



Variability in P2X receptor composition in human taste nerves: implications for treatment of chronic cough

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Molecular composition of receptors in taste nerves in most people makes it difficult to develop more specific antagonists for treating chronic cough while avoiding taste disturbance <https://bit.ly/3JTzQep>

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Abstract

Background Antagonists to the P2X purinergic receptors on airway sensory nerves relieve refractory or unexplained chronic cough (RCC/UCC) but can evoke unwanted dysgeusias because the gustatory nerves innervating taste buds express this same family of receptors. However, the subunit composition of the P2X receptors in these systems may differ, with implications for pharmacological intervention of RCC/UCC. In most species, the extrapulmonary airway nerves involved in cough predominantly express P2X3 subunits that 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.

Methods Since neural P2X subunit composition can differ across species, we used immunohistochemistry to test whether taste nerves in humans and rhesus macaque monkeys express both P2X2 and P2X3 as in mice.

Results In taste bud samples of fungiform papillae and larynx from humans and monkeys, all taste bud samples exhibited P2X3⁺ nerve fibres, but the majority lacked substantial P2X2⁺. Of the 35 human subjects, only four (one laryngeal and three fungiform) showed strong P2X2 immunoreactivity in taste nerves; none of the rhesus monkey samples showed immunoreactivity for P2X2.

Conclusions These findings suggest that for most humans, unlike mice, taste buds are innervated by nerve fibres 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 RCC/UCC 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 fibres present in the airway epithelium, for suppression of RCC/UCC [3, 4]. One receptor subtype, P2X3, is expressed in sensory nerve fibres that innervate the epithelium of extrapulmonary airways (larynx, trachea and 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 P2X3 subunits [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 trialled 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]. Although 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 the P2X3 inhibitor gefapixant at a 45 mg twice-daily dose reported taste-related adverse events 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 end-points 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 signalling between taste buds, the sensory end-organs 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 specialised epithelial taste cells which synapse onto primary afferent gustatory nerve fibres. Following tastant activation, taste cells release ATP as an obligatory neurotransmitter to activate gustatory nerve fibres [7, 14–16]. ATP release from taste cells and subsequent activation of P2XRs on the nerves initiates action potentials in post-synaptic gustatory nerve fibres 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-fibres innervating extrapulmonary airways and which are implicated in cough express P2X3 homomeric receptors, whereas the nodose C-fibres of intrapulmonary airways express P2X3 and P2X2, likely forming heterotrimers [11, 18–20]. In the murine taste system, P2X2 and P2X3 are coexpressed in most gustatory nerve fibres 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 found in airway nerves, one avenue of thought has been that a P2X3 homomeric-specific antagonist might minimise the dysgeusia effects observed in RCC/UCC patients while still reducing objective cough frequency due to blockade of extrapulmonary C-fibres 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 paediatric laryngeal taste buds were obtained from subjects at the University of Colorado Hospital Outpatient Clinical and Translational Research Center (Aurora, CO, USA), the Children's Hospital Colorado (Aurora, CO, USA), and the Smell and Taste Clinic housed at the Technical University of Dresden (Dresden, Germany) (table 1). Fresh frozen de-identified human duodenal tissue obtained from the Biorepository Core Facility at University of Colorado Anschutz Medical Campus following two 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 provided in the supplementary material.

Monkey tissue

Samples of rhesus macaque monkey tongue and larynx from five animals (two females and three males; aged 3–20 years) were obtained through Merck Research (West Point, PA, USA) with approval of the Merck Institutional Animal Care and Use Committee. These samples were fixed in 10% formalin at Merck

TABLE 1 Demographics for the human paediatric and adult samples

	Tissue type	Age	Sex	Race/ethnicity	P2X2 rating	Taste buds rated for P2X2 (n)
Paediatric taste buds (USA)						
1	Laryngeal	3 months	Female	White	Negative	3
2	Laryngeal	22 months	Male	White	Positive	3
3	Laryngeal	8 months	Male	Unknown	Negative	2
4	Laryngeal	3 months	Male	White	Negative	1
5	Laryngeal	3 months	Male	White	Negative	5
6	Laryngeal	3 months	Male	White	Negative	5
7	Laryngeal	4 months	Female	Unknown	Negative	3
8	Laryngeal	5 months	Male	Unknown	Negative	3
9	Laryngeal	5 months	Female	Unknown	Negative	3
10	Laryngeal	25 months	Male	White	Negative	3
Adult taste buds (USA)						
11	Laryngeal	37 years	Female	White	Negative	5
12	Laryngeal	21 years	Female	White	Negative	2
13	Fungiform	32 years	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 years	Female	White	Negative	1
18	Fungiform	28 years	Male	White	Negative	3
19 [#]	Fungiform	25 years	Female	Native American	Negative	2
20 [#]	Fungiform	29 years	Female	East Asian	Negative	1
21 [#]	Fungiform	35 years	Male	White	Negative	5
22 [#]	Fungiform	25 years	Female	East Asian	Negative	5
23 [#]	Fungiform	30 years	Female	White	Negative	5
24 [#]	Fungiform	34 years	Male	White	Negative	4
25 [#]	Fungiform	26 years	Female	Black	Positive	3
26 [#]	Fungiform	31 years	Female	Black	Positive	5
27 [#]	Fungiform	29 years	Female	Black	Negative	1
28 [#]	Fungiform	32 years	Female	Black/Hispanic	Negative	1
29 [#]	Fungiform	23 years	Male	White	Negative	2
30 [#]	Fungiform	32 years	Female	Black	Negative	1
Adult taste buds (Germany)						
31 [#]	Fungiform	33 years	Female	Unknown	Negative	5
32 [#]	Fungiform	21 years	Female	Unknown	Negative	2
33 [#]	Fungiform	53 years	Female	Unknown	Negative	3
34 [#]	Fungiform	45 years	Male	Unknown	Negative	4
35 [#]	Fungiform	39 years	Female	Unknown	Negative	2
Summary demographics						
Paediatric cohort		3–25 months	3 female; 7 male	6 White; 4 Unknown	1/10 positive subjects	3/31 positive taste buds
Adult cohort		21–53 years	18 female; 8 male	11 White, 5 Black (including 1 Black/Hispanic), 2 East Asian, 1 Native American, 8 Unknown	3/25 positive subjects	9/67 positive taste buds

[#]: samples from Subjects 19–35 were collected after the onset of the coronavirus disease 2019 (COVID-19) pandemic and some samples were collected from subjects who had COVID-19 in the past. Among subjects who had COVID-19 in the past, none reported a COVID-associated taste loss or disturbance at the time of sample collection in a survey assessing post-COVID-19 subjective taste and smell disturbances.

for up to 24 h and then shipped overnight to the University of Colorado Anschutz Medical Campus in 0.1 M PBS.

Heterologous expression system

A stably transfected human embryonic kidney (HEK) cell line was obtained from SB Drug Discovery (Glasgow, UK) to test the specificity of P2X2 antibodies in recognising 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: 10 and 50 µM; pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid: 100 µM). 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, 10% formalin or periodate-lysine-parafomaldehyde (PLP: 0.01 M NaIO₄, 0.075 M lysine, 0.0375 M NaPO₄ 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 h using either 4% PFA in phosphate buffer, 10% formalin or PLP. They were then cryoprotected in 20% sucrose in 0.1 M phosphate buffer 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 antibody to P2X2 (AB_2040054). See supplementary material for more details.

Data analysis

Images of human fungiform and laryngeal taste buds taken on an Olympus BX41 epifluorescence microscope (Olympus, Tokyo, Japan) were scored for both P2X3 and P2X2 immunoreactivity by an expert panel comprising five independent scorers, all experienced with taste bud histology. Tuj1 (β -tubulin III), 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 <0.5 were considered negative, i.e. do not show obvious P2X immunoreactivity, and those >1.5 were considered positive. Any taste buds with mean scores between 0.5 and 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 (Leica, Wetzlar, Germany). These images underwent quantitative colocalisation 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 coefficient (r). Pearson's values from 18 scored positive and six scored negative taste buds were used to generate a two-independent-groups mean difference plot to establish a threshold for determining P2X positivity status (supplementary figure S3) [32]. Quantitative colocalisation analysis showed all positively scored images to have $r>0.1$ and negatively scored images to have $r<0.1$ (supplementary tables S3 and S4). The indeterminate images then were analysed quantitatively, and those with $r>0.1$ were scored as positive and those with $r<0.1$ were scored as negative (supplementary 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 (figure 1a and b), rhesus monkeys (figure 1c and d) and humans (figure 1e and f) were stained for markers against Tuj1 (a β -tubulin III marker for neural processes) to examine general innervation, as well as α -gustducin (GNAT3) or phospholipase C (PLC)- β 2 (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 fibres co-stained with Tuj1. For all taste buds, Tuj1 staining showed a dense plexus of gustatory

TABLE 2 Primary and secondary antisera

	Marker for [#] or host [¶]	Company, catalogue ID	RRID	Host; dilution
Primary antisera				
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-terminal)	Alomone, APR-003	AB_2040054	Rabbit; 1:500
P2X2	ATP receptor on taste nerves (460–472 rat C-terminal)	ThermoFisher Scientific, PA1-24624	AB_2157912	Rabbit; 1:500
P2X2	ATP receptor on taste nerves (460–472 rat C-terminal)	Neuromics, RA10108	AB_2236508	Rabbit; 1:500
GNAT3	G-protein subunit in Type II taste cells	Aviva Systems Biology, OAEB00418	AB_10882823	Goat; 1:200, 1:500
PLC-β2	Transduction component in type II taste cells	Custom-made by PhosphoSolutions	AB_2910247	Guinea pig; 1:1000
Tuj1	Nerve fibres	Cell Signaling, (TU-20) 4466	AB_1904176	Mouse; 1:1000
Secondary antisera				
Alexa Fluor 488	Donkey anti-mouse	Invitrogen by ThermoFisher Scientific, A-21202	AB_141607	1:800
Alexa Fluor 647	Donkey anti-mouse	Jackson ImmunoResearch, 715-605-150	AB_2340862	1:800
Alexa Fluor 568	Donkey anti-rabbit	Invitrogen by ThermoFisher Scientific, A-10042	AB_2534017	1:800
Alexa Fluor 647	Donkey anti-goat	Invitrogen by ThermoFisher Scientific, A-21447	AB_141844	1:800
Alexa Fluor 488	Donkey anti-goat	Invitrogen by ThermoFisher Scientific, A-11055	AB_2534102	1:800

[#]: primary antisera; [¶]: secondary antisera. RRID: Research Resource Identifier (www.ncbi.nlm.nih.gov/RRIDs/); GNAT3: α -gustducin; PLC: phospholipase C; Tuj1: β -tubulin III.

nerve fibres running amid the elongate GNAT3⁺ or PLC-β2⁺ cells of the taste bud. Tuj1 also marked perigemmal fibres, *i.e.* non-gustatory, non-P2X-expressing nerve fibres innervating epithelium outside the taste bud. Staining for GNAT3 or PLC-β2 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 fibres present in all fungiform and laryngeal taste buds of mice were heavily immunoreactive for both P2X2 and P2X3, while all five samples from rhesus monkeys and most human taste buds showed only P2X3 immunoreactivity with no or minimal P2X2 immunoreactivity (table 1, figures 2 and 3, and supplementary figure S5). 194 images of taste buds from 35 subjects were evaluated, with 101 being stained for P2X3 and 93 being stained for P2X2. 23 out 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 one taste bud in their sample evaluated for each staining condition (supplementary table 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, eight 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 out of the 194 taste bud images as showing no P2XR staining coincident with Tuj1; all 78 of these had been stained for P2X2. The remaining nine taste bud images that rated as unclassified as well as an additional five taste buds that received discrepant ratings were further evaluated using Coloc2. All these unclassified taste buds were able to be categorised as positive or negative according to their correlation coefficients (supplementary table S3 and supplementary figure S4). In total, only 12 out of 98 human taste bud images evaluated for P2X2 showed the presence of P2X2 in the taste nerves.

The 12 images of P2X2⁺ taste buds were drawn from only four subjects. Of the adult human fungiform (n=23) and paediatric laryngeal (n=12) samples, only three fungiform and one laryngeal sample (Subjects 2, 14, 25 and 26) were judged positive for P2X2 immunoreactivity (figure 4). For all subjects wherein

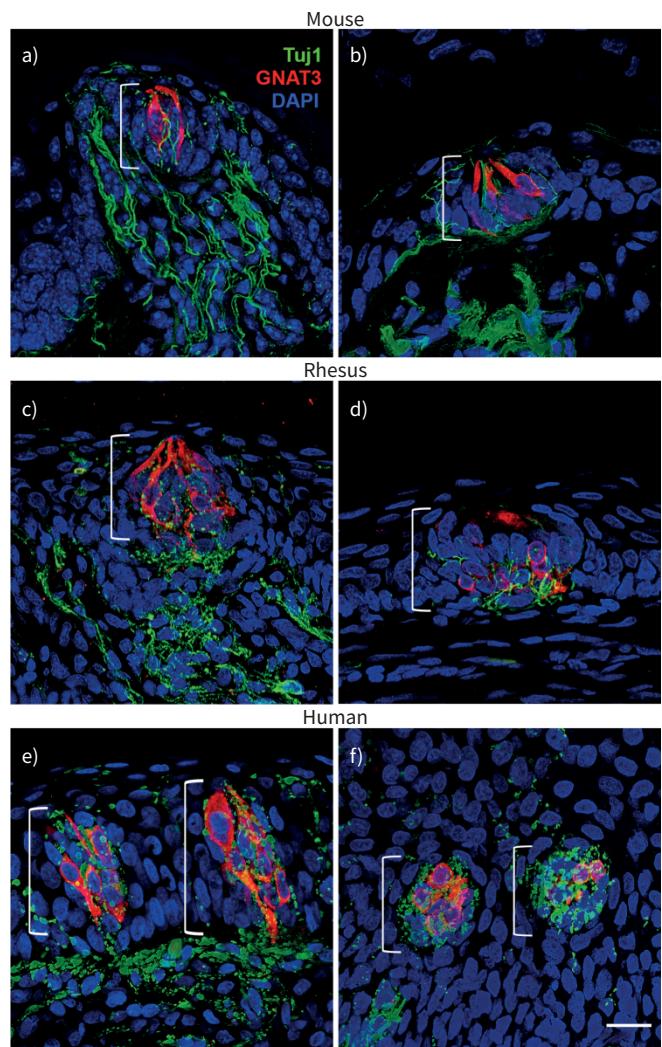


FIGURE 1 Taste buds have a similar structure in a, b) mouse, c, d) rhesus macaque monkey and e, f) human a, c, e) fungiform papillae and b, d, f) larynx. White brackets indicate the region in which a taste bud resides. Elongate taste cells (red) are intimately surrounded by gustatory nerve fibres (green) as stained with Tuj1. Non-gustatory nerve fibres (also in green) innervate the epithelium outside of the taste buds. Scale bar: 20 µm for all panels. Tuj1: β-tubulin III (a marker for neural processes); GNAT3: α-gustducin (a marker for some type II taste cells); DAPI: 4',6-diamidino-2-phenylindole (a general nuclear stain).

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⁺ innervation, they all did and *vice versa* (supplementary figure S2). For example, Subject 26 had five taste buds evaluated and all were positive for P2X2. Given the relative number of total taste buds showing P2X2 immunoreactivity (12 out of 98), the probability of all five being positive in an individual subject by chance is very low ($p=0.0002$). Similarly, Subjects 2 and 25 each had three 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 four out of 35 subjects' samples, *i.e.* ~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 monkey and human samples. While sensory nerves in all mouse taste buds demonstrate immunoreactivity for both

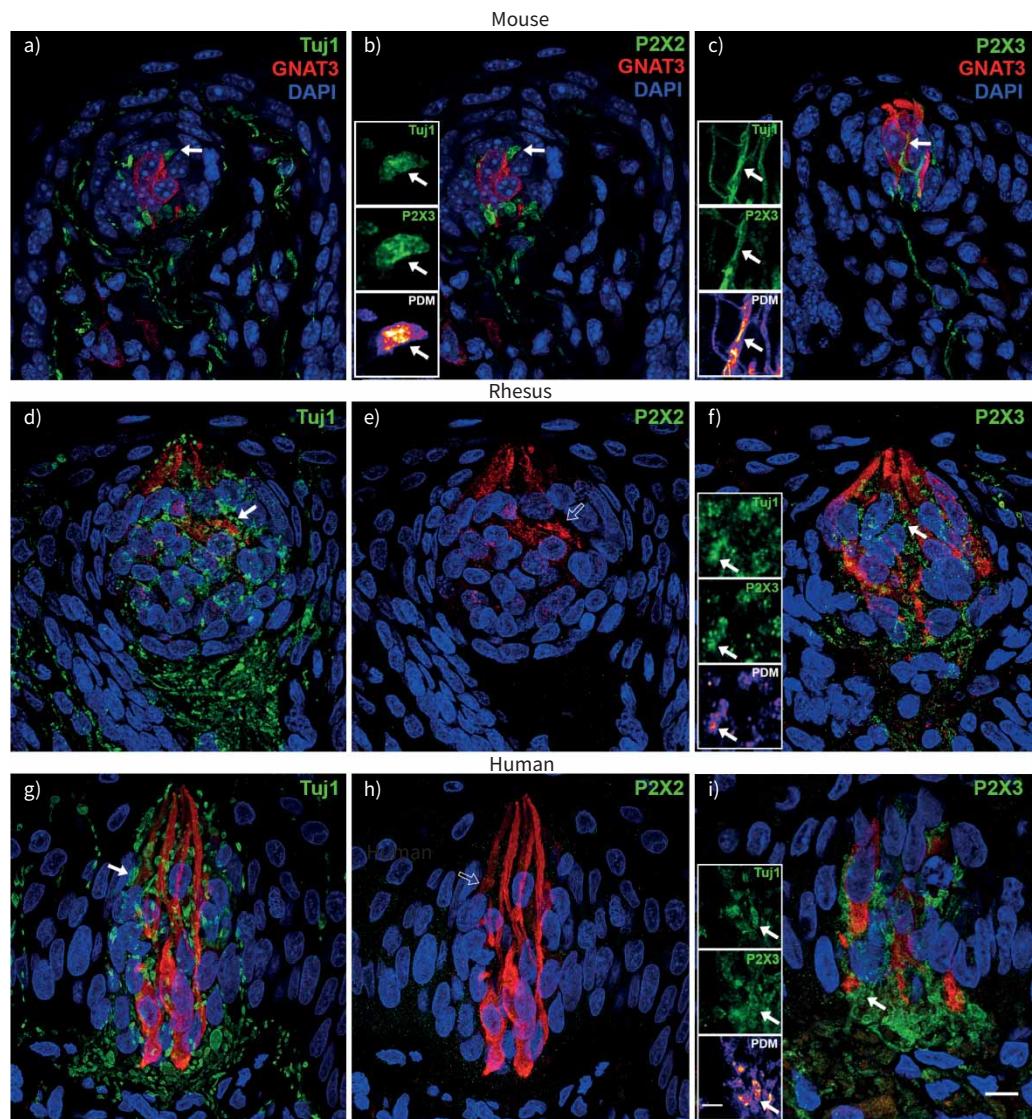


FIGURE 2 Fungiform taste buds. P2X2 and P2X3 receptor staining in **a–c**) mouse, **d–f**) rhesus macaque monkey and **g–i**) human. In all panels, a subset of type II taste cells is marked by GNAT3 (red). **a, d, g)** Across all species, Tuj1 (green) marks both intra- and perigemmal nerve fibres in and around taste buds. **b, e, h)** P2X2 (green) staining in the same taste buds; P2X2 immunoreactivity is present in **b)** mouse but not **e)** monkey or **h)** human taste buds. **c, f, i)** An adjacent section with the presence of P2X3 staining (green) in all species. Solid arrows indicate P2X receptor-positive nerve fibres. Empty arrows indicate an absence of P2X2-stained fibres and match the position of the solid arrows indicating positively stained nerve fibres in the left column. Insets in the right column show colocalisation between Tuj1 and P2X3, which is present in all samples. Colocalisation is quantified by the PDM (product of differences from the mean) image, which indicates colocalisation of Tuj1 and P2X3 or Tuj1 and P2X2 via fluorescence intensity and by pixel, *i.e.* location. PDM values are pseudo-coloured such that blue=modest colocalisation, red=higher, white=highest and black=below average. For all species, Tuj1 and P2X3 show higher to highest colocalisation throughout the taste bud. In mice, Tuj1 and P2X2 also show higher to highest colocalisation. Scale bars: 10 µm for all panels, including insets. Tuj1: β-tubulin III (a marker for neural processes); GNAT3: α-gustducin (a marker for some type II taste cells); DAPI: 4',6-diamidino-2-phenylindole (a general nuclear stain).

P2X2 and P2X3, neither human nor rhesus monkey 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.

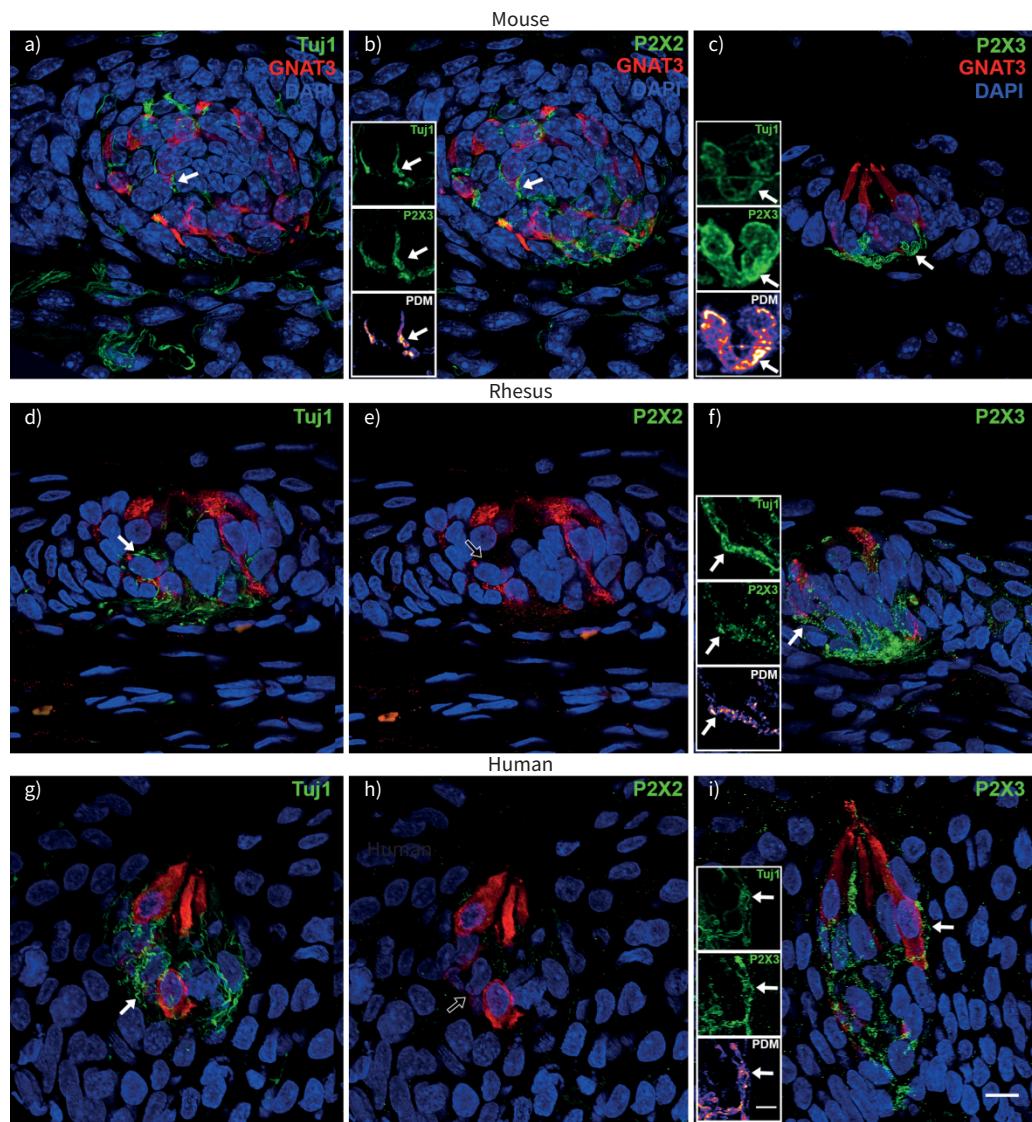


FIGURE 3 Laryngeal taste buds. P2X2 and P2X3 receptor staining in a–c) mouse, d–f) rhesus macaque monkey and g–i) human. In all panels, a subset of type II taste cells is marked by GNAT3 (red). a, d, g) Across all species, Tuj1 (green) marks both intra- and perigemmal nerve fibres in taste buds. b, e, h) P2X2 (green) staining in the same taste buds; P2X2 immunoreactivity is present in b) mouse but not e) monkey or h) human taste buds. c, f, i) An adjacent section with the presence of P2X3 staining (green) in all species. Solid arrows indicate P2X receptor-positive nerve fibres. Empty arrows indicate an absence of P2X2-stained fibres and match the position of the solid arrows indicating positively stained nerve fibres in the left column. Insets in the right column show colocalisation between Tuj1 and P2X3, which is present in all samples. Colocalisation is quantified by the PDM (product of differences from the mean) image, which indicates colocalisation of Tuj1 and P2X3 or Tuj1 and P2X2 via fluorescence intensity and by pixel, i.e. location. PDM values are pseudo-coloured such that blue=modest colocalisation, red=higher, white=highest and black=below average. For all species, Tuj1 and P2X3 show higher to highest colocalisation throughout the taste bud. In mice, Tuj1 and P2X2 also show higher to highest colocalisation. Scale bars: 10 µm for all panels, including insets. Tuj1: β-tubulin III (a marker for neural processes); GNAT3: α-gustducin (a marker for some type II taste cells); DAPI: 4',6-diamidino-2-phenylindole (a general nuclear stain).

Of the human samples, four out of 35 subjects showed strong P2X2 immunoreactivity. Immunoreactivity in one of these P2X2⁺ samples was validated using two separate P2X2 antisera (ThermoFisher and 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

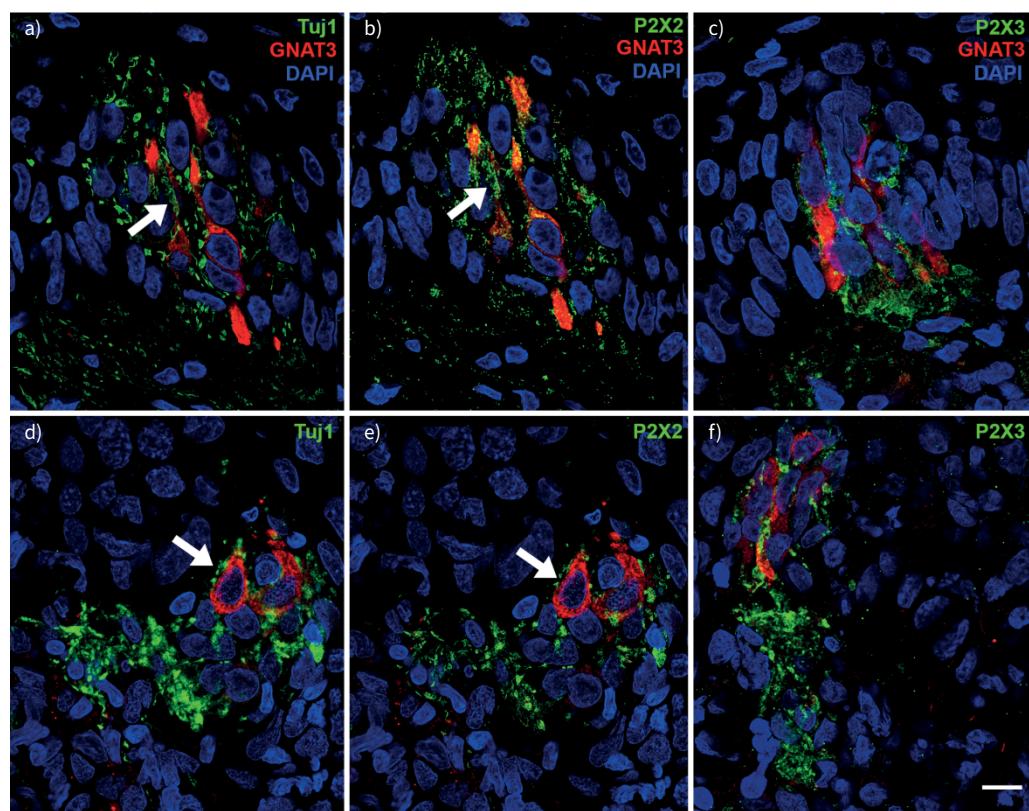


FIGURE 4 Human taste buds showing immunoreactivity for P2X2. **a–c**) Three subjects in the adult fungiform cohort from the USA group demonstrated positive staining for **b)** P2X2 as well as for **c)** P2X3 and **a)** Tuj1. **d–f**) One subject in the paediatric laryngeal group demonstrated positive staining for **e)** P2X2 as well as for **f)** P2X3 and **d)** Tuj1. Scale bar: 10 µm for all panels. Tuj1: β-tubulin III (a marker for neural processes); GNAT3: α-gustducin (a marker for some type II taste cells); DAPI: 4',6-diamidino-2-phenylindole (a general nuclear stain).

plexus across two individuals (supplementary figure 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 fibres 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%) coexpress P2X2 at levels easily detected by immunohistochemistry as used in the present study [22]. It is unlikely that a P2X2 isoform not recognised by the antibodies employed is expressed in the human taste system although that would certainly be a novel finding, as no such P2X2 splice variant is known in any human sensory system [36–38].

In humans, P2X3 antagonists such as gefapixant cause taste-related adverse events at therapeutic doses in 60–70% of patients with RCC, suggesting that P2X receptors in the gustatory nerves contain P2X3 subunits [3, 17]. 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 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 <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 the 45 mg twice-daily clinically effective dose. 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 with 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 formalised 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 treatment due to adverse events including those related to taste. It is possible that alternative routes of administration allowing for more localised 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 a 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.

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