Taste Bud Connectome: Implications for Taste Information Processes

Abbreviated Title: Taste Bud Connectome

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Abstract (250 words maximum, including citations)
Taste buds, the sensory end organs for the sense of taste, consist of 3 types of morphologically identifiable mature cells, 2 of which mediate transduction of specific taste qualities: Type III cells for sour and Type II cells for either sweet, bitter or umami. A long-standing controversy in the field is whether the nerve fibers innervating these cells are wired specifically, in a labeled-line fashion, or non-specifically, leading to broad responsiveness across taste qualities, the so-called cross-fiber system of encoding. Using serial blockface scanning electron microscopy through 5 circumvallate mouse taste buds, we reconstructed the pattern of connectivity of nerve fibers as well as the degree of potential interaction between the two types of transducing taste cells. Type II and Type III cells show few points of contact, which constrains the opportunity for direct information transfer between these cell types. Of the 133 nerve fibers traced in the 5 taste buds, 87 fibers synapsed with Type II cells (n=43), 46 fibers with Type III cells (n=33) with 4 of these fibers (3%) synapsing with both Type II and Type III cells. Since Type II and Type III cells transduce different taste qualities, these few mixed fibers do not follow a labeled line system of transmission of taste quality information although the large majority of fiber connectivity is consistent with the labeled line model.

Significance Statement (120 words maximum)
Taste buds, the sensory end organs for the sense of taste, contain multiple types of sensory cells, with each type responding to one of the primary tastes: salt, sweet, sour, bitter and umami. A long-standing question is whether each type of taste cell is wired specifically to a unique set of nerve fibers conveying a “labeled line” message to the brain. Using serial sections, we delineated the neural connectivity in mouse circumvallate taste buds. The majority of individual nerve fibers connect to a single type of taste cell, but about 3% of the fibers branch to receive input from taste cells of differing response types. Thus taste is not entirely carried along nerve fibers dedicated to single taste qualities.

INTRODUCTION (650 words)

Recognition of stimulus quality requires detection of the nature of the stimulus by the peripheral receptors and transmission of this information to the central nervous system in a decodable form. The sense of taste can distinguish at least 5 principal sensory qualities: salt, sweet, sour, bitter and umami (Ohla et al., 2019). The initial detection of these diverse qualities is accomplished by the specialized epithelial cells of taste buds, called taste cells (Roper and Chaudhari, 2017b). Each transducing taste cell responds principally to one of the five taste qualities according to the molecular nature of the receptor employed. The manner in which this information is encoded and transmitted by the sensory nerves remains open to question.

Although the taste cells themselves generally express molecular receptors for only one taste quality (Chandrashekar et al., 2006), the responses of taste cells are not always restricted to a single quality (Caicedo et al., 2002; Chandrashekar et al., 2006). Similarly, responses of individual gustatory nerve fibers range from specific, responding to a single taste quality, to multimodal, responding to multiple qualities (Wu et al., 2015). The origin of this broader specificity is unclear and may reside in cross-talk between receptor cells (Tomchik et al., 2007; Roper and Chaudhari, 2017a) within the taste bud, lack of specificity in synaptic connectivity, or both.

The "labeled line" hypothesis of information encoding holds that each particular functional class of receptor cell, e.g. sweet, synapses only with a particular subset of quality-specific nerve fibers (Chandrashekar et al., 2006). In this scheme, analysis of the connectivity within a taste bud should reveal specificity, i.e. a particular nerve fiber should receive synapses only from one particular functional type of taste receptor cell. Conversely, the "cross-fiber" hypothesis of taste coding states that each nerve fiber may be broadly responsive and that taste quality can be extracted only by comparing activity across fiber populations. This model does not require precise connectivity within a taste bud and allows for substantial cross-talk between taste cells of differing specificities. In this report, we test these contravening hypotheses by reconstructing the "connectome" of taste buds by means of serial blockface scanning electron microscopy (sbfSEM). The high resolution of this process enables us to identify all sites of synaptic interaction within a taste bud, including taste cell-to-nerve fiber synapses as well as contacts between taste cells themselves indicative of potential information exchange between cells within a taste bud.

Taste cells responsive to sour, Type III cells (Huang et al., 2008; Kataoka et al., 2008; Chang et al., 2010; Teng et al., 2019; Dutta Banik et al., 2020), have molecular and ultrastructural features distinctive from Type II cells -- those responsive to sweet, bitter or umami (Clapp et al., 2004; Chandrashekar et al., 2006; Roper and Chaudhari, 2017b; Yang et al., 2020). Furthermore, these two different types of taste transducing cells exhibit different relationships to the afferent nerves. Type III cells (sour) utilize conventional synapses where a cluster of synaptic vesicles lies apposed to the presynaptic membrane which abuts the postsynaptic nerve process (Kinnamon et al., 1985; Royer and Kinnamon, 1991; Yang et al., 2000). In contrast, Type II taste cells rely on an unconventional synaptic structure (Romanov et al., 2018) characterized by a large "atypical" mitochondrion (Royer and Kinnamon, 1988) closely associated with CALHM1/3 channels (Ma et al., 2018; Kashio et al., 2019) which release the neurotransmitter ATP upon activation of the receptor cell (Murata et al., 2010; Taruno et al., 2013). Using these different ultrastructural features as markers of synaptic loci, we were able to map the patterns of connectivity of taste

cells to nerve fibers to ask the question of whether any nerve fibers synapse with both Type II and Type III taste cells thereby testing specificity of connectivity within the bud.

638 words. (650 maximum)

METHODS

Serial Block-face Scanning Electron Microscopy (sbfSEM) [see (Yang et al., 2020)] was used to generate two datasets from adult (>45 days) mouse circumvallate taste buds: DS2, composed of 563 sections, and TF21, composed of 633 sections. The data used in the present study include 6 different taste buds -- three partial taste buds (two of which were conjoined) from DS2, and two complete and another nearly complete taste bud from TF21. Four of the taste buds of the connectome dataset were included in the data described in (Yang et al., 2020); an additional taste bud described in that work (TF21_TB0) was examined for cell-cell contacts but was excluded from the connectome analysis in that it included too few cells. A different taste bud, (TF21_TB2) is now included in the present work but not described in the previous paper. Cells and fibers that were only partially contained in the sample volume and which showed no evidence of synapses were not included in the connectome data although such cells were cataloged in terms of cell-cell contacts. The image data include a total of 54 Type II cells and 44 Type III cells although only 43 Type II and 33 Type III cells showed synaptic foci within the imaging volume. The original image data are freely available at the Electron Microscopy Public Image Archive, part of European Bioinformatics Institute (EMBL-EBI), in data set EMPIAR-10331.

The two conjoined small taste buds of DS2 (containing within the connectome map 5 Type II cells and 4 Type III cells for TB1; and 4 Type II cells and 2 Type III cells for TB2) were fused along the basal lamina, although each taste cell extended an apical process into only one or the other taste pore. Cells were allocated to these conjoined buds according to which taste pore the cell was associated with. Nerve fibers innervating the taste cells of these conjoined taste buds generally innervated cells in one or the other taste bud, but two nerve fibers received synapses from Type III cells associated with the two different buds.

Every taste cell in each section was assigned a unique identifier and was analyzed using Reconstruct software (Synapse Web Reconstruct, RRID:SCR_002716) (Fiala, 2005) to determine cell types, and whether Type II and Type III cells were in contact. When such contacts were identified, the segmented measuring tool in image J (National Center for Microscopy and Imaging Research: ImageJ Mosaic Plug-ins, RRID:SCR_001935) (Schneider et al., 2012) was utilized to measure the length of each contact and ultimately determine the surface area (using the measured contact lengths and the overall number of sections showing the contact). For taste cells with contacts spanning fewer than 10 sections, the length of each contact in every section was measured. For taste cells with contacts spanning more than 10 sections, the contact length was measured in every fifth section to calculate the surface area of the contact.

The defining characteristics of synapses from Type II cells in mouse circumvallate taste buds are the presence of large, “atypical” mitochondria, with tubular cristae, closely apposed to the plasma membrane of the Type II cells at the area of contact with a nerve fiber (Romanov et al., 2018; Yang et al., 2020). The plasma membranes of both the Type II cell and nerve fiber are slightly thickened and maintain a constant spacing between them. Similarly, the external membrane of the “atypical” mitochondrion maintains a fixed distance from the plasma membrane of the taste cell often with patches of dense material bridging this gap.

As viewed with transmission electron microscopy, synapses associated with Type III cells

in mouse circumvallate taste buds e.g., (Kinnamon et al., 1985; Yee et al., 2001; Yang et al., 2020) have many of the characteristics of synapses seen in the mammalian central nervous system (Palay et al., 1991; Harris and Weinberg, 2012) including clusters of 40-50 nm vesicles in the presynaptic partner at the point of contact with the postsynaptic fiber, and a slight increase in the density of the pre- and postsynaptic membranes associated with the synaptic cleft. However, the increase in the density of pre- and postsynaptic membranes is often not evident using sbfSEM. In addition, at the low voltages used (~2.2 KV) for scanning, only about the top 30 nm of the 75-80 nm thick section is imaged, so synaptic vesicles deeper in the tissue section may not be visible or recognizable. Identification of other types of junctions such as gap junctions would also be problematic because of this limitation on resolution.

In both datasets, we find a type of specialized, non-synaptic contact between nerve fibers and taste cells which has not been described previously. Based upon its appearance, we term this a "Wavy Junction". This type of contact is unlike a gap, macula adherens or desmosomal type of junction. In wavy junctions, closely associated plasma membranes of taste cells and adjacent nerve fibers separate to form a gap of approximately 40-50 nm in the region of apposition. The plasma membranes have the appearance of waves in the regions of contact (arrows, see Figure S1). The contacts can be extensive, sometimes several microns in length and may extend through approximately 10 sections (750-800 nm). This type of junction can occur between a nerve fiber and each of the various types of taste cells as shown in Fig. S1. We do not consider these to be a form of synapse and therefore have not included these wavy junctions in our connectome map.

Data gathering

Type I, II and III cells (criteria for identification given in (Yang et al., 2020) and nerve fibers (NFs) were segmented by several individuals but any questionable identifications were resolved by referral to a panel of 2 or more experienced segmenters for consensus. NFs to segment were chosen in several different ways: by random selection from fascicles in the lamina propria, by random selection of nerve profiles from within taste buds, and by tracing back starting from identified synaptic contacts with taste cells. These contacts included both classical vesicle-type synapses and channel synapses (Taruno et al., 2021) marked by atypical mitochondria. In addition, NFs partially or completely embedded in cells or closely associated with nuclei were noted and traced. All observations involving cells and NFs were recorded in spreadsheets for later analysis. All contacts with nerve fibers were checked by at least one other person not doing the original segmentation.

A limitation of the current data set is that many nerve fibers

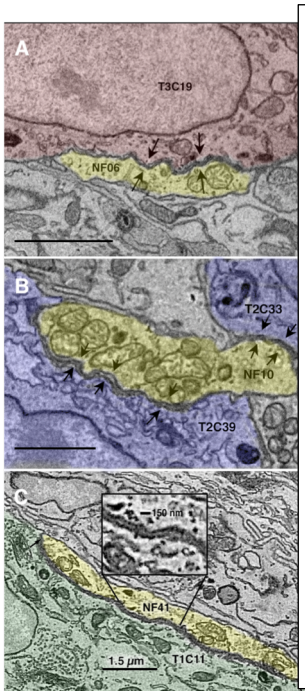

Fig. 1 -S1. Examples of Wavy junctions between nerve fibers (yellow) and diverse cells of taste buds. **A**. From TF21_TB2 : Wavy Junction between a nerve fiber (NF06, yellow) and a Type III cell (T3C19, red). Arrows mark the extent of the contact, in this case about 3 microns. **B.** From TF21_TB1: Wavy Junction between a nerve fiber (NF10, yellow) and two Type II cells, T2C39 and T2C33, blue. Arrows mark the extent of the contacts, in this case about 1 micron for T2C33 and about 3.5 microns for T2C39. **C.** From DS2_TB3: Wavy Junction between a nerve fiber (NF41, yellow) and a Type I cell (T1C11, green). The inset shows an enlargement of the gap region of approximately 40-50 nm. Arrows mark the extent of the contact, in this case about 8 microns. Scale bars = 1.5µm.

could not be followed to their origins below the taste bud, outside of the sampling volume. The connectome data included in this report identify all nerve fibers that formed apparent synapses with taste cells, but does not include nerve fiber fragments which formed no apparent synaptic connections. In three taste buds where we identified all nerve fiber fragments, we found approximately half of the nerve fiber profiles formed synapses (DS2_TB1-2: n=67, 53.7%; DS2_TB3: n=61, 45.9%; TF21_TB1: n=62, 43.5%). The nerve fragments lacking synapses may have been collaterals of fibers that did form connections within the taste bud or may have been independent fibers.

Data analysis and construction of connectomes

Recorded observations were collated in a summary sheet and then used to construct preliminary connectomes using mind mapping software (MindNode or iThoughtsX running on a Macintosh computer). Final connectome diagrams were constructed using Adobe Illustrator (RRID:SCR_010279).

Statistics

Counts of synapses or ATMs in cells were tested using a Chi-square analysis and shown to be not random, i.e. synapses per cell was skewed toward lower values than would occur randomly if the number of synapses is related to a cell's relative age. Pearson's correlation was used to test the relationship between mixed fiber innervation and total cell number in a taste bud and between the number of synaptic sites and the number of post-synaptic nerve fibers innervating a given cell. The relationship between synapses per cell and number of synapses per fiber was tested using Cohen's d. Estimation statistics and calculations are taken from estimationstats.com (Ho et al., 2019). For these tests, 5000 reshuffles of the

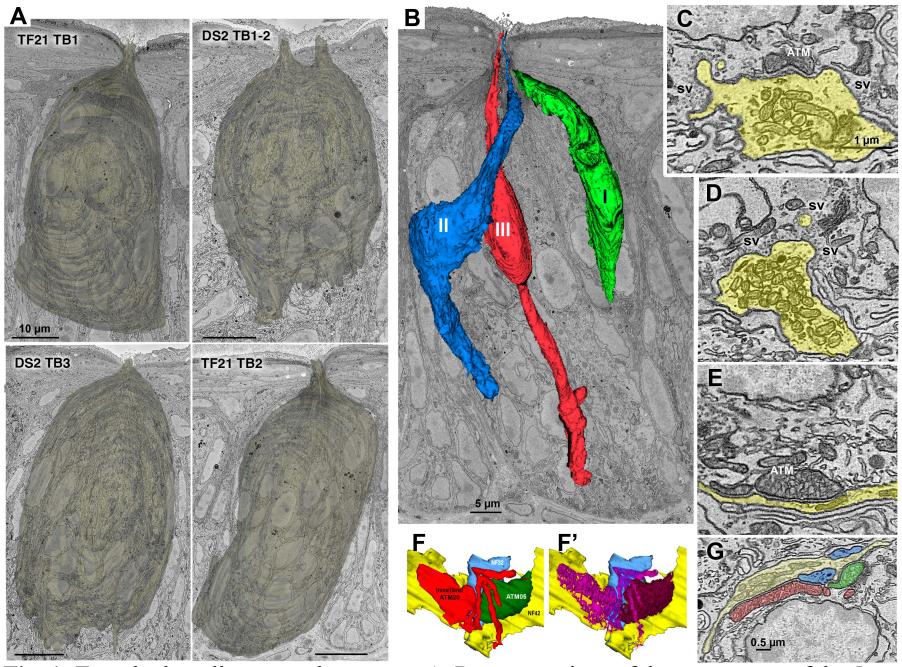

Fig. 1: Taste buds, cell types and synapses. **A.** Reconstructions of the appearance of the 5 segmented taste buds in the connectome data set. The upper right image (DS2_TB1-2) shows the conjoined taste buds with 2 distinct apical pores. Scale bars 10µm. **B**. Taste bud TF21_TB2 showing representatives of the three elongate cell types. **C**. Hybrid type synapse showing both an atypical mitochondrion (ATM) and a cluster of synaptic vesicles (sv) at the region of contact between the nerve fiber (yellow) and Type III taste cell. **D.** Vesicular synapse characteristic of Type III taste cells. **E.** Channel synapse characteristic of Type II cells with ATM apposed to the plasma membrane at the region of contact with the nerve fiber. **F & F':** 3-D reconstructions of a transitional mitochondrion at a synapse between a Type II cell and two nerve fibers (blue and yellow). **F** and **F'.** The transitional mitochondrion (red) with mixed lamellar and tubular cristae (magenta, F') is adjacent to an ATM (green) containing only tubular cristae (dark red, F'). **G.** EM image of the mitochondrial complex reconstructed in panel F. For images of Wavy junctions, see Fig. 1-S1.

control and test labels were taken; the confidence interval is bias-corrected and accelerated.

RESULTS:

Our analysis of taste bud connectivity included 5 complete or partial taste buds from the circumvallate papillae of 2 mice. Cell types and synaptic contacts were identified using ultrastructural criteria established in previous studies. Identifying features of Type II cells include a single large apical microvillus (0.2-0.3 µm width), a broad, regular spindle shape with a large round or oval nucleus, and pale cytoplasm with abundant, sometimes swollen, smooth endoplasmic reticulum. Such cells usually contain one or more "atypical" mitochondria closely apposed to the plasma membrane at points of contact with nerve fibers (Romanov et al., 2018). Some of the atypical mitochondria were continuous with conventional mitochondria with parallel cristae and were interpreted as being transitional (Fig.1F).

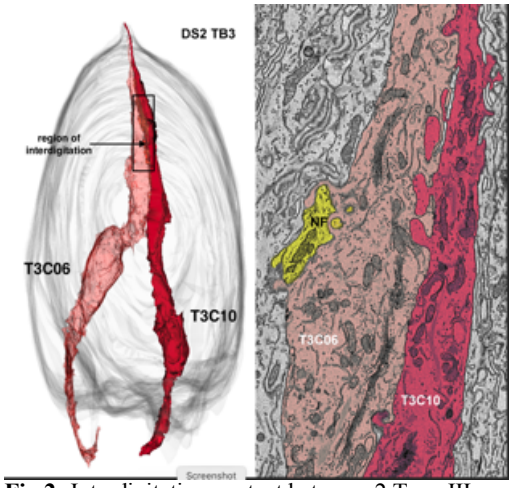

Fig 2: Interdigitating contact between 2 Type III cells. **Left:** 3-D reconstruction of the two Type III cells that form an extensive contact in the apical region of the taste bud. **Right:** Colorized EM image of the region of contact also showing the presence of a nerve fiber (yellow) near the line of contact.

Type III cells were identified by a long, large apical microvillus (0.3-0.4 µm), a generally slender, smooth spindle outline, cytoplasm with evenly dense, smooth ER lacking swellings and an elongate nucleus with one or more deep invaginations. Such cells usually display small accumulations of synaptic vesicles at points of contact with nerve fibers (Kinnamon et al., 1985; Royer and Kinnamon, 1991). A minority (11 of 33) of cells with Type III morphological characteristics also contain small mitochondria with tubular cristae similar to atypical mitochondria of Type II cells, adjacent to sites of synaptic vesicle accumulation (Royer and Kinnamon, 1988) and we now refer to these as "hybrid synapses" (Fig.1C). These mitochondria with tubular cristae in the Type III cells do not display the same fixed, close spacing with the plasma membrane as do the atypical mitochondria of the Type II cells (c.f. Fig. 1C & 1F). The pattern of connectivity of the Type III cells with hybrid synapses does not obviously differ from that of Type III cells with only conventional vesicular synapses and so are not considered separately in our analysis of connectivity below.

Cell-Cell Contacts

Although we emphasize relationships between nerve fibers and the known transducing cells of taste buds (Type II and Type III), we also re-examined points of contact between Type I cells and nerve fibers and between Type I cells and the other taste bud cell types. We observed no membrane specializations between Type I cells and nerve fibers, other than wavy junctions (Fig. S1), although as reported previously (Yang et al., 2020), the Type I cells often enveloped nerve processes within the taste buds, and nerve fibers often closely approach the nuclei of Type I cells.

To test for evidence of cell-cell communication within the taste bud between Type II and Type III cells, which is suggested to play an important role in modulation of taste bud function (Roper, 2013, 2021), we examined areas of direct contact between such heterotypic cells. We

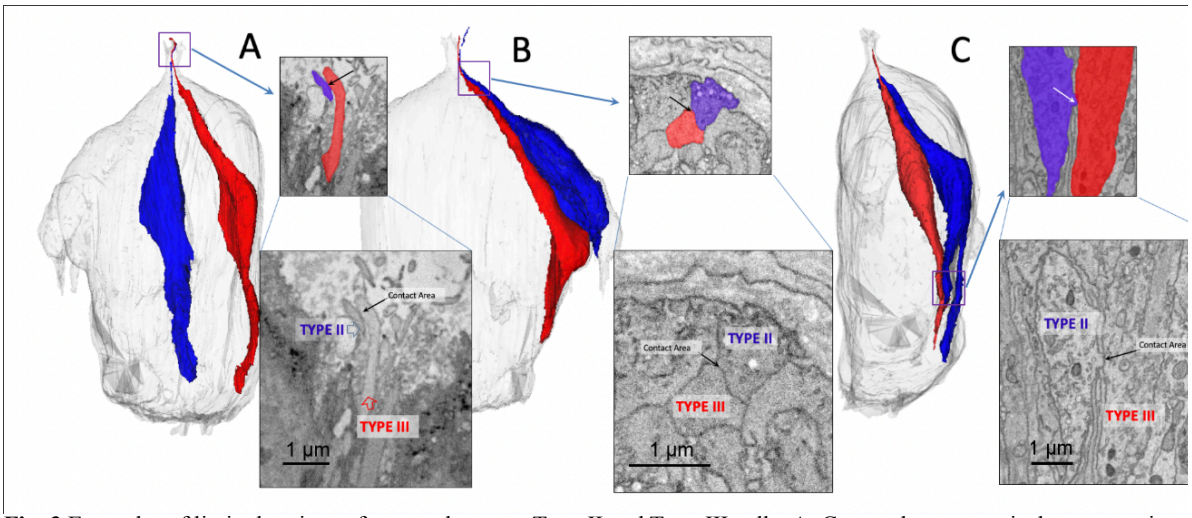

Fig. 3 Examples of limited regions of contact between Type II and Type III cells. **A.** Contact between apical processes in the taste pore. **B.** Apical contact below the taste pore. **C.** Contact in the basal region of the taste bud.

also noted rare examples of homotypic contact between taste cells of the same type, i.e. Type II-Type II contacts and Type III-Type III contacts. Nearly all Type II-Type II contacts involved small (<1µm^2) areas of membrane and exhibited no form of synaptic specialization. A few areas of Type III-Type III cell contact were somewhat more extensive and occasionally involved interdigitation of membrane elements, but exhibited no signs of synaptic transmission such as atypical mitochondria, synaptic vesicle clusters or membrane thickenings (Fig. 2).

In the 6 taste buds examined in the current study, we find only small, non-specialized points of contact between Type II and Type III taste cells with the areas of contact occurring largely in the upper one-third of the bud and especially in the vicinity of the taste pore where the apices of the different cells converge. Out of a total of 54 Type II cells and 44 Type III cells in the sampling volumes, we find 26 points of contact between Type II and Type III cells with 4 cell pairs contacting one another at more than 1 point of contact. Of these 26 contact points, 1 lies in the taste pore, 15 lie apically in the upper third of the taste bud, 6 lie centrally and 4 are in the lower third of the taste bud.

These points of contact exhibit no membrane specializations or accumulations of vesicles adjacent to the membranes of either cell. The zones of contact are small, the 2 largest, situated at the apical quarter of each cell, involve about 2 µm^2 of membrane; 13 of the contacts are less than 0.2 µm^2 (Fig. 3). The cells exhibit no particular organelles or membrane thickenings along these points of contact. The areas of contact are relatively remote from the synaptic sites from both Type II and Type III cells. These findings suggest that direct cell-cell synaptic communication between Type II and Type III cells does not play a role in taste bud function. Technical limitations on the resolution obtainable by this technique do not entirely rule out the possibility that some of these contacts might reflect small, unconventional junctional complexes (Akisaka and Oda, 1978) underlying possible coupling of taste cells (Roper, 2021).

Taste Cell-Nerve Fiber Contacts

We identified synapses between taste cells and nerve fibers based on the criteria described above, i.e. atypical mitochondria closely apposed to the plasma membrane marking channel type synapses (Taruno et al., 2021) for Type II cells and vesicle clusters for conventional synapses in Type III cells (Fig. 1). The post-synaptic nerve fibers were identified and traced to

produce a taste bud connectome for each taste bud.

Conventionally, nerve fibers associated with taste buds are divided into an intragemmal group, within the taste bud, and a perigemmal group which closely surround the taste bud (Zaidi et al., 2016). We find occasional fibers which enter the taste bud as intragemmal fibers to receive a synapse from one or more taste cells and then exit the taste bud more superficially to terminate as free nerve endings in the vicinity of the taste pore (Fig. 4A & B). Conversely, other fibers enter the perigemmal epithelium but continue on to penetrate the taste bud and receive synapses from one or more taste cells (Fig. 4C).

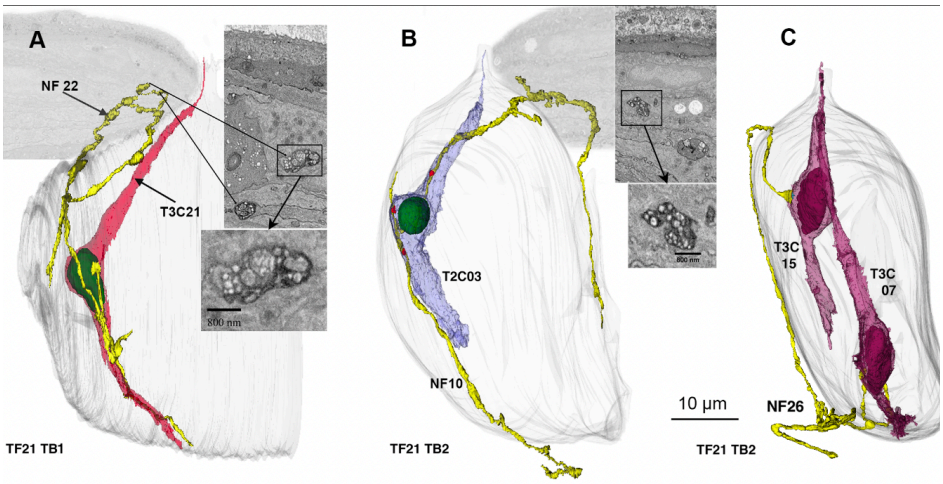

Fig 4: Examples of nerve fibers (yellow) that both synapse with taste cells and traverse perigemmal space. **A.** 3D reconstruction of a fiber that starts as a an intragemmal fiber, synapses with a Type III cell, then exits the taste bud to run in perigemmal space. Inserts at right show EM morphology as indicated. **B.** Similar nerve fiber that synapses with a Type II cell. Adjacent EM images illustrate the nature of the synapses and free endings in the perigemmal epithelium. **C.** An example of a fiber that starts as a perigemmal fiber and then enters the taste bud to form a synapse with 2 Type III cells.

In most, but not all cases, when nerve fibers intimately contact Type II or Type III cells, the taste cells synapse onto the nerve fiber. We do, however, find many examples where a Type II or Type III taste cell contacts a nerve fiber, but no synapse is evident. Such non-synaptic contacts occur without regard to cell type, e.g. a nerve fiber receiving a synapse from a Type II cell may contact other Type II cells without evidence for a synapse, or may contact a Type III cells without evidence for a synapse. Fig. 5 shows an example of a nerve fiber receiving a channel synapse from a Type II cell and then extending downward to become enveloped by a Type III cell but without any evident membrane specialization or synaptic vesicles. The absence of a membrane specialization at such points of contact does not, however, preclude exchange of information at these junctions, e.g. by ephaptic interaction. We did not include such points of non-specialized contact in our connectome map.

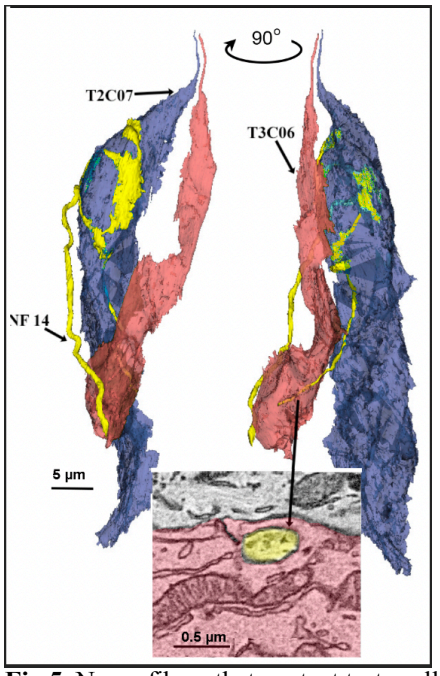

Fig 5. Nerve fibers that contact taste cells do not always form synapses. In this example, showing 2 views rotated about 90°, the nerve fiber (yellow) synapses with a Type II cell (blue) and then runs downward to become fully surrounded by a Type III cell, (see inset below) but without forming a specialized contact with that cell.

A noteworthy feature of taste cell-nerve fiber synapses from both Type II and Type III cells was the variety in number of synapses made by each taste cell and by each nerve fiber (Fig. 6). For Type II cells, the number of channel synapses made by individual taste cells ranged from 1 to over 20, with an average of 6.2 ATM/cell. The number of synapses per cell for Type III cells (mean = 3.8) was also variable but was significantly lower than the number of synapses per Type II cell. The unpaired Cohen's d between vesicular synapses (Ves Syn) and ATM is 0.516 [95.0%CI 0.102, 0.888]. The p value of the two-sided permutation t-test is 0.0294.

Since taste cells turnover continuously, taste cells of a given type include cells of different ages with similar numbers of young, mature and aged cells within each type. If synapses are added as cells mature, then older cells should have more synapses and similar numbers of taste cells should show many synapses as show few synapses. This is not the case. Significantly fewer taste cells show high numbers of synapses than expected for a homogenous distribution of cells of all ages with synaptic numbers increasing with age (For Type II cells: Chi squared equals 52.397 with 10 degrees of freedom; $p < 0.0001$; For Type III cells, Chi squared equals 29.500 with 6 degrees of freedom. $p < 0.0001$).

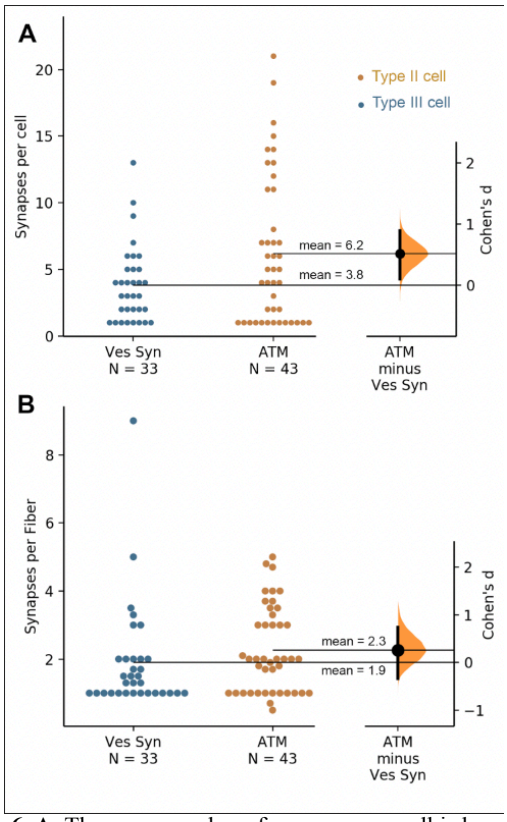

Fig. 6. A: The mean number of synapses per cell is larger for Type II cells (ATM) than for Type III cells (Ves Syn). The Cohen's d between Ves Syn and ATM is shown in this Gardner-Altman estimation plot. Both groups are plotted on the left axes; the mean difference is plotted on a floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a large dot; the 95% confidence interval is indicated by the ends of the vertical error bar.
B: Similar plot showing no significant difference between average number of synapses per fiber for Type II (ATM) and Type III (Ves Syn) cell synapses.

Of the 43 fully characterized Type II cells in our data, 16 showed only 1 or 2 ATM) whereas 4 cells had 15 or more. Cells with higher numbers of ATM tended to be connected to multiple nerve fibers and, even omitting the trivial case for 1 ATM, the number of postsynaptic nerve fibers was proportionate to the number of ATM in any given cell ($r(30) = .799$, $p < 0.001$). Often, a single nerve fiber made repeated synaptic contacts onto a single Type II cell, e.g. in TB3 of the DS2 data set, a nerve fiber (NF04) contacts Type II taste cell (T2C1) at 9 distinct sites. Although Type III cells also displayed multiple synapses with some nerve fibers, the number of such contacts usually was much lower than in Type II cells (1-4 sites per cell). One exceptional Type III cell (T3C2 in TF21_TB1) showed 13 distinct synaptic loci onto 4 different nerve fibers, but the average number of synapses per Type III cell was 3.8 (+/-2.86 s.d.). The number of nerve fibers innervating a particular Type III cell was not significantly correlated with the number of synapses from that cell ($r(16) = .30 = p>0.05$) although individual nerve fibers often received several synapses from the same Type III cell. Conversely, some cells of both types synapse onto many nerve fibers (see below).

Patterns of Connectivity

Within the sampling volume of the largest taste bud in our datasets (TF21_TB2), containing about 100 elongate taste cells (including 12 Type II and 12 Type III synaptically connected cells), we identified over 27 independent nerve profiles forming synapses with taste cells. As mentioned above this does not necessarily equal the total number of independent nerve fibers entering the taste bud since many of the fibers likely branch below the basal lamina in the subgemmal plexus (Kanazawa and Yoshie, 1996). Nonetheless, we discern several distinct patterns of connectivity in this and other taste buds as described below.

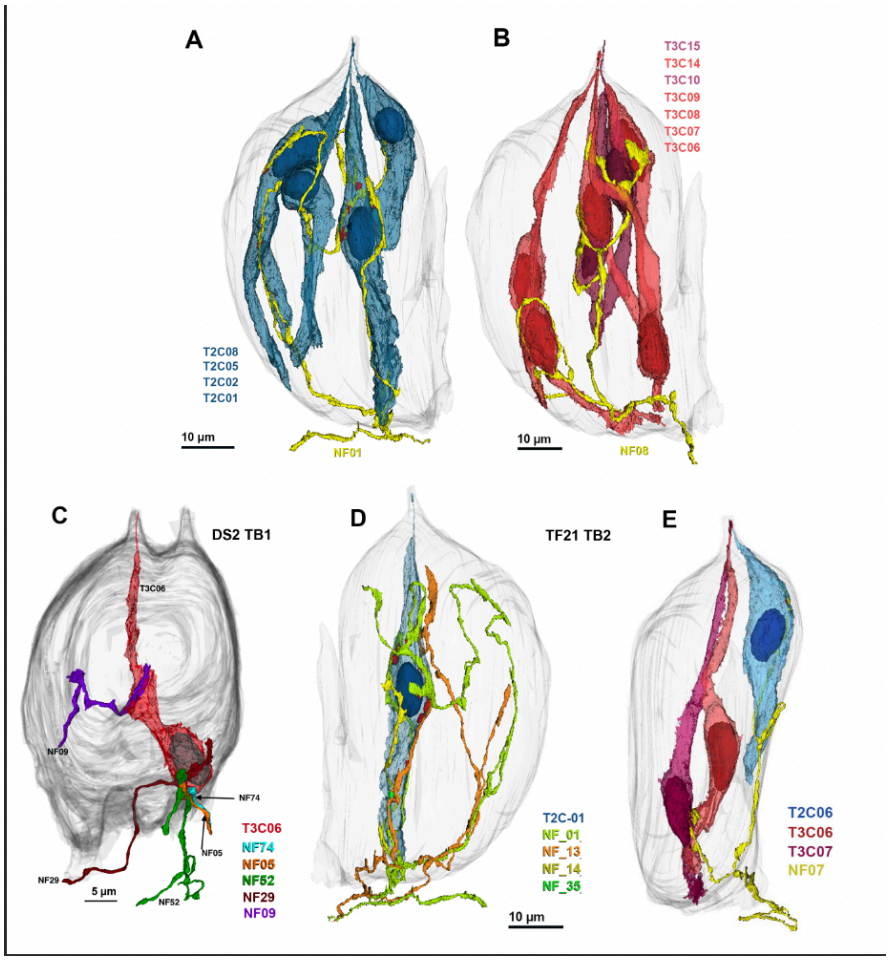

Fig: 7: Examples of different patterns of innervation. **A & B.** Individual nerve fibers may connect to many cells of the same type. **A**. Network of 4 Type II cells (blue) connected to a single nerve fiber (yellow). ATM in red. **B.** Network of 7 Type III cells connected to a single nerve fiber. **C & D.** Individual taste cells innervated by multiple nerve fibers. **C.** Type III cell synapsing with 5 different nerve fibers. **D.** Type II cell forming synapses with 4 distinct nerve fibers. **E.** Example of a single nerve fiber (yellow; NF07) receiving synapses from different cell types. The Type II (blue; T2C06) cell forms channel synapses while the Type III cells (red; T3C06 & T3C07) form vesicular synapses on the same fiber.

With a few exceptions, nerve fibers that synapse with a Type III cell synapse only on other Type III cells; fibers that synapse with a Type II cell generally synapse only with other Type II cells (Fig. 7A & 8). Each nerve fiber generally forms synapses with a small number of taste cells – typically one, two or three cells, but some fibers innervate 4 or more cells of the same type (Fig. 7A, B). Many taste cells synapse with nerve fibers largely dedicated to that cell and only one or two others. For Type II cells, 44.2% of the cells (19 of 43) are innervated by a unique set of unbranched nerve fibers; for Type III cells, 5 of 33 (15.2%) are uniquely innervated. As can be appreciated in the connectome maps (Fig. 8), the remaining cells of each type within a taste bud are interconnected in various ways, especially in the larger taste buds. For example in TF21_TB2, 9 of the 12 Type II cells synapse onto a network of 7 nerve fibers. In DS2_TB3, 5 of 9 Type II cells synapse onto the same pool of 4 nerve fibers. Similarly, Type III

cells may synapse onto a connected network, e.g. in DS2_TB1, the 4 Type III cells all synapse onto the same set of nerve fibers; in DS2_TB3, 4 of 5 Type III cells converge onto 3 nerve fibers; and in TF21_TB2, 8 of 12 Type III cells converge onto 2 nerve fibers while 3 other cells converge onto 2 other nerve fibers. Conversely, a single cell may synapse onto numerous fibers (Fig. 7C, D).

While a single nerve fiber generally forms either all channel synapses with Type II cells or vesicular synapses with Type III cells, some uncommon nerve fibers form both types of connections with different cell types (Fig. 7E). Across all 5 taste buds in the sampling volume, of 87 total fibers forming channel synapses with Type II cells, only 4 fibers of these also form vesicular synapses with Type III cells. These 4 mixed synapse fibers also are a minority of the 46 total fibers receiving synapses from Type III cells. The probability of a taste bud having a mixed synapse fiber is directly related to the total number of Type II and Type III cells within the bud (Pearson's correlation: $r(3)= .89$, $p = .04$). The existence of fibers that form both channel (Type II cell) and vesicular (Type III cell) synapses demonstrates that synaptic structure is not dictated by the post-synaptic nerve fiber. Furthermore, the convergence of Type II cell inputs and Type III cell inputs onto single nerve fibers shows that connectivity within a taste bud is not organized entirely on a labeled-line basis since Type II and Type III cells transduce different taste qualities.

An example of such cross-modal connectivity is NF2 in TF21_TB1, which forms a channel type synapse with a Type II cell (T2C33), a synapse on one Type 3 cell (T3C19), a complex series of synapses with ATMs and 4 vesicle clusters on another Type III cell (T3C15) and 4 vesicular synapses with Type III cell T3C02, which also synapses onto 3 other nerve fibers (Fig. 8). Similarly, another nerve fiber in this same taste bud (NF07) receives a channel synapse from a Type II cell while also receiving vesicular synapses from 2 Type III cells (Fig. 7E). This shows that a given nerve fiber can form different types of synapses with different taste cells, and even different types of synapses with a single taste cell. A similar pattern of complex connectivity occurs for one fiber in taste bud DS2_TB3.

As can be appreciated from the connectome maps in Fig. 8, multiple branches and complex patterns of connectivity are not limited to fibers innervating different types of taste cells. For example, a single Type II cell (T2C40 in TF21_TB1), has 10 sites with channel synapses and these 10 sites form contacts with 8 different nerve fibers. Of the 8 nerve fibers, 6 are uniquely associated with this taste cell while 2 form channel synapses with but one other Type II cell (T2C43), which itself forms multiple synapses onto only one other nerve fiber (NF12). Further, each nerve fiber may be post-synaptic to multiple channel synapses from a single taste cell, e.g. NF5 receives 4 synapses from cell T2C40 sharing one of these sites with nerve fiber NF4. At the other end of the spectrum of connectivity is Type II cell T2C11 in TF21_TB 2 which contains but a single channel synapse connecting to a unique nerve fiber (Fig. 8).

Summary: In general, consistent with a labeled-line system of taste encoding, nerve fibers that receive synapses from a Type III cell are generally post-synaptic only to other Type III cells and conversely nerve fibers post-synaptic to Type II cells are generally post-synaptic only to other Type II cells (Fig. 8). Nerve fibers innervating both Type II and Type III cells represent a little less than 5% (4 of 87) of the fibers innervating Type II cells but about 10% (4 of 46) of those innervating Type III cells. These results suggest that a strict labeled line type of connectivity does not exist and that some fibers will carry an admixture of different quality information from Type II (sweet-umami-bitter) and Type III (sour) cells.

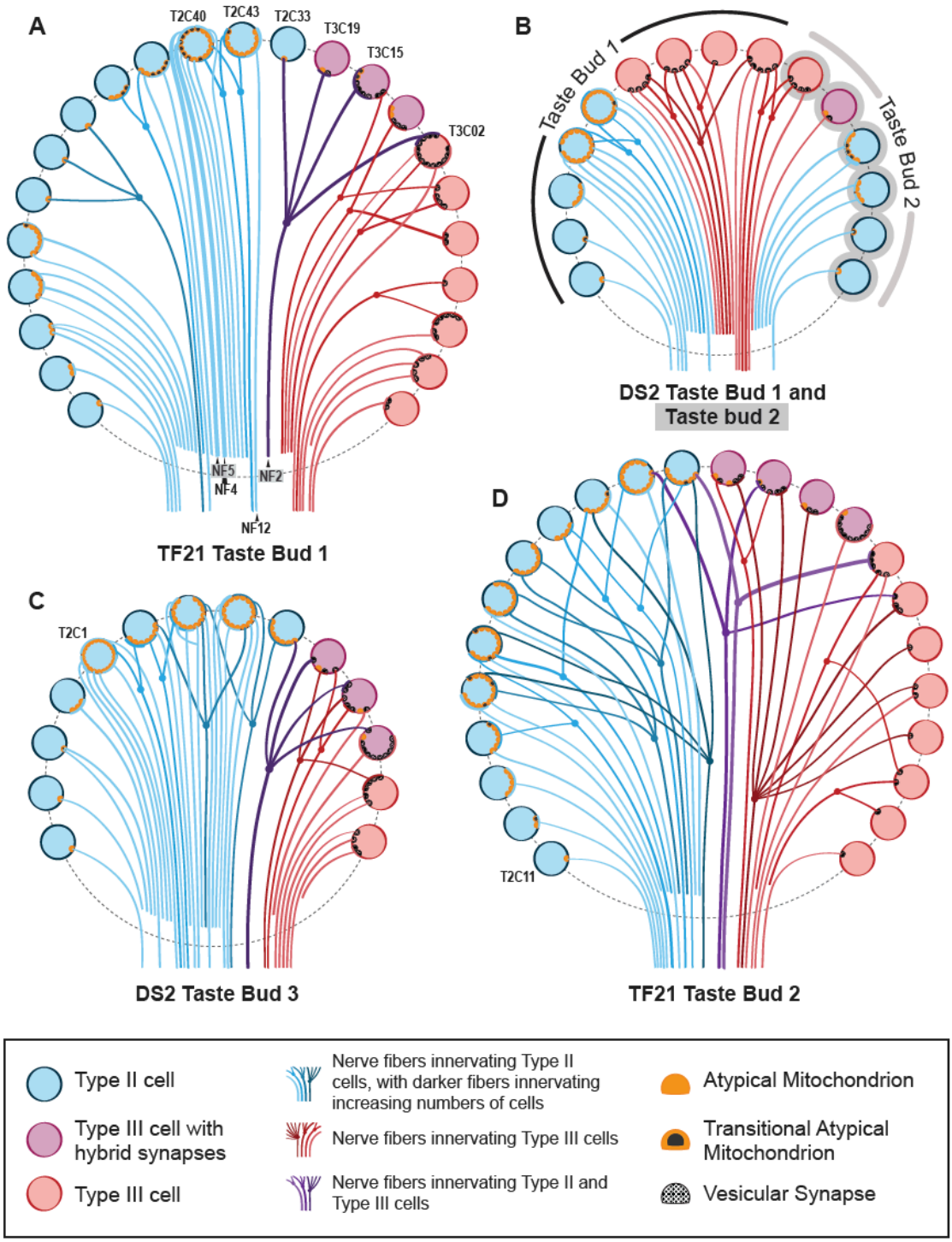

Fig. 8 Connectome maps for the 5 taste buds described in this study. Blue circles = Type II cells; purple circles = Type III cells with hybrid synapses; red circles = Type III cells with vesicular synapses. Each line denotes a single nerve fiber color coded: red fibers innervate Type III cells, blue fibers innervate Type II cells, Purple fibers innervate both Type II and Type III cells. Branched fibers are shown by dots and forked connections. Nerve fragments that could not be traced to exit the taste bud are illustrated as not extending below the dashed line outlining each taste bud. Those cells mentioned in the text are denoted by larger lettering. A larger version of this figure with all cell and fiber designations appears as S2.

DISCUSSION (1494 words; 1500 word limit)

Reconstructions of nerve fibers and taste cells from sbfSEM images reveal details about the anatomical organization of circumvallate taste buds thereby providing a foundational anatomical model (Swanson and Bota, 2010) for rational construction of hypotheses about physiological interactions of cells and neural networks within the system. The different anatomical types of cells within a taste bud directly relate to their function and chemical responsiveness. Accordingly a detailed anatomical understanding of cellular interrelationships and interactions with nerve fibers will enable appropriately constrained models of the taste periphery.

Our principal conclusions relate to two widely-held concepts regarding the functional organization of taste buds (Ohla et al., 2019): 1) that taste information is transmitted to the brain over "labeled lines", i.e. nerve fibers conveying information about a single taste quality, and 2) that the different types of taste cells within a taste bud directly communicate with one another to modulate the transduction and transmission of taste information (Roper, 2007; Dando and Roper, 2009; Chaudhari and Roper, 2010; Vandenbeuch et al., 2020). These two concepts are interrelated in that cross-modal taste information could be generated by cross-talk between different types of taste cells even if the wiring were strictly according to cell type. For example a Type II taste cell expressing sweet receptors might exhibit responses to acids because of modulatory input from adjacent, sour-responsive Type III cells.

Labeled lines: One of the classic debates within the taste field has been the nature of the signal conveyed from the taste bud to the brain. One school, the "labeled line" hypothesis, posits that the taste signal is quality-specific, i.e. a single fiber will carry information about only a single taste quality, e.g. sweet or bitter. The contravening hypothesis, the "cross-fiber pattern theory", holds that individual fibers respond to multiple qualities and that quality information can be derived only by comparing patterns of activity across the population of nerve fibers. Two recent studies directly comparing activity of taste ganglion cells in mice come to opposite conclusions about the responsiveness of the peripheral taste axons. Barretto and colleagues (Barretto et al., 2015) conclude that individual ganglion cells respond to single taste qualities whereas Wu and colleagues using similar techniques (Wu et al., 2015), conclude that most nerve fibers exhibit broader responsiveness, especially at suprathreshold concentrations. Since Type III cells and Type II cells respond to different taste qualities, the labeled line theory would predict that these different types of cells should synapse on different populations of nerve fibers. Indeed, two recent studies suggest that at least some degree of specificity exists in the wiring of taste cells to taste nerves (Lee et al., 2017; Stratford et al., 2017). Conversely a study following branching patterns of nerves in fungiform taste buds finds that 28% of fibers contact both Type II and Type III cells (Huang et al., 2020). We find that only about 10% of the fibers synapsing with Type III cells also receive synapses from Type II cells indicating that wiring specificity in circumvallate taste buds is not absolute – some subset of nerve fibers likely respond to stimuli activating the different taste cell types: Type III cells responding to acids (Huang et al., 2006; Huang et al., 2008; Kataoka et al., 2008) and Type II cells responding to either sweet, umami or bitter. Indeed, Barretto et al report a significant population of ganglion cells responsive to both bitter and acids but attribute the acid responsiveness to direct activation of bitter-responsive cells. But even without considering these sour-bitter responsive ganglion cells, the data still show that nearly a quarter of all ganglion cells that respond to sour also respond to Type II cell qualities such as sweet. Similarly, Wu et al show ganglion cells that respond to both sour and sweet. Such multimodal ganglion cells may represent a population of nerve fibers synaptically connected to both Type III and Type II taste cells. Further, the fact that an individual ganglion cell can be

post-synaptic to both conventional vesicular synapses in Type III cells and channel-type synapses in Type II cells indicates that the nerve fiber does not dictate the type of synapse formed.

Cell-cell Communication: The adjacency of different cell types within a taste bud suggests the possibility of cell-cell intercommunication. Indeed, several studies indicate that neurotransmitters – ATP, serotonin, GABA, acetylcholine – released by one type of taste cell can influence the activity in nearby taste cells of similar or different types (Roper, 2013). Accordingly we had anticipated finding substantial, specialized areas of contact between the Type II and Type III cells as well as between adjacent Type II cells or Type III cells. Such is not the case. Type I cells effectively surround and largely isolate the Type II and Type III cells from one another. We found no specializations – synaptic or otherwise – at the limited points of membrane contact between these cells. Accordingly, the interactions between the different cell types observed in intact taste buds must be due to either paracrine effects of neurotransmitter leaking past the Type I cells, or due to transcellular activation via Type I cells. The role of Type I cells in modulation of activity of sweet responsive taste cells is well characterized. The ectoATPase on Type I cell membranes (Bartel et al., 2006) rapidly degrades ATP released by Type II cells to ADP which is further degraded by nonspecific phosphatases to adenosine. The extracellular adenosine activates A2b receptors on some Type II cells to modulate responses to sweet (Dando et al., 2012; Kataoka et al., 2012). Similar Type I-cell mediated modulation of other taste transducing cells may be via release of transmitters similar to the non-synaptic release of gliotransmitters in the CNS (Newman and Zahs, 1998; Pascual et al., 2005; Araque et al., 2014).

Although Type I cells may play an important role in modulation of taste signaling within a taste bud, they show no membrane specializations typical of synapses at any areas of contact with nerve fibers or other cells. Many Type I cells form intimate contacts with nerve fibers, especially in the nuclear region (Yang et al., 2020), but display neither accumulations of vesicles, membrane thickenings, nor atypical mitochondria at the zones of contact.

Synaptic Efficacy: The number of synapses made by a taste cell onto a single nerve fiber varies greatly and may be a determining factor for effectiveness of information transfer. Many taste cells – both Type II and Type III – form but a single synapse onto a single nerve fiber (Huang et al., 2020). In contrast, other taste cells form multiple synapses onto a single nerve fiber; the maximum observed in our data is 10. Whether the number and size of the synaptic contacts relates to synaptic efficacy is unclear. Different Type II taste cells release different amounts of ATP to similar levels of chemical stimulation (Murata et al., 2010). Cells having more synapses may release more ATP than cells with fewer synapses, thereby activating the postsynaptic nerve fiber more effectively. A taste cell forming 10 synapses onto a nerve fiber would likely exert a much greater influence on the activity of that nerve fiber than would a cell with but a single synapse.

Divergence and convergence: Substantial variety exists in terms of the number of taste cells contacted by individual nerve fibers and the number of nerve fibers synapsed onto by a single taste cell. In all but the smallest taste buds, at least one nerve fiber in each bud branches within the taste bud to connect synaptically with 4 taste cells, often including both Type II and Type III cells. This suggests that these fibers are broadly responsive to both sour (Type III) and one or more GPCR-mediated taste qualities, i.e. bitter, sweet or umami (Type II). Other widely branched fibers connect only with taste cells of a similar type. With the limitations of the techniques employed, we cannot determine whether these interconnected cells all respond to the

same taste quality, or whether they respond to different qualities. If the presynaptic taste cells in the synaptic network respond to different GPCR-mediated taste qualities, such connectivity might underlie the few broadly responsive ganglion cells, e.g. those responding to sucrose and bitter, reported by others (Barretto et al., 2015; Wu et al., 2015).

The number of axons postsynaptic to any given taste cell varies from 1 – 8. How this variability affects the output of the system is unclear. A multiply-connected taste cell might exert a larger influence on total nerve output than would a taste cell synaptic onto but a single fiber. If the presynaptic cells are of different taste specificity, then the more connected cell may drive the response of the nerve fiber at low concentrations of tastant whereas the activity of the less connected cell becomes evident only at higher concentrations. Indeed, differences in specificity of response with concentration (Wu et al., 2015) is reported for taste ganglion cells. A ganglion cell may respond to a single taste quality at low concentrations, but show broader responsiveness at high stimulus concentrations.

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FIIGURE CAPTIONS

Fig. 1: Taste buds, cell types and synapses. **A.** Reconstructions of the appearance of the 5 segmented taste buds in the connectome data set. The upper right image (DS2_TB1-2) shows the conjoined taste buds with 2 distinct apical pores. Scale bars 10µm. **B**. Taste bud TF21_TB2 showing representatives of the three elongate cell types. **C**. Hybrid type synapse showing both an atypical mitochondrion (ATM) and a cluster of synaptic vesicles (sv) at the region of contact between the nerve fiber (yellow) and Type III taste cell. **D.** Vesicular synapse characteristic of Type III taste cells. **E.** Channel synapse characteristic of Type II cells with ATM apposed to the plasma membrane at the region of contact with the nerve fiber. **F & F':** 3-D reconstructions of a transitional mitochondrion at a synapse between a Type II cell and two nerve fibers (blue and yellow). **F** and **F'.** The transitional mitochondrion (red) with mixed lamellar and tubular cristae (magenta, F') is adjacent to an ATM (green) containing only tubular cristae (dark red, F'). **G.** EM image of the mitochondrial complex reconstructed in panel F. For images of Wavy junctions, see Fig. 1- S1

Fig 2: Interdigitating contact between 2 Type III cells. **Left:** 3-D reconstruction of the two Type III cells that form an extensive contact in the apical region of the taste bud. **Right:** Colorized EM image of the region of contact also showing the presence of a nerve fiber (yellow) near the line of contact.

Fig. 3 Examples of limited regions of contact between Type II and Type III cells. **A.** Contact between apical processes in the taste pore. **B.** Apical contact below the taste pore. **C.** Contact in the basal region of the taste bud.

Fig 4: Examples of nerve fibers (yellow) that both synapse with taste cells and traverse perigemmal space. **A.** 3D reconstruction of a fiber that starts as a an intragemmal fiber, synapses with a Type III cell, then exits the taste bud to run in perigemmal space. Inserts at right show EM morphology as indicated. **B.** Similar nerve fiber that synapses with a Type II cell. Adjacent EM images illustrate the nature of the synapses and free endings in the perigemmal epithelium. **C.** An example of a fiber that starts as a perigemmal fiber and then enters the taste bud to form a synapse with 2 Type III cells.

Fig 5. Nerve fibers that contact taste cells do not always form synapses. In this example, showing 2 views rotated about 90°, the nerve fiber (yellow) synapses with a Type II cell (blue) and then runs downward to become fully surrounded by a Type III cell, (see inset below) but without forming a specialized contact with that cell.

Fig. 6. A: The mean number of synapses per cell is larger for Type II cells (ATM) than for Type III cells (Ves Syn). The Cohen's d between Ves Syn and ATM is shown in this Gardner-Altman estimation plot. Both groups are plotted on the left axes; the mean difference is plotted on a floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a large dot; the 95% confidence interval is indicated by the ends of the vertical error bar.
B: Similar plot showing no significant difference between average number of synapses per fiber for Type II (ATM) and Type III (Ves Syn) cell synapses.

Fig: 7: Examples of different patterns of innervation. **A & B.** Individual nerve fibers may connect to many cells of the same type. **A**. Network of 4 Type II cells (blue) connected to a single nerve fiber (yellow). ATM in red. **B.** Network of 7 Type III cells connected to a single nerve fiber. **C & D.** Individual taste cells innervated by multiple nerve fibers. **C.** Type III cell synapsing with 5 different nerve fibers. **D.** Type II cell forming synapses with 4 distinct nerve fibers. **E.** Example of a single nerve fiber (yellow; NF07) receiving synapses from different cell types. The Type II (blue; T2C06) cell forms channel synapses while the Type III cells (red; T3C06 & T3C07) form vesicular synapses on the same fiber.

Fig. 8 Connectome maps for the 5 taste buds described in this study. Blue circles = Type II cells; purple circles = Type III cells with hybrid synapses; red circles = Type III cells with vesicular synapses. Each line denotes a single nerve fiber color coded: red fibers innervate Type III cells, blue fibers innervate Type II cells, Purple fibers innervate both Type II and Type III cells. Branched fibers are shown by dots and forked connections. Nerve fragments that could not be traced to exit the taste bud are illustrated as not extending below the dashed line outlining each taste bud. Those cells mentioned in the text are denoted by larger lettering. A larger version of this figure with all cell and fiber designations appears as S2.

Fig. 1 -S1. Examples of Wavy junctions between nerve fibers (yellow) and diverse cells of taste buds. **A**. From TF21_TB2 : Wavy Junction between a nerve fiber (NF06, yellow) and a Type III cell (T3C19, red). Arrows mark the extent of the contact, in this case about 3 microns. **B.** From TF21_TB1: Wavy Junction between a nerve fiber (NF10, yellow) and two Type II cells, T2C39 and T2C33, blue. Arrows mark the extent of the contacts, in this case about 1 micron for T2C33 and about 3.5 microns for T2C39. **C.** From DS2_TB3: Wavy Junction between a nerve fiber (NF41, yellow) and a Type I cell (T1C11, green). The inset shows an enlargement of the gap region of approximately 40-50 nm. Arrows mark the extent of the contact, in this case about 8 microns. Scale bars = 1.5µm.

Fig. 8. S2: High resolution version of the connectome maps showing all fiber and cell identifiers.

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