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LETTERS

The cells and peripheral representation of sodium taste in mice

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Salt taste in mammals can trigger two divergent behavioural responses. In general, concentrated saline solutions elicit robust behavioural aversion, whereas low concentrations of NaCl are typically attractive, particularly after sodium depletion[1–5]. Notably, the attractive salt pathway is selectively responsive to sodium and inhibited by amiloride, whereas the aversive one functions as a non-selective detector for a wide range of salts[1–3,6–9]. Because amiloride is a potent inhibitor of the epithelial sodium channel (ENaC), ENaC has been proposed to function as a component of the salt-taste-receptor system[1,3,6–14]. Previously, we showed that four of the five basic taste qualities—sweet, sour, bitter and umami—are mediated by separate taste-receptor cells (TRCs) each tuned to a single taste modality, and wired to elicit stereotypical behavioural responses[5,15–18]. Here we show that sodium sensing is also mediated by a dedicated population of TRCs. These taste cells express the epithelial sodium channel ENaC[19,20], and mediate behavioural attraction to NaCl. We genetically engineered mice lacking ENaCα in TRCs, and produced animals exhibiting a complete loss of salt attraction and sodium taste responses. Together, these studies substantiate independent cellular substrates for all five basic taste qualities, and validate the essential role of ENaC for sodium taste in mice.

Sodium is the major cation of extracellular fluids and an essential component of every fluid compartment in the body. It is therefore not surprising that animals have evolved dedicated salt-sensing systems, including prominent detectors in the taste system[1–3]. These salt-sensitive receptors are crucial for the acceptance of low concentrations of sodium (for example, to satisfy the 'salt appetite')[1,3] while simultaneously serving as a warning mechanism against hyper-salinity[2,3], thus helping to maintain ion and water homeostasis. For humans, the 'taste for salt' also has direct bearing on excessive Na^+-consumption, which is believed to be a significant dietary risk factor in hypertension, particularly in the developed world[21]. In mice, the low-concentration, and behaviourally 'attractive' salt-taste pathway has three salient properties: it is activated at NaCl concentrations as low as 10 mM, it is highly selective for sodium versus other cations, and it is blocked by lingual application of the ion-channel inhibitor amiloride[8,9,14,22]. The high-concentration (aversive) pathway, conversely, begins to be significant only at concentrations greater than 150 mM NaCl, it is non-selective for sodium (that is, other salts are equally effective), and it is amiloride-insensitive[9,14,22].

To explore the cellular basis for the taste of NaCl (that is, determine whether the distinct physiological and behavioural responses are mediated by the same or separate TRCs), we developed a new preparation that allows functional imaging of TRCs in response to salt stimulation. In essence, TRCs in fungiform papillae were loaded with the calcium-sensitive dye calcium green[23] *in vivo*, and then were stimulated and imaged, *ex vivo*, with a regime that either preferentially activated the low-concentration pathway (100 mM NaCl), or activated both the high- and low-concentration pathways (500 mM NaCl). To separate the contribution of each of the two salt-sensing systems at high-stimulus concentrations, we examined the salt responses in the presence and absence of 10 μM amiloride (Fig. 1). Receptor cells that are only activated by high concentrations of salt also respond to a wide range of non-sodium salts (for example, from KCl to *N*-methyl-D-glucamine (NMDG)-Cl; Fig. 1 and Supplementary Fig. 1), and their activity is unaffected by the presence of amiloride (Fig. 1b, c). In contrast, low-concentrations of NaCl activate a completely separate population of TRCs; these cells do not respond to non-sodium salts (Fig. 1a, c, see also Supplementary Fig. 1), and their responses are blocked by amiloride (Fig. 1c). These results demonstrate the presence of two anatomically distinct salt-sensing systems, and accordingly suggest that the appetitive and aversive behaviours are mediated by non-overlapping populations of TRCs. As the TRCs activated by low concentrations of NaCl are highly selective for sodium salts, we consider them to be the dedicated sodium-sensing system and thus are the subject of this study.

Because amiloride is an inhibitor of the epithelial sodium channel (ENaC), ENaC has been proposed to be a potential component of the salt-taste-receptor system[1,6,8,10–12,14]. The ENaC channel is made up of three subunits (α, β and γ), and has an important role in regulating trans-epithelial transport of Na^+ in a wide range of tissues, including kidneys, airway cells of the lung, epithelial skin cells, and the ducts of salivary and sweat glands[19,20]. Although the knockout of any ENaC subunit is sufficient to completely abolish ENaC function[20], conventional ENaC knockouts die within a few days of birth[20], precluding their use in physiological and behavioural studies of taste.

To examine the role of ENaCs in the taste system, we used a floxed ENaCα conditional knockout strategy (*Scnn1a*^{flox})[24]. In essence, we generated animals in which ENaC function was selectively eliminated in all differentiated TRCs by using the cytokeratin19 gene—a marker for all mature taste cells (see Supplementary Fig. 2) to drive the expression of Cre-recombinase in the taste system. To investigate the taste responses of the conditional ENaCα knockout mice, we recorded tastant-induced action potentials from nerves innervating the taste cells of the tongue; this physiological assay monitors the activity of the taste system at the periphery and provides a reliable measure of TRC function[9,18,25]. In wild-type mice, NaCl elicits a dose-dependent increase in action potentials in the chorda tympani nerve,

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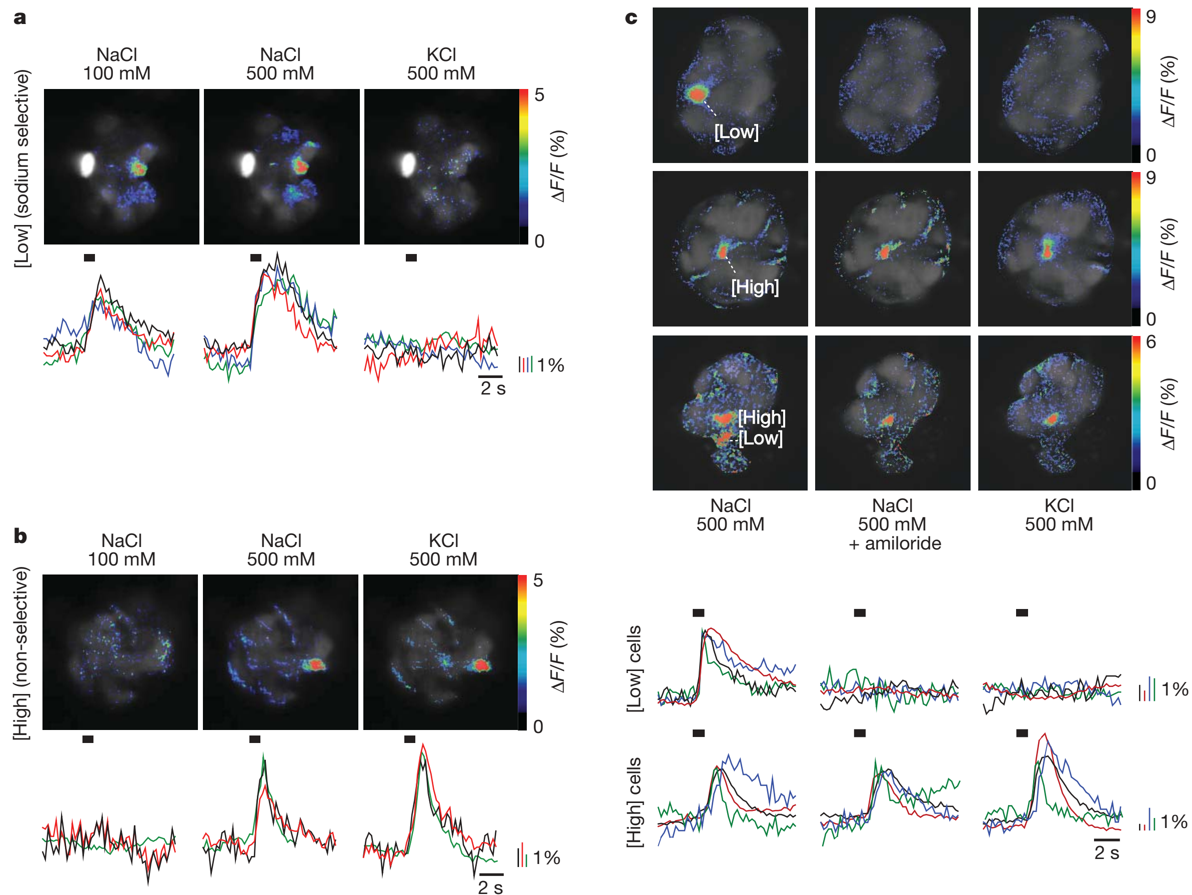

Figure 1 | Two classes of TRCs mediate distinct salt taste responses. Fungiform taste buds loaded with the activity sensor calcium green respond with high selectivity and specificity to different concentrations of salt. **a**, A unique subset of TRCs (labelled as [low]) respond to low concentrations of sodium chloride (100 mM) as well as higher concentrations (500 mM) but not to other salts (KCl). Shown below the imaging data are individual traces from four different TRCs depicting the kinetic and amplitude changes in intracellular calcium levels after salt stimulation; calcium changes were pseudo-coloured as depicted. **b**, A different population of TRCs (labelled as [high]) are activated only at increased NaCl concentrations (500 mM) and are also stimulated by KCl; shown are individual traces for three different TRCs. **c**, Amiloride selectively blocks [low] responses but has no effect on [high] responses; shown are individual traces for four different TRCs; the duration of tastant application is denoted by black bars. See Supplementary Fig. 1 for a diagram of the preparation, quantifications and responses to other salts.

with a physiological response threshold of approximately 10 mM (Fig. 2). Loss of ENaCα in the taste system does not affect responses to four of the five basic taste qualities: sweet, bitter, umami and sour stimuli (Fig. 2d and Supplementary Fig. 3). In contrast, ENaCα knockouts show a complete loss of the responses to low concentrations of NaCl (Fig. 2). As would be expected if ENaC was the sodium sensor, these animals are also missing all amiloride-sensitivity in their NaCl responses (Fig. 2). Importantly, the knockout mice retain all responses to non-sodium salts (Fig. 2d and Supplementary Fig. 3). These results demonstrate that taste responses to salts are mediated by genetically separable components.

Animals ranging from simple invertebrates to mammals readily consume low to moderate concentrations of Na^+, and actively seek it under conditions of salt deprivation[1,2,4,26]. Therefore, we carried out behavioural tests of salt consumption to examine the taste behaviour of the ENaCα conditional knockout animals both under conditions of salt depletion (to test attraction) and under water deprivation (to test aversion)[5]. We reasoned that if ENaC encodes the principal sodium taste sensor, it should mediate all attraction to salt, and consequently, the knockout mice should have a total loss of behavioural attraction to NaCl. Indeed, ENaCα knockout mice show no significant attraction to salt, even under conditions in which control animals have an extraordinary appetite for sodium (Fig. 3a). In contrast, the aversive responses to high concentrations of NaCl (and KCl) are unaltered in the same knockout animals (Fig. 3b, c). Notably, behavioural responses to sweet, sour, umami and bitter tastants are indistinguishable from control animals (Fig. 3d). These results validate ENaC as the mammalian taste receptor responsible for behavioural acceptance of (and attraction to) NaCl.

Our previous studies have shown that sweet, bitter, umami and sour tastes are mediated by independent populations of TRCs, each tuned to a single taste modality[5,15,17,18]. If this labelled-line logic of taste coding at the periphery extends to all five basic taste modalities, then sodium taste should also be mediated by a unique population of TRCs. Thus, we examined whether the amiloride-sensitive salt-sensing cells indeed define a sub-population of TRCs separate from sweet, bitter, sour and umami TRCs. We engineered mice expressing Cre-recombinase under the control of the ENaCα gene, and then crossed them to a floxed green fluorescent protein (GFP) reporter line (*Z/EG*). To validate the fidelity of Cre expression in ENaCα-expressing cells, we analysed progeny from four independent Cre-driver founders and confirmed proper GFP reporter expression in the airway cells of the lung as well as in the kidney cortical collecting duct cells and distal convoluted tubules—well-characterized sites of ENaCα expression[19] (see Supplementary Fig. 4).

Co-labelling with the sweet/umami/bitter TRC marker, TrpM5 (ref. 17), demonstrated that ENaCα-expressing cells are distinct from sweet-, bitter- or umami-TRCs (Fig. 4a and Supplementary Fig. 6). In fungiform and palate taste buds co-localization with a sour cell marker, Car4 (ref. 27), showed the presence of two populations of TRCs: one exhibiting co-expression of ENaCα and Car4 (Fig. 4b), and importantly, a second one expressing ENaCα, but not sour, sweet,

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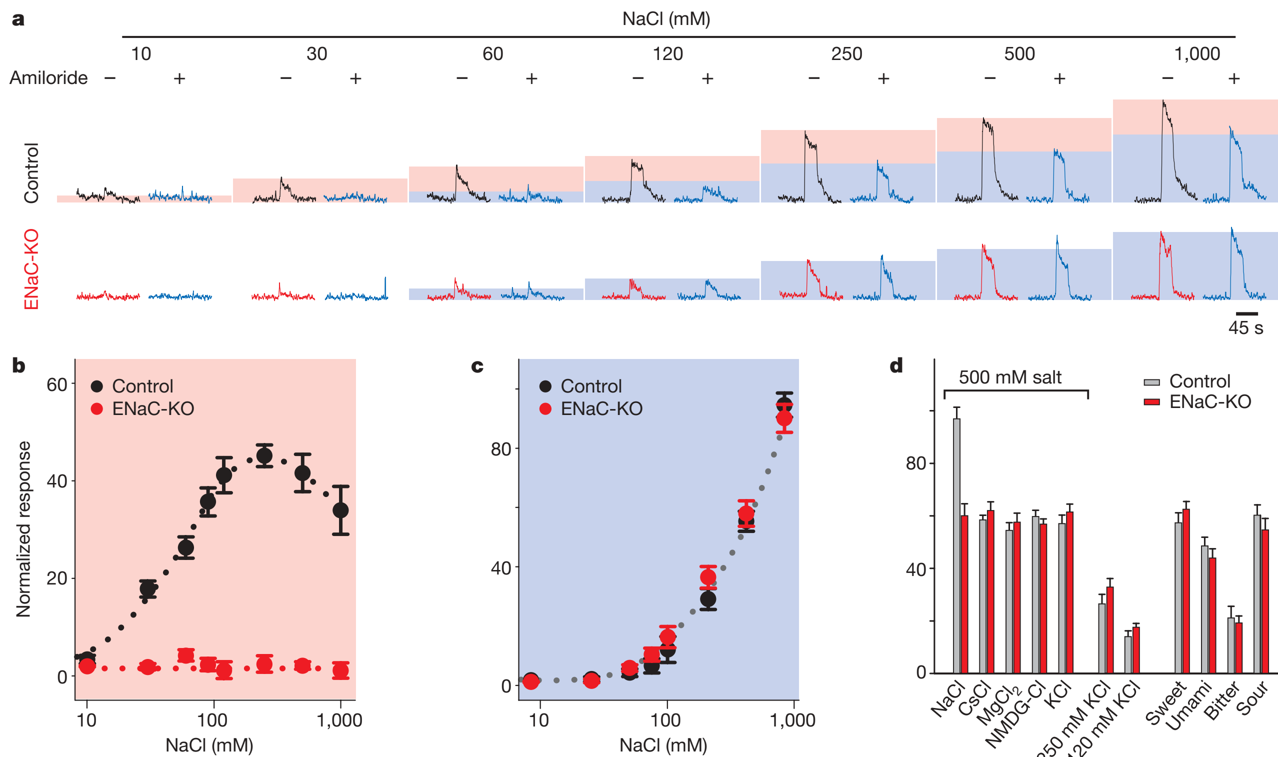

Figure 2 | ENaC is necessary for high sensitivity taste responses to sodium salts. Conditional knockout of ENaCα in TRCs (ENaC-KO) abolishes responses to low NaCl concentrations, and eliminates amiloride-sensitivity. **a**, Integrated neural recordings from the chorda tympani nerve of normal (control) and ENaC-KO mice in the presence (blue traces) or absence of 10 μM amiloride. Shaded boxes illustrate the amiloride-sensitive (pink) and -insensitive (blue) components. **b**, **c**, Quantifications of integrated neural responses of control and knockout animals; the coloured boxes are as in **a** ($n = 4$, mean ± s.e.m., $P < 0.001$ for amiloride-sensitive responses of control and mutant groups at 30–1,000 mM NaCl). **d**, ENaC-KO mice retain normal responses to other salts or other taste qualities ($n = 4$, mean ± s.e.m.); see Methods for details of calculations, tastants used, concentrations, genotype of strains and abbreviations.

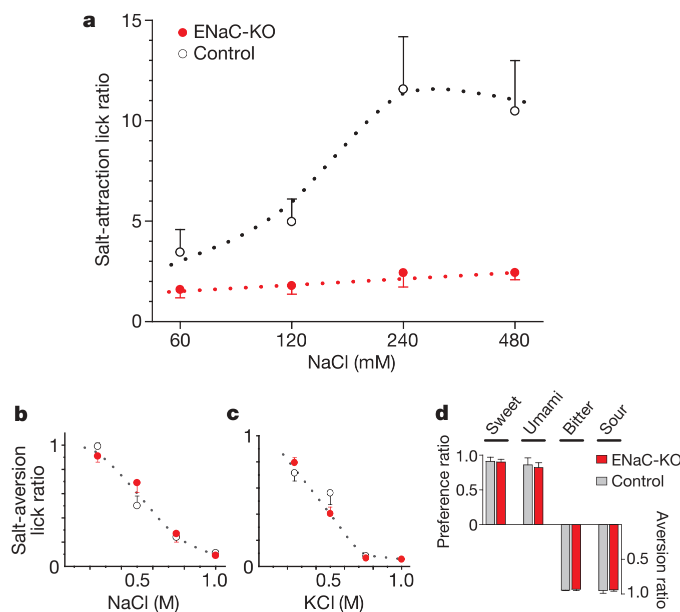

Figure 3 | ENaC function in TRCs is required for behavioural attraction to salt. Conditional knockout of ENaCα in TRCs selectively abolishes the attractive taste of NaCl. **a**, After diuretic-induced Na^{+}-depletion, ENaC-KO mice show little or no preference for NaCl solutions relative to water, whereas littermate controls exhibit very robust attractive responses ($P < 0.02$ between control and mutant groups at 120–480 mM NaCl). **b**, **c**, In contrast, in salt-aversion assays, water-deprived ENaC-KO mice and controls are indistinguishable in their responses to NaCl (**b**) or KCl (**c**). **d**, Behavioural responses to other taste qualities are unaffected in the ENaC-KO animals; shown are means ± s.e.m. ($n \geq 7$); see Methods for further details.

bitter or umami markers (referred to as 'ENaC-alone' cells; Fig. 4b, c). We hypothesized that the ENaC-alone cells are the bona-fide sodium taste sensors, and that the expression of ENaCα in sour cells may just be a consequence (that is, non-functional) of a common lineage between the cells mediating ionic tastes. Thus, we carried out further studies. First, we generated animals lacking ENaCα solely in Car4-expressing cells by using a sour-cell Cre driver to excise the conditional ENaCα knockout allele. As expected, these mice show wild-type responses to sweet, bitter, umami and sour stimuli. Notably, they have normal salt responses that are indistinguishable from wild-type controls (Supplementary Fig. 5), thus demonstrating that the ENaCα expression in the sour cells is in fact not required for salt taste. In a complementary study, we also generated mice entirely lacking sour-sensing cells[15]; these animals show a total loss of sour sensing, yet they maintain normal salt responses (Supplementary Fig. 5). Most critically, we directly imaged salt and sour responses using our new peeled epithelium preparation. Indeed, there is total segregation of the cells responding to salt (low and high concentrations) versus those responding to acid stimulation (that is, sour cells never respond to salt stimuli[13]; see Supplementary Fig. 1). Taken together, these results substantiate the functional and anatomical segregation of sodium-sensing TRCs, and prove that all five basic taste modalities are mediated by separate and dedicated receptor cells at the periphery.

An unusual feature of the physiology of sodium taste in mice has been the observation that the back of the tongue (circumvallate papillae) contains no sodium-selective (amiloride-sensitive) responses[10,22,28], highlighting a strong topographic segregation (front to back) in salt taste (see later). With the identity of the amiloride-sensitive salt taste receptor at hand, we reasoned that it should now be possible to explore the molecular basis of the absence of sodium sensing at the back of the tongue. ENaC channels are composed of

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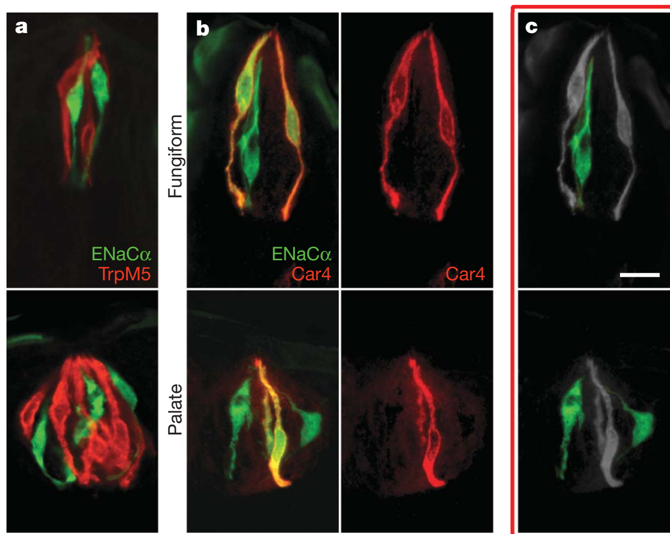

Figure 4 | ENaC defines a novel population of TRCs. Transgenic mice expressing GFP under the control of ENaCα (see Methods for details) were immunostained for markers of known classes of TRC. **a**, No overlap in the expression of ENaCα (green) and TrpM5 (red; a marker of sweet, bitter and umami TRCs) was observed in fungiform or palate taste buds. **b**, In contrast, Car4-expressing sour cells (red) co-express ENaCα (green; compare red- and yellow-labelled cells). However, ENaCα is also expressed in a unique subset of 'ENaC-alone' TRCs (green-only cells in **b** and **c**); see Methods for details of mice and the illustration in **c**. Scale bar, 10 μm. Note that because Car4-expressing sour cells do not express the essential ENaCβ subunit, they do not respond to salt (see Supplementary Fig. 6).

three essential subunits (α, β and γ), thus we anticipated that this Na^+- and amiloride-insensitivity could be easily explained if the functional ENaC heterotrimeric channel was not found in the circumvallate papillae. Indeed, our results show that at the back of the tongue, ENaCα and ENaCβ subunits are found in completely non-overlapping populations of TRCs (Supplementary Fig. 6). Therefore, amiloride-insensitivity at the back of the tongue is due to the lack of functional ENaC channels.

In this study we have shown that ENaC, first proposed to have a role in salt taste more than 25 years ago[8,12], functions as the sodium taste receptor. We also demonstrated that sodium taste is mediated by a dedicated population of TRCs separate from those mediating sweet, umami, bitter and sour taste. Notably, the taste of sodium and non-sodium salts are detected by genetically, pharmacologically and physiologically distinguishable TRCs. The availability of two channels (and cellular pathways) for salt-sensing endows animals with the ability to distinguish sodium-containing salts from other salts; this affords mammals with a powerful mechanism to select food sources containing adequate sodium but at the same time to avoid ingesting excessive amounts of salt.

The presence of salt shakers on dinner tables around the world attests to the appetitive role of salt taste in the human diet. Indeed, salt has been a food additive shared by humans for thousands of years, with empires from the Roman (for salary) to the British (for taxes) valuing it as a precious commodity. Does ENaC function in human salt taste? Physiological recordings in non-human primates have clearly demonstrated an amiloride-sensitive component in taste responses to salt stimuli[29,30]. However, psychophysical experiments in humans remain inconclusive[7], with some reports of amiloride altering salt taste[7,12], and several failing to substantiate a significant effect for amiloride in the perception of saltiness (reviewed in ref. 7). Given the molecular similarities between mice and humans in all other taste modalities[16], a 'human-specific' molecular mechanism for salt taste would be surprising. Perhaps more likely, the contribution of ENaC to human salt taste may be masked as a result of experience, exposure to salt, and diet. Future experiments studying people subjected to controlled salt intake may help clarify the role, if any, of ENaC in human taste.

METHODS SUMMARY

Transgenic animals and mouse strains. *ENaCα-IRES-Cre*, *ENaCβ-IRES-tTA* and *cytokeratin19-IRES-Cre* are bacterial artificial chromosome (BAC)-transgenics engineered to express Cre-recombinase or the tetracycline-dependent transactivator (tTA) by inserting an *IRES-Cre* or *IRES-tTA* transgene 3′ to the *Scnn1a*, *Scnn1b* or *Krt19* stop codon. Other strains have been described previously[15,24].
Calcium imaging. Fungiform TRCs were pre-loaded *in vivo* with Calcium Green-1 dextran 3kD (Invitrogen) by electroporating single taste buds. After 24–36 h, taste epithelium was enzymatically peeled[11,18] and placed on a recording chamber with the apical side of TRCs facing up (Supplementary Fig. 1). Taste stimuli were delivered in artificial saliva by focal application. Changes in intracellular calcium ($[Ca^{2+}]_i$) were imaged using a 5-Live confocal microscope (Zeiss) and the relative change in fluorescence ($\Delta F/F$) from individual TRCs analysed and pseudo-coloured as described previously[23].
Nerve recording, behavioural and localization studies. All procedures were as described previously[5,17,18,25,27].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.C. designed the study, carried out electrophysiological and expression studies, analysed data and wrote the paper; C.K. designed and carried out behavioural experiments and analysed expression in engineered and knockout mice; Y.O. designed and carried out calcium imaging experiments and analysed data; D.A.Y. carried out molecular studies and helped write the paper; E.H. provided essential reagents; N.J.P.R. and C.S.Z. designed the study, analysed data and wrote the paper.

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METHODS

Transgenic animals and mouse strains. The *PKD2L1-IRES-Cre*, *ROSA-DTA*, *Z/EG* and ENaCα conditional knockout (*Scnn1a*$^{flox/flox}$) strains have been described previously[15,24]. *ENaCα-IRES-Cre*, *ENaCβ-IRES-tTA* and *cytokeratin19-IRES-Cre* are BAC-transgenics engineered to express Cre-recombinase or the tetracycline dependent transactivator (tTA) by inserting an *IRES-Cre* or *IRES-tTA* transgene 3′ to the *Scnn1a*, *Scnn1b* or *Krt19* stop codon. The *ENaCβ-IRES-tTA* transgenic mice also carried a *TetO-sapphire* (modified GFP) reporter in the BAC-transgene. Four independent lines of *ENaCα-IRES-Cre* expressed the Cre transgene appropriately in kidney and lung and labelled equivalent populations of taste cells (Supplementary Figs 4 and 6). Similarly, three founder lines expressing *ENaCβ-IRES-tTA* showed equivalent tTA-expression in lung, kidney and taste tissue. Mice were inter-crossed as described in the text to generate appropriate genotypes for physiological, behavioural and anatomical experiments. Control groups were littermates not carrying the *IRES-Cre* transgene and/or animals with at least one copy of the wild-type *Scnn1a* allele. Wild type, heterozygous and the conditional ENaCα taste knockouts had no obvious differences in size, weight, fertility, life expectancy or food consumption.

Calcium imaging. Fungiform TRCs were pre-loaded *in vivo* with calcium green-1 dextran 3kD (Invitrogen) by electroporating single taste buds in the tongue of anaesthetized mice using a 3.5 μA, 50 pulses $s^{-1} \times 16$ cycles regime. On average, 8–9 taste buds were loaded per animal. After 24–36 h of recovery, tongues were removed and the taste epithelium enzymatically peeled as described previously[11]. The epithelium was then placed on a recording chamber with the apical side of TRCs facing up (Supplementary Fig. 1), ensuring that the integrity and polarity of taste buds is maintained. The apical surface of the preparation was bathed in artificial saliva at a constant flow rate of 4.5 ml min^{-1}, and taste stimuli were delivered by focal application using a custom made dispensing pipette (800 μm diameter). Tastant application was for 1 s, with a minimum of 10 s of artificial saliva between stimuli. Changes in $[Ca^{2+}]_i$ were imaged using a 5-Live confocal microscope (Zeiss) using a ×40 C-Apochromat 1.20W objective; images were captured at 4 Hz, and $\Delta F/F$ from individual TRCs analysed and pseudo-coloured as described previously[23].

Nerve recordings. Lingual stimulation and recording procedures were performed as previously described[18,25]. All data analyses used the integrated response over a 25-s period immediately after the application of the stimulus. Tastants used for nerve recordings were: 3–30 mM acesulphameK (sweet); 1–100 mM monopotassium glutamate plus 1.0 mM inosine monophosphate (umami); 1–10 mM quinine hydrochloride (bitter); 1–50 mM citric acid (sour). The responses to 50 mM citric acid were used to normalize responses to each experimental series in control and ENaCα-KO (Fig. 2). To compute the amiloride-sensitive salt component (Fig. 2b), the stimulation regime involved sequential applications of NaCl solutions first without and then with amiloride (in the same experimental series). The amiloride-insensitive component was defined as the response in the presence of amiloride (Fig. 2c). The fraction of the response inhibited by amiloride was defined as the amiloride-sensitive component (amiloride-sensitive component = response without amiloride − response with amiloride; Fig. 2b). Responses in experiments involving *PKD2L1-IRES-Cre Rosa-DTA* (PKD2L1-DTA) and *PKD2L1-IRES-Cre ENaCα flox/flox* (PKD2L1-ENaC-KO) were normalized to responses obtained with 30 mM acesulphameK (Supplementary Fig. 5). NaCl solutions used in dose-response studies for measuring the amiloride-insensitive sodium responses (Fig. 2c) included 10 μM amiloride. Differences between knockout and control responses were analysed for statistical significance using an unpaired, two-tailed Student's *t*-test and 95% confidence limits.

Behavioural assays. Behavioural assays used a custom-made gustometer to measure immediate lick responses as described previously[17,18]. For salt-attraction assays, mice were injected with furosemide (50 mg kg^{-1}) and were placed on a low sodium diet with unrestricted water for 16–20 h to deplete sodium before testing[5]. For salt-aversion assays, mice were water deprived for 24 h before testing[5,17,18]. Control tastants were 32 mM acesulphameK (sweet), 100 mM monosodium glutamate plus 1 mM inosine monophosphate and 0.1 mM amiloride (umami), 1 mM quinine sulphate (bitter), and 150 mM citric acid (sour). Differences between knockout and control responses were analysed for statistical significance using an unpaired, two-tailed Student's *t*-test and 95% confidence limits; for Supplementary Fig. 5b a one-way analysis of variance (ANOVA) with Newman–Keuls posterior test was used to compare data sets.

Immunohistochemistry and cell labelling. Immunostaining, whole-mount imaging (GFP) and *in situ* hybridization were performed as described previously[17,18]; animals were perfused with 4% paraformaldehyde and tissue was post-fixed for 6–48 h to allow localization of GFP. Images were obtained using a Leica SP2 TSC or a Zeiss 510 LSM meta confocal microscope. Anti-TrpM5 and anti-Car4 antibodies were as described previously[17,27]. The illustration in Fig. 4c was composed by converting the red and green channels in Fig. 4b to greyscale and overlaying with the ENaC-alone (green only) cells using Adobe Photoshop.

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