Cellular/Molecular

Knocking Out P2X Receptors Reduces Transmitter Secretion in Taste Buds

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In response to gustatory stimulation, taste bud cells release a transmitter, ATP, that activates P2X2 and P2X3 receptors on gustatory afferent fibers. Taste behavior and gustatory neural responses are largely abolished in mice lacking P2X2 and P2X3 receptors [P2X2 and P2X3 double knock-out (DKO) mice]. The assumption has been that eliminating P2X2 and P2X3 receptors only removes postsynaptic targets but that transmitter secretion in mice is normal. Using functional imaging, ATP biosensor cells, and a cell-free assay for ATP, we tested this assumption. Surprisingly, although gustatory stimulation mobilizes Ca^{2+} in taste Receptor (Type II) cells from DKO mice, as from wild-type (WT) mice, taste cells from DKO mice fail to release ATP when stimulated with tastants. ATP release could be elicited by depolarizing DKO Receptor cells with KCl, suggesting that ATP-release machinery remains functional in DKO taste buds. To explore the difference in ATP release across genotypes, we used reverse transcriptase (RT)-PCR, immunostaining, and histochemistry for key proteins underlying ATP secretion and degradation: Pannexin1, TRPM5, and NTPDase2 (ecto-ATPase) are indistinguishable between WT and DKO mice. The ultrastructure of contacts between taste cells and nerve fibers is also normal in the DKO mice. Finally, quantitative RT-PCR show that P2X4 and P2X7, potential modulators of ATP secretion, are similarly expressed in taste buds in WT and DKO taste buds. Importantly, we find that P2X2 is expressed in WT taste buds and appears to function as an autocrine, positive feedback signal to amplify taste-evoked ATP secretion.

Introduction

Taste buds are specialized chemoreceptive organs that transduce chemical stimuli and transmit the information to gustatory afferent fibers. Several neurotransmitter candidates have been proposed for synapses in taste buds, including acetylcholine, ATP, glutamate, norepinephrine (NE), serotonin (5-HT), GABA, and a number of peptides (Nagahama and Kurihara, 1985; Nagai et al., 1998; Ogura, 2002; Lu et al., 2003; Kaya et al., 2004; Finger et al., 2005; Huang et al., 2005, 2007, 2008a; Shen et al., 2005; Zhao et al., 2005; Romanov et al., 2007; Shin et al., 2008; Cao et al., 2009; Dando et al., 2010; Murata et al., 2010; Starostik et al., 2010; Dvoryanchikov et al., 2011). Of these, definitive evidence for taste-evoked transmitter release only exists for ATP, norepineph-

rine, serotonin, and GABA (Finger et al., 2005; Huang et al., 2005, 2007, 2008a; Romanov et al., 2007; Murata et al., 2010; Huang and Roper, 2011). Specifically, Finger et al. (2005) used a luciferin-luciferase-based assay to detect ATP released from intact taste epithelium while Huang et al. (2007), Romanov et al. (2007), and Murata et al. (2010) measured ATP release on a cellular level to establish that taste bud Receptor (Type II) cells secrete ATP in response to gustatory stimulation. This transmitter release was via a novel mechanism—secretion through gap junction hemichannels, likely composed of pannexin 1 (Huang et al., 2007; Dando and Roper, 2009). Further, Huang et al. (2007, 2008a, 2009) showed that Presynaptic (Type III) taste cells release serotonin, or serotonin with norepinephrine, when they are stimulated by acidic tastants or by ATP released from adjacent taste Receptor cells (also see Dvoryanchikov et al., 2007). These findings highlight the importance of ATP as a taste transmitter.

Sensory axons innervating taste buds express two ionotropic P2X receptor subunits: P2X2 and P2X3 (Bo et al., 1999). In a key paper, Finger et al. (2005) established that ATP signaling via these P2X receptors was necessary for successful transmission of information from taste cells to the sensory axons that innervate taste buds. These studies showed that taste-evoked responses recorded from gustatory nerves innervating taste buds were abolished and behavioral responses to most taste modalities lost in mice lacking both P2X2 and P2X3 purinoceptors [double knock-out (DKO) mice], but not in mice lacking only one of these receptors. These

Table 1. Primers for end-point and quantitative RT-PCR, annealing temperature (Annl., °C), and PCR product length (Prod, bp)

Figure	Protein/gene	Accession #	Forward primer (5′→3′)	Reverse primer (5′→3′)	Prod, bp	Annl., °C
Controls						
7	Keratin8/Krt8	NM_031170	cagagggcatcgttggag	tcatgttctgcatcccagac	250	60
7	Keratin20/Krt20	NM_023256	gaggcccaactgatgcagat	ccttgacttcagaggacacgac	249	58
7	PLCβ2/Plcb2	NM_177568	gagcaaatcgccaagatgat	ccttgtctgtggtgaccttg	163	60
4	Px1/Panx1	NM_019482	cattgaccccatgctactcc	tcagccacagaagtcacagg	249	60
7	SNAP25 /Snap25	NM_011428	ggcaataatcaggatggagtag	agatttaaccacttcccagca	310	58
4	TrpM5/Trpm5	NM_020277	gtctggaatcacaggccaac	gttgatgtgccccaaaaact	234	60
P2X receptors						
7	P2X1/P2rx1	NM_008771	ggtggaggaggtgaatggta	caggttcttctccccgtaca	241	60
7	P2X2/P2rx2	NM_153400	caccaccactcgaactctca	ggtacgcaccttgtcgaact	217	60
7	P2X3/P2rx3	NM_145526	caaagccaggaagtttgagg	gttctgcagcccaaggataa	179	60
7	P2X4/P2rx4	NM_011026	cgctttgacatcatcgtgtt	tgctcgtagtcttccacatactt	200	60
7	P2X5/P2rx5	NM_033321	gggctttcttctgtgtgacctg	gtgatggcttcatgttcaag	294	60
7	P2X6/P2rx6	NM_011028	ttcaggacagccaatcactg	gtaacagggttagcgggtga	314	60
7	P2X7/P2rx7	NM_011027	ccctgcacagtgaacgagta	cgtggagagatagggacagc	217	60

data were interpreted as indicating that postsynaptic receptors for taste transmitters were eliminated by the gene knock-out, thereby implicating ATP as a likely transmitter between gustatory receptor cells and afferent fibers. It was assumed that taste transmitter release was still normal since the P2X2 and P2X3 receptors had been identified previously only on the postsynaptic afferent fibers (Bo et al., 1999).

To test this assumption, we investigated whether transmitter release and other key properties of taste transduction are normal in mutant mice lacking P2X2 and P2X3 receptors. By measuring released ATP using a luciferin/luciferase-based assay as well as Fura 2 Ca^{2+} imaging with cellular biosensors, we examined taste-evoked ATP secretion from taste buds of WT, P2X2, and P2X3 DKO mice. Unexpectedly, our findings indicate that in addition to the previously demonstrated postsynaptic defect in taste afferent neurons, tastant-evoked release of ATP from taste buds themselves is disrupted in mice with genetic deletion of the P2X2 and P2X3 receptors.

Materials and Methods

Animals. We used adult P2X2 and P2X3 DKO mice of either sex, along with the control WT line of similar background (mixed C57BL6 and Ola) as in the previous study (Finger et al., 2005). For some experiments (e.g., validation of antisera), we used C57BL/6J mice. No differences were observed when using the different wild-type control lines. For functional studies, mice were killed with CO_2 followed by cervical dislocation before tongues were removed. For histological studies, mice were anesthetized and perfused transcardially with fixative as detailed below. All experimental protocols were performed following National Institutes of Health guidelines as approved by the Institutional Animal Care and Use Committees of University of Miami, University of Colorado Denver, University of Denver, and Colorado State University.

Luciferin/luciferase experiments. Lingual epithelium containing vallate papillae from adult wild-type and DKO mice were collected for luciferin/luciferase experiments as previously described (Finger et al., 2005). For each experiment, the peeled lingual epithelium was placed onto a plastic sheet such that the basal portion of the vallate papilla protruded through a rectangular area (0.5 mm × 1 mm) into a well containing 100 μl of Tyrode's solution (see below for composition). Tastants were applied to the mucosal surface of the lingual epithelium and Tyrode's solution bathing the basal portion of the vallate papilla was collected following application of the tastant. An equal amount of Luciferase reagent (Roche: ATP Bioluminescence Assay Kit HS II) was added to the samples and the combined solution was placed into a luminometer (Turner Biosystems 20/20 [n]) for analysis. Readings were collected as arbitrary relative light units (RLU) using the software provided with the luminometer. RLU data were converted into ATP concentrations using a calibration curve obtained before each experiment. All solutions were brought to room temperature before the start of each experiment. Taste stimuli consisted

of a mixture of denatonium, 1 mM; cycloheximide, 10 μm; and SC45647, 0.1 mM. Typically, 5 μl of the taste mix was applied to the mucosal surface. As a control for mechanical stimulation, we applied 5 μl of Tyrode's buffer. Experiments were blinded to prevent bias.

Biosensor cells. Chinese hamster ovary (CHO) cells stably expressing 5-HT$_{2C}$ receptors or P2X2 and P2X3 receptors (hereafter, "5-HT-" or "ATP-biosensor cells," respectively) were prepared as described previously (Huang et al., 2005, 2007). Biosensors were transferred into the recording chamber alongside freshly harvested taste buds or taste cells. Individual biosensors were drawn onto a fire-polished glass micropipette and positioned against a taste bud or cell to measure transmitter release during gustatory stimulation. In some experiments, we used CHO cells expressing only endogenous P2Y receptors for biosensors to test whether BzATP, a P2X receptor agonist, elicits ATP secretion from isolated taste buds. In separate experiments, we verified that biosensor cells were not affected by bath-applied KCl, taste stimuli, or any pharmacological agents used in this study. In particular, BzATP did not elicit responses in the P2Y-ATP biosensors.

Isolated taste cells. We removed lingual epithelium containing taste papillae from the tongue and harvested taste buds and taste cells as described previously, with slight modification (Huang and Roper, 2010). Briefly, lingual epithelium was removed from the tongue after subepithelial injection of enzyme mixture. Taste buds were isolated from the epithelium and for preparing individual taste cells, were transferred to a shallow chamber containing trypsin for additional incubation. Taste cells were disassociated from taste buds by gently triturating and transferred to a recording chamber. Taste buds and taste cells were superfused with Tyrode's solution (in mM; 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, 10 Na-pyruvate, 5 $NaHCO_3$, pH 7.4, 310–320 mOsm).

Ca^{2+} imaging. Fura 2 Ca^{2+} imaging was performed using Workbench v5 software (Indec). F340/F380 was converted to $[Ca^{2+}]_i$ as described by Grynkiewycz et al. (1985). The fluorescence ratios of free and Ca^{2+}-bound Fura 2 were determined using a Fura 2 Calcium Imaging Calibration Kit (Invitrogen). Isolated taste cells with resting $[Ca^{2+}]_i > 200$ nM were excluded from the analyses (Hacker and Medler, 2008; Huang and Roper, 2010). The average resting $[Ca^{2+}]_i$ in these experiments was 154 ± 11 nM.

Stimulation. Isolated taste buds and/or cells were stimulated by bath-perfusion of taste mix (cycloheximide, 10 μm; saccharin, 2 mM; SC45647, 0.1 mM; denatonium, 1 mM). For some experiments, taste buds were depolarized with increasing concentrations of KCl (50, 100, and 140 mM substituted equimolar for NaCl), or taste mix paired with 50 mM KCl (Huang and Roper, 2010). All stimuli were in Tyrode's solution and applied at pH 7.2 unless otherwise noted. BzATP was purchased from Sigma. All solutions were at room temperature.

Quantification and data analysis of Ca^{2+} imaging. The quantification of responses is described by Huang et al. (2009). Briefly, we recorded the resting baseline for 30–60 s before each stimulus. Stimuli were applied for 30 s after which the perfusion was returned to Tyrode's solution. A minimum of 3–5 min was allowed between stimuli if repeated tests were
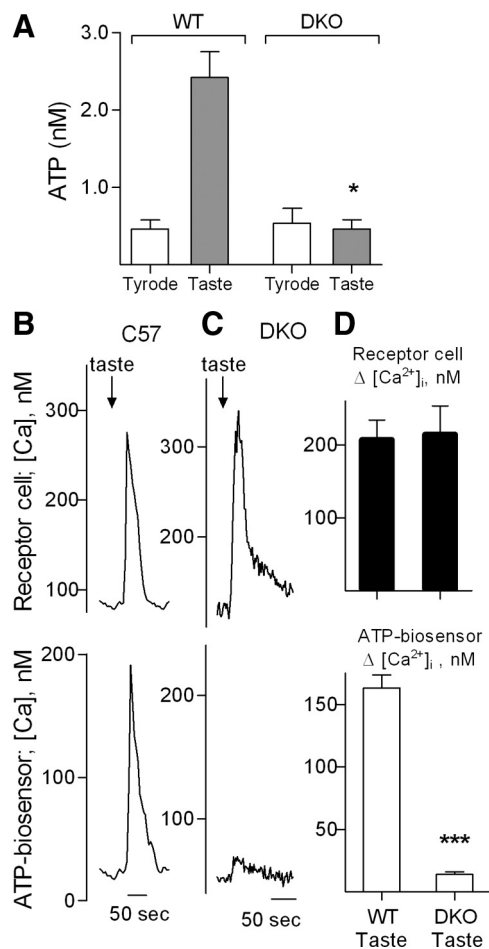
Figure 1. Taste-evoked ATP secretion from taste buds is significantly reduced in double knock-out mice lacking P2X2 and P2X3 receptors (DKO mice). **A**, Luciferase assay of ATP secretion from pieces of lingual epithelium containing vallate papillae. Open bars, Lingual epithelium was stimulated with Tyrode solution; filled bars, stimulation with taste mixture. Data from DKO mice (right) show no taste-stimulated ATP secretion. Ordinate, ATP secreted, nM (WT Taste vs. DKO Taste, $*p < 0.05$, Student's t test). **B–D**, Fura 2 Ca^{2+} imaging and biosensor assay of ATP secreted from identified Receptor (Type II) taste cells. **B**, Traces from a Receptor cell (upper) and concurrent recording from an ATP biosensor (lower) from a C57BJ/6l (wild-type) mouse, showing taste-evoked responses in the Receptor cell and taste-evoked ATP secretion (biosensor trace). Ordinates, Intracellular Ca^{2+} mobilization, nM. Arrows in this and following figures indicate 30 s bath application of stimulus. **C**, A similar experiment from a Receptor cell from a DKO mouse. Although taste stimulation evoked a robust Ca^{2+} response in the Receptor cell, ATP secretion was greatly reduced. **D**, Summary of results. Filled bars (top), Ca^{2+} mobilization in Receptor cells; open bars (bottom), ATP biosensor responses; wild-type mice, $N = 16$; DKO mice, $N = 17$; $***p < 0.001$, Student's t test.

conducted. This protocol is efficient for eliciting responses without desensitization. Statistical analyses using paired Student's t tests were applied to determine whether changes in responses following a given treatment were significant. Data presented in bar graphs show means $\pm$ SEM.

Immunohistochemistry. Mice were anesthetized with pentobarbital. For anti-pannexin, mice were perfused transcardially with PBS followed by 4% paraformaldehyde. The tongue was removed, postfixed for 1 hour, and transferred to 30% sucrose overnight. For anti-TRPM5, mice were perfused transcardially with 0.9% saline solution followed by a periodate-lysine-paraformaldehyde (PLP) fixative containing 75 mM lysine, 10 mM sodium periodate, and 1.6% paraformaldehyde. The tongue was removed, postfixed for 2 h, and transferred into 0.1 M PBS with 25% sucrose overnight. For immunolabeling, tissue blocks were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA).

Sections were incubated with anti-pannexin 1 antibodies (generated against recombinant amino acids sequence corresponding to 135–165 of the intracytoplasmatic loop of the mouse Pannexin1 protein expressed in
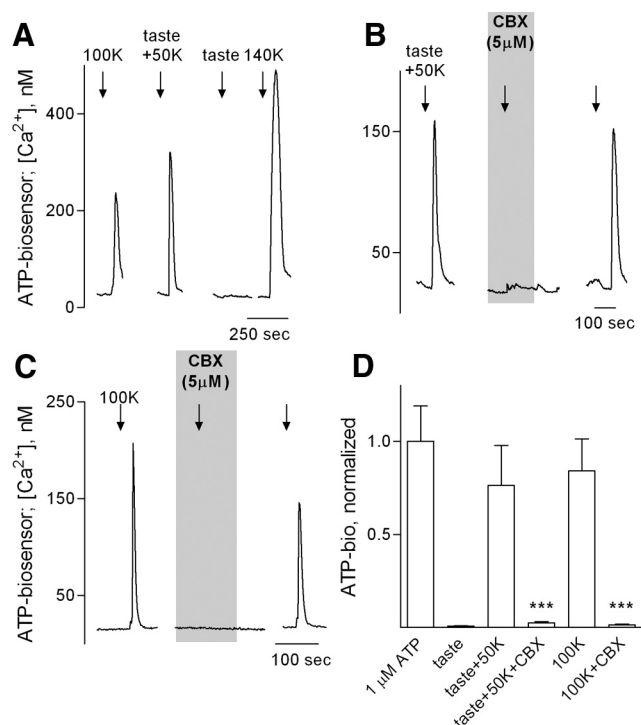

Figure 2. Strong depolarization rescues taste-evoked ATP secretion from taste buds of DKO mice. **A**, Traces show recordings from an ATP biosensor closely apposed to a taste bud isolated from a DKO mouse. Bath-applied KCl (100 and 140 mM) evoked robust biosensor responses (i.e., ATP secretion). If taste stimulation was paired with milder depolarization (50 mM KCl), ATP secretion was also observed (50 mM KCl alone or taste alone did not elicit ATP secretion). **B**, **C**, ATP secretion evoked by taste plus KCl (50 mM) or KCl (100 mM) was abolished by 5 μM carbenoxolone (shaded area), a Px1 hemichannel antagonist at the concentration used. **D**, Summary of data for taste- and KCl-evoked ATP secretion from taste buds of DKO mice. Bars show mean $\pm$ SEM of Ca^{2+} responses in ATP biosensor cells, normalized to biosensor responses to 1 μM ATP. $N = 5$; $***p < 0.001$, Student's t test.

Escherichia coli; chicken anti-Px1, ANT0027; Diatheva; diluted 1:2000 and processed with TSA amplification, Invitrogen) or a 1:500 dilution of TRPM5 antibody (provided by Emily Liman, Department of Biological Sciences, University of Southern California, Los Angeles, CA). Sections were then washed and reacted with secondary antiserum, horseradish peroxidase-labeled goat anti-chicken (Aves Labs Cat H-1004, concentration 1: 500) or Alexa 568 goat anti-rabbit IgG (1:400; Invitrogen) for 2 h at room temperature.

RT-PCR. Tissue was isolated from the foliate and circumvallate papillae and pooled from six adult wild-type and DKO mice. RNA was extracted according to the manufacturer's instructions using the RNeasy Micro kit from Qiagen, including a 2 h DNase I treatment at room temperature for removal of genomic DNA. Reverse transcription was performed using the iScript cDNA Synthesis kit from Biorad using primers in Table 1. Reactions were set up in which the reverse transcriptase enzyme was omitted as a control to test for DNA contamination. Two microliters of cDNA were added to the PCR (Qiagen TaqPCR Core kit). PCR conditions included an initial 5 min denaturation step followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 60°C, and 45 s extension at 72°C; concluding with a 7 min final extension step. We included cDNA from nontaste lingual epithelium and a no template control (water). Amplified sequences were visualized by gel electrophoresis in 2% agarose gels stained with GelRed (Biotium). The nontaste lingual epithelium was determined to be free of taste buds via RT-PCR for α-gustducin and PLCβ2 expression (data not shown). Experiments were repeated three independent times.

Real-time RT-PCR. Real-time RT-PCR for P2X receptors and controls was performed as described previously (Dvoryanchikov et al., 2007) using taste bud cDNA of three DKO and WT mice. Each cDNA was quantified in triplicate for every gene analyzed.

Ecto-ATPase histochemistry. DKO mice were anesthetized and perfused transcardially with 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 Tris-maleate buffer, pH 7.4. After 1 h postfix, lingual tissue was transferred to 20% sucrose in the same buffer. Frozen sections (16 μm) were rinsed in 0.07 M Tris maleate buffer and incubated for 30 min in detection solution containing 2 mM $Pb(NO_3)^2$, 1 mM levamisole, 1 mM ouabain, 50 μM α,β-methylene ADP, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM either ATP or ADP (all from Sigma). The lead precipitate was visualized with 1% ammonium sulfide in buffer. As shown previously (Barry, 1992; Bartel et al., 2006), taste-bud associated precipitate was present only following incubation with ATP and not after ADP, indicating the high specificity for ATP.

Electron microscopy. Mice were anesthetized and perfused for transmission electron microscopy (Kinnamon et al., 1988). The tissues were cut into 200 μm thickness sections with a Vibratome. After rinsing in buffer the sections were osmicated for 1 h in 1% OsO_4 in 0.05 M cacodylate buffer at 4°C. Sections were stained *en bloc* in 1% uranyl acetate and embedded with Eponate 12 (Ted Pella). The sections were re-embedded using the technique of Crowley and Kinnamon (1995). Thin sections (90 – 120 nm) were cut and examined with a HITACHI H-7000 electron microscope.

Results

Using a luciferin/luciferase-based bioluminescence assay, we tested the ability of lingual tissue containing taste buds to release ATP. As shown previously (Finger et al., 2005), stimulating epithelial pieces containing vallate papillae from wild-type mice with tastants elicited a significant release of ATP (2.42 $\pm$ 0.33 nM) relative to stimulation with buffer alone (0.463 $\pm$ 0.118 nM) (means $\pm$ SEM, N = 3 each; p < 0.05). Stimulation of equivalent size pieces of nongustatory epithelium with the taste mixture evoked little or no ATP release above baseline levels (data not shown). In contrast, we observed little or no taste-evoked ATP secretion from taste epithelium of DKO mice (buffer stimulation, 0.57 $\pm$ 0.19 nM vs taste stimulation, 0.460 $\pm$ 0.124 nM; N = 3). The small amount of ATP released from tissue of DKO mice is consistent with mechanical stimulation.

To examine taste-evoked ATP release in greater detail, we isolated individual taste cells from DKO mice and used biosensors to monitor transmitter secretion. We identified isolated taste Receptor (Type II) cells from wild-type and DKO mice by virtue of the fact that Receptor cells show a robust $[Ca^{2+}]_i$ mobilization in response to taste stimulation but do not respond to depolarization with KCl (50 mM) (DeFazio et al., 2006). Isolated taste Receptor cells from wild-type and DKO mice alike responded to taste stimuli with increases in intracellular Ca^{2+} (Fig. 1 *B–D*, top). However, concurrent biosensor measurement of taste-evoked ATP secretion from Receptor cells (Fig. 1 *B–D*, bottom) revealed significantly reduced ATP secretion in DKO mice relative to wild type.

Taste Receptor cells in wild-type mice (C57BL/6J) are capable of secreting ATP in the absence of taste stimulation and without Ca^{2+} mobilization if they are sufficiently depolarized (Romanov et al., 2008; Huang and Roper, 2010). Surprisingly, in taste buds isolated from DKO mice, KCl depolarization (100 mM and higher) elicited ATP secretion as in wild-type mice (Huang and Roper, 2010) (Fig. 2). Depolarization with 50 mM KCl alone does not release ATP from WT or DKO taste buds (data not shown). Furthermore, taste-evoked ATP secretion from isolated taste buds could be rescued in DKO mice when taste stimulation was combined with depolarization (KCl, 50 mM) (Fig. 2*A*). In all cases, ATP secretion from taste buds isolated from DKO mice, whether evoked by strong KCl depolarization ($\geq$100 mM) or taste stimulation plus mild depolarization (50 mM KCl), was blocked by a low concentration of carbenoxolone (5 μM), consistent with
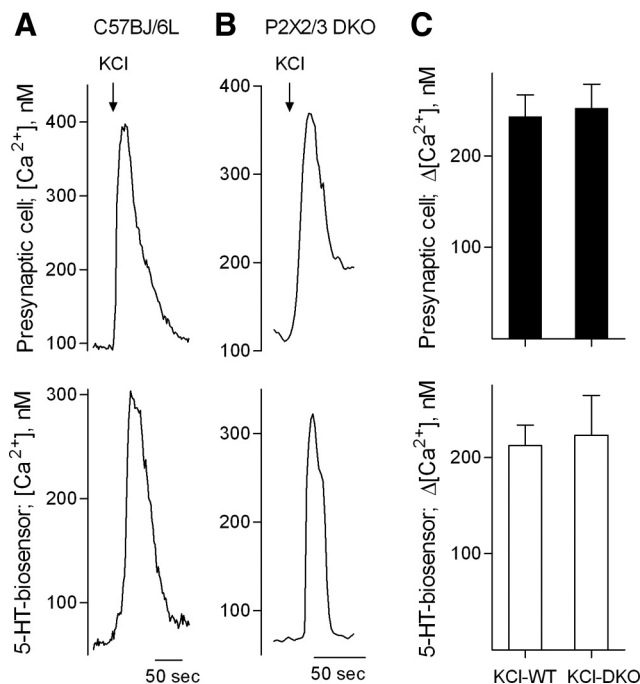
Figure 3. Transmitter (serotonin) secretion from Presynaptic (Type III) taste cells of DKO mice is normal. Serotonin (5-HT) biosensor cells were used to record taste-stimulated 5-HT release from Presynaptic cells in wild-type and DKO mice. *A*, Traces from a wild-type mouse; depolarization with KCl (50 mM) evoked a marked Ca^{2+} response from a Presynaptic cell (upper trace) and concurrent response from a 5-HT biosensor in close proximity to the Presynaptic cell (lower trace). *B*, Data from a DKO mouse: recordings show depolarization-evoked response in the Presynaptic cell (upper) and concurrent 5-HT release (lower), just as in wild-type mouse. *C*, Summary of data from Presynaptic/biosensor cells. Filled bars (above), depolarization-evoked Ca^{2+} responses in Presynaptic cells (50 mM KCl). Open bars (below), 5-HT release (biosensor cell responses). Wild-type mice, N = 14; DKO mice, N = 8. Depolarization evoked 5-HT release from taste cells of DKO mice is indistinguishable from that of taste cells of wild-type mice.

ATP efflux through pannexin1 hemichannels in DKO and wild-type mice alike (Fig. 2 *B–D*) (Huang et al., 2007; Dando and Roper, 2009).

In summary, taste stimulation fails to trigger significant ATP secretion from taste buds of DKO mice. However, taste-evoked ATP release can be rescued in the DKO mice if taste cells are sufficiently depolarized with KCl. Strong depolarization alone also can elicit ATP secretion from Receptor cells isolated from DKO mice.

We next tested whether transmitter release from Presynaptic (Type III) taste cells was similarly depressed in DKO mice. Presynaptic (Type III) taste cells normally release serotonin (5-HT) in response to exposure to sour tastants, KCl depolarization, or ATP released from Receptor (Type II) taste cells (Huang et al., 2007, 2008b) (Fig. 3*A*). Presynaptic cells from DKO mice responded normally to KCl depolarization with 5-HT release (Fig. 3*B*, top). Concurrent measurement of 5-HT release from Presynaptic cells showed no difference between DKO mice and WT mice (Fig. 3, bottom). Similarly, bath-applied ATP (1 μM) also evoked 5-HT release from Presynaptic cells that was indistinguishable between DKO and WT mice (data not shown).

In an attempt to identify why knocking out P2X2 and P2X3 receptor expression eliminated normal taste-evoked ATP secretion, we examined Receptor cells from DKO mice for the presence of two key elements in transmitter secretion—TRPM5 cation channels (Zhang et al., 2007; Huang and Roper, 2010) and pannexin 1 gap junction hemichannels (Huang et al., 2007; Romanov et al., 2007; Dando and Roper, 2009). There were no

obvious differences between wild-type and DKO mice either in the gross histology, in Pannexin 1 immunostaining (Fig. 4A,B), or in TRPM5 immunopositivity (Fig. 4C).

These immunohistochemical results were confirmed by RT-PCR. DKO and WT mice showed similar expression of Px1 and TRPM5 in taste epithelium and their absence from adjacent nontaste epithelium (Fig. 4D).

In WT mice, taste buds express robust ecto-ATPase activity, likely needed to inactivate the ATP released following taste stimulation (Bartel et al., 2006). Because DKO mice are defective in ATP release, we tested whether ecto-ATPase is present and active in taste tissues from DKO mice. Ecto-ATPase histochemistry showed a typical pattern and density of staining in DKO and WT tissue (Fig. 4E).

We next examined the ultrastructure of contacts between taste cells and afferent nerve fibers. In taste buds from WT mice, Presynaptic (Type III) cells form readily identifiable synapses complete with synaptic vesicles and thickened presynaptic and postsynaptic membranes. In contrast, no clear ultrastructural specializations resembling synapses exist between Receptor (Type II) cells and nerve fibers (Murray, 1993), consistent with transmitter release via Px1 gap junction hemichannels (Huang et al., 2007; Romanov et al., 2007; Dando and Roper, 2009). Instead, there often are atypical enlarged mitochondria in Receptor cells where nerve fibers come in proximity (Royer and Kinnamon, 1988). As shown in Figure 5, Presynaptic (Type III) cells in the DKO mice, as in WT mice, have distinct synapses (Fig. 5A) while Receptor (Type II) cells exhibit large mitochondria with tubular cristae at close appositions with nerve processes (Fig. 5B). Thus, there were no ultrastructural abnormalities in the synaptic contacts of DKO mouse taste buds that could explain the absence of taste-evoked neurotransmission.

In a final attempt to resolve why taste-evoked ATP secretion in P2X2 and P2X3 DKO mice is significantly reduced, we reasoned that Receptor (Type II) cells might normally express P2X2 and/or P2X3 receptors that would serve to boost ATP release during taste stimulation. The absence of such an autocrine positive feedback in DKO mice could explain the observation that taste buds in DKO mice fail to secrete ATP upon taste stimulation. We tested for functional P2X receptors by isolating Receptor cells from WT and DKO mice, bath-applying MRS2179 to block P2Y1 receptors that these cells express (Huang et al., 2009), and stimulating the cells with ATP. Any residual ATP-evoked Ca^{2+} mobilization would likely be generated by Ca^{2+} influx through P2X channels, if present. Indeed, in 4 of 11 Receptor cells from WT mice, we observed marked ATP-evoked Ca^{2+} responses under these conditions (Fig. 6A,B). Moreover, the residual ATP-evoked responses were eliminated by removing Ca^{2+} from the bath (i.e., eliminating Ca^{2+} influx) but were unaffected by blocking intra-
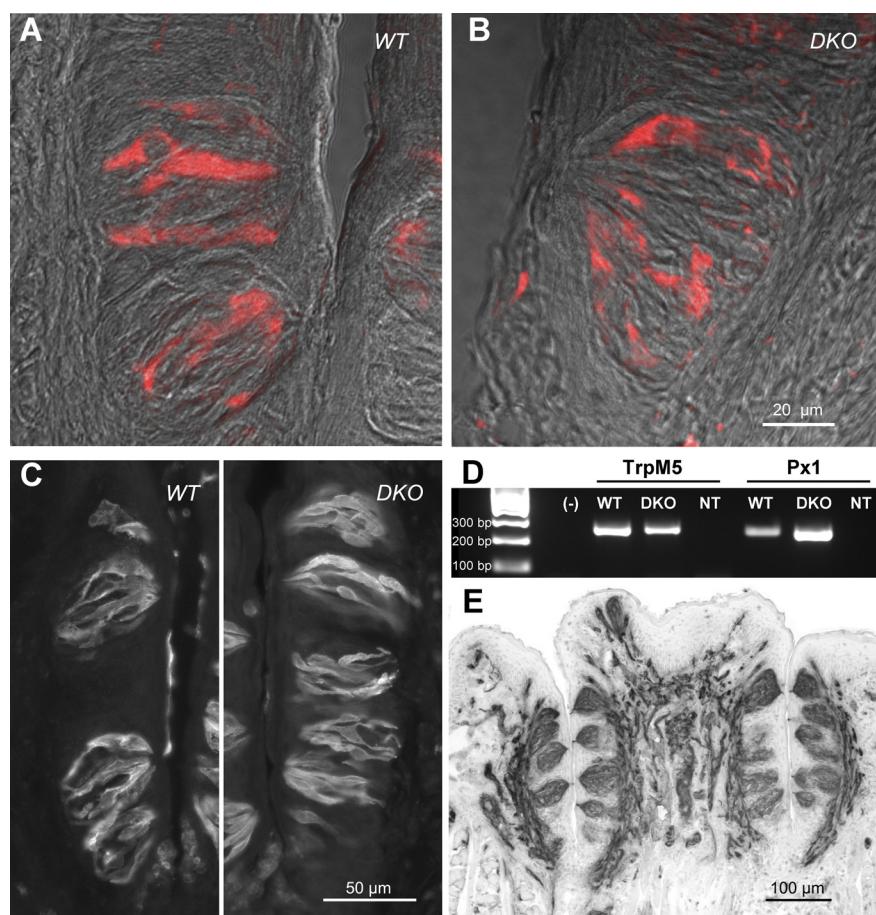
Figure 4. Expression of key elements for taste-evoked ATP release and degradation is similar between wild-type and DKO mice. *A*, *B*, Immunostaining for pannexin 1, a gap junction hemichannel through which ATP is believed to be secreted from taste Receptor (Type II) cells. *A*, Vallate taste buds from wild-type (*WT*) mouse. *B*, Vallate taste buds from DKO mouse. *C*, Photomicrographs of TrpM5 immunoreactivity in vallate papillae of a wild-type and a DKO mouse (*WT*, *DKO*). *D*, PCR for TRPM5 and Px1 from wild-type (WT) and DKO mice, from taste (foliate, vallate) and nontaste (NT) epithelium and no template control (−). *E*, Histochemical staining for ecto-ATPase in vallate papilla from a DKO mouse. The pattern and degree of staining in taste buds and underlying nerve fibers is typical of wild-type mice as has been shown previously (Bartel et al., 2006). None of the markers shown in this figure differ significantly between taste tissue from wild-type and DKO mice.

cellular store-release with the phospholipase C inhibitor, U73122 (i.e., eliminating contribution from P2Y-mediated Ca^{2+} mobilization). Both features are consistent with activation of P2X receptors. In sharp contrast, 0 of 15 Receptor cells from DKO mice tested under identical conditions showed any signs of P2X-mediated responses (Fig. 6C,D). The difference in incidence of P2X-mediated responses between the two samples is significant at $p = 0.022$ (Fisher's exact test).

Certain P2X receptors (most notably, P2X7) are known to interact with Px1 channels (Pelegrin and Surprenant, 2006). In taste buds, Px1 is the conduit for ATP release and P2X7 has been reported to be present (Hayato et al., 2007). To determine whether the expression of P2X7 receptors is altered in DKO mice, we performed highly sensitive and quantitative RT-PCR. As reported by others (Hayato et al., 2007), we found that WT taste buds express P2X2, P2X4, and P2X7 (Fig. 7A). DKO taste buds only express P2X4 and P2X7. Using quantitative RT-PCR, we confirmed that P2X4, and P2X7 are expressed at very similar levels in WT and DKO mouse taste buds (Fig. 7B). Expression levels for control mRNAs, e.g., keratin 8, keratin 20, and Snap25 were also indistinguishable across the genotypes. PLCβ2 expression in DKO mice was lower than that in WT mice (Fig. 7B), a
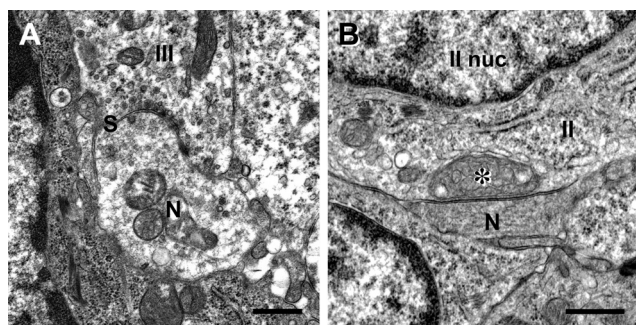
Figure 5. Ultrastructure of synapses in taste buds from DKO mice show no abnormalities. ***A***, Transmission electron micrograph showing a synapse (S) from a Type III (Presynaptic) cell (III) onto a nerve process (N) in a P2X2 and P2X3 DKO mouse. Note the numerous presynaptic pleomorphic vesicles in the Type III cell. Scale bar, 0.5 μm. ***B***, An atypical mitochondrion (*) lies within the Receptor (Type II) cell (II) at its point of apposition with a nerve process (N) in a P2X2 and P2X3 DKO mouse. Note the large size and tubular cristae of the atypical mitochondrion. This appearance is essentially identical to that in a wild-type mouse (Kinnamon et al., 1985). II nuc, Nucleus of the Type II cell. Scale bar, 0.5 μm.
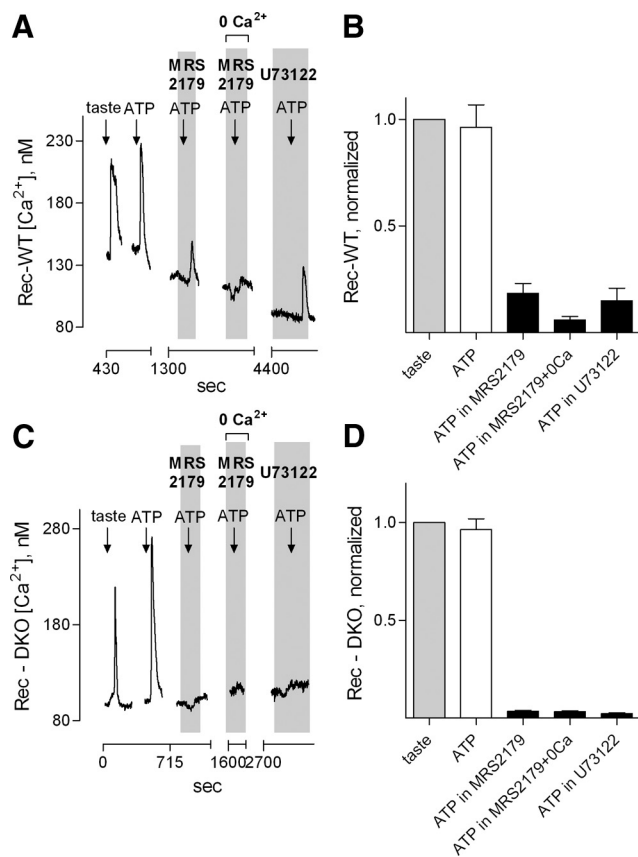

Figure 6. Taste Receptor (Type II) cells in wild-type mice express autocrine P2X receptors. ***A***, Trace showing continuous recording (>1 h duration) of Fura 2 Ca^{2+} imaging from an identified Receptor cell isolated from a wild-type mouse, showing response to taste stimulation (arrow) followed by Ca^{2+} transients evoked by repeated applications of 1 μM ATP (arrows) before and during MRS2179 (10 μM) to block P2Y1 receptors, removal of Ca^{2+} from the bathing solution to eliminate Ca^{2+} influx, and lastly, in presence of U73122 (10 μM) to block phospholipase C. ***B***, Summary of data from experiments as in ***A***, taken from 11 WT mouse Receptor cells. ATP-evoked Ca^{2+} responses were normalized to taste-evoked Ca^{2+} responses. Bars show means ± SEM. ***C***, Ca^{2+} imaging data from a Receptor taste cell isolated from a DKO mouse, parallel protocol as in ***A***. ***D***, Summary of data from 15 DKO mouse Receptor taste cells, as in ***C***.

result that was unexpected and that could contribute to taste differences between the two strains. However, note that taste-evoked mobilization of calcium, in which PLCβ2 participates, was similar in WT and DKO mice (Fig. 1D). Our expression data suggest that a relevant and important difference in taste buds between WT and DKO mice is the absence of P2X2. This channel could be a key contributor to the loss of taste-evoked ATP secretion in DKO mice.

A hypothesis that emerged consequent to these experiments is that during taste stimulation, Receptor cells secrete ATP, which acts postsynaptically as well as presynaptically (i.e., autocrine positive feedback) via P2 receptors, including P2X2. Loss of this positive feedback in mice lacking P2X2 receptors (i.e., DKO) could lead to a significant decline in transmitter output from Receptor cells. This hypothesis predicts that that in WT mice, activating P2X2 on Receptor cells should stimulate ATP release. Thus, we tested the actions of BzATP, a potent P2X2 agonist, on taste buds from WT and DKO mice. Moreover, by using ATP biosensors that expressed only P2Y receptors (insensitive to BzATP), we could monitor ATP release from BzATP-stimulated taste buds. We verified that such biosensors were not directly activated by BzATP (up to 30 μM). Indeed, BzATP (0.3–3 μM) elicited ATP release from Receptor cells in WT mice (Fig. 7C,E). However, BzATP failed to elicit ATP secretion from Receptor cells in DKO mice (Fig. 7D,E). The magnitude of BzATP-induced ATP release was significantly different in WT versus DKO mice (Fig. 7E). BzATP at these concentrations is effective for P2X2 but not for P2X7, which is also present in taste buds from both WT and DKO mice. Together, these data suggest that Receptor cells might boost ATP secretion during taste stimulation by exciting autocrine P2X2 receptors. Loss of P2X2 receptors from Receptor cells as well as afferent nerves constitutes the cellular basis of taste failure in DKO mutant mice.

Discussion

ATP is a transmitter in mammalian taste buds, released from taste Receptor (Type II) cells when taste buds are stimulated by taste compounds. ATP is crucial for communication from taste buds to gustatory nerves (Finger et al., 2005) as well as for cell–cell communication within the taste bud (Huang et al., 2007; Dando and Roper, 2009). Our present results indicate that taste Receptor cells require functional P2X2 receptors for normal transmitter release, most likely as autocrine receptors. When excited by taste stimuli, Receptor cells from mutant mice lacking P2X2 and P2X3 purinoceptors (double knock out, DKO, mice) secrete significantly less ATP than do Receptor cells from WT mice. Mice lacking P2X2 and P2X3 receptors were previously shown to be taste-blind in nerve recordings and taste behavioral assays (Finger et al., 2005; Eddy et al., 2009; Hallock et al., 2009). The loss of taste in DKO mice was interpreted as due to the loss of postsynaptic purinoceptors, hence implicating ATP as a taste transmitter (Finger et al., 2005). The present study emphasizes that presynaptic functions—transmitter release—are also abnormal in DKO mice. Despite the reduction of ATP release, the anatomical and molecular features examined in taste buds from DKO mice appeared normal, and release of transmitter from Presynaptic cells (5-HT) was indistinguishable between DKO and WT mice.

The likely explanation for these unexpected findings is that P2X2 receptors, in addition to their well established expression on postsynaptic sensory afferent fibers (Bo et al., 1999; Cheung and Burnstock, 2002; Finger et al., 2005; Kataoka et al., 2006; Hayato et al., 2007), are also present presynaptically on Receptor cells. Our data show that presynaptic P2X receptors can produce

autocrine positive feedback to stimulate ATP secretion, similar to the role of the autocrine P2Y1 receptors there (Huang et al., 2009). Our data focused on P2X2 for which we could demonstrate expression in WT mice using RT-PCR. Neither we (here) nor Hayato et al. (2007) detected P2X3 in wild-type taste buds. In a similar manner to what is proposed here, autocrine P2X receptors on glucose-sensing pancreatic β cells enhance insulin secretion (Jacques-Silva et al., 2010), and expression of autocrine P2X receptors is a common theme for controlling ATP release at synapses in the nervous system (Dorostkar and Boehm, 2008). Further, P2X7 has been shown to complex with Px1 channels to influence secretion (Pelegrin and Surprenant, 2006), while P2X2 can functionally interact with nicotinic acetylcholine receptors (Decker and Galligan, 2010). Interestingly, P2X2 receptors are slower to desensitize than P2X3, and are much more sensitive than P2X7 (North, 2002). Thus, P2X2 receptors would be optimal candidates for autocrine regulation of ATP secretion.

This interpretation of the findings would readily explain why in the absence of P2X2 receptors (i.e., in DKO mice), taste-evoked depolarization is insufficient to reach levels required to fully open Px1 hemichannels for ATP release (Huang and Roper, 2010). It would also explain why slightly depolarizing taste buds with 50 mM KCl would rescue transmitter release and lead to a burst of ATP release in DKO mice (Fig. 3). Of course, ATP could also be a paracrine stimulus for adjacent Receptor cells: ATP released by one Receptor cell during taste activation might recruit neighboring Receptor cells, leading to two or more cells being activated. In short, taste-evoked transmitter release and signal output from taste buds appears to be controlled by a host of factors, including (1) positive feedback from P2X2 receptors by autocrine stimulation; (2) additional amplification by P2Y1 purinoceptors on Receptor cells excited by ADP generated by the robust ecto-ATPase expressed throughout the taste bud (Bartel et al., 2006; Huang et al., 2009); (3) ultimately, elimination of secreted ATP by the ecto-ATPase; and finally, (4) negative feedback from serotonin released by Presynaptic (Type III) taste cells (Huang et al., 2009).

Additional factors contributing to the loss of taste-evoked transmitter secretion in mice lacking P2X2 and P2X3 receptors include the possibility that knocking out these genes affects the expression of other genes that are coordinately regulated and that participate in taste-evoked transmitter secretion (Iacobas et al., 2003; Sladek and Song, 2008; Singh et al., 2009). Our examination of a few likely candidates [TRPM5 and Px1 (this paper) and gustducin, P2X7, and T1Rs (Finger et al., 2005)] did not reveal any obvious defects. However, we did notice a decrease in PLCβ2 expression in DKO mouse taste buds that might contribute to the loss of taste signaling but may also merely reflect a decreased number of Receptor cells in taste buds. We have not quantitatively tested this possibility. In any event, tastant-evoked calcium responses in DKO
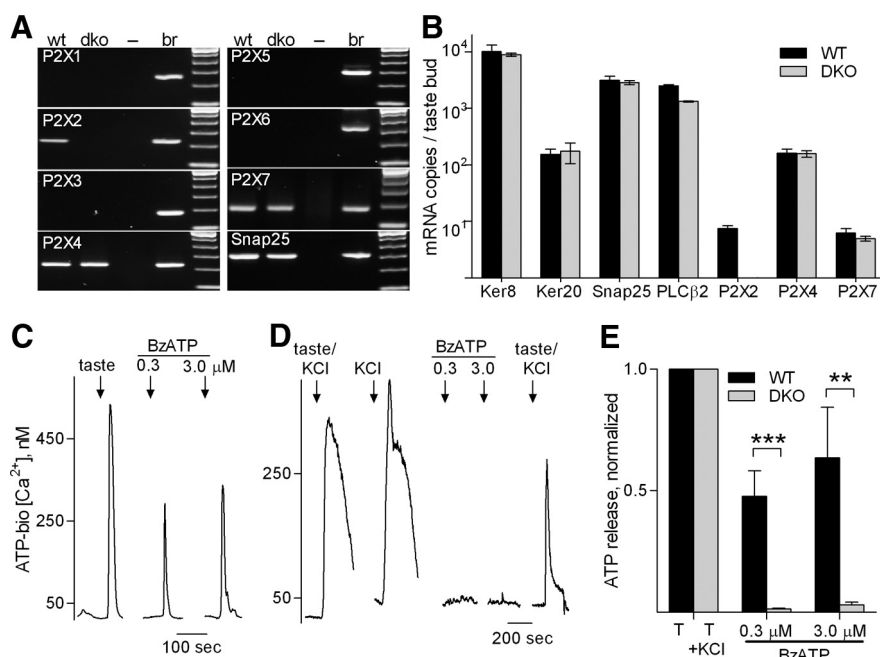

Figure 7. Taste-evoked ATP secretion is regulated via autocrine P2X2 receptors. *A*, RT-PCR for P2X1 to P2X7 receptors in mouse vallate taste buds from WT and DKO mice. Taste buds of WT mice express P2X2, P2X4 and P2X7. Taste buds of DKO mice express P2X4 and P2X7 only. Snap25 served as a control to confirm the presence of taste buds in RNA samples. Control reactions (brain as positive; no template as negative) were run in parallel. *B*, Quantitative RT-PCR on taste buds isolated from WT and DKO mice to assess expression levels of P2X2, P2X4, and P2X7 as well as taste bud-selective keratins (keratin 8 and keratin 20), Snap25, and PLCβ2, all as controls. Each bar represents the mean ($\pm$SEM) of three independent samples. *C*, *D*, Responses of biosensors measuring ATP secretion from taste buds of WT (*C*) or DKO (*D*) mice. ATP release was measured in response to taste stimulation, 0.3 and 3 μM BzATP (arrows above traces). *D*, As in Figure 2, taste stimulation for DKO taste buds, paired with depolarization with 50 mM KCl (arrows; taste/KCl), as well as strong depolarization (100 mM KCl) triggered ATP secretion. (Taste stimulation alone is ineffective in eliciting transmitter secretion in DKO mouse taste buds.) However, in contrast to taste buds from WT mice, BzATP failed to elicit ATP secretion. *E*, Summary of data from experiments as in *C* and *D*, from WT and DKO mouse taste buds. Biosensor responses to BzATP were normalized to taste-evoked responses for the same taste cell/biosensor pair. Bars show means $\pm$ SEM ($N = 3$ for WT; $N = 4$ for DKO). The BzATP-induced ATP release from WT taste buds was significantly larger than from DKO taste buds (***$p < 0.001$ at 0.3 μM BzATP; **$p < 0.01$ for 3 μM BzATP). T, Taste stimuli.

mice did not differ from those in WT mice. Finally, knocking out P2X2 and P2X3 may result in the loss of necessary retrograde signal(s) from the nerve fibers during the development and maintenance of taste buds. Transection of the taste nerves leads to rapid alterations in gene expression in the taste buds (Miura et al., 2004) with ultimate loss of differentiated taste cells (Farbman, 1969). Even simply blocking axoplasmic transport within the taste nerves results in functional and morphological loss of taste buds (Sloan et al., 1983). The absence of P2X2 and P2X3 receptors prevents activation of the taste nerves by taste stimuli (Finger et al., 2005); this loss of activity may lead to insufficient retrograde signals that contribute to taste transmitter secretion. The exact mechanisms by which neural activity may affect taste cell differentiation and function requires further study. In any case, we interpret the main defect transmitter release by taste cells in the DKO mice to be their loss of autocrine P2X2 on Receptor (Type II) taste bud cells with additional contributions as discussed above.

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