Expression of GABAergic Receptors in Mouse Taste Receptor Cells

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Abstract

Background: Multiple excitatory neurotransmitters have been identified in the mammalian taste transduction, with few studies focused on inhibitory neurotransmitters. Since the synthetic enzyme glutamate decarboxylase (GAD) for gamma-aminobutyric acid (GABA) is expressed in a subset of mouse taste cells, we hypothesized that other components of the GABA signaling pathway are likely expressed in this system. GABA signaling is initiated by the activation of either ionotropic receptors (GABA_A and GABA_C) or metabotropic receptors (GABA_B) while it is terminated by the re-uptake of GABA through transporters (GATs).

Methodology/Principal Findings: Using reverse transcriptase-PCR (RT-PCR) analysis, we investigated the expression of different GABA signaling molecules in the mouse taste system. Taste receptor cells (TRCs) in the circumvallate papillae express multiple subunits of the GABA_A and GABA_B receptors as well as multiple GATs. Immunocytochemical analyses examined the distribution of the GABA machinery in the circumvallate papillae. Both GABA_A and GABA_B immunoreactivity were detected in the peripheral taste receptor cells. We also used transgenic mice that express green fluorescent protein (GFP) in either the Type II taste cells, which can respond to bitter, sweet or umami taste stimuli, or in the Type III GAD67 expressing taste cells. Thus, we were able to identify that GABAergic receptors are expressed in some Type II and Type III taste cells. Mouse GAT4 labeling was concentrated in the cells surrounding the taste buds with a few positively labeled TRCs at the margins of the taste buds.

Conclusions/Significance: The presence of GABAergic receptors localized on Type II and Type III taste cells suggests that GABA is likely modulating evoked taste responses in the mouse taste bud.

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Introduction

Chemosensory reception in the peripheral sensory organs of taste is influenced by neuroactive molecules that ultimately regulate signaling to and from taste buds. Taste receptor cells (TRCs), housed in taste buds, transmit signals by forming synaptic connections with sensory afferent fibers and perhaps even with other TRCs within the taste bud [1,2,3]. To date, serotonin (5-hydroxytryptamine; 5HT) and ATP [4,5] have been most definitively identified within the taste bud as neurotransmitters through anatomical localizations, physiological observations, and pharmacological data. Histochemical and immunocytochemical techniques have shown that 5HT is expressed in a subset of Type III TRCs from circumvallate and foliate papillae in mammals as well as in amphibian taste buds [6,7,8,9,10,11]. Other evidence currently exists for acetylcholine, adrenergic neurotransmission, neuropeptides, glutamate, and γ-aminobutyric acid (GABA) expression in taste buds [12,13,14,15,16,17,18,19,20,21,22]. However, the physiological roles for most of these neurotransmitters have not been well defined. Recently, it was determined that expression of glutamate decarboxylase (GAD67), an enzyme which converts glutamate into GABA [23], is expressed in a subset of Type III taste cells in mice [24,25]. While these findings revealed a useful marker to enable the identification of taste cells with chemical synapses, it also indicated that GABA is likely produced and released by these cells.

GABA is well known as an inhibitory mediator of neural transmission in the mammalian central nervous system [26,27,28]. GABA acts through two distinct types of receptors: ionotropic and metabotropic [29]. Ligand-gated GABA_A ion channels are pentameric channels comprised of a combination of subunit subtypes (α1–6, β1–3, γ1–3, δ, ε, π, θ), which determine specific pharmacological and gating properties [30,31,32]. Activation of these channels generates the fast inhibitory actions of GABA [33,34]; the slower, more modulatory actions of GABA are mediated by heterodimers of GABA_B receptors which are G-protein coupled receptors [35,36]. GABAergic transmission is terminated by the uptake of GABA through transporters...
GABA receptors in rat taste buds and provided evidence that was peeled from the connective and muscular tissue. The peeled 1 mM NaOH or 1 mM HCl). Tongues were incubated in 10 mM glucose, and 1 mM pyruvic acid. Adjusted to pH 7.4 with solution containing 0.6 mg of collagenase B (Roche, Indianapolis, IN) per milliliter of Tyrode’s solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM BAPTA, 10 mM glucose, and 1 mM pyruvic acid. Adjusted to pH 7.4 with 1 mM NaOH or 1 mM HCl). Before taste buds were removed with a capillary pipette using gentle suction and frozen for later analysis.

RNA isolation and sample analysis
Total RNA was extracted from multiple isolated taste buds from the circumvallate papillae, non-gustatory lingual epithelium, and the brain (cerebellum) using Trizol (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s protocol. Taste buds and epithelia tissue were each collected from two mice and combined for RNA isolation. Unamplified total RNA was DNase treated and then subjected to reverse transcription using Superscript III (Invitrogen) to yield cDNA. We tested the quality of the cDNA using a PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene that has constitutive and wide-spread expression [43,46]. Only samples that correctly amplified GAPDH products and lacked genomic contamination were used for subsequent experiments (see Figure S1). All materials were purchased from Fermentas (Glen Burnie, MD) unless otherwise noted.

PCR Analysis
Primers were custom made (Integrated DNA Technologies Inc., Coralville, IA) for the GABAAR subunits using the primer design tool OLIGO (Molecular Biology Insights Inc., Cascade, CO) with sequence files deposited in the Eutrept Nucleotides database (www.ncbi.nlm.nih.gov). Primers for GABA transporters (GAT), the subunits of GABAARβ and GABAARγ receptors were taken from previously published studies [47,48,49,50]. PCRs were performed in 25 μL reactions with 2 μL cDNA. Samples were run for 40 cycles at 95°C for 30 sec, specific annealing temperatures for 45 sec (see Table 1) and 72°C for 90 sec. PCR products were separated by electrophoresis on a 1% agarose gel. All PCR products were gel purified and subjected to DNA sequencing to confirm identity. Experiments were repeated three to five times with different cDNA samples to confirm the findings.

Immunocytochemistry
Mice were deeply anesthetized and then perfused transcardially with a solution of 0.025% heparin and 1% sodium nitrite followed by 4% buffered paraformaldehyde/0.1 M phosphate buffer (PB), pH 7.2. Tongues were removed and post-fixed for 1 hour in 4% paraformaldehyde/0.1 M PB, then transferred in 20% sucrose solution at 4°C overnight for cryoprotection. Tongues that were used for the GABAAR antibody experiments were immersion fixed overnight in 4% buffered paraformaldehyde/0.1 M phosphate buffer (PB), pH 7.2 at 4°C. 40 μm sections of mouse circumvallate papillae were cut, washed in 0.1 M phosphate buffered saline (PBS, pH 7.2) and then blocked for 1 hour at RT.

Primary antibodies used in this study were: rabbit polyclonal anti-GABAARγ1 (1:100, Millipore, Temecula, CA) [12], rabbit polyclonal anti-GABAARβ1 subtype (1:200) [51,52,53], rabbit polyclonal anti-GABAARβ2 subtype (1:200) [51,52], and rabbit polyclonal anti-GABAAR transporter-3 (1:100, Millipore) [54,55,56]. Sections were incubated overnight in primary antibodies at 4°C, washed with PBS and then incubated for 2 hours at RT in the dark with the secondary cy-5-anti-rabbit antibody (1:250; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Following this incubation, sections were washed and mounted on slides using Fluormount G (Southern Biotechnology Associates, Birmingham, AL) and coverslipped. Negative controls lacking primary antibody were run with each experiment. A blocking peptide for anti-GABAARγ1 (corresponding to amino acids 28-43) was pre-incubated with the antibody (1 μg peptide with 1 μg antibody) and staining was eliminated.

Sections were viewed with a three-channel laser scanning confocal with a cooled CCD camera, and Axiovision software was used for data acquisition. Images were processed using Adobe Photoshop CS software adjusting only brightness and contrast. Settings for the negative control sections
were matched to the immunoreactive sections, both for the initial collection of the images and during the final adjustment for brightness and contrast.

Antibody characterization

**Anti-GABAA1.** A rabbit polyclonal anti-GABAα1 antibody was produced against the peptide corresponding to amino acids 28–43 from mouse or rat GABAα1 subunit (Accession P18504). This antibody recognizes a single band of molecular size 51kD in western blots of brain and taste tissue [12]. In addition, pre-absorption with the control antigen eliminates staining.

**Anti-GABAB1.** A rabbit polyclonal anti-GABAB1 antibody was produced against amino acid residues 901–960 of rat GABAB1 which recognizes both GABAB1R1a and GABAB1 R1b isoforms in the brain. In COS expressing cells, this antibody reacted specifically with GABAB1 and did not cross react with GABAB2. Labeling was blocked when the antibody was pre-absorbed with the antigen [53]. Control electron microscopy experiments using a GABAB1 knock out mouse brain revealed no labeling. Additional electron microscopy comparing the labeling patterns of this antibody to another GABAB2 antibody that was raised against a different epitope [53] revealed similar sub-cellular distribution within hippocampal neurons for both antibodies.

**Anti-GABAB2.** A rabbit polyclonal anti-GABAB2 antibody was produced against amino acid residues 844–892 of GABAB2 which labeled a single band of 110 kDa in the brain. In receptor expressing COS cells, this antibody reacted specifically with GABAB2 and did not cross react with GABAα1, β1 or GABAα5 [52]. Control electron microscopy experiments using a GABAB2 knock out mouse brain revealed no labeling. Additional electron microscopy comparing the labeling patterns of this antibody to another GABAB2 antibody that was raised against a different epitope [53] revealed similar sub-cellular distribution within hippocampal neurons for both antibodies.

**Anti-GAT3.** A rabbit polyclonal anti-rat GAT3 antibody was produced against a peptide corresponding to amino acids 607–627 in the C terminus of rat GAT3. Anti-GAT3 recognizes a single band at 71kD in the mouse brain and retina and staining was eliminated after preadsorption with the cognate-epitope [54,55,56].

Results

Results from the RT-PCR analysis of the GABA receptors and transporters revealed that multiple isoforms are expressed in mouse circumvallate taste receptor cells. Negative controls consisting of samples without reverse transcriptase lacked any

| Primers | GenBank Access Number | Sequence of Primer | Amplicon size (bp) | Annealing (°C) |
|---------|-----------------------|--------------------|--------------------|---------------|
| GABAα1  | NM_010250.4           | F 5’-cgtctaaacacctttagtga-3’ | 455                | 60.0          |
|         |                       | R 5’-attatgcagctgagctatgt-3’ |                    |               |
| GABAα2  | NM_008066.3           | F 5’-cagctcaagccgagagtaa-3’ | 498                | 60.0          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GABAα3  | NM_008067.3           | F 5’-cagctcaagccgagagtaa-3’ | 511                | 46.1          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GABAα4  | NM_010251.2           | F 5’-cagctcaagccgagagtaa-3’ | 699                | 60.0          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GABAα5  | NM_176942.4           | F 5’-cagctcaagccgagagtaa-3’ | 465                | 60.0          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GABAα6  | NM_001099641.1 (variant 1) | F 5’-cagctcaagccgagagtaa-3’ | 358                | 60.0          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GABAβ1  | NM_008066.2 (variant 2) | F 5’-cagctcaagccgagagtaa-3’ | 655                | 61.4          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GABAβ2  | NM_008070.3           | F 5’-cagctcaagccgagagtaa-3’ | 514                | 58.9          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GABAβ3  | NM_008071.3 (variant 1) | F 5’-cagctcaagccgagagtaa-3’ | 415                | 55.8          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GABAβ4  | NM_01038701.1 (variant 2) | F 5’-cagctcaagccgagagtaa-3’ | 427                | 63.1          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GABAβ5  | NM_019439.3           | F 5’-cagctcaagccgagagtaa-3’ | 993                | 63.1          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GAT1    | NM_178703.3           | F 5’-cagctcaagccgagagtaa-3’ | 697                | 62.1          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GAT2    | NM_133661.3           | F 5’-cagctcaagccgagagtaa-3’ | 438                | 63.3          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GAT3    | NM_144512.2           | F 5’-cagctcaagccgagagtaa-3’ | 354                | 61.0          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |
| GAT4    | NM_172890.3           | F 5’-cagctcaagccgagagtaa-3’ | 681                | 65.0          |
|         |                       | R 5’-cagctcaagccgagagtaa-3’ |                    |               |

GABA Receptors in Taste Cells

Table 1. Primer sequences for mouse GABAα and GABAβ subunits and GABA transporters.

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Expression of GABA_A subunits

The expression of GABA_A receptor subunits in mouse taste cells and non-gustatory lingual epithelium was examined using RTPCR and immunocytochemical analysis. GABA_A shares a high degree of basic structural similarity and functional characteristics with other members belonging to the superfamily of ligand-gated ion channels [58]. We concentrated on the GABA_A with other members belonging to the superfamily of ligand-gated ion channels [58]. We concentrated on the GABA_A with other members belonging to the superfamily of ligand-gated ion channels [58].

In some of the immunocytochemistry studies, we used a transgenic mouse which has GFP linked to the IP3R3 promoter. TRCs that express IP3R3 can be identified by their fluorescence which allows us to recognize Type II taste cells that detect bitter, sweet and umami tastes but lack conventional chemical synapses [44,57]. While IP3R3 is primarily expressed in Type II cells, reports indicate that IP3R3 expression is not absolutely restricted to Type II cells [24,57]. Our characterization of these transgenic mice did not find any overlap with the IP3R3-GFP expression and synaptic markers [44]. However, since its expression in other cell types has not been rigorously characterized and electron microscopy studies have demonstrated that almost all IP3R3 expressing taste cells are Type II cells [57], for this study we are presuming that the presence of IP3R3 identifies the taste cell as a Type II cell. Experiments were also performed in the GAD67-GFP mouse which expressed GFP fluorescence in some Type III taste cells. These taste cells have been shown to express synaptic markers and presumably have conventional synapses [24,25].

Figure 1. RT-PCR analysis of the GABA_A subunits. cDNA from circumvallate TRCs (C), non-gustatory lingual epithelium (E), and brain tissue (B) were subjected to PCR analysis using gene specific primers for the GABA_A subunits 1–6. PCR products were separated by agarose gel electrophoresis. Bands were observed for all subunits in the control brain tissue at the appropriate sizes (1=455 bp, 2=498 bp, 3=511 bp, 4=699 bp, 5=465 bp, 6=358 bp) while only GABA_A1, GABA_A2, GABA_A3, GABA_A4, and GABA_A6 subunits were detected in taste buds. GABA_A1 and GABA_A2 were detected in the non-gustatory lingual epithelium. Results were repeated at least three times and example data are shown.

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Figure 2. RT-PCR analysis of the GABA_Aβ subunits. cDNA from circumvallate TRCs (C), non-gustatory lingual epithelium (E), and brain tissue (B) were subjected to PCR analysis using gene specific primers for the GABA_Aβ subunits 1–3. PCR products were separated by agarose gel electrophoresis. Bands were observed for all subunits in the control brain tissue at the appropriate sizes (1=665 bp, 2=514 bp, 3=415 bp), but only GABA_A3 was amplified in the TRCs and non-gustatory epithelium. PCR products of the appropriate size were amplified the brain control sample for 1 (665 bp), 2 (514 bp) and 3 (415 bp).

Immunocytochemical studies provide further evidence for the presence of the ionotropic GABA receptors in mouse circumvallate papillae. Staining of TRCs with the GABA_A2 antibody showed a spotty distribution of GABA immunoreactivity throughout the taste cell in a subpopulation of TRCs (Figure 3A–D) with no corresponding labeling in the negative control sample (Figure 3E–F). GABA_A immunoreactivity was detected in all taste buds that were labeled with anti-GABA_A antibodies (n = 173 taste buds from IP3R3-GFP mice and n = 64 from GAD-GFP mice). Some GABA_A2-immunoreactivity was detected on the IP3R3-GFP fluorescence in some Type III taste cells. This labeling was abolished when the blocking peptide was incubated with the primary antibody (see Figure 3E) and immunoreactivity was not readily apparent below the taste bud (see Figure 3B), so we concluded the antibody
labeling is specific and that GABA_α1 expression is not restricted to the taste bud. This agrees with our RT-PCR results that found GABA_α1 expression in both the taste buds and non-gustatory lingual epithelium. We also evaluated GABA_α1 expression in Type III cells using the GAD67-GFP to identify this sub-population of taste cells (Figure 4). Some labeling in the taste bud was detected but the strongest labeling was in the surrounding epithelial cells. We saw a few GAD67-GFP labeled TRCs that had some immunoreactivity for GABA_α1, but most fluorescent cells were not labeled.

Expression of GABA_B subunits

We also determined if circumvallate papillae TRCs express metabotropic GABA_B receptors. Native G-protein-coupled GABA_B receptors are heterodimers composed of two subunits, GABA_B1 and GABA_B2 [63,64,65]. In this heterodimer, the ligand binding domain is found only within the GABA_B1 subunit while G protein activation is entirely mediated through the GABA_B2 subunit. Physiological GABA_B receptor responses are inhibited if either subunit is nonfunctional [66,67]. We measured for the expression of both subunits using RT-PCR and immunocytochemistry. PCR products for GABA_B1 and GABA_B2 in the brain control samples were the expected sizes of 427 bp and 923 bp with no amplification in the non-gustatory lingual epithelium. Similar results were obtained with cDNA isolated from mouse circumvallate taste cells, indicating the presence of a GABA_B heterodimer in peripheral taste cells (see Figure 5).

Immunocytochemical analysis of the GABA_B1 expression in TRCs found antibody labeling was expressed in all the circumvallate taste buds that were analyzed (n = 77 taste buds from IP₃R3-GFP mice and n = 43 taste buds from GAD67-GFP mice). Some immunoreactivity overlapped with the IP₃R3-GFP expressing taste cells (Figure 6A–D, see arrowheads) while other IP₃R3 expressing cells (see arrow) were not immunoreactive for GABA_B1. Almost all labeling was restricted to the taste buds with very little to no labeling found in the surrounding epithelium. Negative controls (Figure 6E–F) lacked any non-specific labeling. Similar labeling patterns were detected with anti-GABA_B2 [53] in all of the taste buds tested (Figure 6G–L, n = 109 taste buds from IP₃R3-GFP mice and n = 72 taste buds from GAD67-GFP mice).

Figure 3. Localization of GABA_α1 receptors in the IP₃R3-GFP expressing circumvallate taste buds. A Z-stack of 4 laser scanning confocal micrographs (LSCM, 0.5 μm each, collected 1 μm apart) of circumvallate taste buds from an IP₃R3-GFP mouse labeled with an antibody directed against the GABA_α1 subunit is shown. Panel A shows the GFP fluorescence with the corresponding anti-GABA_α1 immunoreactivity of the same section shown in panel B (red labeling). A DIC bright field image of the taste buds is shown in C. An overlay of the images from A, B, and C is shown in D and demonstrates that some IP₃-GFP expressing taste cells were immunoreactive for GABA_α1 (see arrowheads for example cells). The lack of labeling when the section is incubated with primary antibody that has been pre-incubated with blocking peptide is shown in E. All staining is eliminated when the blocking peptide is present. F shows an overlay of the panel from E with the corresponding DIC image and GFP expression in the taste buds. Scale bars = 20 μm.

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Parallel experiments performed on circumvallate taste buds from the GAD67-GFP mouse revealed heavy co-localization of the GABAB receptors and the GFP fluorescence for both B1 and B2 isoforms (Figure 7). Control immunocytochemical experiments determined that anti-GABAB1 and anti-GABA B2 were co-expressed in the same TRCs in the circumvallate papillae (See Figure S2).

Expression of GAT transporters

In light of these data revealing the molecular machinery for both types of GABA receptors, we reasoned that GATs should be expressed in or near mouse taste buds if GABA receptors in taste buds are involved in GABAergic transmission. RT-PCR analysis (see Figure 8) revealed the presence of GAT1 (697 bp) and GAT4 (681 bp) in mouse circumvallate taste cells, but did not detect GAT2 (438 bp) and GAT3 (354 bp). GAT1 was amplified in the non-gustatory lingual epithelium sample while the other transporters were not (Figure 8).

In mice, there are four different GATs: slc6a1 (GAT1), slc6a12 (GAT2), slc6a13 (GAT3), and slc6a11 (GAT4) while in rats there are only three different proteins: SLC6A1 (GAT1), SLC6A13 (GAT2) and SLC6A11 (GAT3). The GAT1 sequences are the same in both species while the rat GAT2 is orthologous to mouse GAT3 and the rat GAT3 is orthologous to mouse GAT4 [40]. Preliminary immunocytochemical analyses with anti-GAT1 in the circumvallate taste buds were inconsistent. In one experiment, anti-GAT1 labeling was widespread throughout the taste bud and the surrounding non-gustatory lingual epithelium. This corresponds with the amplification of GAT1 mRNA from the non-gustatory lingual epithelium as well as the circumvallate taste buds. However, we were unable to repeat our results and did not include them in this study.

Immunocytochemical analysis of circumvallate taste buds with anti-rat GAT3 is shown in Figure 9 and corresponds to GAT4 immunoreactivity in the mouse. The most intense anti-mouse GAT4 immunoreactivity was localized to a few TRCs in the basolateral portion of the taste bud and in the cells surrounding the taste buds. Most anti-mouse GAT4 immunoreactivity was absent within the taste bud and we did not detect any overlap between anti-mouse GAT4 labeling and IP3R3-GFP expressing TRCs. Similar expression patterns for anti-mouse GAT4 were found in the GAD67-GFP expressing taste cells (Figure 10).
was some overlap between the GAD67-GFP fluorescence and the anti-mouse GAT4 labeling (see arrowheads), but this was very occasional as most anti-mouse GAT4 labeling was localized in the cells surrounding the taste buds. Due to these differences in terminology between rats and mice, our GAT4 findings parallel the report of GAT3 in rat taste cells [43], including the localization of the protein at the basolateral portion of the taste buds.

**Discussion**

This study is the first to systematically analyze the compositional expression of GABA receptors and transporters in mouse TRCs. Our results indicate that circumvallate taste buds express mRNA transcripts for some of the GABAA ionotropic subunits (a1, a2, a3, a4, a6 and a5), both metabotropic subtypes (B1 and B2), and two GABA transporters (GAT1 and GAT4). Since functional GABAA receptors must express both α and β subunits [63,64] and GABAB receptors require both B1 and B2 isoforms to function [66,67], this study confirms that the mouse peripheral taste system has all the necessary components to utilize multiple GABAergic signaling mechanisms. GABA activity can also be mediated by the ionotropic GABAC receptor, a third type of pharmacologically distinct GABA receptor. So far, studies have primarily linked the expression of GABAC receptors to regions of the visual system [68,69] and the hippocampus [70]. Nonetheless, we ran experiments to see if GABAC receptor expression was apparent in TRCs but RT-PCR analysis of mRNA revealed no transcripts for this particular receptor isoform (data not shown).

We followed up our RT-PCR analysis with immunocytochemistry to determine if the GABA receptor and transporter proteins were also expressed in the peripheral taste system. These data would further support the hypothesis that GABA is an important neurotransmitter in the taste bud. Immunocytochemical analysis revealed the protein expression patterns for ionotropic and metabotropic GABA receptors as well as a GABA transporter. Taken with the report that GAD67 is expressed in a subset of mouse TRCs [24], these data suggest that GABA likely contributes to the formation or modulation of output signals from the taste bud.
the mouse taste bud. Recent evidence has also determined that GABA as well as known modulators of GABA activity, significantly affect the physiological properties of rat taste cells [12] which provides further support for the hypothesis that GABA is a physiologically relevant neurotransmitter in the mammalian taste system.

Our immunostaining for GABAA₁ differs from results in the rat, which found GABAA₁ immunoreactivity was restricted to a small subset of TRCs and was not expressed in the surrounding epithelium [12]. Our experiments revealed labeling in the cells surrounding the taste buds as well as the labeling in the taste cells. These results were confirmed with RT-PCR analysis which identified mRNA for GABAA₁ in both taste buds and non-gustatory lingual epithelium. Use of the blocking peptide for anti-GABAA₁ eliminated this staining (Figure 3E-F) which confirms that this staining pattern is specific for GABAA₁. Since we used the same antibody that was used in the Cao et al. [12] study, these differences may be due to species differences or differences in fixation. Cao et al. [12] reported using immersion fixation in either 4% paraformaldehyde or Bouin’s fixative with subsequent tissue embedding in paraffin for sectioning. We transcardially perfused the mice with 4% paraformaldehyde/0.1 M PB and embedded the tissue in OCT. We confirmed our findings using an antibody that recognizes all six GABAA receptor subunits (data not shown) and obtained similar results for both antibodies. This labeling pattern for GABAA₁ in the surrounding epithelia suggests that GABA may have additional functions outside the peripheral taste system. Earlier studies have reported the presence of GABAergic receptors, specifically GABAA receptors, in multiple lung epithelial cell types [71,72,73]. Within this system, these

Figure 7. Localization of GABAB receptors in the circumvallate taste buds from a GAD67-GFP mouse. A Z-stack of 4 LSCMs (0.5 μm each, collected 1 μm apart) from mouse circumvallate taste buds with GFP expression in the GAD67-expressing taste cells is shown in panel A. The corresponding labeling with anti-GABAB₁ is shown in panel B and the DIC image shown in C. D. When images were combined, many GAD67-GFP expressing TRCs were labeled with anti-GABA B₁ (see arrowheads for example cells). Parallel results using anti-GABA B₂ are shown in E–H. Negative controls are the same as those shown in Figure 6. Scale bars = 20 μm.
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GABA receptors play an essential role in regulating mucus production [72]. Their expression in the epithelia cells surrounding the taste buds suggests that these receptors may have similar roles in the tongue. GABA receptors have also been shown to have trophic roles in neuronal maturation [74] and it is possible they are exerting similar effects in the maturation of the peripheral taste receptor cells. Further studies are needed to address these questions.

Specific GABA_A1 immunoreactivity was detected along the plasma membrane of some IP3R3-GFP expressing Type II taste cells which suggests that these cells are sensitive to GABA release. Since GAD67 expression has been localized to Type III taste cells in mice [24] and both Type III and gustducin expressing taste cells in rat [12], the presence of the GABA_A receptor on some Type II cells makes them a potential target for modulation by other taste cell populations in the taste bud. Further studies are needed to address these questions.

Growing evidence suggests that the final stimulus-evoked output signal from taste cells can be influenced in a paracrine manner [2,12,19] and our data support a role for GABA_B in these processes.

Using RT-PCR analysis, we also detected GAT1 and GAT4 expression in mouse circumvallate taste cells (Figure 8). Immunohistochemical analysis using an antibody raised against GAT-1 was inconsistent and was not included. However, our mouse GAT4 immunolabeling was very consistent and was primarily restricted to the basolateral portion of a few taste cells and the cells immediately next to the taste buds with very little to no labeling in the apical portion of the taste bud. Therefore, mouse GAT4 transporters are primarily localized at the site of most synaptic activity and where one would predict most GABA signaling would occur. It has been reported that GAT2 and GAT3 are expressed in taste cells with no discernable expression in the surrounding cells. Only a subpopulation of taste cells exhibited antibody labeling and negative controls were blank, indicating that the antibody labeling was specific. In addition, multiple studies in other cell types have validated that these antibodies are specific for GABA_B1 and GABA_B2 [51,52,53]. Some overlap with IP3R3-GFP expressing TRCs was detected but most anti-GABA_B1/B2 labeling was present in taste cells that do not express IP3R3 and are not Type II cells. Since GABA_B receptors inhibit the activity of their target cells on a relatively slow time scale [75], this labeling pattern suggests that stimulus-evoked GABA release likely causes a longer term inhibition of a specific subset of target cells, including some Type II cells. In the rat, Cao et al [12] reported no overlap with the anti-GABA_B1 and anti-gustducin immunoreactivity which is another Type II cell marker [76,77]. Since gustducin is found in a subset of PLC beta/IRP3 expressing taste cells [78], we predict that the GABA_B2 labeling is likely present in the Type II taste cells that do not express gustducin. We also determined that most, but not all, GAD67-GFP expressing Type III taste cells were labeled with the anti-gustducin antibodies, indicating that GABA can function in an autocrine manner for this sub-population of Type III cells.

Figure 8. RT-PCR analysis of the GABA transporters. cDNA from circumvallate TRCs (C), non-gustatory lingual epithelium (E), and brain tissue (B) were subjected to PCR analysis using gene specific primers for the GAT transporters 1–4. PCR products were amplified for all subunits in the control brain tissue at the appropriate sizes (1–697 bp, 2–438 bp, 3–354 bp, 4–681 bp) while only GAT1 and GAT4 were detected in the taste cells. GAT1 was also detected in the non-gustatory lingual epithelium. Results were repeated at least three times and example data are shown. doi:10.1371/journal.pone.0013639.g008

Figure 9. Mouse GAT4 immunoreactivity in mouse IP3R3-GFP expressing circumvallate papillae. A Z-stack of 5 LSCMs (0.5 μm each, collected 1 μm apart) of mouse circumvallate taste buds with GFP expression identifying the IP3R3-expressing taste cells is shown in panel A. The corresponding image of the taste buds labeled with an anti-rat GAT3 (mouse GAT-4) antibody is shown in B with the corresponding DIC image shown in C. D, An overlay of A, B, and C illustrates that most labeling is localized in the surrounding cells near the basolateral portion of the taste bud and in a few cells in basolateral portion of the taste bud. The lack of labeling in the negative control is shown in E. An overlay of E, the corresponding GFP expression and DIC image is shown in F. Scale bars = 20 μm. doi:10.1371/journal.pone.0013639.g009
in the rat taste system, with GAT3 expressed primarily at the margin of the taste bud and in some TRCs, while GAT2 had more widespread expression [43]. In mice, our RT-PCR analysis did not identify GAT2 expression but did identify the presence of GAT1 which was not reported in rat [43]. It is likely that species-specific gene expression accounts for these differences. Since rat GAT3 is orthologous to mouse GAT4, our finding of mouse GAT4 expression parallels the expression pattern previously reported for GAT3 in the rat [43]. Thus, across mammalian species, these GAT transporters are localized primarily in the cells lining the basolateral portion of the taste bud, presumably where they function to remove GABA that has been released from a synapse. The GAT1 expression in non-gustatory lingual epithelium was found using RT-PCR analysis while GAT4 was not detected. This is likely due to our method of sample collection. We isolated total RNA from epithelial tissue that had been separated from the underlying muscle and was located in an area anterior to the circumvallate papillae where no taste buds are present. While our RT-PCR data indicates that GAT1 and GABAβ subunits are expressed in these epithelial cells, mouse GAT4 was not detected. Based on these data, we conclude that GAT4 is not widely expressed throughout the lingual epithelium but is preferentially associated with the taste buds. Therefore, GAT4 was not detectable in our non-lingual gustatory epithelium sample because there were no taste buds near the area where our epithelial sample was collected. While we removed the taste buds from the surrounding epithelium to isolate taste bud RNA for the RT-PCR analysis, it is possible that some surrounding epithelial cells were also collected. Since we detected very few GAT4 immuno-reactive taste cells with the anti-mouse GAT4 antibody but were able to detect GAT4 using RT-PCR, it is possible that our taste bud sample was contaminated with a few of the cells that surround the taste buds.

The evidence for the role of GABA as a neurotransmitter in the taste system is accumulating. A study of GAD67-GFP knock-in mice found a strong GFP signal in taste cells which was confirmed using immunocytochemical analysis [79]. Physiological actions of GABA activity in GABA A and GABAB receptors have been successfully recorded in rat taste cells [12]. Our data establish the presence of both ionotropic and metabotropic GABA receptors in the mouse taste system, which may play a critical role in its responsiveness to multiple stimuli. Moreover, a GABA reuptake system which is critical to the physiological function of a GABAergic signaling pathway is expressed in the basolateral portion of the taste buds, the primary site of interaction between taste receptor cells and their post-synaptic targets. All of these data are consistent with a role for GABA as one of the neurotransmitters that regulates signaling to and from taste cells.

Supporting Information

**Figure S1** Amplification of GAPDH was used to measure for any genomic DNA contamination. All RNA from the brain and taste samples were DNAse treated and analyzed to ensure that no contaminating genomic DNA was present before being used for PCR analysis. The panel to the left illustrates the lack of GAPDH amplification in a sample that did not have any genomic DNA while the panel to the right reveals the presence of contaminating DNA.
genomic DNA. When genomic DNA was detected, the sample was discarded and not included in the analysis.

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**Figure S2** GABA B1 and GABA B2 are co-expressed in the same taste cells. Sections from mouse circumvallate papillae were subjected to double-labeling using anti-GABA B1 (A) and anti-GABA B2 (B). C, An overlay of the images in A and B revealed similar labeling patterns for each of these antibodies.

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### Author Contributions

Conceived and designed the experiments: MRS MRR KAC KFM. Performed the experiments: MRS MRR KAC KFM. Analyzed the data: MRR KFM. Contributed reagents/materials/analysis tools: AK. Wrote the paper: MRS KFM.
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