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Original research article

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Please cite this article as: High B, Jetté ME, Li M, *et al*. Variability in P2X Receptor Composition in Human Taste Nerves: Implications for Treatment of Chronic Cough. *ERJ Open Res* 2023; in press (https://doi.org/10.1183/23120541.00007-2023).

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Title: Variability in P2X Receptor Composition in Human Taste Nerves: Implications for Treatment of Chronic Cough

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Running Title: P2X2 in Taste Bud Innervation

Acknowledgements:

The authors thank Nestor Barrezueta, Rebecca Klein, Darrell Henze, and Kenneth Lodge at Merck & Co. for providing the Rhesus tissues. The authors also would like to thank Clinical Coordinators Kristi Engle-Folchert and Laura Temple at the University of Colorado Department of Otolaryngology-Head & Neck Surgery for their assistance with coordinating the fungiform and laryngeal taste buds study, Dylan Volz for his assistance with automating image preparation for scoring, and Courtney Wilson, PhD, and Eric D. Larson, PhD, for their assistance with image analysis as well as scoring images. The authors thank Wayne Rasband for development of ImageJ, (U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018). The authors also thank the Anschutz Human Immune Monitoring Shared Resource, in particular Angela Minic and Kimberly Jordan for assisting us with the Vectra straining.

This work was supported by an investigator-initiated grant (MISP# 58359) from Merck Sharp & Dohme Corp.

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Abstract

Antagonists to the P2X purinergic receptors on airway sensory nerves relieve refractory and unexplained chronic cough but can evoke unwanted dysgeusias because the gustatory nerves innervating taste buds express this same family of receptors. The subunit composition of the P2X receptors in these systems may, however, differ with implications for pharmacological intervention. In most species, the extrapulmonary airway nerves involved in cough predominantly express P2X3 subunits which form homotrimeric P2X3 receptors. In contrast, most sensory nerves innervating taste buds in mice express both P2X2 and P2X3 subunits, so the majority of receptors in that system are likely P2X2/P2X3 heteromers. Since neural P2X subunit composition can differ across species, we used immunohistochemistry to test whether taste nerves in humans and Rhesus monkeys express both P2X2 and P2X3 as in mice. In taste bud samples of fungiform papillae and larynx from humans and monkeys, all taste bud samples exhibit P2X3+ nerve fibers, but the majority lack substantial P2X2+. Of the 35 human subjects, only four (1 laryngeal, 3 fungiform) showed heavy P2X2 expression in taste nerves; none of the Rhesus samples showed P2X2. These findings suggest that for most humans, unlike mice, taste buds are innervated by nerve fibers predominantly expressing only P2X3 homomeric receptors and not P2X2/P2X3 heteromers. Thus, antagonists specific for P2X3 homomeric receptors might not be spared from affecting taste function in treated patients.

Introduction

Chronic cough, defined as a cough lasting over 8 weeks, occurs in 5-10% of the population [1]. The pathophysiology of a patient's chronic cough may often be diagnosed clinically, but some patients have a persistent cough despite a full work-up and treatment; such patients are considered to have refractory or unexplained chronic cough (RCC/UCC) [1-3]. Recently, clinical trials have tested the efficacy of purinergic receptor antagonists, which target P2X receptors (P2XRs) on nerve fibers present in the airway epithelium, for suppression of RCC/UCC [3, 4]. One receptor subtype, P2X3, is expressed in sensory nerve fibers that innervate the epithelium of extrapulmonary airways (larynx, trachea, large bronchus) and contribute to initiating cough reflexes [5-8] following local release of ATP from irritated airway epithelium [9, 10]. In addition, intrapulmonary airway nerves, also implicated in cough initiation, express both P2X2 and P2X3subunits [11]. The mechanical stress of coughing itself can trigger local release of ATP, leading to recurrent cough which can be alleviated by pharmacological blockade of the neural purinergic receptors [12].

Among the many therapeutics trialed for RCC/UCC, P2X3 antagonists reduce objective cough frequency rather than just subjective cough frequency, i.e., the patient's perception of how much they cough [3, 13]. Though an important target for cough treatment, P2X3 receptors are widely expressed in diverse sensory nerves and are crucial to transmission of taste information [5, 7]. Two phase 3 trials in which patients received P2X3 inhibitors reported taste disturbances such as altered taste (dysgeusia) or loss of taste sensitivity (ageusia) in 59.3% (COUGH-1) and 68.9% (COUGH-2) of subjects; 14% of these discontinued the drug even though both studies met primary efficacy endpoints of reducing cough frequency and increasing cough-related quality of life scores [3, 4]. Despite these side effects, P2X3 antagonism represents a promising avenue for treating RCC/UCC, as there are no other approved treatments for this condition.

Gustatory disturbances from P2X3 antagonists likely occur because taste transmission requires purinergic signaling between taste buds, the sensory endorgans of gustation, and the post-synaptic gustatory nerves. Taste buds reside in tongue gustatory papillae, the larynx, the soft palate, and other parts of the oropharynx. Each taste bud, regardless of location, comprises 50-100 specialized epithelial taste cells which synapse onto primary afferent gustatory nerve fibers. Following tastant activation, taste cells release ATP as an obligatory neurotransmitter to activate gustatory nerve fibers [7, 14-16]. ATP release from taste cells and subsequent activation of P2XRs on the nerves initiates action potentials in postsynaptic gustatory nerve fibers conveying taste signals to the brain.

P2XRs form trimers, either homomeric, where all three receptor subunits are one isoform, or heteromeric, where the three subunits may be different isoforms, e.g., P2X3 and P2X2 intermingled [6]. P2X3 antagonists exhibit antagonism to any receptor containing a P2X3 subunit, regardless of homo- or heterotrimeric composition [17]. In rodents and primates, the jugular c-fibers innervating extrapulmonary airways and which are implicated in cough, express P2X3 homomeric receptors whereas the nodose c-fibers of

intrapulmonary airways express P2X3 and P2X2, likely forming heterotrimers [11, 18-20]. In the murine taste system, P2X2 and P2X3 are co-expressed in most gustatory nerve fibers and so likely assemble predominantly as heterotrimers [7, 21, 22]. Application of P2X3 antagonists in wild-type rodents eliminates taste responses to all qualities as this class of drug binds to any P2X3-containing trimer while homomeric-selective P2X3 antagonists do not show effects on taste perception in rodents [21, 23]. Consistent with these findings from rodents, P2X3 antagonists often evoke dysgeusia in RCC/UCC patients [3].

Since taste nerves might predominantly express P2X2/P2X3 heteromers rather than P2X3 homomers that are typically in airway nerves, one avenue of thought has been that a P2X3 homomeric-specific antagonist might minimize the dysgeusic effects observed in RCC/UCC patients while still reducing objective cough frequency due to blockade of extrapulmonary c-fibers expressing P2X3 homomers. Indeed, recent phase 2 trials of P2X3-specific antagonists seem to support this approach [17, 24]. However, since P2X subunit composition in peripheral ganglia can vary across species [25-27], it is unknown whether human taste nerves have a P2X subunit composition similar to that in rodents. Here, we describe findings related to the question of whether human taste nerves express both P2X2 and P2X3 or only one of these subunits.

Methods

Human tissue

In this observational study, human adult fungiform and pediatric laryngeal taste buds were obtained from subjects at the University of Colorado Hospital Outpatient Clinical and Translational Research Center, the Children's Hospital Colorado, and the Smell & Taste Clinic housed at the Technical University of Dresden (**Table 1**). Fresh frozen deidentified human duodenal tissue obtained from the Biorepository Core Facility at CU AMC following 2 cases of Roux-en-y gastric bypass surgeries was used for reagent validation since ganglion cells of the submucosal plexus express both P2X2 and P2X3 [28]. Details regarding how these tissues were obtained and processed are in the *Supplementary Methods*.

Monkey tissue

Samples of *Rhesus macaque* monkey tongue and larynx from five animals (2 female, 3 male; ages from 3-20yo) were obtained through Merck Research Labs (West Point, PA, USA) with approval of the Merck Institutional Animal Care and Use Committee. These samples were fixed in 10% formalin at Merck for up to 24 hours, and then shipped overnight to the CU Anschutz Medical Campus in 0.1M PBS.

Heterologous Expression System

A stably transfected HEK cell line was obtained from SB Drug Discovery (Glasgow, UK) to test the specificity of P2X2 antibodies in recognizing human P2X2 isoforms. These cells were generated by transfection of HEK cells with human P2X2, antibiotic selection, single cell dilution, and expansion of surviving clones.

Clones were then assessed by SB Drug Discovery in a functional fluorescence-based assay to identify the clone with the best window of activity over untransfected cells and showing correct pharmacology using a standard reference activator (ATP – 100 µM) and inhibitor (Suramin – 10µM, 50µM; PPAD – 100µM). Both transfected and control HEK cells were shipped frozen to Colorado and prepared either fixed or unfixed for immunocytochemistry.

Mouse tissue

For procedural and reagent validation, samples of tongue, larynx and intestine were obtained from mice with approval of the Animal Care and Use Committee at the University of Colorado Medical School. These tissues were fixed using 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), 10% formalin, or periodate-lysine-parafomaldehyde ([PLP] 0.01M $NaIO_4$, 0.075M lysine, 0.0375M $NaPO_4$ buffer, 2% paraformaldehyde, adjusted to pH 7.2-7.4 with NaOH) to mirror fixative conditions used in both the rhesus monkey and human-sourced tissues.

Immunohistochemistry

All human tissues were immersion-fixed for up to 24 hours using either 4% PFA in PB, 10% formalin, or PLP. They were then cryoprotected in 20% sucrose in 0.1 M PB for at least 3 days at 4° C. These tissues then underwent sectioning and immunostaining with the antibodies described in **Table 2.** Most samples were stained using the Alomone labs antibody to P2X2 (AB_2040054). See *Supplemental Methods* for more details.

Data analysis

Images of human fungiform and laryngeal taste buds taken on an Olympus BX41 epifluorescence microscope were scored for both P2X3 and P2X2-immunoreactivity by an expert panel comprising five independent scorers, all experienced with taste bud histology. Tuj1, a general marker of innervation, was used for comparison with either P2X2 or P2X3. The panel was blinded to condition (P2X2 or P2X3) and each member assigned a score of 0 (no staining), 1 (unclassified), or 2 (positive) to each taste bud. For each taste bud, the mean of the five scores was used to determine whether P2X immunoreactivity was present. Mean scores below 0.5 were considered negative, i.e., do not show obvious P2X immunoreactivity, and those above 1.5 were considered positive. Any taste buds with mean scores between 0.5-1.5 were designated as unclassified, as were any that received both a 0 and 2 score from independent raters.

The unclassified taste buds (as well as representative samples of positively and negatively-scored taste buds) were re-imaged on a Leica SP8 confocal microscope. These images underwent quantitative colocalization analysis between the Tuj1 and P2X2 or P2X3 channels using Coloc2, an ImageJ (Fiji) plugin [29-31]. Coloc2 performs pixel intensity correlation over space methods yielding a Pearson's correlation (*r*) coefficient.

Pearson's values from 18 scored positive and 6 scored negative taste buds were used to generate a two-independent-groups mean difference plot to establish a threshold for determining P2X positivity status (**Fig. S3**) [32]. Quantitative colocalization analysis showed all positively scored images to have Pearson's correlation coefficients greater than 0.1 and negatively scored images to have coefficients <0.1 (Tables **S3, S4**). The indeterminate images then were analyzed quantitatively and those with correlation coefficients >0.1 were scored as positive and those <0.1 were scored as negative (**Table S4**).

Results

Taste bud morphology is similar across species

The morphology of taste buds is generally similar between mice, primates, and humans although minor structural differences exist [25, 33]. Fungiform and laryngeal taste buds from mice (**Fig. 1a, b**), rhesus monkeys (**Fig. 1c, d**), and humans (**Fig. 1e, f**) were stained for markers against Tuj1 – a class III beta-tubulin marker for neural processes – to examine general innervation, as well as GNAT3 or PLCβ – markers for Type II taste cells – to confirm the presence of taste buds. Taste buds were also stained for either P2X2 or P2X3 to test for the presence of these receptors in gustatory nerve fibers co-stained with Tuj1. For all taste buds, Tuj1 staining showed a dense plexus of gustatory nerve fibers running amidst the elongate GNAT3+ or PLCβ2+ cells of the taste bud. Tuj1 also marked perigemmal fibers, i.e., non-gustatory, non-P2X-expressing nerve fibers innervating epithelium outside the taste bud. Staining for GNAT3 or PLCβ2+ expressed in type II taste receptor cells, was also observed in all taste buds in a similar staining pattern across species.

P2X2 and P2X3 immunoreactivity

The majority of gustatory nerve fibers present in all fungiform and laryngeal taste buds of mice are heavily immunoreactive for both P2X2 and P2X3, while all 5 samples from rhesus and most human taste buds show only P2X3 immunoreactivity with no or minimal P2X2 immunoreactivity (**Table 1, Fig. 2, Fig. 3, S5**). 194 images of taste buds from 35 subjects were evaluated, with 101 being stained for P2X3 while 93 were stained for P2X2. Twenty-three of the 35 subjects had multiple taste buds in their sample evaluated under both P2X2 and P2X3 conditions while 12 of the subjects had only 1 taste bud in their sample to be evaluated for each staining condition (**S3**), consistent with known variability in the number of taste buds in human fungiform papillae [34, 35].

A total of 194 taste bud images were rated regarding P2XR staining status. The expert panel rated 107 of the 194 taste bud images as showing definitive presence of P2XR staining coincident with Tuj1 as a nerve marker. Of these 107 positive images, 8 were of taste buds stained for P2X2 and 99 were of taste buds stained for P2X3. All taste buds rated as positive had been stained for P2X3. The panel also rated 78 of the 194 taste bud images as showing no P2XR staining co-incident with Tuj1; all 78 of these had been stained for P2X2. The

remaining 9 taste bud images which rated as unclassified as well as an additional 5 taste buds that received discrepant ratings. were further evaluated using Coloc2. All these unclassified taste buds were able to be categorized as positive or negative according to their correlation coefficients (**S3, S4**). In total only 12 of 98 human taste bud images evaluated for P2X2 showed the presence of P2X2 in the taste nerves.

The 12 images of P2X2 positive taste buds were drawn from only 4 subjects. Of the adult human fungiform (n=23) and pediatric laryngeal (n=12) samples, only three fungiform and one laryngeal sample (Subjects 2, 14, 25, 26) were judged positive for P2X2 immunoreactivity (**Fig. 4**). For all subjects wherein multiple taste buds were scored, the presence or absence of P2X2 staining was consistent within each subject, i.e., if one taste bud showed P2X2 positive innervation, they all did and vice versa (**S2**). For example, subject 26 had 5 taste buds evaluated and all were positive for P2X2. Given the relative number of total taste buds showing P2X2 immunoreactivity (12/98), the probability of all 5 being positive in an individual subject by chance is very low, p=0.0002. Similarly, subjects 2 and 25 each had 3 taste buds positive for P2X2 and the probability of this occurring at random is also low (p=0.015). Therefore, we conclude that the substantial presence of P2X2 in taste innervation varies across individuals and not across taste buds within each subject. P2X2 was present in only 4 of 35 subjects' samples, i.e., approximately 11.4% of those tested. This result yields a 95% confidence interval of 4%-27% incidence for the general population.

Discussion

Using immunohistochemistry, we determined the presence or absence of P2X2 and P2X3 immunoreactivity within gustatory afferents that penetrate taste buds in mouse, rhesus macaque, and human samples. While sensory nerves in all mouse taste buds demonstrate immunoreactivity for both P2X2 and P2X3, neither human nor rhesus taste bud nerves consistently show immunoreactivity for P2X2 although they do all show robust immunoreactivity for P2X3. This pattern is consistent across the locus of taste bud examined, i.e., fungiform or laryngeal. Fixation condition or duration of fixation also did not seem to affect whether P2X2 immunoreactivity was observed.

Of the human samples, 4 of 35 subjects showed strong P2X2 immunoreactivity. Immunoreactivity in one of these P2X2+ samples was validated using two separate P2X2 antisera (ThermoFisher, Alomone) that were also validated in mouse taste buds. Human intestinal tissue also served as a positive control for both these P2X2 antisera and demonstrated robust P2X2 immunoreactivity in the submucosal plexus across two individuals (**S2**). Altogether, these data suggest that humans express P2X2 variably within their gustatory systems and that this individual variability leads to our findings, rather than variability in innervation between the taste buds of a single subject. Based on these results, the incidence of P2X2 expression may lie in the range of 4%-27% of the population, suggesting that P2X2 is expressed in gustatory nerve fibers in a minority of people.

Given the limitations of immunohistochemistry, it is possible that P2X2 is expressed in human taste nerves at levels below the detection threshold for the method, but if so, it is unlikely that sufficient P2X2 would be available to form abundant heteromers with the majority of the far more plentiful P2X3 subunits. In mice, all gustatory neurons express P2X3 and a majority (~87%) co-express P2X2 [22] at levels easily detected by immunohistochemistry as used in the present study. In humans, P2X3 antagonists cause taste alterations at therapeutic doses in 60-70% of patients with refractory chronic cough [3, 17]. Therefore, even if P2X2 is expressed at undetectable levels in most humans, P2X2 expression is not high enough avoid the gustatory side effects of P2X3 antagonists experienced by RCC/UCC patients. It is also unlikely that a P2X2 isoform not recognized by the antibodies employed is expressed in the human taste system – though that would certainly be a novel finding – as no such P2X2 splice variant is known in any human sensory system [36-38].

However, recent trials of two homomeric-selective P2X3 antagonists, BLU-5937 and Eliapixant, do suggest that adverse events related to taste occur less frequently than with other P2X3 antagonists, such as Gefapixant [17, 24]. The Eliapixant trial reported that 5-21% of patients experienced taste alterations as assessed by psychophysical testing ("taste strips") while the trial involving BLU-5937 reported an incidence of less than 6.5% [17, 24]. In contrast, the latest trials using Gefapixant reported adverse taste events in 59.3% of patients in the COUGH-1 trial and 68.9% of patients in the COUGH-2 trials at clinically effective doses. These results could be due to a variety of reasons, including differences in taste effects on patients with mixed P2X2/P2X3 receptors versus those expressing only P2X3 or even pharmacological differences between these antagonists where taste function is affected at different doses compared to effects on cough reduction. Additionally, drug access to taste buds may differ according to physiochemical properties of the compounds due to the reported permeability barrier surrounding taste buds in the lingual epithelium [39]. Finally, quantification of taste loss without psychophysical testing is challenging and so minor taste loss may not be consistently documented [40]. Notably, it is unclear from clinical trials for both Gefapixant and BLU-5937 how patients reported their taste-related adverse events – only one study using Gefapixant describes using a formalized taste assessment questionnaire, which is likely to have increased the reported frequency of taste-related adverse events [41]. Prior work examining the effects of chorda tympani transections in humans, which can occur in dental and middle ear operations, suggests that considerable loss is needed before taste alterations become noticeable [42, 43]. In the absence of rigorous psychophysical testing, a minor quantitative decrease of taste function would likely go unreported by patients [44]. This suggests that diminished taste function may still occur with homomeric-selective P2X3 antagonists but could be less pronounced than with P2X3 antagonists, and therefore may not be noticeable to the patient.

Regardless, P2X3 antagonists do produce dose-dependent adverse side effects on taste across many patients and despite their effectiveness at reducing RCC/UCC patients ' objective cough frequency, some patients discontinue the drug because of these taste-related side effects. For example, in the COUGH-1 and

COUGH-2 studies over 52 weeks of treatment 21.4% and 22.1% of patients, respectively, discontinued the treatment due to adverse events. It is possible that alternative routes of administration allowing for more localized drug delivery, i.e., inhalers or throat sprays, might help mitigate undesirable effects on gustation but further work is required to explore this question. Finally, whether homomeric-selective P2X3 receptor antagonists may indeed have lower frequency of taste side-effects at comparable efficacy to P2X3 antagonists which bind to both homo- and heteromeric receptors remains to be seen in phase 3 studies of the more selective antagonists.

TABLE 1: Demographics for the pediatric and adult samples

Pediatric taste buds (USA)	Tissue type	Age (months)	Sex	Race/Ethnicity	P2X2 rating	# Taste buds rated for P2X2
1	Laryngeal	3	Female	White	Negative	3
2	Laryngeal	22	Male	White	Positive	3
3	Laryngeal	8	Male	Unknown	Negative	2
4	Laryngeal	3	Male	White	Negative	1
5	Laryngeal	3	Male	White	Negative	5
6	Laryngeal	3	Male	White	Negative	5
7	Laryngeal	4	Female	Unknown	Negative	3
8	Laryngeal	5	Male	Unknown	Negative	3
9	Laryngeal	5	Female	Unknown	Negative	3
10	Laryngeal	25	Male	White	Negative	3
Adult taste buds (USA)	**Tissue type**	**Age (years)**	**Sex**	**Race/Ethnicity**	**P2X2 rating**	**# Taste buds rated for P2X2**
11	Laryngeal	37	Female	White	Negative	5
12	Laryngeal	21	Female	White	Negative	2
13	Fungiform	32	Male	White	Negative	1
14	Fungiform	Adult (unknown)	Male	Unknown	Positive	1
15	Fungiform	Adult (unknown)	Female	Unknown	Negative	1
16	Fungiform	Adult (unknown)	Female	Unknown	Negative	1
17	Fungiform	27	Female	White	Negative	1
18	Fungiform	28	Male	White	Negative	3
19	Fungiform	25	Female	Native American	Negative	2
20	Fungiform	29	Female	East Asian	Negative	1
21	Fungiform	35	Male	White	Negative	5
22	Fungiform	25	Female	East Asian	Negative	5
23	Fungiform	30	Female	White	Negative	5
24	Fungiform	34	Male	White	Negative	4
25	Fungiform	26	Female	Black	Positive	3
26	Fungiform	31	Female	Black	Positive	5

27	Fungiform	29	Female	Black	Negative	1
28	Fungiform	32	Female	Black / Hispanic	Negative	1
29	Fungiform	23	Male	White	Negative	2
30	Fungiform	32	Female	Black	Negative	1
Adult taste buds (Germany)	**Tissue type**	**Age (years)**	**Sex**	**Race/Ethnicity**	**P2X2 rating**	**# Taste buds rated for P2X2**
31	Fungiform	33	Female	Unknown	Negative	5
32	Fungiform	21	Female	Unknown	Negative	2
33	Fungiform	53	Female	Unknown	Negative	3
34	Fungiform	45	Male	Unknown	Negative	4
35	Fungiform	39	Female	Unknown	Negative	2
Summary demographics						
	Age range	**Female vs. Male**		**Race/Ethnicity**	**P2X2 positive subjects**	**P2X2 positive taste buds**
Pediatric cohort	3-25 mo	3 female 7 male		6 White, 4 Unknown	1/10	3/31
Adult cohort	21-53 yr	18 female 8 male		11 White, 5 Black incl. 1 Black/Hispanic, 2 East Asian, 1 Native American, 8 Unknown	3/25	9/67

Samples from subjects under the bold line (subjects 19-35) were collected after the onset of the COVID-19 pandemic and some samples have been collected from subjects who have had COVID in the past. Among subjects who had COVID in the past, none reported a COVID-associated taste loss or disturbance at the time of sample collection in a survey assessing post-COVID subjective taste and smell disturbances.

TABLE 2. Primary and secondary antisera

Primary antisera:	Marker for:	Company, Catalog No.	Research Resource Identifier No. (RRID)	Host; dilution
P2X3	ATP receptor on taste nerves and airway afferents	Alomone, APR-016	AB_2313760	Rabbit; 1:500, 1:1000
P2X2	ATP receptor on taste nerves (457-472 rat C-term)	Alomone, APR003	AB_2040054	Rabbit; 1:500
P2X2	ATP receptor on taste nerves (460-472 rat C-term)	ThermoFisher, PA1-24624	AB_2157912	Rabbit; 1:500
P2X2	ATP receptor on taste nerves (460-472 rat C-term)	Neuromics, RA10108	AB_2236508	Rabbit; 1:500
GNAT3 (α-gustducin)	G-protein subunit in Type II taste cells	Aviva Systems Biology, OAEB00418,	AB_10882823	Goat; 1:200, 1:500
PLCβ2	Transduction component in Type II taste cells	Custom-made by Phosphosolutions	AB_2910247	Guinea pig; 1:1000
β-tubulin III (Tuj1)	Nerve fibers	Cell Signaling, TU-20, #4466	AB_1904176	Mouse; 1:1000
Secondary antisera:	**Host**	**Company, Catalog No.**	**Research Resource Identifier No. (RRID)**	**Dilution**
A488	Donkey anti-mouse	Invitrogen by ThermoFisher Scientific, A21202	AB_141607	1:800
A647	Donkey anti-mouse	Jackson ImmunoResearch, 715-605-150	AB_2340862	1:800
A568	Donkey anti-rabbit	Invitrogen by ThermoFisher Scientific, A10042	AB_2534017	1:800
A647	Donkey anti-goat	Invitrogen by ThermoFisher Scientific, A21447	AB_141844	1:800
A488	Donkey anti-goat	Invitrogen by ThermoFisher Scientific, A11055	AB_2534102	1:800

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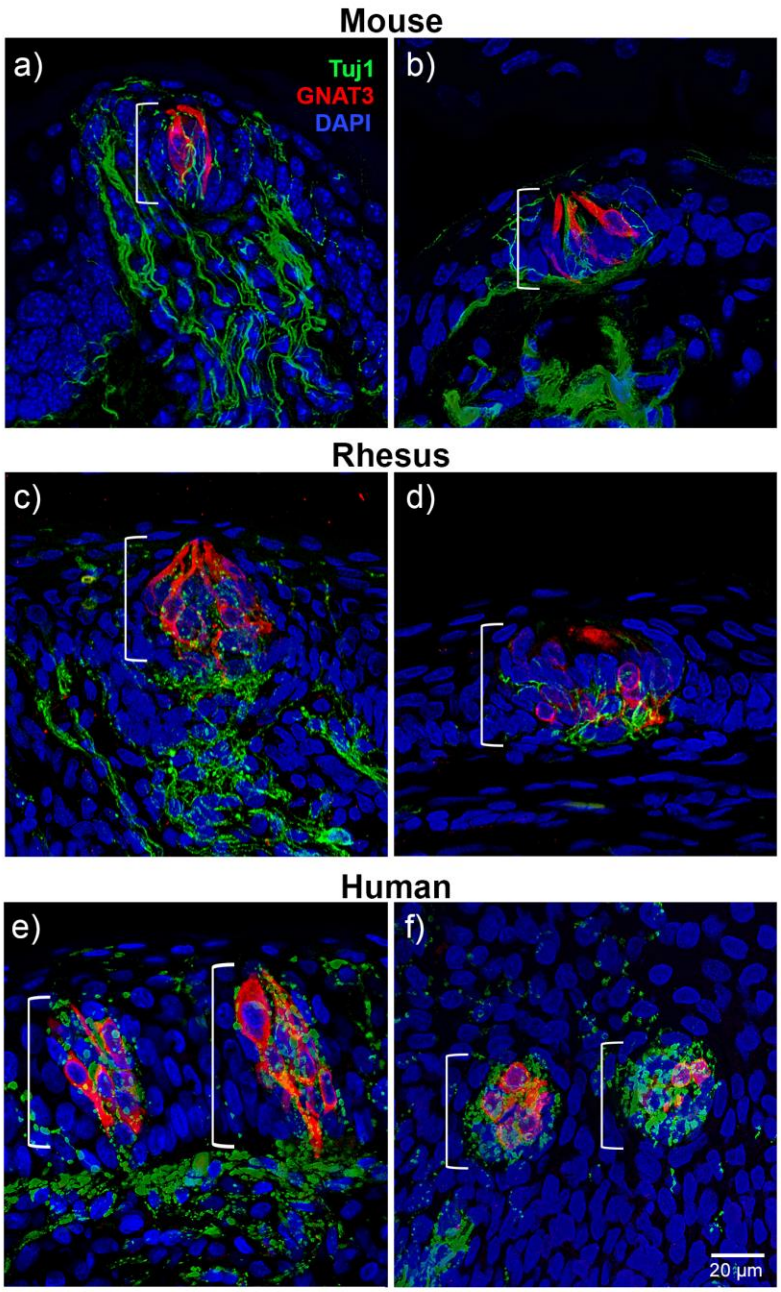
Conflict of interest disclosure

Since 2018 Thomas Hummel did research together with and received funding from Sony, Stuttgart, Germany; Smell and Taste Lab, Geneva, Switzerland; Takasago, Paris, France: aspuraclip, Berlin, Germany; Baia Foods, Madrid, Spain; Frequency Therapeutics, Farmington, CT, USA; Bayer healthcare, Berlin, Germany.

The remaining authors do not have conflicts of interests to disclose for this manuscript.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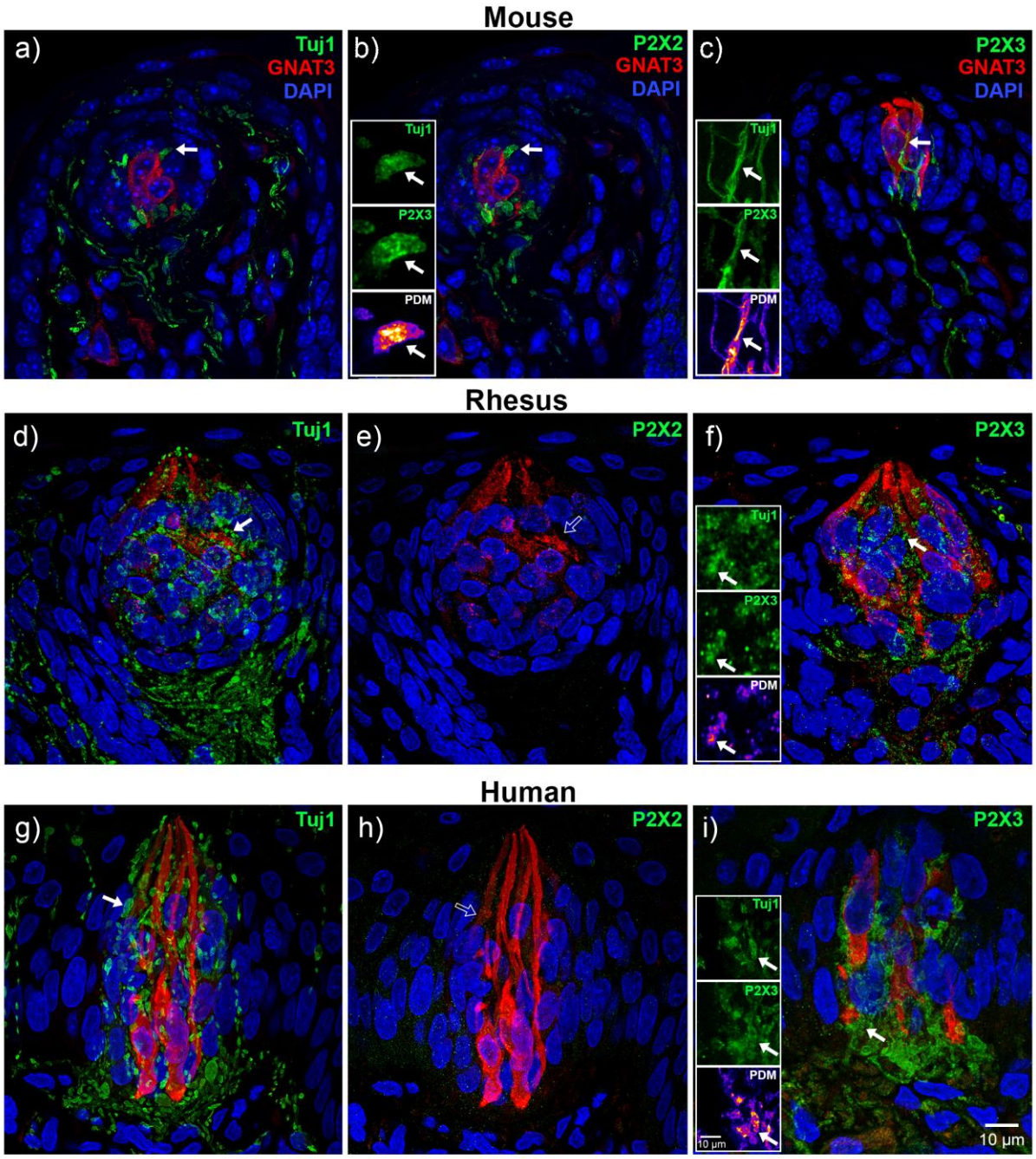
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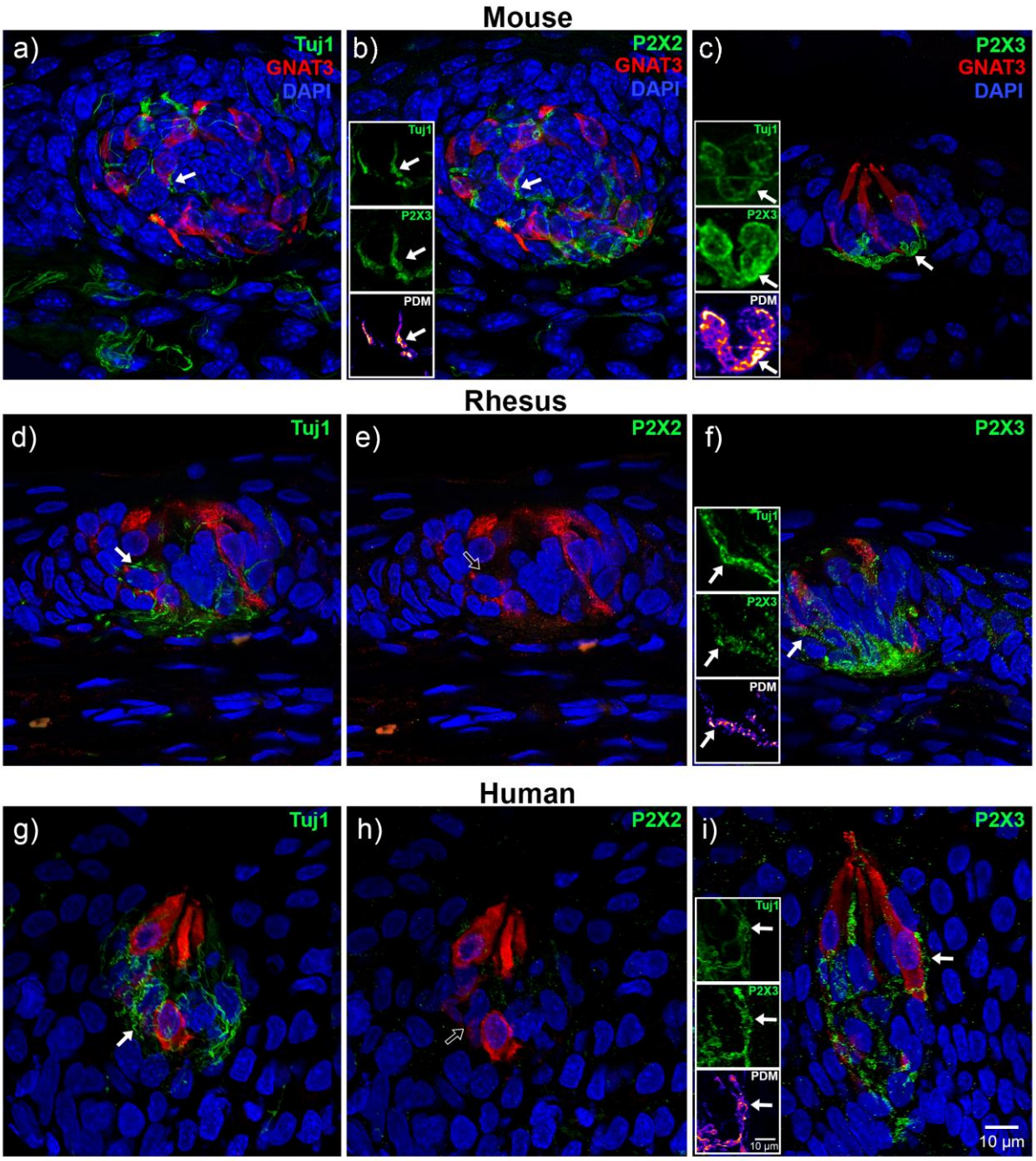
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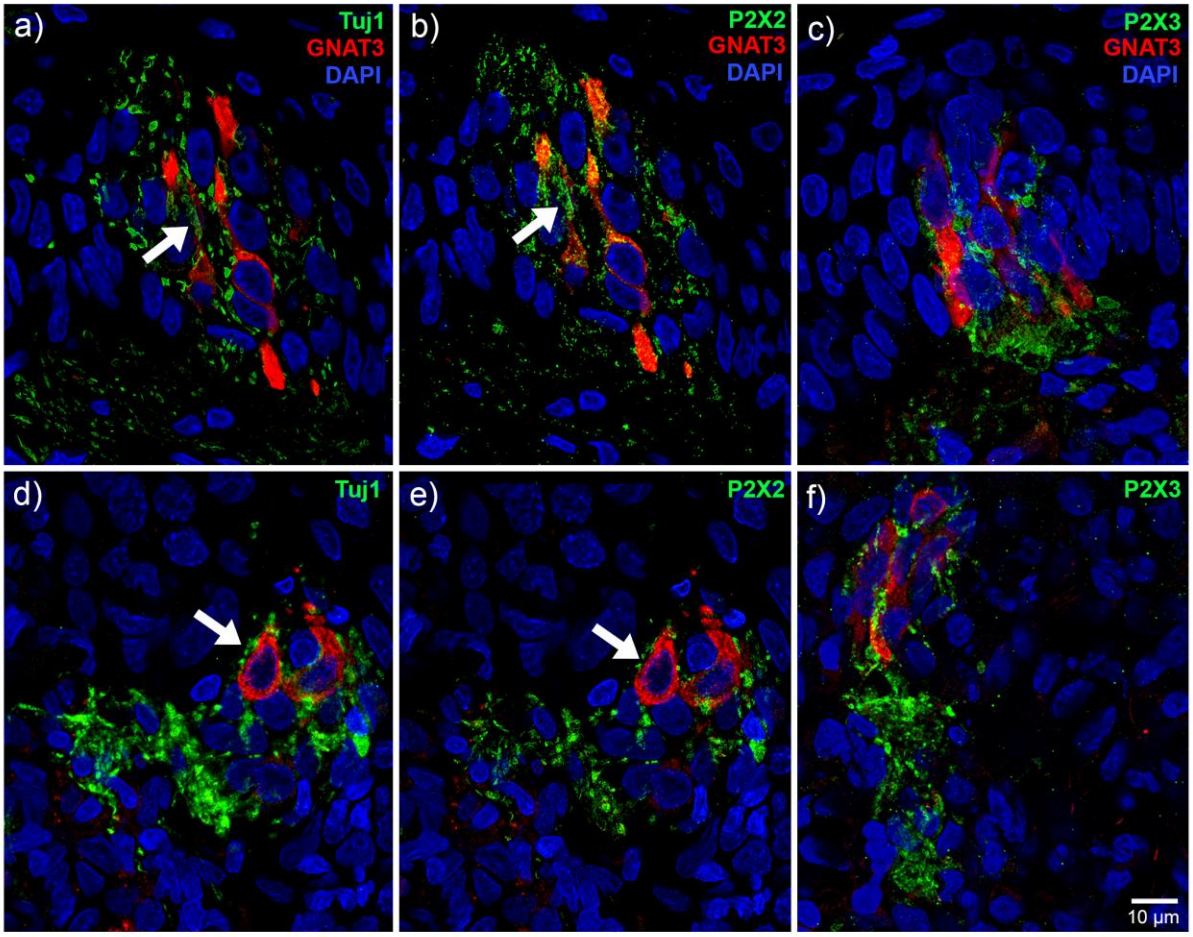
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Supplementary methods

Human tissue

Eighteen human fungiform samples were obtained through word-of-mouth and flyer recruitment on the University of Colorado – Anschutz Medical Campus (CU AMC). After giving informed consent, each subject underwent fungiform papillary biopsies in which 3 fungiform papillae were removed without anesthesia using sterile iridectomy scissors (protocol 14-0439 approved by the Colorado Institutional Review Board). In addition, 5 human fungiform samples were obtained through the Smell & Taste Clinic, Department of Otorhinolaryngology, Technical University of Dresden under the approval of the Ethics Commission of the Technical Univ. of Dresden, #EK499112019. 12 human laryngeal samples were obtained through Children's Hospital Colorado via supraglottoplasties performed for obstructive sleep apnea in children (ages 3-25 months old) in which laryngeal tissue is removed during the normal course of surgery (protocol 14-0776 approved by the Colorado Institutional Review Board). Finally, for the human duodenal tissue, the Pathology Shared Resource has a Colorado Multiple-Institutional Review Board approved protocol (COMIRB 15-1641) to store de-identified tissue from clinical procedures. The provided tissue was remnant archival tissue from a surgical procedure. This research is considered non-human subjects research as the material was provided in a de-identified manner to the study with the Pathology Shared Resource acting as the Honest Broker.

Reagent validation

The P2X2 antisera we employed are directed against a peptide in the C-terminal region of rat P2X2 (457-472 STSTDPKGLAQL). This region of the c-terminus is conserved even in c-terminal variants [36-38] so the antisera that we employed is expected to react with all P2X2 variants described as being functionally present in human tissues. The relevant c-terminal region is lacking in variants f and g which are not of functional relevance and have only been described in rats [36, 37]. The P2X2 antiserum was validated in a heterologous expression system employing human P2X2 expressed in HEK cells (hP2X2- HEK). hP2X2-HEK cells and non-transfected HEK cells were stained with antisera against P2X2 both in the absence and presence of P2X2 blocking peptide (**Table 2, S1**). hP2X2- HEK cells show P2X2 immunoreactivity which is absent in the presence of P2X2 blocking peptide (S1). Non-transfected HEK cells also do not show P2X2 immunoreactivity (**S1**).

P2X2 and P2X3 antisera were also tested in human, monkey, and mouse intestinal tissues, which all express P2X2 and P2X3 in submucosal plexus neurons (**S2**), consistent with previous work [27]. Intestinal tissues were stained with antisera against Tuj1 to identify both ganglion cells and nerve fibers of the submucosal plexus as well as with P2X2 or P2X3 to validate immunoreactivity within fixed, frozen human tissues. Submucosal neurons from humans, mice, and monkeys all demonstrated immunoreactivity for P2X2, P2X3, and Tuj1.

PLCβ2 antiserum, generated against EPLVSKADTQESRL (C-terminal amino acids 1168 to 1181 of mouse sequence (85% identical to human sequence) was validated on mouse taste tissue both by Western blot and by blocking of staining with cognate peptide (0.1µg/ml for antiserum used at 1:1000).

Finally, to account for different fixation conditions (German human samples and rhesus macaque were fixed in 10% formalin while and other human tissues were fixed in 4% PFA or PLP), mouse tissues were fixed under these different conditions. All demonstrated reactivity for both P2X3 and P2X2 regardless of primary fixative employed (data not shown).

Immunohistochemistry

The tissues were sectioned at 14µm-16µm on a cryostat and mounted directly on Superfrost Plus slides (Fisher Scientific) in a 1:3 or 1:4 series. Since taste buds are 40-50 um across, this section thickness allowed a single taste bud to be present in adjacent slides. After drying, slides were rinsed in deionized water, and then underwent antigen retrieval in buffer (1X Tris-EDTA, pH 9.0 for all human samples) at a temperature of 85°C for 10 minutes. After being allowed to cool, the slides were rinsed three times for 5 minutes each in 0.1M PBS, non-specific binding was blocked for at least 1hr at room temperature in blocking solution (2% normal goat serum, 1% bovine serum albumin, 0.3% Triton in PBS). Next, the slides were incubated for at least two nights at 4°C with primary antibodies in blocking solution (**Table 2**). After incubation with primary antibodies, the tissue samples were rinsed with 0.1M PBS three times for 10 minutes per rinse. They were then incubated for 2 hours with fluorescent secondary antibodies at 1:800 dilution (**Table 2**). Finally, the slides were washed twice for 10 minutes each in 0.1M PBS and one time for 10 minutes in 0.05M PB before being coverslipped with DAPI Fluormount (SouthernBiotech – Birmingham, AL, USA).

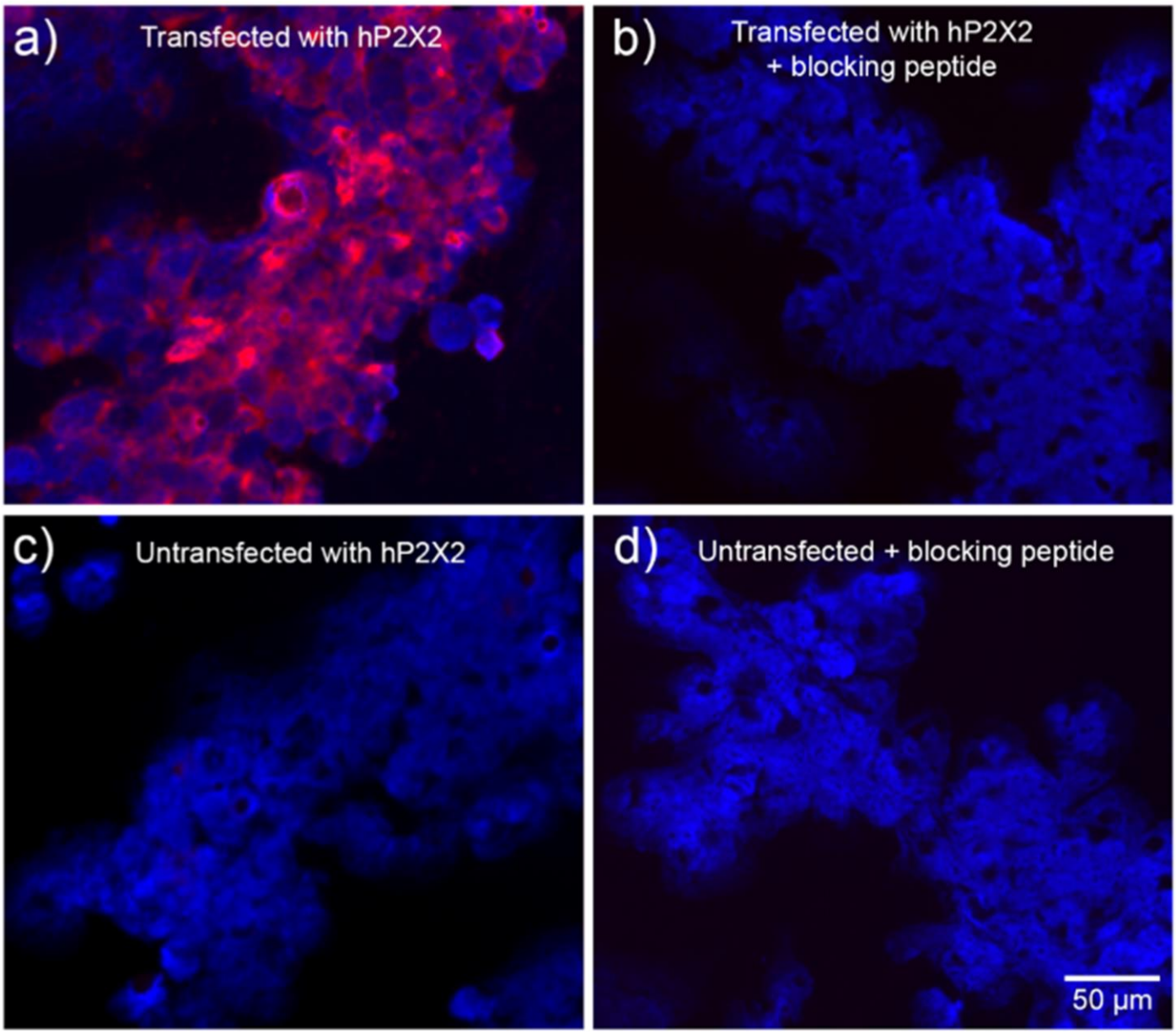
Data analysis

Images of human fungiform and laryngeal taste buds were scored for both P2X3 and P2X2-immunoreactivity by five experienced, independent scorers blinded to condition (0=not present, 1=unclassified, 2=present). When possible, adjacent sections were stained so that P2X2 and P2X3 could be scored across the same set of taste buds per sample. Up to five taste buds were scored per sample per staining condition (i.e. P2X2 vs. P2X3 staining) by these five independent scorers. Staining for the type II taste cell markers GNAT3 or PLCβ2 enabled selection of sections to include the main part of the taste bud. Only sections with taste buds containing two or more GNAT3- or PLCβ2-immunoreactive cells as well as a visible basal plexus stained by Tuj1 were scored to ensure that sections being scored included the central part of the taste bud and were not located at the edge of a taste bud, where nerve fiber density is lower. Any taste bud images receiving average scores between 0.6 and 1.4 or any images with a set of ratings including both a 0 and a 2 were designated as unclassified and further evaluated using confocal microscopy to determine the presence or absence of P2X2 or P2X3. These unclassified samples were imaged at 40x on a Leica SP8 confocal microscope, and scorers were

then asked to rescore these confocal images to make their final determinations. Samples with average above 1.5 were positive and those below 0.5 were considered to be negative. Any taste buds that were still unclassified after their confocal images were re-evaluated underwent quantitative colocalization analysis between the Tuj1 and P2X2/P2X3 channels using Coloc2, a Fiji plugin (**S3**) [29, 30]. A set of known positive and known negative taste buds also underwent colocalization analysis as a means of establishing parameters for Coloc2's Pearson correlation outputs (**S3**). These known positive and negative taste buds were used to generate a two-independent-groups mean difference plot to establish a cutoff value for re-categorizing unclassified samples as being positive or negative for P2X2 staining (**S4**) in combination with their original average scores [31]. Using this cut-off value, unclassified taste buds could then be classified into negative or positive categories. All previously unclassified taste buds were able to be classified as positive or negative using this cut-off value (r = 0.1).

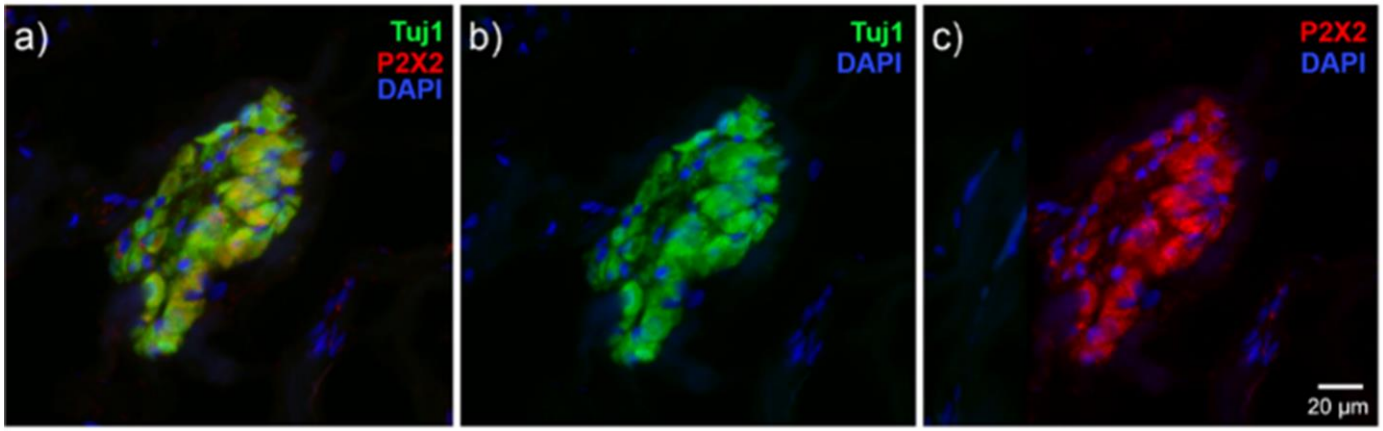
To validate the Pearson's values generated from Coloc2, images of taste buds undergoing this analysis were cropped to a tight square surrounding the taste bud but excluding much of the surrounding epithelium. Two r values were generated using Coloc2 – one with the channels (Tuj1 and P2X2/P2X3) as they were imaged and another with the P2X channel rotated 90° to decorrelate positional information. For all taste buds, when one channel was rotated 90 degrees, the Pearson's above threshold value was near 0, i.e. no correlation. This helped establish that the Coloc2 above-threshold Pearson's value was sufficient to determine colocalization between Tuj and P2X channels. These Pearson's values were used to determine a threshold for P2X immunoreactivity.

Supplementary 1 - figure



Supplementary figure 1. P2X2 antiserum from Alomone labs was validated in HEK cells in both untransfected cells and cells transfected with human P2X2 (a-d). This is the P2X2 antibody used in the majority of the samples. DAPI is shown in blue while P2X2 staining is shown in red. In HEK cells expressing human P2X2, P2X2 immunoreactivity appears red (a). However, with the addition of blocking peptide, no staining is observed (b). Untransfected HEK cells do not demonstrate immunoreactivity for P2X2 with or without peptide (c, d).

Supplementary 2 - figure



Supplementary figure 2. P2X2 antisera were validated in duodenal tissue (a-c). Tuj1 was used to visualize the nerve cells of the submucosal plexus (b), which demonstrated immunoreactivity to two separate antibodies against P2X2 (ThermoFisher, Alomone – **Table 2**) (c).

Supplementary 3 – table. Pearson's above threshold values for unclassified taste buds are bolded.

Subject number	X3 or X2	Taste Bud #	Average panel rating	Pearson's (above threshold)
1	X2	1	0.2	
1	X2	2	0	
1	X2	3	0.4	**0.01**
1	X3	1	2	
1	X3	2	2	
1	X3	3	1.8	0.27
2	X2	1	2	0.67
2	X2	2	2	0.72
2	X3	1	2	0.39
2	X3	2	2	0.38
2	X3	3	2	
2	X3	4	2	
3	X2	1	0	-0.07
3	X2	2	0.2	-0.2
3	X3	1	2	
3	X3	2	2	
3	X3	3	2	
4	X2	1	0.2	
4	X3	1	1.8	
4	X3	2	2	
4	X3	3	2	
5	X2	1	0.2	
5	X2	2	0.2	
5	X2	3	0	
5	X2	4	0	
5	X2	5	0	
5	X3	1	2	
5	X3	2	2	
5	X3	3	2	
5	X3	4	2	
6	X2	2	0.2	
6	X2	3	0	

6	X2	4	0.2	
6	X2	5	0.2	
6	X3	1	2	
6	X3	2	2	
6	X3	3	2	
6	X3	4	2	
6	X3	5	2	
7	X2	1	0	
7	X2	2	0	
7	X3	1	1.8	
7	X3	2	1.8	
7	X3	3	2	

Subject number	X3 or X2	Taste Bud #	Average panel rating	Pearson's (above threshold)
8	X2	1	0.2	
8	X2	2	0.2	
8	X2	3	0.4	
8	X3	1	2	
8	X3	2	2	
8	X3	3	2	
9	X2	1	0	
9	X2	2	0	
9	X2	3	0	
9	X3	2	2	
9	X3	3	2	
10	X2	1	0.2	
10	X2	2	0.2	
10	X2	3	0.2	
10	X3	1	2	
10	X3	2	2	
10	X3	3	2	
11	X2	1	0.4	
11	X2	2	0	
11	X2	3	0.2	-0.06
11	X2	4	0.2	
11	X2	5	0.2	
11	X3	1	1.6	
11	X3	2	2	
11	X3	3	2	
11	X3	4	2	
11	X3	5	2	
12	X2	1	0	0.04
12	X2	2	0	
12	X3	1	1.2	0.24
12	X3	2	1.6	
13	X2	1	0.2	
13	X3	1	2	

14	X2	1	2	0.16
14	X3	1	2	0.28
15	X2	1	0	
15	X3	1	2	
16	X2	1	0.8	**0.05**
16	X3	1	2	0.31
17	X2	2	0	
17	X3	1	2	
17	X3	2	2	
18	X2	1	0	
18	X2	2	0	-0.23
18	X2	3	0	
18	X3	1	2	
18	X3	2	2	
Subject number	**X3 or X2**	**Taste Bud #**	**Average panel rating**	**Pearson's (above threshold)**
19	X2	3	1	**-0.05**
19	X3	1	2	0.43
20	X2	1	0.2	-0.13
20	X3	1	2	
20	X3	2	2	
21	X2	1	0.4	
21	X2	2	1	**0.02**
21	X2	3	0.2	
21	X2	4	0.6	**-0.08**
21	X2	5	0	
21	X3	1	2	
21	X3	2	2	0.25
21	X3	3	2	
21	X3	4	2	0.28
21	X3	5	2	
22	X2	1	0	
22	X2	2	0	
22	X2	3	0	
22	X2	4	0.2	

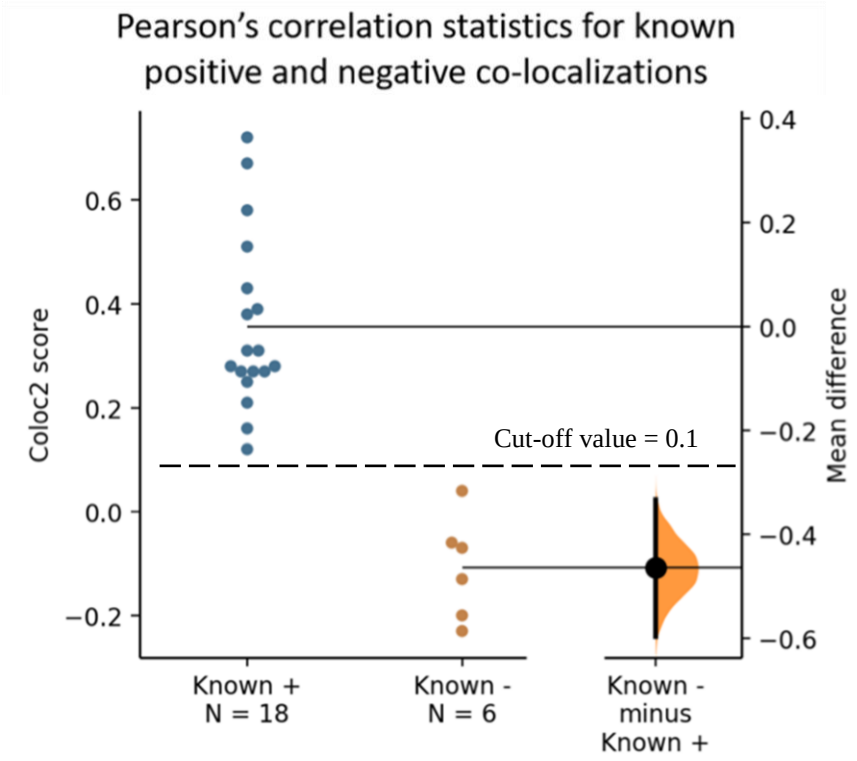
22	X2	5	0	
22	X3	1	2	
22	X3	2	2	
22	X3	3	1.8	
22	X3	4	2	
22	X3	5	2	
23	X2	1	0.2	
23	X2	2	0.2	
23	X2	3	0	
23	X2	4	0	
23	X2	5	0	
23	X3	1	2	
23	X3	2	2	
23	X3	3	2	
23	X3	4	2	
23	X3	5	2	
24	X2	1	0	
24	X2	2	0	
24	X2	3	0.2	
24	X2	4	0.2	
24	X3	1	2	
24	X3	2	2	
24	X3	3	2	
25	X2	1	1.6	**0.2**
25	X2	2	1.6	**0.16**
25	X3	1	2	0.58
25	X3	2	2	0.51

Subject number	X3 or X2	Taste Bud #	Average panel rating	Pearson's (above threshold)
26	X2	1	0.8	**0.17**
26	X2	2	1.4	**0.3**
26	X2	3	1.6	
26	X2	4	1.6	
26	X2	5	2	0.12

26	X3	1	2	0.27
26	X3	2	2	0.27
26	X3	3	2	
26	X3	4	2	
26	X3	5	2	0.31
27	X2	1	0.2	
27	X3	1	2	
28	X2	1	0	
28	X3	1	2	
29	X2	1	0	
29	X2	2	0	
29	X3	1	2	
29	X3	2	2	
29	X3	3	2	
29	X3	4	2	
29	X3	5	2	
30	X2	1	0.8	**-0.05**
30	X3	1	2	0.21
30	X3	2	2	
31	X2	1	0.2	
31	X2	2	0	
31	X2	3	0.2	
31	X2	4	0.2	
31	X2	5	0	
31	X3	1	2	
31	X3	2	1.8	
31	X3	3	2	
31	X3	4	1.8	
31	X3	5	1.8	
32	X2	1	0	
32	X2	2	0	
32	X3	1	1.6	
32	X3	2	1.8	
32	X3	3	1.6	
33	X2	1	0	

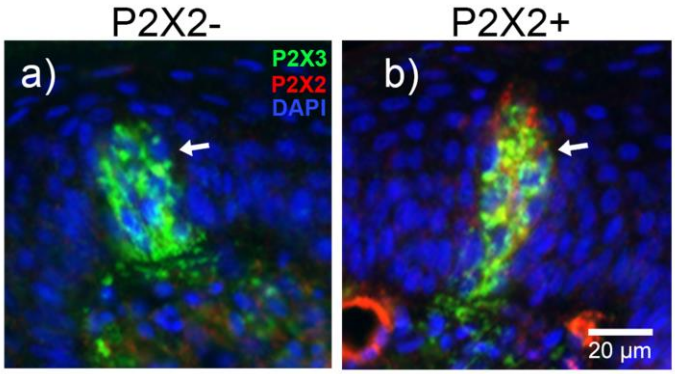
33	X2	2	0	
33	X2	3	0.2	
33	X3	1	1.4	0.26
34	X2	1	0	
34	X2	2	0	
34	X2	3	0	
34	X2	4	0	
34	X3	1	1.6	
34	X3	2	1.8	
34	X3	3	2	
35	X2	1	0.2	
35	X2	2	0	
35	X2	3	0	
35	X3	1	2	
35	X3	2	2	
35	X3	3	2	

Supplementary 4 - figure



Supplementary figure 4. 18 positive and 6 negative taste buds, as determined by the expert panel, were confocaled and analyzed using Coloc2 for correlation between Tuj1 and P2X2 or P2X3 channels. A two-independent-groups mean difference plot shows a significant difference of -0.464 between Pearson's correlation co-efficients for colocalized and non-colocalized samples, p=0.0. We utilized a value of 0.1 as a cut-off for determining the colocalization status for the taste buds in the indeterminate groups following expert panel review.

Supplementary 5 – figure.



Supplementary figure 5. Taste buds from subjects classified as P2X2- (a) and P2X2+ (b), were stained with P2X2 and P2X3 antibodies using a Vectra multispectral imaging system using Akoya's HRP-conjugated secondary polymer and TSA-based Opal fluorophores. Four subjects' taste buds (2 P2X2-, 2 P2X2+) were stained with examples shown in (a) and (b). Note the near absence of red immunolabel denoting P2X2 in panel (a).