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Purinergic Neurotransmission in the Gustatory System

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Abstract

Taste buds consist of specialized epithelial cells which detect particular tastants and synapse onto the afferent taste nerve innervating the endorgan. The nature of the neurotransmitter released by taste cells onto the nerve fiber was enigmatic early in this century although neurotransmitters for other sensory receptor cell types, e.g. hair cells, photoreceptors, was known for at least a decade. A 1999 paper by Burnstock and co-workers (Bo et al. 1999) showing the presence of P2X receptors on the afferent nerves served as a springboard for research that ultimately led to the discovery of ATP as the crucial neurotransmitter in the taste system (Finger et al. 2005). Subsequent work showed that a subpopulation of taste cells utilize a unique release channel, CALHM1/3, to release ATP in a voltage-dependent manner. Despite these advances, several aspects of purinergic transmission in this system remain to be elucidated.

Keywords

Taste bud; geniculate ganglion; serotonin; CALHM1; ATP

Background:

The sense of taste starts with taste buds, which are assemblies of specialized, elongate epithelial cells arrayed across the tongue, palate and larynx. Each taste bud contains roughly 50–100 cells divisible into morphological and functional classes. The most plentiful population, comprising about 50% of the cells in a taste bud are glial-like cells resembling astrocytes in terms of morphology and molecular features(Kinnamon and Finger 2019). The sensory cells of taste buds are so-called “short” receptor cells, lacking an axon. In this sense they are akin to hair cells of the auditory, vestibular and lateral line systems as well as the photoreceptors of the retina. All of these short sensory cells form a synapse onto a neuron which conveys the signal onward into the CNS (Fig. 1).

The nature of the neurotransmitter between these short sensory cells and the next element in the transmission pathway was a matter of some conjecture before the late 1970s (Wu and

Dowling 1978, Schessel and Highstein 1981) and 1980s (Sewell and Mroz 1987). Suggested candidates included acetycholine, catecholamines, GABA, and various amino acids (Sewell and Mroz 1987). Early candidates for the essential neurotransmitter in taste buds focused on acetycholine (DeHan and Graziadei 1973) and serotonin (Nada and Hirata 1976, Morimoto and Sato 1977).

The advent of immunohistochemistry and development of more specific pharmacological tools in the 1980s and 1990s led to more precise characterization of the neurotransmitters and receptors involved in transmission from the short sensory cells to the subsequent neuronal elements in the hair cell and retinal systems. In these systems, glutamate is the key neurotransmitter (Bobbin et al. 1981, Massey and Redburn 1987, Felix and Ehrenberger 1990, Kataoka and Ohmori 1996). Even in somatic sensation, glutamate is the first neurotransmitter utilized by the long-axon receptor cells of the dorsal root ganglia (Jessell et al. 1986, Radhakrishnan and Henry 1993). Hence it was not unreasonable to suggest that glutamate may serve as the afferent neurotransmitter for taste receptor cells as well. Curiously, attempts to localize glutamate or its processing enzymes to taste receptor cells (e.g. (Nagai et al. 1998, Lawton et al. 2000)) were largely not supportive of the hypothesis that taste cells utilize glutamate as a key neurotransmitter. A confound for interpretation of these results in the taste system is that some taste receptor cells respond to glutamate as a taste stimulus, so the presence of glutamate receptors in taste cells was not necessarily indicative of a role in neurotransmission (Brand et al. 1991, Lin and Kinnamon 1999, Caicedo et al. 2000, Eram and Michel 2005). Further, a few taste cells in each taste bud, in particular the Type III cells in mammalian taste buds, take up and release serotonin,, suggesting that this neurotransmitter might play a role in taste transmission (Morimoto and Sato 1977, Jain and Roper 1991, Nagai et al. 1998, Huang et al. 2005). Indeed, recent studies show that serotonin acting on neural 5HT3 receptors does participate in transmission of sour taste information in mice, but that these receptors are not necessary for transmission of taste information to the nervous system (Kaya et al. 2004, Larson et al. 2015). In addition, the scarcity of serotonin-accumulating cells suggested that other transmitters might be involved.

Serotonin had been localized only to one of the three principal types of taste cells in mammalian taste buds: the Type III cell (Nada and Hirata 1975). Type III cells are implicated in transduction and transmission of sour taste information (Huang et al. 2008) but not for other taste qualities, e.g. sweet, umami and bitter which rely on a PLC-mediated transduction cascade found in Type II receptor cells. Further, Type III, but not Type II, taste cells formed classical synapses onto afferent nerve fibers complete with an accumulation of presynaptic vesicles including some dense-cored vesicles typical of serotonergic transmission (Nada and Hirata 1977, Toyoshima et al. 1984). So the problem remained as to what the transmitter might be for the Type II taste cells. These receptor cells do not display typical synaptic junctions with nerve fibers, but rather show a specialized so-called “atypical mitochondrion” (Royer and Kinnamon 1988) closely apposed to the plasma membrane of the taste cell at its point of contact with the nerve fiber. How this arrangement might lead to regulated release of neurotransmitter was entirely unclear.

So by the mid-1990s, even though a primary neurotransmitter had been identified in all other sensory systems, the nature of the neurotransmitter in taste receptor cells remained enigmatic. Previous studies through that time had mostly eliminated potential neurotransmitter candidates rather than suggesting new possibilities.

Personal Reflection:

At about that time, one of us (TEF) was working with Prof. Thomas Dunwiddie at the Univ. Colorado School of Medicine to develop a slice preparation to study transmission of taste information from the sensory nerves to taste centers of the brainstem, e.g. (Finger and Dunwiddie 1992). Indeed, we established that glutamate was indeed the principal neurotransmitter utilized at the first synapse within the brainstem (Smeraski et al. 1998, Smeraski et al. 1999) but did not have the means to test the situation in regard to transmission from taste buds to sensory nerves. Dr. Dunwiddie had been studying purinergic transmission in the hippocampus for quite some time, e.g. (Dunwiddie and Hoffer 1980, Dunwiddie 1985, Masino et al. 2002) and our informal discussions often turned to the idea that perhaps taste buds used some form of purinergic transmission to communicate with the sensory nerves. Unfortunately, we did not then have the tools to investigate that possibility. This became a frequent point of discussion among the investigators of the Rocky Mountain Taste and Smell Center, including Drs. Restrepo, Barlow, John Kinnamon and Sue Kinnamon.

The seminal breakthrough in uncovering the neurotransmitter from taste buds was the paper in 1999 by Geoff Burnstock and colleagues (Bo et al. 1999) reporting the presence of P2X2 and P2X3 receptors in taste nerves. This led to our speculation that ATP might serve as a key neurotransmitter in this system, but how to test it? Looking at the list of key features of a neurotransmitter, demonstration of the presence of the purported neurotransmitter in the presynaptic element came to mind. But of course all cells have ATP. Nonetheless, we attempted experiments relying on quinacrine fluorescence as a surrogate for regions of ATP accumulation. While some taste cells do exhibit quinacrine puncta within their cytoplasm (e.g. (Kataoka et al. 2006)), their relationship to neurotransmission was still unclear.

After several futile attempts to demonstrate ATP release, we found a presentation by an associate of Dr. Burnstock, Debra Cockayne then of the Roche Bioscience Program at the 2002 Soc Neuroscience meeting (Orlando, FL) on P2X2/P2X3 double knockout mice. We realized that if ATP were a key neurotransmitter acting on neural P2X2 and P2X3 receptors, then these double-KO mice should be unable to taste – or unable to taste at least some taste qualities.

Excitedly, we approached Dr. Cockayne at the meeting and asked her if these mice could taste. She responded, “How would I know?”, they eat normally but have a high rate of pup mortality. We told her it was really simple: just offer the mice 2 water bottles – one with just plain water and the other containing sugar water. If they can taste they will drink more of the sugar water; it’s called a 2-bottle preference test. She replied most graciously that she would just send us the mice and we could test them ourselves…which we did, leading up

to our 2005 paper in *Science* establishing ATP as the necessary neurotransmitter in the taste periphery (Fig. 2).

Another important group contributing to that landmark paper was the team of Vika Danilova and Goran Hellekant at the Univ. Wisconsin, who provided the expertise in peripheral taste nerve recording, which we had not then mastered. This is a powerful approach for assessing peripheral taste function because it is a direct readout of the output of the taste bud-taste nerve periphery. Unlike behavioral assays, nerve recording is not reliant on proper functioning of CNS structures involved in preference and motivational states. We arranged to ship the mice to the Hellekant laboratory once we generated sufficient double-KO animals. Shortly after the first lot went out, one of us (TEF) received a call from Dr. Danilova saying that something was wrong because she was unable to record any taste responses in the nerves of even the wildtype mice we had sent although responses to touch and temperature seemed intact. Well, we explained, that is indeed good news in that we had not yet sent any wildtype controls and that the first shipment was only the double knockout animals. Subsequent shipments of both wildtype and knockout mice established that indeed, Dr. Danilova could find taste responses in the wildtype mice but not in the knockouts and it is these data that appear in the 2005 *Science* article (Fig. 2A).

The Science:

Taste buds are complex sensory endorgans containing 3 morphologically and molecularly distinct types of cells with limited life spans. The cells of a taste bud are replaced continuously throughout life with an average replacement time of 14–30 days depending on cell type (Finger and Barlow 2021). The majority of cells in a taste bud are glial-like cells (Type I cells), which surround and isolate the other cell types in a taste bud (Yang et al. 2020). Type II cells rely on GPCR taste receptors for bitter, sweet or umami, coupled to a PLC-mediated signaling cascade to generate an action potential triggering release of neurotransmitter (Kinnamon and Finger 2019). Type III cells transduce sour (acids) via proton influx through the Otop1 ion channel (Liman and Kinnamon 2021) to depolarize the cell, trigger an action potential, and ultimately open voltage-gated Ca^{++} channels to allow for SNARE-protein mediated vesicular release of neurotransmitter.

Our investigation of the P2X2/P2X3 double KO mice (Finger et al. 2005) showed that these purinergic receptors were required for taste-mediated behavior and neural activity for all taste qualities, including both those mediated by Type II cells (bitter, sweet, umami) and those mediated by Type III cells (sour). Recent work shows that at least some components of salty taste are mediated by a unique cell population with some, but not all characteristics of Type II cells (Nomura et al. 2020). Salty tastes also were absent in the P2X2/P2X3 double KO mice and so must affect this novel cell population as well.

In the 2005 paper, we also showed that in taste epithelia from wildtype mice, we could measure taste-mediated ATP release by luciferin-luciferase assay. Curiously, the double-KO mice also showed reduced ATP release from the taste epithelia (Huang et al. 2011) but this may have been a secondary effect of the KO. Taste cells have no measurable expression of P2X3 and only low expression of P2X2. If absence of P2X2 were responsible

for the loss of taste signaling, then a P2X2 single KO should be similarly debilitated. Such is not the case (Fig. S2 in (Finger et al. 2005)); P2X2 single KO mice show taste impairment but not to the extent of the P2X2/P2X3 double KO animals. Subsequent studies established that transmission of all taste qualities was eliminated by application of AF353, an antagonist for P2X3-containing receptors, either injected i.p. or applied directly to the tongue epithelium (Vandenbeuch et al. 2015) (Fig. 2C & D). This pharmacological blockade of P2X3-containing receptors does not result in decreases in taste-evoked ATP release again suggesting that the result from P2X2/P2X3 double-KO mice is a side-effect of the KO.

An important criterion for the establishment of a neurotransmitter candidate is clearance of the substance from the synaptic cleft. In the case of ATP, this clearance is mediated by ectonucleotidases. The presence of high levels of Mg^{++} activated ATPase in taste buds was noted back in the 1960s although the significance of this was unclear at the time (Iwayama and Nada 1967) although these investigators noted that the membrane-associated ectoATPase was absent at the interface between nerve fibers and the Type II or Type III taste cells (Akisaka and Oda 1977). The functional importance of this ectoATPase became clear once we identified ATP as the key neurotransmitter. In 2006, we molecularly identified the taste bud ectoATPase as NTPDase2 which is highly selective for ATP over ADP (Bartel et al. 2006). Genetic elimination of this ectoATPase results in perdurance of ATP in the taste epithelium and reduced responses to tastants, likely due to desensitization of the P2X receptors on the afferent nerves (Vandenbeuch et al. 2013).

Mechanism of ATP release:

Despite the clear demonstration that ATP is released from taste buds to activate P2X receptors on the afferent nerves, until 2013, the mechanism for ATP release was unclear. Type II taste cells do not express several components of the SNARE complex (Yang et al. 2004, Yang et al. 2007, Asano-Miyoshi et al. 2009) and contacts between Type II cells and nerve fibers lack synaptic vesicles. Despite this, the amount of ATP release was clearly related to the number of action potentials in the Type II taste cells (Murata et al. 2010). Several investigators suggested that ATP release might occur via a gated ion channel ((Huang et al. 2007, Romanov et al. 2007) but the identity of this channel was unclear.

The first physiological suggestion that ATP release might be non-vesicular was a study from Dr. Steve Roper’s laboratory showing that ATP release was blocked by carbenoxolone, a rather specific inhibitor of the ATP release channel Pannexin-1 (Panx1), which blocks ATP release in other systems (Ransford et al. 2009). Low concentrations of carbenoxolone blocked taste-evoked ATP release from isolated Type II cells. (Huang et al. 2007) suggesting that Pannexin 1was involved. Using immunocytochemistry and rt-PCR, they went on to show that Panx1 was expressed in nearly all Type II taste cells, and concluded, albeit prematurely, that Panx1 was the ATP release mechanism in the taste bud. Several other studies supported this conclusion, including studies showing carbenoxolone block of ATP signaling in slice preparations of taste buds (Dando and Roper 2009) and carbenoxolone block of tastant-evoked ATP release from single type II cells during loose patch recording (Murata et al. 2010). However, questions remained about whether the Panx1 channels had the right kinetics and voltage dependence to explain ATP release in taste buds (cf, (Romanov

et al. 2008, Romanov et al. 2012) and none of the studies tested Panx1 knockout mice for effects on taste responses. Then in 2013 a seminal paper appeared suggesting a relatively new player, Calcium homeostasis modulator 1 (CALHM1), is the basis of taste-evoked ATP release (Taruno et al. 2013). CALHM1 is a large conductance, voltage-gated channel previously thought to be involved primarily in calcium homeostasis in hippocampal neurons (Ma et al. 2018). Taruno et al showed CALHM1 is expressed in taste buds in Type II taste cells, and that CALHM1 knockout mice have greatly diminished responses to bitter, sweet and umami tastants. However, responses were not totally abolished in the knockout and some features of the channel observed in heterologous systems were not consistent with CALHM1 being the only channel involved in the ATP release from taste buds.

Questions remained, however, about the role of Panx1, since it is expressed in the ATP releasing taste cells and ATP release is blocked by carbenoxolone, which does not affect ATP release via the CALHM channels, at least in heterologous systems. To determine if Panx1 might play some minor role in ATP release in taste buds, two independent labs obtained Panx1 knockout mice and tested them behaviorally and physiologically for deficits related to ATP release in a systems context (Tordoff et al. 2015, Vandenbeuch et al. 2015). However, in both studies, knockouts and wildtype mice had normal chorda tympani responses to all tastants and responded normally in behavioral experiments, confirming that Panx1 plays no direct role in ATP release in taste buds.

So, if pannexin is not involved, what accounts for the discrepancy between properties of CALHM1 and the release channel in taste buds? The discovery of another CALHM subunit in Type II taste cells, CALHM3, resolved this problem (Ma et al. 2018). When CALHM3 was co-expressed with CALHM1 in heterologous systems, the heteromeric channel had the appropriate kinetics and voltage-dependence to account for ATP release in taste buds. Further, double knockout of CALHM1 and CALHM3 completely eliminated responses to bitter, sweet and umami stimuli, confirming the channel as the ATP release channel of Type II cells in taste buds.

So why does carbenoxolone block ATP release in taste buds, if it does not block the CALHM1/3 channel? A recent study by one of us (TEF) in collaboration with the Romanov lab (Romanov et al. 2018) seems to resolve this question. Romanov et al showed that the CALHM1 channel lies on the membrane adjacent to “atypical” mitochondria (Fig. 3) and forms a signaling complex with the afferent nerves containing the P2X receptors. This signaling complex was termed a “channel synapse” by Taruno et al. (Taruno et al. 2021). The mitochondrion appears to be the immediate source of ATP for release by the CALHM1/3 channel and carbenoxolone inhibits the mitochondrial membrane potential, thereby depleting the mitochondrion of ATP resulting in inhibition of ATP release. Thus, the carbenoxolone inhibition is upstream of the ATP release channel and explains the block of ATP release observed by other investigators upon application of carbenoxolone to taste tissues

So, if ATP is released from Type II cells via “channel synapses” and they are exclusively found in Type II taste cells, why is ATP also required for transmission from Type III taste cells, and where is it coming from? As mentioned earlier, Type III cells form conventional

synapses with afferent nerve fibers and release serotonin via vesicles to activate 5-HT3a receptors on afferent nerve fibers (Larson et al. 2015) (Fig. 4A, B). However, although knockout or pharmacological blockade of 5-HTa reduces neural responses to acids, it does not eliminate them and ATP is still required for a full neural response. Yet ATP release has not been detected from identified Type III taste cells, although serotonin release can be detected with biosensor cells by either depolarization or acid stimulation of isolated Type III taste cells (Huang et al. 2007). Further, Type III cells do not contain the ATP vesicular transporter, VNUT, which would be required for transport of ATP into the serotonergic vesicles for co-release (Iwatsuki et al. 2009).

Another recent study suggests that Type II cells are not the source of ATP required for Type III cell transmission, since Skn-1a knockout mice, which completely lack Type II cells, have normal responses to sour stimuli (Larson et al. 2020). So if Type II cells are not the source of ATP required for Type III cell transmission, where is it coming from? Clearly this is one of the main unanswered questions remaining in the field and is a focus of current investigation.

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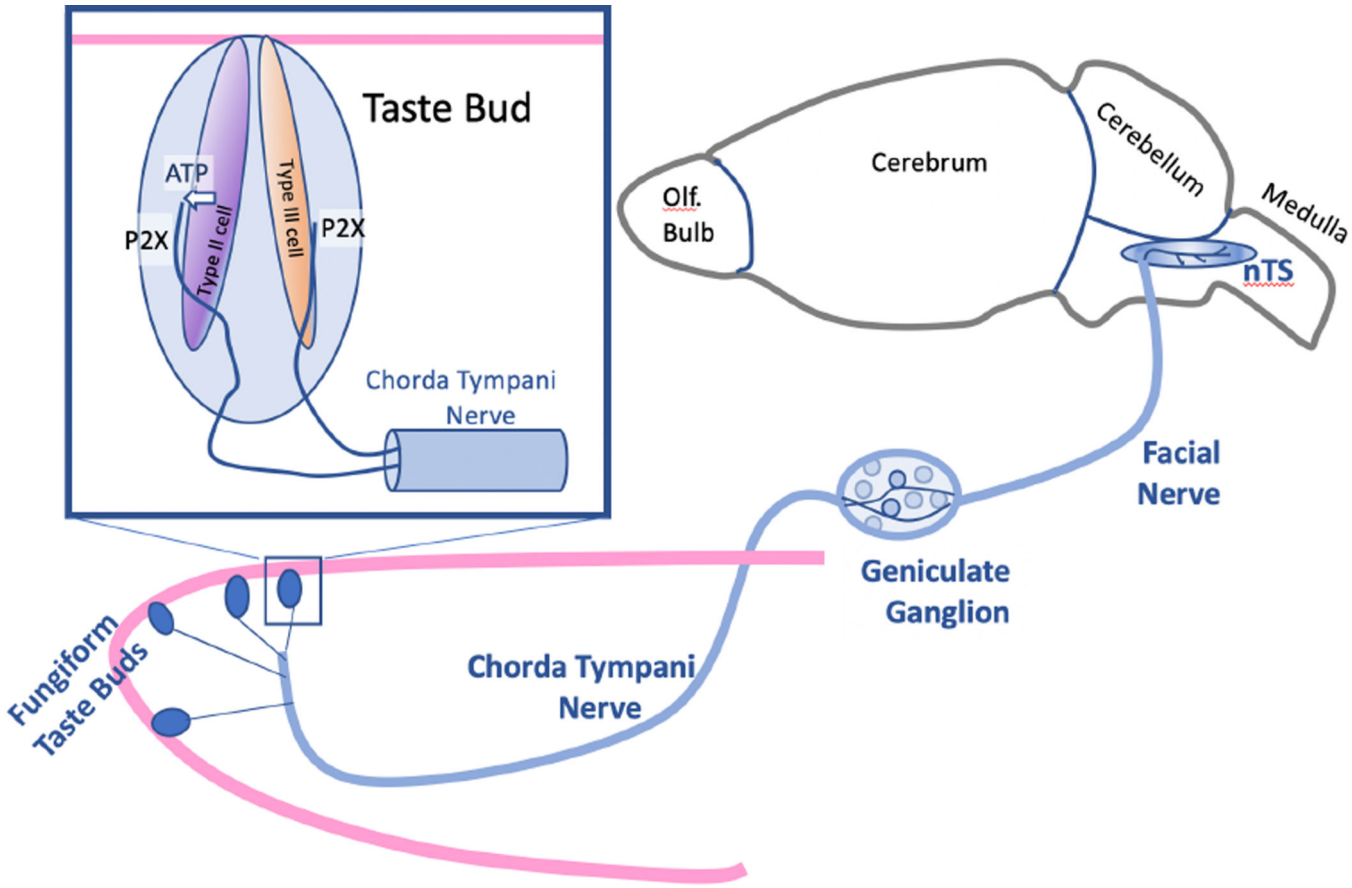

Fig. 1.

Semi-schematic diagram of the relationship between taste buds on the tip of the tongue (Fungiform Taste Buds) and their innervation by fibers of the chorda tympani nerve, a branch of the Facial nerve. The gustatory ganglion cells in the geniculate ganglion extend one branch centrally to terminate in the nuc. of the solitary tract (nTS) of the medulla, and the other branch peripherally, to innervate taste buds. Inset upper left: Each taste bud contains multiple elongate cells including glial-like Type 1 cells [not illustrated], and receptor cells some of which (Type II cells) release ATP through channel synapses to activate the P2X (P2X2 and P2X3 in mice) receptors on the sensory terminals of the afferent nerve fibers. Less clear is how Type III cells (for sour) release ATP to activate the P2X receptors on the afferent fibers.

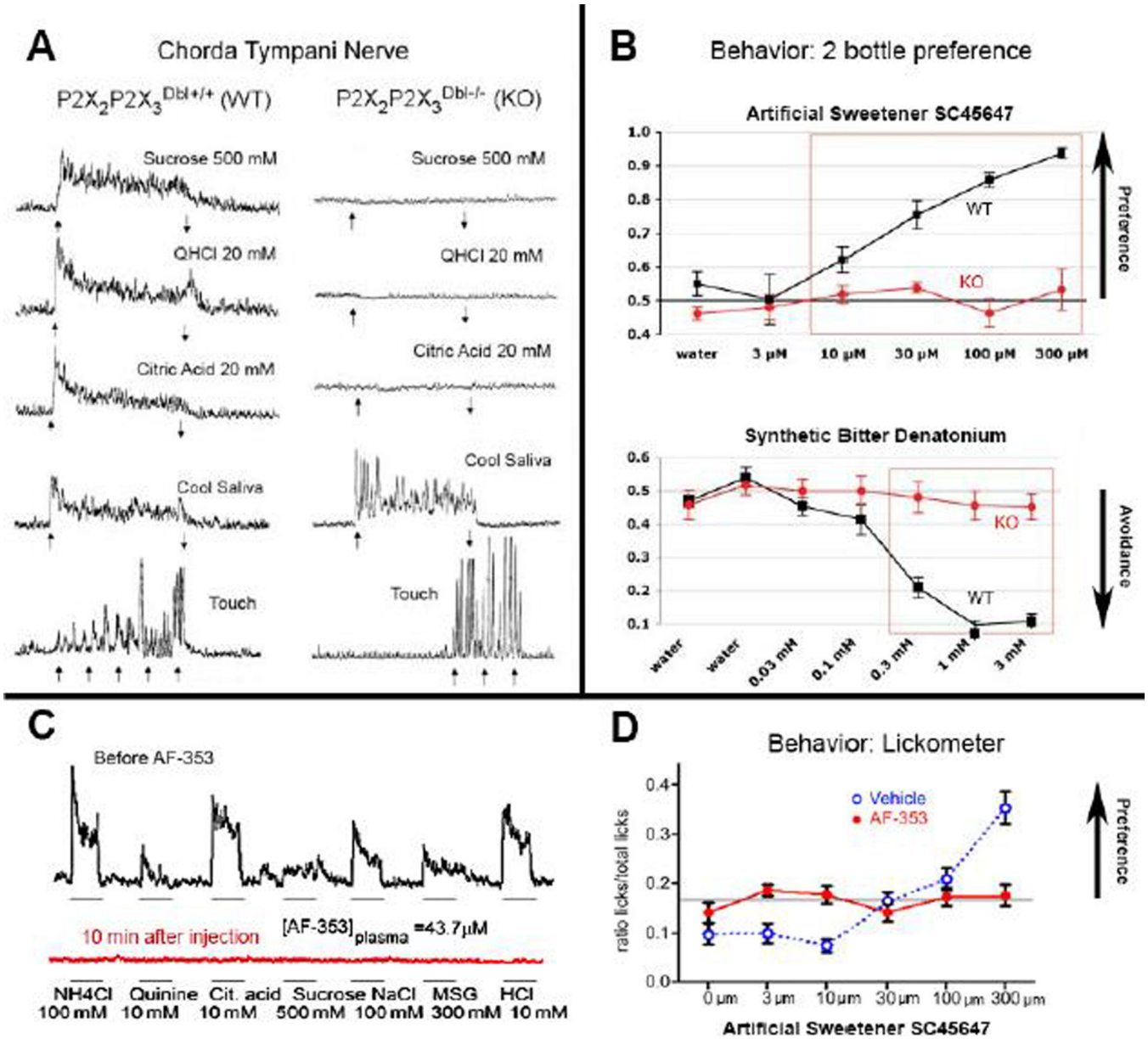

Fig. 2.

Genetic Deletion or Pharmacological Block of P2X3 and P2X2/P2X3 channels eliminates taste responses and behaviors. **A.** Taste nerve (chorda tympani) recordings comparing responses in P2X2/P2X3 double KO mice (Right column) with WT controls (Left column) on the same mixed background for selected taste substance (Sucrose = sweet; QHCl, Quinine HCl = bitter; Citric Acid = sour). Responses in the KO animals are absent although the KO nerves still respond to temperature and touch. **B**. Graphs comparing taste preference behavior in P2X2/P2X3 double KO and WT mice in aa 2 bottle preference test. The ordinate shows the ratio of tastant consumed compared to water. WT animals (black) strongly prefer the artificial sweetener and avoid the bitter compound, denatonium. In contrast, KO animals (red) show neither preference nor avoidance to the respective tastants. **C.** Nerve recordings in WT animals injected with the antagonist AF-353 which inhibits all P2X3 containing

receptors. Top trace (black) shows responses to all tastants; the drug-treated animal shows virtually no taste responses (red) to any tastant. NH_4Cl = ammonium chloride; Cit. Acid = citric acid, MSG = monosodium glutamate. **D.** Tastant-evoked behavior in WT mice treated either with vehicle or with AF-353. Vehicle-treated mice show increasing preference for the artificial sweetener whereas the AF-353-treated animals show no response to this sweetener. Panels A & B adapted from (Finger et al. 2005). Panels C & D adapted from (Vandenbeuch et al. 2015).

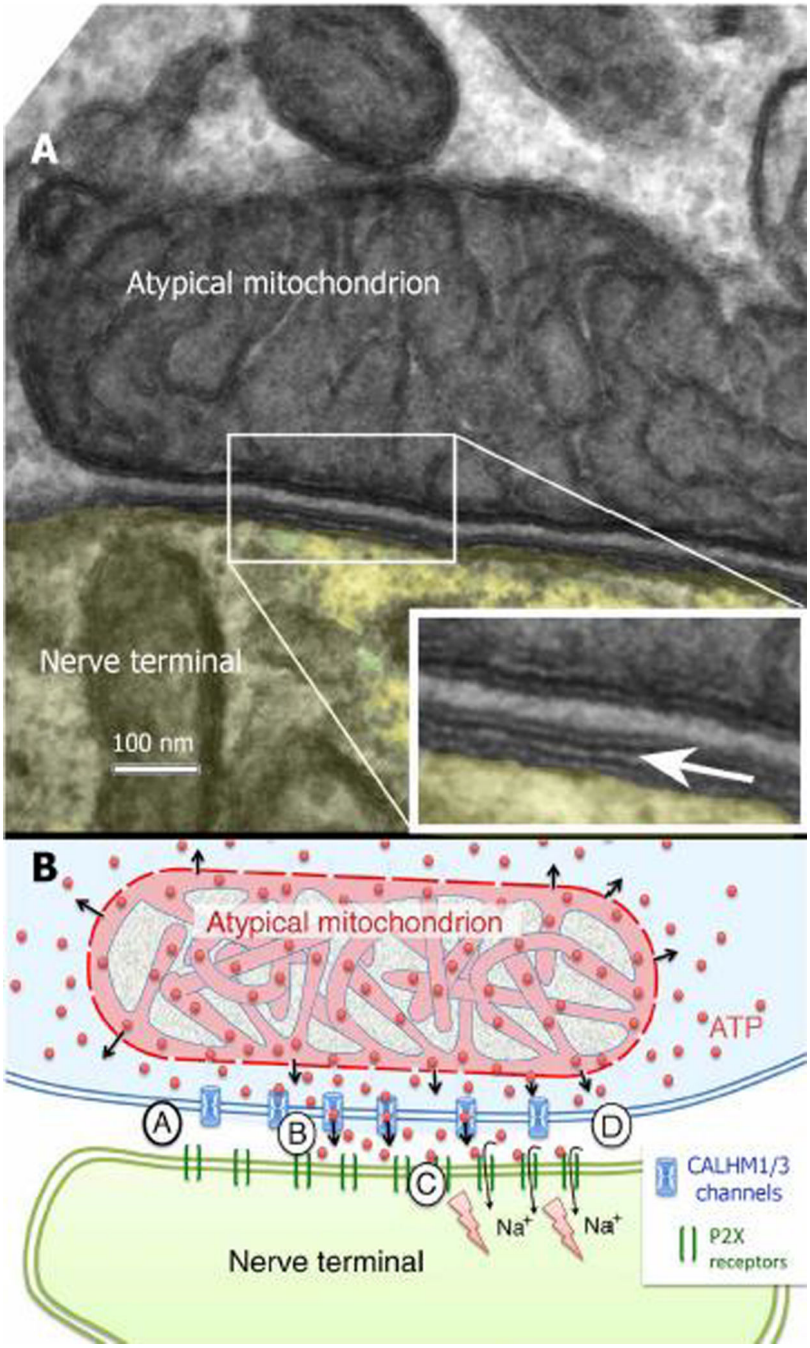

Fig. 3:

Micrograph (A) and Semi-schematic diagram (B) of the channel synapse wherein ATP release is regulated by the voltage-gated CALHM1/3 channels situated in the taste cell membrane. In rodents, a large “atypical” mitochondrion is tightly associated with the release complex with a relatively constant spacing of 20–30 nm between the mitochondrion and the plasma membrane. **A.** Arrow in the inset indicates the intercellular cleft. **B.** Circled letters AA-D indicate the sequence of events leading to ATP release. (A) At rest, the channel is closed; ATP diffuses freely from intermembrane space of the mitochondrion into the

surrounding cytoplasm. (B) Action potentials in the taste cell gate open the CALHM1/3 channel to allow diffusion of ATP into the intracellular cleft where it (C) binds to P2X2/P2X3 receptors in the neural membrane to generate a post-synaptic potential, (D) The CALHM1/3 channel closes and extracellular ATP is degraded by the ectoATPase in the surrounding taste cells. Adapted from (Romanov et al. 2018)

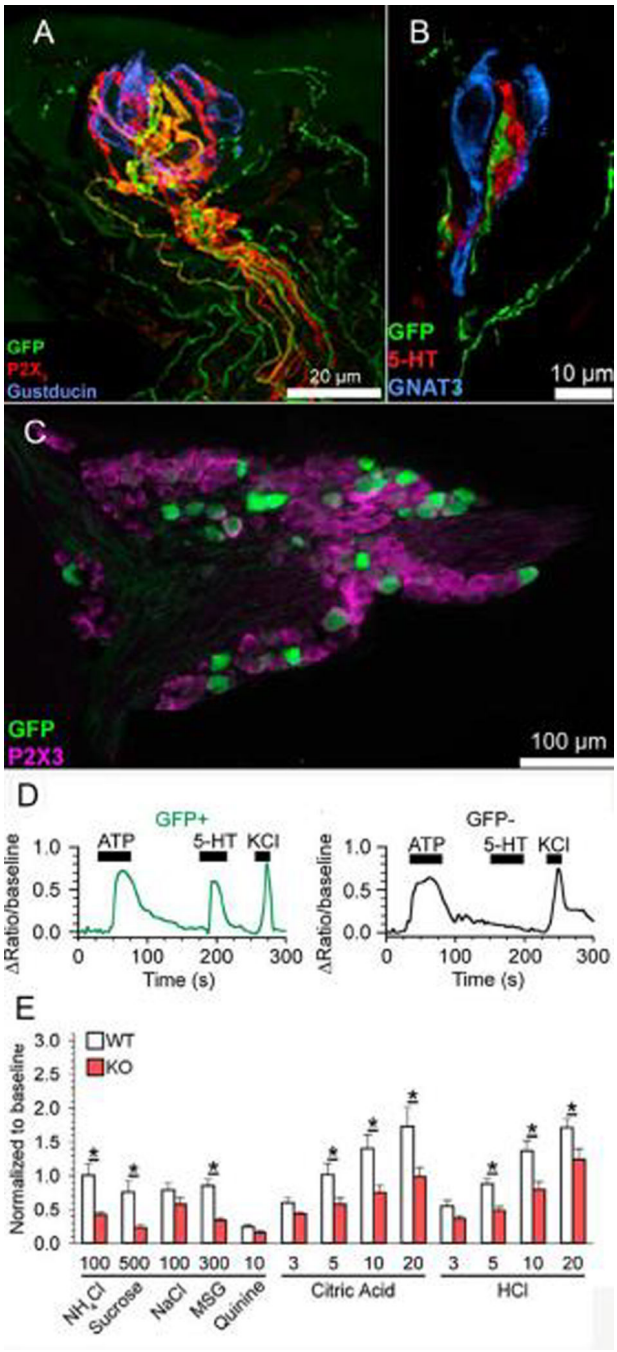

Figure 4.

Role of 5-HT3A in signaling from Type III taste cells to afferent fibers. A. Longitudinal Z stack of a fungiform taste bud from a 5-HT3a-GFP mouse. Intragemmal fibers expressing 5-HT3 (green) are also labeled with an antibody to P2X3 (red). Antibody against gustducin labels Type II taste cells (blue). B. Optical slices from a different fungiform taste bud, showing Type III cells stained with an antibody against 5-HT (red) and Type II cells labeled with an antibody against gustducin (blue). 5-HT3a-GFP fibers (green) preferentially contact the Type III taste cells. C. Optical section through a geniculate ganglion from a 5-HT3a-GFP

mouse. All neurons are stained with an antibody against P2X3 (magenta), while a subset also stain with GFP (green). D. Calcium imaging recordings from geniculate ganglion neurons isolated from a 5-HT3a-GFP mouse. GFP+ neurons responded to both ATP and 5-HT, while GFP- neurons responded only to ATP. KCl was used as a index of cell viability. E. Chorda tympani recordings from 5-HT3a KO mice and their WT littermates. Responses in KO mice were reduced to all acids compared to their WT littermates. Modified from (Larson et al. 2015)