increased magnitude of
CT response to sucrose as a function of the ethanol concentration is most likely due to
increased T1R3 levels in Type II TRCs (Table 3). In mixtures with sweet stimuli, acute
oral ethanol application enhanced CT taste nerve responses to sweet taste stimuli (Fig.
5B and Supplementary Fig. 6). Inhibiting sweet taste transduction by blocking PLCβ2
with U73122 not only inhibited the sweet taste response but also eliminated the
enhancement in the sweet-taste response in the presence of ethanol (Supplementary
Fig. 6A). While ethanol modulates sweet taste responses primarily by interacting with
the sweet-taste receptor (T1R3) (Bachmanov et al. 2001, 2002; Inoue et al. 2004; Lu et
al. 2005; Nelson et al. 2001) other intracellular effectors, such as cell Ca2+, also play a
role in mixture interactions. Chelating TRC [Ca2+] with BAPTA loading, did not affect the
CT responses to sucrose, but inhibited the subsequent increase in the neural response
in the presence of ethanol (Supplementary Fig. 6B). These results suggest that the
modulation of the CT nerve responses to sweet stimuli by ethanol is dependent upon an
increase in TRC Ca2+. We hypothesize that the synergistic effects of alcohol on sucrose
taste involve the Ca2+-activated non-selective cation channel, TRPM5 (Talavera et al.
2005).

It is suggested that the ability of ethanol to stimulate neural pathways involved in
the transduction of sweet taste (Hellekant et al. 1997; Lemon et al. 2004) play an
important role in its consumption (Blednov et al. 2008). Consistent with this, genetic
deletion in mice of T1R3 and its associated G-protein subunit ($\alpha$-gustducin) or the down-
stream non-specific cation channel (TRPM5) significantly decrease alcohol preference
in KO mice (Ellingson et al. 2009). Recent studies suggest that a reciprocal genetic
relationship also exists between the consumption of saccharin and sucrose and self-
administration of alcohol and other drugs of abuse (Carroll et al. 2008). Rats selectively
bred for high saccharin intake demonstrated significantly higher ethanol consumption
relative to rats bred for low saccharin intake (Dess et al. 2005).

The effects of alcohol ingestion on taste preferences arise due to its effects on
the CNS and on the peripheral taste system. The development of alcoholism is
accompanied by alterations in gene and protein expression levels within the brain's
reward neurocircuitry. In P rats, chronic ethanol drinking produced changes in
expression levels for 22 of the 27 identified proteins in the amygdala and 6 proteins in
the nucleus accumbens compared to controls. The proteins could be grouped into
functional categories of chaperones, cytoskeleton, intracellular communication,
membrane transport, metabolism, energy production, or neurotransmission. Thus, it
appears that ethanol drinking and the conditions under which it is consumed,
differentially affect protein expression levels between the nucleus accumbens and
amygdala (Bell et al. 2006). Cyclic-AMP-PKA (protein kinase A) signaling has been
strongly implicated in the CNS effects of ethanol. Ethanol promotes activation and
translocation of the PKA catalytic subunit ($\alpha$) into the cell nucleus. PKA $\alpha$
translocation to the nucleus is followed by cAMP Response Element Protein
phosphorylation (pCREB) and cAMP Response Element (CRE)-mediated gene
expression (Asyyed et al. 2006). The results presented in this study suggest that in addition to the above effects in the CNS, the genetic differences in alcohol preference and chronic ethanol exposure are accompanied by alterations in gene expression levels in chemosensory cells in the taste buds involved in sweet and salty taste transduction.

In our acute studies, in naïve animals, alcohol and other agonists were applied topically to the tongue with the stimulus using a lingual flow chamber and produce their effects on the CT responses instantaneously. Under these conditions, the stimuli applied to the lingual surface do not mix with saliva and are not ingested. Thus it is expected that very little, if any, systemic uptake of alcohol or other drugs occur under these conditions. Therefore, the acute effects of alcohol, RTX and GalA-MRPs on the CT responses to taste stimuli are independent of the post-ingestive effects or their effects on the central nervous system. However, under chronic conditions both of these factors, including additional factors, such as stress, may play a role in modulating CT responses via changes in the taste receptors by themselves and/or in the downstream intracellular signaling effectors.

Randomly bred rats (Kampov-Polevoy et al. 1990), inbred and congenic mice (Blizard 2007; Blizard and McClearn 2000) and the F2 progeny derived from crosses of P and NP rats (Foroud et al. 2002) demonstrate positive correlations between alcohol and sweetener consumption. In F2 progeny generated from the inbred P and NP rats, a quantitative trait loci (QTL) has been identified on rat chromosome 3 which appears to contribute to both alcohol and saccharin consumption (Foroud et al. 2002). The genetic locus that encodes for the T1R3 protein overlaps with a locus that strongly influences alcohol intake (Bachmanov et al. 2002). The allelic variations in the T1R3 gene are
associated with differential sweet preferences in mice (Reed et al. 2004). Recent studies using wildtype and T1R3 knockout mice provide further evidence for the involvement of T1R3 receptor in the sensory detection and transduction of ethanol taste (Brasser et al. 2010). At present QTL studies with a putative TRPV1t-dependent Bz-insensitive salt taste receptor are lacking.

In summary, our studies suggest that genetically-induced alcohol preference in P rats or forced chronic alcohol exposure in NP rats elevate the expression of a putative TRPV1t-dependent Bz-insensitive salt taste receptor in taste cells and increase the responsiveness of the CT nerve to salt taste stimuli in the absence and presence of ethanol. P rats also demonstrated increased expression of T1R3, a sweet-taste receptor component, increased responsiveness of the CT nerve to sweet taste stimuli in the absence and presence of ethanol. Chronic sucrose or ethanol exposure decreased T1R3 mRNA expression that can result in decreased neural responsiveness to sweet stimuli.
ACKNOWLEDGEMENTS

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Fig. 1. Ethanol, sucrose and NaCl consumption and preference ratios in P and NP rats. (A) Two-bottle tests. P and NP rats were given a choice between H2O and 5% ethanol (Group 5; Table 1) or H2O and 10% sucrose (Group 7; Table 1). The fluid intake for 24 hr period was measured to calculate preference ratios for the above stimuli. The data was expressed as either fluid intake in gm/24hr/g body weight or preference ratio (gm of the test solution consumed/24 hr/gm body weight divided by the total fluid intake (gm of H2O/24 hr/gm body weight + gm of the test solution/24 hr/gm body weight). The open bars, filled bars and cross hatched bars represent mean±SEM of H2O intake, 5% ethanol (ETOH) intake or 10% sucrose intake and preference ratios, respectively from 3 P and 3 NP rats in each group. *p <0.002; **p <0.001; ***p <0.0001 (one sample t test).

(B) One-bottle tests. P and NP rats were presented with a single bottle containing 5% ethanol (Group 1; Table 1), 5% sucrose (Group 3; Table 1) or 10% sucrose solutions (Group 4; Table 1). Their fluid intake was expressed as gm/24hr/gm body weight and represented as mean±SEM of n; where n represents the number of rats in each group in the parenthesis. (C) Two-bottle tests. P and NP rats (Group 8; Table 1) were given a choice between H2O and 0.15 M NaCl, H2O+5x10^{-6} M Bz and 0.15 M NaCl+5x10^{-6} M Bz or H2O+5x10^{-6} M Bz+0.25% GalA-MRPs and 0.15 M NaCl+5x10^{-6} M Bz+0.25% GalA-MRPs. The fluid intake for 24 hr period was measured to calculate preference ratios for the above stimuli. The open bars, filled bars and crosshatched bars represent H2O intake, NaCl intake and preference ratios, respectively. The data is expressed as either fluid intake in gm/24hr/gm body weight or preference ratio (gm of the test solution
consumed/24 hr/gm body weight divided by the total fluid intake (gm of H₂O/24 hr/gm body weight + gm of the test solution/24 hr/gm body weight). The values are expressed as mean±SEM of n, where n represents the number of animals in each group. The brackets identify the comparisons being made between the two treatments using unpaired t test and their respective p values. *p <0.002; **p <0.001; #p <0.03; ##p <0.01 and $p = 0.045 (one sample t test).

**Fig. 2. T1R3 and TRPV1/TRPV1t protein levels in the anterior lingual epithelium containing fungiform taste buds in P and NP rats.** (A) Shows Western blots for T1R3 and TRPV1/TRPV1t with bands for two reference genes, β-actin and GAPDH in the anterior lingual epithelium containing fungiform taste buds in P and NP rats maintained on H₂O (Group 2; Table 1). (B) Shows the density of T1R3 and TRPV1/TRPV1t bands computed relative to β-actin or GAPDH.

**Fig. 3. NaCl CT responses in P and NP rats.** (A) Summary of the magnitudes of the tonic CT responses to NaCl (N), N+Bz and the Bz-sensitive component of the CT response in P and NP rats maintained on H₂O (Group 2; Table 1). In each case the CT responses were normalized to the corresponding tonic CT responses obtained with 0.3 M NH₄Cl and presented as Tonic NaCl CT Response/0.3 M NH₄Cl. Each bar represents the mean±SEM values of the normalized tonic CT response from 3 animals. * p = 0.0355; ** p = 0.0133 (unpaired). (B) CT responses were measured while the tongue was stimulated with R and then with N+Bz+RTX (0-10×10⁻⁶M) in P (●) and NP (○) rats maintained on H₂O (Group 2; Table 1). In each animal the tonic CT response was
normalized to the corresponding tonic CT responses obtained with 0.3 M NH$_4$Cl. In NP rats each point represents the mean±SEM values of the normalized tonic CT response from 4 to 9 animals (n) and plotted as a function of log RTX concentration. In P rats each point represents the mean±SEM values of the normalized tonic CT response from 3 animals (n) and plotted as a function of log RTX concentration. The zero RTX concentration is shown as -8.0 on the X-axis. The gray vertical bars represent points at RTX concentration in μM. The right Y-axis represents the percent change in the CT response relative to the response at 0 RTX in NP rats. Significant differences were found for RTX concentration (p = 0.04 for both, two-way ANOVA) and the magnitude of the RTX response in P and NP rats (p = 0.008; Bonferroni corrected) with no significant interaction between effects of strain and concentration. In the rinse solution RTX did not increase the CT response above baseline (data not shown). (C) CT responses were measured while the tongue was first stimulated with the rinse solution R and then with R+ETOH (0-40%) in P (●) and NP (○) rats (Group 2; Table 1). In each case the tonic CT responses were normalized to the corresponding tonic CT responses obtained with 0.3 M NH$_4$Cl. Each point represents the mean±SEM values of the normalized tonic CT response from 3 animals (n) and plotted as a function of percent ETOH concentration. No significant differences were found for ETOH concentration (p >0.05 for both, two-way ANOVA; Bonferroni corrected) and the magnitude of the ETOH response in P and NP rats (p >0.05), with no significant interaction between effects of strain and concentration. (D) CT responses were measured while the tongue was first stimulated with the rinse solution R+ETOH (0-40%) and then with N+Bz+ETOH (0-40%) in P (●) and NP (○) rats (Group 2; Table 1). In each case the tonic CT responses were
normalized to the corresponding tonic CT responses obtained with 0.3 M NH₄Cl. To calculate the magnitude of the N+Bz CT response in the presence of ETOH, the response of ETOH in the rinse was subtracted from the total N+Bz+ETOH response. Each point represents the mean ± SEM values of the normalized tonic CT response from 3 animals and plotted as a function of percent ETOH concentration. The right Y-axis represents the percent change in CT response relative to 0 ETOH in NP rats. Significant differences were found for ETOH concentration (p = 0.027 for both, two-way ANOVA) and the magnitude of the ETOH response in P and NP rats (p = 0.009; Bonferroni corrected), with no significant interaction between effects of strain and concentration.

Fig. 4. (A) Effect of chronic ethanol exposure on RTX versus the NaCl+Bz CT response relationship in P and NP rats. CT responses were measured while the rat tongue was stimulated with R and then with N+Bz+RTX (0-10×10⁻⁶ M) in P rats maintained on H₂O (●) (Group 2; Table 1), in P rats (▲) given 5% ETOH in a choice (Group 5; Table 1) or no choice paradigm (Group 1; Table 1) and in NP (∇) rats given 5% ETOH in a no choice paradigm (Group 1; Table 1). In each animal the tonic CT response was normalized to the corresponding tonic CT responses obtained with 0.3 M NH₄Cl. Each point represents the mean ± SEM values of the normalized tonic CT response from 3 animals and plotted as a function of log RTX concentration. The zero RTX concentration is shown as -8.0 on the X-axis. The gray vertical bars represent points at RTX concentration in μM. The RTX concentration versus the NaCl+Bz CT response relationship in naïve NP rats first shown in Fig. 3B (○) is also plotted for comparison. The right Y-axis represents the percent change in the CT response relative.
to the response at 0 RTX in NP rats. Significant differences were found for RTX concentration (p = 0.0008; Bonferroni corrected) for all three, two-way ANOVA with no significant interaction between effects of strain and concentration. However, no significant difference was observed between RTX-NaCl+Bz CT response relationships in naïve P rats, P rats given 5% ETOH in a no choice or choice paradigm and NP rats given 5% ETOH in a no choice paradigm (p >0.05). All of the data points are shown connected by a common smooth curve. (B and C) Effect of SB-366791 on Bz-insensitive NaCl CT responses in P and NP rats. (B) Shows a representative CT response in a NP rat chronically given 5% ethanol in a no choice paradigm (Group 1; Table 1) while its tongue was stimulated with a rinse solution R and then with 0.3 M NH₄Cl, N, N+Bz or N+Bz+SB-366791 (1x10⁻⁶ M; SB). The arrows represent the time period when different solutions were superfused on the rat tongue. (C) CT responses were monitored in P rats maintained on H₂O (Group 2; Table 1) while their tongues were stimulated with a rinse solution R and then with N+Bz, N+Bz+0.75x10⁻⁶ M RTX or N+Bz+40% ethanol (ETOH) in the absence and presence of SB-366791 (SB). In each case the tonic CT responses were normalized to the corresponding tonic CT responses obtained with 0.3 M NH₄Cl. Each bar represents the mean±SEM values of the normalized tonic CT response from 3 animals. The p values (unpaired) for the differences between the CT responses in the absence (open bars) and presence of SB (grey bars) are shown above grey bars.

Fig. 5. CT responses to sweet taste stimuli in P and NP rats. (A) CT responses were monitored while the P and NP rat tongues (Group 2; Table 1) were first stimulated
with the rinse solution R and then with 0.5 M sucrose or 0.0008 M SC45647. In each case the CT responses were normalized to the corresponding CT responses obtained with 0.3 M NH₄Cl. Each bar represents the mean±SEM values of the normalized tonic CT response from 3 animals. *p = 0.0289; ** p = 0.0013 (unpaired). (B) CT responses were measured while the tongue was first stimulated with the rinse solution R and then with R+0.5 M sucrose in the absence and presence of ETOH (0-40%) in P (●) and NP (○) rats (Group 2; Table 1). To calculate the magnitude of the CT response to sucrose in the presence of ETOH, the response of ETOH in the rinse was subtracted from the total ETOH+sucrose response. In each case the tonic CT responses were normalized to the corresponding tonic CT responses obtained with 0.3 M NH₄Cl. Each point represents the mean±SEM value of the normalized sucrose tonic CT response from 3 animals and plotted as a function of percent ETOH concentration. The right Y-axis represents the percent change in CT response relative to 0 ETOH in NP rats. Significant differences were found for ethanol concentration (p =0.014 for both, two-way ANOVA) and the magnitude of the ethanol+sucrose response in P and NP rats (p = 0.0006; Bonferroni corrected) with no significant interaction between effects of strain and concentration.
 Seventy five P and 71 NP rats were used in this study. Out of these, 59 P and 60 NP rats were given 7 different treatments in a no choice (Groups 1-4) or choice paradigm (Groups 5-7). For the no choice paradigm, Group 1 is the test group and Group 2 is the control group. In the choice paradigm, Group 5 and Group 6 are the test groups and Group 7 is the control group. Additional groups (10-12) received no treatment and were not included in the choice paradigm.

### Table 1
Control and test groups of rats and mice used in the study

<table>
<thead>
<tr>
<th>P &amp; NP rats</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test</strong></td>
<td><strong>Control</strong></td>
<td><strong>Test</strong></td>
<td><strong>Test</strong></td>
<td><strong>Test</strong></td>
</tr>
<tr>
<td><strong>No choice</strong></td>
<td>One bottle</td>
<td>One bottle</td>
<td>One bottle</td>
<td>One bottle</td>
</tr>
<tr>
<td>Days</td>
<td>(Sucrose/ETOH) (%)</td>
<td>Sucrose (%)</td>
<td>Sucrose (%)</td>
<td>Sucrose (%)</td>
</tr>
<tr>
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<td>5</td>
<td>10</td>
</tr>
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<td>3</td>
<td>3/2</td>
<td>3</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>1/4</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0/5</td>
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<td>5</td>
<td></td>
</tr>
<tr>
<td>2-3 weeks</td>
<td>5% ETOH</td>
<td>H2O</td>
<td>5% Sucrose</td>
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</tr>
<tr>
<td>14/4</td>
<td>25/(28)</td>
<td>4/14</td>
<td>5/3</td>
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<td><strong>Choice</strong></td>
</tr>
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<td>Two bottles</td>
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<tr>
<td>Days</td>
<td>H2O:(Sucrose/ETOH) (%)</td>
<td>H2O: Sucrose (%)</td>
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</tr>
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<td>9</td>
<td>H2O:0/5</td>
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<tr>
<td>2-3 weeks</td>
<td>H2O: 5% ETOH</td>
<td>H2O: H2O</td>
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<td>P/NP rats</td>
<td>P/NP rats</td>
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<td>4/4</td>
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<td><strong>Bottle A: Bottle B</strong></td>
<td>Counting TBs</td>
</tr>
<tr>
<td>H2O/0.15 M NaCl⁺</td>
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<td>P/NP rats</td>
<td>P/NP rats</td>
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<th><strong>Group 11</strong></th>
<th><strong>Group 12</strong></th>
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<tbody>
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<td>No treatment</td>
<td>No treatment</td>
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<table>
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<tr>
<th><strong>Two Bottles</strong></th>
<th><strong>Bottle A: Bottle B</strong></th>
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<tbody>
<tr>
<td><strong>H2O:(H2O+Bz)</strong></td>
<td>SD rats: SD rats</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>WT/TRPV1 KO mice</td>
</tr>
<tr>
<td></td>
<td>12/3</td>
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</table>
control group. For the choice paradigm, Group 5 is the test group and Group 6 is the control group. P and NP rats in each of the groups 1, 2, 3, 5 and 6 were either used for CT nerve recordings or were sacrificed and their tissues were harvested for gene expression and protein analysis. The P and NP rats in groups 4 and 7 were used for behavioral studies or for collecting tissues for gene expression studies. In Group 8, P and NP rats were used for 2 bottle preference tests for NaCl, NaCl+Bz and NaCl+Ba+GalA-MRPs. When Bz was used with the salt solution, Bz was also added to the second bottle containing H₂O. The fluid intakes were monitored when Bottle A and Bottle B contained the following solutions (BottleA/Bottle B): (i) (H₂O)/(0.15 M NaCl); (ii) (H₂O+5x10⁻⁶ M Bz)/(0.15 M NaCl+5x10⁻⁶ M Bz); and (iii) (H₂O+5x10⁻⁶ M Bz+0.25% GalA-MRPs)/(0.15 M NaCl+5x10⁻⁶ M Bz+0.25% GalA-MRPs). In Group 9, lingual epithelium was isolated by collagenase treatment from P and NP rats and used for counting the number of taste buds (TBs). In addition to P and NP rats, we used Sprague-Dawley (SD) rats (Groups 10 and 11). Three rats were used to test the effect of Bz on H₂O intake. Additional 6 rats were used to test the effect of U73122 and BAPTA on sweet responses. We also used 3 wildtype (WT; Group 12) and 3 TRPV1 knockout (KO; Group 12) mice to test the effect of TRPV1 agonists on the CT response to 0.15 M NaCl+5x10⁻⁶ M Bz. Additional 5 WT mice (Group 12) were used to construct a cDNA library from fungiform taste buds (FF TBs), circumvallate taste buds (CV TBs) and the non-gustatory lingual epithelium devoid of taste buds (NG Epi) to screen for TRPM5, α-gustducin and TRPV1.
Table 2

Composition of stimulating solutions and drugs used in CT experiments

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition (M)</th>
<th>pH</th>
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<tbody>
<tr>
<td>Rinse (R)</td>
<td>0.01 KCl+0.2 mannitol+0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>Salt stimuli (N)</td>
<td>0.01 KCl+0.1 NaCl+0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>R+RTX</td>
<td>R+0.1$\times$10$^{-6}$ to 10$\times$10$^{-6}$ RTX+0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>N+RTX</td>
<td>N+0.1$\times$10$^{-6}$ to 10$\times$10$^{-6}$ RTX+0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>R+ETOH$^\text{II}$</td>
<td>R+0 to 40% ETOH+0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>N+ ETOH$^\text{II}$</td>
<td>N+0 to 40% ETOH +0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>N+GalA-MRPs</td>
<td>N+0 to 1.5% GalA-MRPs +0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>R+GalA-MRPs</td>
<td>R+0 to 1.5% GalA-MRPs +0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>Control-1</td>
<td>0.3 NH$_4$Cl</td>
<td></td>
</tr>
<tr>
<td>Control-2</td>
<td>0.3 NaCl</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Glycine</td>
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</tr>
<tr>
<td>SC45647</td>
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<td></td>
</tr>
<tr>
<td>Bz</td>
<td>5x10$^{-6}$</td>
<td></td>
</tr>
<tr>
<td>SB-366791</td>
<td>1x10$^{-6}$</td>
<td></td>
</tr>
<tr>
<td>U73122</td>
<td>50x10$^{-6}$ or 150x10$^{-6}$</td>
<td></td>
</tr>
<tr>
<td>U73343</td>
<td>150x10$^{-6}$</td>
<td></td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>33x10$^{-3}$</td>
<td></td>
</tr>
<tr>
<td>Ringer’s solution</td>
<td>0.14 NaCl+0.005 KCl+0.001 CaCl$_2$+0.001 MgCl$_2$+0.01 Na-pyruvate+0.01 glucose+0.01 HEPES</td>
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ETOH = ethanol (200 proof absolute anhydrous from Pharmco-AAPER); GaLA-MRPs (Maillard Reacted Peptides conjugated with galacturonic acid (Katsumata et al. 2008); Bz = benzamil (blocks Na⁺ entry through apical epithelial Na⁺ channel; ENaC); SB-366791 = 4’-Chloro-3-methoxycinnamalilide (blocks Na⁺ entry through TRPV1t) (Gunthorpe et al. 2004), U73122 = 1-[6-[(17β)-3-methoxyestra-1,3,5[10]-trien-17-y]amino]hexyl]-1H-pyrrole-2,5-dione (a non-specific blocker of phospholipase Cs (PLCs) and blocks the hydrolysis of PIP₂ to inositol-1,4,5-trisphosphate (IP₃) + diacylglycerol (DAG) by PLC (Lyall et al. 2010); U73343 = 1-[6-[(17β)-3-methoxyestra-1,3,5[10]-trien-17-y]amino]hexyl]-1H-pyrrole-2,5-pyrrolidine-dione (an inactive analogue of U73122) (Lyall et al. 2010); HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Benzamil and SB-366791 were added to the salt stimuli and produced their effects on the Bz-insensitive NaCl CT responses immediately. However, U73122, U73343 and BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis-(acetoxymethyl) ester) were dissolved directly in 3 ml of dimethyl sulfoxide (DMSO) and were topically applied to the tongue for at least 30 min. DMSO by itself has no effect of the CT responses to sweet or salty taste stimuli (Lyall et al. 1999). GaLA-MRPs were generously provided by Tadayoshi Katsumata of Kirin Kyowa Foods Co. LTD, Ibaraki, Japan. All other drugs were purchased from Sigma. It is important to note that in contrast to 200 proof alcohol used in this study, the beverage grade alcohol (~190 proof) is consumed by humans and used in many previous studies on P and NP rats.
Table 3

T1R3 and TRPV1/TRPV1t mRNA levels in the anterior lingual epithelium containing fungiform taste buds in P and NP rats

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<th>NP rats+H₂O (2⁻ΔCT)</th>
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The 2⁻ΔCT values are expressed as mean±SEM of n, where n represents the number of P and NP rats maintained on H₂O (Group 2; Table 1).

The p values are unpaired comparison of mean±SEM of 2⁻ΔCT values of P and NP rats (Schmittgen and Livak 2008).
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Table 5

T1R3 mRNA levels in the anterior lingual epithelium containing fungiform taste buds and the CT response to sucrose in P and NP rats chronically maintained on H$_2$O, 5% sucrose or 5% ethanol

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The 2$^{-\Delta CT}$ values are expressed as mean±SEM of n, where n represents the number of P and NP rats maintained on H$_2$O (Group 2; Table 1) or chronically exposed to 5% ethanol or 5% sucrose in a no choice paradigm.
The p values are unpaired comparison of mean±SEM of $2^{-\Delta CT}$ values of P and NP rats maintained on H$_2$O or chronically exposed to ethanol or sucrose (Schmittgen and Livak 2008).

In each rat the CT response was normalized to the corresponding CT response obtained with 0.3 M NH$_4$Cl and is presented as the mean±SEM values of the normalized tonic CT response from 3 rats in each group (n). The p values are unpaired comparison of mean±SEM of the normalized tonic CT response between P and NP rats maintained on H$_2$O or after chronic exposure to sucrose or ethanol.
Supplementary Fig. 1. Effect of GalA-MRP on Bz-insensitive NaCl CT responses in P and NP rats. Shows representative CT responses in a NP rat and a P rat maintained on H2O (Group 2; Table 1) while their tongues were first stimulated with the rinse solution R and then with N+Bz+GalA-MRP (0-1%). GalA-MRP (0-1%) did not elicit a CT response in R (Katsumata et al. 2008). The CT responses were normalized to the mean tonic CT responses to 0.3 M NH4Cl obtained in each P and NP rat.

Supplementary Fig. 2. Detection of TRPM5, α-gustducin and TRPV1/TRPV1t in isolated taste buds and non-gustatory epithelium using RT-PCR. cDNA made from C57BL/6J WT mice (Group 12; Table 1) fungiform taste buds (FF TBs), circumvallate taste buds (CV TBs) and the non-gustatory lingual epithelium devoid of taste buds (NG Epi) was screened for TRPM5 (A), α-gustducin (B) and TRPV1 (C). All transcripts were found with bands of expected sizes (DNA ladder on left side in each panel) in the FF and CV taste buds but were not detected in the NG Epi. Negative (-) control was milliQ water. The cDNA from testis (T) was used as a positive control for TRPV1. In addition, cDNA from the isolated CT nerve was used as a negative control for TRPM5 (A) and α-gustducin (data not shown). Beta-actin was found in all tissues (data not shown).

Supplementary Fig. 3. T1R3 and TRPV1/TRPV1t protein levels in the intestinal mucosal cells in P and NP rats maintained on H2O. The figure shows the data from Western blot experiments for T1R3 and TRPV1/TRPV1t in intestinal mucosal cells in 3
P rats and 3 NP rats maintained on H₂O (Group 2; Table 1). The bars represent the density of T1R3 and TRPV1/TRPV1t bands computed relative to the density of β-actin. The p values represent the unpaired comparison between the two groups.

Supplementary Fig. 4. Effects of chronic sucrose exposure on Bz-insensitive NaCl CT nerve responses in P and NP rats. CT responses were measured while the rat tongue was stimulated with R and then with N+Bz+RTX (0-10×10⁻⁶ M) in NP (A) or P (B) rats maintained on H₂O (○) (Group 2; Table 1) or exposed to chronic 5% sucrose (▲) in a no choice paradigm (Group 3; Table 1). In each animal the tonic CT response was normalized to the corresponding tonic CT responses obtained with 0.3 M NH₄Cl. The zero RTX concentration is shown as -8.0 on the X-axis. The gray vertical bars represent points at RTX concentration in μM. The RTX-NaCl+Bz CT response relationship in naïve NP or P rats first shown in Figs. 3B (○) and 4A (○), respectively are also plotted for comparison.

Supplementary Fig. 5. Effect of TRPV1t inhibition on Bz-insensitive NaCl CT responses. CT responses were monitored in WT and TRPV1 KO mice (Group 12; Table 1) while the tongue was stimulated with the rinse solution R and then with N+Bz and N+Bz+TRPV1t agonist (0.25% GalA-MRPs, 1×10⁻⁶ M RTX or 30% ETOH). In each case the tonic CT responses were normalized to the corresponding tonic CT responses obtained with 0.3 M NH₄Cl. Each bar represents the mean±SEM values of the normalized tonic CT response from 3 mice.
Supplementary Fig. 6. (A) Effect of U73122 on the CT responses to SC45647 in the absence and presence of ethanol. CT responses were measured in SD rats while the rat tongue was first stimulated with the rinse solution R and then with R+0.005M SC45647 in the presence of 0 or 30% ETOH under control conditions (Control) and after topical lingual application of 150x10^{-6} M U73122 (Post-U73122). In 3 animals (Group 11; Table 1), in the presence of U73122 the tonic CT response to SC45647 was not different from the baseline (p >0.05) and also inhibited the further increase in the CT response in the presence of ETOH. (B) Effect of BAPTA-AM loading on the CT response to sucrose in the absence or presence of ethanol. CT responses were monitored in SD rats while their tongues were stimulated with a rinse solution and then with 0.5 M sucrose solution containing 0 or 30% ethanol (ETOH) before and after topical lingual application of 33x10^{-3} M BAPTA-AM (Table 2). In 3 animals (Group 11; Table 1), after BAPTA treatment, there was no statistical difference between the CT response to sucrose and sucrose+ETOH (p>0.05).
Supplementary Table 1

T1R3 and TRPV1/TRPV1t mRNA levels in the intestinal mucosal cells in P and NP rats

<table>
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<tr>
<th>mRNA</th>
<th>NP rats+H₂O (2^ΔCT)</th>
<th>P rats+H₂O (2^ΔCT)</th>
<th>Fold change (P/NP)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R3</td>
<td>0.498±0.123</td>
<td>3.686±0.891</td>
<td>7.41</td>
<td>0.0076</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRPV1/TRPV1t</td>
<td>0.00153±0.0004</td>
<td>0.00349±0.0.00054</td>
<td>2.28</td>
<td>0.022</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The 2^-ΔCT values are expressed as mean±SEM of n, where n represents the number of P and NP rats maintained on H₂O (Group 2; Table 1) used for q-RTPCR studies. The p values are unpaired comparison of mean±SEM of 2^-ΔCT values of P and NP rats (Schmittgen and Livak 2008).
Seventy five P and 71 NP rats were used in this study. Out of these, 59 P and 60 NP rats were given 7 different treatments in a no choice (Groups 1-4) or choice paradigm (Groups 5-7). For the no choice paradigm, Group 1 is the test group and Group 2 is the
control group. For the choice paradigm, Group 5 is the test group and Group 6 is the
closest group. P and NP rats in each of the groups 1, 2, 3, 5 and 6 were either used for
CT nerve recordings or were sacrificed and their tissues were harvested for gene
expression and protein analysis. The P and NP rats in groups 4 and 7 were used for
behavioral studies or for collecting tissues for gene expression studies. In Group 8, P
and NP rats were used for 2 bottle preference tests for NaCl, NaCl+Bz and
NaCl+Ba+GalA-MRPs. When Bz was used with the salt solution, Bz was also added to
the second bottle containing H₂O. The fluid intakes were monitored when Bottle A and
Bottle B contained the following solutions (BottleA/Bottle B): (i) (H₂O)/(0.15 M NaCl); (ii)
(H₂O+5x10⁻⁶ M Bz)/(0.15 M NaCl+5x10⁻⁶ M Bz); and (iii) (H₂O+5x10⁻⁶ M Bz+0.25%
GalA-MRPs)/(0.15 M NaCl+5x10⁻⁶ M Bz+0.25% GalA-MRPs). In Group 9, lingual
epithelium was isolated by collagenase treatment from P and NP rats and used for
counting the number of taste buds (TBs). In addition to P and NP rats, we used
Sprague-Dawley (SD) rats (Groups 10 and 11). Three rats were used to test the effect
of Bz on H₂O intake. Additional 6 rats were used to test the effect of U73122 and
BAPTA on sweet responses. We also used 3 wildtype (WT; Group 12) and 3 TRPV1
knockout (KO; Group 12) mice to test the effect of TRPV1 agonists on the CT response
to 0.15 M NaCl+5x10⁻⁶ M Bz. Additional 5 WT mice (Group 12) were used to construct a
cDNA library from fungiform taste buds (FF TBs), circumvallate taste buds (CV TBs)
and the non-gustatory lingual epithelium devoid of taste buds (NG Epi) to screen for
TRPM5, α-gustducin and TRPV1.
## Table 2

### Composition of stimulating solutions and drugs used in CT experiments

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition (M)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinse (R)</td>
<td>0.01 KCl+0.2 mannitol+0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>Salt stimuli (N)</td>
<td>0.01 KCl+0.1 NaCl+0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>R+RTX</td>
<td>R+0.1x10^{-6} to 10x10^{-6} RTX+0.01 HEPES</td>
<td>6.4</td>
</tr>
<tr>
<td>N+RTX</td>
<td>N+0.1x10^{-6} to 10x10^{-6} RTX+0.01 HEPES</td>
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<tr>
<td>R+ETOH¶</td>
<td>R+0 to 40% ETOH + 0.01 HEPES</td>
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<td>Control-1</td>
<td>0.3 NH₄Cl</td>
<td></td>
</tr>
<tr>
<td>Control-2</td>
<td>0.3 NaCl</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.5</td>
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</tr>
<tr>
<td>Glycine</td>
<td>0.25</td>
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</tr>
<tr>
<td>SC45647</td>
<td>0.005</td>
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</tr>
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**TRPV1/TRPV1t mRNA levels in the anterior lingual epithelium containing fungiform taste buds in NP rats chronically maintained on H2O, 5% sucrose or 5% ethanol**

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<td>n</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tonic CT Response/0.3 M NH$_4$Cl</td>
<td>0.274±0.023</td>
<td>0.032±0.011</td>
<td>0.0007</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The 2$^{-\Delta CT}$ values are expressed as mean±SEM of n, where n represents the number of P and NP rats maintained on H$_2$O (Group 2; Table 1) or chronically exposed to 5% ethanol or 5% sucrose in a no choice paradigm.
The p values are unpaired comparison of mean±SEM of $2^{-\Delta CT}$ values of P and NP rats maintained on H$_2$O or chronically exposed to ethanol or sucrose (Schmittgen and Livak 2008).

In each rat the CT response was normalized to the corresponding CT response obtained with 0.3 M NH$_4$Cl and is presented as the mean±SEM values of the normalized tonic CT response from 3 rats in each group (n). The p values are unpaired comparison of mean±SEM of the normalized tonic CT response between P and NP rats maintained on H$_2$O or after chronic exposure to sucrose or ethanol.