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**CONNEXIN-32 AND CONNEXIN-43 IMMUNOREACTIVITY IN  
RODENT TASTE BUDS**

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A Thesis

Presented to

the Faculty of Natural Sciences and Mathematics

University of Denver

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Amanda E. Bond

June 2012

Advisor: Dr. John C. Kinnamon

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Author: Amanda E. Bond  
Title: CONNEXIN-32 AND CONNEXIN-43 IMMUNOREACTIVITY IN RODENT  
TASTE BUDS  
Advisor: Dr. John C. Kinnamon  
Degree Date: June 2012

### **Abstract**

Studies indicate that ATP is one of the primary neurotransmitters in taste transduction. ATP release occurs from taste cells via specific hemichannels such as pannexin/connexin hemichannels (Huang et al., 2007; Romanov et al., 2007). We hypothesize that Type II (receptor) and possibly Type III (presynaptic) cells release ATP at sites containing pannexin/connexin hemichannels. In this study, we examine the presence of connexin-32-LIR (Like Immunoreactivity) and connexin-43-LIR in rodent taste buds through immunocytochemical analysis and DAB (Di-amino-benzidine) immunoelectron microscopy. We observed that connexin-32-LIR co-localizes with P2X2-LIR in nerve fibers and in a small subset of NCAM-LIR cells. Connexin-32-LIR does not co-localize with  $\alpha$ -gustducin-LIR or PLC $\beta$ 2-LIR. We observed that connexin-43-LIR is present in a subset of PLC $\beta$ 2-LIR cells and in a subset of  $\alpha$ -gustducin-LIR cells. Connexin-43-LIR does not co-localize with NCAM-LIR cells or P2X2-LIR nerve fibers. These results are consistent with our results observed using DAB immunoelectron microscopy. Thus, our results indicate that both connexin-32 is expressed in Type III cells and nerve fibers and connexin-43 is expressed in Type II cells in rodent circumvallate taste buds.

## **ACKNOWLEDGEMENTS**

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## Abbreviations

**LIR** – Like Immunoreactivity

**ROMK** - Renal Outer Medullary Potassium Channel

**GLAST** - Glutamate Aspartate Transporter

**T2R** - Taste 2 Receptor

**T1R** – Taste 1 Receptor

**T1R1** - Taste 1 Receptor 1

**T1R2** – Taste 1 Receptor 2

**T1R3** – Taste 1 Receptor 3

**GPCRs** - G-Protein Coupled Receptors

**PLC $\beta$ 2** - Phospholipase C  $\beta$ 2

**Ca<sup>2+</sup>** - Calcium

**IP<sub>3</sub>** - inositol-1,4,5-triphosphate

**IP<sub>3</sub>R3** - inositol-1,4,5-triphosphate receptor type 3

**TRPM5** - transient receptor potential channel M5

**T2R** - Taste 2 Receptor

**PDE** – phosphodiesterase

**PLC** – Phospholipase

**cAMP** - 3'-5'-cyclic adenosine monophosphate

**PIP2** - Phosphoinositol Biphosphate

**DAG** – Diacylglycerol

**mGluR** - metabotropic Glutamate Receptors

**mGluR4** - metabotropic Glutamate Receptor 4

**MSG** - Monosodium Glutamate

**ENaC** - Epithelial Sodium Ion Channel

**PKD2L1** - Polycystic Disease 2 Like 1

**PKD1L3** - Polycystic Disease 1 Like 3

**ASIC** - Acid Sensing Ion Channel

**HCN** - Hyperpolarization Activated Cyclic Nucleotide-Gated Potassium Channel

**NPPB** - 5-nitro-2-(3-phenylpropylamino)- benzoic acid

**Cl<sup>-</sup>** - Chlorine

**LCFA** - Long Chain Fatty Acids

**CD36** – Cluster of Differentiation 36

**ATP** – Adenine Triphosphate

**P2X** – Purinergic 2X

**RT-PCR** – Real Time Polymerase Chain Reaction

**mRNA** – Messenger Ribonucleic Acid

**PFA** – Paraformaldehyde

**OCT** - Optimal Cutting Temperature embedment

**PBS** – Phosphate Buffered Saline

**DAB** – Diaminobenzidine

**ABC** - Avidin Biotin Complex

# INTRODUCTION

## **Mammalian Taste Buds and Gustatory Papillae**

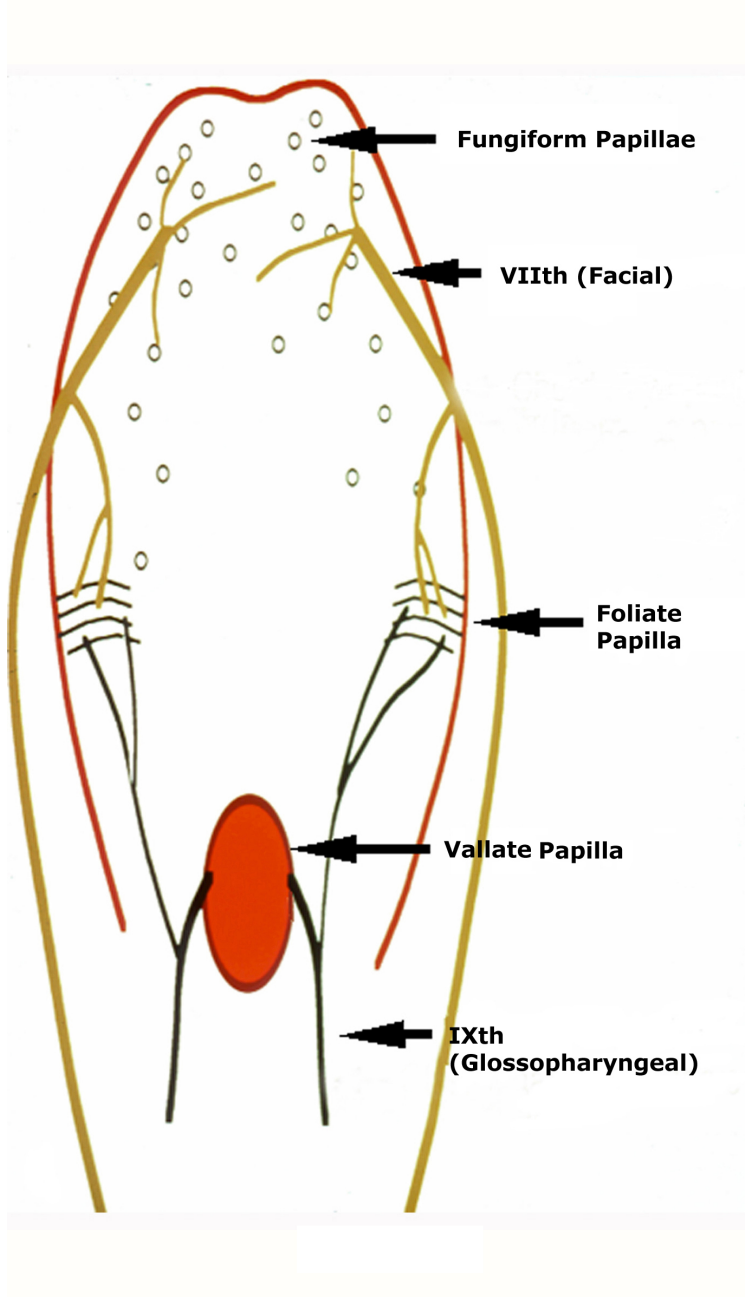
Taste is the most fascinating chemical sense. Not only is it essential for survival, but it can also provoke feelings that range from great euphoria to intense disgust. Detection of gustatory stimuli in the taste bud triggers a series of signal transduction events in taste cells. The mammalian taste bud is an onion-shaped structure surrounded by epithelial tissue. Humans have approximately 5,000 taste buds that sit on the surface of the tongue, epiglottis, and palate (Miller, 1995; Chaudhari & Roper, 2010). Within each taste bud are 50-100 taste cells that detect sour, salty, bitter, sweet, and umami taste stimuli.

Lingual taste buds are found in gustatory papillae on the tongue. In most mammals, these papillae are divided into three major classes; fungiform, foliate, and circumvallate papillae (Whiteside, 1927; Fish et al., 1944; Farbman, 1965; Oakley, 1967; J. Kinnamon, 1987; Miller & Bartoshuk, 1991) (Fig. 1). Each class of papillae is located on a different area of the tongue. Fungiform papillae are mushroom-shaped and they are found on the anterior two thirds of the tongue on the dorsal surface (Gilbertson et al., 2000). Rodents typically have one or two

taste buds in each fungiform papilla. Foliate papillae are found on the posterolateral region of the tongue (Gilbertson et al., 2000). A large number of taste buds line the walls of foliate papillae. In rodents, the circumvallate papillae are located on the medial-posterior surface of the tongue. A deep trench containing approximately 250 taste buds lines this single circumvallate papilla (Gilbertson et al., 2000).

### **Innervation:**

Taste buds interact with a group of sensory neurons whose cell bodies lay in clusters near the brain (Chaudhari & Roper, 2010). In rodents, there are two major cranial nerves that innervate taste buds, depending on their location on the tongue (Whiteside, 1927; Oakley, 1967; Beidler, 1969; Farbman & Hellekant, 1978; Bradley et al., 1985; Whitehead et al., 1985). Innervation of the posterior tongue, which includes vallate and some foliate papillae, is via the glossopharyngeal (IX<sup>th</sup>) nerve. Innervation of the anterior tongue, which includes fungiform and some foliate papillae, is via the chorda tympani branch of the facial (VII<sup>th</sup>) nerve (Gilbertson et al., 2000). Innervation in taste buds extends beyond the major nerves, to a plexus of nerve fibers located under the taste epithelium. In order to enter taste buds, several taste axons penetrate the epithelium (Chaudhari & Roper, 2010). This network of nerve fibers can be divided into intragemmal fibers, which form synaptic connections with taste cells, or perigemmal fibers, which simply surround the taste buds (Nagy et al., 1982; Finger, 1986; Finger et al., 1990).



**Figure 1:** Diagram of the rodent tongue. Large arrows label gustatory papillae and small arrows label cranial nerves (Dunlap, 1997; Yang, 2006).



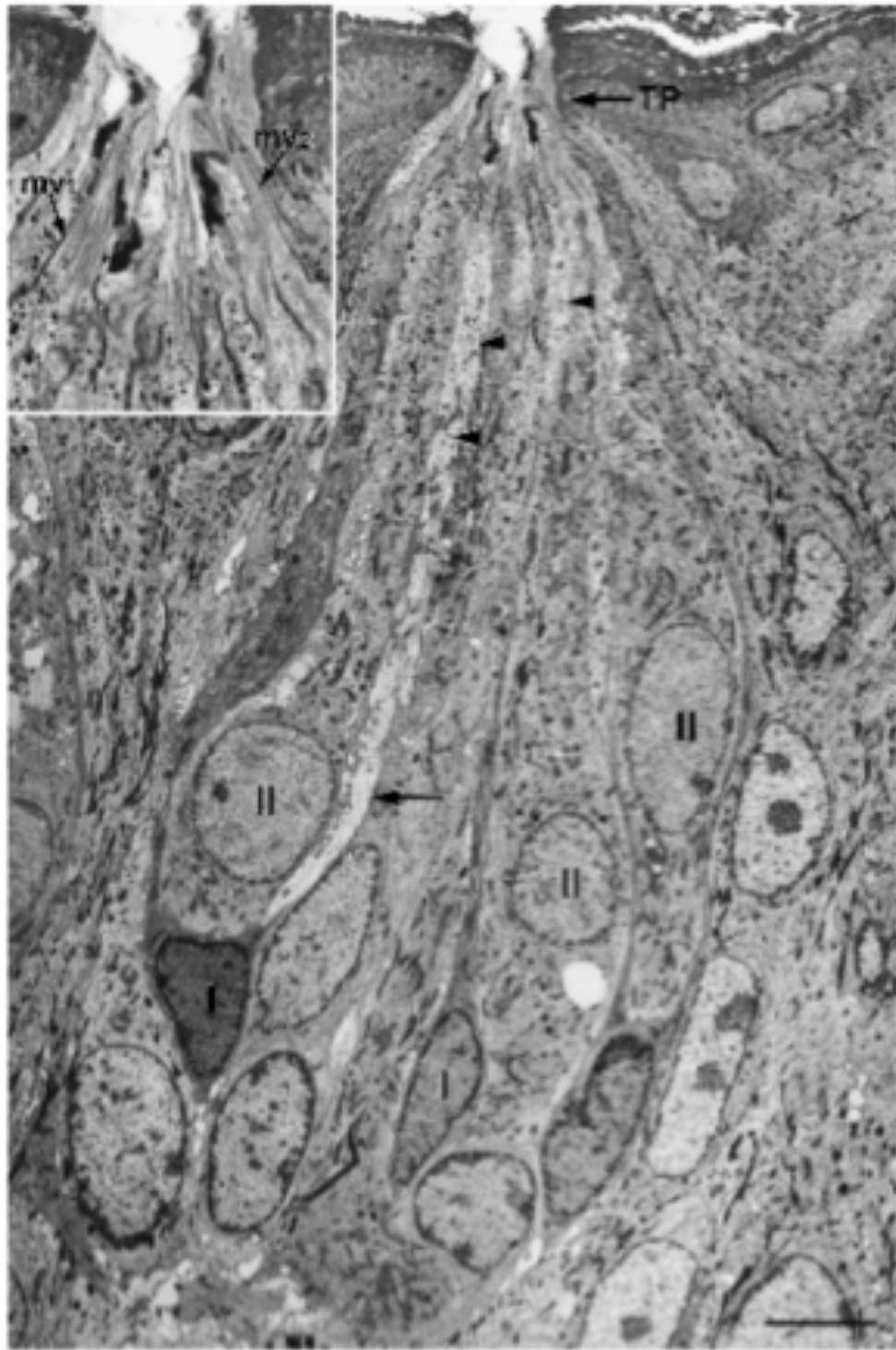
## **Classification of Taste Cells in the Taste Bud**

Taste cells within the taste bud play an important role in cell signaling and transduction. A variety of signaling molecules and transduction mechanisms have been described in taste cells (S. Kinnamon & Margolskee, 1996; Gilbertson et al., 2000; Lindemann, 2001). For these reasons, it is important to use a common system to classify taste cell types. Initially, the characteristics of taste cells observed with cytoplasmic staining using basophilic dyes led to the classification of taste cells as “light” or “dark” (Loven, 1868; Schwalbe, 1868; Wilson & Edin, 1905). Modern techniques, such as electron microscopy, have allowed for a better classification of the ultrastructure of taste cells in the taste bud (Figs. 2 & 3). The classification of taste cell types has been controversial. In a recent review on taste buds by J. Kinnamon & Yang (2008), however, it is clear that after 20 years of investigation, researchers have come to an agreement on the ultrastructure of rodent circumvallate taste buds. Scientists agree that taste cells can be classified into four major cell types: Type I, Type II, Type III, and Type IV.

### ***Type I Cells***

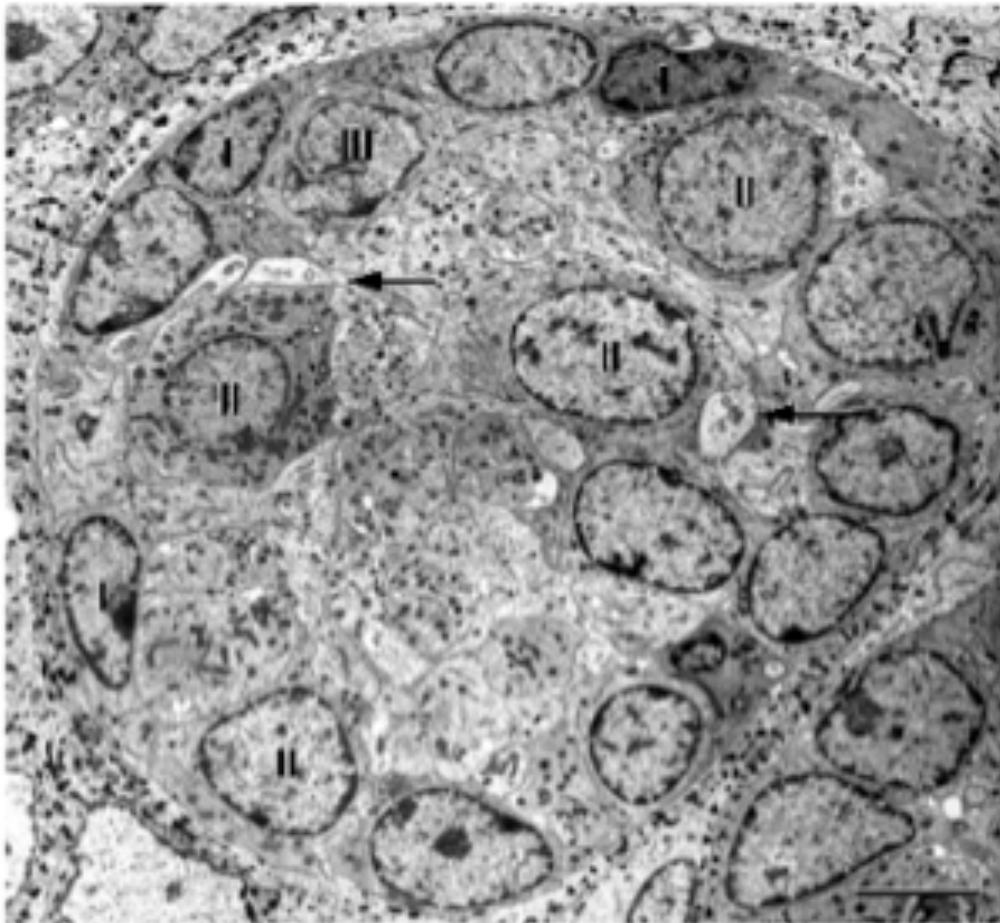
Type I cells, also known as “dark” or glial-like cells, are the most numerous cells in the taste bud, making up 50%-60% of the cell population

Figure 5



**Figure 2:** Longitudinal section through a rat circumvallate taste bud. Type I cells (I) have irregular shaped nuclei, electron dense cytoplasm, and microvilli that terminate in the taste pore. Arrowheads point to dense core vesicles in the apical cytoplasm of Type I cells (I). Type II cells (II) have large circular nuclei. Arrow points to a nerve process. Scale bar: 5  $\mu$ m. **Inset:** In a Type I cell, several long microvilli are present (mv1). In a Type III cell, there is one single microvillus (mv2). (Image from Kinnamon & Yang 2008).

Figure 3



**Figure 3:** Transverse section through a taste bud from a rat circumvallate papilla. Type I cells (I) are characterized by electron-dense nuclei and cytoplasm. Type II cells (II) have large, ovoid nuclei and their cytoplasm is electron-lucent. Type III cells (III) are characterized by nuclei with prominent invaginations. Arrows point to nerve processes. Scale bar = 5  $\mu$ m (Kinnamon & Yang, 2008)

(Farbman et al., 1965; Murray et al., 1973; J. Kinnamon et al., 1985; Delay et al., 1986; J. Kinnamon & Yang, 2008). Type I cells are unique because unlike other taste cell types, Type I cells possess cytoplasmic processes that separate and envelop Type II and Type III cells, as well as intragemmal nerve fibers (Murray, 1973; Royer & J. Kinnamon 1991; Yang & J. Kinnamon, 2008). Type I cells have slender, elongate nuclei filled with heterochromatin (J. Kinnamon & Yang, 2008) (Fig. 2). The apical ends of Type I cells are characterized by many long, slender microvilli. They can also be distinguished by the presence of dense-core granules positioned apically in the cytoplasm (Farbman, 1965; Murray, 1973; Kinnamon et al., 1988; Miller, 1995; Reutter & Witt, 1993; Royer & J. Kinnamon, 1991). Type I cells are found in close apposition to nerve fibers in the taste bud; however, these cells are not believed to form synapses with nerve processes (Farbman, 1965; Murray, 1973; Reutter & Witt, 1993). Type I cells are implicated in both sensory and supportive roles in taste buds (Lindemann, 1996).

Dvoryanchikov et al. (2009) proposed that Type I cells express ROMK (Renal Outer Medullary Potassium Channel), a channel that may maintain K<sup>+</sup> homeostasis within the taste cell (Chaudhari & Roper, 2010). Type I cells also express GLAST (Glutamate Aspartate Transporter), suggesting they may play a role in glutamate uptake (Lawton et al, 2000; Chaudhari & Roper, 2010). It has been proposed that Type I cells play a role in the detection of salt (Vandenbeuch et al, 2008; Chaudhari & Roper, 2010). Researchers continue to explore the role of Type I cells in the taste bud.

## ***Type II Cells***

Type II cells, also known as “light” cells or receptor cells, are characterized by large, ovoid nuclei and an electron-lucent cytoplasm (Farbman, 1965; Murray, 1973; J. Kinnamon et al., 1985, 1988, 1993; Delay et al., 1986; Royer & J. Kinnamon, 1988, 1991, 1994; Pumplin et al., 1997). The apical region of a Type II cell in a rat circumvallate papilla displays short, uniform microvilli that are often referred to as “brush-like” (Yee et al., 2001). Type II cells are the second most abundant cell type in the taste bud, making up approximately 15-30% of cells. (Farbman et al., 1965; Murray et al., 1973; J. Kinnamon et al., 1985; Delay et al., 1986; J. Kinnamon & Yang, 2008). Type II cells detect sweet, bitter and umami gustatory stimuli (Finger et al., 2005a; Tomchik et al., 2007). Detection of these stimuli activates G-protein coupled receptors located on the cell. Cells expressing T2R (Taste 2 Receptor) G-protein coupled receptors are responsible for transducing bitter compounds (Chandrashekar et al., 2000), while cells expressing T1R2 (Taste 1 Receptor 2) and T1R3 (Taste 1 Receptor 3) are responsible for sensing sweet compounds (Nelson et al., 2001; Jiang et al., 2004; Xu et al., 2004; Chaudhari & Roper, 2010). Cells expressing T1R1 (Taste 1 Receptor 1) and T1R3 are believed to signal umami compounds; however, there may be other GPCRs (G-Protein Coupled Receptors) responsible for transduction of umami stimuli (Chaudhari et al., 2000; Li et al., 2002; Nelson et al., 2002; San Gabriel et al., 2009, Chaudhari & Roper, 2010). Type II cells lack

identifiable synapses (Farbman, 1965; Murray, 1973; Yee et al., 2001; Yang et al., 2004). There is evidence, however, suggesting that they form contacts with nerve processes (Royer & J. Kinnamon, 1988; Clapp et al., 2004). Recent studies suggest that Type II cells use non-vesicular signaling mechanisms such as gap junction proteins for cell-cell communication in the taste bud (Romanov et al., 2007; Huang et al., 2007; Romanov et al., 2008; Dando & Roper, 2009).

### ***Type III Cells***

Type III cells, also known as presynaptic cells, are the only taste cells to form identifiable synaptic connections with nerve processes in rat circumvallate taste buds (Yang et al., 2000b; Yee et al., 2001; Yang et al., 2004; J. Kinnamon & Yang, 2007). They are also the only taste cells that express the presynaptic protein, SNAP-25 (Yang et al., 2000a; Finger et al., 2005b), suggesting that Type III cells play a significant role in relaying taste information to the nerve fibers. The nuclei of Type III cells are characterized by prominent nuclear invaginations (J. Kinnamon & Yang, 2008). They constitute the smallest percentage of cells found in the taste bud, constituting only 5-15% of the cells (Delay et al., 1986; Reutter & Witt, 1993; J. Kinnamon, 1987; J. Kinnamon & Yang, 2008). It is presumed that Type III cells are responsible for signaling sour taste stimuli and detecting carbonation (Huang et al., 2006; Tomchik et al., 2007; Huang et al., 2008b; Chandrashekar et al., 2009; Chaudhari & Roper, 2010); however, the pathways through which sour taste and carbonation are transduced remain controversial.



### ***Type IV Cells***

Type IV cells, also known as basal cells, are ovoid-shaped cells found in the basolateral region of the taste bud. They are distinguished by the presence of intermediate filaments that attach to the nuclear envelope of the cell (Delay et al., 1986; J. Kinnamon & Yang, 2008). These cells carry no known responsibility in the detection of taste stimuli. They are, however, considered to be precursors to other cell types in the taste bud (Beidler & Smallman, 1965; Conger & Wells, 1969; Farbman et al., 1980; Stone et al., 2002; J. Kinnamon & Yang, 2008). Basal cells do not have processes that extend to the taste pore and are most likely undifferentiated cells (Farbman, 1965; Chaudhari & Roper, 2010). One of the controversial hypotheses regarding taste cell lineage suggests that basal cells are responsible for the formation of an immature cell, which will ultimately differentiate into a Type I, Type II, or Type III cell (Finger et al., 2005a). Unfortunately, it is difficult to study basal cells because no known cell markers currently exist for this cell type; thus, the significance of basal cells in taste tissue remains a matter of controversy to scientists in the taste field (Chaudhari & Roper, 2010).

## Synapses in Taste Cells

In mouse circumvallate taste buds, approximately 20% of the taste cells form synapses onto nerve fibers (J. Kinnamon et al., 1985). Chemical synapses occur between Type III cells and nerve fibers. Structurally, synapses in the taste bud are classified into two categories; small and macular, or “finger-like” (J. Kinnamon et al., 1985; J. Kinnamon et al., 2005). De Lorenzo (1963) was the first scientist to publish ultrastructural evidence depicting contact between taste cells and nerve fibers. Today, the ultrastructure of a synapse in the taste bud is well studied (Fig. 4). J. Kinnamon et al. (1985, 1988, 2001) developed a defined set of criteria for the purpose of identifying synapses in taste buds. These criteria include 1. Two thickened membranes that are separated by a cleft; these membranes should sit parallel and closely apposed to one another; 2. Small, clear vesicles and large, dense-cored vesicles are present; 3. The postsynaptic thickening is denser and thicker than the presynaptic thickening (Royer & J. Kinnamon, 1991; J. Kinnamon & Yang, 2008). The criteria for identifying a synapse in the taste bud has greatly advanced the study of synaptic connections in taste cells.

## **Other Contacts: Subsurface Cisternae and Atypical Mitochondria**

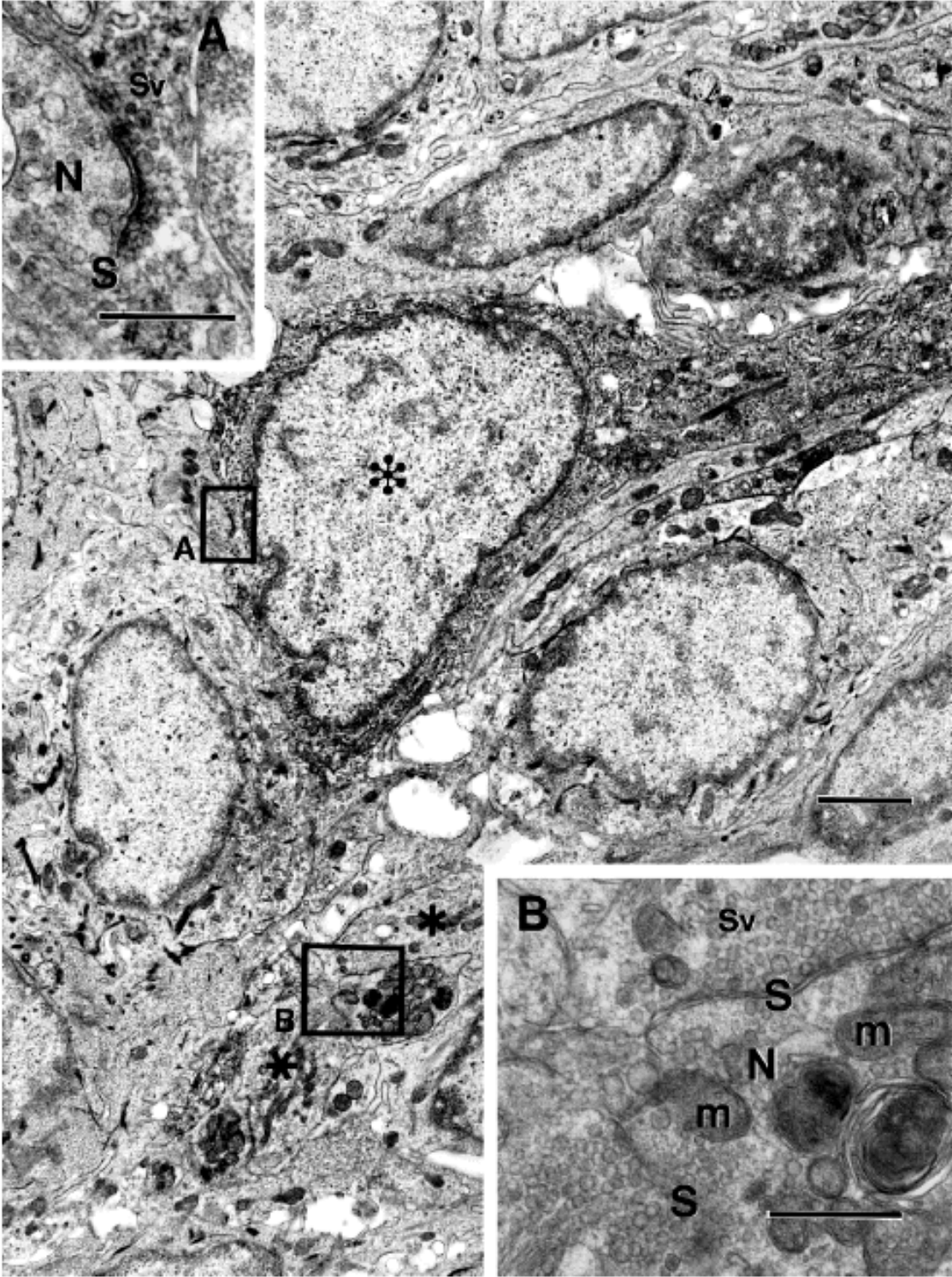
### ***Subsurface Cisternae***

Synapses in gustatory tissue are most commonly afferent; however, it is proposed that subsurface cisternae of the smooth endoplasmic reticulum may also play a role in signaling in taste buds (Ide & Munger; 1980; Clapp et al., 2004; Kinnamon et al., 2005). Subsurface cisternae are located in close apposition to taste cells and nerve fibers. More specifically, they sit near contacts between Type II cells and nerve fibers, next to the cytoplasmic leaflet of the taste cell membrane (Royer & J. Kinnamon, 1988; J. Kinnamon & Yang 2008). In Type II cells, which lack identifiable synapses, it is possible that subsurface cisternae of smooth endoplasmic reticulum are responsible for communication with the nervous system (Clapp et al., 2004).

### ***Atypical Mitochondria***

In taste cells, atypical mitochondria differ from “normal” mitochondria in size and structure. Not only are they larger than “normal” mitochondria, but they also lack the lamellar cristae (Royer & J. Kinnamon, 1988). Instead, atypical mitochondria contain “twisted-energized” or “swollen-twisted-energized” cristae (Green & Baum, 1970; Korman et al., 1970; Williams et al., 1970). These

Figure 4



**Figure 4:** DAB immunoelectron micrograph of a synapse (s) onto a nerve fiber (n) in the taste bud of a rat circumvallate papilla. The presynaptic taste cell (asterisk) exhibits the characteristics of a Type III taste cell (elongate shape, nuclear invaginations). **Inset A:** Synaptic vesicles (sv) are docked at the synaptic membrane. Taste cell (asterisk in low magnification image) synapses (s) onto a nerve process (n). **Inset B:** Presynaptic zone has many clear synaptic vesicles (sv). Dense core vesicles are located near the synapse. Taste cell (Bold face asterisk in low magnification image) synapses (s) onto a nerve process (n). Mitochondria (m) are present. Inset scale bars = 0.5  $\mu$ m. Scale bar for low magnification image = 5  $\mu$ m (Yang et al., 2000).

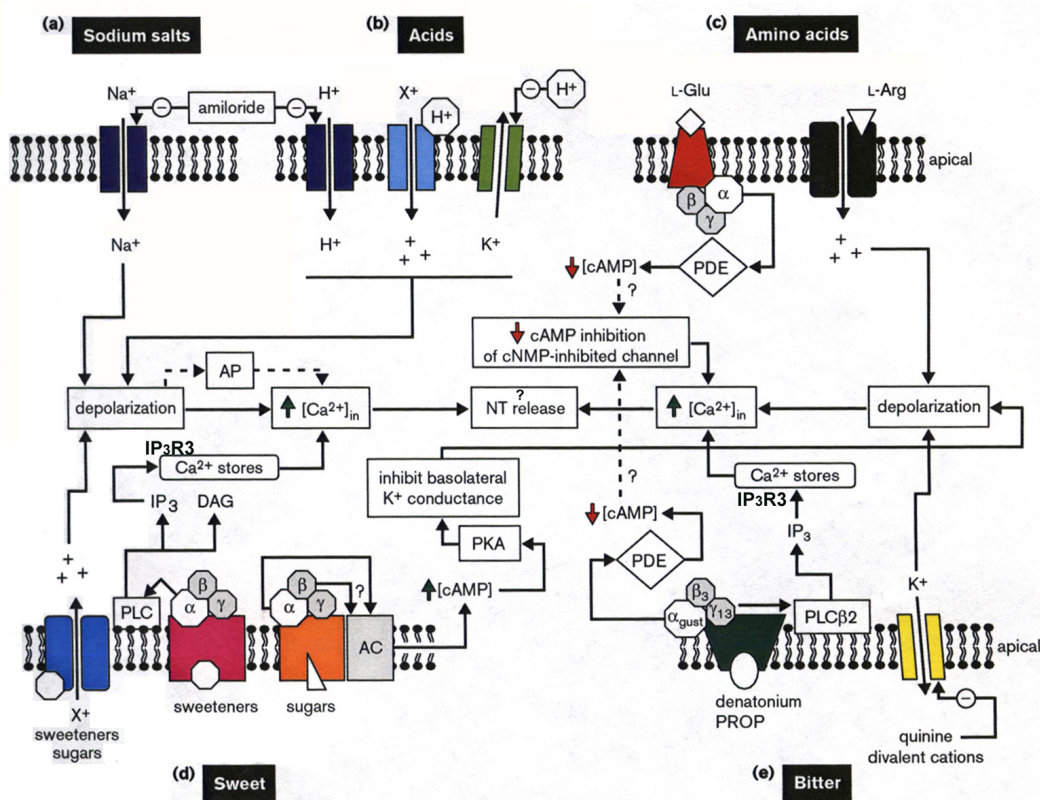
configurations refer to structural changes in mitochondria that occur during swelling. Atypical mitochondria are found at close contact between Type II cells and nerve processes. Atypical mitochondria are sometimes associated with subsurface cisternae (J. Kinnamon & Yang, 2008). They have been proposed to play a role in the uptake and release of  $\text{Ca}^{2+}$  (Hajnoczky et al., 2001; Hawkins et al., 2007).

### **Taste Transduction**

The detection of gustatory stimuli in taste cells initiates interactions between the taste cells and nerve fibers. From the nerve fibers, taste information is transferred to the brain. Taste is transduced through different pathways depending on the type of stimulus detected. The five major taste stimuli are bitter, sweet, salty, sour, and umami. There is also evidence for transduction of fat taste (Khan & Bernard, 2009). Studies show candidate receptors for each basic taste quality (Chandrashekar et al., 2006; Ishimaru, 2006; Niki et al., 2010) that can be divided into two categories: GPCRs and channel type receptors (Niki et al., 2010). Most taste cells can only be stimulated by one of the major taste qualities (Caicedo et al., 2002; Yoshida et al., 2006; Niki et al., 2010). Upon detection of gustatory stimuli, specific signaling pathways are activated

The signaling pathways for sweet, bitter and umami taste are very similar. All three basic tastes use GPCRs; however, the type of GCPR differs with each taste type. When sweet, bitter, or umami tastants bind to receptors they activate

a G-protein, (Hisatsune et al., 2007; Niki et al., 2010), which then stimulates PLC $\beta$ 2 (phospholipase C  $\beta$ 2) (Zhang et al., 2003; Niki et al., 2010). The stimulation of PLC $\beta$ 2 causes IP $_3$  to bind to IP $_3$ R3 (inositol-1,4,5-triphosphate receptor type 3), causing the release of Ca $^{2+}$  and depolarization of taste cells through TRPM5 (transient receptor potential channel M5) channels (Fig. 5) (Zhang et al., 2003; Zhang et al., 2007; Niki et al., 2010).



**Figure 5:** Diagram showing the proposed mechanisms for transduction of taste in vertebrates. It is believed that all pathways result in the elevation of intracellular Ca<sup>2+</sup>, resulting in neurotransmitter release (Gilbertson T.A., S. Damak, and R.F. Margolskee, 2000; Yang, 2006).



### ***Sweet Taste***

The T1R (Taste 1 Receptor) family of GPCRs plays a role in modulation of sweet taste. More specifically, T1R2 and T1R3 function as sweet taste receptors by forming a complex (Nelson et al., 2001, 2002; Niki et al., 2010) that responds to various sweet tastants. The pathway for sweet taste transduction is dependent upon the type of sweet tastant.

### ***Bitter Taste***

T2Rs (Taste 2 Receptor) belong to a family of GPCRs that differ from T1Rs; they play a role in the transduction of bitter taste. Bitter taste is transduced through the common signaling pathway that is also responsible for sweet and umami taste, yet it still exhibits some of its own unique signaling qualities.  $\alpha$ -gustducin has been shown to be a key component in the response of taste cells to bitter compounds (Wong et al., 1996; Ming et al., 1998; Gilbertson et al., 2000). T2Rs activate a specific G $\alpha$  subunit and  $\alpha$ -gustducin (Ruiz-Avila et al., 1995; Chaudhari & Roper, 2010), that is selective to bitter taste.  $\alpha$ -gustducin is believed to activate the PDE (phosphodiesterase) pathway (S. Kinnamon & Margolskee, 1996); however, binding of a bitter tastant to a GPCR also leads to the activation of PLC (Phospholipase C). Once  $\alpha$ -gustducin activates PDE, intracellular cAMP (3'-5'-cyclic adenosine monophosphate) levels are reduced (McLaughlin et al., 1992). Stimulation of the PLC pathway is thought to be triggered by the Gy13

and Gβ3 gamma subunits that are released from G-proteins (Rossler et al., 1998). Through the PLC pathway, PLCβ2 cleaves PIP2 (phosphoinositol bisphosphate) into IP<sub>3</sub> and DAG (diacylglycerol) (Bernhardt et al., 1996, Huang et al., 1999). IP<sub>3</sub> is thought to bind to IP<sub>3</sub>R3, causing a rise in intracellular Ca<sup>2+</sup>, resulting in transmitter release.

Recent studies shows that subsets of partially overlapping T2Rs are expressed in taste cells that respond to bitter tastants (Behrens et al., 2007; Chaudhari & Roper, 2010). This is significant because bitter responsive taste cells can discriminate between bitter compounds (Caicedo & Roper, 2001; Chaudhari & Roper, 2010). The discrimination of bitter compounds is especially important because it plays a role in survival by allowing for the detection of compounds that could be harmful or toxic (Drayna, 2005; Chaudhari & Roper, 2010).

### ***Umami Taste***

Transduction of umami taste, also known as “amino acid” taste, occurs through GPCRs and ligand-gated channels (Chaudhari et al., 2000; Nelson et al., 2002; Li et al., 2002). T1Rs not only play a role in sweet taste, but also function in umami taste. T1R1 (Taste 1 Receptor 1) and T1R3 form complexes that function as umami taste receptors (Nelson et al., 2001; Nelson et al., 2002; Niki et al., 2010). In mice, this complex will respond to various amino acids, while in

humans, glutamate activates this complex (Nelson et al., 2002; Li et al., 2002; Niki et al., 2010).

mGluRs (metabotropic Glutamate Receptors) have been observed in taste cells and are proposed candidates for umami taste receptors (Chaudhari et al., 2000; San Gabriel et al., 2009; Niki et al., 2010). Specifically, mGluR4 (metabotropic Glutamate Receptor 4) has been localized in taste cells (Yang et al., 1999). Glutamate in mGluR4-receptors for taste is proposed to decrease cAMP levels (Zhou & Chaudhari, 1997), possibly triggering an interaction with cyclic nucleotide gated channels (Lindemann, 2001).

In umami taste, a phenomenon known as synergism occurs when 5'-ribonucleotide monophosphates enhance the intensity of MSG (monosodium glutamate) (Yamaguchi, 1967; Yamaguchi, 1991; Niki et al., 2010). Synergism is believed to be a key characteristic of umami taste.

### ***Salty Taste***

Amiloride is an epithelial sodium channel blocker that has been shown to reduce behavioral, neural, and taste responses to sodium chloride (Heck et al., 1984; Spector et al., 1996; Ninomiya, 1998; Yoshida et al., 2009; Niki et al., 2010). It has been proposed that ENaCs (epithelial sodium ion channels) are the receptor for salty taste. Activation of an ENaC by salty tastants causes the depolarization of taste cells, thereby stimulating an action potential (Vaudenbeuch et al., 2008).

### ***Sour Taste***

The transduction pathway for sour stimuli is controversial. It has been suggested that PKD2L1 (Polycystic Disease 2 Like 1) and PKD1L3 (Polycystic Disease 1 Like 3) are responsible for transduction of sour stimuli (Huang et al., 2006; Ishimaru et al., 2006; Lopez Jimenez et al., 2006); however, recent evidence suggests otherwise. Recent studies show that ion channels in the plasma membrane modulated by cytoplasmic acidification are more likely to be a candidate for sour taste transduction (Lin et al., 2004; Richter et al., 2004; Chaudhari & Roper, 2010). ASICs (Acid sensing ion channels) have also been implicated in the detection of sour taste (Ugawa et al., 2003; Niki et al., 2010). HCNs (hyperpolarization activated cyclic nucleotide-gated potassium channels) (Stevens et al., 2001; Niki et al., 2010), and NPPB (5-nitro-2-(3-phenylpropylamino)- benzoic acid) sensitive Cl<sup>-</sup> channels (Miyamoto et al., 1998; Niki et al., 2010).

### ***Fat Taste***

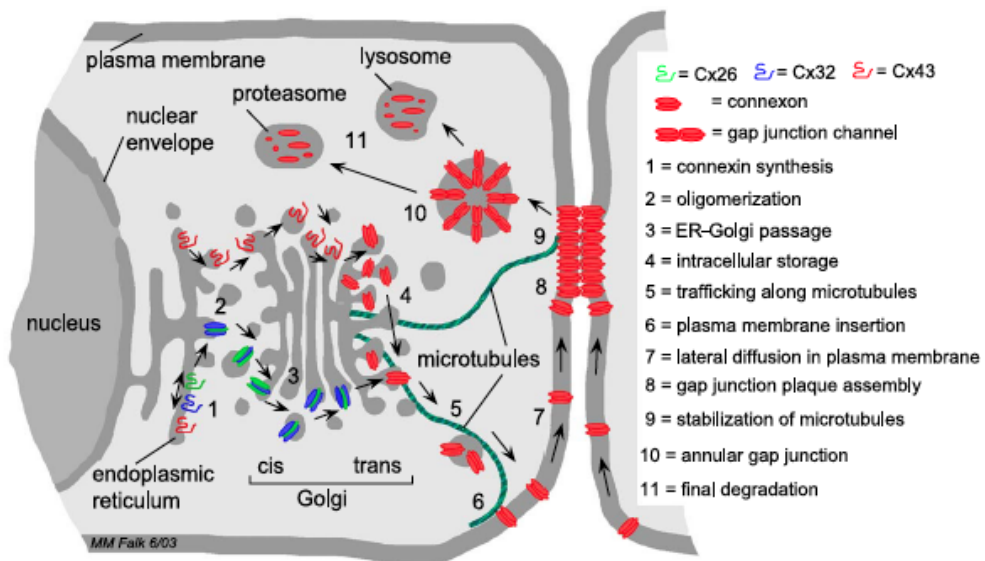
Studies have recently proposed fat taste to be its own basic taste quality (Khan & Bernard, 2009), but the mechanisms behind the transduction of lipids are still unknown. Evidence suggests that upon stimulation of LCFA (Long Chain Fatty Acids), lingual CD36 (Cluster of Differentiation 36) may respond to fat taste,

and activate signaling mechanisms for fat taste transduction (Khan & Bernard, 2009). Future studies should provide further insight into the mechanisms involved in the transduction of fat taste.

### **Gap Junctions and Hemi-channels**

Gap junctions and hemi-channels are formed by connexins in vertebrates and innexins in invertebrates (Hua et al., 2003; Phelan, 2005; Willecke et al., 2002). These channels allow passage of molecules that are less than 1 kDA, such as ions and second messengers, providing a mechanism for cell-cell communication in animal tissues (Bennett et al., 1978; Schwarzmann et al., 1981; Goodenough et al., 1996; Spray et al., 2006; Scemes et al., 2009; Burra & Jiang, 2011). When six connexin subunits oligomerize, they form a hexameric connexon (Fig. 6) (Musil & Goodenough, 1993; Kistler et al., 1995; Sosinsky, 1995; Cascio et al., 1995; Falk et al., 1997; VanSlyke et al., 2000; Ungar et al., 1999; Segretain & Falk, 2004; Burra & Jiang, 2011), which is equivalent to a hemi-channel. Oligomerization occurs in the endoplasmic reticulum, Golgi, or post Golgi compartments (Fig. 6) (Burra & Jiang, 2011). This formation is strongly dependent upon the structure of a connexin. An individual connexin protein subunit contains a transmembrane domain, C and N termini, as well as extracellular and cytoplasmic loops with Cys residues (Panchin, 2005; Scemes, 2009). When a single connexon docks at the cell membrane in close apposition

Figure 6



**Figure 6:** Diagram representing the synthesis and oligomerization of connexin. Connexin synthesis in or near the endoplasmic reticulum results in oligomerization of six connexin subunits to form a hemichannel (connexon). One connexon can dock at the membrane with another connexin to form a gap junction. (Segretain and Falk, 2004).

to a connexon on an adjacent cell, a gap junction is formed (Burra & Jiang, 2011). Essentially, two hemi-channels form a gap junction. Gap junctions have an intercellular space that is approximately 2-4 nm (Panchin, 2005; Litvin et al., 2006). Connexin hemi-channels have been reported to release cytosolic molecules such as ATP (Adenine Triphosphate) and glutamate into extracellular medium (Goodenough & Paul, 2003; Stout et al., 2004; Spray et al., 2007; Scemes et al., 2009). Pannexin is another gap junction protein that is structurally similar to connexins. Like connexin, pannexin can form hexameric channels; however, there is no evidence suggesting that pannexins can form gap junctions (Dahl & Locovei, 2006; Burra & Jiang, 2011).

## **Pannexin**

Panchin et al. (2000) discovered pannexins, a new family of gap junction proteins. Further study confirmed that pannexins are homologs of innexins, the gap junction protein of invertebrates (Baranova et al., 2004). Pannexins are structurally similar to connexins, and in most tissue types, the distribution of pannexin overlaps with connexin (Bao et al., 2004; Scemes et al., 2009). Out of the three known pannexin isoforms; pannexin-1, pannexin-2, and pannexin-3 (Litvin et al., 2006), only pannexin-1 has been observed to form channels (Scemes et al., 2009). Using the same mechanism as connexin, six pannexin subunits oligomerize to form a pannexon, which is often referred to as a “hemi-channel” in pannexin literature. Sosinsky et al. (2011) argues against the use of



the term “hemi-channel” in association with pannexons because this promotes the wrong idea regarding their function. Pannexons are unable to form gap junction intracellular channels; however, associating them with the term “hemi-channels” implies that they are able to form gap junction intracellular channels (Sosinky et al., 2011). It is therefore most accurate to refer to pannexons as “channels”, not “hemi-channels.” Studies implicate pannexin-1 in ATP release in taste cells (Huang et al., 2007; Dando & Roper, 2009; Murata et al., 2010), an idea that has proved to be controversial in the taste field. Previous work in our lab suggests pannexin-1 is present in Type II cells and a small subset of Type III cells in rat circumvallate papillae (Yang et al., 2010).

## **Connexin**

Connexins are members of a large family of proteins responsible for forming gap junctions and hemi-channels in vertebrates. They were first identified as one of the major protein components of gap junctions in the 1980’s (Paul, 1986; Beyer et al., 1987). Today, there are 21 known members of the connexin gene family (Sohl & Willecke, 2003; Burra & Jiang, 2011). Structurally, all connexins are composed of a four transmembrane domain, with cytoplasmic C and N termini, two extracellular loops with Cys residues, and a cytoplasmic loop (Panchin, 2005; Scemes, 2009), yet the connexin family comprises multiple proteins varying in size and function. Scientists have not yet come to an agreement on connexin nomenclature. There are currently two different methods

to distinguish between connexin isoforms: 1. The “CxMW” system; assigns each connexin isoform a name based on their molecular weight (Spray et al., 2006). For example, connexin-43 has a molecular weight of 43 kDa, therefore it will be referred to as Cx43 and connexin-32 has a molecular weight of 32 kDa, so it is named Cx32. 2. The “g<sub>j</sub>αN, g<sub>j</sub>βN, g<sub>j</sub>γN” system in which “g<sub>j</sub>” refers to gap junction; “α,” “β,” or “γ” classifies each connexin into sub-families based on their sequences; and N becomes assigned to each connexin based on order of discovery (Sohl & Willecke, 2003; Spray et al., 2006). For this study, we will employ the use of the “CxMW” nomenclature to differentiate between connexin isoforms.

Connexins are transported to the endoplasmic reticulum following transcription and translation. Oligomerization of six connexin subunits into a connexon can then occur in the endoplasmic reticulum, Golgi, or post Golgi compartments (Spray et al., 2006), depending on the connexin isoform. Connexons are then carried to the plasma membrane in vesicles (Evans et al., 2006). Once inserted into the plasma membrane, one connexon hemi-channel can connect with the hemi-channel of a nearby cell, resulting in the formation of a gap junction (Sohl & Willecke, 2004). Connexins can form three types of gap junction channels: 1. Homomeric channels are formed when a connexon derived from a specific connexin isoform docks with a connexon derived from that same connexin isoform (Goodenough et al., 1996; Jiang & Goodenough, 1996; He et al., 1999; Burra & Jiang, 2011); 2. Heterotypic channels are formed when a connexon derived from a specific connexin isoform docks with a connexon

derived from a different connexin isoform (Barrio et al., 1991; White & Bruzzone, 1996; Bouvier et al., 2009; Burra & Jiang, 2011); 3. Heteromeric channels are formed when a connexon that is derived from different connexin isoforms docks with a connexon that is also derived from different connexin isoforms (Burra & Jiang, 2011). Formation of a heteromeric gap junction is dependent upon compatibility of connexin isoforms.

Connexins have been found to be critical gap junction proteins in other sensory systems. Zhang (2010) demonstrated that connexin hemi-channels impact sensitivity and perception of smell. Altering the structure of connexin-43 in olfactory neurons affected olfactory responses (Zhang, 2010). In the olfactory bulb, the coupling of connexin-36 gap junction hemichannels affects the lateral excitation of mitral cells (Christie & Westbrook, 2006; Zhang, 2010). Connexins are thought to be responsible for the propagation of  $Ca^{2+}$  across the inner ear (Anselmi et al., 2008). Connexins also function in the visual system; they are thought to be mediators of transduction and acuity (Mexeiner et al., 2005; Shubert et al., 2005; Shelley et al., 2006; Anselmi et al., 2008). Connexins have been proposed to mediate the coupling of gap junctions in the retina (Schubert et al., 2005). Schubert et al. (2005) observed that in the mouse retina, the coupling of gap junctions in bi-stratified ganglion cells is mediated by connexin-45. In the skin, a mutation in Connexin-30 causes high amounts of ATP release, resulting in a rare skin disorder, Hidrotic Ectodermal Dysplasia (Clouston Syndrome) (Essenfelder et al., 2004).

In the vital organs of mammals, connexin gap junction proteins are responsible for important cellular activities. In the brain, connexins are thought to play a role in tumor related seizures because astrocytes, oligodendrites, meningeal cells, and ependymal cells from brain tumor tissue express connexin-32 and connexin-43 (Aronica et al., 2001). Connexins have also been described in neuronal cells (Nadarajah et al., 1996; Anselmi et al., 2008). Connexin gap junctions play a critical role in the heart. The mammalian heart expresses multiple connexin proteins, varying in function. Verheule et al. (1997) characterized gap junction channels in the atrial and ventricular myocardium of adult rabbits. They found that specific connexins were expressed in specific areas of the heart; not all connexins were expressed in the same areas. Immunohistochemical studies showed that connexin-43 and connexin-45 were present in the gap junctions associated with ventricular and atrial myocytes, while no immunoreactivity was observed with connexin-40 and connexin-37. Interestingly, they did observe connexin-40 and connexin-37 in endocardial and endothelial tissue of the heart (Verheule et al., 1997). In the liver, connexin-32 is the predominant gap junction protein in hepatocytes (Paul, 1986; Piechocki et al., 1999); however, other components of the liver express different connexins. In biliary epithelial cells of the liver, connexin-43 is the predominant gap junction protein (Neyeu et al., 1994; Piechocki et al., 1999). Based on this evidence, connexins play a role in a diverse array of functions in many different systems.

## ATP Release via Hemi-Channels in Taste Cells

P2X (Purinergic 2X) receptors are present in the gustatory nerve endings that innervate taste buds (Bo et al., 1999). These purinergic receptors are thought to serve multiple functions in taste cells; however, their physiological role in gustation is not yet fully understood. Finger et al. (2005) studied purinergic signaling in taste cells and their results revealed that ATP is a key neurotransmitter in taste transduction. Studies show that in rodent taste cells, ATP release is mediated by hemi-channels, likely to be connexin and/or pannexin hemi-channels (Huang et al., 2007; Romanov et al., 2007). In the taste bud, Type II cells lack identifiable synapses (Clapp et al., 2006). This evidence supports a mechanism for non-vesicular release of ATP in the taste bud, most likely through hemi-channels. The ultrastructure of the taste bud is ideal for cell-cell signaling through hemi-channels. Within the taste bud, Type I, Type II, and Type III taste cells are situated close together and innervated by nerve fibers. The cytoplasm of one taste cell is in close proximity to the cytoplasm of another. Structurally, it seems possible for cells in such intimate contact with one another to signal information via hemi-channels.

Hemi-channels have been observed as sites of ATP release in other sensory cells types. In the retina, ATP release occurs through connexin-43 gap junction hemi-channels in the retinal pigment epithelium (Pearson et al., 2005). Anselmi et al. (2008) found that connexin hemi-channels promote the release of ATP in the inner ear. Locovei et al. (2006) found that elevated  $Ca^{2+}$  and

membrane depolarization causes hemi-channel gates to open, resulting in the release of ATP in erythrocytes. Recent studies show that ATP release occurs in the same manner in taste cells, as they are both mediated by  $\text{Ca}^{2+}$  and voltage-gated channels (Huang & Roper, 2010).

Hemi-channel mediated ATP release in taste cells is controversial. Investigators agree that ATP is a key neurotransmitter in taste cell signaling (Finger et al., 2005b) and is most likely released through hemi-channels; however, there is much debate as to whether pannexin-1 or connexins form these channels (Chaudhari & Roper, 2010). Although pannexins and connexins are structurally similar, they function differently. Pannexins respond to elevated levels of  $\text{Ca}^{2+}$  while connexin channels only open when intracellular  $\text{Ca}^{2+}$  stores are depleted and remain closed if high levels of  $\text{Ca}^{2+}$  are present (Chaudhari & Roper, 2010). Connexin-26, connexin-30, connexin-32, connexin-43 (Stout et al., 2002; Tran Van Nhieu et al., 2003; Romanov et al., 2007) and pannexin-1 (Locovei et al., 2006; Romanov et al., 2007) are the only gap junction proteins that have been implicated in mediating ATP release (Romanov et al., 2007).

Huang et al. (2007) demonstrated that ATP is released via pannexin-1 channels in response to gustatory stimulation. Through the use of a gap junction hemi-channel blocker, carbenoxolone (Davidson & Baumgarten, 1988), they were able to confirm ATP release through hemi-channels. RT-PCR (Real Time Polymerase Chain Reaction) revealed mRNAs (Messenger Ribonucleic Acid) for connexin-30, connexin-43 and pannexin-1 in taste epithelium; however, quantitative RT-PCR revealed that only pannexin-1 is “preferentially enriched” in

taste tissue (Huang et al., 2007). Huang et al. (2007) argues that ATP release through connexin hemi-channels is unlikely because connexin hemi-channels only open if extremely low “non-physiological” levels of  $\text{Ca}^{2+}$  are present (Barbe et al., 2006; Peracchia, 2004). Connexin hemi-channels function best when no intracellular  $\text{Ca}^{2+}$  is present, which is unreasonable because cells typically need  $\text{Ca}^{2+}$  for ATP release to occur. Interestingly, connexin-32 is one of the only connexin that forms channels that open in the presence of high levels of  $\text{Ca}^{2+}$  (Bukauskas et al., 2006; Huang et al., 2007).

Romanov et al. (2007) argued that ATP release in taste cells most likely occurs via connexin hemi-channels; if pannexin channels are involved, they only release a small amount of ATP and voltage-gated current. RT-PCR showed both connexins and pannexins were present in taste tissue (Romanov et al., 2007). They used mimetic peptides,  $^{32}\text{GAP27}$  and  $^{43}\text{GAP26}$ , that are thought to inhibit ATP release from connexin-32 and connexin-43 hemi-channels (Chaytor et al., 1997, 2001; Laybaert et al., 2003) to study the effects on ATP release (Romanov et al., 2007). They observed that  $^{32}\text{GAP27}$  had no effect, while  $^{43}\text{GAP26}$  caused a reduction in outward currents. Octanol, a hemi-channel inhibitor (Eskandari et al., 2002), also reduced outward currents (Romanov et al., 2007). Carbenoxelone, a hemi-channel inhibitor with a high affinity for pannexin-1 (Bruzzone et al., 2005), caused no effect on voltage-gated outwards currents of ATP release, suggesting that ATP release most likely occurs via connexin, not pannexin-1 hemi-channels (Romanov et al., 2007). These data are contrary to a study by Huang et al.

(2007), which suggests that ATP release most likely occurs through pannexin-1 channels.

Connexin-32 and connexin-43 are implicated in cell-cell signaling in multiple tissue types. The ability to demonstrate ATP release via connexin hemichannels was a significant advance (Cotrina et al., 1998; Goodenough & Paul, 2003; Evans et al., 2006). It is now well known that connexin hemichannel opening is dependent upon extracellular  $Ca^{2+}$  levels (Quist et al., 2000; Contreras et al., 2003; Srinivas et al., 2006; Evans et al., 2006).

Connexin-43 has been observed in the mammalian heart, brain, liver and retina. In astrocytes, it has been associated with  $Ca^{2+}$  wave propagation and ATP release (Stout et al., 2002). It has also been observed in ATP release in cardiac myocytes (Clarke et al., 2009) and in other cell types in the heart (Coppen et al., 1999; Kanagaratnam et al., 2002). Localization of connexins in the retina demonstrates connexin-43 to be found at every level (Ball & McReynolds, 1998), and it has also been observed in corneal endothelial cells (Gomes et al., 2005). Our preliminary data suggest that connexin-43 is present in Type II cells in rat circumvallate taste buds (Bond et al., 2012).

Connexin-32 has been studied in multiple mammalian systems. It is found in the brain, where it is expressed in oligodendrocyte and neurons (Aronica et al., 2001), and in the olfactory bulb. Connexin-32 is known to be one of the major gap junction proteins for myelinating glia (Aronica et al., 2001) and the liver (Nicholson et al., 1987; Duffy et al., 2007). Our preliminary data suggest that



connexin-32 is present in a subset of Type III taste cells and the nerve processes in rat circumvallate taste buds (Bond et al., 2012).

### **Hypothesis/Specific Aims:**

Although there has been progress in understanding the release of ATP via hemi-channels in taste cells, the type of hemi-channels found at these sites is still in question. It is known that gap junction proteins are present in taste cells and that they form hemi-channels through which ATP is likely released (Huang et al., 2007; Romanov et al., 2007); however, there is still debate regarding whether ATP release occurs through pannexin or connexin hemi-channels. We propose to test the hypothesis that connexin-32 and connexin-43 are present in taste cells. We believe that Type II (receptor) and some Type III (presynaptic) cells release non-vesicular ATP through connexin hemi-channels; therefore, connexins are present at specific locations in Type II and Type III taste cells, as well as in the nerve processes. With the use of immunocytochemical techniques for confocal and electron microscopy, we will attempt to complete the following goals:

**Aim 1: Use confocal microscopy to test if gap junction proteins connexin-32 and connexin-43 are present in taste cells and/or nerve processes in rat circumvallate taste buds.**

- a. To determine if connexin-32 and connexin-43 label rat circumvallate taste bud.
- b. To determine if connexin-32 and connexin-43 co-localize known taste cell type markers  $\alpha$ -gustducin, PLC $\beta$ 2, NCAM and P2X2

**Aim 2: Use DAB immunoelectron microscopy to test which cell types express connexin-32 and connexin-43.**

- a. To determine if connexin-32 and connexin-43 are present in Type II or Type III cells in rat circumvallate taste buds.
- b. To determine if connexin-32 and connexin-43 are present in the nerve fibers of rat circumvallate taste buds.

## **MATERIALS AND METHODS**

Adult Sprague-Dawley male rats (250-350 g) were used for these studies. Animals were cared for and housed in facilities approved by the Institutional Animal Care and Use Committee of the University of Denver. All animals were anesthetized with a mixture of sodium ketamine (200mg/kg) and xylazine (70mg/kg) (i.p.). Primary and secondary antibodies are listed in Tables 1 & 2.

### **Conventional Immunofluorescence for Confocal Microscopy**

Rats were perfused for ten seconds through the left ventricle with 0.1% sodium nitrite, 0.9% sodium chloride and 100 units sodium heparin in 100 ml 0.1M phosphate buffer (pH 7.3). This was followed by perfusion fixation with 4% PFA (Paraformaldehyde) in 0.1% phosphate buffer for 10 minutes (Weedman et al., 1996). All perfusates were warmed to 42°C before use. After perfusion, the excised circumvallate papillae were fixed in fresh fixative for 3 hours at 4°C. The

tissues were then cryo-protected with 30% sucrose in 0.1 M phosphate buffer overnight at 4°C.

### **Single Labeling**

Tissue was frozen in OCT (Optimal Cutting Temperature embedment) and then sliced on a cryostat into sections 20 µm thick. Cryostat sections containing circumvallate taste buds were washed in 0.1M phosphate-buffered saline (PBS, pH 7.3) for thirty minutes, then blocked in 5% normal goat serum and 0.3% Triton X-100 in 0.1M PBS (pH 7.3) for two hours on ice. The sections were incubated in either mouse monoclonal connexin-32 or mouse monoclonal connexin-43. Both antibodies were mixed with 0.1M PBS (pH 7.3) and refrigerated overnight at 4°C. Tissue was then rinsed in 0.1M PBS (pH 7.3) for thirty minutes. Tissue was treated with Alexa-Fluor 488 goat anti-mouse IgG and 0.1M PBS (pH 7.3) for one hour at room temperature. Following this treatment, the tissues were washed in 0.1M PBS (pH 7.3) for 30 minutes. The tissues were then mounted onto glass slides using Fluoro-Gel with Tris Buffer. All images were viewed using a Zeiss Axioplan II microscope with an Apotome confocal attachment. The Apotome captures multiple images in varying grid positions that results in an optical section through the specimen. Images were taken at 40X magnification using the Axiocam HRm digital camera and video adaptor.

## Double Labeling

Tissue was frozen in OCT and then sliced on a cryostat into sections 20  $\mu\text{m}$  thick. Cryostat sections containing circumvallate taste buds were washed in 0.1M phosphate-buffered saline (PBS, pH 7.3) for thirty minutes, then blocked in 5% normal goat serum and 0.3% Triton X-100 in 0.1M PBS (pH 7.3) for two hours on ice. The sections were incubated in a combination of two primary antibodies: mouse monoclonal connexin-32 was separately incubated with each of the following antibodies: rabbit polyclonal antibody  $\alpha$ -gustducin, rabbit polyclonal PLC $\beta$ 2, rabbit polyclonal P2X2, and rabbit polyclonal NCAM. Connexin-43 was separately incubated with the following antibodies: rabbit polyclonal antibody  $\alpha$ -gustducin, rabbit polyclonal PLC $\beta$ 2, rabbit polyclonal P2X2, and rabbit polyclonal NCAM. All combinations were mixed with 0.1M PBS (pH 7.3) and refrigerated overnight at 4°C. Tissue was then rinsed in 0.1M PBS (pH 7.3) for thirty minutes. The sections were treated in a combination of two secondary antibodies in 0.1M PBS (pH 7.3): Alexa-Fluor 488 goat anti-mouse IgG and Dylight 649 goat anti-rabbit IgG. The tissues were left in this treatment for one hour at room temperature. Following this treatment, the tissues were washed in 0.1M PBS (pH 7.3) for 30 minutes. The tissues were then mounted onto glass slides using Fluoro-Gel with Tris Buffer. All images were viewed using a Zeiss Axioplan II microscope with an Apotome confocal attachment. The Apotome captures multiple images in varying grid positions that results in an optical section through

the specimen. Images were taken at 40X magnification using the Axiocam HRm digital camera and video adaptor.

## **Controls**

Tissues were frozen in OCT and then sliced on a cryostat into sections 20  $\mu\text{m}$  thick. Cryostat sections containing circumvallate taste buds were washed in 0.1M phosphate-buffered saline (PBS, pH 7.3) for thirty minutes, then blocked in 5% normal goat serum and 0.3% Triton X-100 in 0.1M PBS (pH 7.3) for two hours on ice. The sections were incubated in any one of the cell type markers, leaving out connexin-32 and connexin-43. Antibodies were mixed with 0.1M PBS (pH 7.3) and refrigerated overnight at 4°C. Tissue was then rinsed in 0.1M PBS (pH 7.3) for thirty minutes. Tissue was treated with 0.1M PBS (pH 7.3) two secondary antibodies; Alexa-Fluor 488 goat anti-mouse IgG and Dylight 649 goat anti-rabbit IgG, for one hour at room temperature. Following this treatment, the tissues were washed in 0.1M PBS (pH 7.3) for 30 minutes. The tissues were then mounted onto glass slides using Fluoro-Gel with Tris Buffer. The same procedure was used to observe any reactivity when incubating tissue in connexin-32 or connexin-43, leaving out the cell type markers. All images were viewed using a Zeiss Axioplan II microscope with an Apotome confocal attachment. The Apotome captures multiple images in varying grid positions that results in an optical section through the specimen. Images were taken at 40X magnification using the Axiocam HRm digital camera and video adaptor.

## **DAB Staining for Light Microscopy**

Rats were perfused for ten seconds through the left ventricle with 0.1% sodium nitrite, 0.9% sodium chloride and 100 units sodium heparin in 100 ml 0.1M phosphate buffer (pH 7.3). This was followed by perfusion fixation with 4% PFA in 0.1M phosphate buffer for 10 minutes (Weedman *et al.*, 1996). All perfusates were warmed to 42°C before use. After perfusion, the excised circumvallate papillae were fixed in fresh fixative for 3 hours at 4°C. Tissues were then sliced at 80 µm on the vibratome, followed by thirty minutes of washing in 0.1M phosphate-buffered saline (PBS, pH 7.3). The tissues were blocked in 5% normal goat serum and 0.3% Triton X-100 in 0.1M PBS (pH 7.3) for two hours on ice, then incubated in a primary antibody, either mouse monoclonal Connexin-32 or mouse monoclonal Connexin-43 in 0.1M PBS (PH 7.3) overnight at 4°C.

The following day, sections were washed with 0.1M PBS (PH 7.3) for 30 minutes, then incubated in the secondary antibody, biotinylated goat-anti-mouse IgG in 1M PBS (pH 7.3) on ice for two hours. Sections were then washed for 30 minutes in 1M PBS (pH 7.3). Following these washes, the sections were incubated in ABC peroxidase reagent complex (Vector) on ice for two hours. Tissue was washed in 0.1M PBS (PH 7.3) for 30 minutes, then soaked in 0.05% DAB in 0.05M Tris Buffer (pH 7.2) for ten minutes. Hydrogen peroxide was added to the DAB-Tris Buffer mixture to yield a concentration of .003% hydrogen

peroxide in the DAB mixture. Tissue was incubated in this mixture for 5 minutes, followed by thirty minutes of washing in 0.05M Tris Buffer (pH 7.2). Tissue was post-fixed for 15 minutes in 1% osmium tetroxide in 0.1M phosphate buffer. Tissue was then washed in 0.05M sodium maleate buffer (pH 5.2) for forty minutes. A 2% solution of uranyl acetate in water was prepared, then mixed 1:1 with 0.05M sodium maleate buffer (pH 6.0). Sections were incubated in this mixture overnight.

The following day, tissue was dehydrated in a graded alcohol series: 50% ethanol for five minutes, 60% ethanol for five minutes, 75% ethanol for 15 minutes, 85% ethanol for 15 minutes, 95% ethanol for 15 minutes, 100% ethanol for 15 minutes. Tissue was then washed in propylene oxide for 15 minutes. Tissue then underwent infiltration using Lufts 5:5 and propylene oxide. First, tissue was incubated in a 2:1 solution of propylene oxide to Lufts 5:5 for 15 minutes. Then, tissue incubated in a 1:1 solution of propylene oxide to Lufts 5:5 for 15 minutes. Next, tissue incubated in a 1:2 solution of propylene oxide to Lufts 5:5 for 30 minutes, followed by one hour in pure Lufts 5:5 under a heat lamp. Finally, tissue was transferred to a new vial containing pure Luft's for three hours. Individual sections were then mounted onto gelatin subbed slides. All slides were placed in the oven at 60°C overnight.

The following day, slides were removed from the oven, cooled, and separated. Individual sections were viewed under the light microscope and images were taken using the Zeiss Axiocam 1.



## **DAB Staining for Electron Microscopy**

Rats were perfused for ten seconds through the left ventricle with 0.1% sodium nitrite, 0.9% sodium chloride and 100 units sodium heparin in 100 ml 0.1M phosphate buffer (pH 7.3). This was followed by perfusion fixation with 4% PFA in 0.1% phosphate buffer for 10 minutes (Weedman et al., 1996). All perfusates were warmed to 42°C before use. After perfusion, the excised circumvallate papillae were fixed in fresh fixative for 3 hours at 4°C. Tissue was then sliced at 80µm on the vibratome, followed by thirty minutes of washing in 0.1M phosphate-buffered saline (PBS, pH 7.3). The tissue was blocked in 5% normal goat serum and 0.3% Triton X-100 in 0.1M PBS (pH 7.3) for two hours on ice, then incubated in a primary antibody, either mouse monoclonal Connexin-32 or mouse monoclonal connexin-43 in 0.1M PBS (PH 7.3) overnight at 4°C.

The following day, sections were washed with 0.1M PBS (PH 7.3) for 30 minutes, then incubated in the secondary antibody, biotinylated goat-anti-mouse IgG in 1M PBS (PH 7.3) on ice for two hours. Sections were then washed for 30 minutes in 1M PBS (PH 7.3). Following these washes, sections were incubated in ABC peroxidase reagent complex (Vector) on ice for two hours. Tissue was washed in 0.1M PBS (PH 7.3) for 30 minutes, then soaked in 0.05% DAB in 0.05M Tris Buffer (pH 7.2) for ten minutes. Hydrogen peroxide was added to the DAB-Tris Buffer mixture to yield a concentration of .003% hydrogen peroxide in the DAB mixture. Tissue was incubated in this mixture for 5 minutes, followed by thirty minutes of washing in 0.05M Tris Buffer (pH 7.2). Tissue was post-fixed for

15 minutes in 1% osmium tetroxide in 0.1M phosphate buffer. Tissue was then washed in 0.05M sodium maleate buffer (pH 5.2) for forty minutes. A 2% solution of uranyl acetate in water was prepared, then mixed 1:1 with 0.05M sodium maleate buffer (pH 6.0). Sections were incubated in this mixture overnight.

The following day, tissue was dehydrated in a graded alcohol series: 50% ethanol for five minutes, 60% ethanol for five minutes, 75% ethanol for 15 minutes, 85% ethanol for 15 minutes, 95% ethanol for 15 minutes, 100% ethanol for 15 minutes. Tissue was then washed in propylene oxide for 15 minutes. Tissue then underwent the infiltration process using Lufts 5:5 and propylene oxide. First, tissue incubated in a 2:1 solution of propylene oxide to Lufts 5:5 for 15 minutes. Then, tissue incubated in a 1:1 solution of propylene oxide to Lufts 5:5 for 15 minutes. Next, tissue incubated in a 1:2 solution of propylene oxide to Lufts 5:5 for 30 minutes, followed by one hour in pure epon under a heat lamp. Finally, tissue was transferred to a new vial containing pure Luft's for three hours. Individual sections were then mounted onto gelatin subbed slides. All slides were placed in the oven at 60°C overnight.

The following day, slides were removed from the oven, cooled, and separated. All sections were re-embedded using Lufts 5:5, then placed in the oven at 60°C overnight. The next morning, blocks of tissue were removed from the oven, cooled, and separated from the slides. Blocks were trimmed to prepare for slicing on the microtome. Tissue was sliced onto microtome into thin sections. Each section was placed onto a grid and viewed with the Hitachi H-7000 transmission electron microscope at 75 kV.

**Table 1: Primary Antibodies**

Antibodies	Species	Dilution	Source	Code No.
$\alpha$ -Gustducin	Rabbit	1:100	Santa Cruz Biotech.	SC-395
PLC $\beta$ 2	Rabbit	1:100	Transduction Lab.	610313
NCAM	Rabbit	1:100	Sigma	058K4841
P2X2	Rabbit	1:100	Alamone	APR-300
Connexin-32	Mouse	1:100	Sigma	C6344
Connexin-43	Mouse	1:100	Sigma	C8093

**Table 2: Secondary Antibodies**

Antibodies/Stains	Species	Dilution	Source	Code No.
Dylight 649 IgG	Rabbit	1:100	Jackson	111-495-144
Alexa-Fluor 488 IgG	Mouse	1:100	Jackson	115-545-166
Biotin-SP- IgG	Mouse	1:100	Jackson	115-065-166

## **RESULTS**

### **General Features of Connexin-32 and Connexin-43 in Taste Cells and/or Nerve Processes**

Connexin-32 is present in a small subset of taste cells and in the nerve fibers of rat circumvallate taste buds (Fig. 7). The immunoreactive taste cells are slender, with elongate nuclei and prominent nuclear invaginations. Immunoreactivity in the nerve fibers extends the entire span of the taste bud, beginning at the basal lamina and terminating at the taste pore.

Connexin-43 is also present in a subset of taste cells in rat circumvallate taste buds (Fig. 8). The immunoreactive taste cells are spindle-shaped, with large, ovoid nuclei, which is characteristic of Type II cells. Staining is punctate in the apical area of the cell and in the area surrounding the nucleus in some immunoreactive cells.

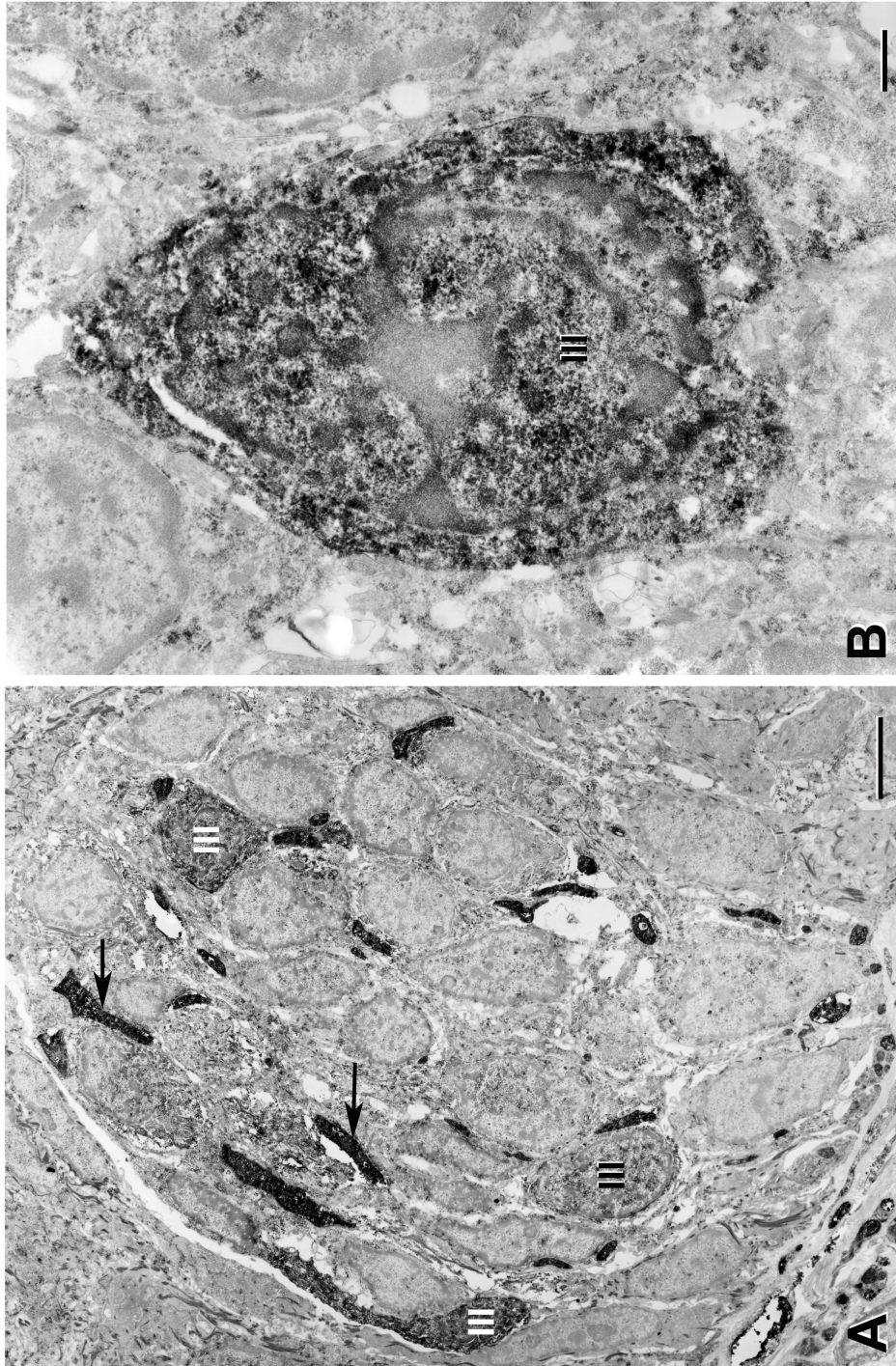
## **Double-Label Studies of Connexin-32 and Connexin-43 with NCAM**

Neural Cell Adhesion Molecule (NCAM) is produced by receptor cells and thought to contribute to the development of specific connections in gustatory tissue (Nelson & Finger, 1993). NCAM is known to label the membrane along the surface of Type III cells.

Connexin-32 is expressed in the cytoplasm and nuclei of immunoreactive cells (Figs. 11). Connexin-32 co-localizes with a subset of NCAM-LIR cells. Connexin-32 immunoreactivity is present in nerve fibers, extending from the basal lamina to the taste pore (Fig. 11).

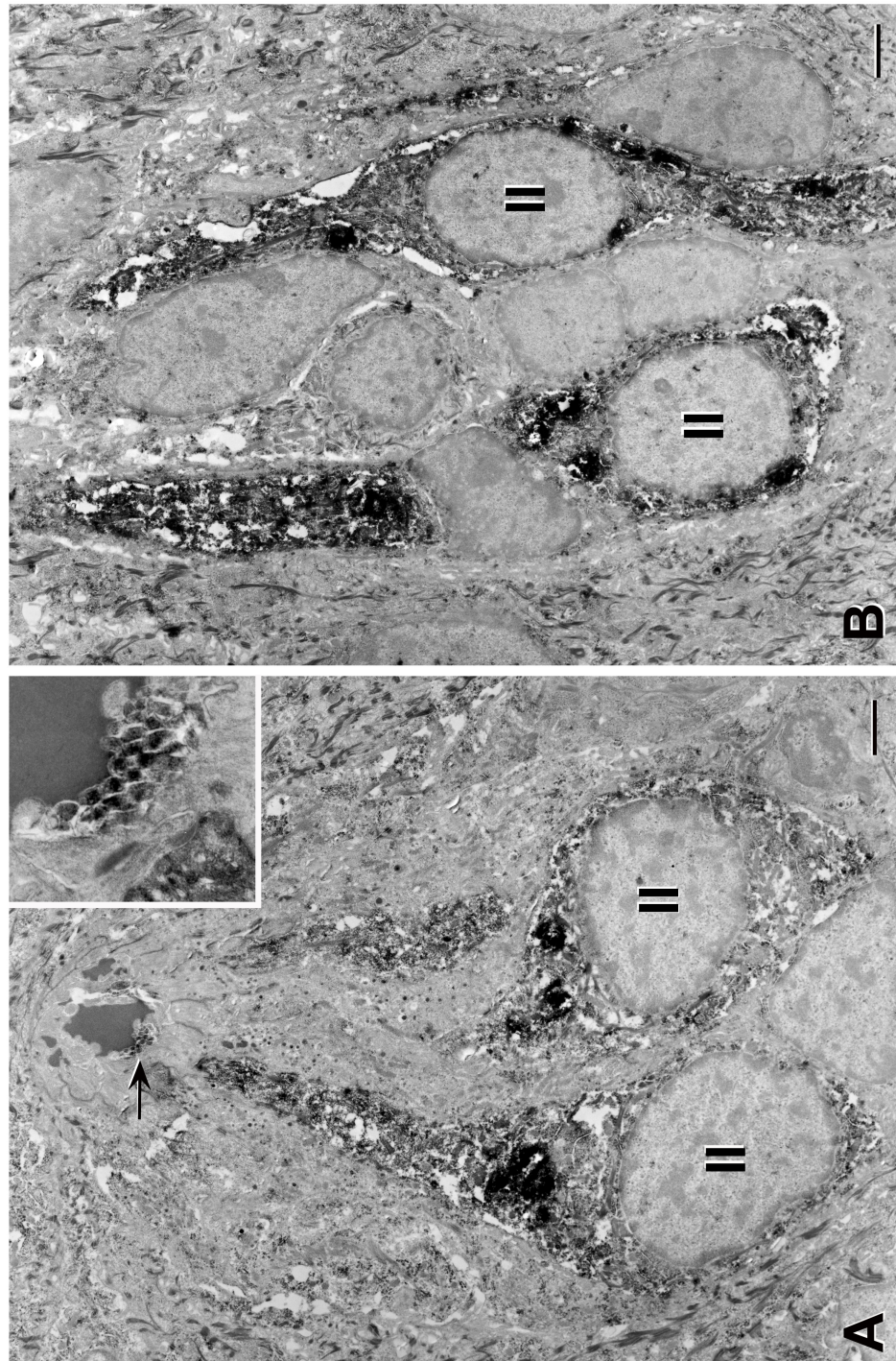
Connexin-43-LIR cells do not co-localize with NCAM-LIR cells (Fig. 12). Connexin-43 immunoreactivity is expressed in a punctate staining pattern in a subset of cells that do not display NCAM immunoreactivity (Fig. 12).

Figure 7



**Figure 7: A & B:** DAB immunoelectron micrographs showing Type III cells (III) and nerve fibers (arrows), displaying connexin-32-LIR. Scale bars = 5  $\mu\text{m}$  (A) and 1  $\mu\text{m}$  (B)

Figure 8





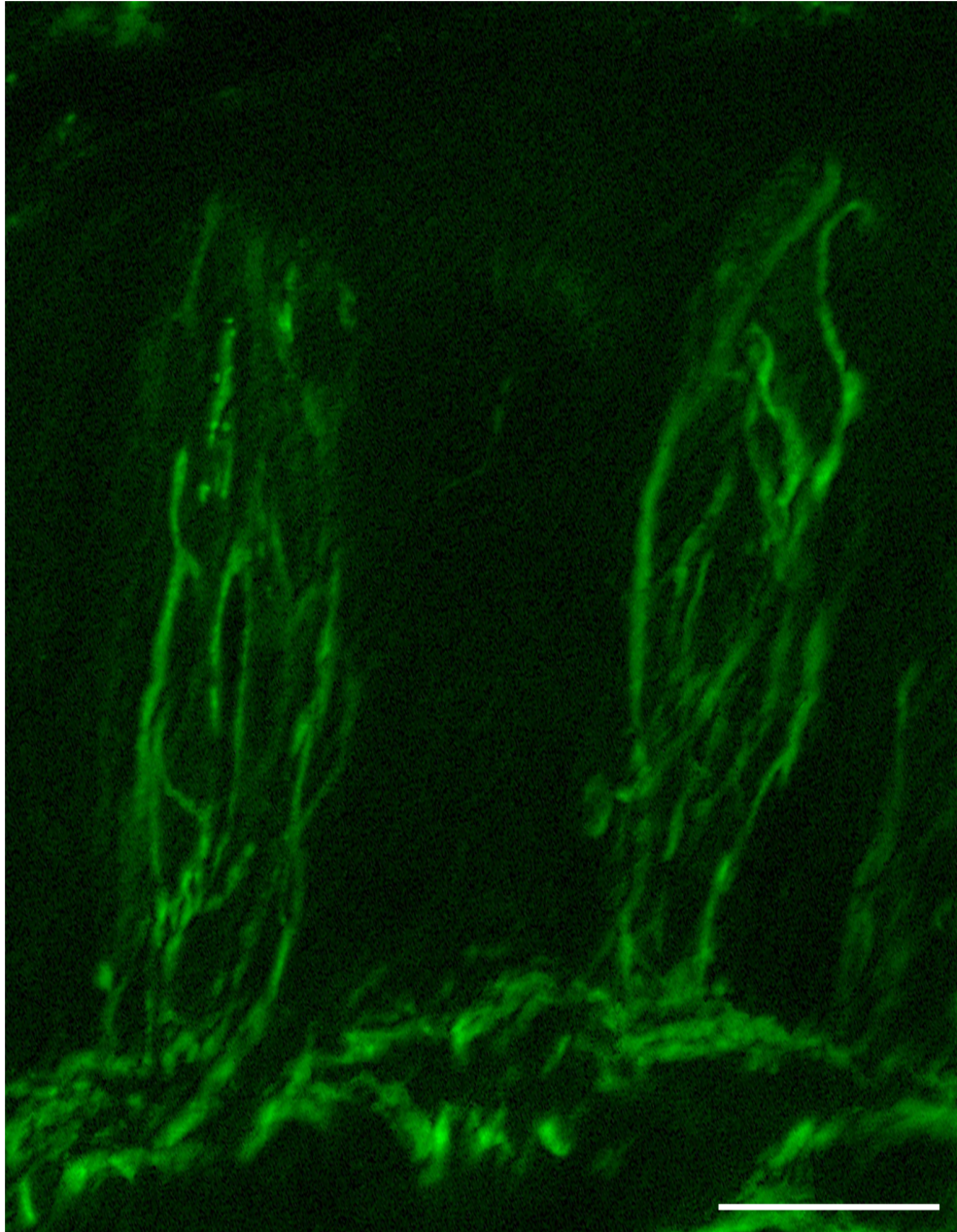
**Figure 8: A & B:** DAB immunoelectron micrographs showing Type II cells (II) displaying connexin-43-LIR. **Inset:** staining of microvilli (arrow). Scale bars = 2  $\mu\text{m}$ .

## **Double-Label Studies of Connexin-32 and Connexin-43 with P2X2**

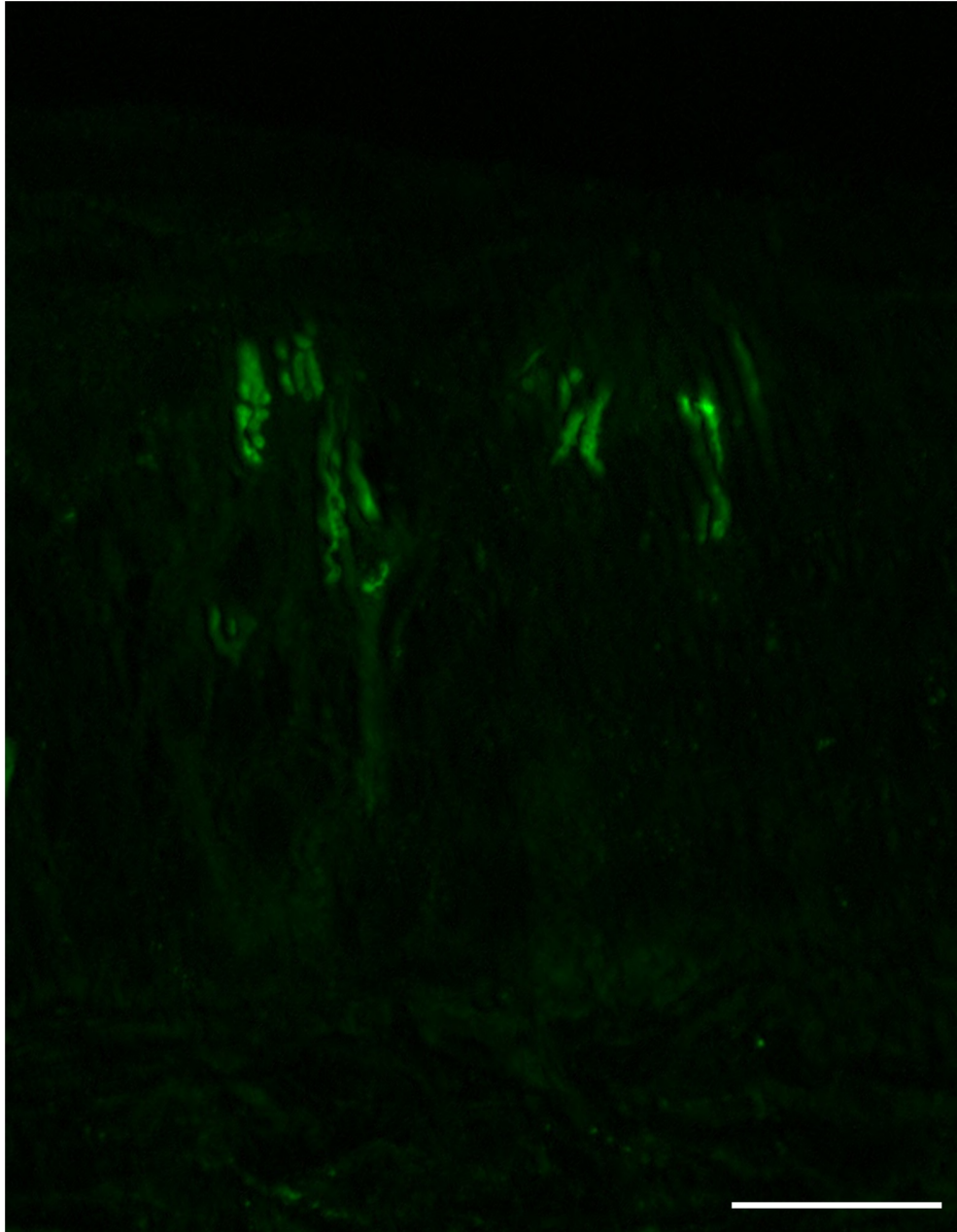
P2X2 is an ionotropic purinergic receptor that is present in gustatory tissue. Recent evidence indicates that P2X2 immunoreactivity is expressed in intragemmal nerve processes in rodent taste buds (Yang et al., 2012 in press). Thus, we use P2X2 in this study as a nerve fiber marker.

Connexin-32-LIR nerve fibers co-localize with P2X2-LIR nerve fibers (Fig. 13). Most of the nerve processes, which extend from the basal lamina to the taste pore, display connexin-32 and P2X2 immunoreactivity (Fig. 13). There is a subset of immunoreactive cells that are slender with elongate nuclei (Fig. 13). The morphology of these immunoreactive cells suggests that they are Type III cells.

Connexin-43-LIR cells do not co-localize with P2X2-LIR nerve fibers (Fig. 14). Connexin-43-LIR immunoreactivity appears as a punctate staining pattern in suggests that connexin-43 is not expressed in the nerve processes of rat circumvallate taste buds.

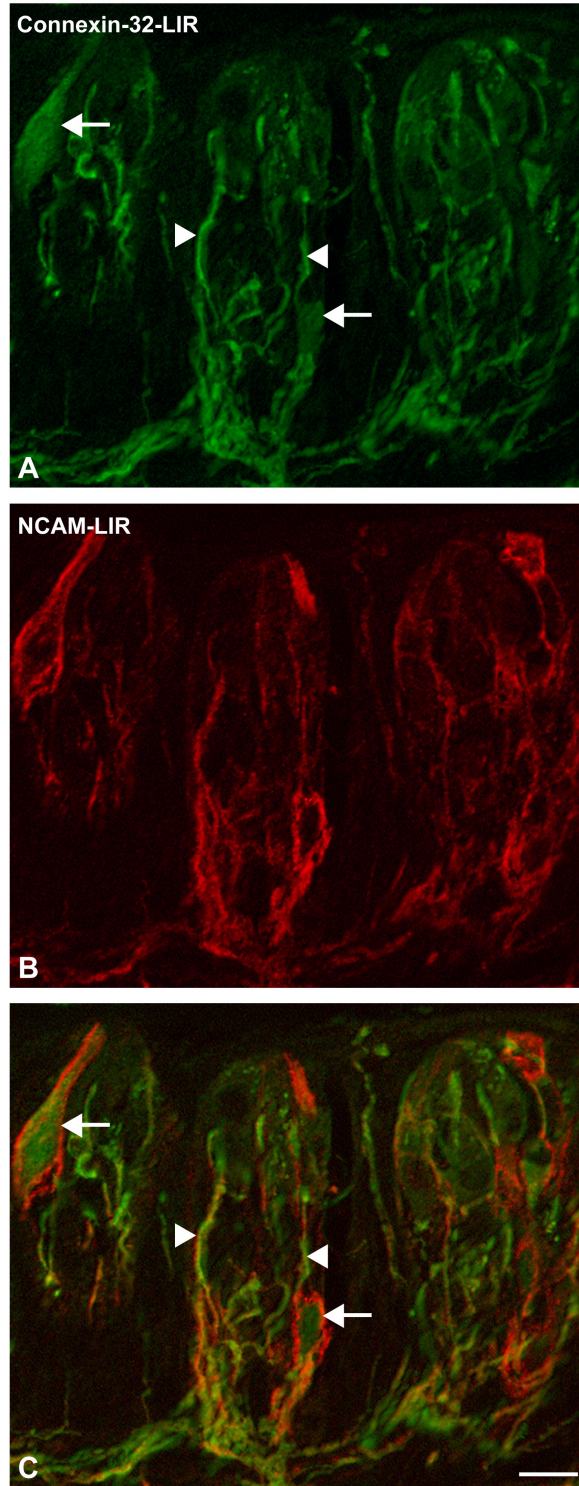


**Figure 9:** Image showing a single label study of connexin-32. Scale



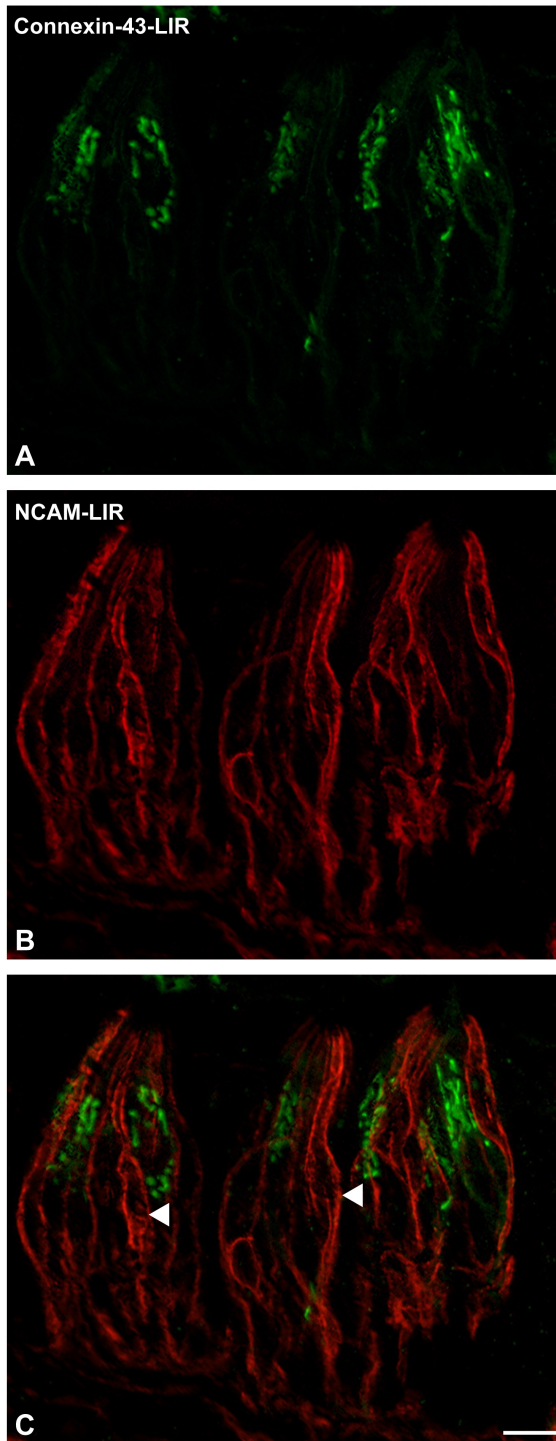
**Figure 10:** Image showing a single label study of connexin-43.

Figure 11



**Figure 11:** Confocal Image showing the co-localization of connexin-32 and NCAM. **A.** Connexin-32-LIR (green). **B.** NCAM-LIR (red). **C.** Merged Image. Type III cells (arrows). Nerve processes (arrowheads). Scale bar = 20  $\mu$ m.

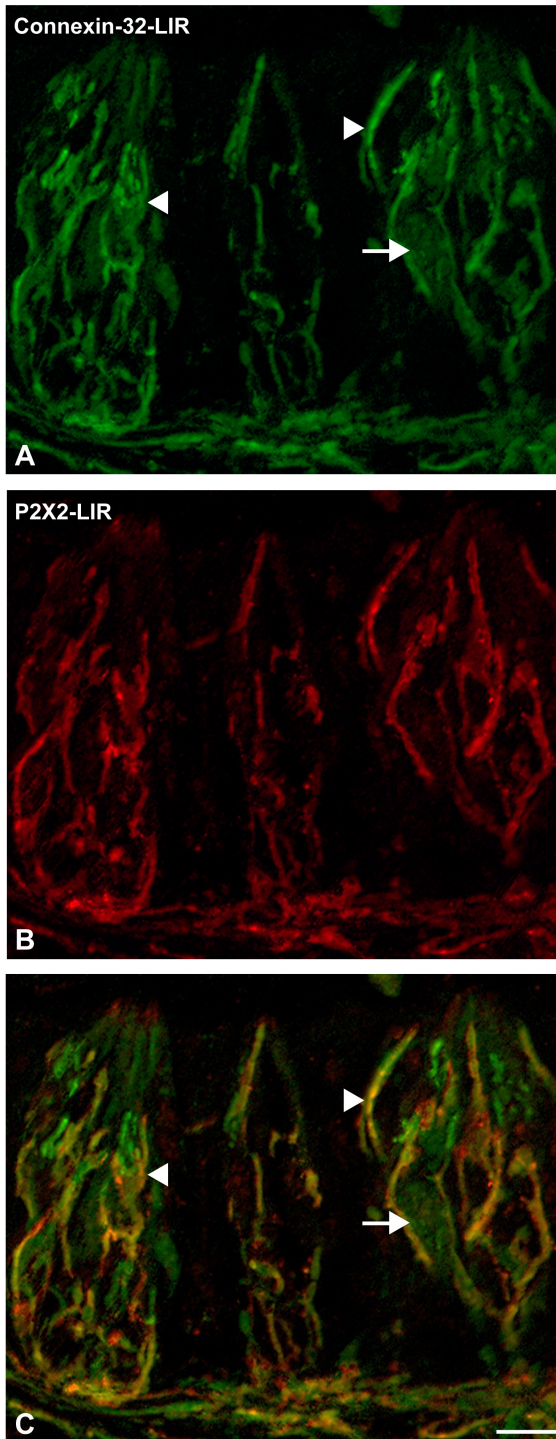
Figure 12



**Figure 12:** Confocal Image showing the co-localization of connexin-43 and NCAM. **A.** Connexin-43-LIR (green). **B.** NCAM-LIR (red). **C.** Merged Image. Type III cells (arrowheads). Scale bar = 20  $\mu$ m.

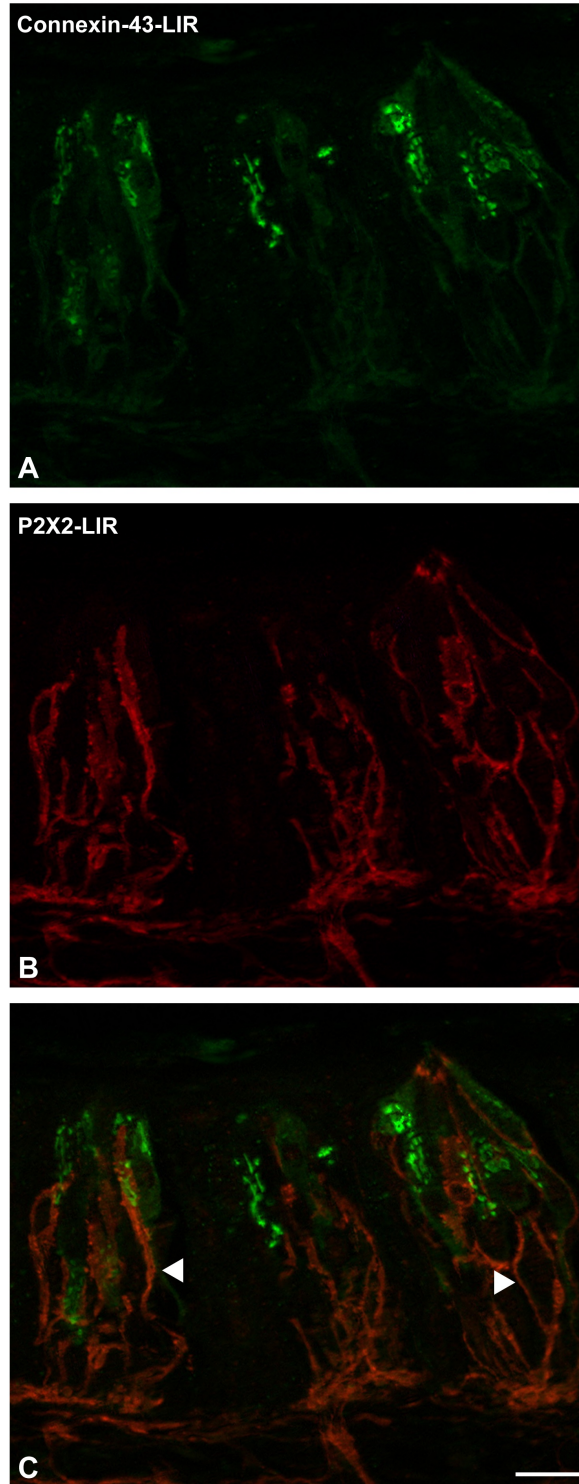


Figure 13



**Figure 13:** Confocal Image showing the co-localization of connexin-32 and P2X2. **A.** Connexin-32-LIR (green). **B.** P2X2-LIR (red). **C.** Merged Image. Type III cells (arrows). Nerve processes (arrowheads). Scale bar = 20  $\mu$ m.

Figure 14



**Figure 14:** Confocal Image showing the co-localization of connexin-43 and P2X2. **A.** Connexin-43-LIR (green). **B.** P2X2-LIR (red). **C.** Merged Image. Nerve processes (arrowheads). Scale bar = 20  $\mu$ m.

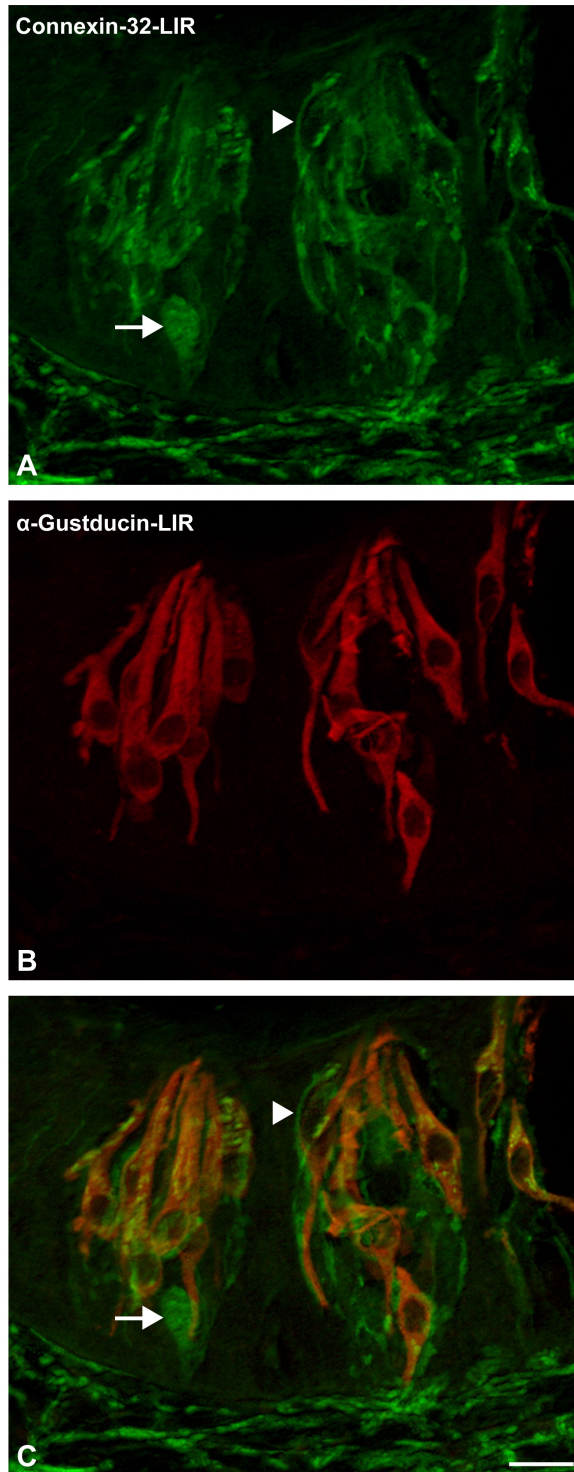
## **Double-Label Studies of Connexin-32 and Connexin-43 with $\alpha$ -gustducin**

$\alpha$ -gustducin is a guanine nucleotide-binding protein (G-protein) that plays a key role in taste transduction. It is expressed in a subset of Type II cells in rat circumvallate taste buds (Yang et al., 2000; Clapp et al., 2001, 2004; Miyoshi et al., 2001). We are using  $\alpha$ -gustducin as a Type II cell marker in this study.

Connexin-32-LIR cells do not co-localize with  $\alpha$ -gustducin-LIR cells (Fig. 15). There is a subset of connexin-32-LIR cells that are slender with elongate nuclei, suggesting that they are Type III cells (Fig. 15). There is also connexin-32 immunoreactivity in the nerve fibers that extend from the basal lamina to the taste pore (Fig. 15). There is a small portion of punctate staining of connexin-32 on some  $\alpha$ -gustducin-LIR cells. This punctate staining is insignificant and can most likely be attributed to non-specific staining of the golgi apparatus. (Fig. 15)

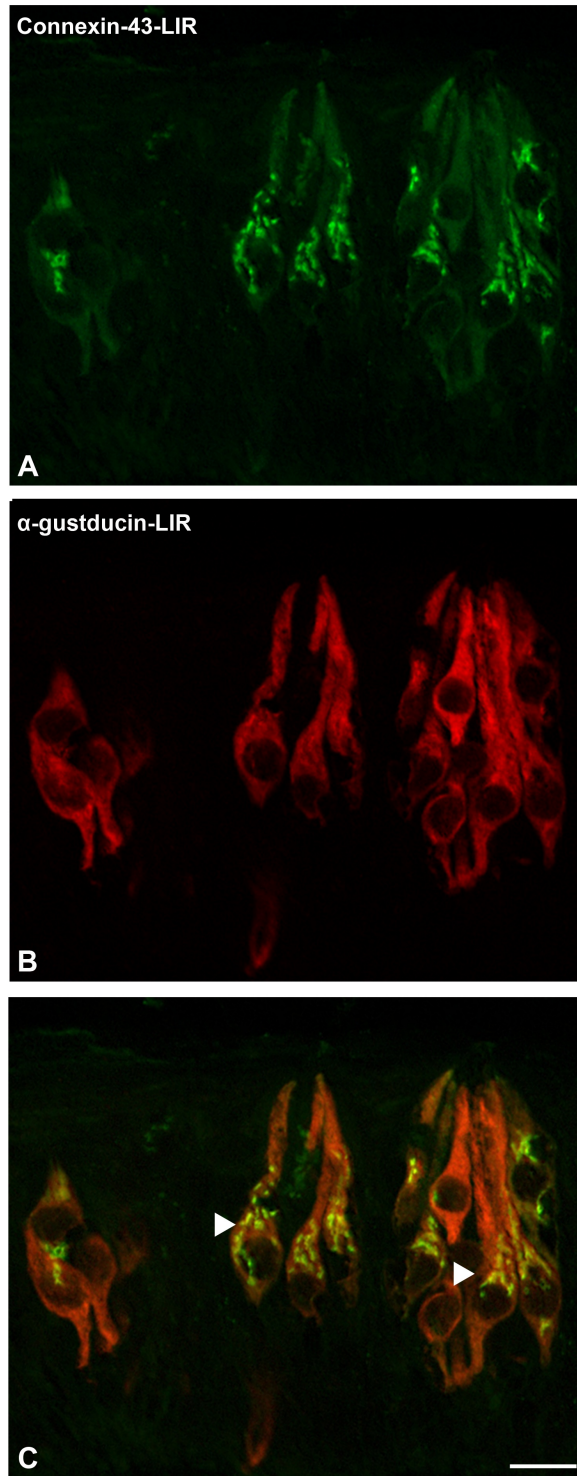
Connexin-43-LIR is present in  $\alpha$ -gustducin-LIR cells (Fig. 16). Connexin-43 labels the cytoplasm of the cell (Fig. 16). In the apical region of the cell and sometimes surrounding the nucleus in connexin-43-LIR cells, the staining has a punctate nature (Fig. 16). There is a small subset of connexin-43-LIR cells that do not display  $\alpha$ -gustducin immunoreactivity (Fig. 16). The connexin-43 staining pattern in these cells is similar to the staining pattern displayed in connexin-43-LIR cells that co-localize with  $\alpha$ -gustducin-LIR cells (Fig. 16). These results suggest that connexin-43 is expressed in a larger subset of Type II cells than  $\alpha$ -gustducin.

Figure 15



**Figure 15:** Confocal Image showing the co-localization of connexin-32 and  $\alpha$ -gustducin. **A.** Connexin-32-LIR (green). **B.**  $\alpha$ -gustducin-LIR (red). **C.** Merged Image. Type III cells (arrows). Nerve processes (arrowheads). Scale bar = 20  $\mu$ m.

Figure 16





**Figure 16:** Confocal Image showing the co-localization of connexin-43 and  $\alpha$ -gustducin. **A.** Connexin-43-LIR (green). **B.**  $\alpha$ -gustducin-LIR (red). **C.** Merged Image. Type II cells (arrowheads). Scale bar = 20  $\mu$ m.

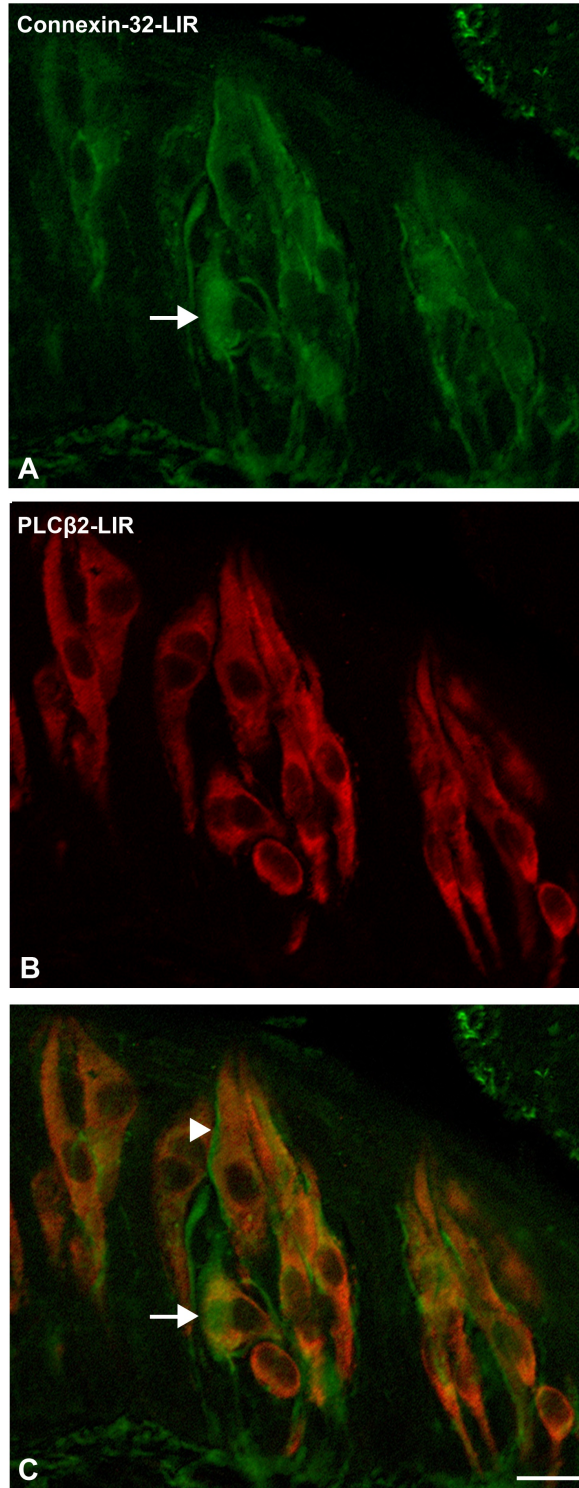
## **Double-Label Studies of Connexin-32 and Connexin-43 with PLC $\beta$ 2**

PLC $\beta$ 2 (phospholipase C  $\beta$ 2) is a key signaling molecule in the transduction of taste. In the taste bud, PLC $\beta$ 2 is a known marker for Type II cells (Yang et al., 2000; Clapp et al., 2001, 2004; Miyoshi et al., 2001). Although PLC $\beta$ 2 and  $\alpha$ -gustducin are both present in Type II cells, PLC $\beta$ 2 is expressed in a much larger subset.

Connexin-32-LIR cells do not co-localize with PLC $\beta$ 2-LIR cells (Fig. 17). Connexin-32 immunoreactive cells are slender with elongate nuclei, suggesting that they are most likely Type III cells (Fig. 17). There is also connexin-32 immunoreactivity in the nerve fibers that extend from the basal lamina to the taste pore (Fig. 17).

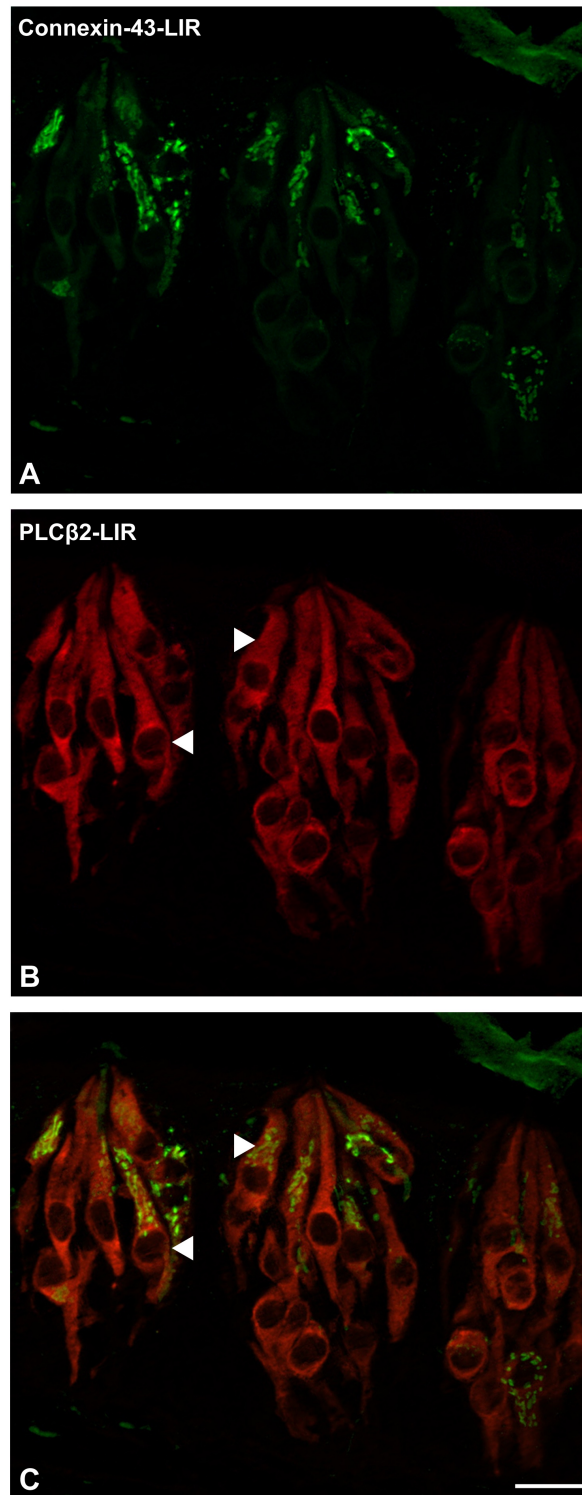
Connexin-43-LIR cells also display PLC $\beta$ 2-LIR (Fig. 18). Connexin-43 appears to label the cytoplasm of the cell (Fig. 18). In some areas of the connexin-43 immunoreactive cells, the staining is punctate in nature (Fig. 18). Connexin-43-LIR is only present in a subset of PLC $\beta$ 2-LIR cells. These results suggest that connexin-43 is expressed in a subset of Type II cells that slightly differs from the population of PLC $\beta$ 2-LIR cells because there are more PLC $\beta$ 2-LIR cells than connexin-43-LIR cells.

Figure 17



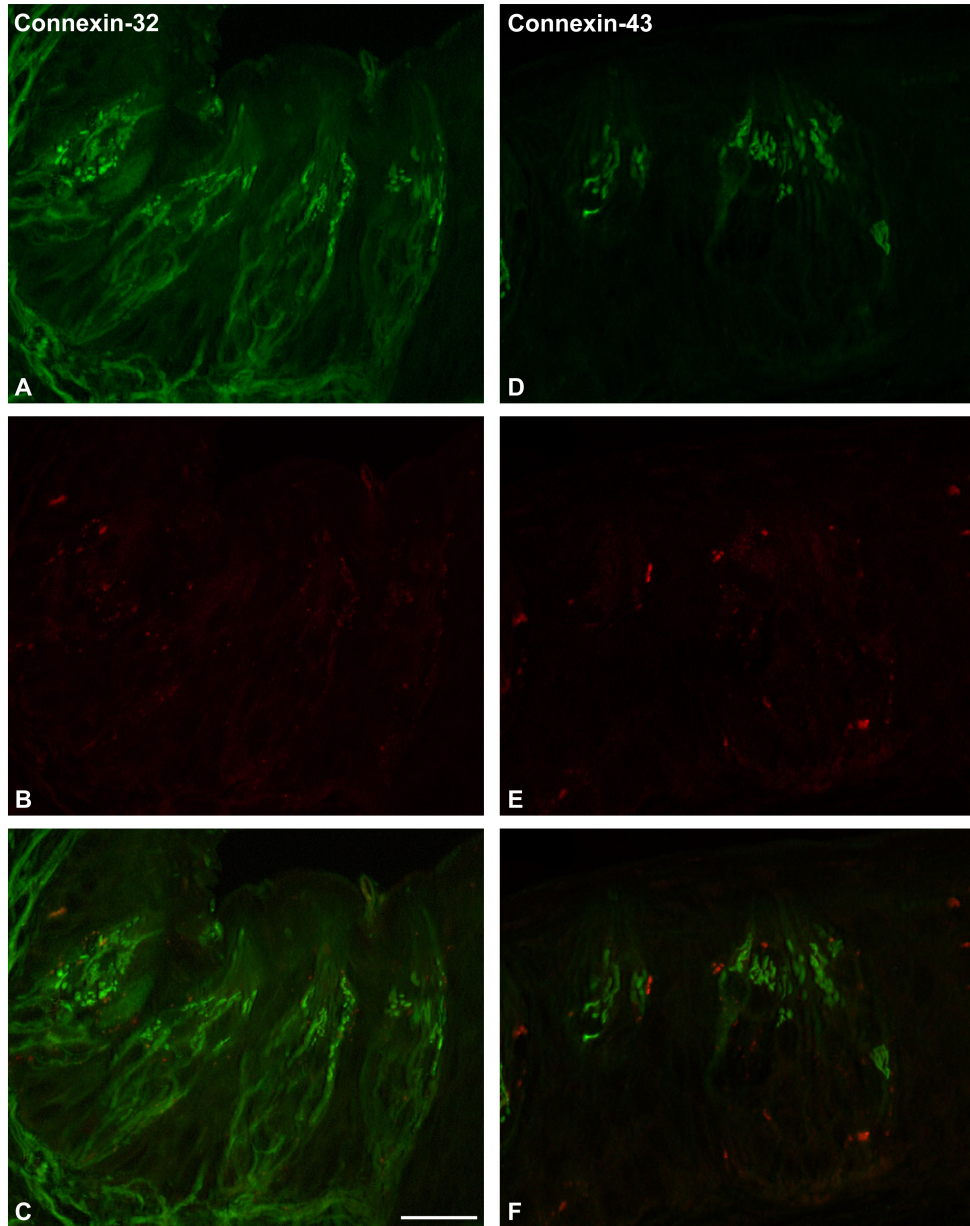
**Figure 17:** Confocal image showing the co-localization of connexin-32 and PLC $\beta_2$ . **A.** Connexin-32-LIR (green). **B.** PLC $\beta_2$  (red). **C.** Merged Image. Type III cells (arrows). Nerve process (arrowhead). Scale bar = 20  $\mu$ m

Figure 18



**Figure 18:** Confocal image showing the co-localization of connexin-43 and PLC $\beta_2$ . **A.** Connexin-43-LIR (green). **B.** PLC $\beta_2$ -LIR (red). **C.** Merged Image. Type II cells (arrowheads). Scale bar = 20  $\mu$ m.

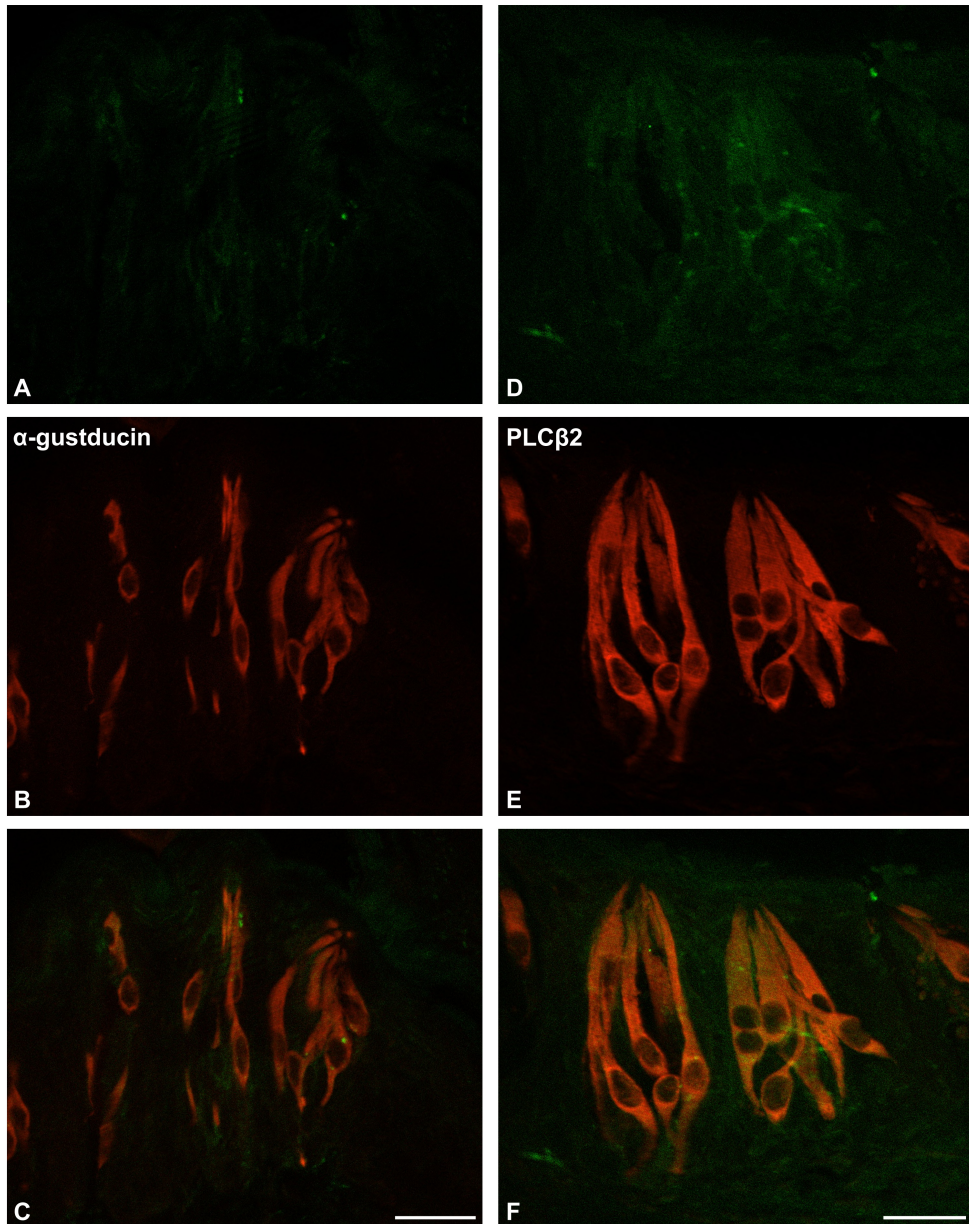
Figure 19



**Figure 19:** Connexin-32 and Connexin-43 control images for immunocytochemical studies. A. Connexin-32 (green). B. No primary antibody (red). C. Merged image of connexin-32 (green + red). D. Connexin-43 (green). E. No primary antibody (red). F. Merged image of connexin-43 (green + red). Scale bar = 20  $\mu$ m.



Figure 20



**Figure 20:** Secondary antibody control images for immunocytochemical studies. **A.** No primary antibody (green). **B.**  $\alpha$ -gustducin (red). **C.** Merged image of  $\alpha$ -gustducin (green + red). **D.** No primary (green). **E.** PLC $\beta_2$  (red). **F.** Merged image of PLC $\beta_2$ -(green + red). Scale bar = 20  $\mu$ m

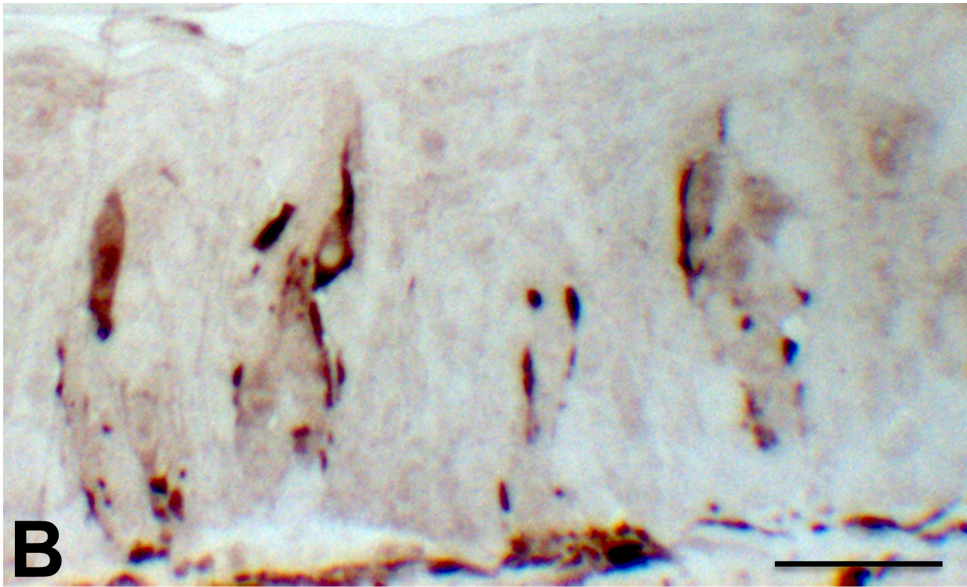
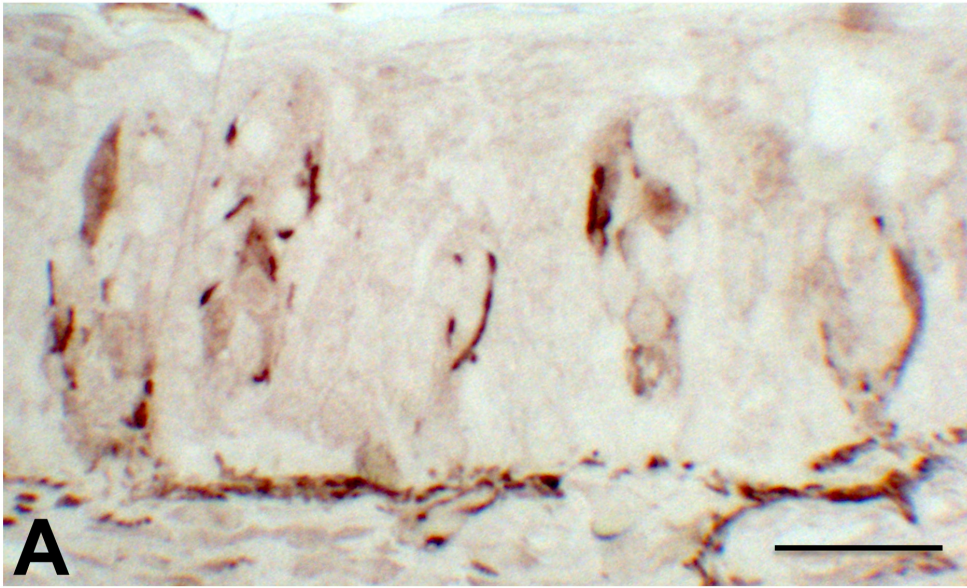
## **DAB for Light Microscopy**

### ***Connexin-32***

DAB results for connexin-32 are consistent with the immunofluorescence double-label studies with  $\alpha$ -gustducin, PLC $\beta$ 2, NCAM, and P2X2. Connexin-32 expression is visible in Type III cells of rat circumvallate papillae and in the nerve processes (Fig. 21). There appears to be a much larger population of connexin-32 immunoreactive cells in the DAB studies than in the immunofluorescence studies. This is most likely due to the fact that the tissue slices for DAB techniques for light microscopy are generally 60-70 microns thick, while tissue slices for immunofluorescence are only 20  $\mu$ m thick. Thicker tissue is expected to have a larger population of taste cells. These results confirm the immunofluorescence data and suggest that connexin-32 is present in Type III cells and nerve processes in rat circumvallate taste buds.

### ***Connexin-43***

DAB results for connexin-43 are consistent with the immunocytochemical findings on the confocal level. The staining pattern expressed by connexin-43



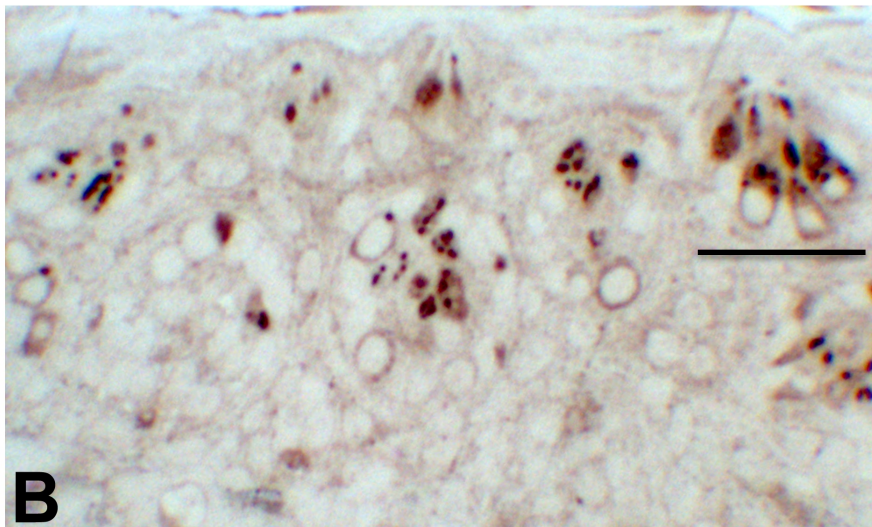
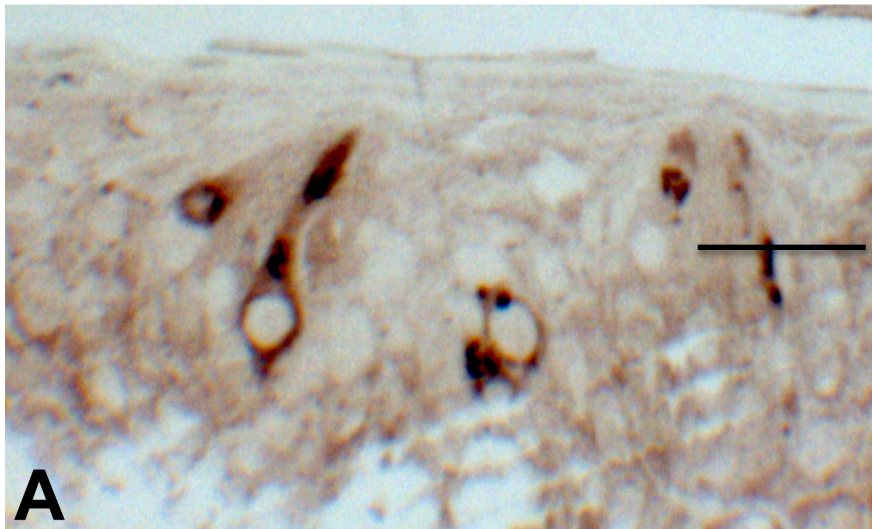
**Figure 21: A & B:** DAB for light microscopy images showing connexin-32-IR. Scale bar = 20  $\mu$ m

when colocalized with  $\alpha$ -gustducin, PLC $\beta$ 2, NCAM, and P2X2 is the same pattern that is expressed using DAB light microscopy. Connexin-43 expression is seen in Type II taste cells (Fig. 22). A punctate staining pattern appears in the apical regions of the Type II taste cells. There appears to be DAB staining in the cytoplasm of the entire cell. These results confirm the findings of the immunofluorescence studies and suggest that connexin-43 is present in Type II cells in rat circumvallate papillae.

## **DAB for Electron Microscopy**

### ***Connexin-32***

In the taste bud, connexin-32 appears to label a subset of Type III cells and nerve fibers (Fig. 7). The nerve fibers appear to innervate cells that are morphologically different. Some of the cells have large, ovoid nuclei while others cells have elongate nuclei. The cells with large, ovoid nuclei have clear, translucent cytoplasm, suggesting that nerve fibers with DAB staining are innervating Type II cells. The cells with elongate nuclei have electron-dense cytoplasm, suggesting that they are Type III cells. The Type III cells are innervated by nerve fibers in the taste bud (Fig.7). In Figure 7A, Connexin-32 appears to stain the cytoplasm and nuclei in Type III cells; however, high



**Figure 22: A & B:** DAB for light microscopy images showing connexin-43-LIR. Scale bar = 20  $\mu$ m.

magnification of a Type III cell suggests that Connexin-32 is only present in the cytoplasm (Fig. 7). In Figure 7A, Connexin-32 appears to stain the cytoplasm and nuclei in Type III cells; however, high magnification of a Type III cell suggests that Connexin-32 is only present in the cytoplasm (Fig. 7). There is DAB staining of the cytoplasm in the nuclear invagination of this Type III cell, while the nucleus itself does not show any DAB staining (Fig. 7).

A high magnification image of DAB staining of connexin-32 shows that connexin-32 is present in a cell with a prominent nuclear invagination, indicating that connexin-32 is present in a Type III cell (Fig. 7B). There appear to be other cells with the ultrastructural features of a Type III cell within the taste bud; however, they don't seem to express connexin-32. The only other reactivity expressed in this group of cells is in the surrounding nerve fibers (Fig. 7). This indicates that connexin-32 is only present in a subset of Type III cells. These results support the findings of the immunofluorescence experiments and DAB for light microscopy, confirming that connexin-32 is present in a subset of Type III cells and the nerve processes of rat circumvallate papillae.

### ***Connexin-43***

In the taste bud, connexin-43 appears to label only Type II cells (Fig. 8). As seen in the immunofluorescence, DAB staining is expressed in the whole cell and has a punctate nature in the apical regions (Fig. 8). High magnification of a group of taste cells shows two cells expressing Connexin-43 (Fig. 8). These cells

can be distinguished as Type II cells based on their large, ovoid nuclei. All of the cells that appear to express connexin-43 have the same characteristic large, round nuclei of Type II taste cells. The staining in the cytoplasm of the nuclei is clear and translucent, further supporting the evidence that cells expressing connexin-43 are Type II cells. The microvilli (Fig. 8, inset) are short and clustered, a characteristic of Type II cell microvilli. Consistent with immunofluorescence studies and DAB for light microscopy observations, staining is expressed in the cytoplasm of the cell and in a punctate nature (Fig 8). There are other taste cell types and nerve processes present within this group of cells, but connexin-43 appears to be present in only the two Type II cells. Interestingly, there are other cells in Figure 8 that exhibit the structural characteristics of Type II cells; however, they do not appear to have any connexin-43-LIR. Since not all Type II cells are positive for connexin-43 DAB staining, it appears that connexin-43 is only present in a subset of Type II cells.

In another high magnification image, two Type II cells, once again distinguished by their large, round, ovoid nuclei, appear to be in close contact with one another (Fig. 8). Similar to the other DAB immunoelectron micrographs, the staining of these Type II cells is expressed in the cytoplasm of the whole cell. There is also a punctate staining pattern that only appears to be present in the apical area of these two cells (Fig.8). It is not known why the staining in a punctate nature or why this staining is expressed in the apical area of the cell. As seen in Figure 8A, there is staining of a short cluster of microvilli, suggesting that the microvilli are in a Type II cell. The staining of the microvilli further support that



connexin-43 is expressed in the entire cell and that connexin-43 is present in Type II cells. Based on these results and immunohistochemical studies, connexin-43 is present in Type II cells in rat circumvallate papillae.

## DISCUSSION

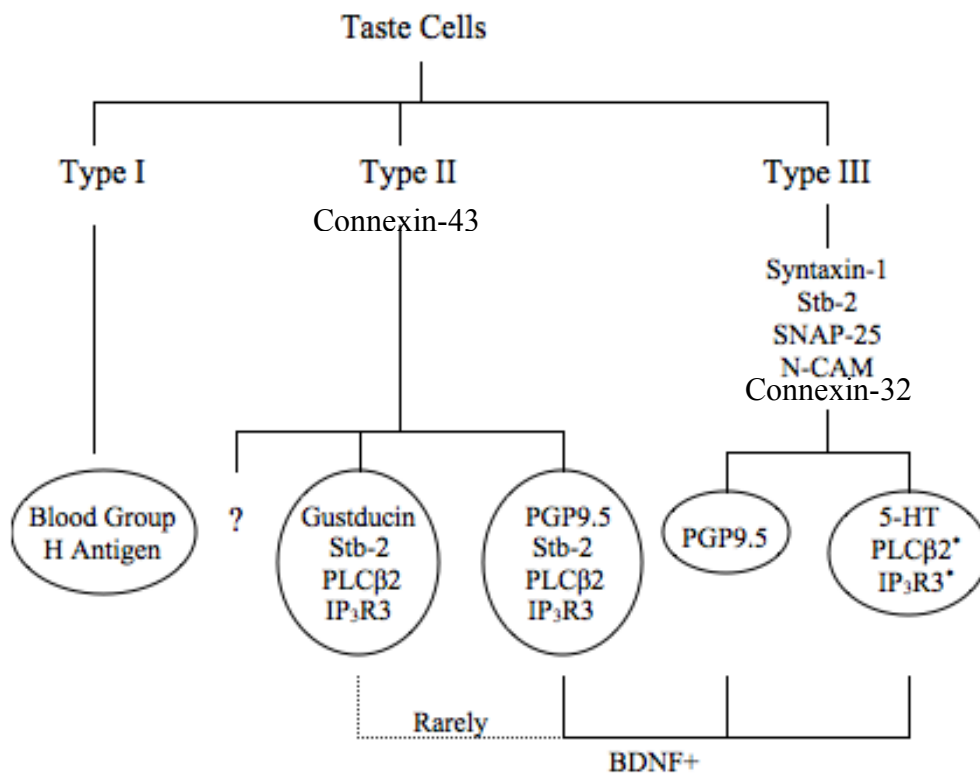
### Summary of the Results

This study demonstrates that taste tissue expresses connexin-32-LIR and connexin-43-LIR. Based on these data, connexin-32 is present in Type III cells and nerve processes. The results also indicate that connexin-43 is present in Type II cells. The co-localization of connexin-32-LIR and connexin-43-LIR with other taste cell markers in circumvallate taste buds is summarized in Figure 23. This diagram also refers to previous studies (Yee et al., 2001, 2003, Clapp et al., 2003, Yang et al., 2004).

Immunofluorescence studies indicate that connexin-32 does not co-localize with  $\alpha$ -gustducin or PLC $\beta$ 2 (Type II cell markers). Connexin-32-LIR is present in a subset of NCAM-LIR cells (Type III cell marker) and P2X2-LIR nerve processes (nerve fiber marker). Connexin-32 appears to label the entire cell and nucleus of a subset of Type III cells, as well as many of the nerve fibers. DAB immunoelectron microscopy studies support these results, confirming that

Figure 23

### MOLECULAR MARKERS IN TASTE CELLS



\*: A few IP<sub>3</sub>R3- and PLCβ<sub>2</sub>-LIR taste cells also display 5-HT-LIR.

**Figure 23:** Diagram showing known taste cell type markers in rat circumvallate papillae (Yang, 2006). In the present study, we used PLC $\beta$ 2, gustducin, and NCAM. Nerve fibers markers are not shown.

connexin-32 is present in a subset of Type III cells and nerve processes. High magnification images of the whole taste bud show connexin-32 DAB staining in Type III cells and in multiple nerve fibers that innervate taste cells (Fig. 7).

Connexin-43 immunoreactivity differs greatly from the connexin-32 immunoreactivity. Immunofluorescence studies show that connexin-43 does not co-localize with NCAM (Type III cell marker) or P2X2 (nerve fiber marker). Connexin-43-LIR co-localizes with  $\alpha$ -gustducin-LIR and PLC $\beta$ 2-LIR (Type II cell markers). Connexin-43-LIR cells appear to be present in the cytoplasm of the cell. There is also a punctate staining pattern that is displayed in some areas of connexin-43-LIR cells. Unlike immunofluorescence studies with connexin-32-LIR cells, there appears to be no labeling of the nuclei in connexin-43-LIR cells. DAB immunoelectron microscopy studies confirm these results. A high magnification electron micrograph (Fig. 8) shows Type II cells showing connexin-43 DAB in the cytoplasm of the cell with a punctate staining pattern.

From these results, we conclude that connexin-32 is expressed in Type III cells and nerve processes and connexin-43 is present in Type II cells in rat circumvallate papillae.

### **Connexin-32 and Connexin-43 in Taste Cell Types**

Connexins are gap junction proteins that have been implicated in a variety of functions in various tissue types. When six connexin subunits assemble

together, they form a connexon, which functions as a hemi-channel. One connexon can associate with another connexon to form a heteromeric or homomeric channel, which results in a gap junction. Connexin gap junctions allow the passage of molecules with a molecular weight that is less than 1kDa (ions, second messengers, etc.) Currently, there are 21 known connexin isoforms in vertebrates. Their cellular functions vary based on tissue type and the system in which they are expressed. Not all connexins are found in every tissue type. For example; connexin-36, connexin-43 and connexin-45 have been localized to olfactory tissue (Rash et al., 2005); however, RT-PCR shows that connexin-45 is not detectable at significant levels in gustatory tissue (Romanov et al., 2007; Huang et al., 2007) and data in this present study indicate that connexin-36 is also lacking in taste tissue (data not shown). It is still unclear as to which connexin isoforms are present in taste tissue. Furthermore, their role in transduction of taste has yet to be fully elucidated. Previous studies implicate connexin-26, connexin-30, connexin-32 and connexin-43 to play a role in mediating the release of ATP (Stout et al., 2001; Tran Van Nhieu et al., 2003; Kim et al., 2005). Romanov et al. (2007) suggests that connexin-43 is likely the connexin responsible for mediating ATP release in taste cells. Huang et al. (2007) argues against this idea, instead proposing that pannexin-1 is more likely to mediate ATP release in taste cells.

Many studies support ATP release through pannexin-1 channels instead of connexin hemi-channels based on the evidence showing that connexin hemi-channels only respond to low levels of intracellular  $Ca^{2+}$  (Dahl & Locovei, 2006;

Dando & Roper, 2009). Interestingly, connexin-32, a connexin that we find in Type III cells and nerve processes in this study, is the only known connexin that responds to elevated levels of  $\text{Ca}^{2+}$  (De Vuyst et al., 2005; Baukauskas et al., 2006).  $\text{IP}_3$  (inositol-1,4,5-trisphosphate) has been implicated in ATP release in brain epithelial cells (Braet et al., 2003, 2004; De Vuyst et al., 2005). In taste cells,  $\text{IP}_3$  acts as a second messenger in the transduction of sweet, bitter and umami taste. It is possible that  $\text{IP}_3$  could play a role in mediating ATP release through connexin hemi-channels in taste cells; however, this has not yet been studied.

There are studies that argue against ATP release via pannexin-1 channels and support connexin hemi-channels as mediators of ATP release based on kinetics. Romanov et al. (2008) reported that Type II cells secrete ATP through channels that are slow-activating with no inactivation. Studies show that pannexin-1 channels are fast-activating with a strong inactivation (Bruzzzone et al., 2003, 2005; Romanov et al., 2008). Only connexin hemi-channels have been observed to be slow-activating (Castro et al., 1999; Valiunas & Weingart, 2000; Valiunas, 2002; Bader & Weingart, 2004; Essendelder et al., 2004; Bruzzzone et al., 2005; Romanov et al., 2007, 2008), leading some investigators to believe that connexin hemi-channels are responsible for mediating ATP release in taste cells. If the experimental models truly mimic the environmental conditions of the cell, then this evidence strongly supports ATP release via connexin, not pannexin-1, hemi-channels.

The present study indicates that connexin-32 and connexin-43 are present in subsets of taste cells and/or nerve processes. Based on the ultrastructural features of the taste bud, connexin-32 is present in nerve fibers. These structures appear to be in intimate contact with cells that structurally represent Type II and Type III cells in the taste bud (Fig. 2). Connexin-32-LIR cells examined in this study most commonly exhibit an elongate shape, with slender nuclei and prominent nuclear invaginations, which are common features of Type III cells. However, connexin-43-LIR cells exhibit different structural features than connexin-32-LIR cells. Connexin-43-LIR cells appear to have large, ovoid nuclei and are spindle-shaped, which is characteristic of Type II cells.

### **Co-localization of Connexin-32 and Connexin-43 with Type II Cell Markers**

In order to determine if the connexins in this study are present in taste cells responsible for transducing a signal (Type II cells), connexin-32 and connexin-43 were tested for colocalization with known Type II cell markers. Tastants interact with G-protein coupled receptors. These receptors contain three important molecules:  $\alpha$ -gustducin,  $\beta$ 3, and  $\gamma$ 13. These molecules activate PLC $\beta$ <sub>2</sub>, which then results in the production of second messengers (IP<sub>3</sub> and DAG) to be produced (Huang et al., 1999; Yang et al., 2004). In the present study, connexin-43 and connexin-32 were co-localized with PLC $\beta$ <sub>2</sub> and  $\alpha$ -gustducin. No co-localization was observed between connexin-32 and these Type II cell markers.



However, connexin-43-LIR cells are present in a subset of PLC $\beta$ 2-LIR cells and a subset of  $\alpha$ -gustducin-LIR-cells.

Type II cells are one of the receptor cells that respond to gustatory stimuli in the taste bud. Only Type II taste cells have been observed to release ATP in response to depolarization (Romanov et al., 2007). Based on this evidence and the hypothesis that ATP is released via connexin hemi-channels, we would expect connexin to be present in Type II cells. This may explain why the results of this study indicate that connexin-43 is present in Type II cells.

Romanov et al. (2007) used the connexin-43 mimetic peptide, <sup>43</sup>GAP26, which is believed to inhibit the release of ATP via connexin-43 hemi-channels (Chaytor et al., 1997, 2001; Laybaert et al., 2003), on taste cells expressing connexin-43. They found that <sup>43</sup>GAP26 caused a reduction in Ca<sup>2+</sup> responses to the ATP biosensor and outward current (Romanov et al., 2007). If ATP is released from Type II (receptor) cells in the taste bud and the connexin-43 mimetic peptide effectively inhibits ATP release, then these data further support our findings that connexin-43 is present in Type II cells. The results of this study combined with evidence from Romanov et al. (2007) indicate that connexin-43 could possibly play a role in cell-cell communication and signal transduction in rat circumvallate papillae.

## **Connexin-32-LIR Co-localizes with a Type III Cell Marker and a Nerve Fiber Marker**

In order to determine if connexins in this study are present in cells that form synapses onto nerve fibers, connexin-32 and connexin-43 were co-localized with NCAM, a known Type III cell marker and P2X2, a known nerve fiber marker. No co-localization was observed between connexin-43 and NCAM. Connexin-43 also did not co-localize with P2X2. However, connexin-32-LIR cells were expressed in a small subset of NCAM-LIR-cells. Also, connexin-32-LIR nerve fibers were expressed in P2X2-LIR-nerve fibers. Based on these results, it seems that connexin-32 is expressed only in Type III cells and nerve fibers in rat circumvallate papillae.

Contrary to our data, RT-PCR in other studies showed that connexin-32 is either not expressed in taste tissue or is expressed in insignificant amounts (Romanov et al.,2007; Huang et al.,2007). The discrepancy in the data could be due to the fact that other studies have been conducted in mice whereas our experiments are conducted on rats. Yang et al. (2007) observed significant differences in the amount of taste cells expressing signaling molecules between rats and mice. These differences are most likely a result of differences in the processing of gustatory stimuli (Yang et al., 2007) and may explain why other studies have not observed connexin-32 expression in taste cells.

Connexin-32 mimetic peptide, <sup>32</sup>GAP27, is believed to inhibit the release of ATP via connexin-32 hemi-channels (Laybaert et al., 2003; De Vuyst et al.,

2006). Romanov et al. (2007), applied  $^{32}\text{GAP27}$  to taste tissue, but no effects were observed. However,  $^{32}\text{GAP27}$  was only applied to receptor cells; the effects of  $^{32}\text{GAP27}$  on Type III cells were never studied. Our results indicate that connexin-32 is present in Type III cells and nerve fibers, so even with this contradictory data, it is possible that connexin-32 is present in taste cells and may play a roll in cell-cell signaling.

Connexin-32 has been observed to play a role in the myelination of nerve fibers (Martini & Carenini, 1998). It is also expressed in some neurons in the brain (Dermietzel et al., 1989). In the female rat, connexin-32 was observed in gonadotropin-releasing hormone neurons, suggesting a mechanism through which gonadotropin-releasing neurons could be synchronized (Hosny & Jennes, 1998). It is possible that in rat circumvallate taste buds, connexin-32 provides a mechanism through which neurotransmitters or other signals can be distributed to neurons and other taste cells.

## **Conclusions**

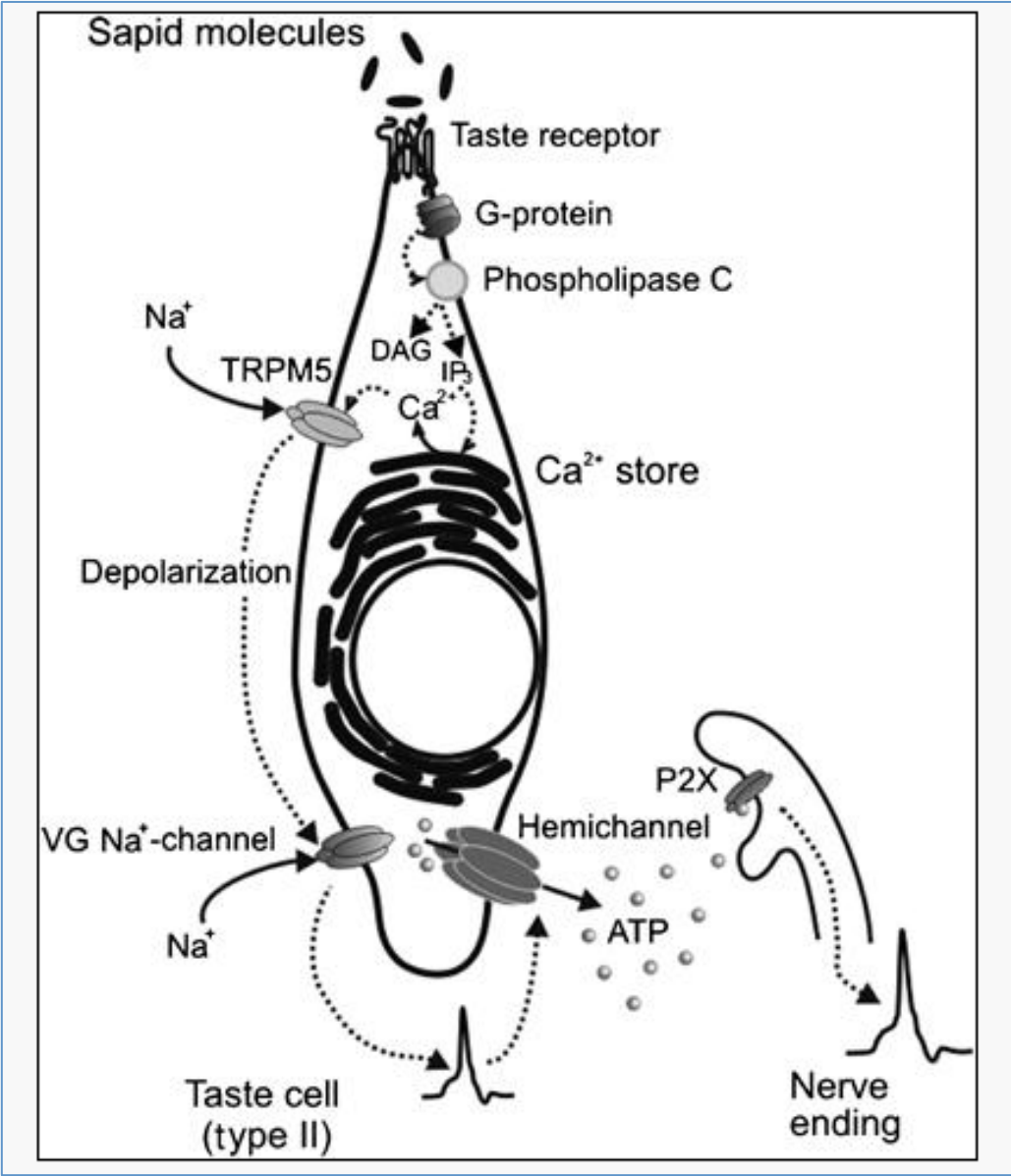
Type II cells lack identifiable synapses and SNAP-25, the presynaptic protein found in gustatory tissue (Clapp et al., 2006; De Fazio et al., 2006). Moreover, studies show that unlike Type II cells, Type III cells form “classical synapses onto nerve processes (Yang et al., 2000a; Yee et al., 2001). Type II cells respond to bitter, sweet, and umami taste stimuli (Clapp et al., 2004, 2006), indicating that Type II cells must utilize non-vesicular mechanisms to

communicate with other cells and nerve fibers. In the gustatory system, ATP is a key neurotransmitter in taste signaling (Finger et al., 2005b) and is released via specific channels in taste cells. The most likely candidates are pannexin and/or connexin hemi-channels (Huang et al., 2007; Romanov et al., 2007). Therefore, cell-to-cell communication is likely mediated through hemi-channels. Based on the ultrastructure of the cells examined in this study, we believe that connexin-32 is present in Type III cells and nerve fibers, and that connexin-43 is present in Type II cells. It is possible that these connexins play a role in cell-cell signaling and transduction in gustatory tissue. Figure 24 depicts a hypothesized mechanism for cell signaling in taste cells.

Romanov et al. (2007) found evidence for a population of taste cells that release ATP in a manner that is voltage dependent and  $Ca^{2+}$  independent. They also found strong evidence that voltage-gated outward currents and ATP release that is elicited by depolarization is mediated by connexin hemi-channels. Based on this research, the presence of connexin-32 and connexin-43 in rat circumvallate papillae may suggest that these connexins play a role in mediating ATP release in taste cells. On the other hand, studies show that pannexin-1 has also been implicated in ATP release in taste cells. Huang et al. (2007) showed that when a gustatory stimulus is applied, receptor cells will secrete ATP through pannexin-1 channels, which triggers the release of 5-HT from presynaptic cells. These findings indicate that pannexin-1 channels, not connexin hemi-channels, mediate ATP release in taste cells. Murata et al. (2010) found that action potentials in Type II cells enhanced ATP release through pannexin-1, not

connexin hemi-channels. Dando & Roper (2009) support these data, showing that pannexin-1 channels blockers reduce cell-cell signaling while connexin hemi-channel blockers have no effect. Previous work in our lab suggests that pannexin-1 is present in Type II and Type III cells (Yang et al., 2010); however, antibody restrictions made it impossible to obtain more of this antibody, thus preventing further study. It is possible that both connexin and pannexin hemi-channels are responsible for mediating ATP release and cell – cell signaling in taste cells.

Unfortunately, much of the evidence used to study the significance of connexin hemi-channels is circumstantial because it relies on gap junction channel blockers and the influx of fluorescent molecules (Spray et al., 2006; Scemes et al., 2007). These characteristics are shared by all gap junction protein family members, making it much more difficult to study gap junction proteins. Another obstacle in studying gap junction proteins is that research is conducted on cells that are isolated and manipulated for experimental use, therefore resulting in skewed environmental conditions and an inability to handle extended periods of ATP release (Goodenough & Paul, 2003; Evan et al., 2006). Future studies will help to clarify the presence of connexins in taste cells and their role in cell-to-cell communication. Colloidal gold studies will provide the ability to localize connexin-32 and connexin-43 in the taste bud. We hope that further study will provide a link to the role of connexin in cell-cell signaling and lead to gap junction research that is less circumstantial.



**Figure 24:** Diagram showing proposed mechanism of ATP release through hemi-channels (Romanov et al.,2007)

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