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Purinergic Receptors in the Circumvallate Papillae of Rats

Alana Marselle Montoya
University of Denver

PURINERGIC RECEPTORS IN THE CIRCUMVALLATE PAPILLAE OF RATS

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Alana M. Montoya

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Advisor: Dr. John C. Kinnamon

Author: Alana M. Montoya
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Abstract

$P2X_2$ receptors and $P2Y_4$ receptors are ATP-activated cell surface receptors that gate movement of K^+, Na^+, and Ca^{2+}. We used immunocytochemistry for $P2X_2$ and $P2Y_4$ receptors as well as taste cell type markers to learn if $P2X_2$ immunoreactivity (IR) is present in nerve processes in close apposition to Type II and/or Type III taste cells. We also tested to see if $P2Y_4$ IR is present in Type II and/or Type III taste cells. Our results indicate that $P2X_2$ is present only in intragemmal nerve processes. $P2X_2$-LIR nerve processes form close contacts with Type II and Type III taste cells. $P2Y_4$ IR is present in Type II cells that also display IP_3R3 IR, and in Type III cells with Syntaxin-1 IR and 5-HT IR. These data suggest that ATP stimulates $P2X_2$ receptors on nerve processes and $P2Y_4$ receptors present on both Type II cells and Type III cells.

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Introduction

The Taste Bud

The taste bud is an onion-shaped structure specialized for the detection of sapid chemical stimuli (S. Kinnamon and Margolskee, 1996; Lindemann, 1996; Gilbertson et al., 2000). A typical taste bud is composed of 50-150 neuroepithelial taste cells, extending from the basal lamina to the taste pore, where apical microvilli extend into the oral cavity and interact with sapid molecules (J. Kinnamon et al., 1985, 1988; S. Kinnamon and Margolskee, 1996; Gilbertson et al., 2000). There are three fields of lingual taste buds: fungiform, foliate and circumvallate (= vallate) (Whiteside, 1927). Fungiform papillae are located on the dorsal surface of the anterior two-thirds of the tongue (Gilbertson et al., 2000). The term, "fungiform", derives from the mushroom-like shape of these papillae. One to two taste buds are contained within a single rodent fungiform papilla. Hundreds of foliate papillae line the walls of epithelial folds on the posterolateral surfaces of the tongue. Rodents also contain a single circular or horseshoe-shaped circumvallate papilla located on the dorsal posterior surface of the tongue. This papilla is surrounded by a trench or moat of epithelium that is lined on both sides with several hundred taste buds.

Taste transduction begins when tastants interact with the apical microvilli of taste receptor cells that project into the oral cavity via the taste pore (Fig. 1) (J. Kinnamon, 1988; S. Kinnamon and Margolskee, 1996; Gilbertson, 2000). Microvilli possess ion

channels and G protein-coupled receptors, as well as other transduction machinery that interact with aqueous chemical stimuli and carry out taste transduction (S. Kinnamon and Margolskee, 1996). Interactions with aqueous chemical stimuli lead to changes in membrane conductance, cellular depolarization, and release of neurotransmitter onto afferent nerve processes (S. Kinnamon and Margolskee, 1996).

Innervation of Rodent Taste Buds

Rodent lingual taste buds are innervated by branches of the facial (VIIth) and glossopharyngeal (IXth) nerves (Whiteside, 1927; Oakley, 1967; Farbman and Hellekant, 1978; Whitehead et al., 1985). The chorda tympani branch of the facial (VIIth) nerve provides sensory innervation to fungiform papillae on the anterior two- thirds of the tongue and anterior foliate papillae (Farbman and Hellekant, 1978). Circumvallate and posterior foliate papillae of rodents are innervated by lingual-tonsillar fibers branching from the glossopharyngeal (IX) nerve (Whiteside, 1927; Oakley, 1967) (Fig. 2).

Taste Cell Types

Taste receptor cells have been classified into types based on species, type of papillae and ultrastuctural differences between the cells (Farbman, 1965; Murray and Murray, 1971; Murray, 1973; J. Kinnamon et al., 1985, 1988; Delay et al., 1986; Pumplin et al., 1997).

Type I Cells

Type I cells are electron-dense, elongate cells with invaginated nuclei and long microvilli extending into the taste pit (Murray, 1973) (Fig. 1). In contrast to other cell types, Type I cells possess sheet-like cytoplasmic processes that separate and envelop

Type II cells, Type III cells, and intragemmal nerves, much like Schwann cells (Murray, 1973; Royer and J. Kinnamon, 1994). Type I cells are thought to play a supportive role in the taste bud, and usually occupy peripheral positions (Lindemann, 1996). Type I cells express blood group antigen H (Pumplin et al., 1999), and are believed to release "pore substance" into the taste pore (Fujimoto, 1973, Ohmura et al., 1989). Bartel et al. (2006) found that the ecto-ATPase, NTPDase2, was present on the plasma membrane of Type I cells, possibly functioning in the degradation of ATP (Vlajkovic et al., 2007), which is now believed to be a neurotransmitter released from Type II cells (Baryshnikov et al., 2003; Finger et al., 2005; Romanov et al, 2007; Huang et al, 2007).

Type II Cells

Type II cells make up ~20% of the taste receptor cell population (J. Kinnamon et al., 1985, 1988; Lindemann, 1996). The cytoplasm of a Type II cell is electron-lucent and has circular to ovoid shaped nuclei (Fig. 1). The cytoplasm of Type II cells often contains swollen cisternae of smooth endoplasmic reticulum and numerous mitochondria. The apical process of a Type II cell terminates in several brush-like microvilli of equal length (Lindemann, 1996). Type II cells express the immunocytochemical markers IP_3R3 (Clapp et al., 2001) and PLCβ2 (Clapp et al., 2004). The G-protein, α-gustducin, is also present in a subset of Type II cells (Boughter et al., 1997, Yang et al., 2000b). These Type II cells are thought to perform a chemosensory function, but do not form classical synapses with nerve processes in the rat (Lindemann, 1996, Yang et al., 2000a; Finger et al., 2005).

Type III Cells

Type III cells have both electron-dense and electron-lucent regions (Fig. 1). Scattered throughout the cytoplasm are mitochondria, granular and smooth endoplasmic reticulum, free ribosomes, and polyribosomes (Royer and J. Kinnamon, 1994). They also have invaginated nuclei, but are best distinguished by their single blunt microvillus. Many small clear vesicles, as well as occasional large, dense-cored vesicles are present within Type III cells (Royer and J. Kinnamon, 1994). Conventional synapses onto nerve processes have been observed associated with Type III cells of the rat (Yang et al., 2000a; Yee et al., 2001). A subset of Type III cells expresses the immunocytochemical marker, serotonin (5-HT) (Kim and Roper, 1995; Yee at al., 2001), the presynaptic membrane protein SNAP-25 (Yang et al., 2000a), and the presynaptic T-SNARE membrane protein Syntaxin-1 (Yang et al., 2007). Clapp et al. (2004) found PLCβ2-immunoreactivity in a small subset of Type III cells. Immunoreactivity for protein gene product 9.5 (PGP 9.5) (Yee at al., 2001), and neural cell adhesion molecule (NCAM) (Nelson and Finger, 1993) is also present in Type III cells. The subsets of Type III taste cells displaying immunoreactivity for PGP 9.5 and 5-HT are mutually exclusive (Yee et al., 2001).

Transduction Pathways

Salt

The prototypical salty stimulus is table salt (NaCl) (S. Kinnamon, 1988; Gilbertson et al., 2000; Roper, 2007). The transduction of salt taste does not require specific membrane receptors (S. Kinnamon, 1988). Most taste cells responsive to salt

have epithelial-type sodium channels (ENaCs) that are blocked by amiloride (S. Kinnamon, 1988; Gilbertson et al., 2000). These amiloride-sensitive sodium channels are unaffected by voltage, and are continually open, thus the constant influx of Na^+ across the membrane depolarizes the cell (S. Kinnamon, 1988; Gilbertson et al., 2000; Roper, 2007).

Bitter

There are several transduction pathways for bitter taste. Bitter compounds such as denatonium and quinine bind directly to K^+ channels, preventing the release of K^+ from the cell, which leads to a depolarization of the cell (S. Kinnamon and Margolskee, 1996). Depolarization of the cell causes release of Ca^{2+} from intracellular stores (Ogura et al., 2002) that trigger the release of neurotransmitter (S. Kinnamon and Margolskee, 1996). Other bitter transduction pathways include specialized membrane receptor proteins for bitter compounds, such as T2R/TRB membrane receptors (Margolskee, 2001; Ogura et al., 2002; Roper, 2007), which activate G-protein coupled second messenger cascades. T2Rs activate the G-protein, gustducin, which is composed of α and β3γ13 subunits (Margolskee, 2001; Ogura et al., 2002). When denatonium binds to a G-protein coupled receptor it activates both the α- and βγ-subunits, causing a separate cascade of events (S. Kinnamon and Margolskee, 1996; Margolskee, 2001). α-Gustducin activates phosphodiesterase (PDE), decreasing cyclic adenosine 5'-monophosphate (cAMP) levels by converting it into adenosine monophosphate (AMP), allowing Ca^{2+} to enter the cell, and causing the release of neurotransmitter (Gilbertson et al., 2000, Ogura et al., 2002; Roper, 2007). β3γ13 activates PLCβ2, triggering inositol 1,4,5-triphosphate (IP_3)

synthesis, releasing Ca^{2+} from internal stores, which then causes release of neurotransmitter (S. Kinnamon and Margolskee, 1996; Clapp et al., 2001; Margolskee, 2001; Ogura et al., 2002).

Sweet

cAMP and IP_3 are believed to be the second messengers involved in the transduction of sweet stimuli (S. Kinnamon and Margolskee, 1996; Margolskee, 2001; Roper, 2007). Sugars bind to receptors (T1R2/T1R3), activating the G-protein G_s through one or more coupled G-protein coupled receptors (Margolskee, 2001; Roper, 2007). The activated G_s α-subunit then activates adenylyl cyclase, which catalyzes the formation of cAMP in the cytoplasm. cAMP may then directly cause influx of cations via cyclic nucleotide monophosphate-gated channels. cAMP may also directly activate protein kinase A (PKA), causing the phosphorylation of a K^+-selective channel, inhibiting K^+ release, depolarizing the cell, which allows Ca^{2+} to flow into the cell, stimulating neurotransmitter release (S. Kinnamon and Margolskee, 1996; Margolskee, 2001). Like bitter transduction, some sweeteners such as saccharin bind to a receptor, the α-subunit of G_q or the Gβγ subunits activate PLCβ2, causing phosphatidylinositol-4, 5-bisphosphate (PIP_2) to split into IP_3 and diacylglycerol (DAG). DAG stays within the plane of the membrane activating the downstream enzyme, protein kinase C. IP_3 synthesis releases Ca^{2+} from internal stores (S. Kinnamon and Margolskee, 1996; Margolskee, 2001; Roper, 2007).

Sour

Sour taste results from acidic stimuli, which help animals to avoid rotten foods or unripened fruits. It may also function to protect against ingesting excess dietary acid that can alter the body's acid-base balance (Roper, 2007). The functional group for sour stimulus is the proton (H^+), or more accurately, the hydronium ion (H_3O^+) (Roper, 2007). Protons are thought to act on ion channels in the membranes of acid-sensitive taste receptor cells, generating inward currents (Richter et al, 2004). Sour foods affect taste receptors in several ways; H^+ permeates the amiloride-sensitive sodium channel, the same channel involved in the transduction of salt, causing an inward H^+ current, depolarizing the cell (S. Kinnamon and Margolskee, 1996). H^+ also binds to and blocks K^+- selective channels, decreasing the K^+ levels across the membrane, depolarizing the cell (S. Kinnamon and Margolskee, 1996). The paracellular pathway also contributes to sour transduction, as protons seep into tight junctions between adjacent taste receptor cells (S. Kinnamon and Margolskee, 1996). Richter et al. (2003a) found that voltage-gated Ca^{2+} channels open in response to intracellular acidification in a small subset of taste cells. Ca^{2+} influx may lead to the release of neurotransmitter from synapses between nerve fibers and Type III cells (Richter et al., 2004). The mechanisms that occur by which cytoplasmic acidification stimulates an influx of Ca^{2+} in sour taste receptor cells remain unclear (Richter et al., 2004).

Umami

Umami is Japanese for "delicious". Umami taste results from amino acid/nucleotide stimuli (L-glutamate, L-aspartate, and the 5'-ribonucleotides, inosine 5'-

monophosphate (IMP) and guanosine 5'-monphosphate (GMP)) (He et al., 2004; Maruyama et al., 2006; Narukawa et al., 2006). There are two putative taste transduction pathways for umami. First, it is thought that umami stimuli depolarize taste cells by inhibiting resting K^+ conductance or by increasing conductance of Na^+ and Ca^{2+} channels (Narukawa et al., 2006). There are three G-protein coupled receptors proposed to be receptors for glutamate; mGluR4 (Chaudhari et al., 2000), the heterodimer T1R1/T1R3, and a truncated mGluR1 (Maruyama et al., 2006). The candidate umami receptor (T1R1/T1R3) is co-expressed with α-gustducin in murine fungiform and circumvallate papillae (Narukawa et al., 2006; Roper, 2007). For the second pathway, umami stimuli bind to the G-protein coupled receptor, T1R1/T1R3. The alpha subunit of the G-protein, perhaps α-gustducin or rod α-transducin (He et al., 2004), stimulates PDE, (Narukawa et al., 2006) which in turn converts cAMP into AMP. A decrease in cAMP levels may modify other channels (S. Kinnamon et al., 2005; Roper, 2007).

Synapses

Conventional synapses in taste buds are functional connections between a presynaptic taste cell (Type III cell) and a postsynaptic nerve process. Criteria for identifying a synapse in a rodent taste bud include; 1) a thickening of the presynaptic membrane, 2) a 15-30nm cleft between the parallel membranes, and 3) vesicle clusters adjacent to the thickened membrane (Royer and J. Kinnamon, 1991; J. Kinnamon and Yang, 2007). Presynaptic cells release neurotransmitters via exocytosis, thereby stimulating or inhibiting a postsynaptic cell. Sites of neurotransmitter release are referred to as active zones. Synaptic vesicles are concentrated in the cytoplasm adjacent to the

active zones and a few are docked onto the presynaptic membrane (J. Kinnamon and Yang, 2007). Postsynaptic neurotransmitter receptors are located just below the postsynaptic membrane in a region referred to as the postsynaptic density. These postsynaptic neurotransmitter receptors transduce the intercellular chemical signal into an intracellular chemical signal within the postsynaptic cell (Clements, 1996).

Approximately 20% of mouse taste cells form synapses onto nerve processes in circumvallate taste buds (J. Kinnamon et al., 1985). Only Type III cells within rodent taste buds have been observed to form synapses with afferent nerve fibers (Yang et al., 2000a, Yee at al., 2001). Type II cells are now thought to transmit taste information via non-vesicular release (Finger et al., 2005; Huang et al., 2007). Fewer synapses are present in fungiform taste buds. Fungiform taste buds possess more vesicles than synapses when compared with circumvallate or foliate taste buds (J. Kinnamon et al., 1993). Taste cell synapses are generally located near the nuclear region of the presynaptic taste cell (Type III cell) (J. Kinnamon et al., 1985, 1988; Yang et al., 2000a).

Type II cells contain the receptors for bitter, sweet, and umami, yet they do not form classical synapses onto nerve fibers (Yang et al., 2000a; Clapp et al. 2004, 2006; Finger et al., 2005). Taste transduction pathways involving Type II cells may use nonsynaptic pathways between taste cells, as well as from taste cells onto nerve fibers (Huang et al., 2007; Romanov et al., 2007; Yang et al., 2008).

Other Potential Functional Contacts

Atypical Mitochondria

Large, atypical mitochondria are often present at close appositions between Type II cells and nerve processes. These atypical mitochondria are long structures that can be two or three times larger in diameter than conventional mitochondria in taste cells (Royer and J. Kinnamon, 1988). The taste cell membrane lies parallel to the outer membranes of atypical mitochondria separated by a gap. Atypical mitochondria do not possess lamellar cristae as do "normal" taste cell mitochondria. Instead, atypical mitochondria possess "twisted-energized" or "swollen twisted-energized" cristae (Green and Baum, 1970; Korman et al., 1970; Williams et al., 1970), which resemble electron-dense sacs or tubules inside the mitochondrion (Royer and J. Kinnamon, 1988). Such cristae have been shown to be present in metabolically active cells such as cancer cells (Watanabe and Burnstock, 1976). Atypical mitochondria may regulate the uptake and release of Ca^{2+} (Hajnóczky et al., 2001; Hawkins et al., 2007), modulating the open/closed state of pannexin/connexin gap junction hemichannels, which would gate ATP release from Type II cells onto nerve processes or other taste cells (Baryshnikov et al.; 2003, Kataoka et al., 2004; Yang et al., 2008).

Subsurface Cisternae

Subsurface cisternae of smooth endoplasmic reticulum lie next to the cytoplasmic leaflet of taste cell membranes at contacts between Type II taste cells and nerve processes (Royer and J. Kinnamon, 1988; Yang et al., 2008). The cytoplasmic leaflet of the taste cell membrane is separated from the outer membranes of the cisternae by a small space

(Royer and J. Kinnamon, 1988; Yang et al., 2008). These subsurface cisternae are sometimes associated with atypical mitochondria and may also be associated with modulating Ca^{2+} levels (Royer and J. Kinnamon, 1988; Yang et al., 2008).

Gap Junctions and Hemichannels

One common form of intercellular communication is the gap junction (Panchin, 2005; Barbe et al., 2006; Litvin et al., 2006). Gap junctions are composed of the membrane protein, connexin, that forms channels permeable to ions and small molecules (Panchin, 2005; Dahl and Locovei, 2006; Litvin et al., 2006). A proposed model for gap junction hemichannels is produced when six connexin subunits oligomerize, forming a hexameric torus (Panchin, 2005). The gap junction that is formed is a pair of hemichannels, connexons, one from each adjacent cell (Panchin, 2005; Barbe et al., 2006; Litvin et al., 2006). The intercellular space is approximately 2-4 nm at a gap junction (Panchin, 2005; Litvin et al., 2006). Gap junctions are present in nearly all tissue types found in the human body, as well as circulating blood cells (Panchin, 2005; Litvin et al., 2006).

Connexins

Connexins are proteins that form hexamers, often referred to as connexons, gap junction hemichannels, or hemichannels (Saez, 2003). Individual connexins contain four membrane-spanning α-helices in addition to intracellular N and C termini (Panchin, 2005). Gap junctions consist of two hemichannels. The hexameric assembly of hemichannels occurs in the endoplasmic reticulum, the Golgi, or post Golgi compartments (Saez, 2005). Vesicles transport connexins to a cell's surface. Connexins

are inserted by means of vesicular fusion. Once inserted, connexins dock with another hemichannel located in a juxtaposed membrane. This mechanism forms a cell-cell connexin hemichannel (Saez, 2005). Connexin hemichannels, or connexons, have also been associated with the release of messengers like ATP and glutamate (Leybaert, 2003). Prior to the formation of gap junctions, connexin hemichannels provide an alternative pathway for the release of ATP, glutamate, NAD+, and prostaglandin E2 from cells, which serve as paracrine messengers (Evans, 2006). Recent evidence indicates that the opening of hemichannels may occur under physiological and pathological circumstances in astrocytes and other types of cells (Saez, 2003). Responses to numerous categories of external changes, such as mechanical, shear, ionic, or ischemic stress may cause a connexin hemichannel to open (Evans, 2006). Intracellular signals like membrane potential, phosphorylation, and redox conditions also influence connexin hemichannel responses (Evans, 2006).

Pannexins

Two families are associated with gap junctions: connexins and pannexins. Connexins are present only in chordates, while pannexins are found in both chordates and invertebrates (Panchin, 2005; Litvin et al, 2006). Connexins were once considered the only class of vertebrate proteins with the capability of forming gap junctions (Litvin et al, 2006). Recent discoveries indicate that pannexins may form gap junctions (Dahl and Locovei, 2006). Failed attempts to clone connexins from invertebrates led to alternative gap junction protein contenders. It has been proposed that invertebrate gap junction assembly may be related to pannexins, proteins not part of the connexin gene family

(Panchin, 2005). Three pannexins have been described in human and rodent genomes, PANX1, PANX2, and PANX3 (Litvin et al, 2006). PANX1 and PANX3 are closely related in mammals, where as PANX2 is not (Baranova et al., 2004). Pannexins expression is found in several brain structures, including both neurons and glial cells (Litvin et al, 2006). Pannexins are highly conserved at the genetic level among worms, mollusks, insects, and mammals (Baranova et al., 2004). The predicted structure for pannexins includes four transmembrane regions, one intracellular loop, two extracellular loops, and intracellular N and C termini (Baranova et al., 2004). Pannexins share similar membrane topology with connexins, including regularly spaced cysteine residues found in the two extracellular loops that connect the transmembrane domains (Barbe et al., 2006).

ATP as a Neurotransmitter

Burnstock (1972) first postulated that ATP acts as a neurotransmitter in taste transduction. Finger et al. (2005) found that taste cells release ATP as a neurotransmitter. $P2X_2/P2X_3$ double knockout mice almost entirely blocked nerve responses to bitter, sweet, and umami stimuli (Finger et al., 2005). These results suggest that ATP may act as a neurotransmitter in taste transduction. Type II cells possessing receptors for bitter, sweet, and umami; do not form classical synapses onto nerve fibers (Yang et al., 2000a; Clapp et al. 2004, 2006; Finger et al., 2005), therefore, taste transduction pathways utilizing Type II cells are thought to transmit taste information by non-vesicular release of ATP (Finger et al., 2005; Huang et al., 2007; Romanov et al, 2007). ATP released through non-vesicular means by Type II cells was observed by Romanov et al. (2007)

and Huang et al. (2007). Results from Romanov et al. (2007) favor voltage-gated outward currents causing depolarization-elicited release of ATP through connexin-43 hemichannels. Huang et al. (2007) prefer a Ca^{2+} and voltage dependent release of ATP through pannexin-1 hemichannels.

Hemichannel Mediated Release of ATP

The identification of afferent neurotransmitters in taste buds remains elusive (Finger et al, 2005). Recent studies have determined that hemichannels may mediate afferent neurotransmission in taste cells among mammals (Romanov et al, 2007). Taste nerves express the ionotropic purinergic receptors $P2X_2$ and $P2X_3$, suggesting that ATP acts as a neurotransmitter in this system (Finger et al, 2005). Recent studies have focused on which taste cell type releases ATP, and the mechanisms (Huang et al, 2007) for transmitter release. Huang et al. (2007) have shown that gustatory stimuli cause receptor cells to release ATP through pannexin-1 hemichannels in the taste buds of mice. ATP may then stimulate serotonin (5-HT) secretion release from Type III taste cells (Huang et al, 2007). Only Type III taste cells (presynaptic cells) release 5-HT, while Type II taste cells (receptor cells) release ATP (Romanov et al, 2007, Huang et al, 2007). Because Type II cells lack classical synapses, non-vesicular methods of ATP release have been proposed (Romanov et al, 2007, Huang et al, 2007). Glial cells and erythrocytes release ATP through gap junction hemichannels, which led to the hypothesis that hemichannels may be involved in the release of ATP from taste receptor cells (Huang et al, 2007). Romanov et al. (2007) postulated that hemichannels are present only in Type II taste cells (Fig. 4 and Fig. 5). Their results argue strongly in favor of connexin hemichannels in

mediating voltage-gated outward currents and depolarization-elicited secretion of ATP (Fig. 3) (Romanov et al, 2007). Huang et al. (2007) found both connexin-30 and connexin-43 were either completely absent from, or expressed at insignificant levels in taste buds. These connexins were expressed in perigemmal cells on the periphery adjacent to taste buds (Huang et al, 2007). Using quantitative RT-PCR, Huang et al, found pannexin-1 in abundance in taste buds when compared with surrounding non-taste epithelium. They proposed an expression pattern of pannexin-1 as the major hemichannel in taste cells, functioning in tastant-elicited ATP release from receptor cells (Huang et al, 2007).

P2 Receptors

P2 receptors are receptors located on a cell's surface that are activated by ATP, ADP, or UTP in order to balance intracellular Ca^{2+} levels (Burnstock, 2006). ATP-responsive taste cells are found in abundance in circumvallate, foliate, and fungiform papillae (Baryshnikov et al., 2003). P2 receptors for extracellular nucleotides are separated into two categories: P2X receptors are ionotropic, whereas P2Y receptors are G-protein coupled receptors (Burnstock, 2006). Seven human P2X ($P2X_{1-7}$) receptor subunits have been cloned (Burnstock, 2006), showing 30-50% sequence identity at the protein level (Erb et al., 2006). Signal transduction in P2X receptors is simple in comparison with P2Y receptors (Erb et al., 2006). Currently, eight P2Y ($P2Y_{1, 2, 4, 6, 11, 12, 13, \text{and } 14}$) receptor subtypes have been cloned (Burnstock, 2006). Functional evidence associates P2Y-like receptors with ATP-dependent Ca^{2+} mobilization and ionic current modulation in mouse taste cells (Kim et al., 2000). The observations made by Kim et al.

also indicate a role for ATP as a neuromodulator that operates in the taste bud (Baryshnikov et al., 2003).

P2X Receptors

P2X receptors are widely distributed and have been observed in neurons, glial cells, bone, muscle, endothelium, epithelium, and hematopoietic cells (Ralevic and Burnstock, 1998; Bo et al., 1999; Abbracchio et al., 2003; Burnstock, 2004; Erb et al, 2006). Studies have shown that P2X receptors may be associated with fast synaptic transmission, neurotransmitter release, and the generation of pain signals in the central and peripheral nervous systems (Ralevic and Burnstock, 1998; Bo et al., 1999; Abbracchio et al., 2003; Burnstock, 2004; Erb et al, 2006). The predicted structure for a P2X subunit includes a transmembrane protein that has two membrane spanning domains (TM1and TM2) (Erb et al., 2006). TM1 and TM2 are involved in gating the ion channel, as well as lining the ion pore, respectively (Erb et al., 2006). The TM regions are detached by a large hydrophilic extracellular loop that contains several conserved amino acids, an ATP-binding site, and a hydrophobic H5 region, that may be important for regulating metal cations (Erb et al., 2006). The intracellular N and C termini contain consensus phosphorylation sites for protein kinases A and C (Burnstock, 1997). These protein kinases have been shown to be involved in the modulation of $P2X_2$ receptor activity (Erb et al., 2006).

P2X receptors are ATP-gated ion channels that act as a go-between for sodium and calcium influx, as well as potassium efflux, leading to the depolarization of the cell. (Dubyak, 1991; Bo et al., 1999; North, 2002; Burnstock, 2006; Volonté et al., 2006). An

extracellular ATP activated channel, composed of P2X receptor subunits, opens allowing cations, such as Ca^{2+}, Na^{+}, and K^{+}, to move across the plasma membrane (Erb et al., 2006). This results in changes of electrical potential of the cell, which then transmits a signal. The depolarization of the membrane activates voltage-gated calcium channels. Activation of P2X receptors causes calcium ions to accumulate in the cytoplasm, and is responsible for the activation of numerous signaling molecules (Erb et al., 2006).

Finger et al. (2005) found $P2X_2$ and $P2X_3$ receptors on nerve fibers that innervate rat taste cells. The trigeminal branch of the lingual nerve and laryngeal taste buds were also found to possess P2X receptors (Rong et al., 2000; Finger et al., 2005). Experiments using $P2X_2$ and $P2X_3$ double knockout mice showed that these knockouts are unresponsive to bitter, sweet, and umami. Such findings suggest that $P2X_2$ and $P2X_3$ receptors are involved in taste signaling (Finger et al., 2005).

P2Y Receptors

P2Y receptors are G-protein coupled receptors that are activated by purine and/or pyrimidine nucleotides (Dubyak, 1991; Ralevic and Burnstock, 1998; Abbracchio et al., 2003; Baryshnikov et al., 2003; Lazarowski et al., 2003; Kataoka et al., 2004; Burnstock, 2006; Bystrova et al., 2005; Erb et al., 2006; Volonté et al., 2006). Currently, eight P2Y ($P2Y_{1, 2, 4, 6, 11, 12, 13, \text{ and } 14}$) receptor subtypes have been cloned (Erb et al., 2006). Within the P2Y family, $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ are receptors that are activated by ADP, $P2Y_6$ is activated by UDP, and $P2Y_{11}$ has a preference for ATP as an agonist, while both human and rodent $P2Y_2$ and $P2Y_4$ are equally receptive to ATP and UTP (Abbracchio et al., 2003; Lazarowski et al., 2003).

The predicted structure of a P2Y receptor includes an extracellular N terminus that has several potential N-linked glycosylation sites, as well as seven transmembrane spanning regions that aid in forming the ligand binding pocket. There is an intracellular C terminus, which contains several phosphorylation sites for protein kinases (Erb et al., 2006).

Signal transduction involving P2Y receptors is more complex compared with P2X receptors. Pathways of communication between P2Y G-protein coupled receptors rely on intracellular signaling cascades. Signaling cascades operate by sequential activation or deactivation of heterotrimeric or monomeric G proteins, phospholipases, protein kinases, adenylyl and guanylyl cyclases, and phosphodiesterases that control cellular processes such as proliferation, differentiation, apoptosis, secretion, metabolism, and cell migration (Ralevic and Burnstock, 1998; Baryshnikov et al., 2003;; Kataoka et al., 2004; Erb et al., 2006). Numerous ion channels, cell adhesion molecules, and receptor tyrosine kinases are modulated by P2Y receptors (Erb et al., 2006). By interacting with G-proteins, P2Y receptors can modulate voltage-gated ion channels, control transmembrane ion transport, and regulate negative feedback of neurotransmitter release by inhibiting Ca^{2+} channels (Kim et al., 2000; Kataoka et al., 2004; Erb et al., 2006; Volonté et al., 2006).

Bystrova et al. (2005) found evidence that mouse taste cells express P2Y receptors that are coupled to the production of IP_3 and the mobilization of Ca^{2+}. ATP and UTP are believed to mobilize intracellular Ca^{2+} in the taste buds of the mouse, which suggests that receptors $P2Y_2$ and $P2Y_4$ may be involved (Bystrova et al., 2005). The

presence of $P2Y_2$ and $P2Y_4$ has been confirmed in taste receptor cells from circumvallate and foliate papillae, using RT-PCR and immunohistochemistry. These results suggest that $P2Y_2$ and $P2Y_4$ receptors play a major role in ATP and UTP signaling within the taste cell (Bystrova et al., 2005). $P2Y_4$ is believed to be expressed by both Type II and Type III cells (Baryshnikov et al., 2003; Kataoka et al., 2004; Yang et al., 2008).

Hypotheses

Hypothesis 1:

Finger et al. (2005) found $P2X_2$ and $P2X_3$ receptors on nerve fibers that innervate rat taste buds. The trigeminal branch of the lingual nerve and laryngeal taste buds also possess P2X receptors (Rong et al., 2000; Finger et al., 2005). Experiments using $P2X_2$ and $P2X_3$ double knockout mice showed that these knockouts were unresponsive to bitter, sweet, and umami. Such findings suggest that $P2X_2$ and $P2X_3$ receptors are essential for taste signaling (Finger et al., 2005).

I hypothesize that $P2X_2$ receptors are located on nerve processes that are closely apposed to Type II taste cells and Type III taste cells. Immunofluorescence microscopy and DAB- immunoelectron microscopy will be used to test for the presence and dispersal of $P2X_2$ receptors on nerve processes within close proximity to Type II and Type III taste cells in the circumvallate papillae of rats.

Hypothesis 2:

Bystrova et al. (2005) proposed that mouse taste cells express P2Y receptors that are coupled to the production of IP_3 and the mobilization of Ca^{2+}. ATP and UTP also mobilize intracellular Ca^{2+} in the taste tissue of mice, which suggests that receptors $P2Y_2$

and $P2Y_4$ may be involved in taste signaling (Bystrova et al., 2005). The presence of $P2Y_2$ and $P2Y_4$ has been confirmed in taste bud cells from circumvallate and foliate papillae, using RT-PCR and immunohistochemistry. These results suggest that $P2Y_2$ and $P2Y_4$ receptors play a major role in taste cell responses to ATP (Bystrova et al., 2005). $P2Y_4$ is hypothesized to be expressed in both Type II and Type III cells (Baryshnikov et al., 2003, Kataoka et al., 2004).

I propose to determine if $P2Y_4$ receptors are present on both Type II and Type III taste cells (Fig. 5). Immunofluorescence microscopy and DAB- immunoelectron microscopy will be used to test for the presence and dispersal of $P2Y_4$ receptors on Type II and Type III taste cells in the circumvallate papillae of rats.

Figure 1. Electron micrograph of a mammalian taste bud showing three types of taste cells. Type I (I) cells are electron-dense, elongate cells with invaginated nuclei and several slender, long microvilli. Type II (II) cells are electron-lucent, have circular to ovoid shaped nuclei, and several brush-like microvilli of equal length. Type III (III) cells have both electron dense and electron lucent regions. These cells have invaginated nuclei, a single blunt microvillus, as well as dense-cored vesicles and small clear vesicles. Type III cells form conventional synapses onto nerve processes. Basal lamina (BL). Taste pore (TP). Connective tissue (CT). Nerve processes (arrows). X 2,250. (From Royer and Kinnamon, 1991).

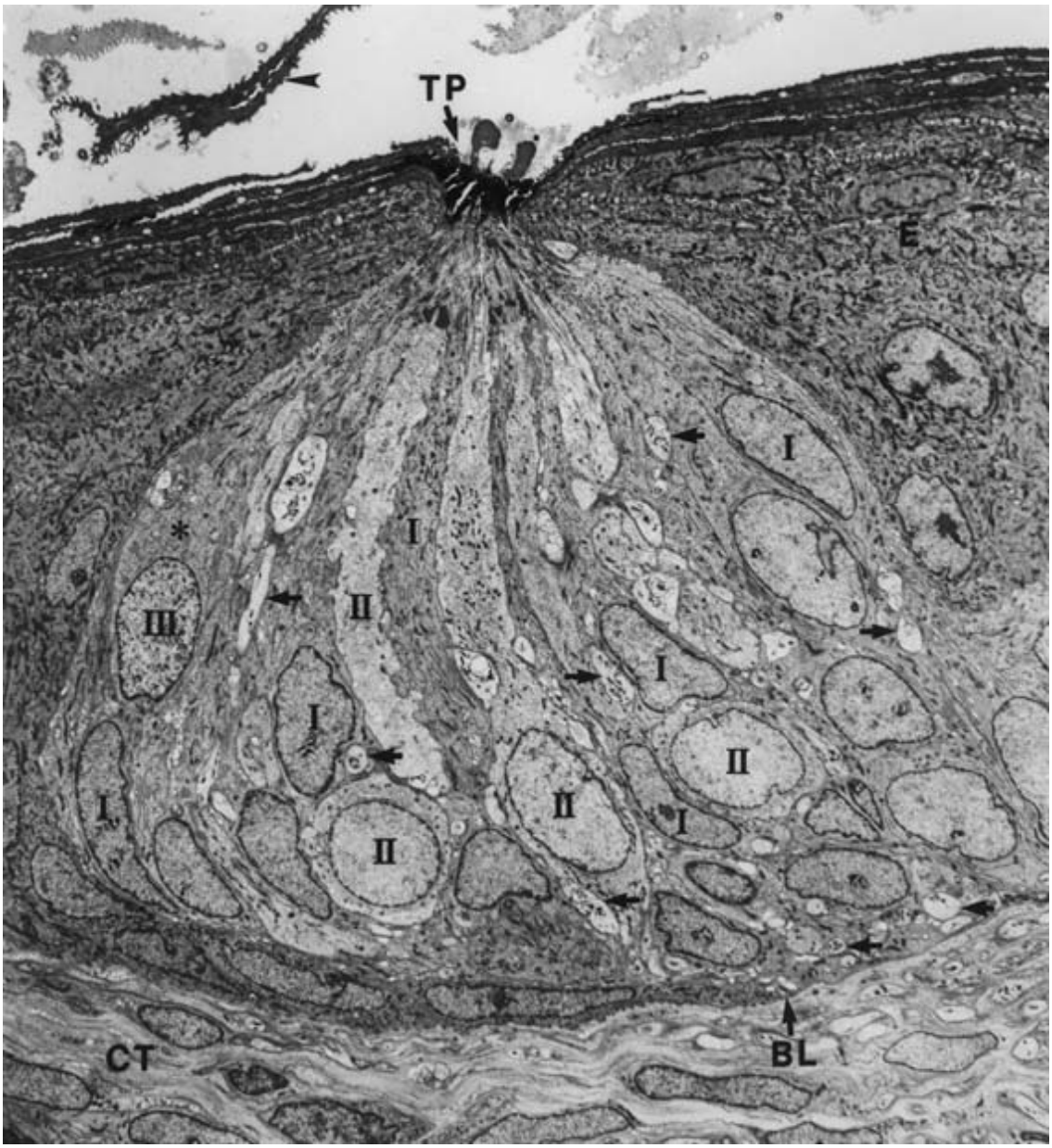

TP
E
I
I
*
III
II
I
I
II
II
I
II
I
CT
BL

Figure 2. Innervation of rodent lingual taste buds occurs via the chorda tympani branch of the facial (VIIth) and glossopharyngeal (IXth) nerves (Whiteside, 1927). The chorda tympani branch of the facial (VIIth) nerve innervates rodent fungiform papillae on the anterior two- thirds of the tongue and anterior foliate papillae. The glossopharyngeal (IXth) nerve innervates the circumvallate and posterior foliate papillae (From Kinnamon and Yang, 2007).

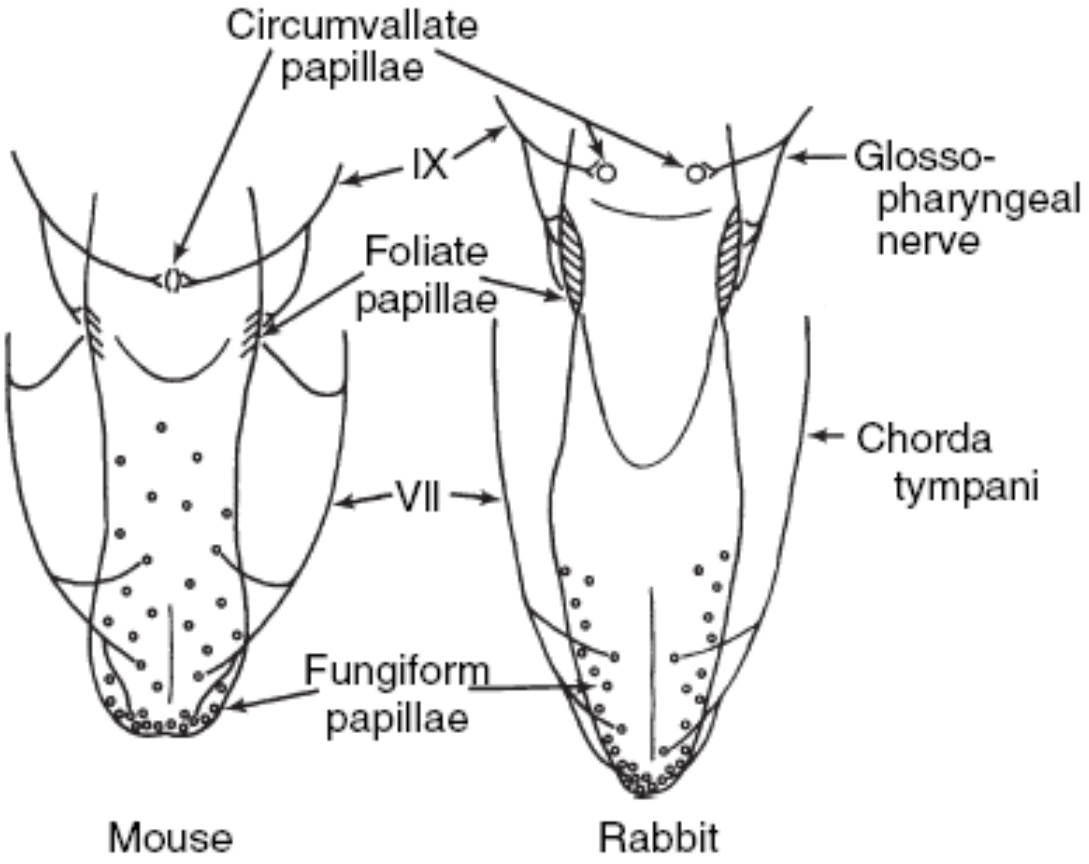

Circumvallate papillae
IX
Glosso-pharyngeal nerve
Foliate papillae
Chorda tympani
VII
Fungiform papillae
Mouse
Rabbit

Figure 3. Proposed series of intracellular events resulting from gustatory stimulation of Type II cells that result in the release of ATP via hemichannels. Sapid stimuli bind to a taste receptor, activating a G protein, upregulating the enzyme phospholipase C. Phospholipase C catalyzes a reaction that produces diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 binds to the IP_3R3 receptors present in smooth endoplasmic reticulum, releasing Ca^{2+} from internal stores. Ca^{2+} opens TRPM5 channels, allowing an influx of Na^+, causing depolarization of the cell. The depolarization results in the opening of voltage-gated Na^+ channels, allowing for greater influx of Na^+, depolarizing the cell. The depolarization opens the hemichannels present on the plasma membrane, allowing the efflux of ATP from the cell. ATP then binds to $P2X_2$ receptors present on an adjacent intragemmal nerve process. (From Romanov et al., 2007).

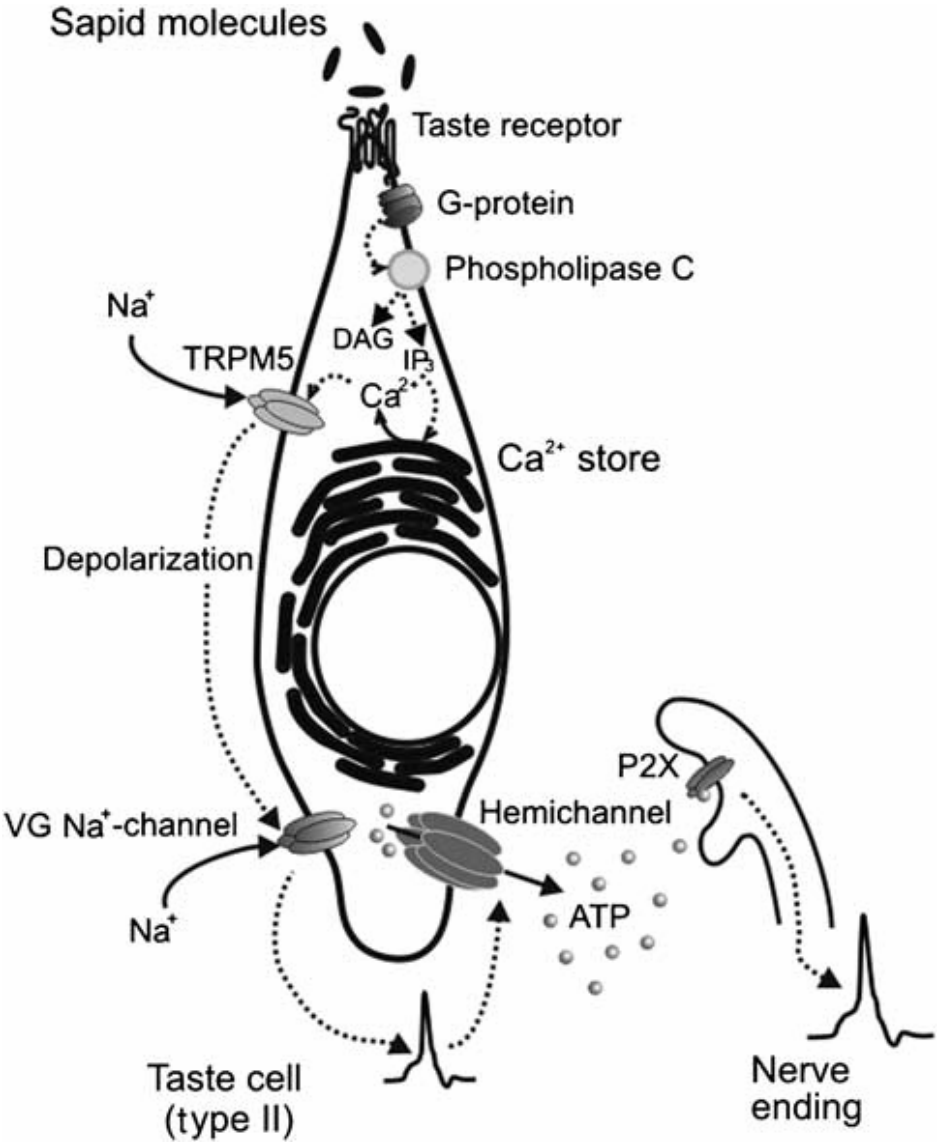

Sapid molecules
Taste receptor
G-protein
Phospholipase C
Na+
TRPM5
DAG
IP3
Ca2+
Ca2+ store
Depolarization
P2X
VG Na+-channel
Hemichannel
ATP
Na+
Taste cell
(type II)
Nerve
ending

Figure 4. Diagram showing proposed contacts between pannexin/connexin hemichannels on Type II cells and $P2X_2$ receptors found on adjacent nerve processes. AM- Atypical mitochondria. (From J. Kinnamon Grant, 2008). Ca^{2+} release from atypical mitochondria, present in Type II cells, stimulate the opening of pannexin/connexin hemichannels, present on the cell membrane. Opening of pannexin/connexin hemichannels elicits release of intracellular ATP which binds to $P2X_2$ receptors present on a "postsynaptic" nerve fiber.

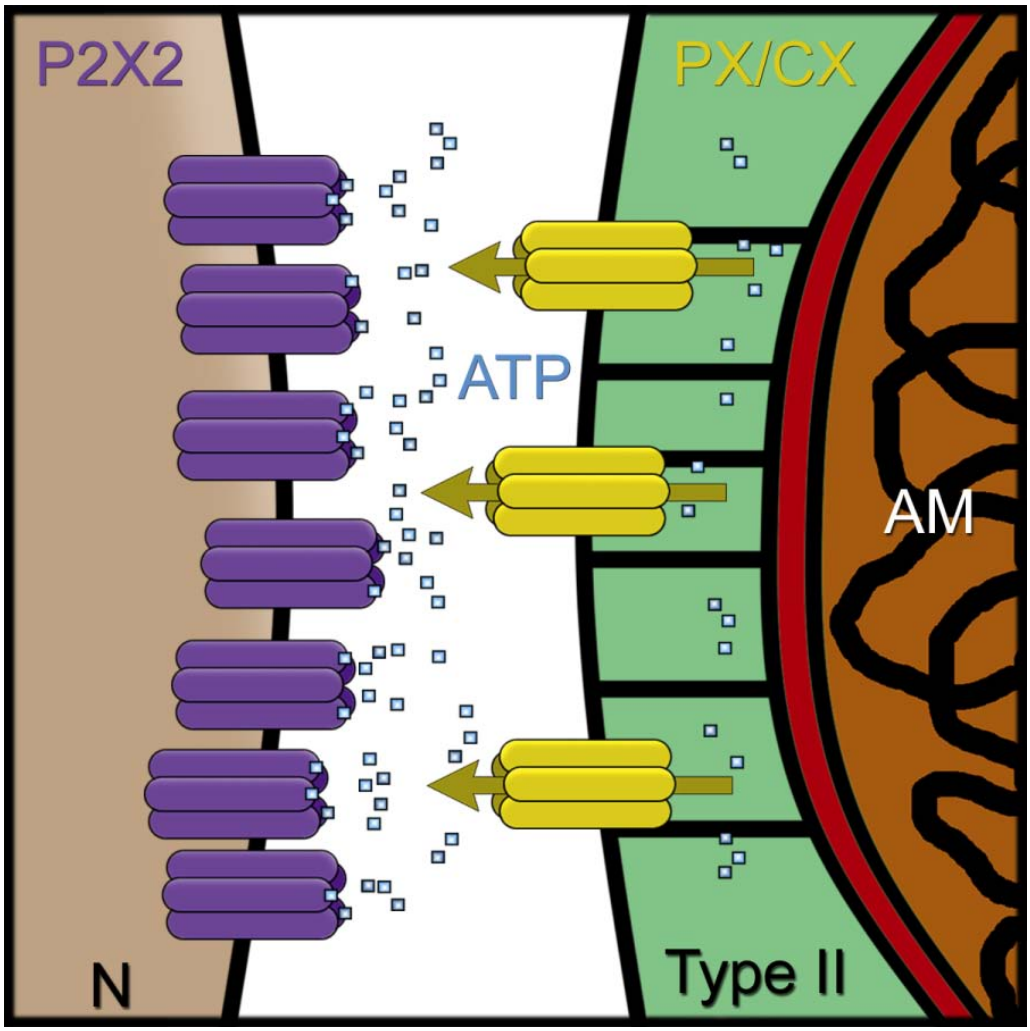
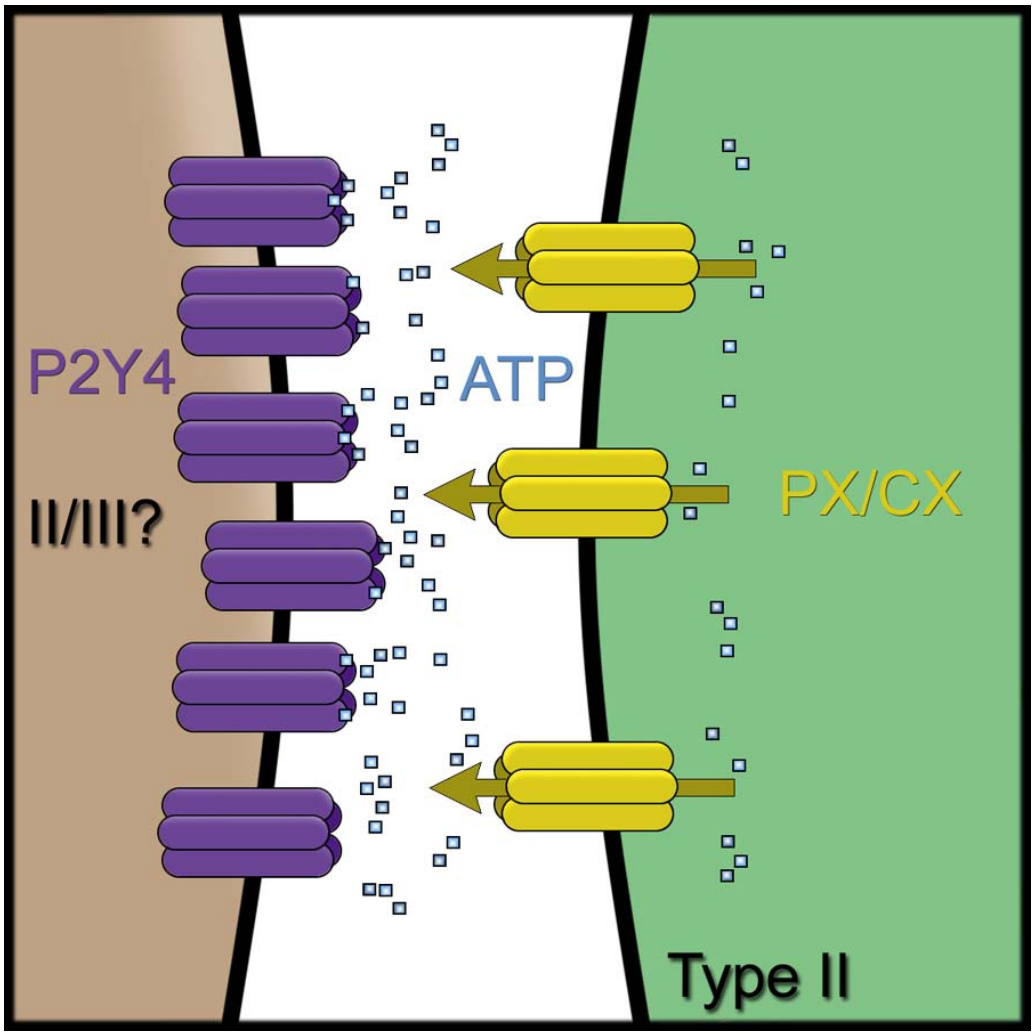

P2X2
PX/CX
ATP
AM
N
Type II

Figure 5. Diagram showing schematic model of contacts between pannexin/connexin hemichannels on Type II cells and $P2Y_4$ receptors found on nearby Type II or Type III cells. (From J.Kinnamon Grant, 2008). Intracellular Ca^{2+} release in Type II cells activates the opening of pannexin/connexin hemichannels, releasing ATP that binds to $P2Y_4$ receptors present on nearby Type II or Type III taste cells.


P2Y4
ATP
II/III?
PX/CX
Type II

Materials and Methods

All experiments were done using adult Sprague-Dawley male rats (250-350g). Rats were housed and cared for in facilities supported by the Institutional Animal Care and Use Committee of the University of Denver. Each rat was anesthetized with an injection of 240 mg/kg body weight of ketamine-HCl. $P2X_2$ and $P2Y_4$ colocalizations done with serotonin (5-HT) included pre-injection of 80 mg/kg body weight of 5-hydroxytryptophan, 1 hour prior to perfusion, in order to increase 5-HT-LIR. The primary and secondary antibodies used in these experiments are listed in tables 1 and 2, respectively.

Immunocytochemistry

Twenty-one rats were perfused for 1 minute through the left ventricle with 0.1% sodium nitrite, 0.9% sodium chloride, and 100 units of sodium heparin in 100 mL 0.1M PO_4 buffer (pH 7.3). Animals were fixed for 10 minutes with 4% paraformaldehyde in 0.1M PO_4 buffer (Weedman et al., 1996). All perfusion solutions were warmed to 42 °C before use. Tongues were removed and fixed for 3 hours at 4°C in fresh 4% paraformaldehyde in 0.1M PO_4 buffer. After fixing, tongues were cryoprotected in 30% sucrose in 0.1M PO_4 buffer overnight at 4 °C.

Single Labeling

Sections were cut 20 µm thick using a cryostat (HM 505E, Micron; Laborgeräte GmbH). Next, sections with circumvallate papillae were washed three times for 10

minutes in 0.1M phosphate buffered saline (PBS; pH 7.3) at room temperature. The sections were then blocked in a solution of 5% normal goat serum, 1% bovine serum albumin, and 0.3% Triton X-100 in 0.1M PBS for 1.5 hours on ice. Sections were incubated in rabbit polyclonal $P2X_2$ primary antibody or rabbit polyclonal $P2Y_4$ primary antibody in 0.1M PBS, overnight at 4 °C. After incubation with the primary antibodies, the sections were washed three times for 10 minutes in 0.1M PBS at room temperature, and were then placed in Cy5 goat anti-rabbit IgG secondary antibody in 0.1M PBS for 1 hour at room temperature. Following incubation with secondary antibodies, sections were washed three times for 10 minutes in 0.1M PBS at room temperature, and were mounted on slides. Images were obtained using an Axioplan 2 fluorescence microscope under 40X oil objective using a Zeiss apotome for confocal imaging (Carl Zeiss Inc.)

Double Labeling

Sections were cut 20 μm thick using a cryostat (HM 505E, Micron; Laborgeräte GmbH). The sections that included circumvallate papillae were washed three times for 10 minutes in 0.1M PBS at room temperature. For experiments that involved IP_3R3, antigen retrieval was performed using 10mM sodium citrate (pH 9.0) at 80 °C for 20 minutes. Sections were then blocked in a solution of 5% normal goat serum, 1% bovine serum albumin, and 0.3% Triton X-100 in 0.1M PBS for 1.5 hours on ice. Next, sections were incubated in either rabbit polyclonal primary $P2X_2$ antibody or rabbit polyclonal primary $P2Y_4$ antibody and mouse monoclonal primary serotonin (5-HT), IP_3R3, or Syntaxin-1 antibody in 0.1M PBS overnight at 4°C. The sections were washed three times for 10 minutes in 0.1M PBS at room temperature, and were placed in Cy5 goat

anti-rabbit IgG secondary antibody and FITC goat anti-mouse IgG secondary antibody in 0.1M PBS for 1 hour at room temperature. Sections were then washed three times for 10 minutes in 0.1M PBS at room temperature, and were mounted on slides. Images were obtained under 40X oil objective with a Zeiss Axioplan 2 fluorescence microscope using Axiovision software with an Apotome for confocal imaging (Carl Zeiss Inc.). To adjust the brightness and contrast of the images, Adobe Photoshop CS (San Jose, CA) was used.

Controls

Control experiments involved the procedures listed above, but excluded the primary antibody in order to check for species related cross-reactivity. No cross-species immunoreactivity was observed. For a peptide $P2X_2$ control, 3μL of Alomone's polyclonal rabbit primary $P2X_2$ antibody was pre-incubated with 3μL of antigen peptide for 1 hour at room temperature, as instructed by the manufacturer. The same peptide control procedure was used for Alomone's polyclonal rabbit primary $P2Y_4$ antibody.

Diaminobenzidine Electron Microscopy

Eight rats were perfused as for immunocytochemistry. Following perfusion, the tongues were fixed in 4% paraformaldehyde in 0.1M PO_4 buffer for 3 hours at 4°C. Sections (70μm thick) were sliced using a vibratome (Vibratome Series 1000; Ted Pella Inc., Redding, CA), and were blocked in a solution of 5% normal goat serum, and 1% bovine serum albumin in 0.1M PBS for 1.5 hours on ice. Sections were incubated with either rabbit polyclonal primary $P2X_2$ antibody or rabbit polyclonal primary $P2Y_4$ antibody in 0.1M PBS (pH7.3) overnight at 4°C. After washing three times for 10 minutes in 0.1M PBS, sections were incubated with secondary affinity-purified

biotinylated goat anti-rabbit IgG antibody in 0.1M PBS for 2 hours at room temperature. The sections were incubated with avidin-biotin complex (Elite Vectastain; Vector Laboratories Inc., Burlingame, CA) in 0.1M PBS for 1 hour at room temperature. Sections were then washed three times for 10 minutes in 0.1M PBS, and were treated with 0.05M Tris buffer (pH7.3) containing 0.05% diaminobenzidine (DAB) for 10 minutes. Stain was visualized by floating sections for 2-4 minutes in fresh DAB mixture, which was made active by the addition of hydrogen peroxide to a final concentration of 0.002%. Sections were washed three times for 5 minutes in 0.1M PO_4 buffer (pH 7.3), and postfixed in 1% osmium tetroxide in 0.1M PO_4 buffer for 15 minutes. After being washed in 0.05M sodium maleate buffer (pH 5.2) four times for 15 minutes, the sections were stained in 1% uranyl acetate in 0.025M sodium maleate buffer (pH 6.0) overnight at 4°C. Sections were then dehydrated in an alcohol series; 50% EtOH for 5 minutes, 60% EtOH for 5 minutes, 3 times in 75% EtOH for 5 minutes each, 3 times in 85% EtOH for 5 minutes each, 3 times in 95% EtOH for 5 minutes each, 3 times in 100% EtOH for 5 minutes each, processed through propylene oxide three times for 5 minutes, and embedded with Eponate 12 (Ted Pella Inc.). Sections were then reembedded using the technique of Crowley and Kinnamon (1995). Sections (90-120 nm thick) were sliced with a diamond knife on a Reichert Ultracut E ultramicrotome, and were imaged with a Hitachi H-7000 transmission electron microscope at 75kV.

Antibodies and Antibody Validation

$P2X_2$: Polyclonal antibody $P2X_2$ receptor was raised from rabbit against the peptide (C) SQQDSTSTD PKGLA QL, corresponding to residues 457-472 of rat $P2X_2$, located at intracellular C-terminus. Western blot showed specific band of expected size at 75kDa (Alamone Labs, manufacturer's technical information).

$P2Y_4$: Polyclonal antibody $P2Y_4$ receptor was raised from rabbit against the peptide (C) HEES ISRWA DTHQD, corresponding to residues 337-350 of rat $P2Y_4$, located at intracellular C-terminus. Western blot showed specific band of expected size (Alamone Labs, manufacturer's technical information).

Syntaxin-1: Monoclonal anti-syntaxin clone HPC-1 (mouse IgG1 isotype) was raised against a synaptosomal plasma membrane fraction from adult rat hippocampus (Inoue et al., 1992) and recognizes an epitope of HPC-1 antigen in the cytoplasmic surface of plasma membrane. Western blot showed a specific band of expected size at 35kDa (Sigma, manufacturer's technical information, Barnstable et al., 1988).

5-HT: Serotonin (5-HT) antiserum was generated in a rabbit against serotonin coupled to bovine serum albumin with paraformaldehyde. This antibody was quality control tested using standard immunohistochemical methods (manufacturer's technical information).

IP_3R3: The monoclonal antibody IP_3R3 is raised against an immunogen corresponding to amino acids 20-230 of human IP_3R3. Western blot showed specific band of expected size at 300kDa (BD Transduction Lab, manufacturer's technical information).

Table 1. Primary Antibodies

Antibodies	Species	Dilution	Source	Code No.
$P2X_2$	Rabbit	1:100	Alomone Labs	APR-003
$P2Y_4$	Rabbit	1:100	Alomone Labs	APR-006
Serotonin	Mouse	1:1	Biomeda	066D
Syntaxin-1	Mouse	1:100	Sigma	S 0664
IP_3R3	Mouse	1:100	BD Trans Lab	610313

Table 2. Secondary Antibodies

Antibodies	Dilution	Source	Code No.
Cyanine (Cy5) Goat anti-Rabbit IgG	1:100	Jackson	111-175-144
Fluorescein (FITC) Goat anti-Mouse IgG	1:100	Jackson	115-095-166
Biotin-conjugated Goat anti-Rabbit IgG	1:200	Jackson	111-065-144

Results

$P2X_2$ and $P2Y_4$ are cell surface receptors that are activated by ATP, which mediate K^+, Na^+, and Ca^{2+} levels (Kim et al., 2000; Burnstock, 2006). In order to determine which taste bud cell types, or nerve processes express these receptors, several immunocytochemical and DAB-immunoelectron experiments were carried out. Immunofluorescence experiments make use of antibodies targeted against specific, established cell type markers, such as IP_3R3 for Type II cells (Clapp et al., 2001) and serotonin (5-HT) for Type III taste cells (Yee et al., 2001), as well as synaptic proteins, such as Syntaxin-1 for Type III cells and nerve processes (Yang et al., 2007) involved in the mechanisms for synaptic transmission of the taste cell.

Controls

Control experiments involve deleting the primary antibody to check for any non-specific staining that is associated with the secondary antibody. Cyanine (Cy 5) goat anti-rabbit IgG is the secondary antibody used for the control experiments (Table 2). No cross-species immunoreactivity is seen (Fig. 6A). Peptide controls for the primary antibodies, $P2X_2$ and $P2Y_4$, include preabsorption with their analogous peptides (Fig. 6B and Fig. 6C, respectively). The $P2X_2$ antibody has been previously verified (Finger et al., 2005). Please see antibody validation section for additional information (Materials and Methods).

Colocalization of $P2X_2$ and Type II Cell Markers

If Type II cells have functional contacts with $P2X_2$-LIR nerve processes, then Type II cells and nerve processes should be in close apposition with each other. Double-label immunocytochemistry tests were performed using antisera directed against $P2X_2$ and IP_3R3, a known Type II cell marker (Clapp et al., 2001) to determine if $P2X_2$-LIR is present in nerve fibers that come into close contact with Type II cells. IP_3R3-LIR cells have large, circular nuclei, which are non-immunoreactive, indicating that they are probably Type II cells. IP_3R3-LIR is found in the cytoplasm of these cells, extending from the taste pore to the basal lamina, and is found only in taste cells (Fig. 7A). In contrast, $P2X_2$-LIR is present only in the nerve processes (Fig. 7B) and is in close apposition to IP_3R3-LIR cells, partially enveloping the cell (Fig. 7C). These data support the hypothesis, but do not prove that $P2X_2$-LIR nerve processes may communicate with Type II cells.

Colocalization of $P2X_2$ and Type III Cell Markers

If Type III cells form functional contacts with nerve processes, then they should have close contacts with $P2X_2$-LIR nerve processes. Further double-label immunocytochemistry experiments were done using $P2X_2$ and Syntaxin-1, a presynaptic T-SNARE membrane protein we have used as a cell marker for Type III cells as well as nerve processes (Yang et al., 2007). Syntaxin-1-LIR cells are Type III cells, and are slender, fusiform cells with intense cytoplasmic staining. The nuclei, however, do not display immunoreactivity. Structurally, the nuclei are roughly cylindrical in shape with

prominent invaginations (Yang et al., 2007). Syntaxin-1-LIR is also present in both subgemmal and intragemmal nerve processes (Yang et al., 2007). Syntaxin-1-LIR is found throughout the cytoplasm of the cell, spreading from the basal lamina to the taste pore, and is present in not only taste cells, but also nerve processes (Fig. 8A). Cells immunoreactive for Syntaxin-1 colocalized with $P2X_2$ -LIR (Fig. 8C), suggesting that $P2X_2$-LIR nerve processes are in close contact with a subset of Type III cells.

Experiments using $P2X_2$ and 5-HT, a known cell marker observed in a small subset of Type III cells (Yee et al., 2001) were also performed to determine if $P2X_2$-LIR nerve processes have close appositions with Type III cells. Serotonin (5-HT)-LIR cells are narrow and fusiform shaped, typical of Type III cells. Immunoreactivity in the cytoplasm reaches from the basal lamina to the taste pore (Ren et al., 1999; Yee et al., 2001), and is present only in taste cells. 5-HT–LIR cells resemble Syntaxin-1-LIR cells in both shape and structure, and have been shown to colocalize with a subset of Syntaxin-1-LIR (Type III) cells. 5-HT-LIR is present in a small subset of taste cells (Yee et al., 2001) (Fig. 9A). No colocalization occurs between 5-HT-LIR and $P2X_2$-LIR (Fig. 9C), and no 5-HT-LIR immunoreactivity is present in the nerve processes. All nerve processes that are in close apposition with Type III cells display $P2X_2$-LIR (Fig. 9B, C). These data suggest that a pathway from Type III cells to nerve processes is present.

Colocalization of $P2Y_4$ and Type II Cell Markers

Double-label immunocytochemistry experiments were done to determine the extent of colocalization between $P2Y_4$-immunoreactive cells and the Type II cell marker IP_3R3 (Clapp et al., 2001), in order to determine if $P2Y_4$ is present in Type II cells. Most

IP_3R3-LIR cells contain large, non-immunoreactive, circular nuclei, typical of Type II cells, with IP_3R3-LIR present throughout the cytoplasm of the cell. A large subset of IP_3R3-LIR taste cells display $P2Y_4$-LIR (Fig. 10C), suggesting that $P2Y_4$ is present in most, but not all IP_3R3-LIR cells (i.e. Type II cells). Some $P2Y_4$-LIR cells however do not display IP_3R3-LIR, suggesting that $P2Y_4$ receptors are also found in taste cell types other than Type II cells.

Colocalization of $P2Y_4$ and Type III Cell Markers

Double-label immunocytochemistry experiments were carried out to determine if $P2Y_4$ is also present in Type III cells. Two colocalizations were performed. First, $P2Y_4$ was colocalized with Syntaxin-1, a presynaptic T-SNARE protein present in Type III cells (Fig. 11). Next, $P2Y_4$ was colocalized with 5-HT (Fig. 12), a cell marker present in a subset of Type III cells (Yee et al., 2001). As described above, we have previously demonstrated that Syntaxin-1-LIR cells are Type III cells (Yang et al., 2007). Syntaxin-1-LIR is in the cytoplasm, extending from the basal lamina to the taste pore, as well as nerve processes (Fig. 11A). Most Syntaxin-1-LIR cells display $P2Y_4$-LIR (Fig. 11C), suggesting that $P2Y_4$-LIR is present in Type III cells. However, not all $P2Y_4$-LIR cells display Syntaxin-1-LIR.

5-HT-LIR cells are slender, fusiform shaped cells. Immunoreactivity extends the length of the taste bud, existing in the cytoplasm of the cell (Ren et al., 1999; Yee et al., 2001), and was found only in taste cells. Staining was seen throughout the nuclei of 5-HT-LIR cells (Fig. 12A). No staining was observed in the nerve processes, but Type III cells with 5-HT-LIR are known to form synapses with nerve fibers. Cells with 5-HT-LIR

display $P2Y_4$-LIR (Fig. 12C), supporting the notion that $P2Y_4$ is located in a subset of Type III cells. Most 5HT-1-LIR cells indicate $P2Y_4$-LIR, but not all $P2Y_4$-LIR cells indicate 5HT-1-LIR (Fig. 12C).

Ultrastructural Features of $P2X_2$-LIR Nerve Processes and $P2Y_4$-LIR Taste Cells

Our immunoelectron microscopy shows that $P2X_2$-LIR is present within most, if not all, intragemmal nerve processes in rat circumvallate taste buds (Fig. 13). No $P2X_2$-LIR was observed within actual taste cells. $P2X_2$-LIR nerve processes are in close apposition with both Type II and Type III taste cells. Type II taste cells have numerous mitochondria found adjacent to swollen smooth endoplasmic reticulum. These cells form close contacts with $P2X_2$-LIR nerve processes (Fig. 13). Dense patches of heterochromatin are present within the nuclei of Type III cells, and are also located on the inner leaflet of the nuclear membrane (Fig. 14). $P2X_2$-LIR nerve fibers are closely apposed to these cells (Figs. 13, 14). Mitochondria are often found in abundance within the $P2X_2$-LIR nerve processes (Figs. 13, 14A, 14B). Type III taste cells form synapses onto $P2X_2$-LIR nerve processes (Fig. 14B). Synapses from Type III taste cells onto $P2X_2$-LIR nerve processes are classical synapses, having parallel, thickened membranes of the presynaptic taste cell and postsynaptic nerve process, separated by a cleft (J. Kinnamon et al., 1985, 1988). Numerous clear vesicles are juxtaposed with the presynaptic active zone of the synapse (Fig. 14B), some clear vesicles seem to be docked at the presynaptic membrane. Abundant mitochondria are present within the postsynaptic $P2X_2$-LIR nerve process as well (Fig. 14B).

Both Type II taste cells and Type III taste cells express $P2Y_4$ (Figs. 15A, 15B, respectively). $P2Y_4$-LIR taste cells display immunoreactivity throughout their cytoplasm, but have non-immunoreactive nuclei (Figs. 15, 16). $P2Y_4$-LIR Type II cells possess numerous mitochondria throughout their cytoplasm, adjacent to swollen smooth endoplasmic reticulum (Fig. 15A). No synapses were observed between $P2Y_4$-LIR Type II cells and adjacent nerve processes (Fig. 15A). $P2Y_4$-LIR Type III taste cells were observed to form synapses with adjacent nerve processes (Fig. 16A, B). Synapses from $P2Y_4$-LIR taste cells onto nerve processes are classical synapses, exhibiting parallel, thickened membranes of the presynaptic taste cell and postsynaptic nerve process, separated by a cleft (J. Kinnamon et al., 1985, 1988). Several clear vesicles are juxtaposed with the presynaptic active zone of the synapse (Fig. 16B). Some clear vesicles appear to be docked at the presynaptic membrane. Numerous mitochondria are located within the postsynaptic nerve process as well (Fig. 16B). Rough endoplasmic reticulum can be seen adjacent to nerve processes (Fig. 16B). $P2Y_4$-LIR was not seen within nerve processes (Figs. 15, 16A, 16B).

Figure 6. Control images. A) Primary antibody had been omitted to show no cross-species immunoreactivity. B) $P2X_2$ preabsorption with its analogous peptide. C) $P2Y_4$ preabsorption with its analogous peptide. The secondary antibody used for all control experiments was cyanine (Cy 5) goat anti-rabbit IgG. No cross-species immunoreactivity was observed. Scale bar = 20μm.

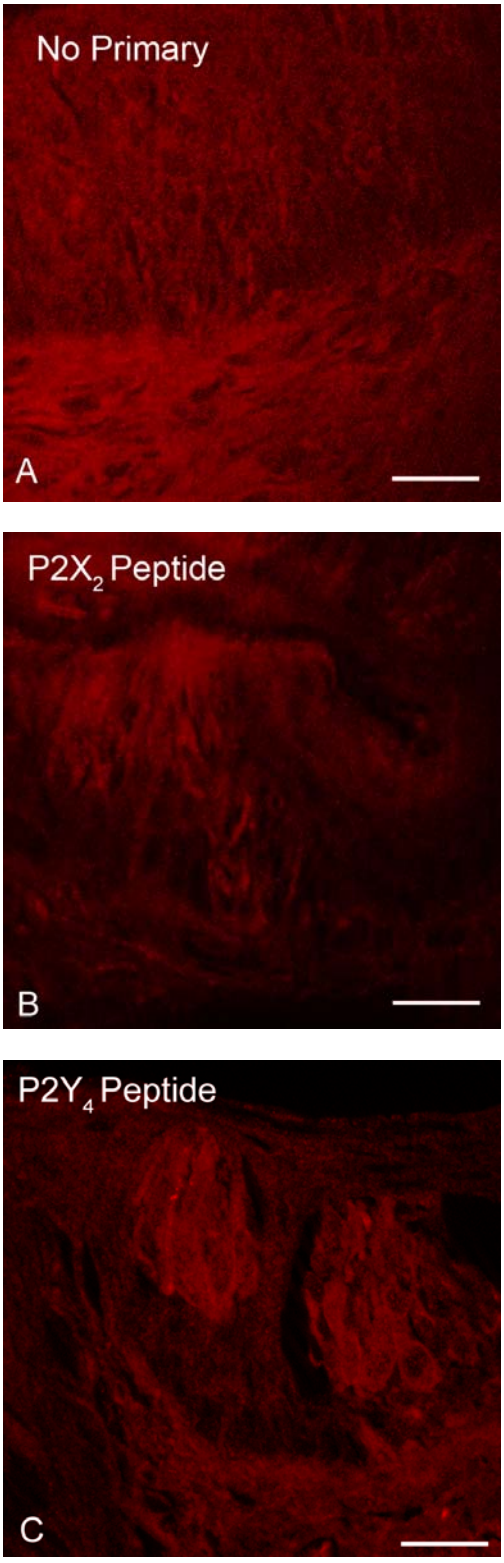

No Primary
A
P2X2 Peptide
B
P2Y4 Peptide
C

Figure 7. Immunofluorescence images showing colocalization of IP_3R3-LIR (green) and $P2X_2$-LIR (red) in circumvallate taste buds of the rat. A) Two taste buds (TB1, TB2) contain subsets of IP_3R3-LIR taste cells. The immunoreactive taste cells display intense cytoplasmic staining, but the nuclei are free of immunoreactivity. The cell shape and large ovoid nuclei are typical of Type II taste cells. These results are consistent with the known specificity of IP_3R3-LIR for Type II cells. B) $P2X_2$ -LIR nerve processes. Notice $P2X_2$-LIR fibers surround or come in close contact with IP_3R3-LIR (Type II cells). C) Merged image of IP_3R3-LIR (green) and $P2X_2$-LIR (red). Arrows indicate nerve processes. TP- Taste pore. Scale bar = 20μm.

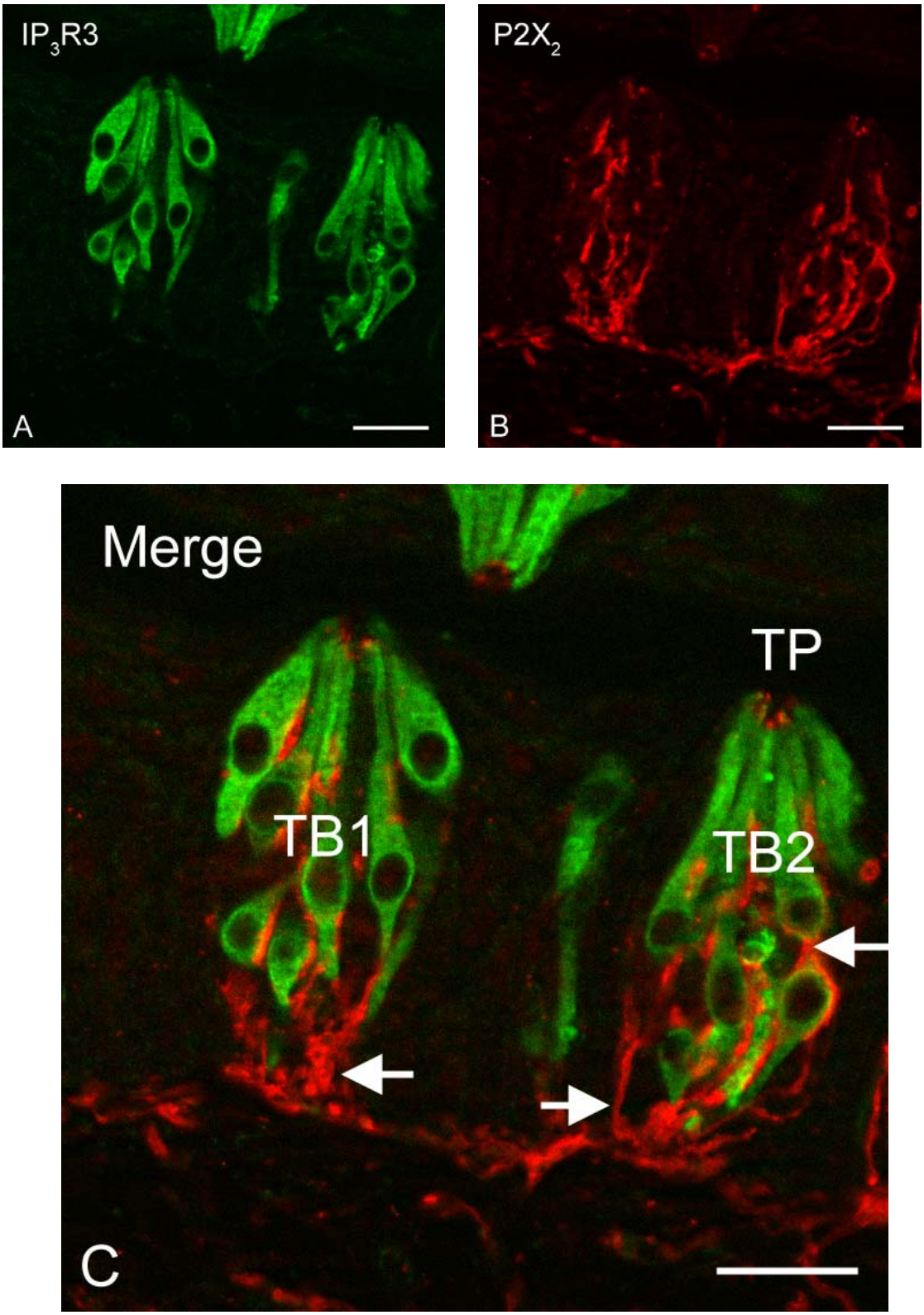

IP3R3
A
P2X2
B
Merge
TP
TB1
TB2
C

Figure 8. Immunofluorescence images showing colocalization of Syntaxin-1-LIR (green) and $P2X_2$-LIR (red) in circumvallate taste buds of the rat. A) A taste bud contains a small subset of Syntaxin-1-LIR taste cells. Immunoreactive taste cells are slender, fusiform cells with intense cytoplasmic staining, and the nuclei do not display immunoreactivity. The cell shape and invaginated nuclei are typical of Type III taste cells. B) $P2X_2$-LIR nerve processes colocalize or come in close contact with Syntaxin-1-LIR (Type III cells). C) Merged image of Syntaxin-1-LIR (green) and $P2X_2$-LIR (red). These results are consistent with the known specificity of Syntaxin-1-LIR for Type III cells and nerve processes. Scale bar = 20μm.

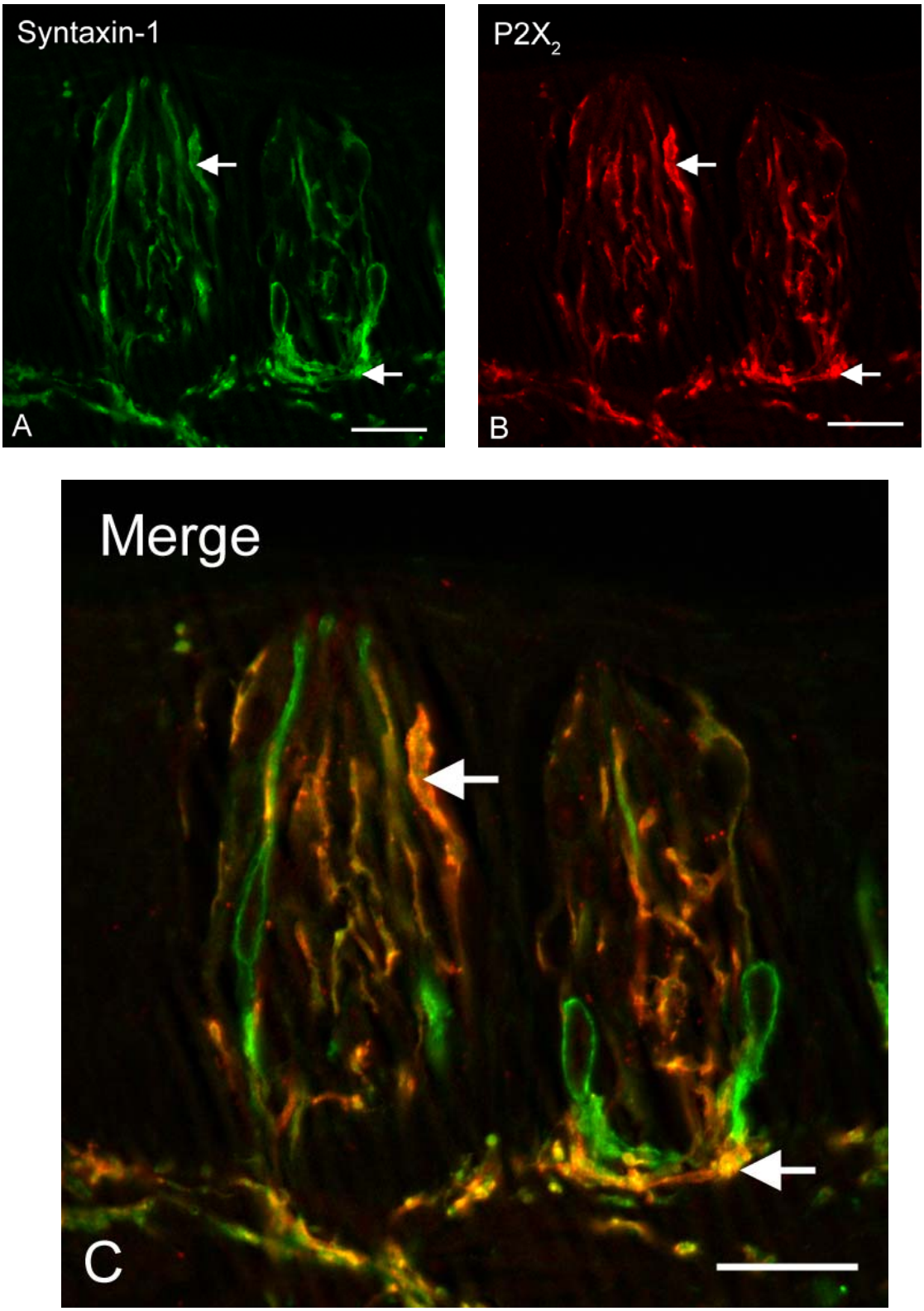

Syntaxin-1
A
P2X2
B
Merge
C

Figure 9. Immunofluorescence images showing colocalization of 5-HT (green) and $P2X_2$-LIR (red) in circumvallate taste buds of the rat. A) Taste buds containing a small subset of 5-HT-LIR taste cells. Immunoreactive taste cells are slender, fusiform cells with intense cytoplasmic staining. Note the nuclei of these cells display immunoreactivity. The cell shape and invaginated nuclei are characteristic of Type III taste cells. B) $P2X_2$-LIR nerve processes envelop 5-HT-LIR cells (Type III cells). C) Merged image of 5-HT-LIR (green) and $P2X_2$-LIR (red). These results are consistent with the known specificity of 5-HT-LIR for Type III cells. Arrow indicates P2X2-LIR nerve processes in close contact with 5-HT-LIR cell. TP- Taste pore. Scale bar = 20μm.

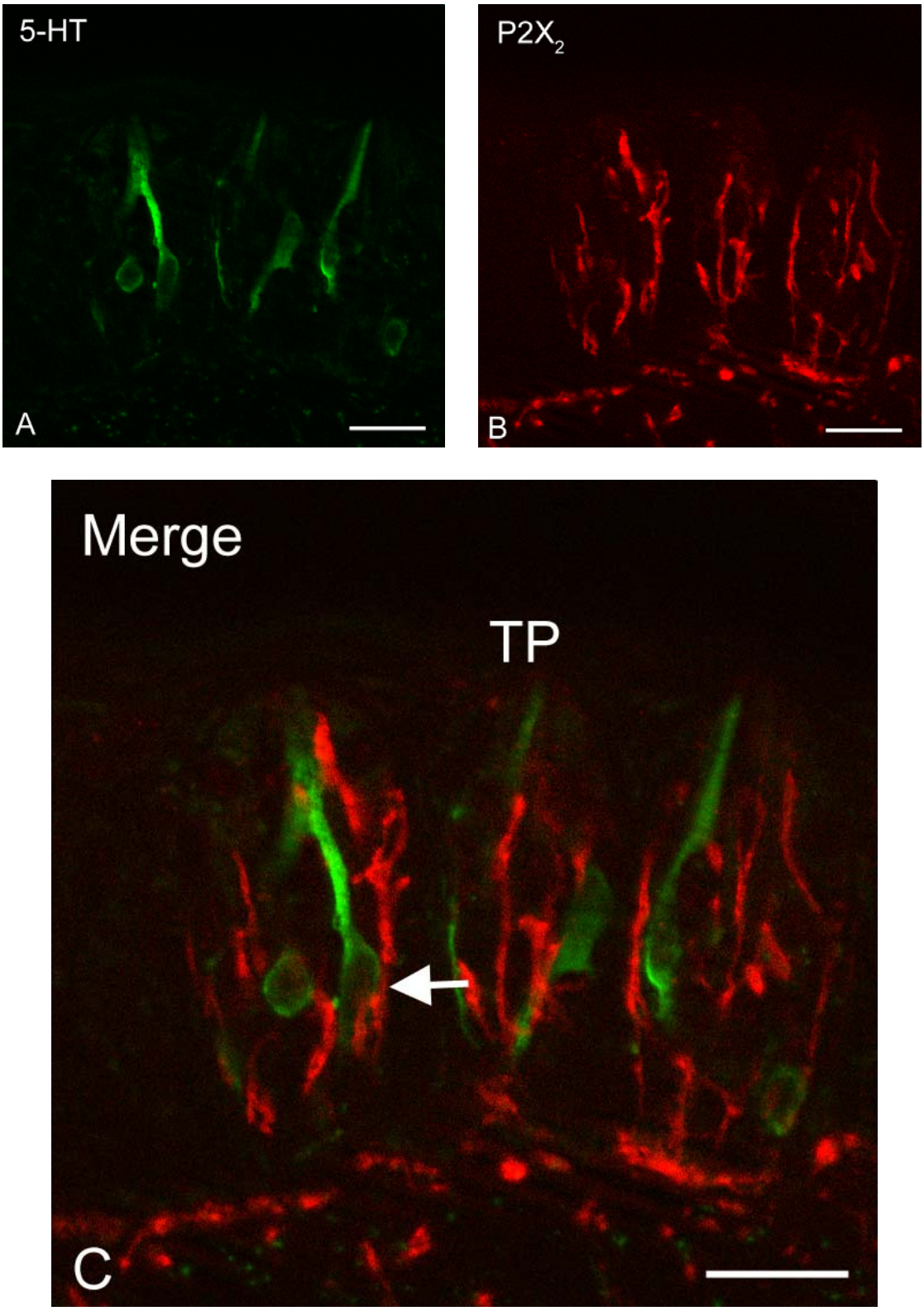

5-HT
A
P2X2
B
Merge
TP
C

Figure 10. Immunofluorescence images showing colocalization of IP_3R3-LIR (green) and $P2Y_4$-LIR (red) in circumvallate taste buds of the rat. A) Taste buds containing a large subset of IP_3R3-LIR taste cells. The immunoreactive taste cells display intense cytoplasmic staining, but the nuclei are free of immunoreactivity. The cell shape and large ovoid nuclei are typical of Type II taste cells. These results are consistent with the known specificity of IP_3R3-LIR for Type II cells. B) $P2Y_4$-LIR taste cells. Intense staining throughout the cytoplasm is observed, and nuclei do not display immunoreactivity. C) Merged image of IP_3R3-LIR (green) and $P2Y_4$-LIR (red). Most IP_3R3-LIR cells indicate $P2Y_4$-LIR, but not all $P2Y_4$-LIR cells indicate IP_3R3-LIR. Arrows indicate colocalization of $P2Y_4$-LIR and IP_3R3-LIR. Arrowheads indicate $P2Y_4$-LIR cells only. TP- Taste pore. Scale bar = 20μm

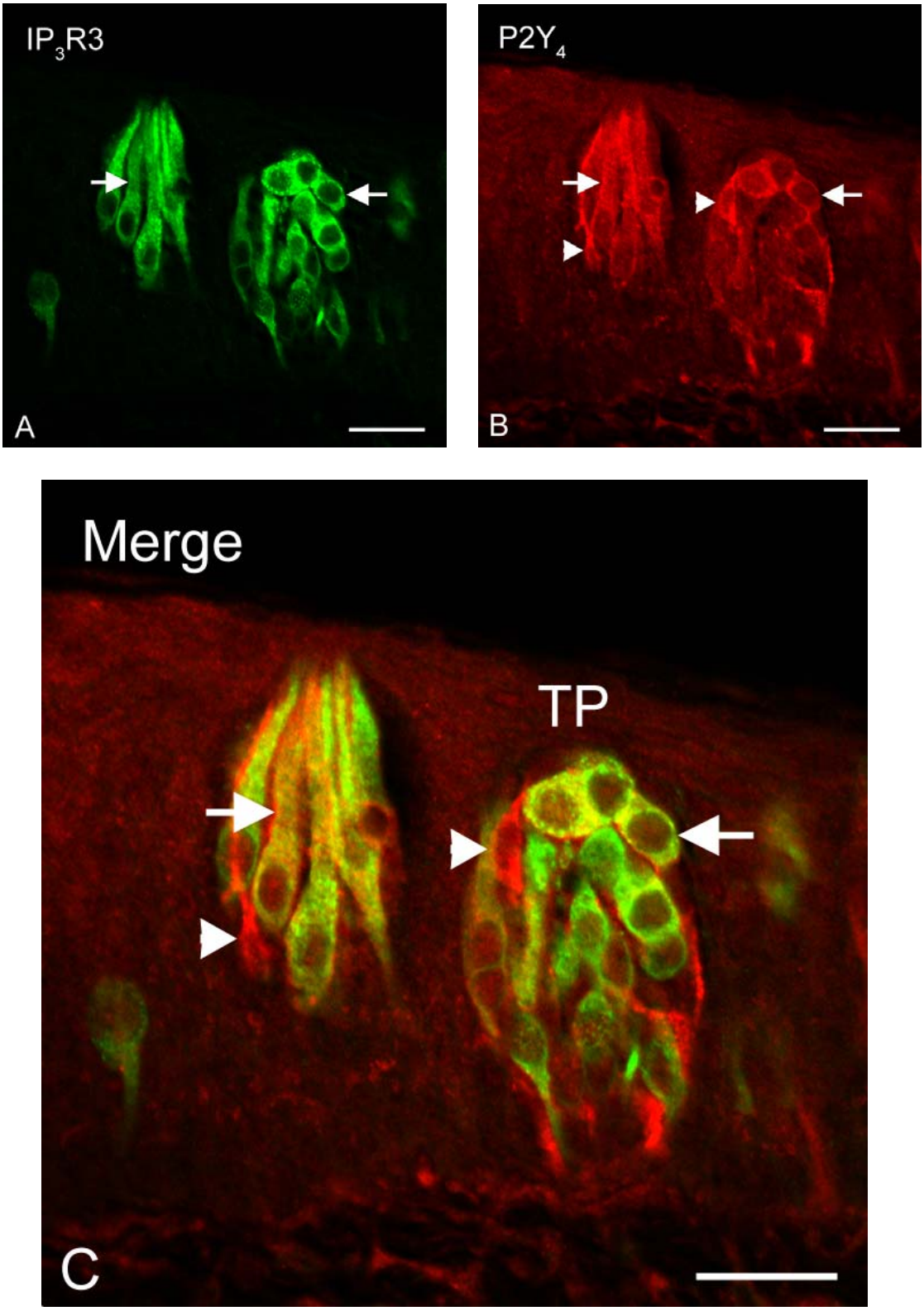

IP3R3
A
P2Y4
B
Merge
TP
C

Figure 11. Immunofluorescence images showing colocalization of Syntaxin-1-LIR (green) and $P2Y_4$-LIR (red) in circumvallate taste buds of the rat. A) A taste bud contains a small subset of Syntaxin-1-LIR taste cells. Immunoreactive taste cells are slender, fusiform cells with intense cytoplasmic staining, and the nuclei do not display immunoreactivity. The cell shape and invaginated nuclei are typical of Type III taste cells. Syntaxin-1-LIR is also seen in nerve processes. B) $P2Y_4$-LIR taste cells. Intense staining throughout the cytoplasm is observed, and nuclei do not display immunoreactivity. C) Merged image of Syntaxin-1-LIR (green) and $P2Y_4$-LIR (red). These results are consistent with the known specificity of Syntaxin-1-LIR for Type III cells and nerve processes. Most Syntaxin-1-LIR cells display $P2Y_4$-LIR, but not all $P2Y_4$-LIR cells indicate Syntaxin-1-LIR. $P2Y_4$-LIR is not seen in the nerve processes. Arrow indicates colocalization. Scale bar = 20μm.

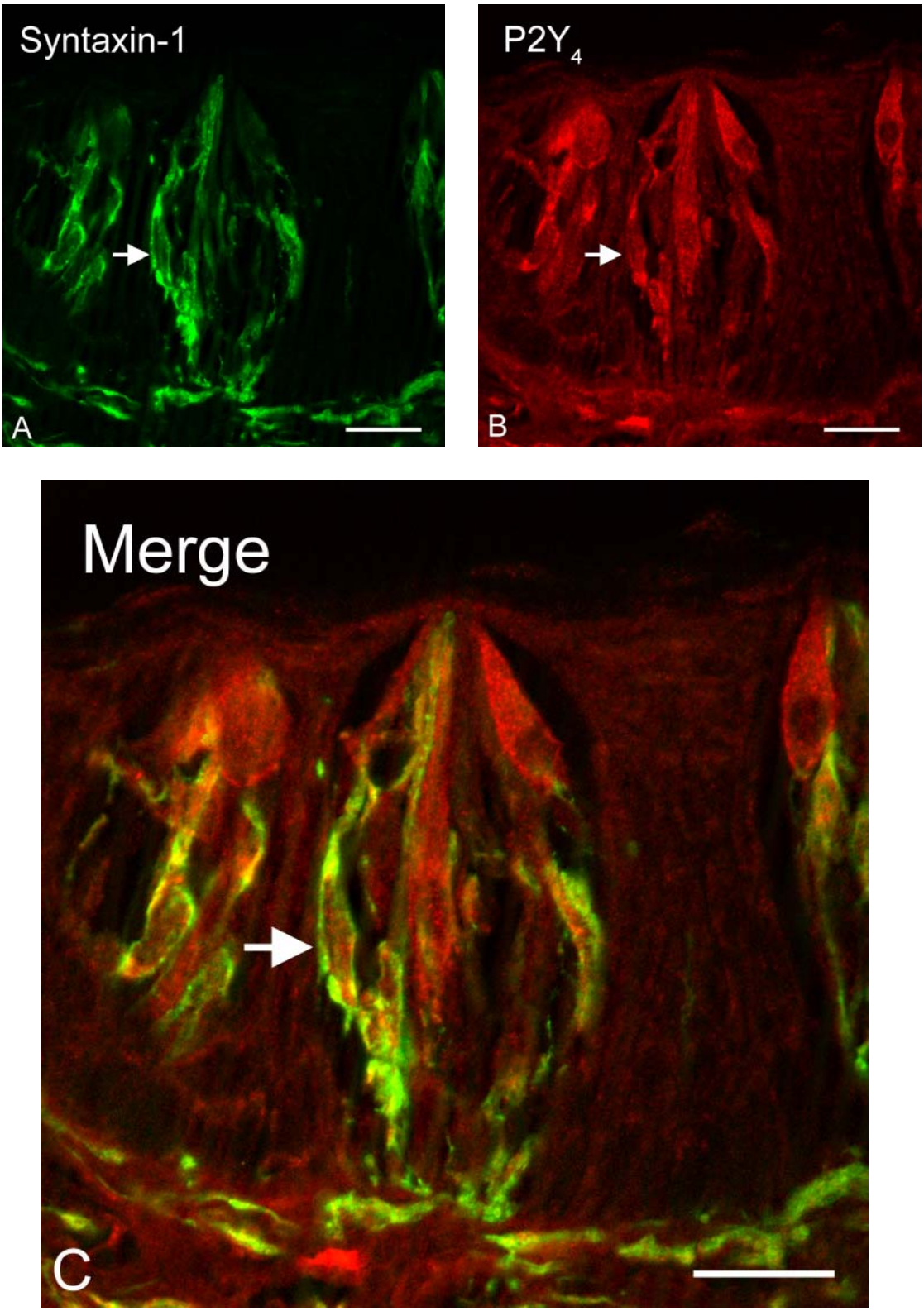

Syntaxin-1
A
P2Y4
B
Merge
C

Figure 12. Immunofluorescence images showing colocalization of 5-HT (green) and $P2Y_4$-LIR (red) in circumvallate taste buds of the rat. A) A taste bud containing a small subset of 5-HT-LIR taste cells. Immunoreactive taste cells are slender, fusiform cells with intense cytoplasmic staining. Note the nuclei of these cells display immunoreactivity. The cell shape and invaginated nuclei are characteristic of Type III taste cells. B) $P2Y_4$-LIR taste cells. Intense staining throughout the cytoplasm is observed, and nuclei do not display immunoreactivity. C) Merged image of 5-HT-LIR (green) and $P2Y_4$-LIR (red). These results are consistent with the known specificity of 5-HT-LIR for Type III cells. Arrow indicates $P2Y_4$-LIR and 5-HT-LIR colocalization. Most 5-HT-LIR cells indicate $P2Y_4$-LIR, but not all $P2Y_4$-LIR cells indicate 5-HT-LIR. TP- Taste pore. Scale bar = 20μm.

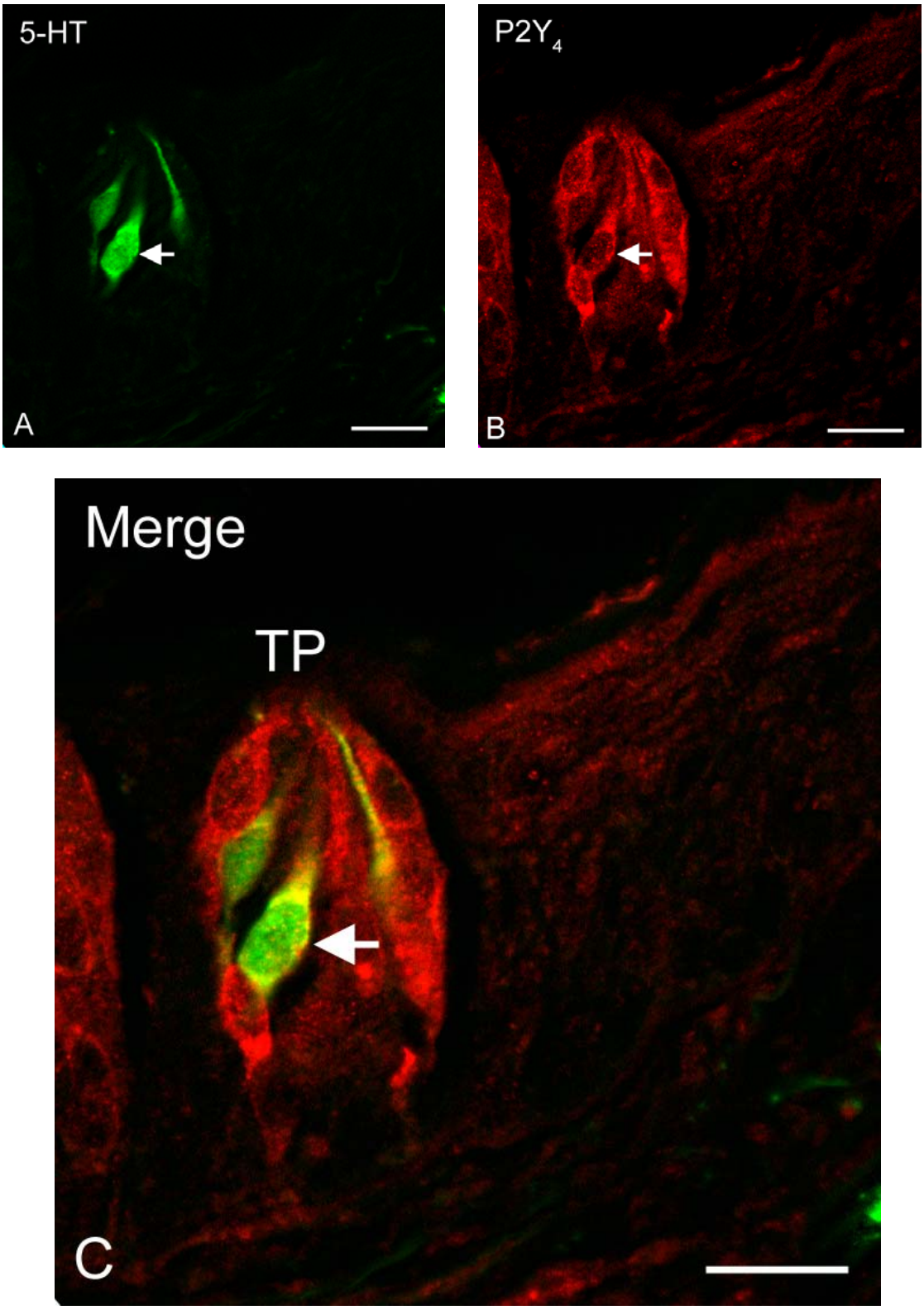

5-HT
A
P2Y4
B
Merge
TP
C

Figure 13. Low magnification DAB immunoelectron micrograph showing $P2X_2$-LIR in intragemmal nerve processes (N). Intense immunoreactivity is seen within the nerve process only. Numerous mitochondria are present within the $P2X_2$-LIR nerve process. Rough endoplasmic reticulum is observed to the right of the $P2X_2$-LIR nerve process, and swollen smooth endoplasmic reticulum can be seen to the left of the $P2X_2$-LIR nerve process. No immunoreactivity is seen within the taste cell. Patchy heterochromatin is seen throughout the nucleus of the nearby taste cell, which is characteristic of a Type III taste cell, as well as on the inner leaflet of the nuclear membrane. X 22,500. Scale bar = 1μm.

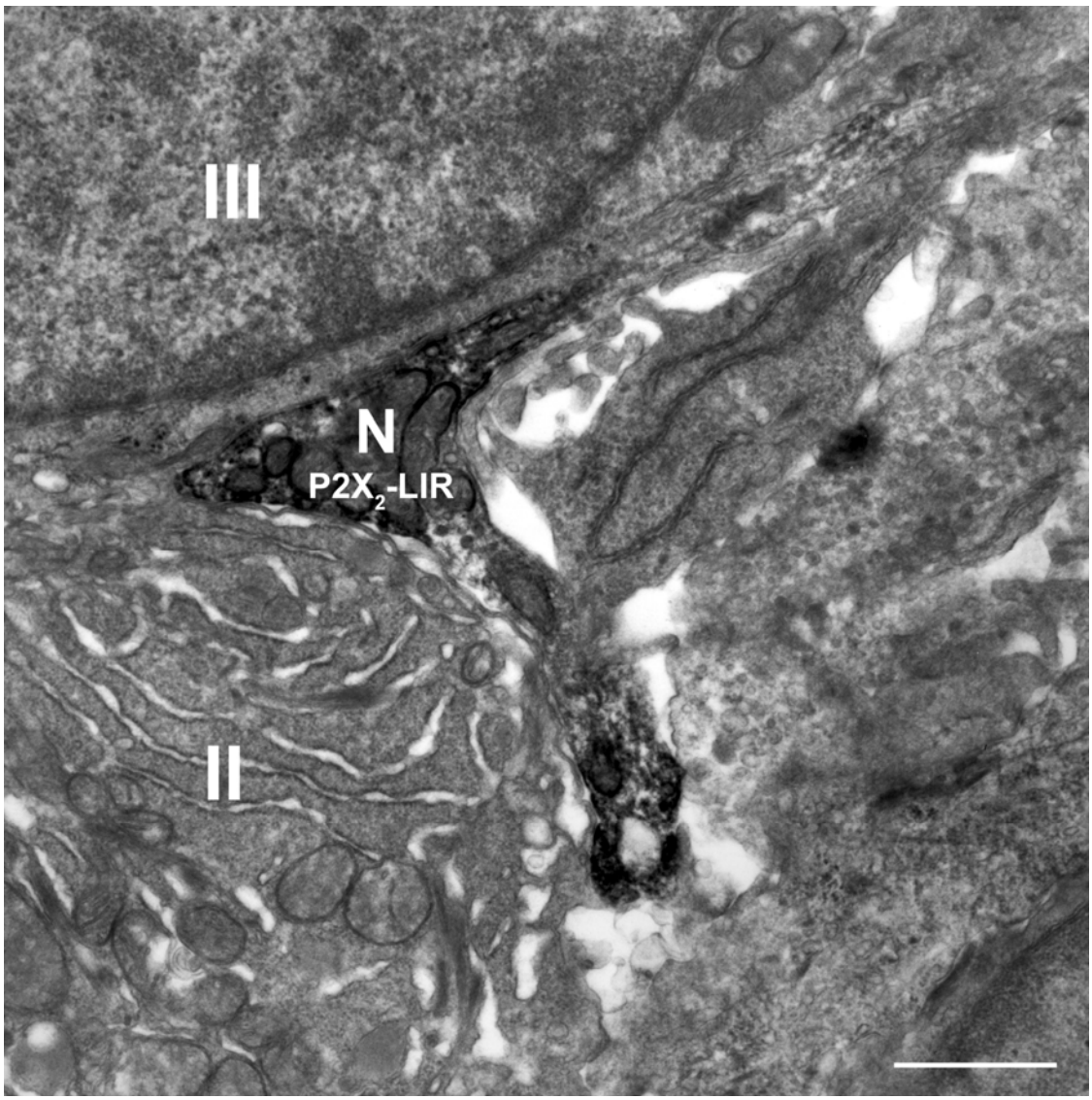
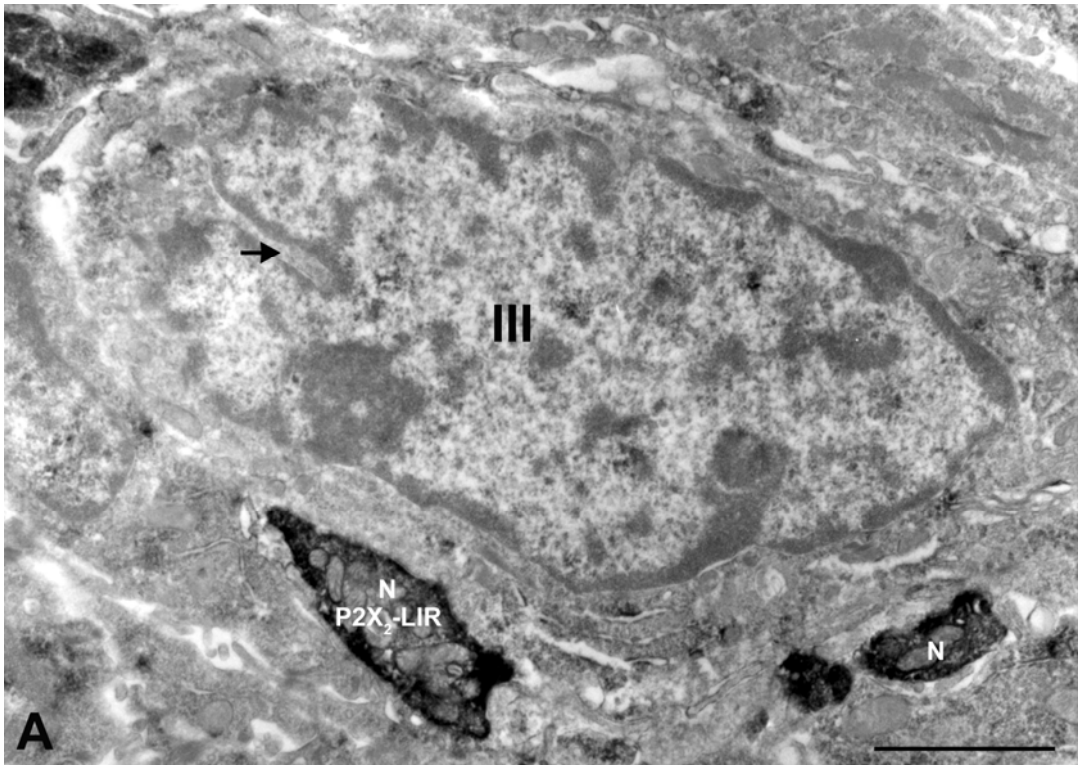

III
N
P2X2-LIR
II

Figure 14. A) Low magnification DAB immunoelectron micrograph showing $P2X_2$-LIR in intragemmal nerve processes (N). Intense $P2X_2$-LIR immunoreactivity is seen within the nerve process only. Numerous mitochondria are present within the $P2X_2$-LIR nerve process. No immunoreactivity is seen within the Type III taste cell. X 15,000. Scale bar = 2μm. B) High magnification DAB immunoelectron micrograph showing Type III taste cell forming a synapse (S) onto $P2X_2$-LIR intragemmal nerve processes (N). Criteria for identifying a synapse in a rodent taste bud include; 1) a thickening of the presynaptic membrane, 2) a 15-20nm cleft between the parallel membranes, and 3) vesicle clusters adjacent to the thickened membrane (Royer and J. Kinnamon, 1991; J. Kinnamon and Yang, 2007). Many synaptic vesicles (Sv) come into close contact with the presynaptic membrane. Intense immunoreactivity is seen within the nerve process only. Numerous mitochondria (M) are present within the $P2X_2$-LIR nerve process. No immunoreactivity is seen within the Type III taste cell. X 37,500. Scale bar = 1μm (Images taken by Dr. Ruibiao Yang).


III
N
P2X2-LIR
N
A

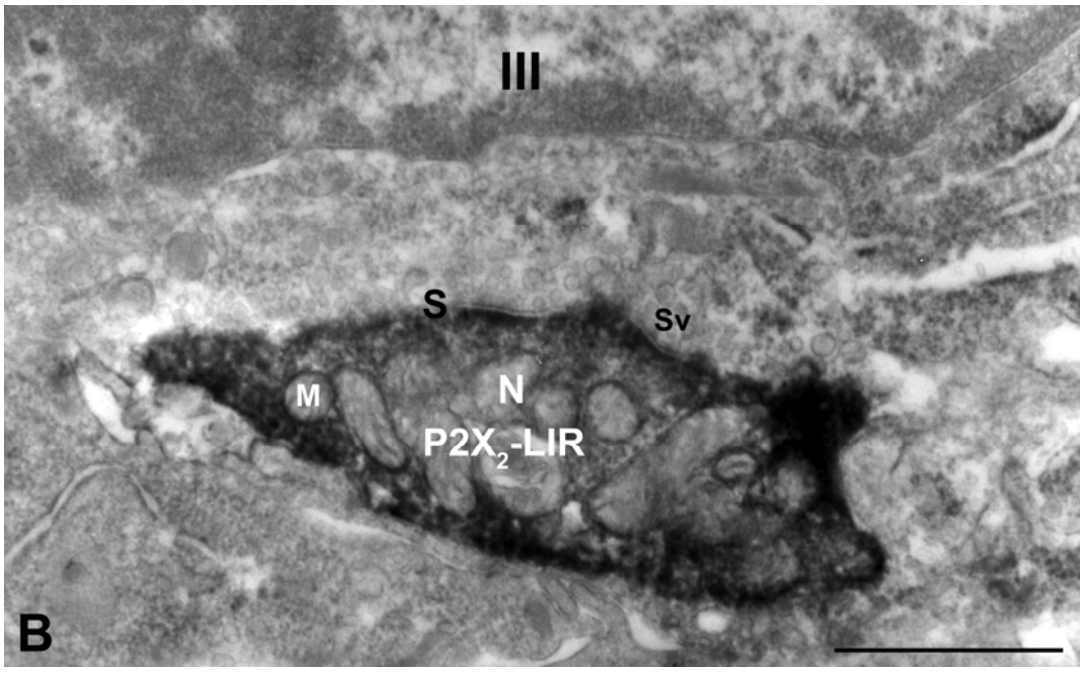

III
S
Sv
M
N
P2X2-LIR
B

Figure 15. Low magnification DAB immunoelectron micrographs showing $P2Y_4$-LIR. A) $P2Y_4$-LIR in a Type II taste cell. Note the round, electron lucent nucleus characteristic of a Type II cell. Swollen smooth endoplasmic reticulum can be seen adjacent to numerous mitochondria throughout the cytoplasm. TC- Taste cell. X 12,500. Scale bar = 2μm. B) $P2Y_4$-LIR in a Type III cell. Heterochromatin patches are observed throughout the nucleus of the Type III cell, as well as on the inner leaflet of the nuclear membrane. The adjacent nerve process (N) contains numerous mitochondria. Rough endoplasmic reticulum is found to the upper right of the nerve process. Keratin fibers are observed just below the $P2Y_4$-LIR Type III cell, characteristic of a Type I taste cell. Arrow indicates nuclear invagination, typical of a Type III cell. X 15,000. Scale bar = 2μm.

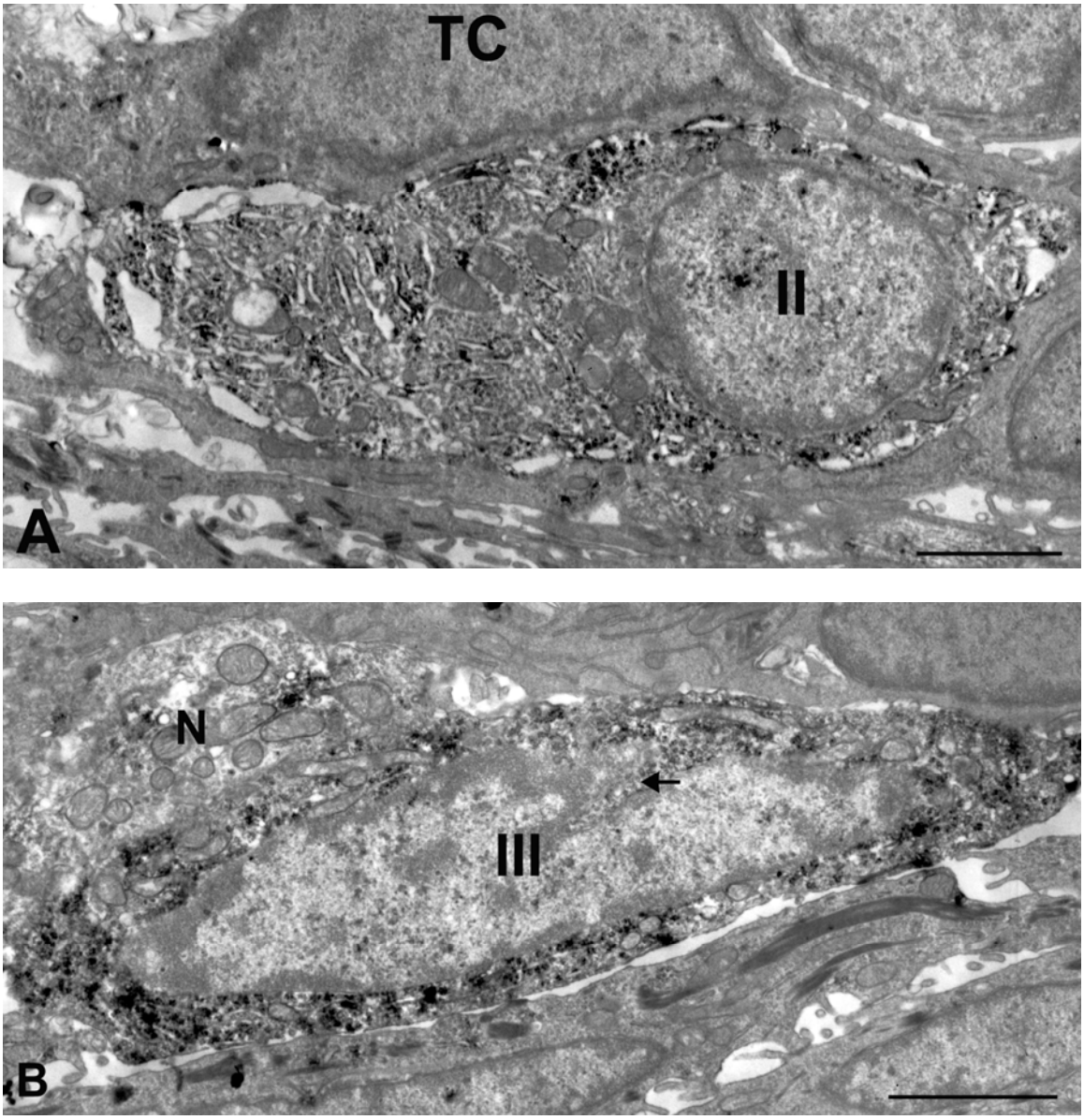
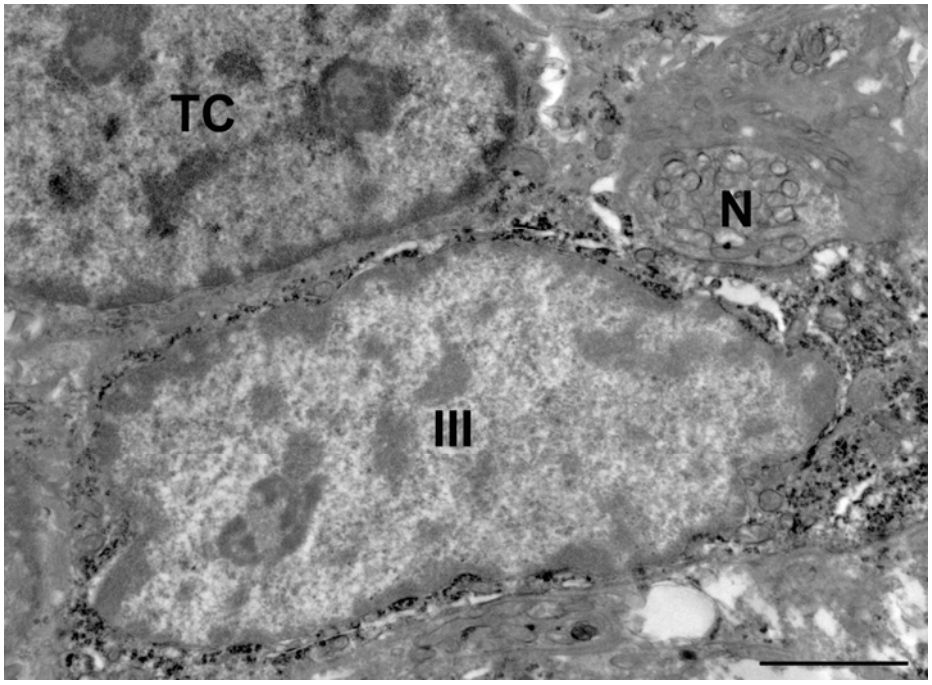
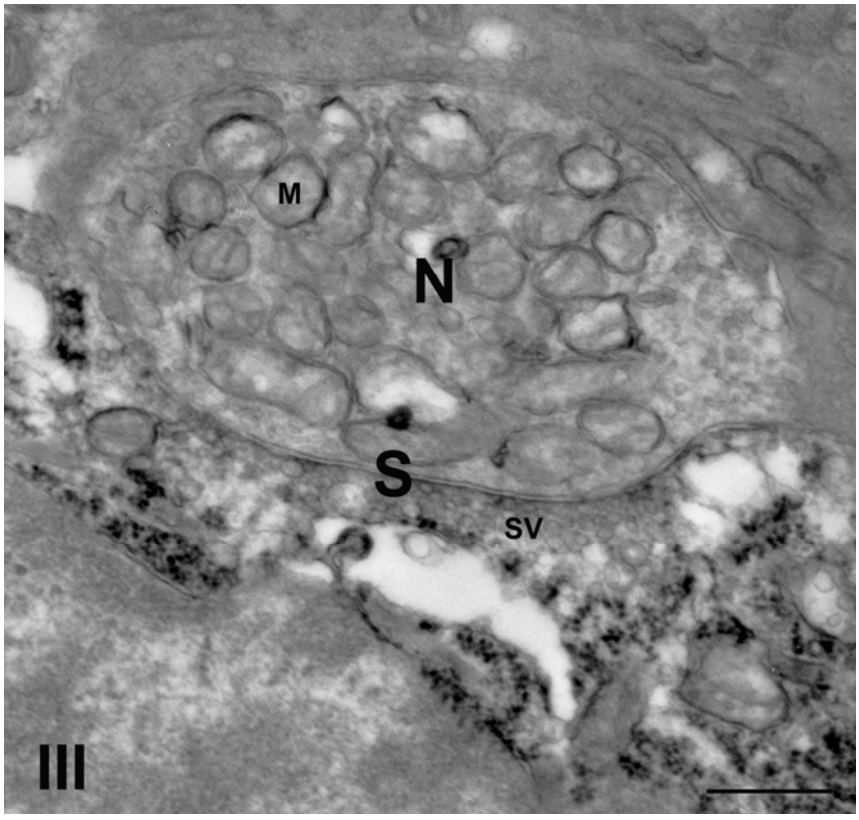

TC
II
A
N
III
B

Figure 16. A) Low magnification DAB immunoelectron micrograph showing $P2Y_4$-LIR in a Type III cell. Patchy heterochromatin is seen throughout the nucleus, as well as on the inner leaflet of the nuclear membrane. The adjacent taste cell (TC) also has heterochromatin patches throughout its nucleus, indicative of a Type III cell. The adjacent nerve process (N) contains numerous mitochondria. X 12,500. Scale bar = 2μm. B) High magnification DAB immunoelectron micrograph showing $P2Y_4$-LIR in a Type III cell. $P2Y_4$-LIR is present throughout the cytoplasm of the Type III cell. Patchy heterochromatin is found throughout the nucleus of the Type III taste cell, as well as on the inner leaflet of the nuclear membrane. The adjacent nerve process (N) contains numerous mitochondria (M), as well as synaptic vesicles (Sv) that come into close contact with the presynaptic membrane. Rough endoplasmic reticulum can be seen to the upper right of the nerve process. Criteria for identifying a synapse in a rodent taste bud include; 1) a thickening of the presynaptic membrane, 2) a 15-20nm cleft between the parallel membranes, and 3) vesicle clusters adjacent to the thickened membrane (Royer and J. Kinnamon, 1991; J. Kinnamon and Yang, 2007). S- Synapse. X 42,500. Scale bar = 0.5μm.


TC
N
III


M
N
S
SV
III

Discussion

Summary of Results

$P2X_2$ receptors are present on afferent nerve processes that are in close contact with both Type II and Type III taste cells in circumvallate papillae of the rat (Figs. 7, 8, 9, 13, and 14). IP_3R3-LIR taste cells (Type II cells) do not display $P2X_2$-LIR (Fig. 7). Intragemmal nerve processes that contact Type II cells possess $P2X_2$-LIR (Figs. 7, 13). Syntaxin-1-LIR taste cells (Type III cells) lack $P2X_2$ –LIR (Fig. 8). Syntaxin-1-LIR also colocalizes with $P2X_2$-LIR in intragemmal nerve processes (Fig. 8). DAB-immunoelectron microscopy shows that $P2X_2$-LIR is not present in Type I, Type II, or Type III cells and is present only in nerve processes (Figs. 13, 14). This is further confirmed by our results with a second Type III cell marker, 5-HT. 5-HT-LIR taste cells lack $P2X_2$-LIR, but are in close apposition with $P2X_2$-LIR nerve processes (Fig. 9). In sum, these results demonstrate that $P2X_2$ receptors are associated with afferent nerve fibers that contact Type I, Type II, and Type III taste cells, but are not associated with any taste cells. Thus, potential pathways exist for the communication of gustatory information from taste cells to nerve processes.

Type II taste cells with IP_3R3-LIR also display $P2Y_4$-LIR (Fig. 10). 5-HT- and Syntaxin-1-LIR (Type III cells) also display $P2Y_4$-LIR (Figs. 12, 11, respectively). These results suggest that $P2Y_4$ receptors are located on both Type II and Type III taste cells.

This indicates that a pathway exists for the possible communication of taste information from Type II cells to Type III taste cells and/or from one Type II cell onto other Type II cells. DAB-immunoelectron microscopy shows that $P2Y_4$-LIR is present within the cytoplasm of both Type II and Type III taste cells (Figs. 15A, 15B, respectively). $P2Y_4$-LIR was not observed in nerve processes (Figs. 15, 16). These results suggest that gustatory information may be transmitted via the putative neurotransmitter, ATP to: 1) adjacent nerve processes (that contain $P2X_2$ receptors), 2) Type II cells, and 3) Type III cells, both of which possess $P2Y_4$ receptors.

$P2X_2$ Receptors

$P2X_2$ receptors are ATP-gated ion channels that modulate Ca^{2+} and Na^+ influx, and K^+ efflux, leading to the depolarization of the cell. Activating $P2X_2$ receptors causes an accumulation of calcium ions in the cytoplasm, and is responsible for the activation of numerous signaling molecules (Dubyak, 1991; Bo et al., 1999; North, 2002; Burnstock, 2006; Erb et al., 2006; Volonté et al., 2006). We found that $P2X_2$-LIR was observed in all intragemmal nerve processes. This observation that $P2X_2$-LIR is present in all intragemmal nerve fibers has functional significance because these results suggest that ***all intragemmal nerve processes are stimulated by ATP***. Taken together with the well-documented observations of conventional synapses from Type III cells onto intragemmal nerve processes, this is strong evidence that intragemmal nerve processes are probably activated by more than one type of neurotransmitter.

$P2X_2$ and Type II Taste Cells

Type II cells comprise approximately 20% of the taste receptor cell population (Delay et al., 1986). Type II cells are thought to function as chemoreceptor cells in the taste bud (Farbman, 1965; Murray and Murray, 1971). The receptors for bitter, sweet, and umami are expressed on Type II cells (Clapp et al., 2004, 2006). Type II cells have been shown to possess the molecular machinery used for bitter, sweet, and umami taste transduction, but do not form classical synapses with adjacent nerve fibers (Farbman, 1965; Lindemann, 1996; Royer and Kinnamon, 1994; Yang et al., 2000a).

Type II cells express the immunocytochemical marker IP_3R3 (Figs. 7, 17) (Clapp et al., 2001). Studies have shown that $P2X_2$-LIR nerve processes are often in close apposition with taste cells that display IP_3R3-LIR (Fig. 7). Our DAB immunoelectron microscopy shows that $P2X_2$-LIR is not present within Type II cells, but is only present within nerve processes (Fig. 13). Physiological studies from other laboratories suggest that Type II cells possess pannexin/connexin hemichannels, which are believed to be the sites where ATP release occurs (Huang et al., 2007; Romanov et al., 2007). Our immunofluorescence and immunoelectron microscopic observations show that $P2X_2$ receptors are present on all nerve fibers in contact with Type II cells (Figs. 7, 8, 9, 13, 14). Thus, our studies support previous physiological studies suggesting that Type II cells may release ATP onto intragemmal nerve processes.

$P2X_2$ and Type III Taste Cells

Approximately 15% of taste cells are Type III cells (Delay et al., 1986). Conventional synapses onto nerve processes have been observed to be associated with

Type III cells (Yang et al., 2000a; Yee et al., 2001). Type III cells have also been termed the *presynaptic* cell in taste buds (Roper, 2006, 2007; Tomchik and Roper, 2007). A subset of Type III cells contains the immunocytochemical marker serotonin (5-HT) (Kim and Roper, 1995; Yee at al., 2001). Our laboratory has previously shown that the presynaptic T-SNARE membrane protein, Syntaxin-1, is present in Type III cells and nerve processes (Yang et al., 2007) (Fig. 17). Although 5-HT has been proposed to be a neuromodulator in taste buds (Roper, 2006; Roper, 2007), 5-HT-LIR is not thought to be present in the vesicles at the synapses from Type III cells onto nerve processes. Thus, the nature of the neurotransmitters released at Type III cell – nerve process synapses is unknown.

Our immunocytochemical studies show $P2X_2$-LIR nerves are in intimate contact with Type III cells (Figs. 8, 9, 13, 14). Our DAB-immunoelectron microscopy shows that $P2X_2$ is limited to nerve processes and is not present in Type III cells (Figs. 13, 14). Significantly, Type III taste cells form conventional synapses with $P2X_2$-LIR nerve processes (Fig. 14). Because all of the nerve processes in taste buds display $P2X_2$-LIR, we infer that the nerve processes are stimulated by ATP. Since Type III taste cells form conventional synapses with the same $P2X_2$-LIR nerve processes, we consider this to be compelling evidence that there is a convergence of input from taste cells using multiple transmitters—ATP from Type II cells and unidentified neurotransmitter(s) released by exocytosis from Type III cell synapses. If this is the case, then there are significant ramifications regarding neural coding of gustatory information. Based on our results, we now speculate that labeled line coding for different gustatory stimuli is not likely because

of the putative convergence of input onto the $P2X_2$-LIR nerve processes from both Type II and Type III cells.

$P2Y_4$ and Type II Taste Cells

Immunocytochemical studies done using $P2Y_4$ and IP_3R3 showed partilar colocalization (Fig. 10). Not all $P2Y_4$-LIR cells show IP_3R3-LIR, suggesting that $P2Y_4$-LIR is not present only in Type II taste cells. DAB-immunoelectron microscopy shows that $P2Y_4$-LIR is present in Type II cells, indicating that $P2Y_4$ receptors are present in Type II taste cells (Fig. 15A). The presence of these ATP receptors on Type II cells suggests that ATP may transmit the taste signal to other Type II or Type III taste cells that possess $P2Y_4$ receptors. ATP may also act in an autocrine manner via $P2Y_4$ receptors present on a Type II receptor cell.

$P2Y_4$ and Type III Taste Cells

$P2Y_4$-LIR is also present in Type III taste cells (Figs. 11, 12, 15B, 16). Colocalizations were seen between $P2Y_4$ and the Type III cell markers 5-HT and Syntaxin-1 (Figs. 12, 11, respectively). Not all $P2Y_4$-LIR cells show 5-HT-LIR or Syntaxin-1-LIR, suggesting that $P2Y_4$-LIR may be present in the PGP 9.5 subset of Type III cells, as well as in Type II taste cells (Figs. 12, 11). Our DAB-immunoelectron microscopy also indicates that $P2Y_4$-LIR is present in Type III cells (Figs. 15B, 16). Therefore, $P2Y_4$ receptors are probably present on Type III taste cells. It should also be noted that $P2Y_4$-LIR Type III taste cells form conventional synapses onto intragemmal nerve processes (Fig 16B).

Conclusions

The transduction mechanisms and processing of gustatory information in taste cells are similar in many ways with neurons. One can think of a taste receptor cell as being like a neuron that is "postsynaptic" to gustatory stimuli (which can be considered to be analogous to neurotransmitters). Within the circumvallate papillae of rats, only Type III taste cells form "classical" synapses onto afferent nerve fibers (Yang et al., 2000a; Yee et al., 2001). Type II cells; however, are known to respond to bitter, sweet, and umami stimuli (Clapp et al., 2004, 2006). Thus, Type II cells must communicate with nerve processes (and possibly other taste cells) by non-vesicular mechanisms. Bo et al. (1999) showed that taste nerves expressed two ionotropic receptor subunits ($P2X_2$ and $P2X_3$), suggesting that ATP functions as a neurotransmitter in taste transduction. Finger et al. (2005) found that $P2X_2/P2X_3$ double knockout mice were almost completely unresponsive to bitter, sweet, and umami stimuli, further supporting the notion that these receptors play an important role in the taste transduction of these stimuli. Finger et al. (2005) also showed that ATP is secreted from gustatory epithelium upon taste stimulation from bitter, sweet, and umami tastants. Huang et al. (2007) and Romanov et al. (2007) furthered the findings of Finger et al. (2005), showing that Type II taste cells, receptor cells, release ATP via hemichannels present on Type II cells. Huang et al. (2007) also showed that ATP acts as a mediator between Type II cells and Type III cells. The release of ATP from receptors cells triggered the release of 5-HT from presynaptic cells (Huang et al., 2007).

Hemichannel-mediated release of ATP from Type II cells is believed to convey taste information to $P2X_2$ receptors found on afferent nerve processes adjacent to the receptor cell (Roper, 2007). Our results support those of Roper (2007) and Finger et al., (2005) in that our DAB immunoelectron microscopy shows that $P2X_2$-LIR nerve processes are closely apposed to Type II cells. These results provide support for the hypothesis that there is a pathway for ATP release via Type II cells onto $P2X_2$-LIR nerve processes (Roper, 2007). This pathway provides Type II cells with a non-synaptic means of carrying a taste signal from bitter, sweet, or umami stimuli directly to nerve processes. It is not known, however, whether bitter, sweet and umami signaling Type II cells provide input to separate nerve processes (labeled-line) or have convergent input onto a single nerve process, which would require across-fiber coding.

Taste cells responsive to ATP are present in circumvallate, foliate, and fungiform papillae (Baryshnikov et al., 2003). P2Y-like receptors were found to be present in mouse taste receptor cells (Kim et al., 2000; Baryshnikov et al., 2003). ATP activates $P2Y_4$ cell surface receptors to gate intracellular Ca^{2+} levels (Baryshnikov et al., 2003; Burnstock, 2006). Based on our immunocytochemical experiments, we have provided compelling evidence that $P2Y_4$ receptors are present on both Type II and Type III cells. Thus, one can speculate that complex cell-to-cell communications occur between Type II and Type III cells within the taste bud. Physiological and molecular biological experiments from other laboratories have also suggested that ATP receptors may be present on taste cells (Baryshnikov et al., 2003; Kataoka et al., 2004; Y. Huang et al., 2006; Bystrova et al., 2006; Tomchik and Roper, 2006; Roper, 2007), but did not

positively demonstrate which cell types are involved, as our immunoelectron microscopical experiments have done.

It is possible that ATP receptors serve as autoreceptors on Type II cells (Baryshnikov et al., 2003). Because Type II cells lack identifiable synapses it is presumed that ATP release occurs via non-vesicular release (Huang et al, 2007; Romanov et al., 2007). Huang et al. (2007) and Romanov et al. (2007) proposed that pannexin and/or connexin hemichannels mediate ATP release in taste cells. Whether pannexin, connexin, or a combination of both hemichannels functions in releasing ATP from Type II cells remains controversial (Huang et al., 2007; Romanov et al., 2007).

ATP released via hemichannels from Type II cells may also transmit taste information to $P2Y_4$ receptors present on Type III cells, stimulating 5-HT secretion from Type III cells (Fig.18) (Tomchik and Roper, 2006; Huang et al, 2007; Roper, 2007). Thus, this signaling pathway could indirectly transmit taste information to the central nervous system. $P2Y_4$ receptors present on a Type II cell may be associated with signaling from one Type II cell to another Type II cell via paracrine ATP signaling pathways (Fig. 19). Alternatively, release of ATP from a Type II cell might modulate the activity of the same cell via autocrine pathways (Baryshnikov et al., 2003). Kim et al. (2000) suggested that P2Y-mediated inhibition of Ca^{2+} channels in Type II cells may provide negative feedback regulation of ATP release from Type II cells. One Type II cell with pannexin/connexin hemichannels may act like a "presynaptic" cell onto an adjacent Type II cell with $P2Y_4$ receptors (Fig. 19).

For decades it has been assumed that the processing of gustatory information in the taste bud was simple——stimulation of a receptor cell causes release of a transmitter at a synapse from the receptor cell onto a nerve process. The results of the present study, together with recent data from other laboratories, indicate that the processing of gustatory information in the taste bud is much more complex than previously thought. Much more research needs to be done to better understand the role of ATP as a neurotransmitter in the taste bud. Some possible areas of future research include:

1. The specific localization of $P2X_2$ and $P2X_3$ receptors on intragemmal nerve processes using colloidal gold immunoelectron microscopy. Are these receptors evenly distributed over the nerve cell membrane or are they concentrated at the close appositions between the taste cell and the nerve process?

2. The specific localization of $P2Y_4$ receptors on Type II and Type III cells. Are these receptors evenly distributed over the taste cell membrane or are they concentrated at the close appositions between adjacent taste cells?

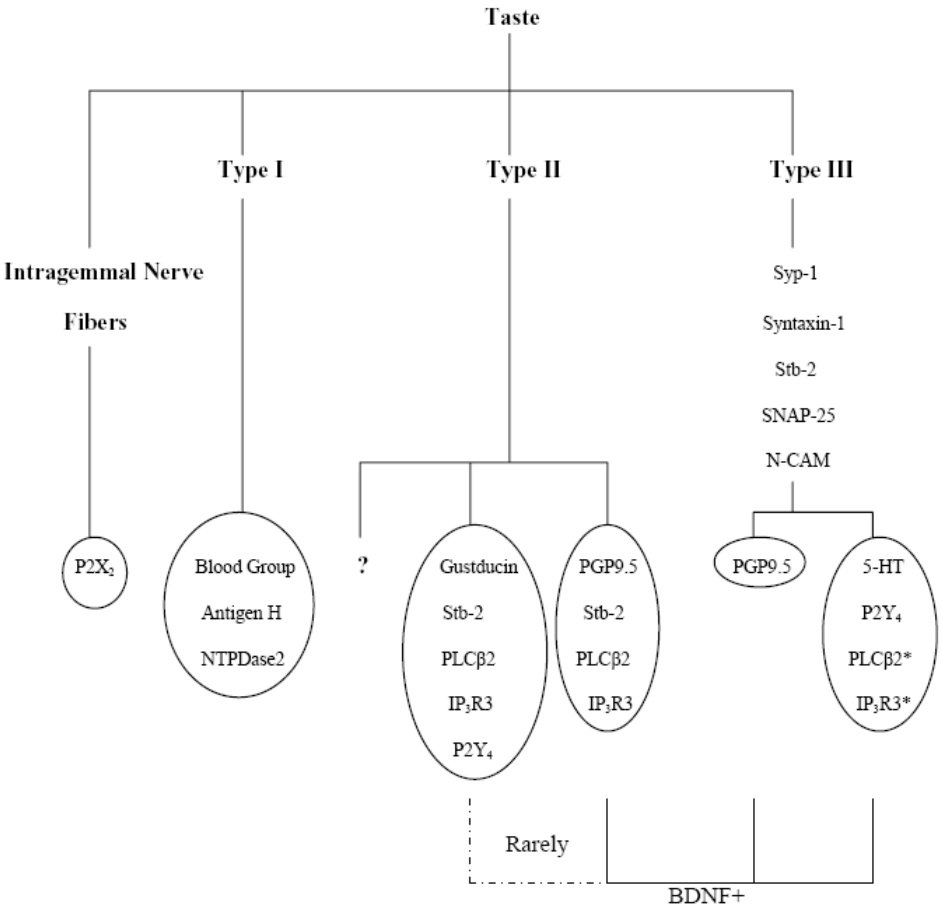
3. Quantitation of $P2Y_4$ on Type II and Type III cells. What percentages of taste cells contain $P2Y_4$ receptors? Are these receptors more abundant in a specific cell type?

Gustation is one of the most complex senses, utilizing a diversity of transduction mechanisms. This contrasts with olfaction, vision, and audition——all of which employ single transduction pathways. On a larger scale, results obtained from these experiments

will help to broaden our understanding of the fundamental workings of taste and the mechanisms involved. The results of our studies will facilitate the understanding and treatment of gustation-related diseases and other diseases of the senses. Understanding gustatory mechanisms will also provide a better understanding of the mechanisms underlying the nervous system as a whole.

Figure 17. Immunocytochemical markers present in Type I, Type II, and Type III taste cells. $P2X_2$ has been added as a marker for intragemmal nerve processes. $P2Y_4$ has been added as a marker in Type II and Type III taste cells (From Clapp et al., 2004; Yang et al., 2004; Yee et al., 2001).

MOLECULAR MARKERS IN TASTE CELLS



*: A very low percentage of IP_3R3- and PLCβ2-LIR taste cells also display 5-HT-LIR.

Figure 18. Type II cell-Type III cell communication. Type II cells may secrete ATP, directly acting on adjacent $P2X_2$-LIR sensory afferent fibers, providing a non-vesicular pathway for taste transduction from Type II cells onto nerve processes. Type II cells may also secrete ATP, acting on nearby $P2Y_4$-LIR Type III cells, stimulating the release of 5-HT from the Type III cell (Adapted from Roper, 2007).

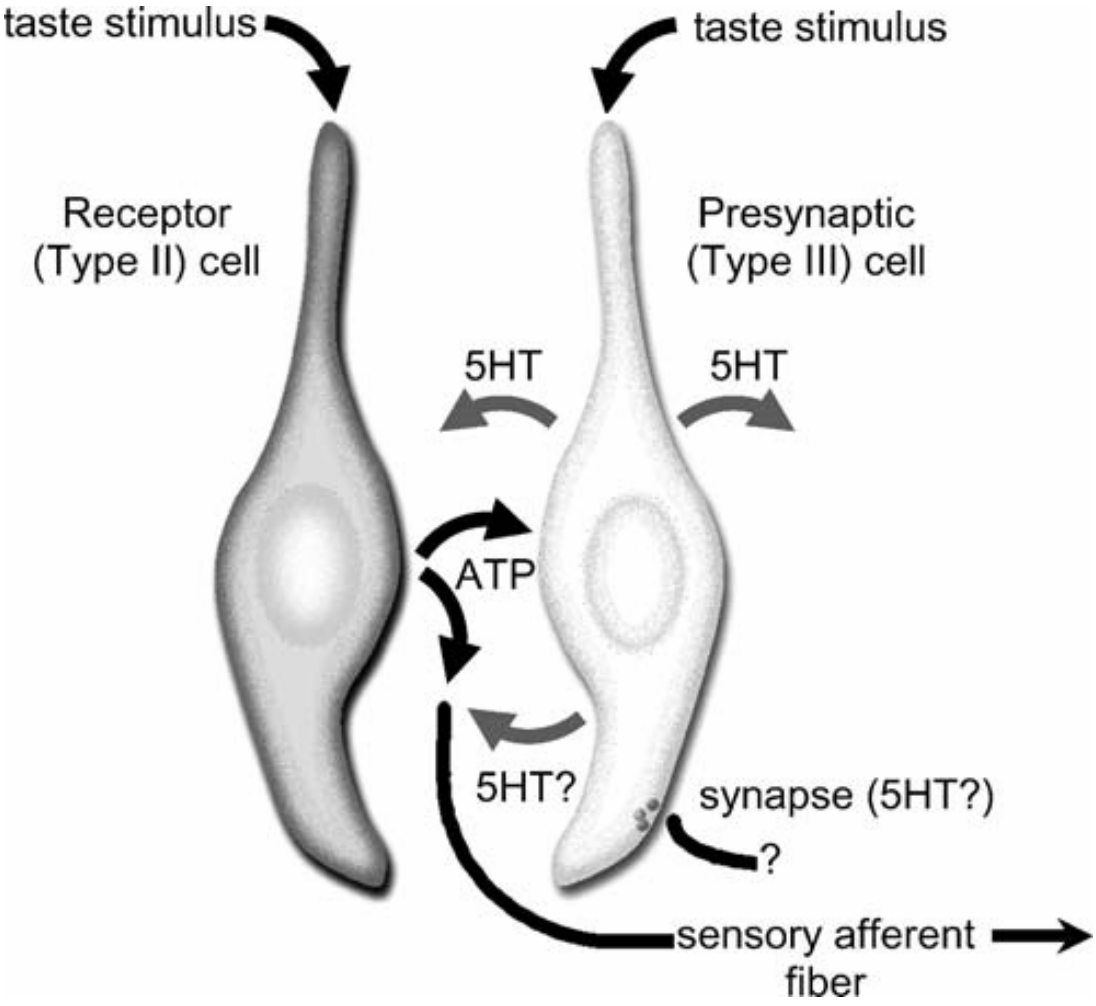
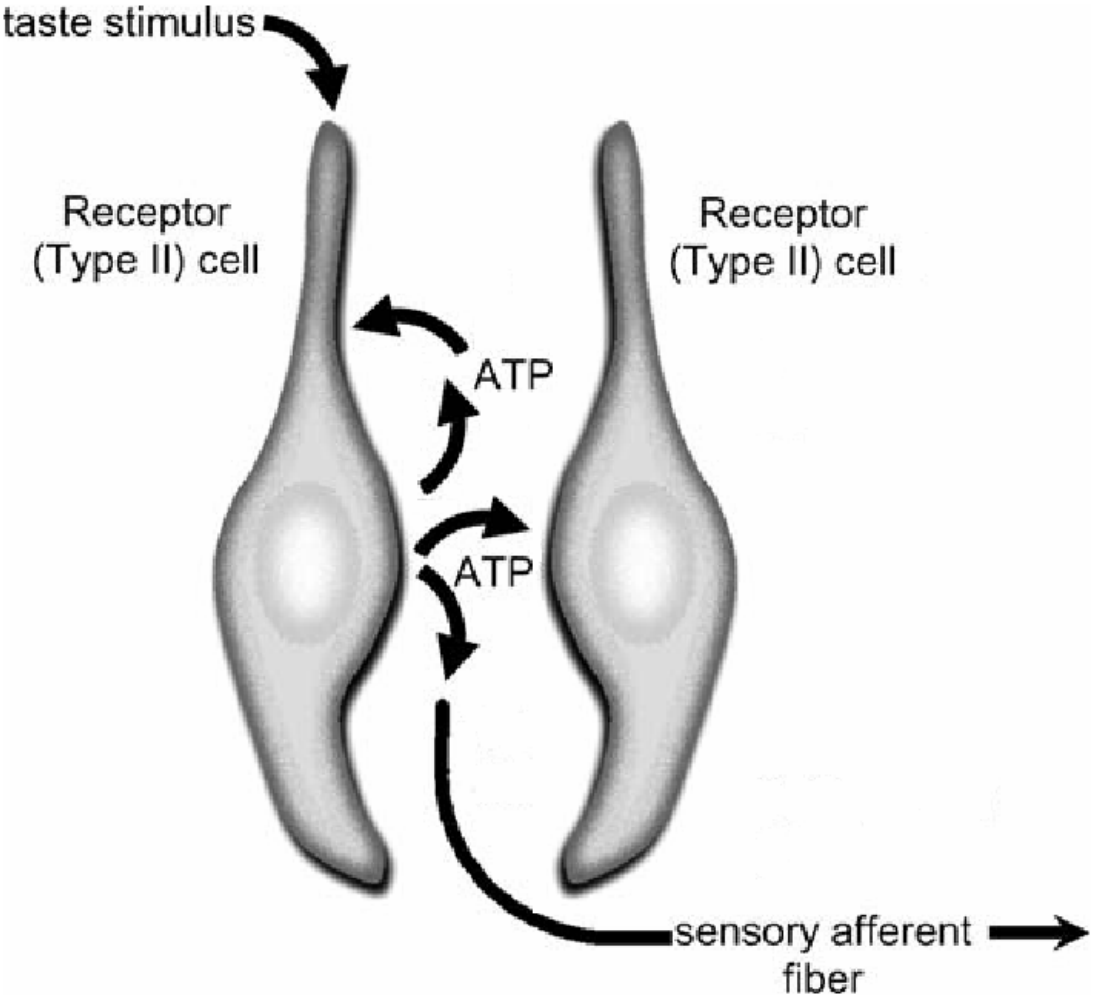

taste stimulus
taste stimulus
Receptor
(Type II) cell
Presynaptic
(Type III) cell
5HT
5HT
ATP
5HT?
synapse (5HT?)
?
sensory afferent
fiber

Figure 19. Type II cell-Type II cell communication. Type II cells may secrete ATP, directly acting on adjacent $P2X_2$-LIR sensory afferent fibers, providing a non-vesicular pathway for taste transduction from Type II cells onto nerve processes. Type II cells may also secrete ATP, acting on nearby $P2Y_4$-LIR Type II cells, up-regulating the release of ATP from adjacent Type II cells. Type II cells may also secrete ATP back onto themselves, acting as a negative feedback mechanism for the down-regulation of ATP secretion (Adapted from Roper, 2007).


taste stimulus
Receptor
(Type II) cell
Receptor
(Type II) cell
ATP
ATP
sensory afferent
fiber

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