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Expression of the Voltage-Gated Potassium Channel KCNQ1 in Mammalian Taste Bud Cells and the Effect of Its Null-Mutation on Taste Preferences

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Abstract

Vertebrate taste buds undergo continual cell turnover. To understand how the gustatory progenitor cells in the stratified lingual epithelium migrate and differentiate into different types of mature taste cells, we sought to identify genes that were selectively expressed in taste cells at different maturation stages. Here we report the expression of the voltage-gated potassium channel KCNQ1 in mammalian taste buds of mouse, rat and human. Immunohistochemistry and nuclear staining showed that nearly all rodent and human taste cells express this channel. Double immunostaining with antibodies against type II and III taste cell markers validated the presence of KCNQ1 in these two types of cells. Co-localization studies with cytokeratin 14 indicated that KCNQ1 is also expressed in type IV basal precursor cells. Null mutation of the *kcnq1* gene in mouse, however, did not alter the gross structure of taste buds or the expression of taste signaling molecules. Behavioral assays showed that the mutant mice display reduced preference to some umami substances, but not to any other taste compounds tested. Gustatory nerve recordings, however, were unable to detect any significant change in the integrated nerve responses of the mutant mice to umami stimuli. These results suggest that although it is expressed in nearly all taste bud cells, the function of KCNQ1 is not required for gross taste bud development or peripheral taste transduction pathways, and the reduced preference of *kcnq1*-null mice in the behavioral assays may be attributable to the deficiency in the central nervous system or other organs.

Keywords

cell turnover; human biopsy; coexpression; gene knockout

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INTRODUCTION

Mammalian taste buds are the major peripheral end organs of taste, where the initiation of taste perception occurs (Lindemann, 1996; Margolskee, 2002). The onion-shaped taste buds consist of 50–100 individual cells, which, based on their morphology and ultra-structure, can be classified into four cell types (Farbman, 1965; Kinnamon et al., 1985; Yee et al., 2001). Each cell type appears to play distinct roles in taste bud physiology. For example, type I cells are thought to be supporting cells as well as candidate salty taste receptor cells (Bartel et al., 2006; Lawton et al., 2000; Pumplin et al., 1997; Vandenbeuch et al., 2008). Type II cells are receptive cells for sweet, bitter and umami taste stimuli (DeFazio et al., 2006; Yang et al., 2000b). Type III cells are the only taste bud cells that form conventional synapses with afferent gustatory nerves; and these cells seem to be important to sour taste transduction as well (DeFazio et al., 2006; Huang et al., 2006; Kataoka et al., 2008; Yang et al., 2000a). Type IV cells are precursor cells, replacing aged and/or damaged cells during the rapid cell turnover in taste buds (Stone et al., 2002).

One of the characteristics common to many intragemmal taste bud cells is their electrical excitability. These cells can generate action potentials, spontaneously or in response to taste or electrical stimuli (Avenet and Lindemann, 1991; Chen et al., 1996; Cummings et al., 1993; Gilbertson et al., 1992; Kashiwayanagi et al., 1983; Roper, 1983; Yoshida et al., 2006). Electrophysiological studies have recorded various voltage-gated ion channels present in taste bud cells, including: tetrodotoxin (TTX)-sensitive Na^+ channels, tetraethylammonium (TEA)-sensitive delayed rectifier K^+ channels, inward rectifier K^+ channels, outward rectifier Cl^- channels, low-/high-voltage activated Ca^{2+} channels (Bigiani et al., 2003). Although some of these channels are detected only in certain types of taste bud cells, the voltage-gated K^+ conductance seems to be the most ubiquitous among taste bud cells, and has been recorded even from basal cells (Mackay-Sim et al., 1996). It is largely unclear whether the intragemmal electrical activities such as the spontaneous action potentials or any particular voltage-gated ion channels play a role in taste bud development and taste cell differentiation.

In mature functional taste cells, stimulation of G protein-coupled taste receptors by bitter, sweet and umami substances or ion channels by sour and salty stimuli triggers intracellular signaling cascades, leading to the generation of receptor potentials. These tastant-evoked receptor potentials presumably regulate the activity of the voltage-sensitive hemichannels and their release of taste transmitters such as ATP or other bioactive molecules onto gustatory nerve fibers (Finger et al., 2005; Huang et al., 2007; Romanov et al., 2007; Zhao et al., 2005). However, it remains unknown which, if any, voltage-gated ion channels may contribute to the taste sensation by setting or resetting taste receptor cells' resting and receptor potentials. Even less is known whether the taste signal transduction-induced electrical activity in taste bud cells sends the feedback signals to progenitor cells and controls these cells' fate determination and differentiation.

Molecular identification and characterization of the voltage-gated ion channels expressed in taste bud cells is a prerequisite to elucidating their possible contribution to taste bud cell turnover and perhaps to taste signal transduction and transmission as well. So far a few of these channels have been molecularly characterized (Lin et al., 2004; Liu et al., 2005; Richter et al., 2004; Stevens et al., 2001). The messenger RNAs for two additional voltage-gated potassium channels, KCNQ1 and KCNH2, have been found in taste buds as well (Ohmoto et al., 2006). In our endeavor to identifying genes that are differentially expressed in taste bud cells vs. extragemmal cells, we independently discovered the expression of KCNQ1 in taste bud cells (Wang et al., 2006). Here we report the co-expression of the channel proteins in mouse, rat and human taste bud cells with other taste signaling

molecules and the characterization of behavioral and nerve responses of *kcnq1*-null mutant mice to taste stimuli. Our results indicate that although it is expressed in nearly all taste bud cells, the function of KCNQ1 is not be required for gross taste bud development or peripheral taste transduction pathways, and the reduced preference of *kcnq1*-null mice to some umami stimuli may be attributable to the deficiency in the central nervous system, the digestive system or other organs. Part of the preliminary data was published in abstract form (Wang et al., 2006).

MATERIALS AND METHODS

Animals

All studies involving animals were performed according to protocols approved by the Monell Chemical Senses Center Institutional Animal Care and Use Committee. C57BL/6 and other genetically modified mice and Sprague-Dawley rats were housed in a climate-controlled environment at the Monell Chemical Senses Center's Animal Care Facility.

The generation and characterization of the transgenic mice that carried the gene for green fluorescent protein (GFP) driven by an 8.4 kb α-gustducin promoter were previously described (Huang et al., 1999). The expression of GFP and α-gustducin in taste bud cells largely overlapped.

The generation and breeding of *kcnq1*-null mice were described previously (Casimiro et al., 2001). The mutant animals derived from 129/Sv × 129/Sv-CP embryonic stem cells were backcrossed with C57BL/6J mice for at least four generations. Heterozygous progeny were interbred to generate $kcnq1^{+/+}$, $kcnq1^{+/-}$ and $kcnq1^{-/-}$ animals used for two bottle preference tests and gustatory nerve recordings.

Human tissue

Biopsies from fungiform and circumvallate papillae were obtained from human subjects undergoing tonsillectomy or uvulopalatopharyngoplasty surgery. Human subjects provided consent in accordance with an experimental protocol approved by the Thomas Jefferson University Institutional Review Board. All subjects were between 21 and 50 years of age and denied taste or smell difficulties. Subjects with a history of tobacco use, head and neck cancer or head irradiation were excluded from the study. All subjects exhibited normal gustatory function as assessed via forced-choice threshold testing using sucrose, sodium chloride, citric acid, quinine sulfate, and monosodium glutamate or denatonium benzoate solution, followed by suprathreshold testing to assess taste identification and intensity discrimination.

Biopsies were performed with subjects under general endotracheal anesthesia without infiltration of local anesthetic. Fungiform papillae biopsies were obtained from the dorsal surface of the anterior one third of the tongue using a curved spring micro scissors. Circumvallate papillae biopsies were obtained from the back of the tongue using a pair of cup forceps. All biopsies were briefly rinsed in PBS (pH 7.2) before fixation.

Isolation of kcnq1 cDNA from taste bud cells

Construction and screening of single taste bud cell cDNA libraries—Single taste bud cell cDNA libraries were prepared and screened as described previously (Huang et al., 2005; Huang et al., 1999; Perez et al., 2002). Briefly, tongues were removed from euthanized GFP transgenic mice and injected subepithelially with a mixture of dispase II and collagenase A; the lingual epithelium was peeled off from the rest of tongue; circumvallate and foliate papillae, and a piece of non-gustatory lingual epithelium devoid of taste buds

were excised and dissociated into individual cells. Single green fluorescent cells, non-fluorescent taste cells from taste papillae and non-taste cells from non-gustatory epithelium were picked individually and their messenger RNAs were reverse transcribed, then amplified by the polymerase chain reaction (PCR). A portion of the amplified products from fluorescent or non-fluorescent taste cells was used to construct single cell cDNA libraries, which were then differentially screened with self or non-self probes that were prepared by radiolabeling the amplified products from the same taste bud cell or a non-gustatory lingual epithelial cell, respectively. The inserts of the differentially expressed clones were sequenced and analyzed by bioinformatics. One of these clones was identified by sequence as being a partial kcnq1 cDNA (Figure 1).

Isolation of full-length kcnq1 sequence—Circumvallate and foliate papillae from C57BL/6 mice were excised from the peeled-off lingual epithelium as described above. Total RNA was extracted using Absolutely RNA Microprep kit (Strategene, Cedar Creek, TX) from the excised taste papillae and used to synthesize 1st strand cDNA. PCR amplification was carried out with the synthesized cDNA and the PCR primers that were designed based on the reference sequence (GenBank accession: NM_008434) to cover the entire coding region (Sense primer: CTGCCTTCACCTCAGCTCCGAG; antisense primer: TGAGAACCAGGTGGGTGTG). PCR products were subcloned into pGEM T-easy vector (Promega, Madison, WI) and confirmed using DNA sequencing analysis.

Immunocytochemistry

Tissue Preparation—Both rodent tongues and human biopsies were fixed in 4% paraformaldehyde in PBS for 1 hour, cryoprotected in 20% sucrose overnight and sliced into sections with a cryostat.

Single labeling with one antibody—Sections of 10 microns were blocked in the blocking buffer (3% BSA, 0.3% Triton X-100, 2% horse serum and 0.1% sodium azide in PBS) for 1 hr at room temperature and then incubated overnight at 4 ^{o}C with diluted primary antibody in the blocking buffer. After washing four times in PBS containing 0.3% Triton X-100, the sections were incubated with Cy3- or Alexa 488-conjugated secondary antibody at room temperature for 30 minutes. To preserve the fluorescence, the tissue sections were covered with Vectashield Mounting Medium (Vector Laboratories, H-1000) and fluorescence micrographs were taken using a fluorescence or confocal microscope.

To visualize the nuclei, 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories, H-1200) was used to cover the tissue sections.

Double labeling with two antibodies—After the incubation with the first primary antibody and the first secondary antibody as described above for the single labeling, sections were blocked again in the blocking buffer, followed by incubation with a second primary antibody, and with Cy3- or Alexa 633-conjugated second secondary antibodies. Fluorescence micrographs were taken using a confocal microscope.

Leica TCS SP2 Spectral Confocal Microscope and Leica Scanware software (Leica Microsystems Inc.) were used to acquire confocal images. These images were then arranged and adjusted for contrast and brightness using Photoshop v8 (Adobe Systems Inc.).

Antibodies—Source (manufacturer, catalog number, host species and immunizing antigen), characterization and dilutions of the antibodies used are as follows: Two polyclonal antibodies against KCNQ1 were used: one is an affinity-purified rabbit antibody (Chemicon, AB5932, raised with a synthetic peptide corresponding to amino acids 585 to

604 of human KCNQ1 C-terminal sequence: SNTIGARLNRVEDKVTQLDQ, and cross-reacting with rat and mouse KCNQ1, used in immunohistochemistry at 1:1,000 dilution); its specificity has been confirmed by the manufacturer via Western blotting recognizing a protein of approximately 72 kD from adult mouse ventricles, and verified by previous reports (Liao et al., 2005); and the other is an affinity-purified goat antibody (Santa Cruz, SC-10646, also raised against a synthetic peptide of human KCNQ1 C-terminal 20 amino acid residues: NTLPTYEQLTVPRRGPDEGS; cross-reacting with rat and mouse KCNQ1, used in immunohistochemistry at 1:1,000 dilution), its specificity has been confirmed by the manufacturer via Western blotting recognizing a human KCNQ1-fusion protein, and verified by other researchers via immunofluorescent staining of the COS-1 cells transiently transfected with the KCNQ1 cDNA constructs, and via Western blotting of the transfected COS-1 cell extracts recognizing a single band of the expected size (Rasmussen et al., 2004); and the specificity of the above two KCNQ1 antibodies was also validated by this study with taste tissue sections from *kcnq1*-null mice, which showed non-staining by the antibodies (Figure 2).

Anti-synaptobrevin-2 (Wako, 018-15791) was raised in rabbit with a KLH-conjugated synthetic peptide of N-terminal 20 amino acid residues of synaptobrevin-2 of rat origin: MSATAATVPPAAPAGEGGPP, and used in immunohistochemistry at 1:200 dilution, and its specificity has been confirmed in previous studies demonstrating its immunoreactivity within synaptic vesicles and colocalization with other taste signaling molecules in subsets of taste bud cells (Yang et al., 2004).

Anti-α-gustducin antibody (Santa Cruz, SC-395) is an affinity-purified rabbit polyclonal antibody, raised against a synthetic peptide corresponding to amino acid residues 93-112 of α-gustducin of rat origin, used in immunohistochemistry at 1:1,000 dilution, and its specificity has been confirmed by pre-absorption with its immunizing peptide (SC-395p) and by other studies (Yang et al., 2007).

Anti-TRPM5 antibody is a polyclonal antibody, raised in rabbit with a KLH-conjugated synthetic peptide of amino acid residues 1028–1049 of mouse TRPM5, used in immunohistochemistry at 1:1,000 dilution, and its specificity has been confirmed by: 1) the abolition of its immunostaining on rodent taste tissue sections following pre-incubation with the immunizing peptide (Perez et al., 2002); 2) the absence of its immunoreactivity with taste tissue sections from *Trpm5*-null mice (Damak et al., 2006).

The affinity-purified anti-Tas1R3 antibody was raised in rabbit with a hemocyanin-conjugated synthetic peptide of amino acid residues 45–62 of mouse Tas1R3, used in immunohistochemistry at 1:1,000 dilution, and its specificity was confirmed by blocking its immunoreactivity with the immunizing peptide and by the absence of immunoreactivity on taste tissue sections from T1R3-knockout mice (Damak et al., 2003; Reed et al., 2004).

Anti-NCAM (Millipore, AB5032) is an affinity purified antibody raised in rabbit with highly purified chicken NCAM protein, cross-reactive with NCAM from human, mouse and rat, used in immunohistochemistry at 1:500 dilution, and its specificity was confirmed by Western blotting of mouse brain homogenates recognizing proteins of 200–250 kD, or 140–180 kD after treatment with neurminidase.

Anti-SNAP-25 antibody (Sternberger Monoclonals, SMI81) is a mouse monoclonal IgG, raised with the whole SNAP-25 proteins, used in immunohistochemistry at 1:500 dilution, and its specificity has been confirmed by Western blotting of bovine brain extracts recognizing proteins of 25-kDa and its epitope has been determined to lie within the C-terminal peptide (Keller and Neale, 2001; Keller et al., 1999; Mehta et al., 1996).

Anti-cytokeratin 14 antibody (Chemicon, MAB3232) is a mouse monoclonal IgG raised by immunizing purified proteins from epithelial cells, and its specificity has been confirmed by Western blotting of human buccal epithelial cell extracts recognizing 50 kD proteins (Wetzels et al., 1991).

The following secondary antibodies were used at 1:500 dilution: FITC-conjugated donkey-anti-goat, Cy3-conjugated goat-anti-rabbit, FITC-conjugated donkey-anti-mouse and Tetramethyl Rhodamine-conjugated donkey-anti-goat antibodies (Jackson ImmunoResearch, West Grove, PA), Alexa fluor 488-conjugated donkey-anti-rabbit, Alexa fluor 488-conjugated donkey-anti-goat and Alexa fluor 633 goat-anti-mouse antibodies (Invitrogen).

Control experiments were carried out with the omission of one or two primary antibodies, preincubation of primary antibodies with antigenic peptides, or taste tissue sections from gene knockout mice. Unspecific staining of primary antibodies or cross-reactivity between two secondary antibodies was not found.

Two bottle preference tests

Two bottle preference tests were conducted as described previously (Bachmanov et al., 2001; Bachmanov et al., 1998; Wong et al., 1996). Three groups of age- and sex-matched young adult animals (3–12 weeks of age) with 9–10 mice per group representing three genotypes were used: wild-type (*kcnq1*$^{+/+}$), heterozygous mutant (*kcnq1*$^{+/-}$) and homozygous mutant (*kcnq1*$^{-/-}$). Individually caged animals were presented with two bottles: one containing deionized water, the other taste solution. The bottle positions were switched after 24 hours to eliminate any positional effect. The volume of consumed liquid in each bottle was recorded and preference scores were calculated for each animal by dividing the consumption of the test solution by the total intake of fluid.

Five test solutions representing the five basic taste qualities were used initially: SC45647 (sweet), denatonium benzoate (bitter), citric acid (sour), inosine-5'-monophosphate (IMP, umami) and NaCl (salty). To confirm animals' response to umami taste, two additional umami substances were used: monopotassium L-glutamate (MPG), and a mixture of IMP and monosodium L-glutamate (MSG). To determine concentration-dependent preferences, a range of concentrations for each compound was presented in ascending order. To minimize any carryover effects from a previous test, animals were given only distilled water for 4–7 days between tests of two different taste substances.

Preference ratios for each concentration of the test solutions over a 2-day period were calculated by dividing the intake of test solution by the total fluid consumption, i.e. the sum of solution intake and water intake. Means and standard errors were obtained by averaging the preference ratios from individual animals of the same genotype group. A two-way analysis of variance (ANOVA) was performed with genotype as a between-group factor and concentration as a within-group factor using the Statistica software package to assess the effect of genotypes on taste preferences for each test compound. If there was a significant effect, then post hoc pair-wise comparisons of means at each test solution concentration were conducted with student *t*-tests between two genotype groups, i.e., kcnq1$^{-/-}$ vs. kcnq1$^{+/+}$ and kcnq1$^{-/-}$ vs. kcnq1$^{+/-}$. The criterion for significance was set to a P value <0.05.

Gustatory Nerve Recordings

The procedures for the mouse chorda tympani nerve recordings were described previously (Inoue et al., 2007; Inoue et al., 2001). Briefly, two *kcnq1*-null mice and three wild-type littermates were anesthetized with an intraperitoneal injection of a mixture of ketamine (10.7

mg/kg), xylazine (2.2 mg/kg) and acepromazine (0.35 mg/kg). A cannula was inserted in the trachea, and the animal was placed supine in a non-traumatic headholder. The right chorda tympani nerve at the jaw was exposed at its exit from the lingual nerve by removal of the internal pterygoid muscle. The chorda tympani nerve was then dissected free from surrounding tissues and placed on a platinum wire electrode. A few drops of mineral oil were placed in the wound site to prevent desiccation of the nerve. An indifferent electrode was positioned in nearby muscle tissue.

For chemical stimulation of the fungiform taste papillae, the anterior part of the animal's tongue was enclosed in a flow chamber. Taste substances (NaCl: 100 mM; HCl: 10 mM; Denatonium: 20 mM; IMP: 10 mM; MPG: 100 mM; a mixture of 100 mM MSG + 0.5 mM IMP) were delivered at room temperature into the flow chamber by gravity flow at a rate of 0.5 ml/sec for 30 sec. Between applications of the taste stimuli, the tongue was rinsed with distilled water for at least 1 min. Ammonium chloride (NH_4Cl) at 100 mM was presented frequently throughout the recordings to serve as a reference stimulus. The whole nerve responses to the lingual application of taste substances were amplified, integrated at a time constant of 1 second, converted to digital signals and analyzed offline. The magnitudes of the responses to tastants were determined as the areas below the curves and normalized by the response to 100 mM NH_4Cl from the same animal, and then averaged for animals from the same genotype group. Paired comparisons of the averaged values were analyzed between the wild-type control and kcnq1-null mice using student t-tests. *P* value <0.05 was the criterion for statistical significance.

RESULTS

1) Isolation of KCNQ1

To isolate genes that are specifically or more abundantly expressed in intragemmal taste bud cells than in non-gustatory lingual epithelial cells, we modified a single cell procedure that had been employed to successfully identify several taste signaling molecules, including the G protein subunits, Gβ3γ13, the transient receptor potential ion channel TrpM5 and the voltage-gated chloride channel ClC-4 and its variant ClC-4A (Huang et al., 2005; Huang et al., 1999; Perez et al., 2002). We utilized a transgenic mouse line that labeled the α-gustducin-expressing taste bud cells with the green fluorescent proteins (GFP). Green fluorescent, non-fluorescent taste bud cells and non-gustatory lingual epithelial cells were isolated from the circumvallate and foliate papillae, and from a piece of lingual epithelium devoid of taste buds. cDNAs were synthesized from mRNAs of individual cells, amplified by polymerase chain reactions (PCR), and used to construct single taste bud cell cDNA libraries. These libraries were subtractively screened with probes from the same taste bud cell as well as with probes from a non-taste epithelial cell to isolate differentially expressed clones, which were sequenced and searched using the Blast program against GenBank databases (Figure 1). Blast search results indicated that one of these clones matched the 3'-end cDNA sequence of the voltage-gated potassium channel, KCNQ1. To isolate the full-length coding cDNA sequence, PCR reactions were carried out with cDNAs from taste papillae and a set of PCR primers covering the full coding sequence. Sequencing analysis of the sole PCR product confirmed that the *kcnq1* cDNA expressed in taste buds is identical to the reference sequence (GenBank Accession number: NM_008434).

2.) Expression of KCNQ1 in taste bud cells

To determine the localization of the KCNQ1 channel protein in taste buds, we performed immunocytochemistry with two anti-KCNQ1 antibodies from rabbit and from goat, respectively. The specificity of these two antibodies was proven in previous studies (Liao et al., 2005; Rasmussen et al., 2004). In this paper we again validated with 4 taste tissue

sections from each of 6 animals from three genotype groups: 2 wild-type ($kcnq1^{+/+}$), 2 heterozygote ($kcnq1^{+/-}$) and 2 homozygote ($kcnq1^{-/-}$). It seemed that the expression of KCNQ1 proteins was normal in both wild-type and heterozygous mice, but undetectable in homozygous mice, whereas the expression of the taste signaling molecules Tas1R3 receptor, α-gustducin and TRPM5 appeared to be normal (Figure 2).

The immunostaining pattern in Figure 2 indicated that the expression of KCNQ1 proteins was restricted to taste buds. To determine how widely the KCNQ1 expression is in taste bud cells, we double stained 10 taste sections from 2 human subjects, 30 more taste sections from 3 rats and another 30 from 3 mice with both anti-KCNQ1 antibody and 4',6'-diamidino-2-phenylindole (DAPI) to visualize both KCNQ1-expressing cells and all nuclei present on each section. Examination of the confocal laser scanning microscope images indicated that nuclei were largely absent near the taste pores (Figure 3), which was consistent with our previous observation (Wang et al., 2007). The KCNQ1 staining covered the entire taste bud, which was surrounded by cells with long nuclei orientated along the sides. All cells in a taste bud, including those near the serosal bottom, displayed KCNQ1 antibody staining, indicating that all human and rodent mature taste bud cells and possibly some precursor cells express KCNQ1 (Figure 3).

However, since DAPI stained the nuclei while the vast majority of KCNQ1 channels were localized to the cytoplasmic membrane, the two fluorescent signals did not overlap. Therefore it was possible that the KCNQ1 staining might be from cell membranes of wrapping type I cells instead of those of the same cell. To rule out that possibility, we carried out double immunostaining with antibodies against type II, III and IV taste cell markers.

Specific antibodies against the following three type II cell markers were utilized: 1) Tas1R3, one of the two subunits of the dimeric receptors for sweet and umami tastes (Kitagawa et al., 2001; Li et al., 2002; Max et al., 2001; Montmayeur et al., 2001; Nelson et al., 2002; Nelson et al., 2001; Sainz et al., 2001); 2) α-gustducin, a G protein subunit that is involved in bitter, sweet and umami taste transduction (McLaughlin et al., 1992; Wong et al., 1996); 3) TRPM5, a transient receptor potential ion channel that is a common denominator for bitter, sweet and umami signaling cascades (Hofmann et al., 2003; Liu and Liman, 2003; Perez et al., 2002; Prawitt et al., 2003; Zhang et al., 2003). Confocal laser scanning microscopy images of 60 taste tissue sections from 5 mice indicated that nearly all Tas1R3-, α-gustducin- or TRPM5-expressing taste bud cells also express the KCNQ1 channel (Figure 4). This result demonstrates that almost all type II taste receptor cells express this channel.

To confirm that KCNQ1 is also present in synapse-forming taste bud cells, double immunostaining was performed with antibodies against the type III cell marker neuronal cell adhesion molecule (NCAM) (Nelson and Finger, 1993), and synaptic proteins: SNAP-25 and synaptobrevin-2; the former, like NCAM, is found only in type III cells whereas the latter is present in both type II and III taste bud cells (Yang et al., 2000a; Yang et al., 2004). Since the primary antibody against SNAP-25 is a mouse monoclonal antibody, 60 taste tissue sections from 5 rats were used in this double-staining experiment to minimize any intrinsic background signals. Confocal laser scanning microscopy images showed that NCAM, like KCNQ1 channels, seemed to be present only on the cytoplasmic membranes of taste bud cells whereas SNAP-25 and synaptobrevin-2 were found largely in the cytosol with some proteins on the cytoplasmic membranes (Figure 5). The imaging results indicated that nearly all taste bud cells immunoreactive to the antibodies against NCAM, SNAP-25 or synaptobrevin-2 were immunoreactive to the anti-KCNQ1 antibody whereas many more cells exhibited the KCNQ1-like immunoreactivity (Figure 5). The results also showed that these two synaptic proteins, especially SNAP-25, occur in intragemmal and perigemmal

nerve fibers, while KCNQ1 is absent in these fibers, suggesting that KCNQ1 channels only function within the taste bud cells, and do not participate in the activity of these innervating and surrounding nerve fibers.

To determine whether the co-expression patterns of KCNQ1 with other taste cell markers also exist in human taste buds, we performed similar double immunolabeling experiments with 10 taste tissue sections of 2 human subjects, and antibodies against NCAM and SNAP-25. Confocal laser scanning microscopy images indicated that KCNQ1 was co-localized with NCAM and SNAP-25 to human taste bud cells (Figure 6).

To verify the expression of KCNQ1 in taste bud precursor cells, we carried out the double immunostaining on 30 taste tissue sections from 3 rats with cytokeratin 14 antibodies. Cytokeratin 14 is a member of the keratin family that is a group of intermediate filaments. Cytokeratin 14 is known to be present in immature taste bud cells (Asano-Miyoshi et al., 2008). Confocal laser scanning microscopy images showed that cytokeratin 14 is mostly detected in the intragemmal epithelial cells, but also in some basal cells in taste buds (Figure 7). Most cytokeratin 14-expressing taste bud cells also expressed KCNQ1 (arrows in Figure 7). But occasionally, we observed that in a few cytokeratin-14 positive basal cells the KCNQ1 signal was undetectable (arrowhead in Figure 7).

3) Characterization of responses of *kcnq1*-null animals to taste stimuli

The widespread expression of the KCNQ1 channel among taste bud cells across different species suggests that this channel may play a fundamental role in taste bud development and physiology. Immunostaining of taste tissue sections from the *kcnq1* null mutant mice with antibodies against taste signal transduction components indicated that the gross structure of the taste buds seemed normal and no noticeable changes in the expression patterns of these components were detected (Figure 2).

To characterize the possible roles of KCNQ1 channel in taste sensation, we carried out two experiments: 48-hour two bottle preference tests and chorda tympani nerve recordings. We used young adult knockout animals at age from 3 to 12 weeks. Older knockout animals tended to exhibit circling symptoms resulting from a deficient vestibular system, which could exacerbate bottle-position effect in the 48-hour two bottle preference tests. The behavioral data from individually caged mice showed that the *kcnq1*-knockout animals had a significantly reduced preference for IMP than did their heterozygous or wild-type littermates at the concentrations of 10 and 30 mM ($p<0.05$)(Figure 8). To confirm this diminished preference is true for other umami substances, additional tests were performed with MPG and with a mixture of MSG with 0.5 mM IMP. MPG at 10 and 100 mM were significantly less preferred by the knockout mice than by the heterozygous or wild-type littermates. Interestingly, the mixtures of MSG and IMP tended to be less preferred as well, but the reduction in preference was not statistically significant. However, the preferences of the mutant, heterozygous and wild-type mice for solutions of other taste qualities: SC45647 (sweet), sour (citric acid) and denatonium (bitter) were similar (Figure 8). The preference tests for NaCl were also performed at three representative concentrations (37.5, 75, 150 mM) before the animals started to display circling behavior and no significant differences were found among these three groups of mice (data not shown).

To examine whether the diminished preference of umami substances in *kcnq1*-null animals was attributable to the deficiency in the peripheral taste transduction, we performed gustatory nerve recordings from two *kcnq1*-knockout mice and three wild-type littermates. The integrated responses of chorda tympani nerves from the knockout animals seemed to be as robust as those from their wild-type littermates and no significant difference was observed in these responses (Figure 9).

DISCUSSION

To better understand taste bud cell turnover and taste signal transduction, particularly the involvement of voltage-gated ion channels in these processes, it is necessary to identify genes that are selectively expressed in taste bud cells at various maturation stages. The single cell approach has been effective in isolating cell type-specific genes such as pheromone receptor genes (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Pantages and Dulac, 2000). We have utilized this approach previously to isolate several taste signaling components, including the G protein subunits Gβ3, Gγ13, the transient receptor potential ion channel TRPM5, and the voltage-gated chloride channels ClC4 and ClC-4A (Huang et al., 2005; Huang et al., 1999; Perez et al., 2002). In this study, we identified the expression of the voltage-gated potassium channel KCNQ1 (Figure 1). The occurrence of the KCNQ1 transcripts and some preliminary studies were reported previously (Ohmoto et al., 2006; Wang et al., 2006). Here we found that KCNQ1 is present in nearly all cells throughout taste buds, including basal precursor cells, from both rodents and humans, that knockout of this gene seemed to impact only on rodent preference for umami substances in 48-hour two bottle preference tests, and that electrical recordings failed to detect significant change in the rodent chorda tympani nerve responses to all taste compounds tested, including umami substances. To our knowledge, this is the first study to demonstrate the expression of a voltage-gated ion channel in human taste papillae, and KCNQ1 is the only voltage-gated channel so far found in precursor taste bud cells.

1) Wide expression of KCNQ1 in taste buds

Immunocytochemistry with KCNQ1 antibody showed that the ion channel was present in many taste bud cells (Figure 2). Double staining with DAPI showed that nearly all DAPI-stained nuclei in taste buds were surrounded by KCNQ1 antibody-stained cytoplasmic membranes and the two fluorescent signals hardly overlapped (Figure 3). Since it is known that type I taste bud cells have sheetlike cytoplasmic processes that envelope nerve fibers and other taste cells (Pumplin et al., 1997), the non-overlapping pattern made it difficult to determine whether the KCNQ1 staining enveloping type II and III cells' nuclei was from their own plasma membranes or from those of type I cells. Double immunostaining with antibodies against type II and III cell markers showed that KCNQ1 is co-localized to subsets of human and rodent taste bud cells with transmembrane or membrane-bound proteins: TRPM5, α-gustducin, Tas1R3, SNAP-25, synaptobrevin-2 and NCAM (Figures 4, 5 and 6), demonstrating that the KCNQ1-immunoreactivity encompassing type II and III cells' nuclei was from their own cell membranes. On the other hand, since no any other cells are known to wrap type I cells, we can basically conclude from the double staining pattern of KCNQ1 antibody and DAPI (Figure 3) that type I cells also expressed this channel.

Cytokeratin 14 is a cytoskeleton protein that is present only in undifferentiated precursor cells in taste buds (Asano-Miyoshi et al., 2008). Double immunostaining showed that the majority of cytokeratin 14-expressing basal cells also express KCNQ1 channels. However, a few cytokeratin 14-immunoreactive cells that situated at the very bottom of taste buds were not labeled by KCNQ1 antibody (Figure 7). The apparent explanation for this observation is that these rare cells belong to an even younger group that has not started to transcribe this gene yet. But they are likely to synthesize this channel protein when they mature since this study has shown that all mature taste bud cells carry this channel.

KCNQ1 seems to be the most widely expressed voltage-gated ion channel found so far in taste bud cells. In situ hybridization data, which indicated the wide presence of KCNQ1 transcripts in taste bud cells, supported this notion (Ohmoto et al., 2006; Wang et al., 2006). A large body of electrophysiological data accumulated over the last two decades showed that almost all taste bud cells exhibited voltage-gated potassium currents (Bigiani et al.,

2003; Chen and Herness, 1997). It is possible that KCNQ1 is the channel that renders all taste bud cells the voltage-gated potassium currents, although it is possible that additional voltage-gated potassium channels may contribute to these currents as well.

2) Possible physiological roles of KCNQ1 in gustation

The Shaker-type K^+ channel KCNQ1 is known to be critical to the normal function of cardiac and auditory systems. Mutations in KCNQ1 have resulted in deafness (Jervell and Lange-Nielsen (JLN) syndrome) and cardiac arrhythmia (long QT syndrome) (Neyroud et al., 1997; Schwartz et al., 1975). *Kcnq1*-null animal models also display similar symptoms of the long QT and JLN syndromes (Casimiro et al., 2001). The widespread expression of KCNQ1 in taste buds suggests that as in cardiac and auditory systems, KCNQ1 may play a fundamental role in taste bud development and function. However, two bottle behavioral tests indicated that the sole altered taste preference by the kcnq1-null mutation was the diminished liking for umami substances. Yet the gustatory nerve recordings suggested that even this change may not stem from the taste transduction in taste buds. One explanation for this apparently striking difference in the KCNQ1's role between gustatory system and cardiac and auditory systems is that neither the long-term 48-hour two-bottle preference tests nor the whole gustatory nerve recordings were sensitive enough to detect subtle effects of the channel on taste bud cells' physiology, for example, cytoplasmic membrane potential repolarization, which can be discerned using taste bud cell recordings. Another possible explanation is that the role of KCNQ1 in taste bud cells has been compensated by other potassium channels. Indeed other members of the KCNQ channel subfamily as well as the KCNQ modulators--the KCNE proteins are also expressed in taste bud cells (Wang, Zhou and Huang, unpublished data). Multiple gene knockout, such as that for ATP receptors (Finger et al., 2005), may be needed to reveal the contribution of KCNQ channels or other potassium channels to taste bud cells' electrical activity.

3) Reduced preference for umami substances in the *kcnq1*-knockout mice

The *Kcnq1* knockout animals displayed reduced preference for the umami substances IMP and MPG (Figure 8). Their preference for MSG in the presence of IMP tended to be reduced as well although the reduction did not seem statistically significant. These knockout animals did not differ from the wild-type mice in preferences for substances of other taste qualities. The reduction in preference for the umami substances did not appear attributable to the dysfunction of anterior taste system since the chorda tympani nerve recordings showed unaltered responses to all orally applied umami compounds as well as other taste stimuli (Figure 9). Interestingly, similar discrepancy between behavioral and nerve recording data in response to umami stimuli have been reported in other mutant mice: animals with null mutations in α-gustducin or IP3 receptor IP3R3 showed a nearly abolished behavioral preference but displayed considerable nerve responses to umami compounds (He et al., 2004;Hisatsune et al., 2007). It is not certain what causes this discrepancy. However, in the case of kcnq1 knockout, it is possible that the reduction in behavioral response may result from postingestive effect in the gut as explained below.

KCNQ1 is known to be prominently expressed in the mouse kidney and gastrointestinal tract (Dedek and Waldegger, 2001). Disruption of the *kcnq1* gene resulted in gastric hyperplasia, impaired Cl^- secretion and Na^+/K^+ reabsorption (Lee et al., 2000; Vallon et al., 2005). And these symptoms may have led to reduced preference or increased aversion to some umami substances. Interestingly, umami, sweet and bitter receptors are also found in the GI tract (Dyer et al., 2005; Wu et al., 2002). The taste receptor Tas1R3, which is the common subunit of both umami and sweet receptors, plays an important role in the regulation of expression of the sodium-dependent glucose transporter isoform 1 in the absorption of dietary sugars (Margolskee et al., 2007). It is tempting to postulate that Tas1R receptors and

KCNQ1 channels directly or indirectly act together regulating amino acid absorption whereas the *kcnq1* knockout may diminish a rewarding postingestive effect, resulting in reduced preference for umami compounds. Further studies are needed to characterize the role of KCNQ1 in postingestive effect.

In summary, we have identified a voltage-gated potassium channel, KCNQ1, which is expressed in almost all taste bud cells, including the immature basal cells. Initial characterization of *kcnq1*-null mice indicated that the mutant mice had normal gross taste bud structure and peripheral taste transduction. Further studies using finer methods such as electron microscope and single fiber recordings may reveal subtle impact of the *kcnq1* mutation on taste bud structure and physiology. Its wide expression in taste bud cells suggests that this channel is responsible for the previously recorded potassium currents in taste bud cells. Identifying additional genes that are preferentially expressed in taste bud cells can facilitate our understanding of taste bud cell differentiation and physiology.

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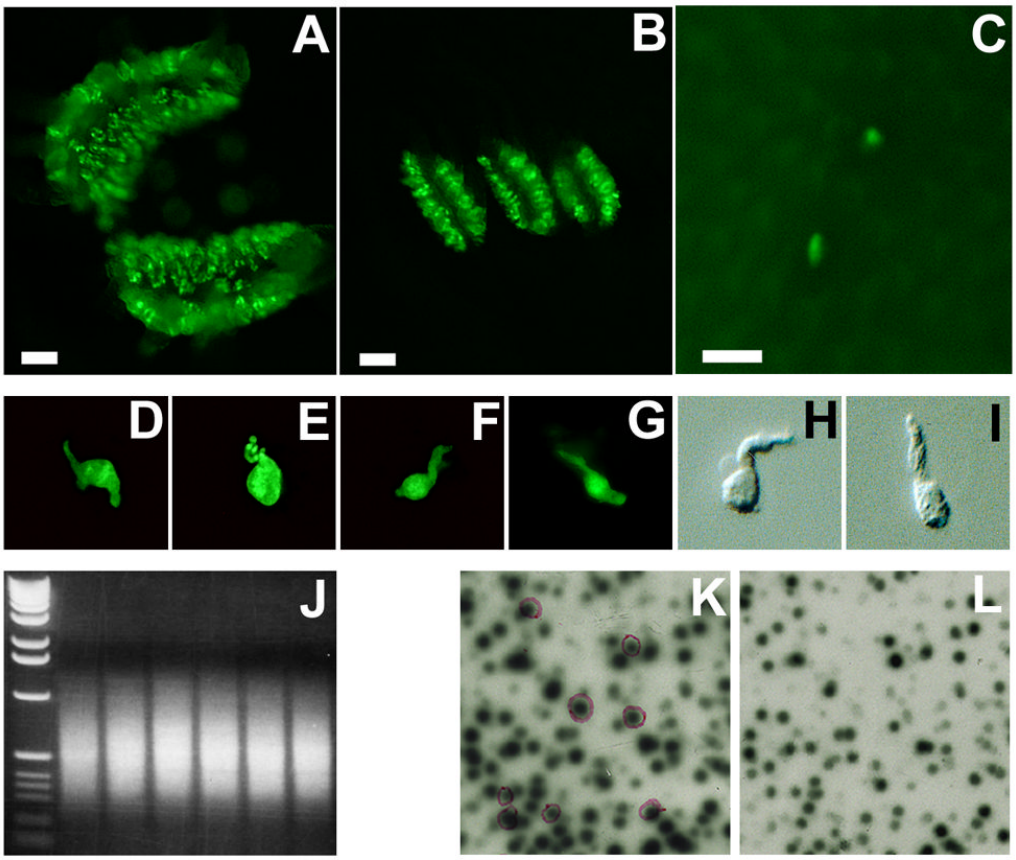

Figure 1.
Isolation of taste cell-selective genes. GFP-labeled taste papillae (A: circumvallate; B: foliate; and C: fungiform. Scale bar: 250 μm) were isolated from GFP transgenic mice and dissociated into individual taste bud cells (D to G: four green fluorescent cells; H and I: two non-fluorescent cells). Transcripts from each cell were amplified by PCR with an average size of 600 bp (J: an electrophoretic gel image of amplified products from the six single cells; left lane: 1 kb DNA molecular weight marker). A portion of the amplified products was used to construct single cell cDNA libraries, which were subtractively screened: λ bacteriophage double lift was screened with self-probe (K) and non-taste-probe (L). Differentially expressed clones with stronger signals on the left lift (K) and weaker or no signals on the right lift (L), shown in purple circles, were identified and sequenced.

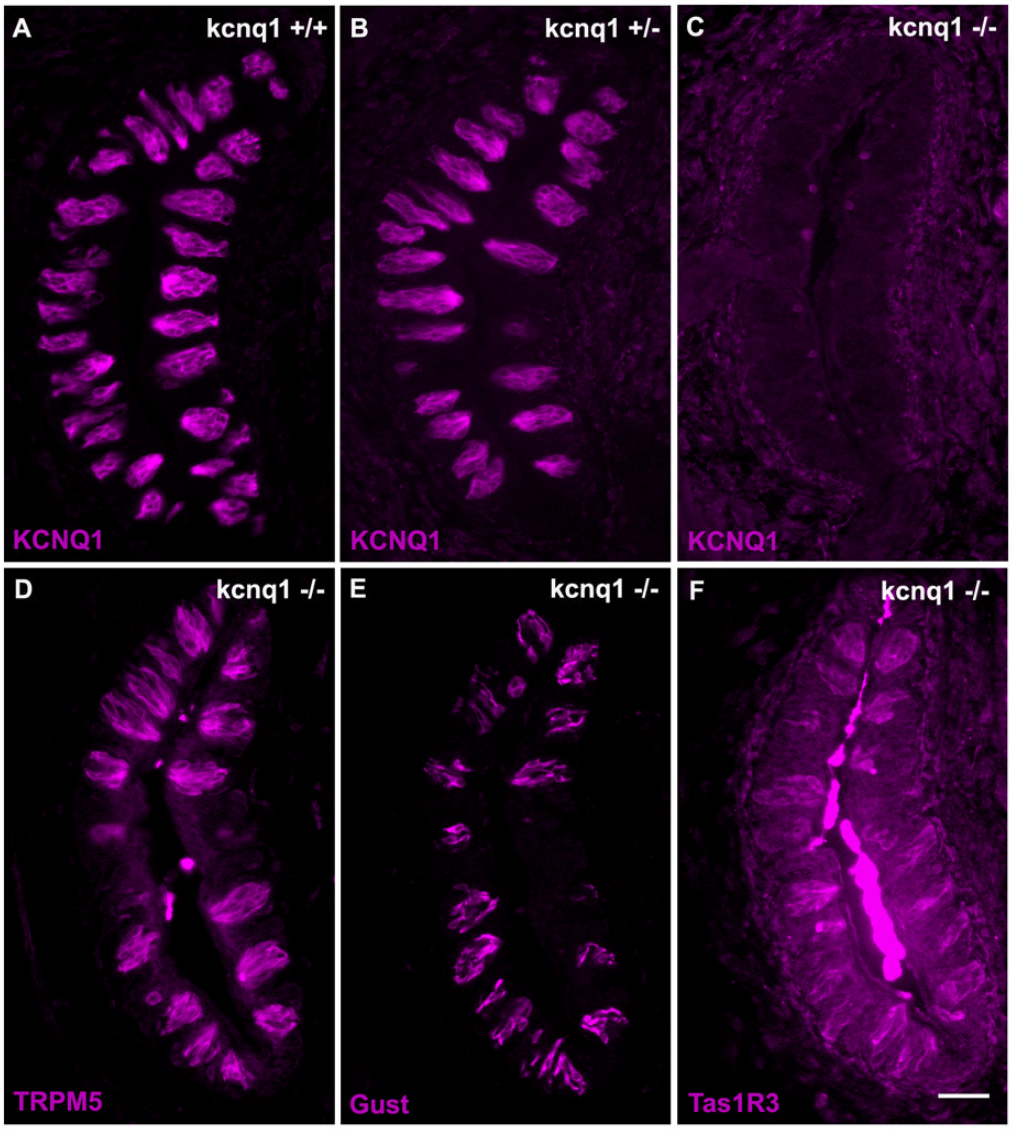

Figure 2.

Expression of KCNQ1 and taste signaling molecules in the *kcnq1*-deficient and wild-type mice. Immunostaining of taste sections confirmed the expression of KCNQ1 in wild-type (A, kcnq1+/+) and heterozygous (B, kcnq1+/−) mice but the absence of the protein in the *kcnq1*-null mutant (C, kcnq1−/−). The gross structure of taste buds and the expression of TRPM5 (D), α-gustducin (E, Gust) and Tas1R3 (F) appeared normal in the knockout animals. Scale bar: 100 μm.

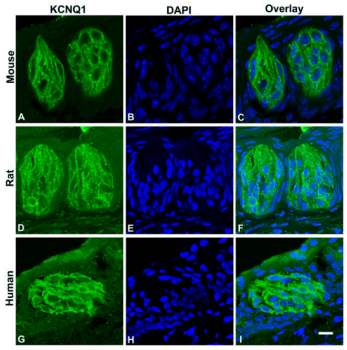

Figure 3.

Single plane confocal images of double stained taste tissue sections with anti-KCNQ1 (green) antibody and DAPI (blue). Longitudinal sections of mouse (top panels: A, B and C) and rat (middle panels: D, E and F) circumvallate taste buds, and an oblique section of a human circumvallate taste bud (bottom panels: G, H and I) showed that nearly all intragemmal nuclei were enveloped in a KCNQ1-stained cell membrane. Scale bar: 20 μm.

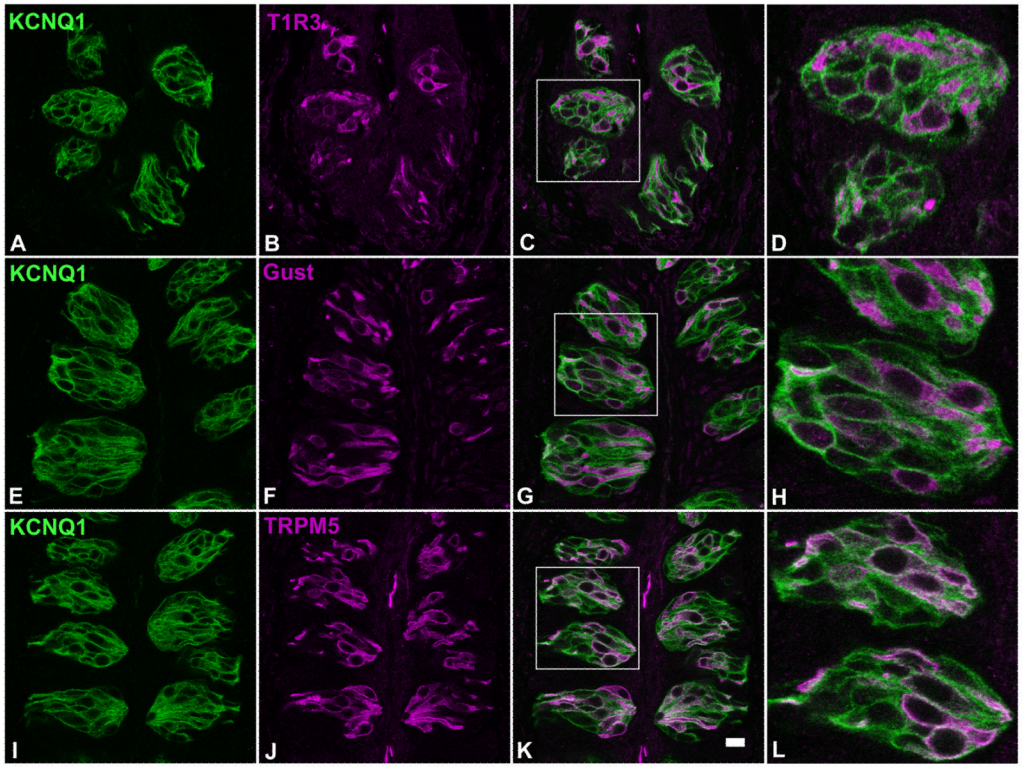

Figure 4.

Single plane confocal images showed that a large number of mouse circumvallate taste bud cells were immunoreactive to an anti-KCNQ1 antibody (left panels A, E and I). Double immunostaining with antibodies against Tas1R3 (T1R3, panel B), α-gustducin (Gust, panel F) and TRPM5 (panel J), indicated that KCNQ1 was present in nearly all cells that were immunoreactive to these three antibodies (superimposed images: C, G and K, and their high magnification images: D, H and L). Scale bar: 20 μm.

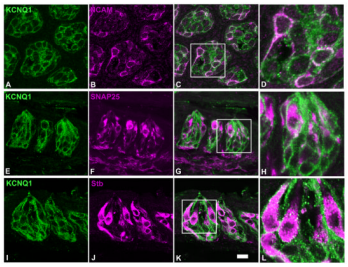

Figure 5.

Single plane confocal images of double immunostaining of rat taste sections with antibodies against KCNQ1 (panels A, E and I), NCAM (panel B), SNAP-25 (panel F) and synaptobrevin-2 (Stb, panel J). To clearly display subcellular colocalization of the immunostaining, one transverse (top row) and two longitudinal (middle and bottom rows) sections were used. Overlays (panels C, G and K) and their high magnification images (panels D, H and L) indicated that the vast majority of SNAP-25 or synaptobrevin-2-expressing taste bud cells also expressed KCNQ1. Note: SNAP-25 and synaptobrevin-2 antibodies also stained extragemmal nerve fibers whereas KCNQ1 staining was largely restricted to the intragemmal cells. Scale bar: 20 μm.

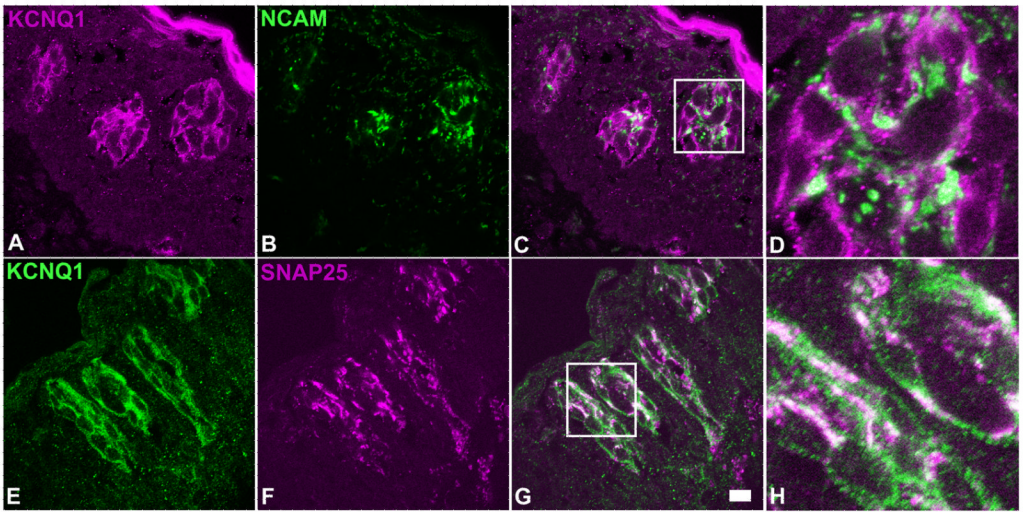

Figure 6.

Single plane confocal images of double immunostaining of human circumvallate sections with antibodies against KCNQ1 and two type III cell markers: NCAM and SNAP-25. Upper panels: a transverse section stained with anti-KCNQ1 (A) and anti-NCAM (B) antibodies; Lower panels: a longitudinal section stained with anti-KCNQ1 (panel E) and anti-SNAP-25 (panel F) antibodies. Overlay of the images (panels C and G) and their high magnification images (panels D and H) showed that all NCAM or SNAP-25 -immunoreactive human taste bud cells displayed KCNQ1 antibody immunoreactivity. Note: To rule out any possible fluorophore effect on imaging, the secondary antibodies conjugated with different fluorophores were used to visualize the KCNQ1 staining on the sections. Scale bar: 20 μm.

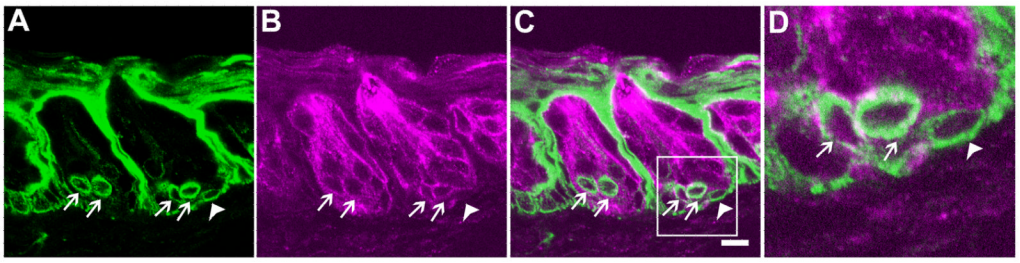

Figure 7.

Single plane confocal images of double immunostaining of rat circumvallate sections with antibodies against cytokeratin 14 (A) and KCNQ1 (B). The overlay (panel C) and its high magnification image (panel D) showed that most of cytokeratin 14-immunoreactive intragemmal cells were also KCNQ1-immunoreactive (arrows). And one cell at the very bottom of a taste bud was not labeled by KCNQ1 antibody (arrowhead). Note the prolonged exposure of panel B to visualize any residual KCNQ1 antibody staining in the section. Scale bar: 20 μm.

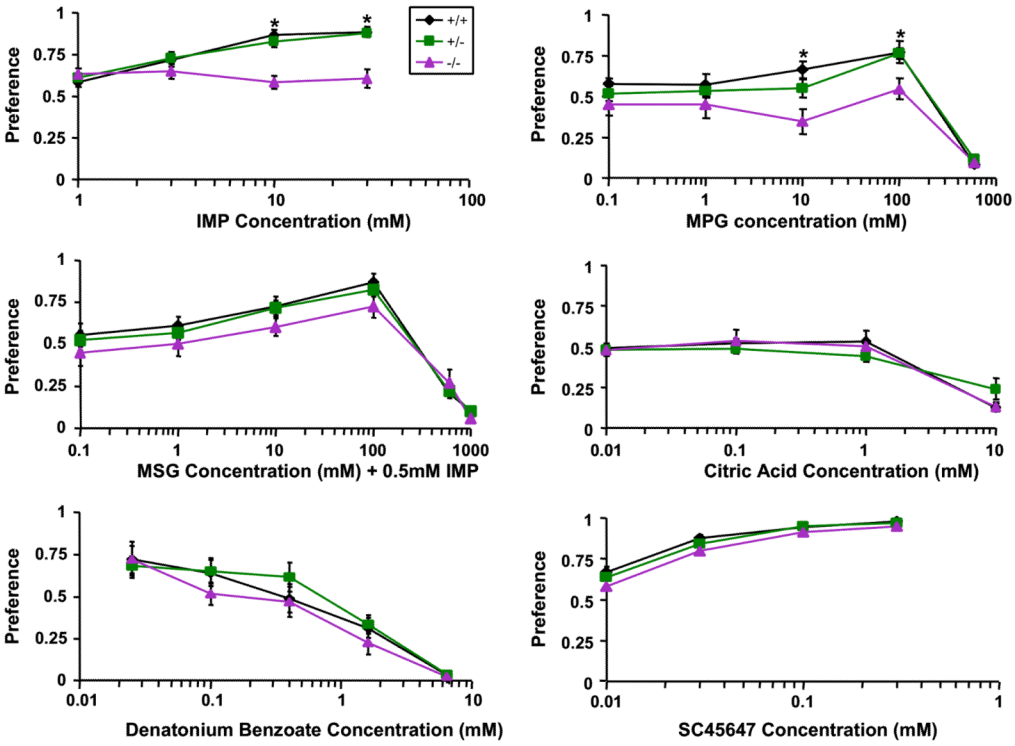

Figure 8.
Preferences of *kcnq1*-null, heterozygous and wild-type mice for taste solutions. *Significant difference between the knockouts and heterozygotes or wild-types.

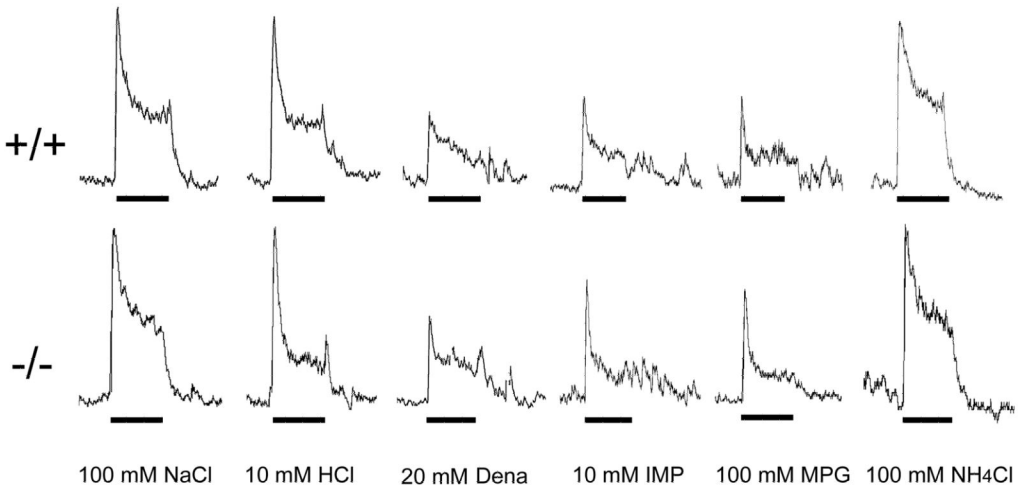

Figure 9.

Integrated responses of the chorda tympani nerve of wild-type (+/+) and kcnq1-null (−/−) mice to tastants. Both wild-type and kcnq1-knockout mice showed robust responses to 100 mM NaCl, 10 mM HCl, 20 mM denatonium (Dena), 10 mM IMP, 100 mM MPG and the reference stimulus: 100 mM NH_4Cl, and no significant differences in these responses between the two genotypes were found. Horizontal bars under nerve recordings show 30-second periods of taste stimulus application to the tongue.