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Dopamine-beta-hydroxylase and Norepinephrine Transporter Immunoreactivity in Rat Taste Buds

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University of Denver

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DOPAMINE-β-HYDROXYLASE AND NOREPINEPHRINE TRANSPORTER IMMUNOREACTIVITY IN RAT TASTE BUDS

A Thesis

Presented to

the Faculty of Natural Sciences and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Jenna A. Walton

June 2012

Advisor: Dr. John C. Kinnamon
Abstract

Of the handful of neurotransmitters modulating taste response, norepinephrine (NE) may be the least understood. Although the physiological role of NE has been tested, it is still unclear whether the rat taste bud may serve as an endogenous source of neurotransmitter or if it must be taken up from outside the taste bud.

Immunocytochemical analysis of DBH has shown that DBH-LIR is present in taste cells of the rat circumvallate papillae. Specifically, DBH-LIR is present in a subset of Type II taste cells. Nearly all (98%) DBH-LIR cells express PLCβ2-LIR, and about half (41%) of PLCβ2-LIR cells express DBH-LIR. DBH-LIR does not co-localize with NCAM, and thus, is not present in Type III cells.

NET-LIR is present in the taste cells of the rat circumvallate papillae. Double labeling of NET-LIR and IP₃R3-LIR indicates that NET is present in Type II taste cells. A large proportion (77%) of NET-LIR cells express IP₃R3-LIR and all (100%) IP₃R3-LIR cells express NET-LIR. A small proportion (7.8%) of NET-LIR cells also express syntaxin-1-LIR in Type III cells. About a third (32.8%) of syntaxin-1-LIR cells express NET-LIR.
Acknowledgements

I would like to first thank my advisor, Dr. Kinnamon, for all his help and guidance. In my time here at the University of Denver, Dr. Kinnamon taught me how to think critically about science and how to become an excellent scientific researcher myself. I would also like to thank the other members of my committee: Dr. Scott Barbee, Dr. Nancy Lorenzon and Dr. Dwight Smith for their guidance, advice and critical comments of my thesis.

The entire Kinnamon lab has served as such a strong and wonderful support system for me through out my entire time in graduate school. Ms. Lulu Yip and Ms. Amanda Bond provided excellent technical assistance and always helped make the laboratory a wonderful place to be. A big thank you to Mr. HoanVu Nguyen for countless hours spent helping with the formatting of this thesis and for the design of several figures. And finally, I would like to thank Dr. Ruibiao Yang who served as my mentor in the laboratory and patiently trained me in the techniques of immunohistochemistry. He always made himself readily available to assist me in anyway possible and for that, I greatly appreciate all of his time and effort.

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## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AADC</td>
<td>aromatic L-amino acid decarboxylase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-o-methyl transferase</td>
</tr>
<tr>
<td>DBH</td>
<td>dopamine-β-hydroxylase</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial sodium channel</td>
</tr>
<tr>
<td>GAD67</td>
<td>glutamic acid decarboxylase 67</td>
</tr>
<tr>
<td>GAT-3</td>
<td>GABA transporter type III</td>
</tr>
<tr>
<td>GLAST</td>
<td>glutamate aspartate transporter</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>IP$_3$R3</td>
<td>IP$_3$ receptor type III</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>-LIR</td>
<td>-like-immunoreactivity</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NET</td>
<td>norepinephrine transporter</td>
</tr>
<tr>
<td>NTPDase-2</td>
<td>ecto-nucleoside triphosphate diphosphohydrolase type II</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PLCβ2</td>
<td>phospholipase C β2</td>
</tr>
<tr>
<td>PLP</td>
<td>periodate-lysine-paraformaldehyde</td>
</tr>
<tr>
<td>PNMT</td>
<td>phenylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>ROMK</td>
<td>renal outer medullary potassium channel</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>synaptosomal-associated protein 25</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>VMAT</td>
<td>vesicular monoamine transporter</td>
</tr>
<tr>
<td>5HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>5HTP</td>
<td>serotonin precursor</td>
</tr>
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</table>
INTRODUCTION

Taste, or gustation, is one of the five traditional senses and, more specifically, is a form of chemoreception. Chemoreception refers to the mechanism by which an organism detects chemical stimuli in both the internal and external environments. The ability to perceive this information in one’s environment has important consequences not only for thirst and satiety, but also for being able to recognize poisonous substances that may be harmful to the organism if ingested. Gustation refers to the perception of the five basic taste qualities: bitter, sweet, salty, sour and umami. The experience of flavor, however, is multimodal. It pulls sensory information from mechanoreceptors, which detect tactile stimuli; thermoreceptors, which detect changes in temperature; nociceptors, which detect painful stimuli; and receptors in the olfactory epithelium, which detect various odors. Together, these sensory inputs in combination with those from gustation allow for the experience of the many flavors organisms encounter in their environments.

Gross Anatomy and Innervation

Lining the dorsal surface of the tongue, soft palate, upper esophagus and epiglottis are gustatory papillae (Miller, 1995). There are three different types of papillae involved in gustation: the fungiform, foliate and circumvallate papillae. The fungiform papillae are mushroom-shaped structures and concentrated on the anterior one-third of the tongue, as
well as a small region along the side of the tongue. These papillae are innervated by the facial/chorda tympani nerve, VIIth cranial (Purves et al., 2008). The foliate papillae consist of distinct ridges and grooves along the side of the tongue as well as the posterior portion of the roof of the mouth. Those papillae located more anterior in the mouth are innervated by the facial nerve, whereas papillae located more posterior are innervated by the glosopharyngeal nerve, IXth cranial (Purves et al., 2008). The final group of gustatory papillae, the circumvallate, is present at the back of the oral part of the tongue and is also innervated by the glosopharyngeal nerve (Purves et al., 2008). They are arranged in a chevron-shaped row just in front of the sulcus terminalis of the tongue and are few in number—most humans only have 10-14 of these papillae. Papillae located on the epiglottis and along the throat are innervated by the vagus nerve, Xth cranial (Purves et al., 2008). Although many structural differences exist between papillae, all three types possess taste buds with equal ability to perceive each of the five primary taste qualities. Taste stimulation results in a series of action potentials and the release of neurotransmitter onto afferent nerve fibers. Sensory signals reach the first synaptic connection in the nucleus of the solitary tract located in the medulla, where the signal then proceeds to the ventral posterior medial (VPM) nucleus of the thalamus until it finally reaches one of two cortical destinations: the insula or the frontal operculum of the parietal lobe (Purves et al., 2008).
Taste Cell Structure and Function

Taste buds are collections of 50-100 polarized epithelial cells that are embedded in the oral cavity and are responsible for the detection of nutrients and other chemical compounds. Each taste bud is a complex and dynamic system of intracellular and intercellular signaling pathways. This idea first became clear when distinct, morphological differences were observed amongst cells within the taste bud (Fig. 1 & 2). Early light microscopic studies distinguished two types of taste bud cells designated as sensory (dark) and sustentacular (light) cells (Kolmer, 1927). However, later studies using electron microscopy revealed further differences in ultrastructure amongst taste cells, and eventually led to the reversal of this nomenclature, proposing that Type I (dark) cells are supporting in nature, and that Type II (light) cells perform a sensory function (Farbman, 1965; Murray, 1971, 1973). Light cells display an electron-lucent cytoplasm and contain large, ovoid nuclei, whereas dark cells have an electron-dense cytoplasm with irregularly shaped nuclei (Delay et al., 1986; Nelson & Finger, 1993; Pumplin et al., 1997). Both dark and light cells are often in close apposition to nerve fibers and extend apically into the taste pore. Other cells, sharing characteristics of both dark and light cells, have been termed “intermediate” cells, or more commonly Type III cells (Kinnamon et al., 1985; Delay et al., 1986; Roper, 1989; Nelson & Finger, 1993). Research suggests that conventional synapses are restricted to Type III cells (Murray & Murray, 1970; 1971, Murray, 1971; 1973, Royer & Kinnamon, 1991; 1994), also known as “Presynaptic” cells (Tomchik et al., 2007).
Figure 1
**Figure 1.** Electron micrograph showing a longitudinal section of a rat taste bud. Type I cells are slender in shape and display an electron dense cytoplasm (I). These cells also contain dense granules (arrowheads). Type II taste cells are often in close apposition to nerve fibers (arrow) and display large, ovoid nuclei and an electron-lucent cytoplasm (II). These cells contain several short microvilli (MV1) near the taste pore (TP). In contrast, Type III cells display one, blunt microvillus (MV2). Scale bar = 5 µm. Image courtesy of Dr. Ruibiao Yang.
Figure 2
Figure 2. Electron micrograph showing a transverse section of a rat taste bud. Type I cells display an elongate nucleus (I). Type II taste cells display large, ovoid nuclei (II) and are often in close apposition to nerve fibers (arrow). Type III cells display a characteristic nuclear invagination (III). Scale bar = 5 µm. Image courtesy of Dr. Ruibiao Yang.
Recently, the field has moved away from this nomenclature system as further research has shown that certain proteins are restricted to different cell types (Table 1). Characterization using cell type markers has led to the present day nomenclature system of Type I, Type II (“Receptor”), Type III (“Presynaptic”) and basal cells.

**Type I (“dark”) cells**

Previously referred to as “dark cells”, Type I cells are electron-dense, slender in shape (Fig. 1) and have several, long microvilli extending into the taste pore (Murray, 1973). These cells also contain lamellate processes that envelop other types of taste cells and are thought to be glial-like in nature (Farbman, 1965; Murray, 1971, 1973). Another characteristic of this cell type is the presence of dense, cytoplasmic granules (Murray, 1973) that can clearly be seen in electron micrographs (Fig. 1). This cell type expresses GLAST, a glial glutamate transporter (Lawton et al., 2000) as well as NTPDase-2, an ecto-ATPase that hydrolyzes extracellular ATP (Bartel et al., 2006). Thus, Type I cells may be involved in the termination of synaptic transmission via ATP. These cells also express ROMK, a $\mathbb{K}^+$ channel thought to be involved in $\mathbb{K}^+$ homeostasis (Dvoryanchikov et al., 2009). The presence of these channels suggests that Type I cells may help eliminate excess $\mathbb{K}^+$ from the taste bud, which would otherwise lower the excitability of the surrounding cells. Recently, it has been suggested that, in addition to their glial role in the taste bud, Type I cells may also serve as receptor cells for salty qualities, as Vandenbeuch et al. (2008) observed $\mathbb{Na}^+$ currents in Type I cells that may be implicated in salt transduction. It has long been hypothesized that salty taste transduction is achieved
Table 1. Cell Types and Molecular Markers

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Molecular Marker</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NTPDase-2</td>
<td>Bartel et al., 2006</td>
</tr>
<tr>
<td></td>
<td>GLAST</td>
<td>Lawton et al., 2000</td>
</tr>
<tr>
<td></td>
<td>ROMK</td>
<td>Dvoryanchikov et al., 2009</td>
</tr>
<tr>
<td>II</td>
<td>α-gustducin</td>
<td>Yang et al., 2000; Clapp et al., 2001, 2004; Miyoshi et al., 2001</td>
</tr>
<tr>
<td></td>
<td>PLCβ2</td>
<td>Yang et al., 2000; Clapp et al., 2001, 2004; Miyoshi et al., 2001</td>
</tr>
<tr>
<td></td>
<td>IP₃R3</td>
<td>Yang et al., 2000; Clapp et al., 2001, 2004; Miyoshi et al., 2001</td>
</tr>
<tr>
<td>III</td>
<td>5HT</td>
<td>Nada &amp; Hirata, 1975; Takeda, 1977; Takeda et al., 1981; Kim &amp; Roper, 1995; Yee et al., 2001</td>
</tr>
<tr>
<td></td>
<td>NCAM</td>
<td>Nelson &amp; Finger, 1993</td>
</tr>
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</table>
through interaction with the amiloride-sensitive epithelial Na⁺ channel, ENaC (Heck et al., 1984; Lin et al., 1999; Lindemann, 2001). This was finally confirmed by knocking out a critical subunit of the ENaC channel in taste buds, diminishing the ability to detect salty stimuli (Chandrashekar et al., 2010).

**Type II (“light”) cells**

Type II taste cells (“light cells”) are electron-lucent, contain several short microvilli of uniform length (Kinnamon & Yang, 2008) have characteristic large, ovoid to round nuclei (Fig. 1), and express the receptors and signaling pathways for the transduction of bitter, sweet and umami taste qualities (Boughter et al., 1997; Hoon et al., 1999; Yang et al., 2000; Miyoshi et al., 2001, Clapp et al., 2004). Each Type II cell is specific for only one of these qualities, effectively tuning each cell to either sweet, bitter or umami reception (Nelson et al., 2001; Tomchik et al., 2007). Initial events of signal transduction for these tastants occur at the plasma membrane of the microvilli that extend into the taste pore from the Type II cell. Embedded in the microvillar membrane are various G-protein coupled receptors (GPCR’s). Those receptors falling in the T2R family sense bitter compounds (Chandrashekar et al., 2000). T2Rs are co-expressed with and activate the α subunit, α-gustducin (Ruiz-Avila et al., 1995). Type II cells expressing the heterodimer T1R2-T1R3 respond to sweet tastants (Nelson et al., 2001; Jiang et al., 2004; Xu et al., 2004). T1R3 KO mice, however, have displayed persistence of sensitivity to some sugars, suggesting that additional receptors for sweet tastants may exist (Damak et al., 2003). Heterodimer receptors composed of T1R1 and T1R3 subunits respond
exclusively to umami taste stimuli (Li et al., 2002; Nelson et al., 2002). This heterodimer appears to be co-expressed with and activates Gα14 (Tizzano et al., 2008). Although many taste GPCR’s activate Gα subunits, signal transduction is primarily achieved through the Gβγ subunit (Huang et al., 1999) through its interaction with phospholipase Cβ2, PLCβ2 (Rossler et al., 1998). The second messenger, inositol triphosphate (IP₃), the cleavage product of the interaction of PLCβ2 with transmembrane phospholipid phosphatidylinositol 4,5-bisphosphate, PIP₂, then frees Ca²⁺ stores within the smooth endoplasmic reticulum upon activation of the IP₃ receptor type III, IP₃R3 (Simon et al., 2006; Roper, 2007). The rise in intracellular Ca²⁺ stimulates opening of TRPM5 cation channels located on the plasma membrane, producing a depolarization in the receptor cells (Liu & Liman, 2003). The elevated levels of intracellular Ca²⁺ and the depolarizing force produced by the opening of TRPM5 channels result in the release of ATP through gap junction hemichannels into the extracellular space (Huang et al., 2007, Romanov et al., 2007, Huang & Roper, 2010). An increasing amount of evidence suggests that this release occurs through pannexin-1 hemichannels (Huang et al., 2007), although some evidence also exists for a role of connexin-34/36 hemichannels in taste as well (Romanov et al., 2007). Interestingly, Type II cells do not display classical synapses. Nerve fibers are, however, often located in close apposition to these cells (Murray, 1973; Yang et al., 2000; Yee et al., 2001; Clapp et al., 2004) and express the purinergic receptor P2X₂ and P2X₃ (Bo et al., 1999). Therefore, Type II cells do not transmit neurological signals via synaptic vesicles but rather through unconventional mechanisms, likely involving the non-vesicular release of ATP.
**Type III (“Presynaptic”) cells**

Type III cells, once called intermediate cells, are now often referred to as “Presynaptic cells” (Defazio et al., 2006). They are characterized by a morphology that is intermediate between that of Type I and Type II cells (Kinnamon & Yang, 2008). These cells display an electron-lucent cytoplasm, are slender and spindle-shaped, contain elongate nuclei with prominent invaginations (Fig. 2) and possess a single, large, blunt microvillus that extends into the taste pore (Murray, 1973). Type III cells are the only gustatory cells to display classical synaptic contacts with the nerve fibers and express the synaptic membrane proteins SNAP-25, synaptobrevin, syntaxin-1 and synaptophysin (Kim and Roper, 1995; Yang et al., 2000, 2004, 2007), as well as the neural cell adhesion molecule, NCAM (Nelson and Finger, 1993). These findings suggest that the presynaptic cells are involved in the transmission of sensory information to the central nervous system. A subset of Type III cells also display immunoreactivity for serotonin, 5HT (Yee et al., 2001). These cells release 5HT upon stimulation, likely leading to a paracrine action on adjacent Type II cells (Huang et al., 2005, 2006; Heath et al., 2006). Norepinephrine, NE, is also released from Type III cells, being coupled to 33% of all serotonergic release (Huang et al., 2008). Type III cells also appear to be involved in the transduction of sour (acidic) taste qualities (Tomchik et al., 2007). In response to sour stimulation, Type III cells release gamma-aminobutyric acid (GABA), which then exerts an inhibitory action on Type II cells preventing the release of ATP (Huang et al., 2011). The nonselective cation channels PKD2L1 and PKD1L3 were once proposed to be the most likely receptors for sour taste (Huang et al., 2006; Ishimaru et al., 2006; Lopez-
Jimenez et al., 2006, Kataoka et al., 2008); however, more recent studies have begun to invalidate this claim. For example, PKD1L3 knockout mice remain fully capable of detecting sour stimuli (Nelson et al., 2010). Researchers are now investigating the possibility that plasma membrane channels modulated by cytoplasmic acidification (such as various $K^+$ channels) may serve as receptors for sour tastants (Lin et al., 2004; Richter et al., 2004). Type III cells also detect carbonation as a result of carbonic anhydrase and the production of protons, leading to an acidification of the environment (Graber and Kelleher, 1988; Simons et al., 1999; Chandrashekar et al., 2009). It has also been shown that carbonated stimuli specifically activate a subpopulation of trigeminal neurons expressing TRPA1 in a HEK-293 cell line (Wang et al., 2010).

**Basal Cells**

The final cell type, basal cells, are round, non-polarized cells located near the base of the taste bud (Farbman, 1965). These cells are not thought to be involved in events of signal transduction, but rather serve as progenitor cells (Delay et al., 1986; Roper, 1989).

**Signaling in Taste Buds**

In the past decade, there have been great advances in the working knowledge of taste transduction and its internal mechanisms for various signaling pathways. In particular, the downstream events of bitter, sweet and umami stimulation have become clearer. As a result of stimulation by these tastants, ATP is released from the Type II taste cell. ATP then has three targets: 1) directly onto afferent nerve fibers via P2X receptors...
(Finger et al., 2005), 2) onto P2Y$_2$ and/or P2Y$_4$ receptors located on adjacent Type III cells, whose action stimulates release of 5HT and/or NE (Huang et al., 2009) and 3) back onto the Type II cell via P2Y$_1$ receptors, acting as a positive feedback to increase secretion of ATP and to potentially overcome ecto-ATPase activity (Kataoka et al., 2004; Huang et al., 2009). Taste cells have been shown to express P2Y receptors and to respond to exogenously applied ATP in a manner reflective of P2Y mediated signaling, offering further support for ATP as a paracrine signaling molecule (Kataoka et al., 2004; Bystrova et al., 2005). 5HT released by the Type III cells has been proposed to be stored in both small and large, dense-cored vesicles; however, little support for this claim exists (Takeda & Kitao, 1980). Recent studies suggest that 5HT may have multiple targets. Huang et al. (2009) showed that 5HT released from Type III cells inhibits receptor cells via 5HT$_{1A}$ receptors. This negative feedback exerted by 5HT may work in tandem with the positive feedback of ATP to effectively shape sensory outputs, although the details of this interaction are poorly understood (Huang et al., 2009). It is also possible that 5HT may mediate lateral inhibition, effectively enhancing the output of a stimulated cell by suppressing the activity of surrounding cells. 5HT may also have effects at nerve fibers that form synaptic connections with Type III cells.

GABA, a less well-studied neurotransmitter, has also been implicated in gustatory transduction. The synthetic enzyme to GABA, glutamic decarboxylase-67 (GAD67), has recently been localized to a subset of Type III cells in mice (DeFazio et al., 2006; Tomchik et al., 2007). In rats, immunocytochemical studies have shown GABA immunoreactivity in taste cells and have identified the GABA transporter type III, GAT-3,
as the primary transporter in the gustatory system to facilitate GABA reuptake (Obata et al., 1997). To further investigate the distribution of the GABA signaling machinery, Starostik et al. (2010) examined various GABAergic receptors and their expression patterns in rat circumvallate papillae. GABA$_A$-and GABA$_B$-LIR was found in subsets of both Type II and Type III cells. As it has been hypothesized that GABA is released from Type III cells, these data suggest that GABA may have both paracrine (onto Type II cells) and autocrine (back onto Type III cells) actions in the taste bud (Starostik et al., 2010). Although evidence for GABAergic signaling in the gustatory system is slowly starting to grow, the details of this mechanism are still poorly understood.

**Norepinephrine (NE)**

Like GABA, a role for NE in the gustatory system has slowly begun to take shape. NE is a neurotransmitter belonging to the catecholamine family, which also includes dopamine and epinephrine. NE is synthesized from dopamine via dopamine-β-hydroxylase (DBH) and then serves as the precursor for the synthesis of epinephrine via phenylethanolamine N-methyltransferase (PNMT) (Fig. 3). It is released from the adrenal medulla into the blood as a hormone, and is also a neurotransmitter in the central nervous system and sympathetic nervous system, where it is released from neurons in the locus coeruleus, typically inducing the “flight or fight” response (Guyton & Hall, 2006). As a neurotransmitter, NE most often has effects at post-synaptic contacts and nerve endings. Movement of NE into synaptic vesicles is accomplished by the vesicular monoamine transporter (VMAT) and is then followed by vesicle docking via V- and T-SNARE
proteins (Berridge & Waterhouse, 2003). During synaptic transmission, an action potential coupled to a rise in intracellular Ca$^{2+}$ levels triggers vesicle fusion on the presynaptic membrane and exocytosis of NE into the synaptic cleft. NE then binds to receptor sites on the post-synaptic cell to continue signal propagation until the signal reaches its final destination. Adrenoceptors belong to three families: $\alpha_1$, $\alpha_2$, and $\beta$ receptors (Fig. 4). The $\alpha_1$ adrenoceptor activates the $G_q$ subunit of the GPCR, initiating the PLC/IP$_3$ signaling pathway ultimately leading to an increase in intracellular Ca$^{2+}$ levels (Schmitz et al., 1981). The $\alpha_2$ adrenoceptor activates the $G_i$ subunit of the GPCR, inhibiting adenylyl cyclase and thus, down-regulating cyclic adenosine monophosphate (cAMP) (Schmitz et al., 1981). Finally, the $\beta$ adrenoceptors activate the $G_s$ subunit of the GPCR and up-regulate both adenylyl cyclase and cAMP (Chen-Izu et al., 2000).
Figure 3. Synthetic pathway for catecholamine neurotransmitters.
Figure 4. Adrenoceptor subtypes and downstream effectors. A. $\alpha_1$ receptors activate the $G_q$ subunit and initiate the PLC/IP$_3$ signaling pathway and an increase in intracellular Ca$^{2+}$ levels. B. $\alpha_2$ adrenoceptors activate the $G_i$ subunit and inhibit AC/cAMP. C. $\beta$ receptors activate the $G_s$ subunit and up-regulate both AC/cAMP. Image courtesy of Mr. HoanVu Nguyen.
Noradrenergic Signaling in Taste Buds

Nagahama and Kurihara (1985) investigated noradrenergic signaling in a frog model, in which catecholamine depleting agents were found to decrease taste response in the glossopharyngeal nerve. NE antagonists such as desipramine and imipramine also most robustly inhibited these responses, suggesting a prominent role for norepinephrine in the gustatory system. Observations at the level of electron microscopy have suggested that taste responses modulated by norepinephrine may be accomplished through dense-cored vesicles, as application of reserpine depletes nearly all visible, dense-cored vesicles in the frog taste organ, with repletion achieved by application of norepinephrine (De Han & Graziadei, 1973).

In rat taste cells, noradrenergic enhancement of the outwardly rectifying chloride current with the noradrenergic agonist isoproterenol was observed and was effectively blocked by the noradrenergic antagonist propranolol (Herness & Sun, 1999). To follow up this work, Herness et al. (2002) examined noradrenergic modulation of various potassium currents in rat taste cells. Both beta and alpha noradrenergic receptor agonists effectively inhibited outward $K^+$ currents while increasing intracellular $Ca^{2+}$ levels. Together, these data suggest that noradrenergic modulation helps bring the taste cell to an excited state and/or maintain it at this state (Roper 2007). Norepinephrine has also been implicated in the modulation of human taste thresholds, as it has previously been noted that individuals suffering from depression or anxiety, both of which result from alterations in 5HT and NE, often show mild to moderate disturbances in taste (Heath et al., 2006). Research has shown that increasing systemic norepinephrine lowers bitter taste
thresholds by 39% and sour thresholds by 22% (Heath et al., 2006). These data not only offer support for noradrenergic modulation in the gustatory system, but also suggest that this modulation may occur at both Type II and Type III cells, due to the selectivity of tastants. Zhang et al. (2010) provided further support for this claim by localizing various noradrenergic receptors in the rat taste bud. Most of these receptors display $\alpha$-gustducin-LIR, a known Type II cell marker, while a very small percentage display NCAM-LIR, a known Type III cell marker.

Serotonin (5HT), one of the most well understood neurotransmitters of the gustatory system, has been physiologically linked to norepinephrine in its release patterns. Huang et al. (2008) showed a 33% concordance in the co-release of 5HT and NE from Type III taste cells in mice. Intracellular calcium levels seem to play a significant role as well, suggesting that this release is $Ca^{2+}$ dependent. Previously it was hypothesized that NE is taken up from the interstitial spaces, as RT-PCR analysis indicated the absence of the synthetic enzymes for NE—tyrosine hydroxylase (TH) and dopamine $\beta$-hydroxylase (DBH)—in the mouse taste bud (Dvoryanchikov et al., 2007). It was proposed that this movement occurred via the norepinephrine transporter (NET) and was further supported through immunocytochemical localization to Type III cells (Dvoryanchikov et al., 2007). However, more recent studies of immunocytochemistry suggest that taste receptor cells could serve as an endogenous source of NE within the rat taste bud (Herness et al., 2002). In addition, DBH-LIR has been found in gustatory cells within the frog taste disc. DBH has been localized to Type II cells as well as Type III cells in the frog (Ando et al., 2007). Non-gustatory epithelium has also shown noradrenergic activation in the presence of the
neurotransmitter (Huang et al., 2008). Collectively, noradrenergic transmission may serve as a paracrine signaling pathway in peripheral, gustatory physiology (Nagahama & Kurihara, 1985).

**Specific Aims**

Although progress has been made in understanding the role of norepinephrine in gustatory transduction, a substantial amount of work needs to be done in order to fully elucidate the mechanism of noradrenergic modulation in this system. To begin addressing such a complex question, the various proteins that contribute to this machinery need to be identified and localized. Protein localization can, then, begin to elucidate the roles of such effectors based on in what type of cell a protein is expressed and with what other proteins it may localize. The noradrenergic system includes many proteins that are essential for the transport (NET, VMAT), degradation (COMT, MAO, PNMT) and synthesis (TH, DBH) of the neurotransmitter itself, as well as the various transmembrane proteins that act as receptors ($\alpha_1$, $\alpha_2$, and $\beta$ subtypes) for noradrenergic modulation. We propose to test the hypothesis that norepinephrine is synthesized in the rat taste bud and that movement of this neurotransmitter is accomplished via NET. We will localize DBH as well as NET through techniques of indirect immunocytochemistry and characterize the expression of both using quantitative techniques.
Aim 1. Using confocal microscopy to test if DBH is present in the rat circumvallate taste bud.

a. To determine the presence of DBH with taste signaling molecules α-gustducin, PLCβ2 and 5HT, as well as with the taste cell type marker NCAM in taste buds.

b. To determine the percentages of taste cell types that express DBH in taste buds.

Aim 2. Using confocal microscopy to test if NET is present in the rat circumvallate taste bud.

a. To determine the presence of NET with the taste signaling molecules IP_{3}R3 and 5HT, as well as the cell type marker syntaxin-1 in taste buds.

b. To determine the percentages of taste cell types that express NET in taste buds

Although the physiological role of norepinephrine has been studied, it is still unclear whether the taste bud may serve as an endogenous source of neurotransmitter or if it must be taken up from outside the rat taste bud. Our goals are to determine: 1) which cell types contain the synthetic enzyme, DBH, and the transporter, NET, in the rat taste bud; 2) the subset of taste cells to which DBH and NET belong. Our results will help better understand the role of norepinephrine in the rat taste bud.
MATERIALS AND METHODS

Adult Sprague-Dawley male rats (250-350g) 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. For co-localizations with 5HT, animals were injected with 5-hydroxytryptophan (5-HTP, H 9772; Sigma, St. Louis, MO; 80 mg/kg, intraperitoneal, i.p.) 1 hour prior to sacrifice (Kim & Roper, 1995; Yee et al., 2001; Clapp et al., 2004; Yang et al., 2004). This procedure is commonly used because endogenous levels of serotonin in taste cells are quite low and difficult to detect with IHC. All rats were anesthetized with an intraperitoneal injection of ketamine KCL (80 mg/kg body weight) and xylazine (5 mg/kg body weight).

Antibodies

Primary and secondary antibodies used for these studies are listed in Tables 1 and 2. Monoclonal DBH was raised against purified bovine DBH in mouse. Antibody specificity was determined using immunohistochemistry (Appendix 1).

NET is an affinity purified rabbit polyclonal antibody raised against amino acids 551-617 mapping within a C-terminal cytoplasmic domain of NE transporter of human origin. Western blot shows a distinct band of expected size at 58 kDa (manufacturer’s
technical information). Secondary antibody specificity was also determined using immunohistochemistry (Appendix 1).

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

Serotonin antiserum was generated in a rabbit (polyclonal) or mouse (monoclonal) against serotonin coupled to bovine serum albumin (BSA) with paraformaldehyde. This antibody was quality control tested using standard immunohistochemical methods (manufacturer’s technical information).

\( G_{\alpha_{\text{gust}}} \) (I-20) is an affinity-purified rabbit anti-gustducin polyclonal antibody raised against a peptide corresponding to amino acids 93-112 mapping within a highly divergent domain of \( G_{\alpha_{\text{gust}}} \) of rat origin. Antibody specificity was determined using immunohistochemistry and has been previously validated (Yang et al., 2000; Clapp et al., 2001, 2004; Miyoshi et al., 2001).

PLC\( \beta_2 \) (Q-15) is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 1170-1181 mapping near the C-terminus of PLC\( \beta_2 \) of human origin. Western blot analysis of this antibody showed a major band at 120 kDa in RAW 264.7 whole cell lysate (manufacturer’s technical information and Choi et al., 2001).
IP$_3$R3 is an affinity-purified mouse anti-IP$_3$R3 antibody raised against a peptide mapping near the N-terminus of IP$_3$R3 of human origin. Antibody specificity was determined using immunohistochemistry and has previously been validated (Yang et al., 2000; Clapp et al., 2001, 2004; Miyoshi et al., 2001).

NCAM is an affinity-purified rabbit anti-NCAM antibody raised against amino acids 1-300 mapping at N-terminus of NCAM of human origin. Antibody specificity was determined using immunohistochemistry and has previously been validated (Nelson & Finger, 1993).

The NTPDase-2 polyclonal antibody was raised in rabbit by direct intramuscular and intradermal injection of the complementary DNA (cDNA), encoding the entire mouse Entpd2 gene (GenBank accession No. AY376711) ligated into pcDNA3.1/V5-His (Invitrogen, Ontario, Canada). The antibody specificity was determined by both Western blot and immunocytochemistry.

**Immunohistochemistry for Confocal Microscopy**

All rats were perfused following the technique of Weedman et al. (1996) intracardially for 10 seconds with 0.1% sodium nitrite, 0.9% sodium chloride, and 100 units sodium heparin in 100 mL 0.1 M phosphate buffer (PB, pH 7.3). This was followed by perfusion fixation in either paraformaldehyde (PFA: 4% paraformaldehyde in 0.1 M PB) or periodate-lysine-paraformaldehyde (PLP: 1.6% paraformaldehyde, 10 mM sodium periodate and 75 mM lysine in 0.1 M PB) 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 cryoprotected with 30% sucrose in 0.1 M PB overnight at 4°C.

**Single labeling.** Cryostat sections (20 µm thick) containing circumvallate taste buds were blocked in 5% normal goat serum, 1% BSA and 0.3% Triton X-100 in 0.1 M phosphate-buffered saline (PBS) (pH 7.3) for 2 hours on ice, then incubated in primary antibody monoclonal DBH or primary antibody polyclonal NET in 0.1 M PBS (pH 7.3) overnight at 4°C. After washing, the sections were exposed to affinity-purified secondary antibody fluorescein (FITC) goat anti-mouse IgG for monoclonal antibody DBH or affinity-purified secondary antibody Cyanine 5 (Cy'5) goat anti-rabbit IgG for polyclonal antibody NET in 0.1 M PBS (pH 7.3) for 2 hours on ice.

**Double labeling.** Cryostat sections (20 µm thick) containing circumvallate taste buds were blocked in 5% normal goat serum, 1% BSA and 0.3% Triton X-100 in 0.1 M phosphate-buffered saline (PBS) (pH 7.3) for 2 hours on ice, then incubated in a combination of two primary antibodies, monoclonal DBH and one of the following polyclonal antibodies: Gαgust, PLCβ2, 5HT, NCAM; or polyclonal NET and one of the following monoclonal antibodies: syntaxin-1, 5HT in 0.1 M PBS (pH 7.3) overnight at 4°C. After rinsing in 0.1 M PBS for 30 minutes the sections were treated in a cocktail of two secondary antibodies consisting of Cy'5-conjugated to goat anti-rabbit IgG and FITC-conjugated to goat anti-mouse IgG in 0.1 M PBS for 2 hours on ice. For NET double-labeling with IP_3R3, cryostat sections (20 µm thick) containing circumvallate taste buds were blocked in 5% normal goat serum, 1% BSA and 0.3% Triton X-100 in 0.1 M phosphate-buffered saline PBS for 2 hours on ice, then incubated in NET primary
antibody in 0.1 M PBS overnight at 4°C. After rinsing in 0.1 M PBS for 30 minutes the sections were treated with Cy’5-conjugated to goat anti-rabbit IgG in 0.1 M PBS for 2 hours on ice. The sections were then exposed to IP$_3$R3 primary antibody in 0.1 M PBS for 8 minutes followed by FITC-conjugated mouse IgG in 0.1 M PBS for 8 minutes using the Ted Pella Biowave for microwave processing.

**Imaging.** Images were collected using a 40x objective with a Zeiss Axioplan 2 fluorescence microscope using Axiovision software with an Apotome for confocal imaging (Zeiss). Adobe Photoshop CS (San Jose, CA) was used to adjust brightness and contrast of the images.

**Controls.** Primary antibodies were excluded from the processing to check for any species-related cross-reactivity. Elimination of one of the primary antibodies with application of both secondary antibodies confirmed secondary antibody specificity. No immunoreactivity was observed under these conditions (Appendix 1).

**Quantitation of Immunoreactive Taste Cells**

Twenty adult Sprague-Dawley male rats were perfused for IHC and confocal microscopy following the technique previously published by Yang et al. (2004). A cryostat (HM 505E, MICRON, Laborgerate, Germany) was used to cut 20 µm-thick transverse sections. Transverse sections were used for several reasons: 1) There is less confusion distinguishing cells caused by profiles from different cells being superimposed on top of each other, 2) Identification of nuclear and cytoplasmic immunoreactivity is
easier to distinguish; 3) It is easier to identify taste cell types in transverse sections. The primary disadvantage of transverse sectioning is identifying and tracking nerve processes.

Five rats were used for each experiment. Approximately 25 serial transverse sections (20 µm thick) were taken from the circumvallate papilla from each rat. The 25 sections from each papilla were divided into groups of five sections based on the following sampling procedure (Yang et al., 2004): Every fifth section was saved into a group, the first section for each group was determined using a new random number (1, 2, 3, 4, or 5). For example, sections 3, 8, 13, 18, and 23 formed Group 1 from rat #1, while sections 2, 7, 12, 17, and 22 formed Group 2. Rat #1 contained Groups 1–5; rat #2 contained Groups 6–10, etc. One group of five sections was selected from each rat using the same random number procedure as described above, creating a set of 25 sections (e.g., Group 3 from rat #1, Group 8 from rat #2, Group 13 from rat #3, Group 18 from rat #4, and Group 23 from rat #5). The set of 25 sections from the randomly selected groups were used for each experiment, in which DBH was tested for co-localization with each of the following antibodies: PLCβ2 and NCAM. NET was tested for co-localization with each of the following antibodies: IP₃R3 and syntaxin-1. Approximately 6–14 taste bud profiles were examined from each section. Cells were scored as immunoreactive only if the area included a nuclear profile.

**Quantitation.** Sections were stained using double labeling for immunofluorescence microscopy. Approximately 130 circumvallate taste buds were analyzed from each rat. The number of taste cells expressing each of the antibodies of interest was counted and then an average co-localization was calculated.
## Table 2. Primary Antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Immunogen</th>
<th>Source, species, CAT #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{\alpha}^{gust}$</td>
<td>A peptide mapping within a highly divergent domain of $G_{\alpha}^{gust}$ of rat origin</td>
<td>Santa Cruz Biotech, rabbit polyclonal, affinity purified IgG, sc-395</td>
<td>1:100</td>
</tr>
<tr>
<td>IP$_3$R3</td>
<td>A peptide mapping near the N-terminus of IP$_3$R3 of human origin</td>
<td>BD Transduction Laboratories, mouse monoclonal, affinity purified IgG, 610312</td>
<td>1:100</td>
</tr>
<tr>
<td>PLC$\beta$2</td>
<td>A peptide mapping near the C-terminus of PLC $\beta$2 of human origin</td>
<td>Santa Cruz Biotech, rabbit polyclonal, affinity purified IgG, sc-206</td>
<td>1:100</td>
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<tr>
<td>Syntaxin-1</td>
<td>The synaptosomal plasma-membrane fraction from adult rat hippocampus</td>
<td>Sigma, mouse monoclonal, affinity purified IgG, S0664</td>
<td>1:100</td>
</tr>
<tr>
<td>NTPDase-2</td>
<td>cDNA encoding the entire mouse Entpd2 gene ligated into pcDNA3.1/V5-His</td>
<td>Invitrogen, Ontario, Canada, rabbit polyclonal, affinity purified IgG, mN2-35I$_5$</td>
<td>1:100</td>
</tr>
<tr>
<td>NET</td>
<td>Amino acids 551-617 mapping within a C-terminal cytoplasmic domain of NE transporter of human origin</td>
<td>Santa Cruz Biotech, rabbit polyclonal, affinity purified IgG, sc-67216</td>
<td>1:100</td>
</tr>
<tr>
<td>D$\beta$H</td>
<td>Purified Bovine D$\beta$H</td>
<td>AbD serotec, mouse monoclonal, affinity purified IgG, 3960-0506</td>
<td>1:100</td>
</tr>
<tr>
<td>5HT</td>
<td>5 hydroxytryptamine hydrochloride</td>
<td>Abcam, mouse monoclonal, affinity purified IgG, ab16007</td>
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<tr>
<td>5HT</td>
<td>5 hydroxytryptamine hydrochloride</td>
<td>Abcam, rabbit polyclonal, affinity purified IgG, ab10385</td>
<td>1:100</td>
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<tr>
<td>NCAM</td>
<td>Amino acids 1-300 mapping at N-terminus of NCAM of human origin</td>
<td>Santa Cruz Biotech, rabbit polyclonal, affinity purified IgG, sc-10735</td>
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### Table 3. Secondary Antibodies

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<th>Dilution</th>
<th>Source</th>
<th>Code No.</th>
</tr>
</thead>
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<tr>
<td>Cyanine (Cy5) goat anti-rabbit, IgG</td>
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<td>Jackson Laboratory</td>
<td>111-175-144</td>
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<tr>
<td>Fluorescein (FITC) goat anti-mouse, IgG</td>
<td>1:100</td>
<td>Jackson Laboratory</td>
<td>115-095-100</td>
</tr>
</tbody>
</table>
RESULTS

DBH-LIR in Taste Buds

General features of DBH-LIR taste cells

Single label immunofluorescence reveals DBH-like-immunoreactivity (-LIR) in rat gustatory tissue (Fig. 5). Staining is restricted to the taste bud, as no fluorescence was detected in the surrounding connective tissue or in nerve fibers. DBH-LIR is present in a small subset of taste cells in the circumvallate papillae. DBH-LIR is found heterogeneously with a punctate pattern in the cytoplasm of taste cells. Longitudinal sections show that staining is typically more concentrated near the nucleus, while apical and basal regions of the cell are typically devoid of fluorescence (Fig. 5A). A large percentage of these cells show intense supranuclear staining (arrow), although this is not seen in all cells. Immunoreactive cells extend from the basal lamina to the taste pore and contain large, ovoid nuclei. Images taken from transverse sections highlight this nuclear feature (Fig. 5B). A similar punctate staining pattern is also seen in transverse profiles.
Double-labeling studies of DBH and α–Gustducin

DBH-LIR co-localizes with the Type II taste cell marker α-gustducin (Fig. 6C). The marker α-gustducin is the G protein involved in transduction of bitter stimuli and is found exclusively in a subset of Type II cells; however, it is only found in a subset of the population (Yang et al., 2000; Clapp et al., 2001, 2004; Miyoshi et al., 2001). Labeling for α-gustducin displays diffuse, cytoplasmic fluorescence that is homogeneous in nature (Fig. 6B). It appears that nearly all α-gustducin-LIR cells also express DBH-LIR, as indicated by the arrow (Fig. 6C), suggesting that DBH-LIR is present in the α-gustducin positive subset of Type II cells. A large proportion of DBH positive cells do not show co-expression with α-gustducin, as indicated by the arrowhead, suggesting that DBH-LIR is present in a different and/or larger subset of Type II cells or is possibly present in other cell types (Fig. 6C).

Double-labeling studies of DBH and PLCβ2

DBH-LIR also shows co-localization with PLCβ2-LIR (Fig. 6F). PLCβ2 is an important enzyme expressed in the IP3 signaling pathway and is found most exclusively in a subset of Type II taste cells (Yang et al., 2000; Clapp et al., 2001, 2004; Miyoshi et al., 2001). Labeling for PLCβ2 displays diffuse, cytoplasmic fluorescence that is homogeneous in nature (Fig. 6E). DBH-LIR is present in a large percentage of PLCβ2-LIR cells, although not all cells, as indicated by the arrowhead (Fig. 6F). It appears that PLCβ2-LIR is present in all or nearly all DBH-LIR cells, as indicated by the arrow (Fig. 6F). Because PLCβ2-LIR represents the largest population of Type II cells, this marker
was used for quantitation of DBH-LIR co-localization. Five rat circumvallate papillae were sectioned transversely and scored for co-expression of DBH-LIR and PLCβ2-LIR (Fig. 8C). An average of 127 taste buds were counted and analyzed per rat (N=637). In a single taste bud profile, an average of 10 cells displayed PLCβ2-LIR and an average of 4 cells displayed DBH-LIR (Table 4). On average, 41.45% of PLCβ2 expressing cells also expressed DBH-LIR (Table 4). On average, 98.6% of DBH-LIR cells express PLCβ2-LIR (Table 4).

**Double-labeling studies of DBH and serotonin**

Serotonin has been shown to be released from Type III cells in rodents (Huang et al., 2008). Serotonin positive cells represent a subset of Type III taste cells and display diffuse, cytoplasmic staining that is homogeneous in nature (Fig. 7B). The nuclei of these cells are also stained with equal intensity as the cytoplasm. Nerve processes do not show serotonin-LIR. DBH positive cells are located in close proximity to serotonin positive cells, although DBH-LIR does not show co-localization with serotonin-LIR, as indicated by the arrowheads (Fig. 7C).

**Double-labeling studies of DBH and NCAM**

NCAM immunofluorescence is localized to the entire population of Type III taste cells. Staining of the taste cells is homogeneous in nature, but the nuclei are devoid of fluorescence (Fig. 7E). Initial analysis of longitudinal sections suggests no co-localization between DBH-LIR and NCAM-LIR, as indicated by the arrowheads (Fig. 7F).
Quantitation of co-localization was carried out to determine if this observation was valid. Five rat circumvallate papillae were sectioned transversely and scored for co-expression of DBH-LIR and NCAM-LIR (Fig. 8F). On average, 125 taste buds were counted and analyzed per rat (N=625). Each taste bud profile displayed an average of 4 DBH-LIR cells and 3 NCAM-LIR cells (Table 5). No co-localization was observed.

**NET-LIR in Taste Buds**

**General features of NET-LIR taste cells.**

Single label immunofluorescence reveals NET-LIR in rat gustatory tissue (Fig. 9). The most robust staining is restricted to the taste bud. Some background fluorescence is detected in the surrounding connective tissue and in the basal lamina. NET-LIR is present in a subset of taste cells in the circumvallate papillae. NET-LIR is found homogeneously through the cytoplasm of taste cells, including the nucleus. Longitudinal sections show immunoreactivity in both long, slender cells (arrow) and in large, spindle-shaped cells (Fig. 9A). Transverse sections more clearly show NET-LIR in spindle-shaped cells (arrowhead), which are most likely Type II taste cells (Fig. 9B).

**Double-labeling studies of NET and IP$_3$R3**

IP$_3$R3 is the IP$_3$ receptor involved in the release of intracellular calcium stores in response to taste stimulation of the Type II taste cell (Simon et al., 2006; Roper 2007). Labeling for IP$_3$R3 displays diffuse, cytoplasmic fluorescence that is homogeneous in nature (Fig. 10B). The IP$_3$R3-LIR cells represent a large subset of Type II taste cells. It
appears that all or nearly all IP$_3$R3-LIR cells co-express NET-LIR (arrows), but not all NET-LIR cells express IP$_3$R3-LIR, as indicated by the arrowhead (Fig. 10C). This may indicate that NET is present in other cell types. Five rat circumvallate papillae were sectioned transversely and scored for co-expression of NET-LIR and PLC$\beta$2-LIR (Fig. 12C). An average of 130 taste buds were counted and analyzed per rat (N=648). In a single taste bud profile, an average of 9 cells displayed IP$_3$R3-LIR and an average of 12 cells displayed NET-LIR (Table 6). One hundred percent of IP$_3$R3-LIR cells express NET-LIR (Table 6). On average, 77.49% of NET-LIR cells also express IP$_3$R3-LIR (Table 6).

**Double-labeling studies of NET and serotonin**

Serotonin positive, Type III cells display diffuse, cytoplasmic staining that is homogeneous in nature (Fig. 11B). The nuclei of these cells also show immunoreactivity. NET-LIR co-localizes with serotonin-LIR in the rat circumvallate papillae (Fig. 11C). It appears that all or nearly all serotonin-LIR cells express NET-LIR, as indicated by the arrow (Fig. 11C). Though a very large subset of NET positive cells do not express serotonin-LIR, as indicated by the arrowhead (Fig. 11C).

**Double-labeling studies of NET and syntaxin-1**

Because serotonin-LIR cells only represent a small subset of Type III taste cells, NET-LIR was also co-localized with syntaxin-1-LIR, a presynaptic protein that labels all Type III cells. Two types of syntaxin-1 fluorescence are observed. The first is diffuse,
cytoplasmic immunoreactivity of Type III taste cells and nerve fibers that is homogeneous in nature, as indicated by the small arrowhead (Fig. 11E). The second is heterogeneous, punctate immunoreactivity of the Golgi Bodies in Type II taste cells, as indicated by the large arrowhead (Fig. 11E). NET-LIR is co-expressed with syntaxin-1-LIR; however, this co-expression is restricted to a subset of immunoreactive cells (arrow, Fig. 11F). Five rat circumvallate papillae were sectioned transversely and scored for co-expression of NET-LIR and syntaxin-1-LIR (Fig. 12F). Taste buds were only counted if at least one syntaxin-1-LIR cell was present, as many transverse sections only yielded syntaxin-1-LIR in nerve processes. An average of 79 taste buds were counted and analyzed per rat (N=394). In a single taste bud profile, an average of 8 cells displayed NET-LIR and an average of 2 cells displayed syntaxin-1-LIR (Table 7). On average, 7.86% of NET-LIR cells also expressed syntaxin-1-LIR (Table 7). On average, 32.85% of syntaxin-1-LIR cells also express NET-LIR (Table 7).
Figure 5. Confocal images showing DBH immunoreactivity in rat circumvallate papillae.

A: Longitudinal section showing DBH-LIR in a subset of taste cells. Immunoreactivity is heterogeneous in nature and displays punctate pattern. This immunoreactivity is typically more concentrated in the supranuclear region (arrow). B: Transverse section showing DBH-LIR. Scale bars = 20 µm.
Figure 6. Confocal images from longitudinal sections of rat circumvallate papillae showing co-localization of DBH-LIR (green) with Type II cell markers α-gustducin-LIR (red) and PLCβ2-LIR (red). A: DBH-LIR is present in a subset of taste cells. B: α-gustducin-LIR is present in a subset of taste cells. C: Double label image showing DBH-LIR and α-gustducin-LIR. Arrow indicates co-localization of DBH-LIR and α-gustducin-LIR. Arrowhead indicates DBH-LIR cells that do not co-express α-gustducin-LIR. D: DBH-LIR is present in a subset of taste cells. E: PLCβ2-LIR is present in a subset of taste cells. F: Double label image showing DBH-LIR and PLCβ2-LIR. Arrow indicates co-localization of DBH-LIR and PLCβ2-LIR. Arrowhead indicates PLCβ2-LIR cells that do not co-express DBH-LIR. Scale bars = 20 μ
**Figure 7.** Confocal images from longitudinal sections of rat circumvallate papillae showing co-localization of DBH-LIR (green) with Type III cell markers serotonin (5HT)-LIR (red) and Neural Cell Adhesion Molecule (NCAM)-LIR (red). **A:** DBH-LIR is present in a subset of taste cells. **B:** 5HT-LIR is present in a subset of taste cells. **C:** Double label image showing DBH-LIR and 5HT-LIR. Arrowheads indicate immunoreactive cells that do not show co-localization of DBH-LIR and 5HT-LIR. **D:** DBH-LIR is present in a subset of taste cells. **E:** NCAM-LIR is present in a subset of taste cells. **F:** Double label image showing DBH-LIR and NCAM-LIR. Arrowheads indicate immunoreactive cells that do not show co-localization of DBH-LIR and NCAM-LIR. Scale bars = 20 μm.
Figure 8. Confocal images from transverse sections of rat circumvallate papillae showing DBH-LIR (green) with PLCβ2-LIR (red) and NCAM-LIR (red). A: DBH-LIR is present in a subset of taste cells. B: PLCβ2-LIR is present in a subset of taste cells. C: Double label image showing DBH-LIR and PLCβ2-LIR. Arrow indicates co-localization of DBH-LIR and PLCβ2-LIR. Arrowhead indicates PLCβ2-LIR cells that do not co-express DBH-LIR. D: DBH-LIR is present in a subset of taste cells. E: NCAM-LIR is present in a subset of taste cells. F: Double label image showing DBH-LIR and NCAM-LIR. Arrowheads indicate immunoreactive cells that do not show co-localization of DBH-LIR and NCAM-LIR. Scale bars = 20 μm.
Table 4. Quantitation of DBH-LIR and PLCβ2-LIR Co-localization

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<th>No. Rat</th>
<th>No. TB</th>
<th>DBH-LIR cells</th>
<th>PLCβ2-LIR cells</th>
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<th>SD</th>
<th>% PLCβ2 express DBH</th>
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Table 5. Quantitation of DBH-LIR and NCAM-LIR Co-localization

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<th>No. TB</th>
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Figure 9. Confocal images showing NET immunoreactivity in the rat circumvallate papillae. A: Longitudinal section showing NET-LIR in a subset of taste cells. Arrow indicates labeling of a slender taste cell. B: Transverse section showing NET-LIR. Arrowhead shows labeling of an ovoid taste cell. Scale bars = 20 µm.
Figure 10. Confocal images from longitudinal sections of rat circumvallate papillae showing co-localization of NET-LIR (red) with Type II cell marker IP$_3$R3 (green). 

A: NET-LIR is present in a subset of taste cells. 

B: IP$_3$R3-LIR is present in a subset of taste cells. 

C: Double label image showing NET-LIR and IP$_3$R3-LIR. Arrows indicate co-localization of NET-LIR and IP$_3$R3-LIR. Arrowhead shows a NET-LIR cell that does not co-express IP$_3$R3-LIR. Scale bar = 20 µm.
**Figure 11.** Confocal images from longitudinal sections of rat circumvallate papillae showing co-localization of NET-LIR (red) with Type III cell markers serotonin (5HT)-LIR (green) and syntaxin-1-LIR (green).  
**A:** NET-LIR is present in a subset of taste cells.  
**B:** 5HT-LIR is present in a subset of taste cells.  
**C:** Double label image of NET-LIR and 5HT-LIR. Arrows indicate co-localization of NET-LIR and 5HT-LIR. Arrowhead shows a NET-LIR cell that does not co-express 5HT-LIR.  
**D:** NET-LIR is present in a subset of taste cells.  
**E:** Syntaxin-1-LIR is present in Type III taste cells (small arrowhead), as well as in nerve fibers. Punctate staining is also present in Type II taste cells (large arrowhead).  
**F:** Double label image of NET-LIR and syntaxin-1-LIR. Arrow indicates co-localization of NET-LIR and syntaxin-1-LIR. Arrowhead shows a syntaxin-1-LIR cell that does not express NET-LIR. Scale bars = 20 µm.
Figure 12. Confocal images from transverse sections of rat circumvallate papillae showing NET-LIR (red) with IP$_3$R3-LIR (green) and syntaxin-1-LIR (green). A: NET-LIR is present in a subset of taste cells. B: IP$_3$R3-LIR is present in a subset of taste cells. C: Double label image showing DBH-LIR and IP$_3$R3-LIR. Arrow indicates co-localization of NET-LIR and IP$_3$R3-LIR. Arrowhead indicates NET-LIR cells that do not co-express IP$_3$R3-LIR. D: NET-LIR is present in a subset of taste cells. E: Syntaxin-1-LIR is present in a subset of taste cells. F: Double label image showing NET-LIR and syntaxin-1-LIR. Arrow shows co-localization of NET-LIR and syntaxin-1-LIR. Arrowhead indicates immunoreactive cells that do not show co-localization of NET-LIR and syntaxin-1-LIR. Scale bars = 20 μm.
Table 6. Quantitation of NET-LIR and IP$_3$R3-LIR

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<tr>
<th>No. Rat</th>
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<th>IP$_3$R3-LIR cells</th>
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Table 7. Quantitation of NET-LIR and Syntaxin-1-LIR

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<th>Syntaxin-1-LIR cells</th>
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DISCUSSION

Summary of the results

The principal findings of this study are: 1) DBH and NET are present in subsets of taste cells in the rat circumvallate papillae; 2) DBH-LIR co-localizes with $\alpha$-gustducin-LIR and PLC$\beta$2-LIR. Approximately 99% of DBH-LIR cells express PLC$\beta$2-LIR and 41% of PLC$\beta$2-LIR cells express DBH-LIR; 3) DBH-LIR does not co-localize with 5HT-LIR or NCAM-LIR; 4) NET-LIR co-localizes with IP$_3$R3-LIR. Approximately 77% of NET-LIR express IP$_3$R3-LIR and 100% of IP$_3$R3-LIR cells express NET-LIR; and 5) NET-LIR co-localizes with 5HT-LIR and syntaxin-1-LIR. Approximately 8% of NET-LIR cells express syntaxin-1-LIR and 33% of syntaxin1-LIR cells express NET-LIR.

Based on double-labeling studies, we believe that DBH localization is restricted to Type II taste cells and, more specifically, to a portion of the PLC$\beta$2-LIR subset. We also believe that NET is expressed in both Type II and Type III taste cells; however, the majority of NET-LIR is present in a subset of Type II cells, namely, the IP$_3$R3 subset.
DBH-LIR taste cells

α-gustducin-LIR, PLCβ2-LIR

Double-labeling experiments indicate that DBH-LIR co-localizes with α-gustducin-LIR and PLCβ2-LIR in rat taste buds, suggesting that DBH is present in Type II taste cells. No immunoreactivity was present in nerve fibers or in the connective tissue, further supporting this observation. Quantitative analysis indicates that nearly half (41%) of PLCβ2-LIR cells express DBH-LIR, suggesting that DBH is only found in a subset of these cells. It is possible that this is actually an underestimation of co-localization. The unbiased technique of sampling selects every fifth section so that each taste cell is only counted once. Depending on the random number chosen as the starting place for sampling, sections collected may be more apically or basally located. Due to the heterogeneous staining pattern of DBH-LIR, and the fact that fluorescence is typically more concentrated near the nucleus, it is possible that more apical or basal sections would show no immunoreactivity in cells that are actually DBH positive.

In the mouse taste bud, DBH-LIR is not present (Dvoryanchikov et al., 2007). RT-PCR and immunocytochemistry confirmed the absence of this enzyme in the mouse taste bud, and researchers concluded that norepinephrine is not synthesized within the mouse taste bud and thus must be taken up from outside the taste bud. This movement has been proposed to be accomplished via NET, which was localized to Type I and Type III taste cells in mice (Dvoryanchikov et al., 2007). Interestingly, Herness et al. (2002) provided data that suggested otherwise, as immunocytochemical localization of NE indicated that the Type II cell in rat might serve as an endogenous source of
neurotransmitter for the taste bud. Together, these data may suggest a distinct difference in the noradrenergic machinery between rat and mouse.

**5HT, NCAM**

DBH-LIR does not co-localize with 5HT-LIR or NCAM-LIR in rat taste buds. We do not believe that DBH localization to Type III cells is likely, as analysis of 637 taste buds indicated 0% co-localization of DBH-LIR and NCAM-LIR, a molecular marker for all Type III cells. This finding is intriguing, as physiology studies conducted in mouse show that norepinephrine is released from Type III taste cells upon KCl depolarization (Huang et al., 2008). Together, these data suggest the possibility that norepinephrine is synthesized in Type II taste cells and is then transported into Type III taste cells for synaptic release.

**Expression by cell type**

We believe that expression of DBH-LIR is exclusive to Type II taste cells, as no co-localization was observed with Type III taste cell markers 5HT or NCAM. Nearly all (99%) DBH-LIR cells express PLC\(\beta 2\)-LIR. It is possible that the remaining 1% of DBH-LIR cells belong to a different subset of Type II taste cells. Although possible, we believe it is unlikely that this percentage of DBH-LIR localizes to Type I taste cells, as the expression pattern for DBH-LIR does not show characteristics of Type I cell immunoreactivity. DBH-LIR is found heterogeneously through spindle-shaped taste cells. Type I cells typically display diffuse, membranous immunoreactivity that resembles
nerve processes because Type I cells have extensions of their membranes that envelop other cells (Appendix 2). This type of immunoreactivity was not seen for DBH-LIR.

**NET-LIR taste cells**

*IP$_3$R3*

Double-labeling experiments indicate that NET-LIR co-localizes with IP$_3$R3-LIR in rat taste buds, suggesting that NET is present in a subset of Type II taste cells. Expression of NET-LIR is not exclusive to this cell type, as some NET-LIR cells did not co-localize with IP$_3$R3-LIR (approximately 23%). However, 100% of IP$_3$R3-LIR cells express NET-LIR, suggesting a strong correlation between the movement of NE and the IP$_3$ signaling pathway in Type II cells in rats. In order to carry out double-labeling of NET-LIR and IP$_3$R3-LIR, an unconventional technique of immunocytochemistry was performed to preserve the immunoreactivity of both antibodies. Immunofluorescence of the IP$_3$R3 antibody requires incubation in 10 mM sodium citrate (pH 9, 87° C) prior to blocking for antigen retrieval. This step, however, completely abolishes NET-LIR. IP$_3$R3-LIR can also be achieved by using microwave processing for labeling of the primary antibody; however, this technique also abolishes NET-LIR. To overcome this obstacle, labeling of the NET primary antibody and its subsequent secondary antibody was first carried out conventionally. This was then followed by microwave processing of the IP$_3$R3 primary antibody and its subsequent secondary antibody labeling. Although the fluorescence pattern for NET-LIR in this double-labeling experiment is very similar to that of other experiments in which microwave processing was not employed, there are
still some slight differences. NET-LIR in the IP$_3$R3-LIR double-label shows more fluorescent puncta and the signal is not nearly as robust. In this experiment, it also appeared that more NET-LIR cells were present (2-3 more per taste bud on average). Because of this, we believe that our estimates from the quantitation experiments may be slightly inflated. However, the data still clearly show that NET-LIR is present in the IP$_3$R3-LIR subset of cells.

Interestingly, Dvoryanchikov et al. (2007) did not find NET localization to Type II cells in mice. Once again, it is possible that this finding simply illustrates one of many potential differences between the rat and the mouse. Typically, transporters (such as NET) are found on the presynaptic cell membrane to facilitate reuptake of extra neurotransmitter left in the synaptic cleft. However, several studies indicate the presence of non-synaptic transporters in different brain regions (Somogyi et al., 1989; Baude et al., 1995; Yung et al., 1995; Venkatesan et al., 1996; Descarries et al., 1997; Nusser et al., 1998) that are accessible for endogenous ligands. These, being located extra-synaptically, may play a physiological role in accepting chemical messages from distant neurons or from adjacent cells. Ultimately, these data illustrate a rare case of non-synaptic reuptake present in the rat taste bud.

5HT, syntaxin-1

Double-labeling experiments show that NET-LIR co-localizes with 5HT-LIR and syntaxin-1-LIR in rat taste buds, indicating that NET localizes to Type III taste cells. This finding is more consistent with the data presented by Dvoryanchikov et al. (2007).
Although this group did not carry out quantitative analysis of NET expression by cell type, it appears that a much larger percentage of Type III cells in mice express NET-LIR than in the rat. We found that approximately 8% of NET-LIR cells express syntaxin-1-LIR and 33% of syntaxin1-LIR cells express NET-LIR.

Expression by cell type

We believe that NET is present in both Type II and Type III taste cells, based on double-labeling immunofluorescence studies. Only a small proportion of NET-LIR cells express syntaxin-1-LIR (8%), compared to the 77% of NET-LIR cells that express IP$_3$R3-LIR. This difference indicates that NET is far more prevalent in Type II taste cells as opposed to Type III taste cells. NET immunoreactivity in Type III cells only represents a small subset, as only 33% of syntaxin-1-LIR cells express NET-LIR. Dvoryanchikov et al. (2007) identified the presence of NET in Type I taste cells. Although we did not carry out co-localization experiments with NET-LIR and a Type I cell marker, such as NTPDase-2, we do not believe NET to be present in Type I cells in the rat taste bud due to the staining pattern of NET-LIR. Staining for NTPDase-2-LIR in the rat taste bud (Appendix 2) shows a distinct staining pattern that does not resemble NET-LIR.

Functional relevance in the gustatory system

The present study has only further demonstrated that the role of norepinephrine in the gustatory system may be far more complex than initially hypothesized. In order to fully elucidate the role of noradrenergic modulation, we must first rectify the
inconsistencies in the literature. It seems possible that these contradictions are simply a result of distinct differences between rats and mice. Our lab has observed differences in immunoreactivity of certain antibodies between rats and mice. For example, ROMK-LIR in the mouse is concentrated in the apical region of taste cells, whereas in rats, immunoreactivity is diffuse and cytoplasmic. We have also shown previously that the number and distribution of taste cells in both animals differs significantly (Ma et al., 2007). Although it is possible that these differences are due to differences in species, one cannot ignore the possibility that these differences are actually due to other variables. In order to ensure that this is not the case, it is crucial that we validate the DBH and NET antibody. We would like to do this in two ways: first, perform western blot analysis of both antibodies and second, perform immunolabeling of our antibodies on protein specific knock-outs. We are currently looking into the availability of such knock-outs.

Our present work suggests that NE is synthesized in the Type II taste cell population; however, previous work has shown synaptic release of NE only from Type III taste cells in mice. These data suggest that, although NE is synthesized in the Type II cell, it is not released from this cell type in response to taste stimulation. This would indicate that NE is only released from Type II cells in order to be taken up by Type III cells for release in response to taste stimuli (specifically, sour tastants). Following this mechanism, the Type II cell would only serve as a storage pool of neurotransmitter. Interestingly, Herness et al. (2002) identified NE-LIR only in Type II taste cells, which our initial IHC data also suggest (Appendix 3). This finding coincides with our immunolocalization of DBH to the Type II cell, but does not support noradrenergic release from Type III cells,
as no immunoreactivity was found in this cell type. It is possible, however, that the levels of neurotransmitter in the Type III cell are much lower than that in the Type II cell and, thus, may be below the threshold for detection using IHC.

Aromatic L-amino acid decarboxylase (AADC) is an enzyme that acts on L-DOPA to synthesize dopamine, the precursor for NE. Previous studies in both rats and mice have found the presence of this protein only in Type III taste cells (DeFazio et al., 2006). It is interesting that this enzyme does not appear to co-occur with DBH, as both enzymes are involved in the synthetic pathway for catecholamines. More specifically, the actions of both enzymes are important in the synthesis of NE. If AADC is acting in the synthesis of NE in the taste bud, it seems unlikely that it would be present in Type III cells and that DBH would be present in Type II cells. Following the proposed mechanism of NE synthesis in Type II cells and taste-evoked release from Type III cells, it is even more peculiar that dopamine would be synthesized in the Type III cell, transported to the Type II cell for the synthesis of NE and then transported back to the Type III cell for synaptic release. AADC has another action, though, as it also serves as the immediate synthetic enzyme for 5HT. If AADC is in fact only present in Type III cells, it is possible that the action of this enzyme in the taste bud is specific for 5HT, and not NE. The presence of 5HT in Type III cells has been shown to be both valid and reliable (Nada & Hirata, 1975; Takeda, 1977; Takeda et al., 1981; Kim & Roper, 1995; Yee et al., 2001), making this a probable scenario. If the action of AADC is specific to 5HT, this eliminates the possibility of dopamine synthesis occurring in the taste bud, suggesting that dopamine must be taken up from outside the taste bud (likely by the
dopamine transporter, DAT) to serve as the precursor for NE synthesis. Figure 13 displays a schematic of our proposed mechanism for the synthesis, action and reuptake of NE in the rat taste bud. Dvoryanchikov et al. (1997) described TH-LIR and AADC-LIR in rat taste buds, although this data was not provided. In order to gain a more complete understanding of this mechanism, we would like to localize TH and AADC in the rat taste bud for analysis and comparison with the findings of our present study. In addition to localization of these enzymes, localization of NE itself is also imperative. Small clear vesicles have been identified in Type II taste cells (Yang et al., 2004) and large dense cored vesicles, along with small clear vesicles have been identified in Type III taste cells (Kinnamon & Yang, 2008). Using colloidal gold electron microscopy, we can potentially identify the contents of these vesicles. By localizing the vesicular location of NE, we can then begin to better understand the role of noradrenergic modulation in the gustatory system.

Summary

1. Our results suggest that DBH, the immediate synthetic enzyme for NE, is present in Type II taste cells. DBH is observed exclusively in Type II cells and is not found in Type III cells. It is still unclear whether the taste bud contains all enzymes of the catecholamine synthetic pathway. We will next use IHC to localize TH and AADC in the rat taste bud to further investigate this question.

2. Our results suggest that NET, the NE transporter, is present on both Type II and Type III taste cells. This observation indicates that both cell types are
capable of taking up neurotransmitter from the extracellular space. Type III taste cells release NE in response to depolarization; however, the mechanism of NE release from Type II taste cells has yet to be identified. We will next use colloidal gold electron microscopy to localize NE to vesicles in both the Type II and Type III taste cell to further support a mechanism of noradrenergic release and modulation in the rat taste bud.
Figure 13. Proposed mechanism for the synthesis, action and reuptake of NE in rat taste buds. A. Dopamine (DA) is taken up from the extracellular space and taken up by Type II taste cells via the dopamine transporter (DAT). Dopamine-β-hydroxylase (DBH) then synthesizes norepinephrine (NE) from DA. B. NE is released from Type II taste cells and can then elicit an autocrine effect on α/β adrenoceptors located on Type II cells (C) or elicit a paracrine effect on adjacent Type III cells (D). E. Type III cells release NE in response to stimulation and can then elicit a paracrine effect on α/β adrenoceptors located on adjacent Type II cells (C) or elicit an autocrine effect on Type III cells (D). Both Type II cells (F) and Type III cells (G) are capable of taking up NE from the extracellular space via the norepinephrine transporter (NET). Figure courtesy of Mr. HoanVu Nguyen.
LITERATURE CITED


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Appendix 1

**Negative Controls:** Negative controls showing species specificity of the secondary antibodies for DBH (species: mouse) and for NET (species: rabbit). **A.** DBH-LIR is detected by goat anti-mouse FITC. **B.** DBH-LIR is not detected by goat anti-rabbit Cy’5. **C.** Overlay. **D.** NET-LIR is detected by goat anti-rabbit Cy’5. **E.** NET-LIR is not detected by goat anti-mouse FITC. **F.** Overlay. Scale bars = 20 µm.
Type I cell immunoreactivity: NTPDase-2-LIR of Type I taste cells in the rat circumvallate taste bud. Scale bar = 20μm.
NE immunoreactivity: NE-LIR is present in a subset of taste cells within the taste bud. These cells appear to display morphology consistent with that of Type II taste cells (arrow). Scale bar = 20μm.