A paracrine signaling role for serotonin in rat taste buds: expression and localization of serotonin receptor subtypes

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Kaya, Namik, Tiansheng Shen, Shao-gang Lu, Fang-li Zhao, and Scott Herness. A paracrine signaling role for serotonin in rat taste buds: expression and localization of serotonin receptor subtypes. Am J Physiol Regul Integr Comp Physiol 286: R649–R658, 2004. First published January 8, 2004; 10.1152/ajpregu.00572.2003.—Recent advances in peripheral taste physiology now suggest that the classic linear view of information processing within the taste bud is inadequate and that paracrine processing, although underemphasized, may be an essential feature of peripheral gustatory transduction. Taste receptor cells (TRCs) express multiple neurotransmitters of unknown function that could potentially participate in a paracrine role. Serotonin is expressed in a subset of TRCs with afferent synapses; additionally, TRCs respond physiologically to serotonin. This study explored the expression and cellular localization of serotonin receptor subtypes in TRCs as a possible route of paracrine communication. RT-PCR was performed on RNA extracted from rat posterior taste buds with 14 primer sets representing 5-HT1A, 5-HT1B, and 5-HT1D receptor subtype families. Data suggest that 5-HT1A and 5-HT1D receptors are expressed in taste buds. Immunocytochemistry with a 5-HT1A-specific antibody demonstrated that subsets of TRCs were immunopositive for 5-HT1A. With the use of double-labeling, serotonin- and 5-HT1A-immunopositive cells were observed exclusively in nonoverlapping populations. On the other hand, 5-HT3-immunopositive taste receptor cells were not observed. This observation, combined with other data, suggests that 5-HT3 is expressed in postsynaptic neural elements within the bud. We hypothesize that 5-HT release from TRCs activates postsynaptic 5-HT3 receptors on afferent nerve fibers and, via a paracrine route, inhibits neighboring TRCs via 5-HT1A receptors. The role of the 5-HT1A-expressing TRC within the taste bud remains to be explored.

IN VERTEBRATES, TASTE RECEPTOR CELLS (TRCs) are organized into taste buds, cloistered structures that place individual TRCs in close apposition with one another. This unique morphology may play an essential role in gustatory function as the substrate for requisite cell-to-cell communication among TRCs during gustatory stimulation (e.g., 22). Several lines of evidence now suggest that the classic linear concept of information processing within the taste bud, i.e., a single TRC responding to a gustatory stimulus by eliciting an action potential and the same individual TRC subsequently releasing neurotransmitter onto an afferent nerve fiber, is outdated. Only a minority of TRCs has afferent synapses, often referred to as the type III cell (e.g., 45), yet many more TRCs are responsive to taste stimuli (7, 19) and most can elicit action potentials (10). Additionally, TRCs expressing the taste-specific G protein gustducin, thought to be important in bitter and sweet transduction cascades, or the T2R family of bitter receptors, are subsets of type II cells (1, 5, 66), cells that lack synapses with the afferent nerve fiber. Thus, as these TRCs are not directly connected with the afferent nerve, they must, when stimulated by tastants, utilize indirect mechanisms to produce an afferent neural discharge. While such communication could include either electrical or chemical routes, electrical communication through gap junctions, although plausible, has yet to be definitively demonstrated in mammalian taste buds. On the other hand, multiple routes of chemical communication among TRCs have recently been reported. It has recently been documented that TRCs respond to neurotransmitters such as 5-HT (11, 15, 20, 21, 31, 30), norepinephrine (NE; 23 24), ACh (42, 49), and the neuropeptide CCK (25). Hence, in addition to transmitting information to the central nervous system (CNS) via afferent nerve fibers, neurotransmitters in taste buds may function to signal neighboring TRCs.

To date, serotonin is the best studied neurotransmitter within the taste bud with extant anatomic, physiological, and pharmacological data. Serotonin is expressed in a subset of type III TRC taste buds of circumvallate and foliate papillae of mouse, rat, rabbit, and monkey (16, 36, 46, 51, 57, 67). Given its putative role as a transmitter with the afferent fiber, reports that TRCs respond to serotonergic stimulation were unexpected. In rat posterior TRCs, inhibition of a calcium-activated potassium current (20) and of voltage-dependent sodium current (21) were observed. Both were mimicked by agonists of the 5-HT1A receptor subtype. These data imply that serotonin may also play a paracrine role in information processing within the taste bud.

Thus, examining expression and cellular localization of serotonin receptor subtypes in the taste bud is essential not only to understand serotonergic transmission but also to firmly establish paracrine neurotransmission within the bud. Given that serotonin receptors are comprised of seven major families with at least 30 distinct members (2, 27, 61), the task of characterizing their expression within the bud is particularly complex. With the use of the techniques of RT-PCR and immunocytochemistry, this communication presents evidence that both 5-HT1A and 5-HT3 subtypes are expressed within the taste bud and that 5-HT and 5-HT1A are expressed in different subpopulations of TRCs, confirming a paracrine pathway for serotonergic processing, and presents suggestive evidence that 5-HT3 is most likely confined to the postsynaptic neural elements within the bud.

MATERIALS AND METHODS

Anesthesia and tissue/taste bud preparation. Experiments were performed on adult male Sprague-Dawley rats. All procedures were...
approved by the University’s Laboratory Animal Care and Use Committee and adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were brought to a surgical level of anesthesia by intraperitoneal injection of a 0.9 ml/100 g body wt ketamine (91 mg/ml; Fort Dodge Laboratories)-acepromazine (0.09 mg/ml; Butler Laboratories) mixture before death and excision of foliate and circumvallate papillae.

For PCR analysis, whole taste buds were isolated from posterior taste papillae by enzymatic dissociation. Approximately 1 ml of enzyme solution was injected submandibularly using a 30-gauge needle. Enzyme solution consisted of 2.0 mg/ml elastase and 2.0 mg/ml dispase dissolved in mammalian physiological saline (MaPS; 120 mM NaCl, 20 mM KCl, 10 mM HEPES, 2 mM BAPTA, pH 7.4). After injection, excised lingual tissue was incubated in this injection buffer for 20–30 min at room temperature or at 37°C. The entire posterior tissue was peeled away under a dissecting microscope. Lingual epithelium was gently agitated in fresh MaPS, and dissociated cells and taste buds were allowed to settle in a chamber on an inverted microscope. With the use of a suction pipette and an inverted microscope, individual taste buds were harvested and collected in a 1.5-ml microtube containing 100–200 μl of TRIzol reagent (Invitrogen-Life Technologies, Carlsbad, CA). Taste buds from several animals were pooled before RNA extraction.

For immunocytochemistry, excised circumvallate or foliate papillae were quickly dissected and fixed by immersion in 4% paraformaldehyde for 5 h at 4°C and then cryoprotected in 30% sucrose in PBS and frozen in methylbutane and dry ice before sectioning. For 5-HT immunocytochemistry, cells were preloaded with serotonin precursor 5-hydroxytryptophan (80 mg/kg, Sigma, St. Louis, MO) by intraperitoneal injection of animals 1 h before death (cf. Refs. 36, 67). All immunocytochemical experiments were performed on tissue obtained from at least three different animals.

**RT-PCR reactions.** Total RNA was extracted from experimental or control tissue using a standard protocol involving cellular disruption in a guanidinium-based buffer followed by organic extraction in phenol-chloroform mixture and alcohol precipitation. Commercial RNA extraction kits, TRIzol (Invitrogen-Life Technologies) or Total RNA Isolation Kit (AMBIon, Austin, TX), were employed according to manufacturer’s instructions. Fifty to 200 isolated taste buds served as starting material for isolation to total RNA from gustatory tissue. Pooled taste buds were subsequently disrupted. Adult whole rat brain was included as a positive control tissue. Control tissues were carefully but quickly removed from the animal, cleaned of adhering tissues, and immediately either snap-frozen and stored at −80°C or homogenized in the kit’s denaturing buffer.

Total RNA (0.1–1 μg) was digested with amplification grade DNase I for 15 min at room temperature (Invitrogen). DNase I activity was inactivated by the addition of 1 μl of 25 mM EDTA solution and subsequent incubation at 65°C for 10 min. RT-PCR was performed using OneStep RT-PCR Kit (QiAGEN) according to the manufacturer’s instructions. Fifty to 100 nanograms of RNA (DNase I digested or undigested as control) served as template for RT-PCR reactions. Additional RNase inhibitors were also employed. For each tube, RnAGuard RNase Inhibitor (Human Placenta; 20–40 U/μl; Amer sham Biosciences) and Prime RNase Inhibitor (30 U/μl; Eppendorf Scientific) were added to the reaction at the concentration of 15 U for each inhibitor.

The targeted serotonin receptor subtypes were the 5-HT₁ₐ, 5-HT₁₆, 5-HT₁₀, 5-HT₁₂, 5-HT₁₇, 5-HT₂₅, 5-HT₂₆, 5-HT₃C, 5-HT₅, 5-HT₄, 5-HT₅₆, 5-HT₆, and 5-HT₇ receptors. The sequences for these 14 primer sets were based either on previously published primer sequences used on rat tissue or were designed from the published sequences for each of the serotonin receptor subtypes expressed in rat tissue. The design of the primer sequences was optimized with regard to primer dimer formation, false priming sites, and their efficiency to anneal to the sites within the target. The total RNA isolation procedure from pure circumvallate and foliate papillae taste bud populations included a DNase treatment to destroy all contaminating DNA likely to present in these preparations, thus minimizing genomic contamination in the PCR reactions. In routinely performed control experiments, where the PCR reaction was carried out on RNA without including the reverse transcription step (RT−), no observable PCR product was produced, confirming that total RNA was free from genomic DNA. The functionality and specificity of the primers were controlled by reverse transcribed total RNA isolated from control tissue using gene-specific sense and antisense primers. Primer sets are described in Table 1. RT was performed at 50°C for 30 min. PCR cycle consisted of an initial step of 95°C for 15 min to activate the HotStarTaq enzyme and 35–50 subsequent cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min and a final extension step of 72°C for 10 min. For RT− controls, the RT step was omitted during the RT-PCR.

PCR products were separated by gel electrophoresis in a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide, observed under UV light, and photographed. To verify the specificity of the bands, PCR products were purified by adsorption of double-stranded DNA on a silica-based membrane and subsequent elution in warm Tris-EDTA buffer (Concert Rapid PCR Purification System; Invitrogen). Products were either sequenced directly or cloned in pCR 2.1 vector (Original TA Cloning Kit; Invitrogen) and sequenced at the Plant Biotechnology Genomics Facility at The Ohio State University. Identity of the bands was confirmed with BLAST search at the National Center for Biotechnology Information.

**Conventional immunocytochemistry protocol.** Primary antisera were obtained to serotonin (Oncogene Research Products, Cambridge, MA), to the 5-HT₄A receptor subtype (DiaSorin, Stillwater, MN), and to the 5-HT₅ receptor subtype (Oncogene Research Products, Cambridge, MA) from commercial sources and were run at optimized dilutions (subsequently described).

Fixed frozen tissue blocks containing either circumvallate or foliate papillae were cryosectioned at 8-μm thickness and then collected onto Fisher Superfrost Plus slides. Sections containing taste buds were rinsed in 0.1 M PBS (pH 7.4). Before immunocytochemistry, sections were incubated with a solution of 0.5% hydrogen peroxide in methanol for 30 min to eliminate endogenous peroxidase activity and subsequently washed in three changes of PBS at 5 min each. To reduce nonspecific antibody binding, sections were then incubated for 1 h at room temperature in blocking solution containing 10% normal goat serum and 0.3% Triton X in PBS. Sections were incubated in primary antisera specific at the specified dilution. Slides were housed in a closed moist chamber for 36 h at 4°C. The sections were rinsed in PBS (3×10 min), incubated for 1 h (room temperature) in secondary biotinylated goat-anti-rabbit IgG diluted 1:800, rinsed in PBS, and then incubated for 1 h in avidin-biotin-peroxidase complex (Vectastain “Elite ABC” kit, Vector Laboratories) at a dilution of 1:50. Tissue-bound peroxidase was visualized by incubating sections in a freshly prepared solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride (in 0.05 M Tris buffer, pH 7.6) containing 0.01% hydrogen peroxide for 3 min. Subsequently, sections were rinsed in distilled water, dehydrated through a series of ethanol, cleared in xylene, and coverslipped with Permount.

A similar protocol was employed for immunocytochemical examination using epifluorescence. The peroxidase blocking step was omitted. After incubation in the primary antibody, sections were rinsed in PBS and then detected using Cy3-conjugated goat anti-rabbit IgG serum (1:800, room temperature, 1.5 h, in the dark). Slides were mounted in Cytosol 60 (Electron Microscopy Sciences, Washington, PA).

Rat hippocampus was used as positive control tissue for 5-HT₄A immunoreactivity. Rat cortex served as a positive control tissue for 5-HT₅A immunoreactivity. Immunocytochemical experiments using the 5-HT₄A and the 5-HT₅A antibody were also performed using the tyramine signal amplification (TSA) method for the purposes of double labeling and increased sensitivity, respectively.

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Table 1. Summary of gene specific primer sets employed for varying serotonin receptor subtypes

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Primer Sequences (5'-sense-3'; 5'-antisense-3')</th>
<th>Product Size, bp</th>
<th>Position, bp</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
<td>5'-AGC TTA GGA ACT TCG TCG GCA-3' 5'-CAG AGG AGG GTC TCT TTC TTG-3'</td>
<td>200</td>
<td>721–741</td>
<td>GenBank NM_012585</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2B&lt;/sub&gt;</td>
<td>5'-GTG GAC TTT GCA ATG GCA TTT-3' 5'-AGC AGG AAT ATA AAG AA-3'</td>
<td>200</td>
<td>1644–1662</td>
<td>Chen et al. (9)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2D&lt;/sub&gt;</td>
<td>5'-AAC AAA AAA CCG TGG TGC AC-3' 5'-TGT GAG TGG TGT CAG AGG CT-3'</td>
<td>367</td>
<td>1824–1843</td>
<td>Chen et al. (9)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;6&lt;/sub&gt;</td>
<td>5'-AAC TCA GGG AAT ATT AAC-3' 5'-TGC TAC CTA CCG CTT-3'</td>
<td>198</td>
<td>600–619</td>
<td>Demchyshyn et al. (13)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;6&lt;/sub&gt;</td>
<td>5'-AGC AGA GGA TAA GTG TTG TT-3' 5'-GTC TGG TTT TCA ATG GCA T-3'</td>
<td>350</td>
<td>683–702</td>
<td>Lovenberg et al. (41)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
<td>5'-GAT GAT TGC TTT TAC TTT TAC-3' 5'-AGC CTT AAC GGA CTA CTC-3'</td>
<td>410</td>
<td>1015–1033</td>
<td>Liu et al. (40)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2A&lt;/sub&gt;</td>
<td>5'-ACG GCC TTC TGC AGA ACG AAC-3' 5'-TGT TAG TGG TTG TG-3'</td>
<td>318</td>
<td>826–844</td>
<td>Kursar et al. (38)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2C&lt;/sub&gt;</td>
<td>5'-GTA GCA ATA GAT CTT ATT GAC-3' 5'-TTG AGC CAC ATG TGC TTT-3'</td>
<td>170</td>
<td>1150–1173</td>
<td>Julius et al. (35)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;3&lt;/sub&gt;</td>
<td>5'-CGT CTG GCA GGA GGT TGG TCG AG-3' 5'-GTC TGA CAT GAT GAG GAA-3'</td>
<td>248</td>
<td>585–608</td>
<td>Isenberg et al. (32)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;4&lt;/sub&gt;</td>
<td>5'-GAC GGC AAC ACC AGC ACC-3' 5'-GTC TGA AGC ACG CAC GTG GTG TT-3'</td>
<td>285</td>
<td>813–833</td>
<td>Gerald et al. (18)</td>
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<tr>
<td>5-HT&lt;sub&gt;5&lt;/sub&gt;</td>
<td>5'-GGC AAA ACA TCT CCC CAT AA-3' 5'-GGG AAG AAG AAG CAC AAA-3'</td>
<td>115</td>
<td>281–300</td>
<td>Erlander et al. (14)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;6&lt;/sub&gt;</td>
<td>5'-AAA GTC AGG ACT AGC ACT CG-3' 5'-GGC GGG GGG GGG GGG CCG-3'</td>
<td>563</td>
<td>1035–1054</td>
<td>Erlander et al. (14)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;7&lt;/sub&gt;</td>
<td>5'-ATG CTC TGG CAT GGT ACG-3' 5'-GAG AAC AGC GAG GCG CTT-3'</td>
<td>370</td>
<td>1578–1597</td>
<td>Raat et al. (53)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;9&lt;/sub&gt;</td>
<td>5'-TAG CGG TCC AGG CTG ATG A-3' 5'-GTC TGG TCT TCA AGG GGC-3'</td>
<td>674</td>
<td>325–342</td>
<td>McLaughlin et al. (43)</td>
</tr>
<tr>
<td>Gustducin</td>
<td>5'-TGG TGC TGC CTA GAT TAC CTA-3' 5'-TTG AGC ACG CAG GGC TCT-3'</td>
<td>325</td>
<td>1058–1076</td>
<td>Raat et al. (54)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AGT GGT GGT GGA GTG TGA TGC-3' 5'-GGT GTG CAG GAG GCG TGG TCA-3'</td>
<td>197</td>
<td>711–730</td>
<td>Zhao et al. (68)</td>
</tr>
</tbody>
</table>

For each receptor subtype, 5'-sense-3' is shown above 5'-antisense-3' primer sequence.

TSA-amplified immunocytochemistry protocol. Experiments using either 5-HT<sub>1A</sub> or 5-HT<sub>3</sub> primary antibodies were also conducted using TSA-amplification method (65). Fixed frozen tissue blocks containing either circumvallate or foliate papillae were cryosectioned at 8-μm thickness. Sections containing taste buds were rinsed in 0.01 M PBS (pH 7.4). Before immunocytochemistry, sections were incubated with a solution of 0.5% hydrogen peroxide in methanol for 30 min to eliminate endogenous peroxidase activity and subsequently washed in three changes of PBS at 5 min each. To reduce nonspecific antibody binding, sections then were incubated for 1 h at room temperature in blocking solution containing 10% normal goat serum and 0.3% Triton X in PBS. Antiserum to 5-HT<sub>1A</sub> at a dilution of 1:200 or 5-HT<sub>3</sub> at a dilution of 1:50 was applied to the sections and the slides were housed in a closed moist chamber for 36 h at 4°C. Sections were rinsed in PBS (3× 10 min) and then incubated with biotin-conjugated goat anti-rabbit Fab fragment (1:1,000, room temperature, 1 h). Sections were rinsed in three changes of TNT (0.1 M Tris, 0.15 M NaCl, 0.05% Tween-20, pH 7.5) for 5 min each and incubated for 30 min at room temperature with TMB buffer (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.6, with 0.5% blocking powder provided in the TSA kit; indirect NEL 700A, NEN Life Science Products, Boston, MA). Excess TMB buffer was blotted, and sections were incubated with horseradish peroxidase (HRP)-conjugated streptavidin (1:300 in TMB buffer, room temperature, 30 min, in the dark, provided in the TSA kit). Sections were rinsed in TNT and then incubated with biotinyl tyramide (1:50 in amplification diluent, provided in the TSA kit) for 10 min (room temperature, in the dark). After being washed in PBS, immunoreactivity was visualized with streptavidin-fluorescein (1:400; Jackson ImmunoResearch Labs, West Grove, PA). Slides were mounted in Cytoseal 60 (Electron Microscopy Sciences) and observed under a Nikon microscope equipped with epifluorescence. In control experiments, omission of either primary antibody or secondary antibody eliminated staining.

Double-labeling immunocytochemistry protocol. An indirect immunofluorescence double-labeling protocol was modified to allow localization of two antigens in the same preparation when both primary antibodies are raised in the same species. This protocol relies on a combination of the methods in previously published papers and involves using TSA with a Fab fragment secondary antibody for detection of the first primary antibody (3, 29, 55, 62). With the use of TSA, the first primary antibody can be used at very low concentration so that the antigen can only be detected by TSA but not by a conventional fluorophore-conjugated secondary antibody, which prevents the cross-reaction between the first primary antibody and the second secondary antibody (referred to as interference I), while the use of a Fab fragment instead of the whole IgG molecule or F(ab)_2 fragment as the second antibody prevents the capture of the second primary antibody by the first secondary antibody (interference II). Therefore, this modified protocol prevents cross-reactions between the primary and the unintended secondary antibodies.

Two control experiments were performed to ensure this cross-reactivity did not occur. To control for interference I (the second secondary with the first primary), after incubation with the first primary, sections are reacted using the second secondary antibody and the standard (non-TSA) protocol (Cy3-conjugated goat anti-rabbit IgG serum; 1:800, room temperature, 1 h 30 min). No fluorescence was observed, indicating that the first primary was too dilute to be detected with unamplified means. To control for interference II (the first secondary with the second primary), a substitute second primary (also from rabbit) whose antigen is not expressed in lingual tissue was used, and the first primary was detected by the usual conditions (immunohistochemistry for 1 h, rinsed, TSA treatment). No fluorescence was observed, indicating that interference II was prevented by the combined procedures.
lingual epithelium. If there were interference binding, the second secondary would be visualized. This was not evident in control experiments. Parallel experiments were performed in inverted order (i.e., switching primary 1 and primary 2) and produced equivalent results.

Fixed frozen 8-μm sections containing taste buds were rinsed in 0.01 M PBS (pH 7.4). To reduce nonspecific antibody binding, the sections then were incubated for 1 h at room temperature in blocking solution containing 10% normal goat serum and 0.3% Triton X-100 in PBS. Primary antiserum directed against 5-HT1A (Diasorin) was applied at a dilution of 1:200, and the slides were housed in a closed moist chamber for 36 h at 4°C. At this dilution, the 5-HT1A antigen could not be detected by Cy3-conjugated goat anti-rabbit IgG serum (1:800, room temperature, 1.5 h) in the conventional immunofluorescence method but was still detectable after TSA.

The sections were rinsed in PBS (3 × 10 min) and then incubated with biotin-conjugated goat anti rabbit Fab fragment (1:1,000, room temperature, 1 h). After being rinsed in TNT (3 × 5 min each), the sections were incubated for 30 min at room temperature with TNB buffer, excess TNB buffer was blotted, and then the sections were incubated with HRP-conjugated streptavidin (1:500 in TMB buffer, room temperature, 30 min, in the dark, provided in the TSA kit, indirect NEL 700A, NEN Life Science Products, Boston, MA). The sections were rinsed in TNT and then incubated with biotinyl tyramide (1:50 in amplification diluent, provided in the TSA kit) for 10 min (room temperature, in the dark). After being washed in TNT, visualization of 5-HT1A immunoreactivity was observed with streptavidin-fluorescein (1:400; Jackson ImmunoResearch Labs) in PBS, which was applied for 1 h at room temperature in the dark. After being rinsed in PBS, the sections were incubated for 36 h at 4°C in the dark with the second primary antibody, rabbit polyclonal anti-5-HT antibody (Oncogene Research Products), at a dilution of 1:1,000 and then detected using Cy3-conjugated goat anti-rabbit IgG serum (1:800, room temperature, 1.5 h, in the dark). Slides were mounted in Cytoseal 60 (Electron Microscopy Sciences). To control for the ability of Cy3-conjugated secondary antibodies to detect the first primary antiserum (anti-5-HT1A), after the TSA and streptavidin steps, Cy3-conjugated secondary antibodies diluted 1:800 in PBS were applied for 1.5 h at room temperature. It was observed that with omission of 5-HT primary antibody, no signal could be detected. Slides were visualized on a Nikon microscope equipped with epifluorescence.

RESULTS

Analysis of serotonin receptor expression in circumvallate and foliate taste papillae by RT-PCR. Detection of serotonin receptor subtypes in taste buds isolated from circumvallate and foliate papillae was investigated using the technique of RT-PCR. To ensure that putative expression of receptor subtypes would be limited to taste buds, rather than surrounding lingual epithelium, taste buds were enzymatically dissociated from surrounding epithelium and individually collected under visual inspection. Thus this procedure limits cellular elements containing RNA to TRCs, basal cells within the bud, and postsynaptic endings of intragemmal nerve fibers. As each taste bud was harvested under visual inspection, starting material was assured of being free from surrounding epithelial cells. Total RNA was isolated from batches of approximately 50 to 100 isolated and pooled taste buds. To minimize genomic contamination, RNA was treated with DNase. RNA purity was checked using UV absorption ratio (260:280 nm) on all RNA preparations. Values ranged between 1.7 and 2.0, indicating that protein contamination was minimal.

After cDNA synthesis, oligonucleotide primer sets specific to particular serotonin receptor subtypes were employed for PCR analysis. Expression of 14 individual subtypes was targeted (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, 5-HT1F, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT3, 5-HT4, 5-HT5A, 5-HT5B, 5-HT6, and 5-HT7) that represented all 7 of the major 5-HT receptor families, as well as isoforms known to be expressed in rat tissues (Table 1). Before testing on cDNA derived from taste bud RNA, each primer set was optimized on brain tissue known to express a particular receptor subtype. In the case of primer sets for the 5-HT1E receptor subtype, where receptor expression is not well known, genomic DNA (which was isolated in parallel with total RNA) served as template for its optimization. Results are presented in Fig. 1. For each PCR reaction, a parallel reaction was conducted that omitted the reverse transcriptase step [columns labeled (–)] or, in the case of the 5-HT1E primer set, with omission of template (column labeled H2O). These reactions ensured that observed PCR products were not derived from genomic template. In all cases, reactions yielded amplification products of expected size for each one of the serotonin receptor subtypes (indicated below each corresponding lane). Size markers (M; 100-bp ladder) are in the left lane of each gel.

Parallel experiments were performed on total RNA isolated from taste buds. Fourteen primer sets were run under optimized conditions and included both positive and negative control reactions. All experiments included parallel reactions with (RT+) or without (RT–) the RT enzyme step. In no case were...
bands observed in RT− reactions, indicating the absence of interfering DNA contamination. In addition, reactions were run that omitted template (H2O) to control for outside contamination and for PCR carryover. As a positive control, a primer set for GAPDH was included. For cDNA derived from taste buds, a primer set for the G protein gustducin was also included. Gustducin is a constitutively expressed gene in a subset of TRCs. With the use of optimized conditions of all 14 primer sets for 5-HT receptor subtypes on total RNA extracted from pure taste buds, only reactions using 5-HT1A subtype or the 5-HT3 subtype primers yielded amplification products of appropriate size (Fig. 2). Expected sizes of the PCR products are indicated below each lane. The identity of the PCR products from taste buds was confirmed by first purifying the PCR products and then directly sequencing them at the Plant-Microbe Genome Facility at The Ohio State University. The sequences were analyzed using a BLAST search of GenBank and were found to correspond to published sequences for the 5-HT1A and 5-HT3 receptor subtypes in rat tissue. The presence or absence of product for PCR reactions using these 14 serotonin receptor subtypes on template derived from taste buds is summarized in Table 2.

Immunocytochemical localization of 5-HT1A or 5-HT3 receptors in rat lingual tissue. With the suggestion that 5-HT1A and 5-HT3 mRNA is expressed in taste buds, verification of the cellular localization of these putatively expressed receptor subtypes in TRCs was performed using immunocytochemistry with commercially available primary antibodies directed against epitopes of either the 5-HT1A or 5-HT3 receptor subtype. Experiments were performed on taste buds of rat foliate and circumvallate papillae using frozen sections and immunofluorescence. Distinctly different patterns of immunopositive and circumvallate papillae using frozen sections and immunocytochemistry was performed on frozen rat brain and tongue tissue. The outline of the lingual epithelium is clearly evident in negative control experiments included omission of the primary antibody or omission of the secondary antibody. In both cases, no immunoreactive product was observed.

Antiserum to 5-HT1A receptor was applied to 8-µm frozen sections of fixed rat foliate and circumvallate papillae. An optimal dilution of primary antibody at 1:200 was empirically determined. Primary antibody binding was visualized with a biotin-conjugated goat anti-rabbit Fab fragment and strepavidin-fluorescein under light microscopic epifluorescence. Discrete cellular immunofluorescent localization of 5-HT1A receptors in subsets of TRCs was evident. An example from rat circumvallate papillae is presented in Fig. 3, bottom. The outline of the lingual epithelium is clearly evident in negative control experiments, whereas in gustatory tissue the taste buds have more particulate appearance, suggestive of membrane staining. Negative control experiments included omission of the primary antibody or omission of the secondary antibody. In both cases, no immunoreactive product was observed.

Cellular detection of the 5-HT3 receptor subtype was tested with a polyclonal antibody generated by immunizing rabbits with a synthetic peptide corresponding to amino acids 444–457 from the rat serotonin 5-HT3 receptor protein (Oncogene, Research Products, Cambridge MA). This antiserum was previously used to analyze a detailed distribution of 5-HT3 receptors in rat brain tissue (44), and staining is reported to be low-level antigen expression, these experiments were performed using a tyramine amplification protocol. Whereas immunoreactive cells were observed in rat brain tissue (Fig. 4F) that was eliminated with omission of the primary antibody (Fig. 4G), no immunoreactive taste receptor cells were observed in the examined tongue tissue (Fig. 4, A, B, and D).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>1A</th>
<th>1B</th>
<th>1C</th>
<th>1D</th>
<th>2A</th>
<th>2B</th>
<th>2C</th>
<th>3</th>
<th>4</th>
<th>5A</th>
<th>5B</th>
<th>6</th>
<th>7</th>
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<tbody>
<tr>
<td>mRNA</td>
<td>+</td>
<td>−</td>
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+, Receptor subtype mRNA detected; −, receptor subtype mRNA not detected.

Fig. 2. PCR analysis of the expression of 5-HT receptor subtypes in rat taste buds. PCR products were separated by agarose gel electrophoresis stained with ethidium bromide. Of the 14 primer sets tested, PCR products were only observed with taste bud-derived template using primer sets for 5-HT1A or 5-HT3 receptor subtypes. PCR products derived from taste bud template and rat whole brain (positive control) are illustrated. In rat brain tissue, a primer set for the housekeeping gene GAPDH was also tested, whereas in gustatory tissue the taste cell-specific G protein gustducin (GUST) served as an internal control. All reactions were either run with RT (+) or the RT step was skipped (−). Far left lane (M) indicates 100-bp ladder of A markers.
Labeling in the posterior papillae was confined to large ganglion cells (Fig. 4B) and nerve fibers in the dermal core of the papillae. Only subsets of taste receptor cells within an individual taste bud were observed as immunopositive. Control sections illustrate immunocytochemical reactions run with omission of the primary antibody. Scale bar, 50 μm.

Fig. 4. Immunocytochemistry using an antibody directed against the 5-HT3 receptor subtype is illustrated using rat foliate papillae. Numerous taste buds are evident in A and B, all displaying an absence of reaction product in taste receptor cells. On the other hand, Remark’s ganglion cells, in the core of the dermal papillae, display obvious label (B). Additionally, bundles of nerve fibers at the base of the papillae were immunopositive (D, arrow and E). C: immunocytochemical reaction run with omission of the primary antibody. F: positive control tissue illustrates a labeled neuron in rat cortex. G: negative control (omission of primary antibody). Scale bar, 50 μm.

Immunocytochemical double labeling of 5-HT and 5-HT1A receptors. Double-labeling epifluorescence experiments were conducted to determine if 5-HT and 5-HT1A immunoreactivity occurs in overlapping, partially overlapping, or nonoverlapping subsets of TRCs within the taste bud. A commercial
polyclonal antibody raised in rabbit against repeated immunization of rabbits with serotonin coupled to BSA was employed (ImmuNoStar, Hudson, WI). Staining was reported to be completely abolished by preabsorption with 5-HT/BSA but not with 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, or dopamine. As commonly employed to enhance 5-HT immunoreactivity in TRCs (e.g., 36), the animal was pretreated with precursor 5-hydroxytryptophan before death.

Two examples of sections containing posterior taste buds demonstrating the immunocytochemical staining pattern for the 5-HT1A receptor (labeled with FITC-green) and for 5-HT (labeled with Cy3-red) are presented in Fig. 5. The overlay, demonstrating the double-labeling pattern, is illustrated at the far right of Fig. 5. Typically, taste buds displayed cells positive for both the 5-HT1A receptor and for 5-HT. The overlay demonstrates that these cells were observed in nonoverlapping cell populations, demonstrating that serotonin-concentrating cells and 5-HT1A receptor expressing cells do not colocalize.

DISCUSSION

The present study is the first thorough analysis of serotonin receptor subtype expression in TRCs. An initial screening using RT-PCR with 14 5-HT receptor subtype-specific primers demonstrated the presence of 5-HT1A and 5-HT2 receptor mRNAs in pure taste bud total RNA. Additionally, data suggest the expression of 5-HT1B, 5-HT1D, 5-HT1E, 5-HT1F, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT4, 5-HT5A, 5-HT5B, 5-HT6, and 5-HT7 in rat posterior TRCs to be unlikely. Because these primers were first optimized on rat positive control tissue, even low-level expression of these subtypes in rat taste buds would likely have been detected. However, if some tissue-specific postgenomic modification, such as alternative splicing, exists in TRCs, detection of these mRNAs might not have been evident with these primer sets. Moreover, our 14 primer sets comprise a minority of all serotonin receptor subtypes, whose exhaustive exploration would be a prohibitively large undertaking. Also, it is always possible that rare expression of receptor subtype mRNAs precluded their accurate detection with this technique. Therefore while these data are strongly suggestive of the expression of both 5-HT1A and 5-HT3 receptor subtypes, they do not prove the nonexpression of other receptor subtypes.

Multiple lines of evidence conclusively establish the expression of 5-HT1A receptors in a subset of rat posterior TRCs. This evidence includes RT-PCR demonstration of 5-HT1A mRNA expression in taste buds, immunocytochemical demonstration of 5-HT1A protein in a subset of TRCs, and patch-clamp studies of 5-HT1A functional roles in these cells. A major advantage of the RT-PCR is the purity of starting material. Isolation of taste buds excludes lingual epithelium and its associated cell types, such as epithelial and endothelial cells, ganglion cells, skeletal muscle, and cells of von Ebner’s glands, ensuring expression of these receptor subtypes to cell types of the taste bud. Taste buds, which served as starting material, are composed of elongate TRCs, basal cells, and postsynaptic fragments of intragemmal sensory nerve fibers. Thus cellular localization with immunocytochemistry is required to localize expression to elongate receptor cells. This confirmation was clearly provided by the immunocytochemical localization of 5-HT1A receptors to a subset of TRCs. Additionally, separate studies in our laboratory, using single-cell physiological analysis with patch-clamp analysis, have demonstrated that rat posterior TRCs respond to 5-HT, to 5-HT agonists, and to 5-HT1A-specific agonists (20, 21). The combination of these approaches, i.e., mRNA localization, peptide expression, and physiological analysis, all point to 5-HT1A expression in a subset of TRCs.

As well, these data are consistent with the notion that 5-HT3 expression in taste buds is confined to sensory afferent terminals within the bud. However, taken alone they are not conclusive, and other confirmatory evidence, e.g., immunocytochemistry at the level of the electron microscope, will be required to prove the cellular localization of the 5-HT3 subtype within the taste bud. Whereas immunocytochemistry using
5-HT1A primary antibodies confirmed cellular localization in a subset of TRCs, reaction product for 5-HT3 receptor immunocytochemistry was not observed in the taste buds. Staining outside of the taste bud in Remack’s ganglion cells as well as stained regions of neural bundles within the core of the papillae was observed suggesting that if TRCs expressed 5-HT3 receptor subtype, immunocytochemistry would have detected it. On the other hand, if 5-HT3 expression was confined to postsynaptic terminals within the taste bud, it would be expected that the resolution of the light microscope would have precluded its detection. Three observations support this view. First, in the periphery 5-HT3 receptors are expressed in sensory afferent nerve fibers, including the myenteric plexus, submucous plexus, nodose ganglion, superior cervical ganglion, and the dorsal root ganglion (33). Thus their expression in gustatory primary afferents could be expected. Second, a recent publication reports that using immunocytochemistry a subset of cell bodies within the rat petrosal ganglion stains positively for 5-HT3 (63). These cell bodies are pseudounipolar neurons whose peripheral branches innervate taste buds of the circumvallate/foliate papillae and the chemosensory cells/baroreceptors of the carotid body. Because cell bodies innervating the carotid body tend to be located at the distal portion of the petrosal ganglion and 5-HT3-immunoreactive cell bodies were widely distributed throughout the ganglion, the likelihood that some 5-HT3-immunoreactive neurons innervate taste buds is high. Finally, there are numerous examples of the localized expression of neurotransmitter mRNA in postsynaptic endings (e.g., 17, 34, 52). Therefore, it is conceivable that the detection of 5-HT3 mRNAs in the taste buds may be due to gustatory afferent nerve terminals rather than by the TRCs in the harvested taste bud populations.

Even more compelling than the observation of 5-HT1A and 5-HT3 receptor subtype expression within the taste bud is the localization of 5-HT and 5-HT1A receptors to different populations of taste receptor cells. Using antibodies directed against 5-HT and 5-HT1A in double-labeling immunocytochemistry experiments on rat posterior taste buds, exclusively nonoverlapping populations were observed. These observations require a reexamination of how serotonin may function within the mammalian taste bud. Previous work established that serotonergic TRCs comprise a subset of the type III TRC (the type forming synaptic contact with the afferent nerve) in variety of mammals such as mouse, rat, rabbit, and monkey taste buds (16, 36, 46, 57, 67). Hence, serotonin has been thought of as a neurotransmitter initiating afferent neural output. In addition to that role, one must now consider paracrine serotonergic cell-to-cell communication within the taste bud. Hence, serotonin release may not only excite the peripheral afferent nerve fiber but, in addition, may act to inhibit neighboring TRCs via activation of 5-HT1A receptors and resultant inhibition of sodium currents.

Physiological implications of serotonergic processing within the taste bud. To date, physiological actions of serotonin on taste receptor cells have been reported in amphibians and mammals, suggesting it may play a role in gustation. In mudpuppy, serotonin application alternately increased or decreased a calcium current (11, 15). In frog, serotonin inhibited sodium and potassium current in ~50% of TRCs (30, 31). In both species, a subset of merklike basal cells, rather than TRCs, expresses serotonin (12, 37). These basal cells are hypothesized to release serotonin onto TRCs during tastersnonic stimulation where they modulate electrical properties of the postsynaptic TRC (11, 15). Preliminary analysis suggests these effects to be mediated by the 5-HT1A receptor subtype.

In mammals, on the other hand, a number of studies have localized 5-HT to a subset of the type III cells in posterior taste buds (16, 36, 46, 51, 57, 67). One study (36) suggests 5-HT may also be present in some TRCs of the fungiform papillae. In the mammalian taste bud, inhibitions of both calcium-activated potassium current and voltage-gated sodium current were observed in TRCs using patch-clamp recordings (20, 21). These effects could be mimicked by the use of serotonergic agonists. N-(trifluoromethylphenyl)piperazine, a general serotonergic agonist, 1-(1-naphthyl)piperazine, with agonist properties at 5-HT1 and antagonistic properties at 5-HT2 receptors, and (±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene, a specific 5-HT1A receptor agonist, were all as effective as 5-HT in producing these effects. However, no effects on these ionic currents were noted when the 5-HT3 agonist phenylbiguanide was applied. Thus the physiological data of prior investigations on rat TRCs and the molecular data presented in this paper are in excellent agreement on the expression pattern of these two serotonergic receptors.

In the mammalian taste bud, serotonin release from type III cells during active gustatory stimulation would excite the peripheral nerve fiber and in addition act to inhibit neighboring TRCs via activation of 5-HT1A receptors and resultant inhibition of sodium currents. Postsynaptic actions of 5-HT3 receptors, since it is nonspecific cation channel, result in rapid depolarization. 5-HT1A receptors, on the other hand, are metabotropic receptors that are often coupled to Gs proteins, which negatively regulate adenylyl cyclase, thus lowering cAMP levels (28, 59). Their postsynaptic actions are often inhibitory. The mechanism underlying the 5-HT-mediated inhibition of ion currents in TRCs remains unknown. However, considered alone, the inhibition of sodium current would obviously reduce the excitability of the 5-HT1A-expressing TRC. This could be analogous to a lateral inhibition, i.e., neighboring 5-HT1A-expressing TRCs would be inhibited by the release of serotonin from the serotonergic type III, which is simultaneously exciting the peripheral afferent nerve fiber via 5-HT3 receptors.

The functional result of the inhibition of a subset of TRCs by serotonin rests largely in the identity of the 5-HT1A-expressing TRCs. One possibility is that they may be another subset of type III cells, the nonserotonergic type III cell. The transmitter of this cell type is presently unknown. Hence, serotonergic paracrine communication would directly shape the afferent output in a manner analogous to lateral inhibition. Another possibility is that serotonin acts to tune the quality of the signal by inhibiting cells contributing (either directly or indirectly) to an antagonist quality (e.g., bitter and sweet). In the absence of additional characterization data on the 5-HT1A-expressing such as its chemical sensitivity or cell type, such mechanisms can only be speculated. Since the serotonin-expressing TRCs colocalize with NCAM but not with PGP9.5, a protein marker found in subpopulations of type III and type II cells (67), it would be interesting to explore whether 5-HT1A expressing TRCs overlap with PGP9.5 expression. This overlap would suggest inhibition of type II cells, which contain much of transductive machinery, and/or overlap with the nonserotoner-
gie type III cell, whose transmitter(s) remains unknown. Further phenotyping of the 5-HT1A-expressing TRC will be required.

GRANTS

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REFERENCES


