LETTER

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High salt recruits aversive taste pathways

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In the tongue, distinct classes of taste receptor cells detect the five basic tastes; sweet, sour, bitter, sodium salt and umami[1,2]. Among these qualities, bitter and sour stimuli are innately aversive, whereas sweet and umami are appetitive and generally attractive to animals. By contrast, salty taste is unique in that increasing salt concentration fundamentally transforms an innately appetitive stimulus into a powerfully aversive one[3–7]. This appetitive–aversive balance helps to maintain appropriate salt consumption[3,4,6,8], and represents an important part of fluid and electrolyte homeostasis. We have shown previously that the appetitive responses to NaCl are mediated by taste receptor cells expressing the epithelial sodium channel, ENaC[8], but the cellular substrate for salt aversion was unknown. Here we examine the cellular and molecular basis for the rejection of high concentrations of salts. We show that high salt recruits the two primary aversive taste pathways by activating the sour- and bitter-taste-sensing cells. We also demonstrate that genetic silencing of these pathways abolishes behavioural aversion to concentrated salt, without impairing salt attraction. Notably, mice devoid of salt-aversion pathways show unimpeded, continuous attraction even to very high concentrations of NaCl. We propose that the 'co-opting' of sour and bitter neural pathways evolved as a means to ensure that high levels of salt reliably trigger robust behavioural rejection, thus preventing its potentially detrimental effects on health.

Sodium is an essential ion, and animals have evolved dedicated salt-sensing systems, including prominent detectors in the taste system. Salt taste in mammals can trigger two opposing behavioural responses; low concentrations of salt (<100 mM NaCl, referred to as 'low salt') are generally appetitive and elicit behavioural attraction, but high concentrations (>300 mM NaCl, referred to as 'high salt') are aversive and provoke strong behavioural rejection. Notably, the attractive salt pathway is selectively responsive to sodium (underscoring the key requirement of NaCl in the diet), whereas the aversive salt pathway functions as a non-selective detector for a wide range of salts[3,4,6,7].

For many years, the sensitivity of ENaC (epithelial sodium channel; also known as SCNN1) to the diuretic amiloride[9–12] has been used as a powerful means to block ENaC function and separate the contributions of the appetitive and aversive salt pathways[8,10,13]. We reasoned that if we could identify an equivalent pharmacological blocker for the high-salt-sensing pathway, this might provide a valuable tool to dissect the cellular basis of high-salt taste. We therefore recorded chorda tympani taste responses in the presence or absence of various compounds that are known to affect ion-channel function (Supplementary Table 1) and found that allyl isothiocyanate (AITC), a component of mustard oil (and the source of its pungency) significantly suppressed responses to high concentrations of NaCl (Fig. 1a, top panel), without affecting responses to low concentrations of NaCl (see Methods). Identical suppression was observed for KCl, which selectively activates the high-salt pathway (Fig. 1a and Supplementary Fig. 1). Interestingly, AITC also inhibited responses to bitter stimuli without significantly impacting any other taste modality (Fig. 1a, bottom panel, and Supplementary Fig. 2; see Methods for details on conditions). These results suggested that taste receptor cells (TRCs) for bitter taste might be the target of AITC, and a constituent of the high-salt sensing pathway. Thus, we next asked whether bitter-sensing cells are activated by high-salt stimuli.

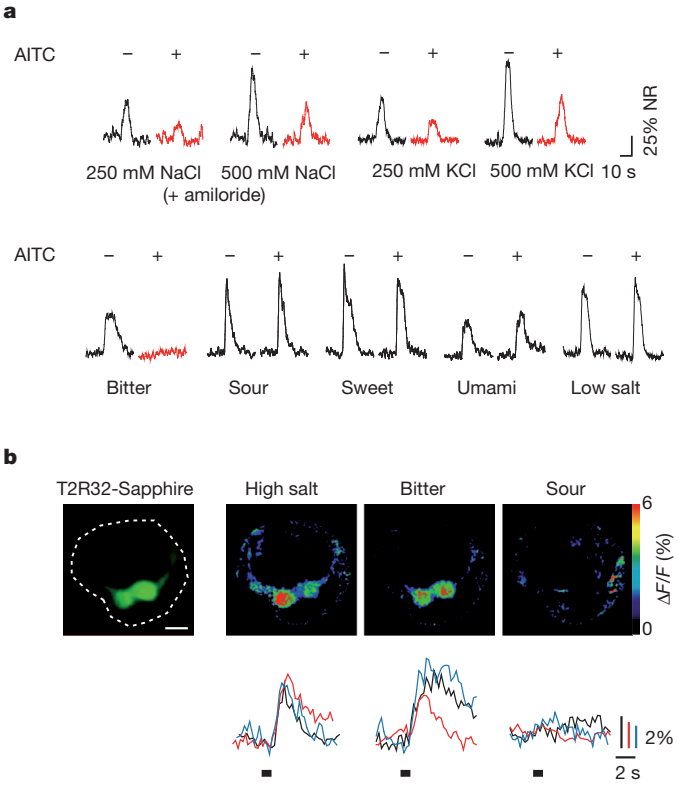

Figure 1 | Bitter receptor cells mediate high-salt taste responses. AITC acts as a selective inhibitor of bitter and high-salt taste responses. **a**, Integrated chorda tympani responses to taste stimuli (see Methods for details) before (−) and after (+) application of AITC; amiloride was used to selectively eliminate the contribution of the ENaC-dependent, low-salt pathways. AITC completely inhibited bitter responses (0.1 mM cycloheximide) and significantly suppressed high-salt responses (250 or 500 mM NaCl + amiloride, and 250 or 500 mM KCl; highlighted in red) but did not affect responses to low-salt (60 mM NaCl) or other taste qualities; representative responses from multiple animals are shown (see also Supplementary Fig. 1). NR, normalized response. **b**, Calcium imaging of taste-cell responses confirmed that T2R32-Sapphire-positive taste cells respond to bitter stimuli (mixture of 1 mM cycloheximide, 1 mM quinine and 10 mM denatonium) and high salt (500 mM KCl), but not to sour stimuli (100 mM citric acid). Shown is a taste bud overlaid with Sapphire fluorescence (dotted circle, left), and three pseudo-coloured images (right panels) depicting its responses to high salt, bitter and sour stimuli, respectively. Scale bar, 10 μm. Below the imaging panels are representative $\Delta F/F$ traces for these tastants from three additional T2R32-Sapphire-positive cells; black bars beneath the traces denote the window of tastant application. In total, 15 and 12 T2R32-Sapphire-positive cells were activated by bitter and KCl respectively; among these, 11 cells were activated by both compounds but not by sour stimuli (see Supplementary Fig. 4).

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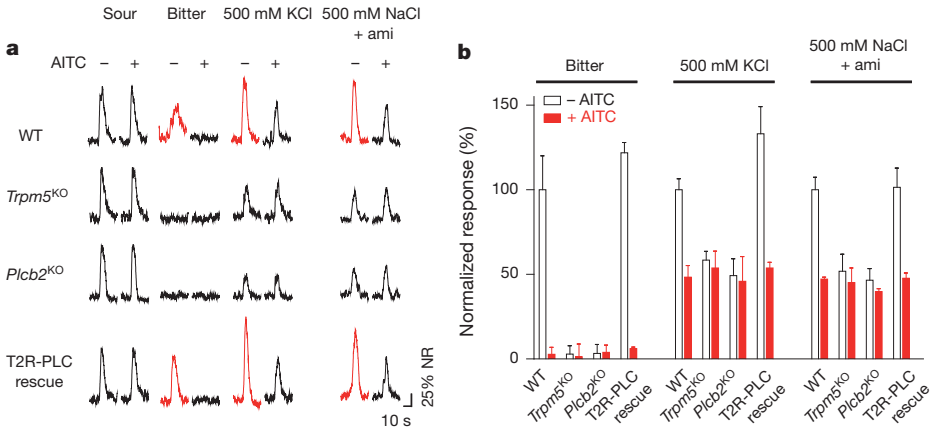

Figure 2 | High-salt responses in bitter cells are TRPM5- and PLC-β2-dependent. **a**, Representative chorda tympani responses from control (wild type; WT), *Trpm5* knockout (*Trpm5*^{KO}), *Plcb2* knockout (*Plcb2*^{KO}) and T2R32-PLC-β2-rescue (T2R-PLC rescue) mice before (−) and after (+) application of AITC. Note that both *Trpm5* knockout and *Plcb2* knockout mice lose bitter responses and a significant part of their response to high salt together with all sensitivity to AITC. Expressing PLC-β2 in just the bitter cells of *Plcb2* knockout mice (T2R-PLC rescue) fully restores normal bitter and high-salt responses as well as AITC sensitivity (shown by red traces); responses to 500 mM NaCl plus amiloride (ami) were from different animals. **b**, Quantification of normalized responses, before (open bars) and after (red bars) application of AITC (mean ± s.e.m., $n \geq 3$ mice; see Methods for normalization). AITC treatment almost completely suppressed responses to 0.1 mM cycloheximide and reduced by half the responses to 500 mM KCl and 500 mM NaCl in the presence of 10 μM amiloride in control and T2R-PLC rescue animals (Student's *t*-test, $P < 0.05$).

We examined salt responses directly using a peeled epithelium preparation that enables functional imaging of TRCs—at single-cell resolution—in response to tastant stimulation[8]. In essence, TRCs from fungiform papillae were loaded with the calcium-sensitive dye, Calcium Green-1 *in vivo*, and then stimulated and imaged *ex vivo*[8]. To ensure that we focused on bitter-sensing cells, we used mice expressing a sapphire variant of the green fluorescent protein (GFP) selectively in T2R-positive cells[14] (T2Rs are the family of G-protein-coupled receptors that mediate bitter-taste sensing) (Supplementary Fig. 3). In these animals, high concentrations of salt indeed activated the GFP-positive cells, which in turn responded to bitter tastants (Fig. 1b and Supplementary Fig. 4).

The finding that high salt activates bitter-sensing cells, and the observation that high salt and bitter stimuli are both blocked by AITC suggest that bitter and high-salt taste may share a common pathway (for example, through the T2R pathway). If this is the case, we would expect *Trpm5* or phospholipase Cβ2 (*Plcb2*) knockout knockout mice[15], which lack key components for bitter-taste signalling, to be also defective in high-salt sensing. Indeed, Fig. 2 shows this to be the case: the nerve responses of the knockout animals to high salt are significantly reduced, and are no longer sensitive to AITC. To confirm that the TRPM5- and PLC-β2-dependent high-salt responses are mediated by bitter receptor cells, we conducted a selective-rescue experiment in which PLC function was restored only to bitter-taste receptor cells of *Plcb2* knockout mice. As expected, expressing a wild-type PLC transgene in bitter receptor cells fully rescued the electrophysiological responses to both bitter and high salt (for example, KCl) to levels indistinguishable from those in wild-type mice (Fig. 2a, b). These results show that bitter-sensing cells mediate the PLC-β2-dependent high-salt responses, and support the proposal that the aversion to high salt is mediated, at least partly, by activation of the bitter-sensing pathway.

AITC, and *Trpm5* or *Plcb2* knockouts eliminate only approximately 50% of the high-salt neural responses (Fig. 2). Not surprisingly, these animals still retain strong behavioural aversion to high salts[15]. Which additional cells mediate the remaining neural responses and behaviour? Given that high salt is strongly aversive and recruits one of the primary aversive taste pathways, we considered that sour taste—the other principal aversive pathway—may mediate the remaining responses.

To examine the involvement of sour-sensing cells in high-salt detection, we inactivated the sour TRCs (PKD2L1-expressing cells[16]) by silencing their synaptic machinery. We engineered animals in which tetanus toxin light chain (TeNT) was targeted to PKD2L1-expressing cells[17,18] and then assayed their tastant-evoked neural activity in response to salt stimulation. As shown previously[17], silencing PKD2L1-expressing

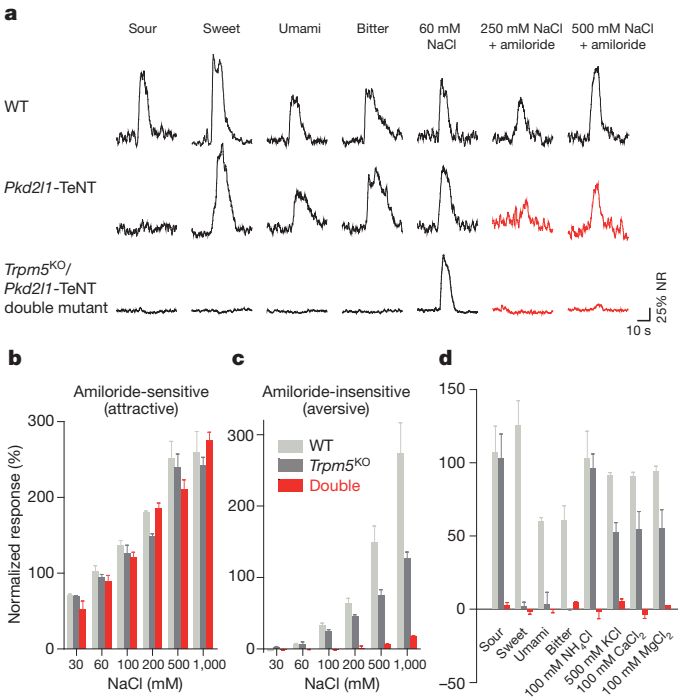

Figure 3 | PKD2L1-expressing cells mediate the residual TRPM5- and PLC-β2-independent high-salt responses. **a**, Integrated chorda tympani recordings show that silencing PKD2L1 sour-sensing cells affects high-salt taste responses. *Pkd2l1*-TeNT mice have severe deficits in their responses to high salt, but *Trpm5*^{KO}/*Pkd2l1*-TeNT double-mutant animals completely lose all amiloride-insensitive NaCl (high-salt) responses (highlighted as red traces). **b**, **c**, Quantification of neural responses shows that double-mutant mice (double) exhibit normal neural responses to low salt (**b**), but lack responses to high salt (**c**) (Student's *t*-test, $P < 0.001$). By contrast, the single mutants (*Trpm5*^{KO}) only show partial loss of responses to high salt. **d**, The double-mutant animals also fail to respond to sweet, bitter, sour and umami as well as non-sodium salts. Data (**b**–**d**) were normalized to the response of 60 mM NaCl and are mean ± s.e.m., $n \geq 3$ mice.

cells eliminates acid-evoked taste responses (Fig. 3a). However, as shown in Fig. 3, these animals also display a major reduction in their high-salt electrophysiological responses, and further treatment with AITC effectively abolished their remaining high-salt (KCl) responses (Supplementary Fig. 5). Therefore, we reasoned that high-salt taste responses are mediated by the combined action of bitter- and sour-sensing cells, and proposed that genetically blocking both pathways should eliminate high-salt responses. Indeed, double-mutant mice expressing *Pkd2l1*-TeNT and harbouring a *Trpm5* mutation show a near-complete loss of electrophysiological taste responses to a variety of high salts (Fig. 3), including concentrations of NaCl as high as 1,000 mM.

Importantly, if these two cellular pathways are the mediators of behavioural aversion to high salt, then simultaneous silencing of the T2R- and PKD2L1-expressing cells should abolish rejection of concentrated salt solutions. As shown in Fig. 4 and Supplementary Fig. 6, single-mutant mice (*Trpm5*$^{-/-}$ or *Pkd2l1*-TeNT) still retain strong aversion to high salt, demonstrating that activation of either pathway on its own is sufficient to trigger behavioural rejection to salt. However, double-mutant animals show no salt aversion even at concentrations at which controls are strongly repelled. Remarkably, these double mutants are not simply indifferent to high salt but exhibit unimpeded attraction, even to very high concentrations of salt (for example, levels equivalent to ocean water; approximately 500 mM NaCl; Fig. 4b). Thus, under normal conditions the appetitive–aversive balance for salt, which collectively tunes the animal's behavioural response to sodium salts, must be orchestrated by the combined activity of the attractive ENaC pathway (which remains in the bitter and sour double mutants) and the repulsive T2R and sour pathways.

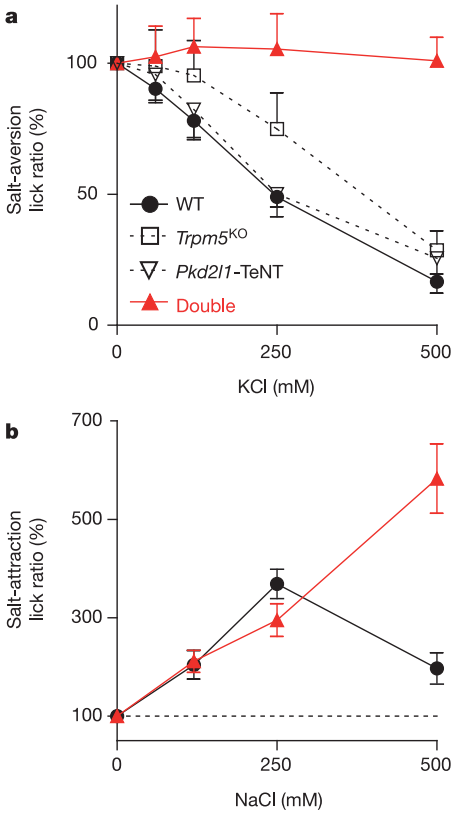

Figure 4 | *Trpm5*^{KO}/*Pkd2l1*-TeNT double-mutant mice show no taste aversion to high salt. Immediate lick assays were used to measure behavioural responses to KCl and NaCl. **a**, Control mice (WT, solid black line) show robust dose-dependent behavioural aversion to increasing concentrations of KCl. By contrast, *Trpm5*^{KO}/*Pkd2l1*-TeNT double-mutant animals do not avoid high-salt stimuli; single mutants behave in the same way as control animals. Two-way analysis of variance (ANOVA) with post-hoc test for individual concentrations revealed significant differences at 500 mM KCl between the double mutants and other genotypes ($P < 0.001$) and at 250 mM KCl between the double mutants and control or *Pkd2l1*-TeNT mice ($P < 0.001$). **b**, After sodium depletion, control mice (black line) show powerful attractive responses to NaCl (see also Supplementary Fig. 7) but the attraction is considerably reduced at higher concentration (500 mM). By contrast, double-mutant animals (red line) show a continuous increase in attraction to NaCl even at concentrations as high as 500 mM NaCl (two-way ANOVA with post-hoc test, $P < 0.001$). Values are mean ± s.e.m., $n \geq 6$ mice. Horizontal dashed line in **b** denotes baseline (no preference relative to water).

The mechanisms by which high salt activates the bitter- and sour-taste receptor cells are not known. However, given that the primary effectors of T2R signalling in bitter cells, PLC-β2 and TRPM5, are also required for high-salt sensing by the bitter cells, we suggest that either a signalling component in bitter cells (for example, an ion channel), or 1 or more of the approximately 30 T2Rs may be sensitive to high concentrations of salt (perhaps causing the serendipitous transition between the receptor's inactive and active states)[19,20]. With respect to the sour-sensing cells, one salient feature is the prominent expression of carbonic anhydrase 4 (CA4)[17], a membrane-bound isoform of carbonic anhydrase. CA4 is likely to be involved in buffering the pH around taste receptor cells ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$), and therefore its activity may impact local proton concentration and acid sensing. Notably, carbonic anhydrases are known to be sensitive to high-ionic-strength environments, with high-salt concentrations markedly inhibiting their enzymatic activity[21,22]. This raises the possibility that CA4 may function as a 'translator' of external salts into local pH changes, and thus operate as an important component of the high-salt receptor in sour-sensing cells. Indeed, our results shown in Supplementary Figs 8 and 9 support this proposal by demonstrating that pharmacological inhibition of tongue carbonic anhydrases, or the knockout of CA4, greatly impairs high-salt sensing by the sour-taste receptor cells.

Taken together, our studies reveal that salts activate three different classes of TRCs: the appetitive responses are mediated through the sodium-selective ENaC pathway[1], whereas the rejection of high salt results from the recruitment of the sour- and bitter-taste-sensing pathways. At a cellular level, these results resolve the conundrum of a 'valence change' by showing that it is a simple matter of having distinct cell types with well-defined but opposing valences responding to salt. At a physiological level, these findings now provide a simple explanation for the long-standing observation that bitter and sour afferent fibres behave as 'generalists', responding not only to bitter and acid stimuli but also to a variety of salts[23]. Importantly, we note that the fact that T2R and PKD2L1 cells are also activated by high salt does not imply a change in the logic of taste coding, or in the valence or quality encoded by these TRCs. In fact, if we assume that rodents and humans use similar taste-signalling mechanisms, then it is possible that the bitterness[24] and 'ionic' taste associated with high concentrations of non-sodium salts in humans may be mediated by the concurrent activation of T2R- and PKD2L1-expressing cells. But why does high salt not taste like a mix of bitter and sour? Sourness represents the detection of protons by at least two separate signalling pathways in the oral cavity: taste (PKD2L1 cells) and non-taste (for example, through TRPV1 or ASIC)[25–27], we therefore suggest that the activation of PKD2L1 cells, in the absence of the non-taste acid-sensing pathway, may instead evoke the ionic taste characteristic of high concentrations of non-sodium salts. This proposal re-casts PKD2L1 cells and their corresponding neural line as sensors of ions (for example, protons and potassium), orchestrating different percepts whether activated alone (for example, ionic taste) or in combination (PKD2L1 + non-taste acid sensors = sour taste, whereas PKD2L1 + T2Rs = the taste of KCl and other non-sodium salts).

Future studies using specific inhibitors and activators of each pathway should help to address the contributions of the ENaC-, T2R- and PKD2L1-expressing taste cells to human salt-taste perception, and may serve as a springboard for the development of selective receptor-cell modulators to help to control (and even satisfy) our strong appetite for high salt, but without the potential ill effects of too much sodium.

METHODS SUMMARY

Mice and nerve recordings. T2R32-Sapphire mice are transgenically engineered to express the blue-shifted GFP-derivative, Sapphire[28], under the control of the T2R32 promoter (T2R32 is also known as TAS2R139). All other strains have been described previously[14–17]. Lingual stimulation and recording procedures were carried out as described previously[8,29]. For pharmacological blocking experiments, 3 mM AITC was applied to the tongue for 5 min before initiation of the recording session; use of higher doses and/or repeated application of AITC often led to less specific inhibition of responses.

Calcium imaging. Calcium imaging from fungiform TRCs was carried out as described previously[8,30].

Behavioural assays. Behavioural assays used a custom-made gustometer to measure immediate lick responses as described previously[8,14,15]. For salt-attraction assays, mice were injected with furosemide (50 mg kg^{-1}) and were placed in their home cage for 3 h without food or water before testing. For salt-aversion assays, mice were deprived of water for 24 h before testing. Three (attraction assay) or two (aversion assay) different concentrations of tastant and water were presented to animals in each experimental session. Data were analysed for statistical significance using a two-way ANOVA with a Bonferroni post-hoc test.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions Y.O. designed the study, carried out electrophysiological, biochemical, pharmacological and behavioural experiments, analysed data and wrote the paper; M.B. carried out nerve recordings and behavioural studies; L.v.B. carried out nerve recordings and localization studies. N.J.P.R. and C.S.Z. designed the study, analysed data and wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to C.S.Z. (cz2195@columbia.edu) or N.J.P.R. (nick.ryba@nih.gov).

METHODS

Mice. All procedures were carried out in accordance with the US National Institutes of Health (NIH) guidelines for the care and use of laboratory animals, and were approved by the Columbia University or National Institute of Dental and Craniofacial Research Animal Care and Use Committees. T2R32-Sapphire mice are transgenically engineered to express the blue-shifted GFP-derivative, Sapphire[28], under the control of the T2R32 promoter (also known as TAS2R139). These mice, generated by K. Mueller, contained 10 kilobase pairs upstream of the T2R32 start codon fused to the GFP reporter. Expression of Sapphire in taste tissue was characterized using double-label *in situ* hybridization (see Supplementary Fig. 3). All other mouse strains have been described previously[14–17].

Calcium imaging. Calcium imaging from fungiform TRCs was carried out as described previously[8,29]. In brief, fungiform TRCs were loaded *in vivo* with 3 kilodaltons Calcium Green-1 dextran (Invitrogen) by electroporating individual taste buds. Tongues were removed 24–36 h after dye loading and the epithelium was peeled enzymatically and placed in a custom recording chamber. The apical surface of the preparation was bathed in a constant flow of artificial saliva, and taste stimuli were delivered by focal application to individual taste buds. Tastants were applied for 1 s, with a minimum of 10 s of artificial saliva between stimuli. Changes in $[Ca^{2+}]_i$ (intracellular calcium concentration) were monitored using a 5-Live confocal microscope (Zeiss) equipped with a ×40 C-Apochromat 1.20 W objective; images were captured at 4 Hz, and $\Delta F/F$ from individual TRCs were analysed and pseudo-coloured as described previously[8]. To identify Sapphire-positive cells in T2R32-Sapphire mice, we used a 405-nm excitation laser to separate Sapphire and Calcium Green-1 fluorescence. Mean cellular fluorescence intensity (F) was calculated for the individual TRCs, and the basal fluorescence (F_o) was assigned to each cell by averaging fluorescence intensity over 3 s just before tastant application. $\Delta F/F$ was calculated as $(F - F_o)/F_o$; taste cells were considered to have responded when $\Delta F/F$ exceeded 3 standard deviations above F_o within 5 s of tastant application.

Nerve recordings. Lingual stimulation and recording procedures were carried out as described previously[8,30]; data analysis used the integrated response during the 5 s of tastant stimulation. Compounds used for nerve recordings were: 0.03–1 M NaCl (with and without 10 μM amiloride) or 0.03–1 M KCl (salty); 20 mM acesulfameK (sweet); 50 mM monopotassium glutamate plus 0.5 mM inosine monophosphate (umami); 0.1 mM cycloheximide (bitter); and 20 mM citric acid (sour). Responses to 20 mM citric acid (Fig. 1 and Supplementary Fig. 1), 60 mM NaCl (Fig. 3 and Supplementary Figs 7, 8b and 9) or 250 mM KCl before AITC (Aldrich) application (Supplementary Fig. 8a) were used to normalize responses for each experimental series. For Fig. 2, data were normalized to 20 mM citric acid and then scaled to wild-type responses before AITC application. Data were analysed for statistical significance using an unpaired, one-tailed Student's t-test and 95% confidence limits.

To compute the amiloride-sensitive salt component, the stimulation regime involved sequential applications of NaCl solutions, first without and then with amiloride (from 5 s before to 5 s after stimulation) in the same experimental series. The amiloride-insensitive component was defined as the response in the presence of amiloride. The fraction of the response inhibited by amiloride was defined as the amiloride-sensitive component (amiloride-sensitive component = response without amiloride − response with amiloride).

For pharmacological inhibition studies using AITC, responses to a series of taste stimuli were measured. Then 3 mM AITC was applied to the tongue at a rate of 6 ml min^{-1} for 5 min. The tongue was washed with artificial saliva for 1 min and nerve responses to the same series of taste stimuli were measured; responses before and after AITC were compared for each animal. To minimize effects of recovery, responses after AITC were recorded within 15 min of AITC treatment.

For pharmacological studies using bicarbonate, taste responses were measured in the presence or absence of 30 mM $KHCO_3$, pH 7.4 (applied 5 s before and during tastant stimulation). In dorzolamide (DZA) experiments, responses were monitored before and after incubation of the tongue with 0.5% DZA (w/v) for 5 min. To study the effects of pH on nerve responses, we adjusted the pH of artificial saliva (7.4) to 5.5 with hydrochloric acid.

Behavioural assays. Behavioural assays used a custom-made gustometer to measure immediate lick responses as described previously[8,14,15]. For salt-attraction assays, mice were injected with furosemide (50 mg kg^{-1}) and were placed in their home cage for 3 h without food or water before testing. For salt-aversion assays, mice were deprived of water for 24 h before testing. Three or four (attraction assay) or two (aversion assay) different concentrations of tastant and water were presented to animals in each experimental session. Differences between knockout and control mice were analysed for statistical significance using a two-way ANOVA with a Bonferroni post-hoc test.