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In vivo analysis of the cellular interactions during taste sensory organ assembly in zebrafish

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για την ανιδιοτέλεια και το θάρρος τους

«*Το φάρμακό μας είναι η τροφή μας*»

Περί Αισθήσεως και Αισθητών

Αριστοτέλης

«*Let thy food be thy medicine*»

On sense and the sensible

Aristotle

A rose by any other name would scent as sweet

William Shakespeare

Sequí naturam

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Through the centuries, numerous definitions have been given for taste from a physiological, sociological, chemical and gastronomic point of view. A physiologist would describe taste as one of the five senses, which defines the ability to distinguish the quality of a substance placed in the oral cavity, including the upper surface of the tongue and the epiglottis. Taste is produced after stimulation of the taste receptors located on taste sensory organs by flavoured substances. Along with olfaction and trigeminal nerve stimulation (registering texture, pain, and temperature), taste determines and interprets food quality.

A sociologist would say that taste is a personal and cultural pattern of choice and individual preference in food. According to Littré dictionary taste comes from Latin “gustus”, meaning « act of tasting, degustation; savour ». Gustus may also derive from Greek and the Greek term of Sanskrit “gush” meaning « like, good». Social and cultural phenomena concerning taste are closely associated to environmental resources and social relations between people; therefore culture and food are closely related.

A romantic would admit that the sense of taste is capable of the development of "an extreme delicacy” of the palate, which allows the appreciation, in tasting of a full range of flavors far beyond the register of basic sensation. Such sentiments are repeated and embroidered many times in the rich literature of gastronomy that blossomed in the ensuing decades [1, 2].

According to the great gastronome Brillat-Savarin, taste is a chemical process that occurs in a liquid phase where sapid molecules dissolve in any fluid. These molecules are consequently absorbed by the taste-related organs, the taste buds that coat the interior of the taste apparatus. For instance, pure water itself, as a neutral liquid, does not cause any gustatory effect on the taste buds but once a grain of salt or some drops of vinegar are diluted in it the result is really appreciated [3].

Gastronomes use the term "taste" to refer to the multi-sensory experience of the flavour and texture of food and drink. Taste however does not function alone outside the laboratory as it is strongly related to olfaction and vision. Texture and colour of food are among the basic reasons for feeding behaviour and curiously, who would choose to eat a blue apple?

Contrasting though the olfactory system of an average person that is capable to recognize approximately 50 different smells, of a chemist's that can distinguish more than 500 and a perfumer's up to 5000 scents, their taste system is limited to only 5 principal groups of savouriness: sweet, umami, bitter, sour and salty.

Sweet: As soon as we are born, we are attracted to sweet nutrients (initially to the breast milk in which 6,89% is sugars) indicating us the importance of this energy (and not only) rich nutrient. When sugars are consumed they transform into glucose in the body. Glucose is then used for immediate energy or stored in the liver and muscles in order to be used for energy at a later time, depending on body needs. Simple sugars, also known as simple carbohydrates, get digested quickly and provoke a fast burst of energy. The sweetness of sugars in fact represents a neuronal burst in the brain related to the pleasant access of these caloric-rich sources of metabolic energy.

Umami: A Japanese word is used to describe the internationally recognised taste produced by L-amino acids and more precisely L-glutamate and aspartate in human. Umami in general can be related to the taste of meat and protein-rich nutrients.

Bitter: While sweet and umami function in order to satisfy our papillae by determining energy rich-nutrients, bitter represents the repugnant but concurrently protective role to eliminate every noxious substance that could have fatal results, simply by aversion. Bitter can be bearable when weak, as indicated by the fact that many people enjoy a cup of coffee but can be really aversive when strong. In contrast to the three parsimonious taste receptors for sweet and umami, nature was quite generous for bitter detecting receptors, as they are quite numerous and reaches up to 30.

Sour: Although small amounts of low concentrated acid substances are quite pleasant but also useful e.g lemon juice (rich in vitamin C), the importance of sour taste lays on the protection of the organism by low-pH compounds which tend to be unpleasant and sometimes poisonous for our body. Sour is also necessary to regulate multiple functions of our body fluids especially within the brain. Although the transduction channels and/or

receptors involved in sour detection has been quite enigmatic, there are still some indications about molecules involved in the process.

Salty: The source of life according to the ancient Greek **αλάτι, ** ἅλς (háls) Proto-Indo-European **séh₂l, *séh₂l.* Salty taste governs intake of sodium and other salts, essential for maintaining the body's water balance and blood circulation. Salts in our body function as catalysts.

Nevertheless, it could be due to the importance, albeit neglected in function sense of taste that eating is a pleasurable experience. Furthermore, the human body homeostasis highly depends on this sense, as the ability to taste and smell food properly is of vital meaning. Apart for its nutritional aspect, taste is related with others emotions. People that lose their ability to taste properly tend to lose weight, become depressed or enrich their food with strong tastants such as salt or acid to obtain a taste sensation. This incapacity may lead to poor nutritional status, impaired immunity, and/or aggravation of several diseases. So what the world would have been without taste?

INTRODUCTION

I. THE ORGANISATION OF THE TASTE SYSTEM

Taste, one of the five senses plays the role of a security guard for the organism by regulating our feeding behaviour. It evaluates the nutritious elements of food that will satiate the body with energy and prevents ingestion of toxic substances that could be potentially harmful. The sense of taste is interpreted by the taste system, which is composed of the taste sensory organs called taste buds, the taste sensory ganglia and taste-specific brain nuclei (Fig. I-1). The taste system has been extensively studied in jawed vertebrates, (especially in mammals but also in fish and amphibia) and *Drosophila*. To introduce the present research work on the taste buds, selected key elements of i) the organisation and ii) development of the taste system in mouse and zebrafish are described in Chapters I.1, I.2 and I.3 respectively.

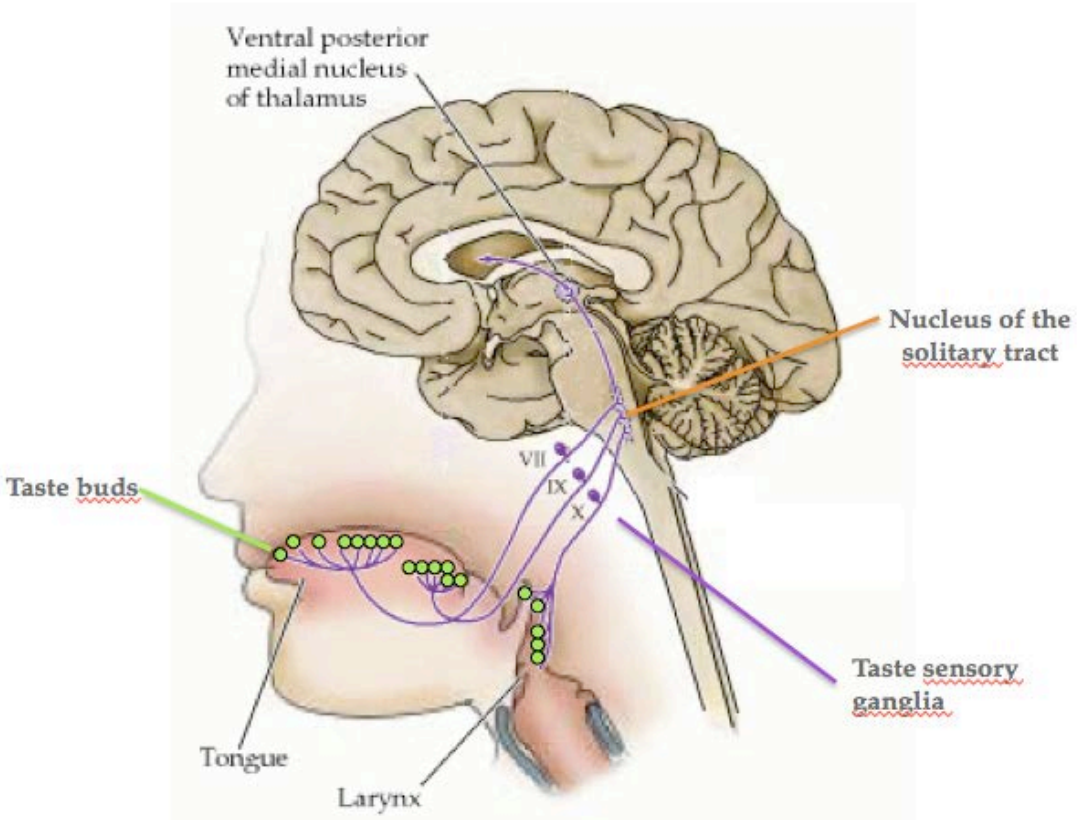


Figure I-1. Organisation of the human taste system. The drawing shows the relationship between the taste sensory organs in the oral cavity and the three taste sensory ganglia (VII, IX, X) and taste-related nuclei in the brain [4].

I.1) ANATOMY AND FUNCTION OF THE MAMMALIAN TASTE SYSTEM

I.1.1) The mammalian taste buds

I.1.1.1) General morphology of the mouse taste bud

In mammals, the taste buds are distributed within the oropharyngeal epithelium in a patterned manner, along the antero-posterior axis of the tongue, palate, epiglottis, pharynx and larynx. Several hundreds of taste buds are found in the mouse oropharyngeal cavity. Each one of them is an onion-shaped structure containing 50-100 cells [5] that possess microvilli on their apical end and contact taste sensory ganglia processes on their basal part. The microvilli project into the *taste pore*, a small opening on the surface of the tongue permitting the taste cells to be exposed to and get in contact with the contents of the mouth [6, 7].

On the surface of the mammalian tongue, the taste buds reside in papillae, 'pockets' formed from the epithelial cells that surround the taste buds. Three different types of lingual papillae are distinguished (Fig. I-2), according to the tongue region:

- *Fungiform:* they have the shape of a mushroom and are located on the anterior part of the upper surface of the tongue [8].

-*Foliate:* they have an elongated fold representing a leaf on the edge and they are clustered into two groups one on each side of the posterior lingual epithelium. There are about 4-5 in mammals and their size and shape varies.

-*Circumvallate:* they are inverted V-shaped structures, situated on the posterior surface of the tongue. There is a single one in mice but 8-10 circumvallate in human.

In addition, filiform papillae are located on the tongue; these are the most numerous, but not associated with taste buds. They have a mechanical and protective function for the tongue serving as a defensive coat complemented by the antibacterial action of components of the saliva [9].

I.1.1.2) The main taste bud cell types and their function

To understand how taste buds function, we set to delineate the cell types that compose the taste buds and how these cells transduce the chemical signals upon stimulation from tastants. A functional taste bud is consisted of three morphologically distinct cell types that detect taste stimuli; Type I cells, also called support, they are glial-like cells, Type II cells, or taste receptor cells, they detect bitter, sweet and umami and Type III cells or presynaptic cells, which sense sour and carbonated substances.

I.1.1.2a) Type I (Support Cells)

The Type I (support) cells represent the greatest number of cells (almost half of all taste bud cells) in mammalian taste buds. They are elongated cells with cytoplasmic protrusions and extensive lamellar processes allowing wrapping around and protecting other cell types. Type I cells do not possess voltage-gated calcium channels and do not form synapses. They express transporters for glutamate (GLAST for glutamate aspartate transporter /SLC3A for solute carrier 3A), as well as NTPDase2, a nucleotidase bound on the plasma membrane that hydrolyses extracellular ATP, a key transmitter released upon bitter, umami or sweet stimulation of the Type II cells. This enzyme is critical for the transduction of the taste stimuli since genetic deletion of NTPDase2 results in decreased neural responses to taste stimuli. The decrease in responsiveness reflects in the lack of degradation and elevated levels of ATP. Given that ATP is necessary for the activation of P2X receptors, deletion of NTPDase2 results in disruption of purinergic transmission in the synapses [10].

The function of the support cells remains poorly understood. It is believed that they detect NaCl but also other salts that provoke not necessarily salty but bitter, metallic or astringent taste. Salts utilize apically located ion channels for transduction (fig.I-6). A subset of Type I cells in the anterior-tongue taste buds express the amiloride-sensitive, epithelial sodium channel (ENaC) [11, 12] and most taste buds contain the three of its subunits α, β, and γ. The ENaC subunits are expressed in a similar manner on the anterior and posterior lingual epithelium in mammals but in greater extent in fungiform than in circumvallate papillae. α-ENaC alone can produce amiloride-sensitive sodium currents, and co-expression with β-ENaC and γ-ENaC enhances these currents

(fig.I-7). Despite the expression of ENaC in Type I cells and its capacity to detect sodium, a clear link between salty taste detection and Type I cells has not been established yet [13]. Another potentially taste-related molecule could be the renal outer medullary potassium channel (ROMK), which is an ATP-dependent potassium channel that exports potassium from the cells to retain potassium homeostasis [14, 15].

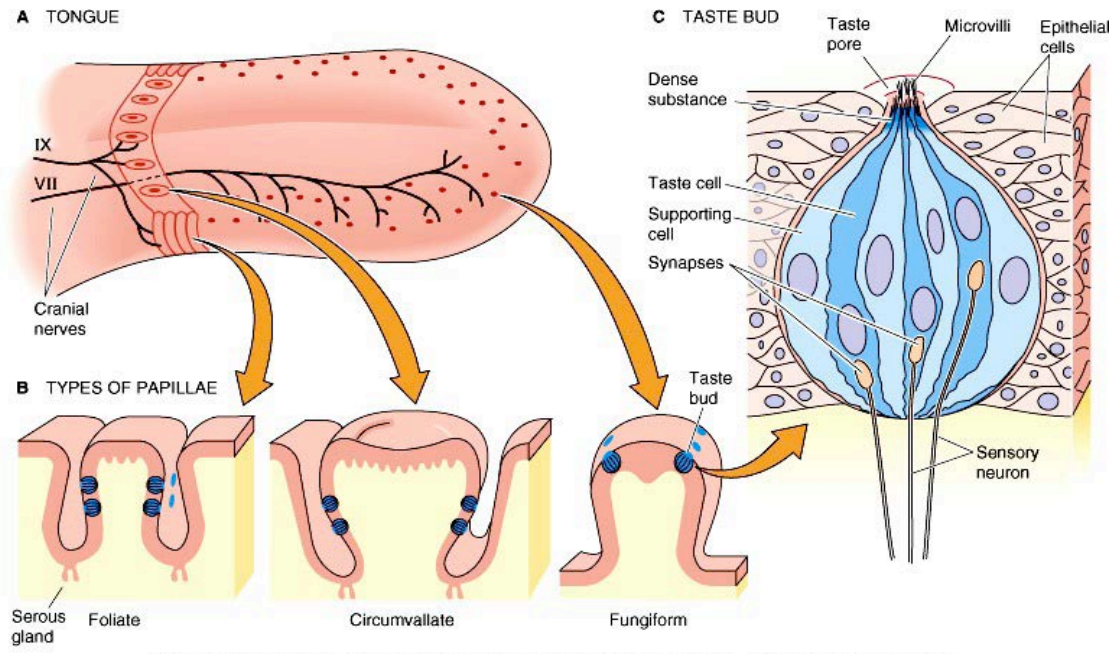


Figure I-2. (A). Distribution of taste buds on the mammalian tongue concentrated within small but visible projections called *papillae.* (B). Papillae are shaped like ridges, pimples, or mushrooms each of a few millimetres in diameter consisting of microvilli, a taste pore and distinct cell types. (C). Each papilla in turn has numerous *taste buds*. Each taste bud is innervated by afferent neurons [9].

I.1.1.2b) Type II (Taste receptor cells)

Type II (taste receptor) cells have large ovoid nuclei and electron–lucent cytoplasm. They express G protein-coupled receptors (GPCRs) with seven transmembrane domains that are located on their plasma membrane. They have been renamed taste receptor cells due to the capacity of these GPCRs to bind ligands that provoke sweet, umami and bitter responses. Historically, the first locus identified in mice that determined the detection of sweet substances was *Saccharin (Sac)* [16-20]. *Sac* encodes the GPCR Tas1R3 and it was Hoon and colleagues that identified two distinct families of taste GPCRs, the taste receptor type 1 (Tas1Rs or T1Rs) dedicated to

sweet and umami responses and the taste receptor type 2 (Tas2Rs or T2Rs) that bind bitter compounds [21].

I.1.1.2bi) Taste specific receptors

The Tas1Rs form heterodimers, Tas1R1/Tas1R3 responding to amino acids (umami taste) and Tas1R2/Tas1R3 responding to sweet. Tas1R1/Tas1R2 receptors respond to amino acids such as glutamate, aspartate and glucine. Ligands specific for Tas1R2/Tas1R3 receptors are sugars (sucrose, fructose, glucose, lactose) and artificial sweeteners (saccharin, sucralose, aspartame). In contrast, the Ta2Rs do not dimerize and respond to bitter tastants such as denatonium, caffeine, quinine, phenythiourea and humolone. Tas1Rs are receptors with long N termini containing globular extracellular ligand-binding domain whereas Tas2Rs have short extracellular N termini (fig.I-7).

Inactivation of the *tas1r2* and *tas1r3* genes in mice revealed that these receptors are required in sweet taste detection. *tas1r2-/-* or *tas1r3-/-* mice present weak preference to sweet tastants though *tas1r2-/-;tas1r3-/-* eliminate completely sweet tastants response. Similarly *tas1r1-/- or tas1r3-/-* knockout mice fail completely to recognise umami tastants [22, 23]. Mice null for specific *tas2rs* lose their sensitivity to bitter compounds. Additionally, genetically engineered mice expressing specifically human receptors for a subset of bitter substances such as salicin and phenylthiocarbamide showed a repulsive behaviour for these two substances. Furthermore, mice expressing κ-opioid receptor (RASSL;receptor activated solely by a synthetic ligand) in T1R2+T1R3 expressing cells become specifically attracted to a κ-opioid antagonist spiradoline, a normally tasteless compound, demonstrating that the activation of sweet–receptor expressing cells (and not the receptor itself) results in sweet perception. Similarly, when the RASSL receptor was expressed in bitter expressing cells the genetically engineered mice showed aversion for the tasteless spiradoline [23, 24].

But how only these two families of receptors (Tas1Rs and Tas2Rs) are able to distinguish the wide variety of tastants exposed in our everyday life? The differences in

the sequences of Tas1R and Tas2R among species reflect their sensitivity and selectivity of sweet or bitter compounds respectively (fig.I-3). For instance, an elegant study used chimeric human-rodent Tas1R2 and Tas1R3 receptors to map regions of the T1R2/T1R3 dimer that are required for responsiveness to some of these sweeteners. These experiments, largely performed using heterologous cells, revealed that the extracellular domain of human Tas1R2 is required for receptor responses to aspartame and neotame [25], the cysteine-rich domain (CRD) of human Tas1R3 is required for responses to the protein brazzein [26], and the transmembrane domain (TMD) of human Tas1R3 is necessary for responses to cyclamate and neohesperidin dihydrochalcone [27]. A creative *in vivo* experiment in which a mouse was "humanized" with the transgenic expression of the human Tas1R2 subunit showed that this protein confers human-like taste sensitivity to aspartame, monellin and other sweeteners [23].

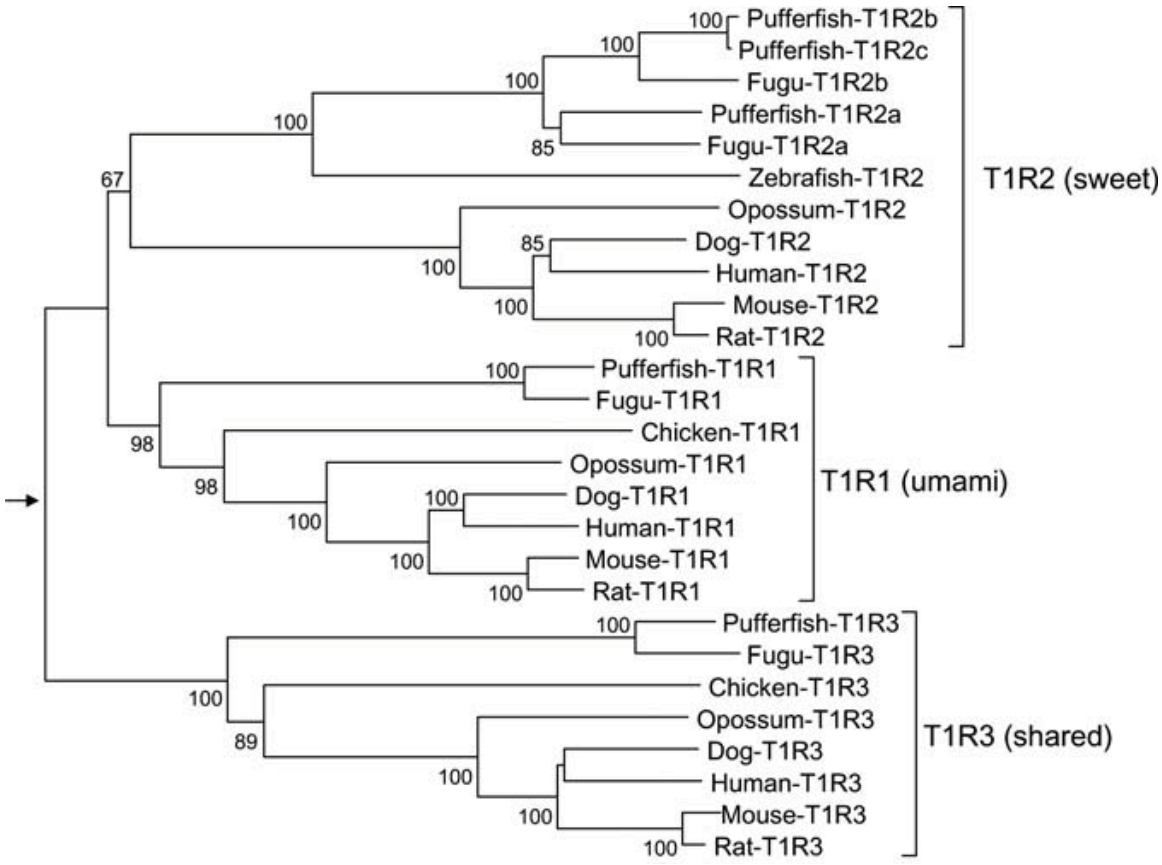


Figure I-3. Phylogenetic relationships of vertebrate intact T1R genes. The arrow points to the tree root, which is determined by using vertebrate V2R genes as outgroups [28]. The T1R1 + T1R3 heteromeric receptor can detect L-amino acids and MSG, which is the taste of umami, whereas the T1R2 + T1R3 heteromeric receptor can respond broadly to sweet tastants [29].

I.1.1.2bii) Taste transduction in Type II cells

Among the G proteins, gustducin is coupled to the TasR in Type II cells and involved in responses to sweet, bitter, and umami compounds [30-33]. Inactivation of the *α-gustducin* gene in mice results in reduced responses to bitter (denatonium benzoate, quinine sulfate) or sweet compounds (sucrose). When a tastant binds to a taste receptor TasR, the G-protein gustducin subunits G β 3 and G γ 13 are released and activate the phospholipase beta2 (Plcβ2), a membrane-bound enzyme specifically expressed in the Type II cells. When Plcβ2 is activated it catalyses the plasma membrane phospholipids into two products diacylglycerol (DAG) and 1,4,5 inositol triphosphate (IP3) that serve as secondary messengers. DAG activates a protein kinase C, which phosphorylates and thus activates a great variety of proteins. IP3 regulates intracellular levels of calcium by opening calcium channels of the endoplasmic reticulum. Cytoplasmatic calcium release can regulate the function of taste-related channels. Trpm5, a member of the transient receptor potential (Trp) channel family, is expressed selectively in Type II cells [12, 22, 34, 35]. Electrophysiological studies of heterologously expressed Trpm5 showed that it is a Ca^{2+}-activated cation channel [36-38] suggesting that Trpm5 functions within the TasR-gustducin-PLCβ2-IP3 pathway to depolarize the Type II cells and lead to neurotransmitter release. Trpm5-/- mice had markedly diminished, but not abolished, responses to bitter, sweet, and umami compounds. Sodium influx through TRPM5 channels depolarizes the cell membrane and stimulates voltage-gated Na^+ channels, promoting the generation of an action potential (AP) (fig. I-8). In response to tastant-evoked membrane depolarization, Cx43/Panx1 (gap junction) hemichannels open, promoting ATP release [39] (fig.I-8).

I.1.1.2biii) ATP, the Type II cell transmitter

The outcome of Plcb2, Trpm5 and sodium channel activation is ATP release. ATP is considered as the main transmitter released from Type II cells upon sweet, umami or bitter stimulation. However, there are still two debated arguments concerning ATP. First, about its release, it was thought that is secreted via pannexin 1 hemichannels (Panx1). More recently it was shown that calcium homeostasis modulator 1 (CALHM1), a voltage-gated ion channel 2,3 is specifically expressed in Type II cells. In *calhm1 -/-*

mice voltage-gated currents are severely reduced in Type II cells, ATP release is compromised and these animals have severely impaired perceptions of sweet, bitter and umami compounds, whereas sour and salty taste recognition remains mostly normal [40]. Second, the role of ATP receptors was examined. P2X2 and P2X3 receptors for purinergic ATP are present on the taste sensory ganglia processes that contact taste bud cells. However, P2X2 is also expressed in Type II cells themselves [41-44]. In *p2rx2-/-;p2rx3-/-* mice there is dramatic disruption of sweet bitter and/or umami sense. Finger and col. proposed that this is due to the purinergic receptors located on the sensory ganglia. However, Huang et al, have shown that P2X purinoceptors are necessary for taste transmitter release from Type II cells since in *p2x2-/-;p2x3-/-* mice, normal taste-evoked transmitter secretion is prevented [45] [51]. Therefore, it is unclear whether P2X receptors are required for taste signal transmission either in the Type II cells that release ATP or in the target sensory ganglia, or both (fig.I-6).

In addition, ATP is degraded into ADP and AMP by nucleoside triphosphate diphosphohydrolase 2 (NTPDase2) expressed on the surface of the Type I cells. ADP further stimulates P2Y1 receptors in Type II and AMP is further degraded by other ectonucleotidases [46]. Adenosine, the final product of this degradation, is neuromodulatory signal acting on adenosine A2B receptors expressed also on Type II cells [47, 48]. Binding of ATP and its degradation products on purinergic receptors located on Type II cells themselves results in increase of cytoplasmic Ca^{2+} released from intracellular stores and thus further ATP release from these cells. Finally, ATP does not bind only to autoreceptors in Type II cells and to receptors in sensory ganglia but also on purinergic receptors (P2Y4) in Type III cells (fig. I-4). Therefore, Type II stimulation and consequent ATP release, stimulates indirectly the Type III cells [10] [49] [50] [51] [92] (fig.I-4).

I.1.1.2b.iv) L-glutamate, the 'unknown' in Type II signal taste bud transmission

Despite the debate on the sites of action of ATP during Type II stimulation, it is considered as the main taste transmitter of Type II cells. However, the role of the classic excitatory neurotransmitter L-glutamate remains unclear. Taste receptor cells are stimulated by glutamate as an umami tastant that binds to Tas1R1/Tas1R3. But these

cells express also ionotropic and metabotropic glutamate receptors (fig. I-4) [52-55]. Studies in isolated taste buds by Caicedo et al showed that glutamate targets the taste buds and not released by them (fig. I-5) [56].

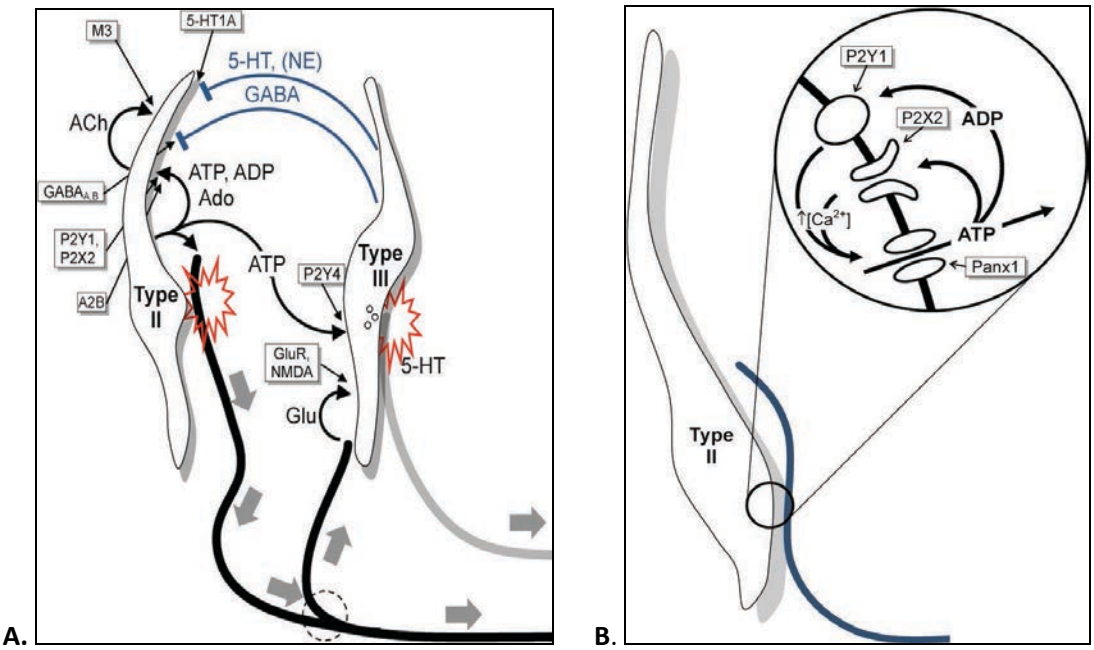


Figure I-4. (A) Synaptic, paracrine, autocrine efferent transmitters and their receptors identified in taste buds [57]. Type II (Receptor cells) respond and transduce sweet, bitter or umami stimuli by GPCRs and secrete ATP to excite sensory afferent fibers (black lines, gray arrows). ATP also excites neighboring Type III (presynaptic) cells. NTPDase degrades ATP within the taste bud to ADP. These purines act as autocrine transmitters to promote ATP release from Type II cells via P2Y1 and P2X2 receptors. Type II also release acetylcholine (ACh), which stimulates receptors on Type II cells. Type III cells respond directly to acid (sour) taste stimuli and indirectly, via ATP, to sweet, umami and bitter stimuli. They also secrete serotonin (5-HT), norepinenephrine (NE) and GABA. 5-HT and GABA are inhibitory transmitters, acting on postsynaptic 5-HT$_{1A}$ and GABA$_{A,B}$ receptors on Type II cells, as shown. 5-HT is also believed to be the neurotransmitter at the synapses thatType III cells form with axon terminals (gray line). Actions of NE have been investigated [58] but the cellular targets and functions for this transmitter are not well established. (B) ATP exerts positive feedback via presynaptic autocrine receptors to promote secretion during taste stimulation. This diagram shows a Type II cell releasing ATP next to a sensory afferent fiber. Type II cells secrete ATP likely via pannexin 1 gap junction hemichannels in the plasma membrane (Panx1). ATP excites postsynaptic targets as well as autocrine P2X2 and P2Y1 receptors on the Type II cell itself. Stimulation of P2X2 and P2Y1 receptors initiates Ca^{2+} influx and Ca^{2+} release from intracellular stores, respectively. Both these actions increase intracellular $[Ca^{2+}]_i$ which further enhances Panx1 activity and promotes ATP release. Genetic ablation of P2X2 (P2X2 knockout) reduces taste-evoked ATP secretion from Type II cells and thus taste reception [44, 45].

In particular, they exposed mouse lingual epithelium to glutamate and recorded the responses of the cells where glutamate could be differentially distributed on the apical part of the taste bud (tip) or the basolateral part that corresponds to synaptic region. They found that ionotropic glutamate receptors (AMPA/Kainate, NMDA receptors) are present on the basolateral surface of taste receptor cells, suggesting that these are postsynaptic sites. In contrast, vesicular transporters for glutamate (VGLUT1, 2) are present on the afferent fibers that end up and innervate taste buds, leading to the speculation that taste sensory ganglia may release L-glutamate that binds potentially binds to taste bud-located receptors [59]. It must be reminded, that the appropriate concentration to activate synapses is micromolar, opposing the concentration needed for taste receptor stimulation, which is millimolar. Huang et al showed that when glutamate is applied on isolated taste buds at synaptic concentrations, the presynaptic cells secrete serotonin [60], further supporting a glutamate synaptic-related role [59] [60].

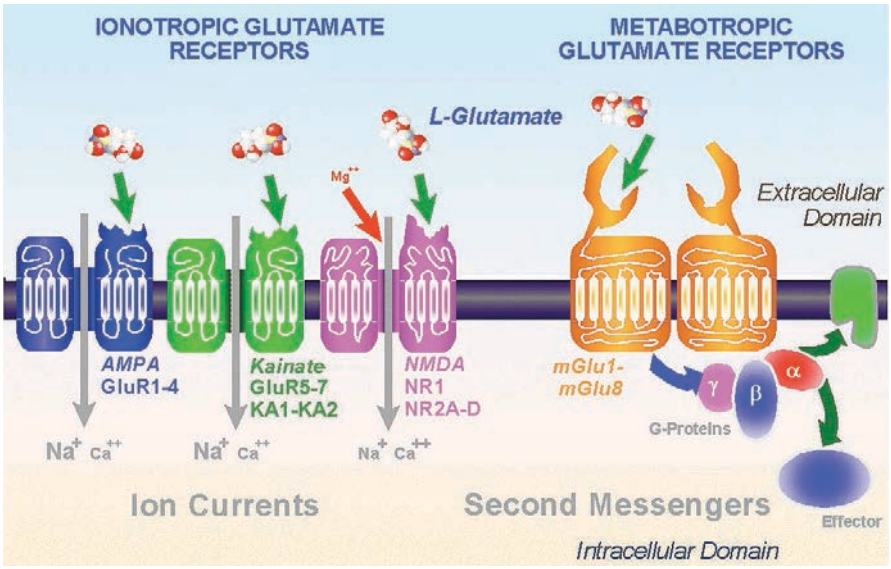


Figure I-5.Ionotropic and metabotropic glutamate receptors. Activation of ionotropic glutamate receptors lead to message transmission via ion currents whereas activation of metabotropic glutamate receptors activate second messengers.

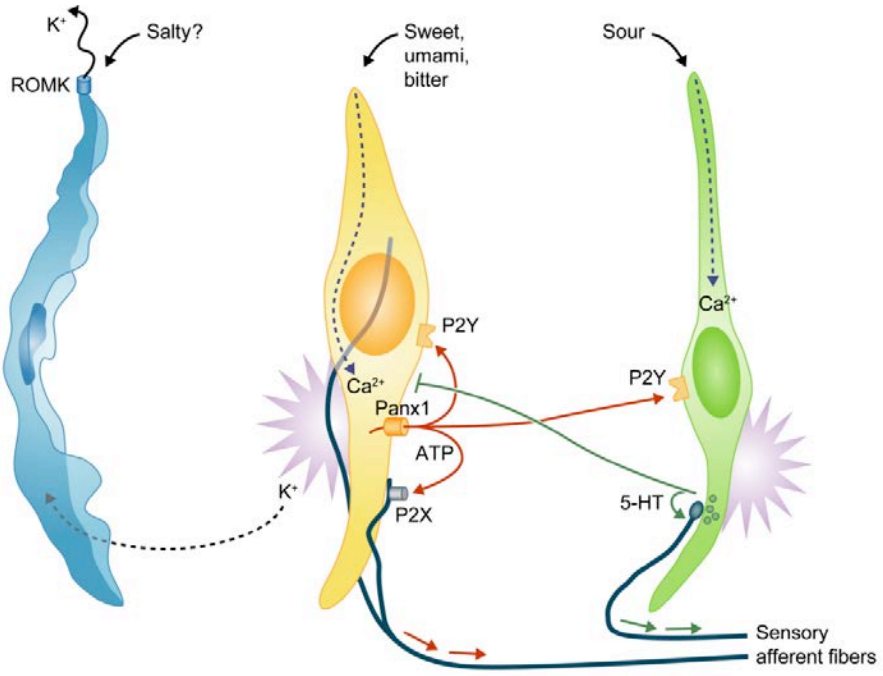


Figure I-6. Three principal classes of taste cells. This categorization includes ultrastructural features, patterns of gene expression and the function of each cell type. Type I cells (Support) in blue degrade or absorb neurotransmitters and might evacuate extracellular K^+ that accumulates after action potentials in Type II cells (Taste receptor) in yellow and Type III cells (Presynaptic) in green. K^+ may be released through apical K channels such as ROMK. Some Type I cells may transduce salty taste, but this still remains uncertain. Sweet, bitter, and umami tastants activate receptor cells, inducing them to release ATP through pannexin1 (Panx1) hemichannels. The extracellular ATP excites ATP receptors (P2X, P2Y) on sensory nerve fibers and on Type II cells. Type III cells, in turn, release serotonin (5-HT), which inhibits Type II cells. Sour stimuli (and carbonation) activate directly Type III cells. Only Type III cells form synapses with nerves.

I.1.1.2c) Type III cells (Presynaptic)

The Type III (presynaptic) cells detect sour and carbonated tastants. In general, acids can block, permeate or activate ion channels and change intracellular pH but the molecules involved in the acid transduction machinery in taste buds are not identified. PKD2L1 and PKD1L3 (for polycystic kidney disease 2-like 1 and 1-like 3 respectively) have been proposed as candidate taste receptors for sour stimuli [61-63]. These channels are sensitive in extracellular pH changes but not to cytoplasmic pH, which is the essential stimulus for sour transduction [64, 65] (fig.I-7). Additionally, PKD1L3 knock-out mice are able to detect sour stimuli indicating that other molecules are required for sour detection at the taste bud level [66].

Type III cells form synapses and express neural-like genes such as neural adhesion molecule (NCAM), synaptosomal-associated protein 25 (SNAP-25) enzymes for neurotransmitter synthesis, transporters and voltage-gated calcium for the release of neurotransmitters [67-70]. They also express sodium and potassium channels and generate action potentials. Upon acid, KCl or ATP stimulation, presynaptic cells secrete 5-HT and GABA [68, 69, 71, 72] and in some cases norepinephrine via calcium influx-dependent vesicular exocytosis [73]. 5HT-1A receptors reside on the surface of the Type II cells and 5-HT binding to them depresses the responses of taste receptor cells to sweet, bitter or umami. When GABA is released, it targets $GABA_A$ and $GABA_B$ receptors also located in Type II cells and promotes GABAergic inhibition of taste-evoked transmitters (Fig. I-4,6).

Type III express also carbonic anhydrase 4 (Car4), a member of a large family of enzymes implicated in sensing, acting on and responding to CO_2 in various systems [74]. Chandrashekar et al developed engineered mice in which specific taste cell types were genetically ablated i.e. sweetless, umamiless or sourless mice and their responses to CO_2 were recorded. Mice without their sour-responsive cells did not respond to CO_2 either, indicating that these sour-responsive cells are essential for CO_2 detection. Since the CO_2 responsive cells are identified, the next step would be to identify the receptors on these cells that respond to CO_2 at the taste bud level.

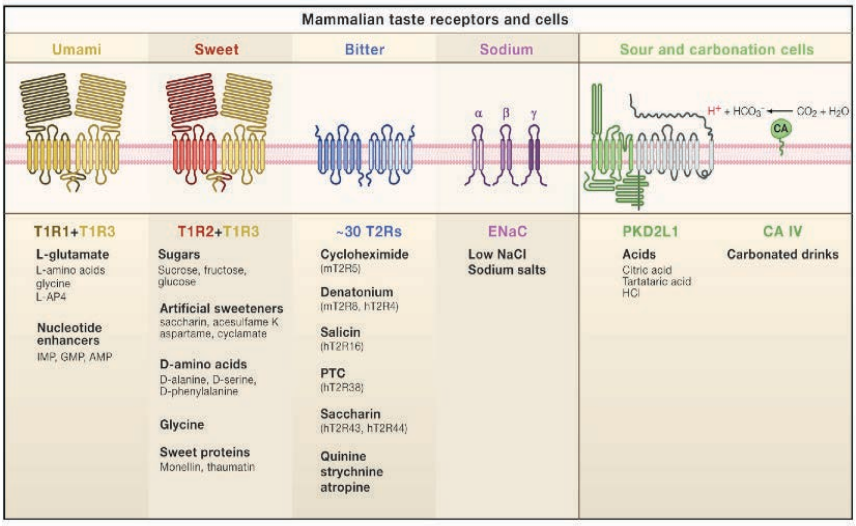


Figure I-7. Distinct classes of mammalian taste receptors, cell types and ligands. Sweet and umami are detected by T1R heterodimers [19] [54] [75] and bitter by T2R receptors [24] [76] [77]. Salt is detected by sodium channel (ENaC) [78]. PKD2L1 is involved in sour detection [61] and carbonation is mediated by membrane-tethered carbonic anhydrase Car4 [74].

THE MAMMALIAN TASTE BUDS IN SUMMARY.

In summary, the three distinct cell types in the taste bud are dedicated to detect different taste stimuli. These taste bud cell types release several neurotransmitters (i.e. type II, ATP; Type III, 5-HT, GABA) that bind to corresponding receptors located either on the same, or neighboring taste bud cell types or the taste ganglia neurons. Taste bud cells communicate with each other and as a result the first level of signal integration takes place within the taste bud (fig.I-8).

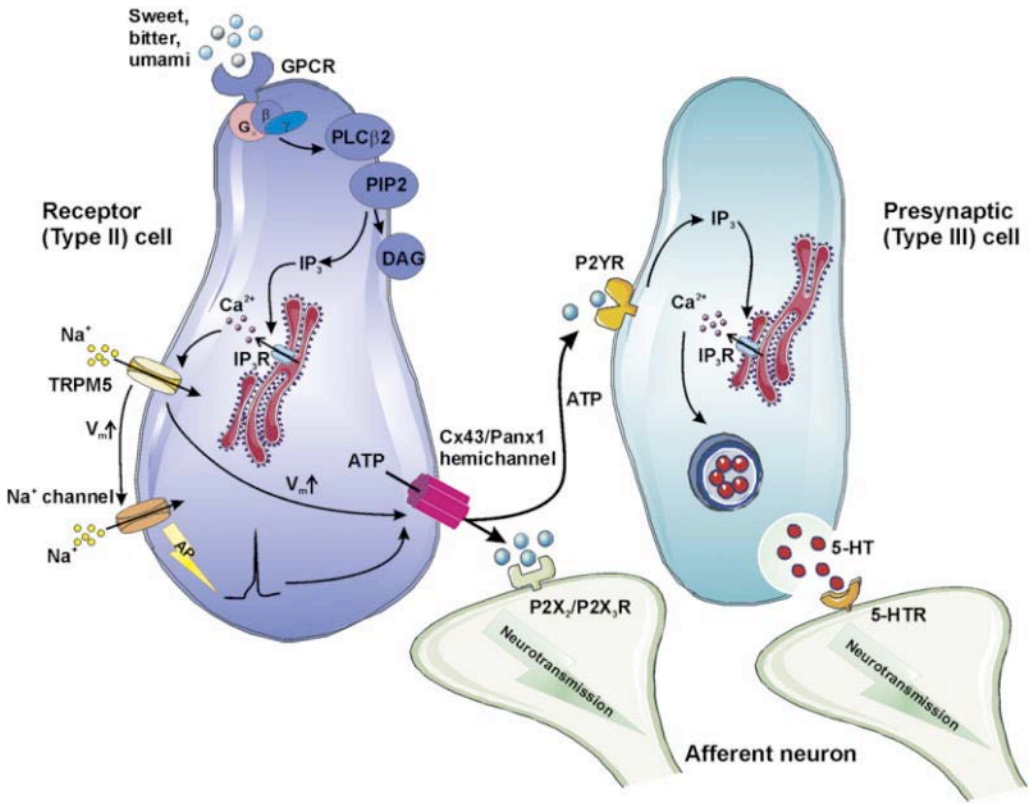


Figure I-8. The paracrine signaling in taste transduction. In taste buds, G-protein coupled receptors (GPCRs) for bitter, sweet and umami tastants are located at the apical membrane of Type II cells. Activation of the GPCR signaling pathway leads to the production of IP3 and diacylglycerol (DAG) through phospholipase Cβ (PLCβ)-mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2). An increase of $[Ca^{2+}]i$ by IP3-induced Ca^{2+} release opens transient receptor potential cation channel subfamily M member 5 (TRPM5). Na^+ influx through TRPM5 channels depolarizes the cell membrane and stimulates voltage-gated Na^+ channels, promoting the generation of an action potential (AP). Available evidence indicates that in response to tastant-evoked membrane depolarization, Cx43/Panx1 hemichannels open, facilitating ATP release. Extracellular ATP is thought to function as a neurotransmitter, targeting P2X2/P2X3R at postsynaptic afferent nerves. Alternatively, ATP may diffuse and bind to P2YR at adjacent presynaptic type III receptor cells. Following elevation of $[Ca^{2+}]i$ by IP3, a second taste transmitter 5-HT is secreted from the presynaptic cells through vesicular release [39].

I.1.2) From the periphery to the CNS: How is taste information transmitted and encoded in mammals

I.1.2.1) Mammalian taste sensory ganglia

In rodents, taste buds receive processes from pseudo-unipolar neurons located in the facial (VI), the glossopharyngeal (IX) and the vagal (X) cranial nerves. This organisation is conserved in jawed vertebrates [79]. In particular, taste buds distributed in the anterior tongue (fungiform papillae) and palate receive processes of the sensory neurons located in the geniculate ganglia (VIIth). These processes invade within the *chorda tympani* nerve and the greater petrosal nerves, respectively. Taste buds in the posterior oral cavity (foliate, circumvallate papillae) receive glossopharyngeal processes from the petrosal ganglia (IXth) (fig.I-9). A small number of taste buds in the epiglottis receive superior laryngeal afferents from the nodose ganglia (Xth) and respond more specifically to the reflex of rejection or ingestion (gag reflex) [80].

Processes from 3-14 sensory neurons reach each taste bud depending on the species. However, each taste bud cell is in contact with a single sensory neuron. Based on the genetic and functional studies described in chapter I, it is evident that each taste bud cell detects one type of taste quality (bitter, sweet, umami, sour or salty), depending on the receptors/channels it expresses. Therefore, taste qualities are encoded in the periphery according to a labelled line model; taste bud cells are tuned to single taste modalities and are connected with individually tuned processes of sensory neurons [81].

However recordings from the *chorda tympani* nerve fibers can show multiple sensitivity upon stimulation and respond to several types of stimuli, suggesting that taste qualities are encoded by an across-fiber pattern. In addition, within the ganglia, neurons that are broadly tuned have been found. [82]. Additional intriguing aspects are that a single T1R2/T1R3 or a T2R expressing taste bud cell can recognise several sweet or bitter types of stimuli, respectively, and that taste bud cells communicate with each other; for instance Type III cells are indirectly activated by Type II -ATP release, suggesting again the existence of some level of broad tuning. Recordings from single units, the gangnlia sensory neurons themselves, but also simultaneous calcium imaging

at the level of the taste buds, ganglia and the primary taste center in the hindbrain (nucleus of the solitary tract) and forebrain taste-related areas in optically clear animals such as zebrafish, could further shed light in the manner that taste information is encoded.

I.1.2.2) Mammalian brain taste-related nuclei

The gustatory efferents of the VII, IX, X nerves synapse on the rostral part of the NTS (rNTS). The caudal part receives also innervation from branches of the vagus nerve, which control gastric motility (visceral) and end of the ipsilateral parabranchial nuclei (PbN). This last respond to the sapid stimuli and continue by sending axons via the ipsilateral reticular formation to the thalamic gustatory cortex [83]. Fibers from the parabranchial gustatory neurons extend to different nuclei; for e.g. the central nucleus of lateral hypothalamus and amygdala as a second (ventral) pathway, but also to midbrain dopaminergic neurons. In primates, the projections of the NTS synapse directly to the ventral posteromedial thalamic nucleus (VPMpc) and PBN transfer visceral data (vagal afferents) to specific regions in the thalamus. The gustatory fibres from the VPMpc terminate in the primary gustatory cortex (GC). GC neurons, in turn, project (reciprocally) to the PBN, to the primary somatosensory cortex, and to the orbitofrontal cortex. The orbital network receives sensory inputs from several modalities related to the intake of food, including olfaction, taste, visceral afferents, somatic sensation and vision (fig. I-9,10) [6] (fig.I-10).

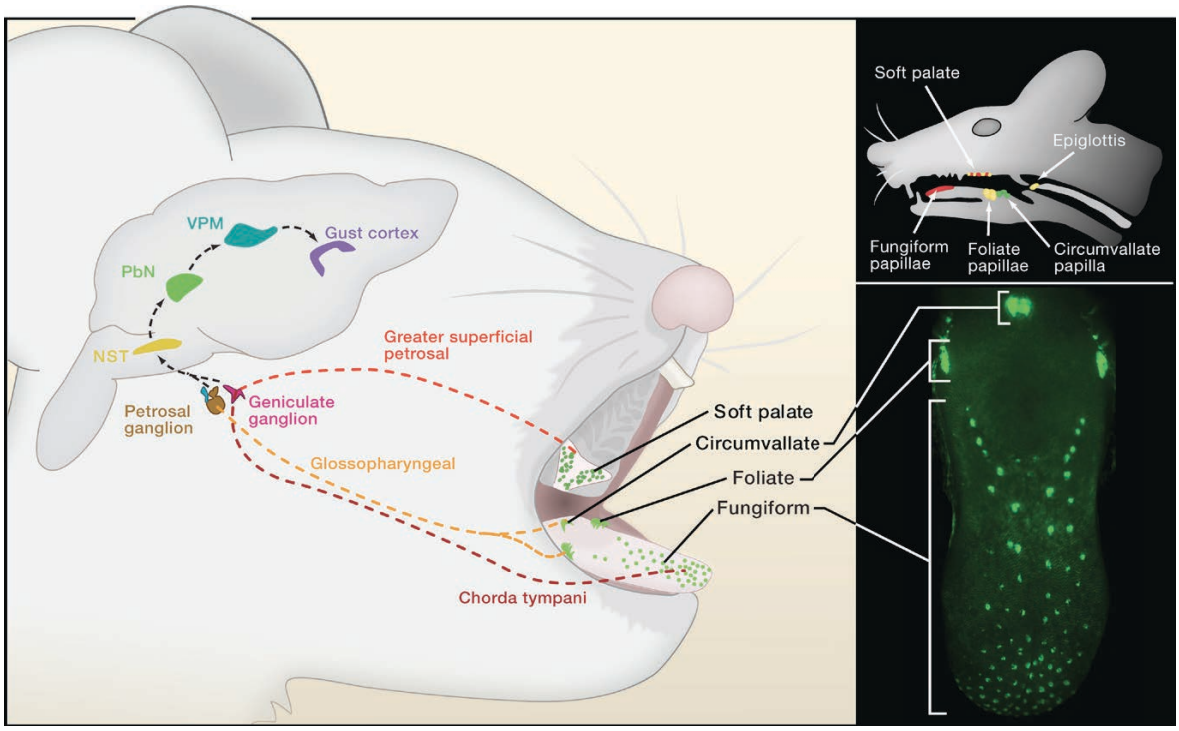


Figure I-9. The anatomy of the taste: Taste buds are distributed on the tongue and soft palate localized into three classes of papillae: In mice, the single circumvallate papilla is found at the very back of the tongue; foliate papillae are at the posterior lateral edges, and fungiform papillae are distributed in the anterior tongue; these three classes of papillae can be highlighted in mice engineered to express green fluorescent protein in taste bud areas. The taste buds on the tongue and palate are innervated by three afferent nerves: the *chorda tympani*, greater superficial petrosal, and glossopharyngeal. These nerves carry taste information from the Type II cells to the nucleus of the solitary tract (NST) in the brain stem. From the NST, taste responses are transmitted (and processed) through the parabrachial nucleus (PbN) and the thalamus (VPM) to the primary gustatory cortex in the insula. Behavioral responses to food (and perceptions of flavor) are ultimately choreographed by the integration of gustatory information with other sensory modalities (such as olfaction, texture, etc.)

The extent of selectivity within the NST, PBN, thalamic nuclei and primary gustatory cortex is variable; some neurons respond to only one stimulus whether others to several. Therefore, 'higher' order neurons seem to be more broadly tuned than those in the periphery. In other sensory systems (visual, auditory), there are spatial maps; neurons in a given area, respond to particular type of stimulus; and this topographic organisation could be expected for the taste system (fig. I-9,10, 11). Studies using optical imaging of intrinsic signals initially identified four distinctive spatial patterns representing the four distinct taste modalities (sweet, bitter, salty and sour), but no region was clearly specific to a single modality. In humans, a functional magnetic resonance imaging (fMRI) study showed, despite a considerable inter-individual

variability, that the five taste modalities evoke specific patterns with some overlap [84].

Using a combination of genetic tracing, extracellular recordings and calcium imaging to achieve single cell resolution, it was shown that with the exception of the sour taste, each taste quality is represented in a seperate cortical field, and thus a taste map is present in the brain [85].

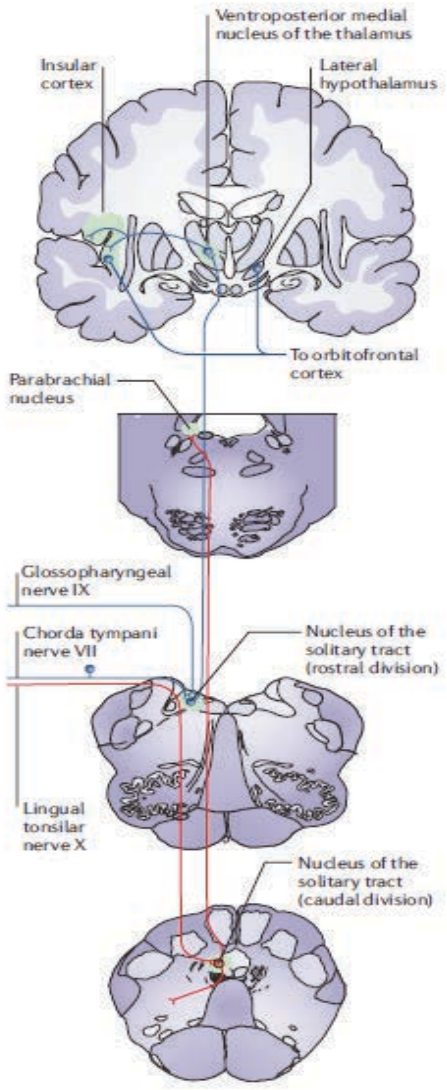


Figure I-10. Anatomical overview of the central taste pathways. Electrical signals from cranial nerves VII, IX and X that contain information on the chemical properties of tastants are conveyed to the rostral division of the nucleus of the solitary tract (rNTS) of the medulla, the principal visceral sensory nucleus of the brainstem. In the rat, second- order fibres (rNTS efferents) project ipsilaterally to gustatory centres in parabrachial nuclei (PBN) of the pons, from where a first (dorsal) pathway projects to the parvicellular part of the ventroposterior medial nucleus of the thalamus (VPMpc), the taste thalamic nucleus. The second (ventral) pathway includes direct projections from PBN to the central nucleus of the amygdala and lateral hypothalamus. In primates, however, the NTS projection fibres bypass the PBN join the central tegmental tract and synapse directly into the VPMpc, whereas the PBN seems to convey general visceral information (mainly through vagal afferents) to specialized thalamic nuclei. In either case, thalamic afferents then project to the primary gustatory cortex, which is defined as the VPMpc cortical target. The VPMpc also sends projections to regions neighbouring the primary somatosensory cortex, adjacent to the precentral gyrus, and that overlap with cortical somatotopic sites for the face and oral cavity. The primary taste cortex projects to the central nucleus of the amygdala, from where gustatory information reaches the lateral hypothalamus and midbrain dopaminergic regions. The primary taste cortex also projects anteriorly to the caudolateral orbitofrontal region, called the secondary taste cortex. Taste neurons in the caudolateral orbitofrontal cortex converge with cells receiving projections from the primary olfactory cortex, which might have implications for flavour perception. The orbitofrontal cortex is also targeted by projections from the lateral hypothalamus, allowing taste responses to be modulated by satiety states. Finally, cortical taste areas send afferents to the rNTS/PBN, allowing for top-down modulation of gustatory processing at the level of the brainstem. Blue, projections to rNTS; green, primary taste areas; red, projections to caudal NTS [86].

I.2) ANATOMY AND FUNCTION OF THE TELEOST FISH TASTE SYSTEM

I.2.1) Teleost fish taste buds

The taste system in teleosts is organised at three levels as described in mammals; the taste buds, the taste sensory ganglia and the taste-related brain nuclei. [87, 88]. The zebrafish taste buds are patterned along the anteroposterior axis of the mouth, palate and pharyngeal arches. In the adult, there are also located on the barbels and epidermis. In contrast to mammals, they are located directly on the oropharyngeal epithelium, and not ensheathed into papilla.

According to their electron-density, shown by electron microscopy analysis, the zebrafish taste cells are separated in three distinct groups; the dark cells, with small microvilli, the light cells with one large microvillus on the apical end and the intermediate or basal cells on the bottom of the taste bud cluster adjacent on the other two types [89-99]. A clear comparison between these morphological characteristics and the mammalian Type I, II and III cell types has not been clearly established. However, several molecular characteristics of the zebrafish taste bud cell types support that the structure and possibly function of these organs is conserved between teleost and mammals.

First of all, zebrafish and in general teleost taste bud cells express taste receptor genes that belong to the same family as their mammalian orthologs. Fish have single *Tas1r1* and *Tas1r3* gene orthologs and several types of *Tas1r2* genes in their genome [100, 101]. But due to the expansion of *Tas1r2* genes, the expression patterns of Tas1r proteins in fish taste buds are diverse compared to those in rodents [100, 101].

An important difference between rodents and fish is that fish do not sense sweet. Indeed fish taste-related facial nerves did not respond to any taste substances that humans perceive as sweet [102]. Consistently, cultured cells expressing any combination of Tas1r proteins from zebrafish and medaka do not respond to “sweet” substances but are activated by L-amino acids in the same way as mammalian *Tas1r1*/*Tas1r3*-expressing umami taste cells [102].

The lack of sweet taste perception in fish is rather not an exception. *tas1r2* gene is a pseudogene in feline genome (cats do not prefer sugar either) [103] and the chicken genome lacks the *Tas1r2* gene entirely [104]. Together with the fact that fish have multiple *Tas1r2* genes, it is intriguingly evident that *Tas1r2* genes are far more divergent than are *Tas1r1* and *Tas1r3* genes. Tas1r-mediated taste may be originally due to L-amino acids and sweet taste may be a newly acquired taste in some mammalian species through the evolution of *Tas1r2* [105].

tas2r-expressing cells in zebrafish function as detectors of harmful chemicals and may trigger self-defensive responses such as avoidance. There are 5 Tas2r genes in zebrafish but as the genome has not been fully annotated, other genes may belong to this subfamily. Intriguingly, *Tas2r* genes in teleost fish are phylogenetically different from tetrapod *Tas2r* genes, and the fish denatonium receptors zfT2R5 and mfT2R1 are not orthologs of the mouse denatonium receptor mTas2r108 (mouse T2R8) [106]. However, zebrafish avoid eating a diet that contains denatonium [102] suggesting that Tas2rs of some type are involved in avoidance feeding behaviors in fish as well as in mammals.

In zebrafish taste buds, a minor but significant population of taste cells expresses both *Tas1rs* and *Tas2rs* [107]. Considering that zebrafish prefers amino acids that are received by various Tas1r heterooligomers [102] the activation of taste cells coexpressing *Tas1rs* and *Tas2rs* should result in attraction. It is unclear whether zebrafish would like or dislike the substances detected by zfT2Rs other than zfT2R5. Interestingly, the expression of *zfT2R5* is completely segregated from that of *Tas1rs* [102] so the cells expressing *zfT2R5*, causing averse responses, are distinct from those causing attraction responses, even in zebrafish (fig. I-11).

A second argument in favour of the conservation of taste bud Type II cells in fish is that a specific subset of fish taste bud cells expresses Plcβ2, Trpm5 and calcium-binding proteins suggesting that the transduction machinery is similar to mammals. Third, *tph1b/*5-HT cells have been described in zebrafish but they are located in a basal position relative to the other cell types within the taste bud [108]. This type of taste bud organisation is also present in amphibians. There are no physiological data available

about the cell type that detects sour in fish. Nevertheless, there is a clear response to acid and pH changes described in channel fish linked to feeding behaviour. The Japanese sea catfish *Plotosus japonicus* senses local pH-associated increases in H^+/CO_2 equivalent to a decrease of ≤0.1 pH unit in ambient seawater provoked by polychaete worms and detects prey in this way [109]. Perhaps more enigmatic is the presence of Type I (support cells) in fish as GLAST, NTPdase and EnaC expression and /or function have not been analysed in these species.

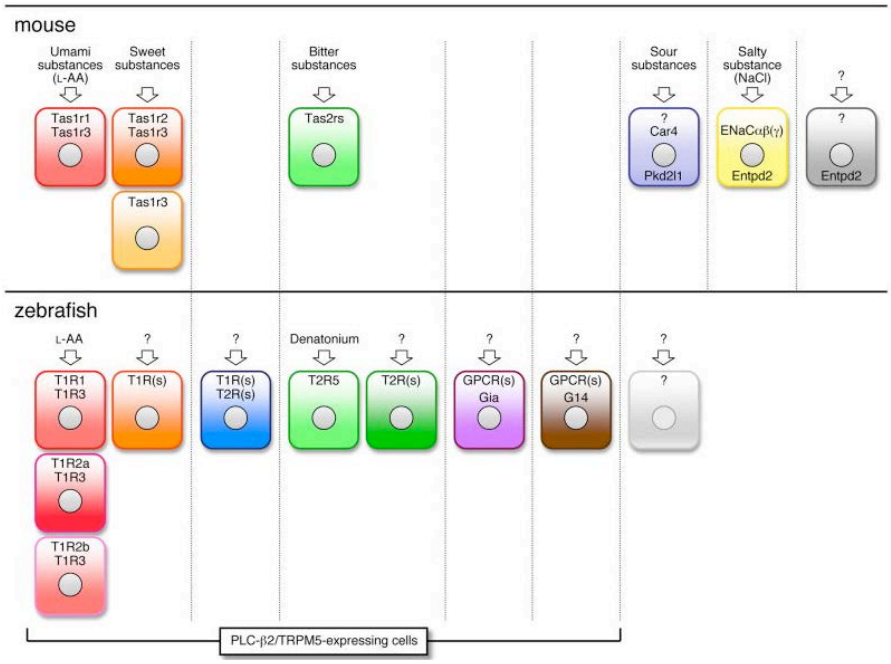


Figure I-11. Diverse array of taste cells in mouse and zebrafish. Types of taste cells are illustrated with specific molecular features of taste receptors and corresponding markers. The identified ligands are indicated though unidentified receptors and ligands are shown by question marks. The unidentified receptors in PLC-β2/TRPM5-expressing taste cells are presumably GPCRs [32]. Pkd2l1 is not a sour receptor channel, because its knockout had little effect to sour response in gustatory neurons [70]. ENaC is necessary for NaCl attraction [48], but it remains unclear whether is the specific salty receptor. Many types of T1R(s)-expressing and T1R(s)/T2R(s)-expressing cells exist in zebrafish, depending on the expression pattern of receptor genes. Taste buds in zebrafish have many cells that do not express PLC-β2 or TRPM5, although little is known for their molecular features [110].

I.2.2) Taste innervation and brain nuclei in teleost fish

As in mammals, three taste sensory ganglia (VII, IX, X) convey taste information in fish. In particular, taste buds on the body surface and oral regions are innervated by the facial (VIIth) nerve, while taste buds located in the pharyngobranchial regions are innervated by the glossopharyngeal (VIIth) and vagal (Vth) nerves respecting an anteroposterior order [111].

Within the brain, silurian and cyprinid teleost fish (including zebrafish) have elaborated specialized nuclei called facial, glossopharyngeal and vagal lobes that are homologous to the primary taste center in the mammalian hindbrain which is the anterior part of nucleus of the solitary tract. The taste-related sensory fibers of the facial nerve terminate in the facial lobe, those of the glossopharyngeal in the glossopharyngeal lobe and those of the vagal nerve into the vagal lobe. In some species such as goldfish, the vagal lobe has an extremely elaborated organisation in layers (like a mammalian cortex). The sensory layers (homologous to the mammalian anterior nucleus of the solitary tract) are coupled to motor layers (homologous to nucleus ambiguus) via a simple reflex arc and serve to rapid food sorting and intake [112].

The taste-related fish lobes are connected with more rostrally located nuclei including the secondary gustatory nucleus in zebrafish (ventral to the cerebellum), the tertiary preglomerular complex nucleus (possibly homologous to taste-related thalamic relay in mammals) and the medial part of the dorsal telencephalon (Dm). In catfish, the vagal lobe has reciprocal connections with the ipsilateral lobobulbar nucleus and facial lobe. However, how taste information is transmitted and encoded in the teleost brain is unknown.

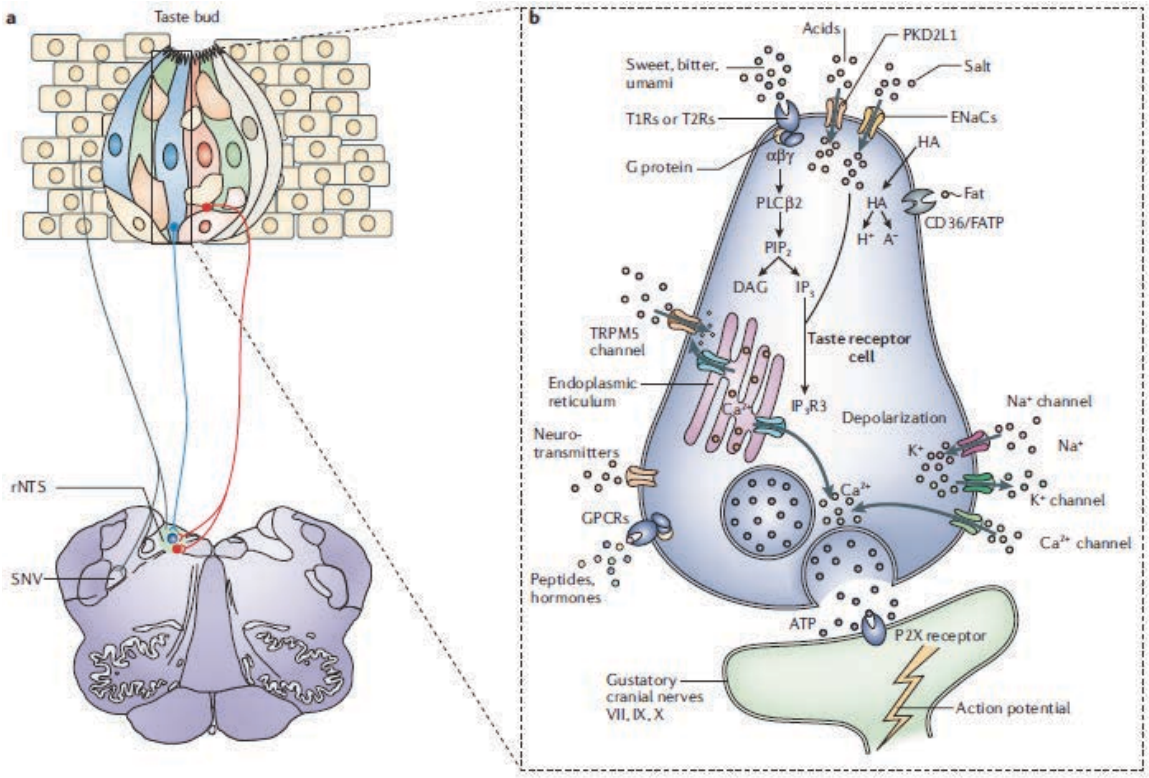


Figure I-12. **(*a*)** Schematic diagram of a taste bud, taste receptor cell and associated neurons. a) Illustration of a taste bud that is embedded in an epithelium. The different types of Type II cells are indicated by different colours as they can contain different types of receptor and intracellular modulator. The gustatory neurons with the associated colours that match the associated Type II cells indicate that they might respond best to stimuli that activate these particular Type II cells. These primary gustatory neurons project ipsilaterally to the rostral nucleus of the solitary tract (rNTS). The black coloured axon that is embedded in the epithelia that surrounds the taste bud is likely to be a nociceptor. These neurons project ipsilaterally to the spinal nucleus of the trigeminal cranial nerve (SNV) and have collaterals that project to the rNTS. **(*b*)** Diagram of a generic Type II cell with an associated neuron. The apical membrane of this cell contains receptors for tastants that are not necessarily in the same Type II cell. These receptors include G-protein-coupled receptors (GPCRs) for amino acids (T1R1/T1R3), sweet tastants (T1R2/T1R3), bitter tastants (T2Rs) and for long chain fatty acids (CD36/FATP). The GPCRs and ion channels in the basolateral membrane are responsive to peptides and hormones, and neurotransmitters, respectively. Ion channels that are likely to be involved in salt taste (epithelial sodium channels, ENaCs) and acid taste (PKD2L1) are also on the apical membrane. The undissociated form of the acid (HA) diffuses into the Type II cells, and protons, sodium and calcium could permeate through PKD2L1 channels. The basolateral membranes of selected TRCs contain TRPM5 channels. Also shown are intracellular pathways that include α-gustducin and PLCβ2, which degrades phosphatidylinositol-4,5-bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). IP3 could then bind to and activate IP3R3 receptors on the endoplasmic reticulum that release calcium. The increase in calcium could activate TRPM5 receptors and cause transmitters such as ATP to be released from synaptic vesicles to bind to their receptors on primary neurons. In other Type II cells, such as those activated by NaCl, their depolarization might evoke action potentials through the activation of voltage-dependent sodium, potassium and calcium channels.

I.3) DEVELOPMENT OF TASTE SENSORY ORGANS

Although the taste system is important for the feeding behaviour little is known about the taste bud development. The cellular and molecular interactions underlying the development of taste buds have been the focus of several studies during the last twenty years. The formation of the taste bud organ could be divided in three phases, the induction of epithelial cells into a taste bud fate, the differentiation of distinct cell types and their assembly in a functional sensory organ. As taste bud cells are renewed throughout life and destroyed under pathological conditions and treatments (i.e. chemotherapy), an increasing amount of research work is dedicated into the mechanisms of their formation (Fig. I-13-15).

I.3.1) The embryonic origin of taste bud cells

The taste buds are unique sensory organs in the sense that they are generated from epithelial cells deriving from two different tissues, ectoderm or endoderm, depending their oropharyngeal localization, anterior or posterior, respectively [113]. Despite the different tissue of origin only a few differences are observed between anterior and posterior taste buds, concerning the expression of paralog genes [114, 115]. Therefore, it seems that although the tissue of origin and perhaps to some extent the signaling molecules differ during the process of taste bud formation, the outcome is rather similar in terms of organization and function of these sensory organs.

The epithelial origin of taste buds was firstly supported by studies done in amphibians and mice. In amphibians, ectopic isotopic and isochronic grafts of placodal ectoderm or neural folds, failed to generate taste buds. In contrast, DiI labelling of the presumptive oropharyngeal endoderm during gastrulation, resulted in DiI labelling of pharyngeal taste buds. Additionally, Stone et al analysed tongues of X chromosome-inactivation mosaic mice to examine whether taste buds originate from local epithelium or neurogenic ectoderm. The *β*-gal phenotype of both fungiform (anterior) and circumvallate (posterior) taste buds matched with the surrounding epithelium suggesting that both tissues share the same taste progenitor cells derived from local epithelium [116, 117]. More recently, Rothova et al, used Sox17-2A-iCre/Rosa26

reporter mice to follow the fate of the endoderm and found that circumvallate and foliate papillae taste buds are of endodermal origin. As all taste buds are derived from epithelial cells and the anterior tongue epithelium is ectodermal derivative, it is assumed that the anterior taste buds are of ectodermal origin [118].

I.3.2) Taste bud placode : the first morphological evidence of a mammalian taste bud

In mammals, taste buds start to develop at E11.5 to E12.5 where epithelial cells invaginate at particular sites of the developing tongue and form the so-called 'taste placodes'. Placodes or papilla placodes refers to a group of cells of the early stages of papilla development and independent from the neurogenic placodes. The precise steps of the morphogenetic movements that lead to the taste bud formation from the initial placode are poorly understood. From fixed mouse tissue, it seems that the epithelial placode thickens and surrounds the proximal mesenchyme. Additionally, a cluster of epithelial cells presents different morphology with a denser cytoplasm and fewer microvilli and this is considered as an indication of taste bud and surrounding papilla formation [119, 120]. During these early developmental steps, taste placodes are identifiable by the expression of Shh. Using inducible *Shh*creERT2 mice, Thirumangalathu et al, showed that Shh-expressing embryonic taste placodes are taste bud progenitors, which give rise to at least two different adult taste cell types, but do not contribute to taste papillae [121, 122].

I.3.3) The role of mesenchyme and innervation during taste bud development

In fish and amphibians, where taste buds lay within the epithelium, and not included in papillae, mesenchyme and neural crest derivatives in general, are not thought to contribute to taste bud development [123]. Indeed, so far all known signaling molecules required for taste bud development are provided by the epithelium itself [108, 124]. In contrast in mammals, subepithelial mesenchyme secrets several signals such as Fgf and Follistatin necessary for taste bud development and therefore although it does not give rise to taste bud cells, it is required for their development.

In fish, taste sensory ganglia processes reach the developing taste buds after the formation of Type III (5-HT) cells, the earliest forming taste bud cells that have been identified so far (*Soulika et al.; unpublished results*). This observation suggests that taste bud formation in these species is independent of the ganglia. In this respect, ectopic endodermal grafts in the axolotl give rise to taste buds without the presence of innervation [125]. In mice, intact innervation is not required for taste placodes formation or taste bud induction but it is necessary for taste bud differentiation during the first postnatal week. In particular, if the nerve is injured but allowed to reinnervate the circumvallate papilla, the number of taste buds is permanently diminished[126-128].

I.3.4) Taste bud progenitor cells

The taste bud cells in mammals have a very short half-life in the local epithelium; for presynaptic taste cells is approximately 22 days and for taste receptor cells, 8 days. Therefore, the formation of taste bud cells is a rather rapid process to efficiently compensate their loss. Mitotically active cells have been observed within the basal part of the epithelium, in proximity or adjacent to the papilla and to a lesser extent within the taste buds themselves [113].

Given the diverse localisation of these cells, the first question raised is whether they contribute to taste bud cells, to papilla cells or to keratinocytes of the tongue. Perea-Martinez et al evaluated the incorporation of the newly born cells into the circumvallate papilla of adult mice by using 5-ethylnil-2'-deoxyuridine (EdU), a highly sensitive thymidine analogue. After injection, most EdU incorporated cells were found in the epithelium outside the taste buds. Only a few EdU cells were found within the taste bud (20 cells in 226 taste buds, 4h post injection). The majority of EdU cells appeared within the taste bud with a delay (1 day or more) after injection. These data suggest that the majority, but not all, of taste bud progenitors are located in the epithelium proximal to the taste bud.[113, 129] (fig.I-13).

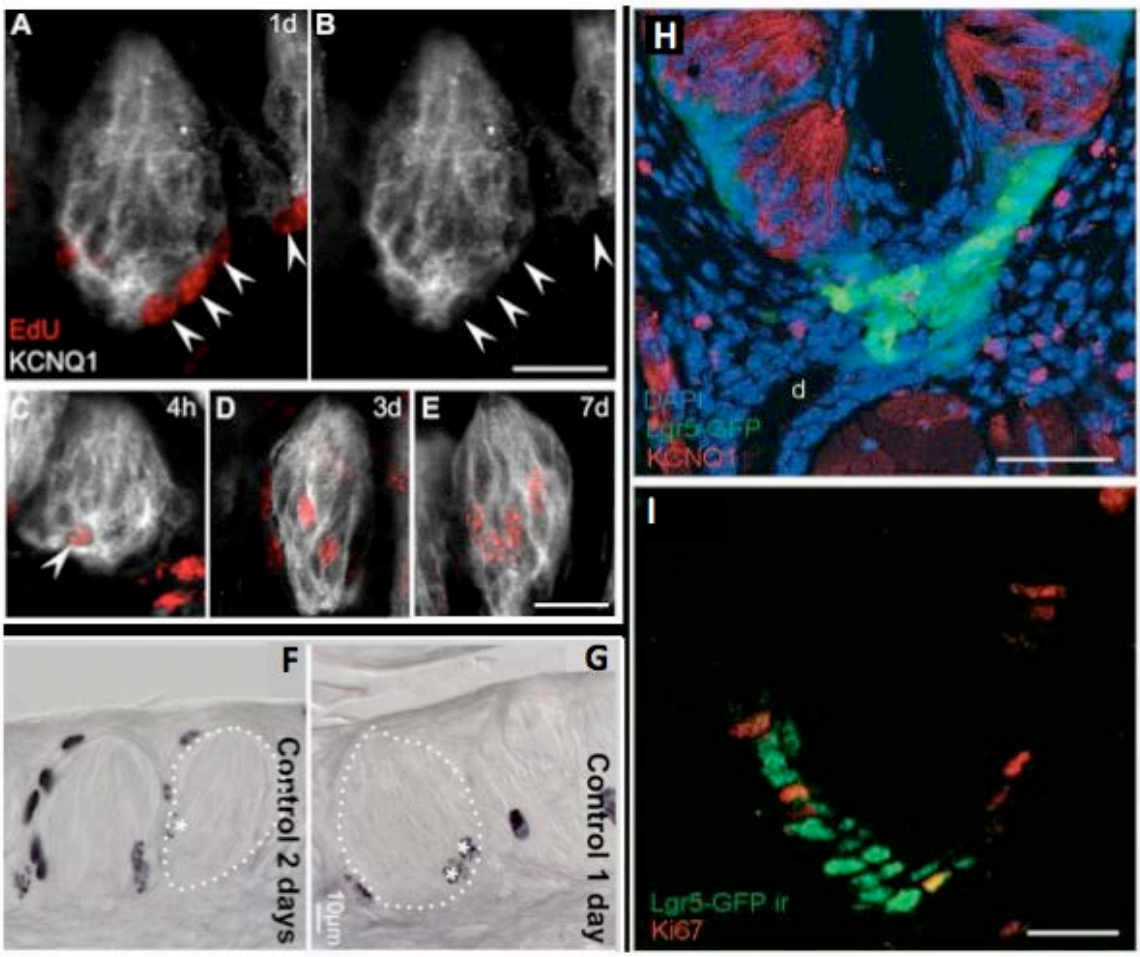


Figure I-13. Taste progenitor cells labelled with EdU (A-E) or BrdU (F,G) or Lgr5-gfp (H,I) are located mainly outside the taste bud.

Genetic mapping analysis using a tamoxifen inducible Keratin-14 Cre recombinase showed that bipotential progenitors located adjacent to the taste buds express Sox2 and generate both taste bud and keratinized epithelial cells. Within this progenitor pool, cells of the keratinocyte lineage lose Sox2 expression whereas cells with taste bud fate upregulate Sox2 [130]. Consistent with this, in the hypomorph Sox2EGFP/LP embryos, filiform papillae are normal but the number of fungiform and palatal taste buds is gradually reduced and then completely absent at birth, indicating that Sox2 activity is necessary for taste bud formation [131].

Finally, lineage-tracing experiments using an inducible Lgr5 (leucine-rich repeat-containing G-protein-coupled receptor) Cre knocking allele in combination with Rosa26-LacZ and Rosa26-tdTomato reporter strains showed that Lgr5-expressing cells gave rise

to taste bud, papilla and self-renewing cells at the bottom of trench areas at the base of CV and foliate papillae.

The second question was whether taste bud progenitor cells represent a unique lineage or will give rise to more than one cell types. Using a fate mapping approach based on the ShhcreER mouse line, Yee et al, showed that Shh-expressing embryonic taste placodes are taste bud progenitors, which give rise to at least two different adult taste cell types (Type I and II). Lgr5-expressing cells co-express markers of Type I, II and III (fig. I-13) [132]. Therefore, a pool of progenitor epithelial cells generates all taste bud cell types but their clonal relationship, if any, remains unknown.

I.3.5) Molecular signaling during taste bud development

Although the function of the taste system has been extensively studied the past 20 years, little is known about the first developmental stages of this sensory system. Several signalling pathways, already known for their role in a variety of tissues throughout development, are likewise involved in taste bud development.

I.3.5.1) Wnt/beta-catenin: key signal for taste placode formation

The Wnt/β-catenin signaling pathway regulates cell fate, cell proliferation and self-renewal of stem and progenitor cells in many tissues [133]. In brief, in the absence of Wnt ligands, β-catenin is degraded after phosphorylation by kinase activity of cytoskeletal-kinase complex that comprises casein kinase 1a (CK1a), glucogen synthase kinase 3 (GSK3), Axin and adenomatous polyposis coli (APC). Upon Wnt ligand binding to Frizzled receptor (seven transmembrane domain-GCPR), the degradation complex becomes inactive, β-catenin is accumulated in the nucleus, binds on lymphoid enhancer-binding factor (LEF) and T-cell factor (TCF) that promote transcription of target genes.

Genetic ablation of β-catenin in the lingual epithelium results in loss of taste placodes. In contrast, when β-catenin is conditionally activated, the entire tongue surface is transformed into an array of taste bud precursors (Shh+). Therefore, Wnt/beta-catenin activity is necessary and sufficient for taste placode formation. The nature of Wnt ligands that regulate this process remains unclear. Wnt10b, although expressed in taste bud placodes, is not required for their formation, since placodes are

intact in Wnt10b-/- mice, suggesting that other ligands should be involved. In addition to *shh,* Wnt/β-catenin signaling promotes *sox2* expression in the lingual epithelium, a transcription factor expressed in taste bud progenitor cells [119, 134, 135].

I.3.5.2) Taste placode patterning is regulated by BMP and Follistatin

BMPs are a large subgroup of secreted factors of the TGFβ superfamily regulating cell proliferation, differentiation, apoptosis and migration [136-138]. BMPs are known to act in organ patterning, for example, as inhibitors of feather placode formation that contribute to spatial distribution of feather buds [139, 140]. BMPs 2, 4, and 7 are expressed in the developing tongue epithelium in a spatiotemporal pattern consistent with a role in taste placode induction and patterning. Their inhibitor, follistatin is secreted by the adjacent mesenchyme [141-144]. Among the BMP antagonists are noggin and follistatin. Noggin binds BMP2, 4 and 7 with high affinity whereas follistatin binds activin with high affinity and BMPs with lower affinity [136, 137]. The inhibitory mechanism of noggin is different from that of follistatin, or chordin, another BMP antagonist [136, 145].

In fst-/- mice, the spatial patterning of taste papillae is lost as revealed by altered Shh expression. The loss of the antagonist *fst* in the mesenchyme results in increased BMP7 expression and signaling within the epithelium. Therefore, the interaction between epithelial BMP7 and mesenchymal FST is required for the correct patterning of taste bud placodes. This interaction must be occurring during a particular time window of taste bud placode induction. Indeed, incubation or beads with BMPs on tongue explants results in increased or decreased number of placodes, depending whether the treatment occurred before or after the stage of placode induction. In the same line, follistatin to cultured tongue explants does not repress taste placode development [135, 146].

I.3.5.3) Shh and taste placode formation

Shh expression is initially broad throughout the developing tongue epithelium, but its expression consolidates in taste placodes as they form. Shh is among the earliest markers expressed in taste bud placodes. Ptch1, a Shh receptor and target, and Gli1, a

downstream target gene, are likewise expressed early in both epithelial and mesenchymal cells of the tongue. When tongue explants are taken prior to formation of taste placodes and cultured in the presence of a function-blocking antibody against Shh [143] or with a Shh pathway inhibitor [147], fungiform taste placodes increase in both size and number. Moreover, when Shh signaling is inhibited, taste placodes form in a posterior domain of the tongue surface, which, in control tissue and intact animals, is always devoid of taste buds. Consistent with this, repressive function of Shh, when tongue explants are cultured with excess Shh ligand, results in dramatically reduced taste placodes [148].

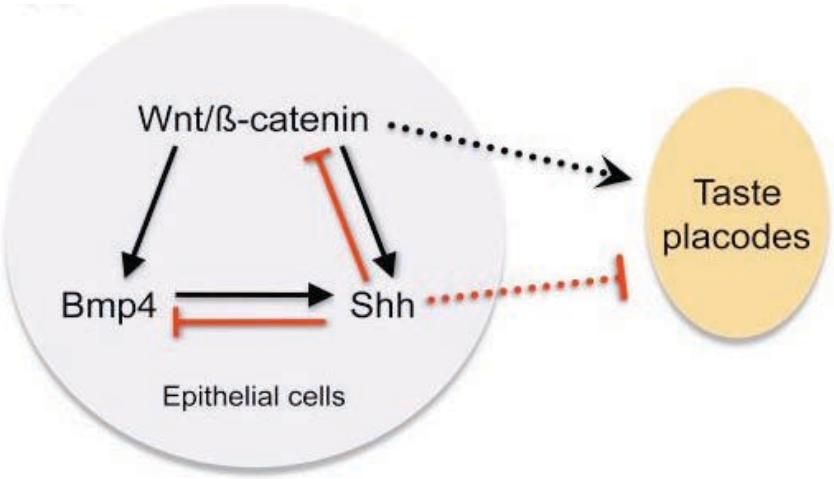


Figure I-14. Wnt/β-catenin signaling is required upstream of Shh and Bmp4 for taste placode specification. Shh represses Wnt and Bmp4, as well as its own expression, while Bmp4, at least early on, is a positive regulator of Shh. Black arrows = positive regulation; red lines = negative regulation. Dashed lines indicate that the Wnt pathway promotes, while Shh inhibits taste placode formation.

Interestingly, when Shh function is inhibited, Shh expression is expanded in the more numerous and enlarged taste placodes [143], indicating that Shh's impact on its own expression is indirect. One candidate for this intermediary is the Wnt/β-catenin pathway. Specifically, β-catenin is required for Shh expression by taste placode cells, placing Shh downstream of Wnt [149]. However, Wnt signaling in taste placodes is also negatively regulated by Shh; in vitro inhibition of Shh results in expanded placode-specific Wnt activity, in an increased number of taste placodes, whereas excess of Shh activity has the opposite effect (fig. I-14) [148]. Remarkably, Shh plays an opposite role in taste bud formation in the adult. It was recently shown that misexpression of SHH transforms adult lingual epithelial cell fate, such that daughter cells of lingual epithelial

progenitors form cell type-replete, onion-shaped taste buds, rather than non-taste, pseudostratified epithelium. These SHH-induced ectopic taste buds contain all cell types and are found in regions of the adult tongue previously thought incapable of generating taste organs [149].

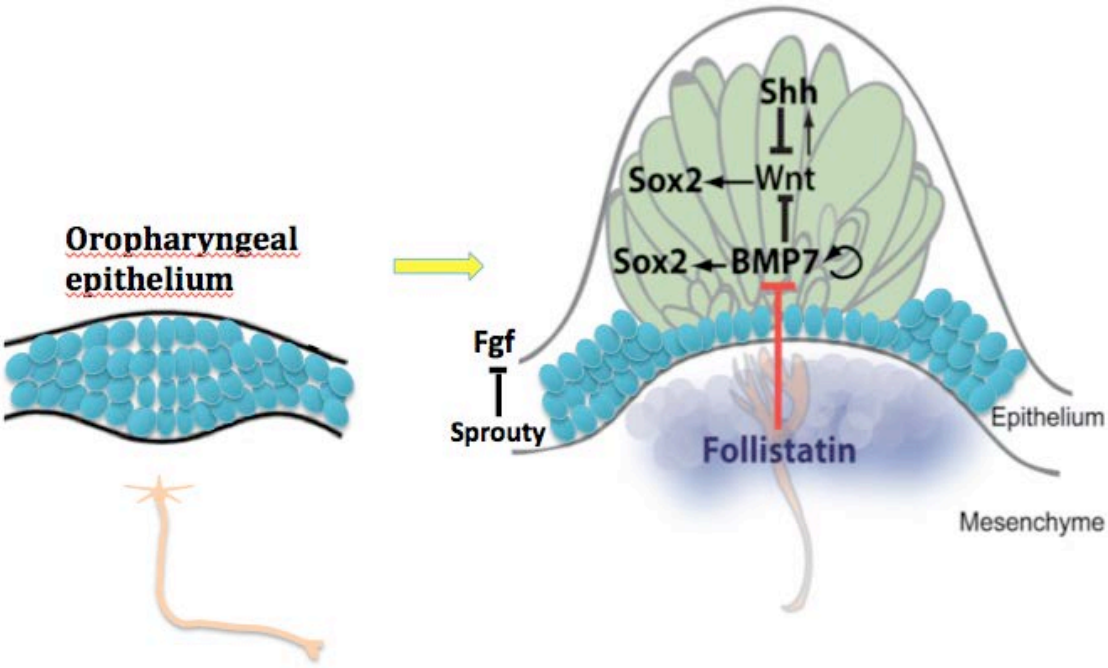


Figure I-15. Taste bud induction in mammals. Fgf and its spouty inhibitors control the number of the progenitor cells. Sox2 is a key molecule for inducing the epithelium into a taste bud fate. Upstream of Sox2 is Wnt/β-catenin. Wnt signaling is inhibited by BMP and Shh and thus pattern the taste buds in the lingual epithelium.

I.3.6) Key signals for the differentiation of taste bud cell types

I.3.6.1) FGF pathway necessary for the differentiation of taste receptor cells

Fgf signaling is activated when two heparan sulfate-connected Fgf ligands bind to extracellular domains of tyrosine kinase receptors [150] and activate many intracellular cascades such as Ras/Raf/Erk1/2, PLCgamma/PKC and PI3K/AKT. Among the Fgf ligands, Fgf10 is expressed in the mouse tongue mesenchyme and Fgf8a in the zebrafish oropharyngeal epithelium and taste bud cells. Among the receptors, Fgfr2b and Fgfr1c are expressed in the mouse tongue at E11 to E13 and *fgfr1, fgfrl1a, fgfrl1b* and *fgfr2* are expressed in the zebrafish pharyngeal arch epithelium at 2dpf [151-155].

Fgf induce the expression of four Sprouty proteins that block the tyrosine kinase activity of the Fgf receptors. In Spry2-/- mice the number of anterior taste placodes (fungiform) is reduced while in the posterior placode (circumvallate) is bigger and the number of taste papillae is increased. Conversely, in fgf10-/- mice the posterior placode is absent and in spry2-/-;fgf10-/- mice the posterior taste bud placode formation is restored. Therefore, Fgf signaling regulates the formation of taste buds but differentially along the anteroposterior axis [156].

In addition to this early requirement for Fgf signaling during taste bud formation, Fgf regulates the cell fate of taste bud cells. Time-controlled, targeted inactivation of Fgf receptor signaling within the pharyngeal epithelium of the zebrafish larva results in severe reduction of Type II cells showing that Fgf signaling is required for the formation of this cell type [108].

I.3.6.2) Notch signaling pathway during taste bud cell differentiation

Upon ligand binding on Notch receptor, this transmembrane protein is cleaved by gamma secretase, and the intracellular part of it is translocated to the nucleus, activates the CSL transcription factor and regulates target gene expression. Notch signaling activates within the cell that expresses this protein, the Hairy enhancer of split (Hes) transcription factors, which inhibit proneural genes such as achaete-scute orthologs (Mash in mouse, Ascl in fish) that promote neuronal fate in the cells that express Notch ligands [157, 158]. Notch ligands are ubiquitinated by RING-type E3 ligases including Mindbomb and get endocytosed.

The role of Notch signaling during taste bud development is not completely elucidated. Among Notch pathway members, Hes1 binds to promoters of both Plcβ2 and Ip3r3 (both expressed by Type II taste receptor cells), while Hes1 protein is localized in the nucleus of undifferentiated taste bud cells. Therefore, Ota and colleagues [159] proposed that Hes1 might repress expression of taste cell specific molecules in precursor cells. Indeed, in Hes1-null mice, the number of Type II/taste receptor cells is increased 5-fold compared to controls suggesting that in the absence of Hes1, aberrant transcription of taste receptor-specific genes takes place in precursor cells.

When Notch activity is blocked in Mindbomb zebrafish mutants, excess Serotonin-expressing taste cells develop, the taste progenitor cell pool is reduced and the number of Calretinin-expressing taste receptor cells is decreased [108]. In a complementary manner, Notch activation just prior to cell differentiation has the opposite effect; Serotonin-expressing cells are absent while the Calretinin-expressing taste receptor cell population is maintained but not increased. These results support the hypothesis that cells with activated Notch signaling maintain a progenitor state as predicted by the mouse model in which Hes1 and therefore its Notch-repressing activity is lost [159]. Consistent with the role of Notch in the formation/maintenance of a progenitor pool for taste bud cells, the severe decrease of taste receptor cells provoked by compromised Fgf signaling in fish is restored by Notch activation [108].

In mouse and zebrafish, *Mash1* and *ascl1*a are specifically expressed in 5-HT cells, respectively. Mash null mice and zebrafish ascl1a-/- mutants are devoid of 5-HT cells. Although in mice, the 5-HT cell loss is not accompanied with any particular taste bud phenotype, in zebrafish, there is slight disorganisation and increase of taste bud Type II cells [160].

I.3.6.3) Mir200 promotes taste bud cell formation

MicroRNAs (miRNAs) are small, non-coding RNA molecules (about 20-22 nucleotides) that regulate gene expression post-transcriptionally. More frequently, they bind to complementary sequence on the 3'UTR of the targeted mRNA, block translation or provoke mRNA degradation.

During taste bud development, the miR-200 family is expressed in Type II cells.. mir-200 knock-down results mainly in severe reduction of type II cells. As Sox2 is a direct miR-200 target [161] and Sox2 expression is excluded from differentiated Type II cells [108] [130, 131, 162], it was proposed that Type II cell differentiation requires downregulation of Sox2 by miR-200. Furthermore, miR-200 expression is regulated by Notch and Fgf activity, suggesting that this miRNA family is at the crossroad of signalisation during Type II cell differentiation [108].

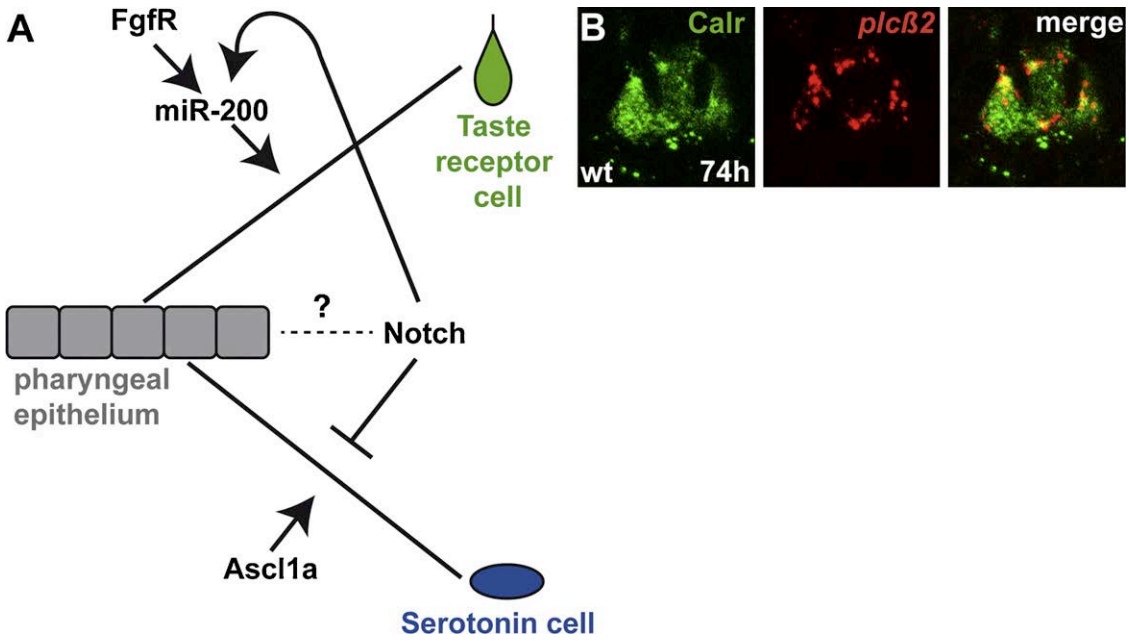


Figure I-16. (A) Schematic representations of the key signals that are required for the late steps of development of taste receptor and Serotonin-expressing cells of the zebrafish taste buds. Fgf receptor (FgfR), miR-200 and Ascl1a activity are required within pharyngeal epithelial cells to drive taste receptor and Serotonin cell formation, respectively. Activation of Notch signalling is necessary for taste receptor cell formation whereas inhibition of this signal is required for Serotonin cell formation. Notch likely has additional functions in taste bud development within the pharyngeal epithelium. (B) A 2µm optical section through a wild-type zebrafish taste bud reveals taste receptor cells co-expressing Calretinin (Calr) and plcβ2.

I.4) Project Goal: in vivo analysis of the cellular interactions during taste bud organ formation

Work over the last two decades has shed light in the molecular interactions that are required for taste placode induction within the oropharyngeal epithelium. There is also increasing evidence about the molecular identity of progenitor cells and notably Shh and Sox2 are considered as faithful markers of early developing taste bud cells. However, it is unclear whether the expression of these genes is maintained throughout the differentiation taste bud process. Indeed, the molecules that regulate specifically the differentiation of each of the three taste bud cell types, has only started to be addressed. Another intriguing aspect of taste bud development is the cellular interactions that are required to assemble differentiating cells into an organ. Most of the progenitor cells are basal and/or external to the forming taste bud organ. Since the

taste bud organ is a functional microcircuit, one would expect that cell position within the organ is not random. Instead, differentiating cells once born, they must reach the forming organ and through interactions with the other cells form an anatomical and functional unit.

During my PhD I addressed precisely this question: how do differentiating taste bud cells, derived from the oropharyngeal epithelium assemble into a functional organ. To achieve this goal, I selected fluorescent transgenic lines with the potential to be expressed in early differentiating taste bud cells. Based on the observation of the in vivo analysis, I combined these lines with cellular and molecular loss of function approaches, to further dissect the role of the Type III cell type during taste bud organ formation.

MATERIALS and METHODS

II.1) Zebrafish lines

The fish were raised in fresh water in a constant temperature of 28°C with a light cycle of 11h of darkness and 13h of light. The produced embryos were raised at 28°-30°C in embryo medium according to the described conditions in the "Zebrafish book" [163]. The transgenic lines used were constructed in the lab or obtained through collaborations. *tg(tph1b :mCherry)* was constructed by using a previously isolated 5kb fragment [108] (*Anna-Lila Kaushik*). mCherry expression was found similar to 5HT expression in the developing taste buds. *tg(fgf8a.enhancer:gfp)* was obtained through collaboration with TS Becker and Anna-Zofia Komicsarsuk [164] (University of Sydney). The *pia*^{t25215} mutation (*ascl1a-/-)* [165] generates a *Hind*III restriction site, which was used as a restriction fragment length polymorphism (RFLP) for genotyping. A 523 bp *ascl1a* fragment containing the polymorphic restriction site was amplified from genomic DNA of single embryos, using primers 5'-TCAGAGCATCCAACTCAGCC-3' and 5'-CGAACGCTCAAAACCAGTTG-3', followed by *Hind*III digest. On wild-type DNA, this results in two fragments of 203 and 320 bp, whereas the mutant PCR product is cleaved into three fragments of 320, 139 and 64 bp. Triple *tg(fgf8a.enhancer:gfp); tg(fgf8a.enhancer:gfp);ascl1a-/-* transgenic embryos were selected through tail genotyping at 24hpf before timelapse or processing of fixed tissue.

II.2) Phenotypic analysis

II.2.1) In situ hybridization

Whole embryos are fixed in 4% parafolmadehyde in Phosphate Buffer Sodium (PBS) for 48 hours. After permeabilisation in methanol, the embryos undergo digestion in proteinase K (10µg/mL). Next to post-fixation the embryos are incubated in pre-hybridization buffer (HYB4 : SSC 5x, formamide 50% , torula yeast RNA (sigma) 5mg/mL, heparine 50µg/mL, Tween-20 0,1%) for one hour at 47, 56 or 60-62°C depending on the probe of interest. Then the embryos are incubated with antisens riboprobes(0,5ng/µL) coupled to digoxygenine (DIG) (Roche, France) and synthetised with RNA polymerase SP6, T7 or T3 (Promega, France). Next, the embryos are stringently washed, followed by an immunohistochemistry with a-DIG antibody coupled with alcaline phosphatase (AP) (Roche) diluted in blocking reagent (Maleic acid buffer 100mM pH 7.2, 150mM NaCl, 0,1%

Tween-20). The embryos are washed in development solution (100mM Tris-HCl pH 8,2, fast red tablets (Roche). After mRNA detection, the embryos are processed for immunohistochemistry.

II.2.2) Immunohistochemistry

Whole embryos were fixed in different stages of development in a fixation solution (paraformaldehyde 4%-PBS 1x) for 1 ½ or 2 hours. After 3 washes in PBTtr the embryos were incubated in the blocking solution. The primary antibodies were: α-GFP (Torrey Pines Biolabs, 1 :1000, rat), α-calretinin (Swant 1 :1000, rabbit or mouse), α-5-HT (Sigma, 1 :500, rabbit), α-pH3 (Beckton Dickinson, 1 :500, rabbit), α-caspase3 active (Beckton Dickinson, 1 :500, rabbit). A secondary antibody coupled to a fluorochrome AlexaFluor A488, A568, A647 (Invitrogen 1 :500) was used to detect the primary antibody. Then the embryos were post fixed, the pharynx was dissected, mounted in glycerol and observed in binocular or confocal microscope.

II.3) Fixed tissue image acquisition and time-lapse imaging

Fluorescent images were obtained by SP5 confocal microscope using 40x oil immersion objectives. Series of images were acquired at 0.5 mm intervals. ImageJ (NIH) was used to analyse stacks, and Adobe Photoshop/Illustrator to adjust brightness/contrast and mount images. Three-dimensional reconstructions and cell counting were carried out using Imaris (Bitplane).

Imaging of embryos' mouth was performed on a Nikon inverted spinning disk equipped with an evolve camera (Photometrics) and Metamorph software. The embryos were mounted in a mix of plasma-thrombine in embryo medium with PTU 0,003% and Tricaine and placed at 28°-30°C on a thermostatic Nikon inverted spinning disk Yokogawa. z-stacks were acquired by an EMCCD Evolve camera through the Metamorph Premier software. To achieve optimal imaging without bleaching and cell damage for long time periods (until 10hpf), z-stacks were acquired every 8 minutes. Filming above this time-length was challenging as it obviously delayed development and therefore was excluded. Embryos were staged according to www.zfin.org. However, between 55hpf and 70hpf, time-period that taste bud organs are initiated, landmarks to stage precisely the embryos

at this age are unavailable. Therefore, the time period cannot be precisely compared between movies.

All the analysis was performed on timelapse of embryos staged between 60hpf and 70hpf on data obtained from images of the first forming taste bud on the most anterior median part of the mouth and its neighbouring (more lateral) taste buds. Similar qualitative phenotypes were observed on palate and anterior mandibular arch. However, timelapse on these areas is more delicate as non taste-bud related epithelium interferes with imaging of the palate and GFP signal from the heart of the *tg(tph1b:mCherry)* line does not allow to follow pharyngeal arch taste buds.

II.4) Timelapse statistical analysis

Timelapse z-stacks were analysed in Imaris (Bitplane). Data on cell or cell group position (center of mass) and speed initially obtained through Imaris were manually verified, combined with cell contact observation and then processed in R to calculate Euclidean distance. All statistical analyses were performed in R (http://www.r-project.org/).

II.5) Two-photon laser ablation

All experiments were performed with a custom-built random-access two-photon laser-scanning microscope [166]. In this instrument, both X and Y scanning are operated by acousto-optic deflectors (AODs). These custom-built nonmechanical beam-steering devices (A-A Opto-Electronic) can redirect the laser beam in 10 µs. To operate the AODs and run the scanning procedures, a custom-made user interface was programmed in LabView (National Instruments). The AOD acoustic frequency drive was generated by a Direct Digital Synthesizer and a fast (10 ns) power amplifier (A-A Optoelectronics). Two-photon excitation was produced by an infrared Ti-Sa pulsed chameleon vision laser (Coherent) tuned to 825 nm coupled to the microscope (Slicescope, Scientifica).

The microscope was equipped with a 25x LUMPlanFL/IR objective with 0.95 numerical aperture (Leicamicrosystems). Fluorescence photons were detected by cooled AsGaP H10769PA–40 photomultipliers in the transfluorescence and epifluorescence

pathways, with 641-75nm bandpass filter (semrock) and a 510-84nm bandpass filter (Semrock) for both pathways. The ablation was produce in confine optical resolution spot (xy 300nm, z 1µm) with 500mw-/+ 10% laserpower and 200µs dwell time. The live stacks were imaged with 3 to 4 µs dwelltime scanning and 20mW laser power.

RESULTS

III) RESULTS

III.1) The *fgf8a.enhancer* is expressed in all developing taste bud cell types.

To get insight into taste bud organ formation we searched for regulatory elements that drive reporter expression at early steps of their development. As *fgf8a* is expressed in the developing oropharyngeal epithelium and taste bud cells and contributes to taste bud formation [108], we examined whether any of the known [164] *fgf8a* regulatory elements drives expression in taste buds. We found that from 55hpf onwards - that is prior and during taste bud cell differentiation - the enhancer *tg(fgf8a.enhancer:gfp)* drives GFP expression in oropharyngeal epithelial cells that express *sox2,* a transcription factor expressed in presumptive taste bud cells and adjacent epithelium [167] (**Fig**.**1a-c**).

To further characterize the identity of *tg(fgf8a.enhancer:gfp)* cells, we analysed taste bud related gene expression at later stages. From 58-60hpf onwards, within the oral epithelium, groups of cells co-express *tg(fgf8a.enhancer:gfp)* and Prox1 marker of early differentiating taste bud cells (ref) (Fig.1d,j,p). From 60hpf onwards, 5-HT, Calb2 (≥64hpf), *plcß2* (≥68hpf), *ntpdase2* (≥70hpf) or GABA (5dpf, **Fig**.**1**) can be expressed in *tg(fgf8a.enhancer:gfp)* cells. Therefore, during zebrafish embryonic development, *tg(fgf8a.enhancer:gfp)* cells express molecules that are characteristic of the oropharyngeal epithelium (*sox2*), Type I (support, *ntpdase2*, GABA), Type II (taste receptor, Calb2, *plcß2*) or Type III (5-HT, GABA) taste bud cell types.

III.2) *tg(fgf8a.enhancer:gfp)* expressing cells form taste bud organs and generate 5-HT cells.

Given the early and wide *tg(fgf8a.enhancer:gfp)* expression in the oral epithelium and taste bud cells, we used this transgenic line to follow in vivo taste bud organ formation. To distinguish between sites where taste bud organs form and the rest of the epithelium, we combined *tg(fgf8a.enhancer:gfp)* with *tg(tph1b:mCherry)*, that is expressed in the earliest differentiating taste bud cell type in zebrafish, the 5-HT cell [108]. We mainly focused on the rostral part of the oral (mouth) epithelium that is easily accessible to film.

In vivo imaging showed that *tg(fgf8a.enhancer:gfp)* is expressed in oral epithelial cells in a dynamic manner. At 55hpf, epithelial cells, located at the most anterior median part of the mouth, express low amounts of *tg(fgf8a.enhancer:gfp)* (**Fig**.**1a**). By 60hpf, at distinct sites (rostro-median and rostro-lateral part of the mouth), GFP expression is intense in cells that contact each other and form apparent groups (**Fig**. **1b, Movie 1**).

Within a forming *tg(fgf8a.enhancer:gfp)* cell group, one *tg(fgf8a.enhancer:gfp)* cell starts expressing *tg(tph1b:mCherry)* and progressively loses *tg(fgf8a.enhancer:gfp)* expression (**Fig.1b,2, Movie 1**). Therefore, *tg(fgf8a.enhancer:gfp)* cells generate *tph1b* expressing (5-HT) cells. This result combined with the *tg(fgf8a.enhancer:gfp)* expression pattern analysis, (**Fig**.**1**) shows that, first, *tg(fgf8a.enhancer:gfp)* expression precedes the expression of a differentiation marker (*tph1b*/5-HT) in a taste bud cell and second, the *tg(tph1b:mCherry)* expressing cell and the adjacent *tg(fgf8a.enhancer:gfp)* cells in contact with it, correspond to a developing taste bud organ. The maximal distance between contacting cells is ≤29.1µm (n=131, 5 embryos, **Fig. 3**) and the distance between neighbouring developing taste-bud organs varies between 36µm and 118.88µm in wild-type embryos staged 60-69hpf. (**Fig. 4** n=10, 5 embryos).

III.3) *tg(fgf8a.enhancer:gfp)* expressing cells are motile, join and quit developing taste bud organs.

As oral epithelial development proceeds, additional strongly expressing *tg(fgf8a.enhancer:gfp)* cells appear in between the taste-bud groups. These *tg(fgf8a.enhancer:gfp)* cells without any contact with the *tg(tph1b:mCherry)* expressing cell, are surprisingly motile; they move to contact *tg(fgf8a.enhancer:gfp)* or *tg(tph1b:mCherry)* cells located in a neighbouring taste-bud group(**Movies 1,2,3**). The contact between a 'newly-arrived' cell with a taste-bud cell results in the maintenance of this cell into the group. However, the time period of maintenance depends on the cell-type contact; it is significantly longer when the contact is established and maintained with a *tg(tph1b:mCherry)* rather than a *tg(fgf8a.enhancer:gfp)* expressing cell (n=15, **Fig**.**5**). Accordingly, grouped *tg(fgf8a.enhancer:gfp)* cells that lose contact with the *tg(tph1b:mCherry)* cell quit the group and either re-join it or move to a neighbouring developing taste bud (**Movies 2,3 Fig**. **6**).

Altogether the analysis of taste bud formation in a live vertebrate shows for the first time that taste bud cells are motile. *tg(fgf8a.enhancer:gfp)* cells form taste-bud groups but also form outside them, within the proximal oral epithelium, and move to join the developing taste buds. *tg(fgf8a.enhancer:gfp)* cells are maintained within or quit the taste bud, depending on their contact with a synthetising 5-HT or *tg(fgf8a.enhancer:gfp)* expressing cell, respectively.

III.4) The 5-HT cell is required for the maintenance of *tg(fgf8a.enhancer:gfp)* expressing cells into taste bud organs.

To address whether a *tg(fgf8a.enhancer:gfp)* or *tg(tph1b:mCherry)* cell is required for *tg(fgf8a.enhancer:gfp)* cell maintenance in the taste buds, we focally ablated them with a multiphoton laser and subsequently time-lapsed taste bud development. The ablation of a grouped *tg(fgf8a.enhancer:gfp)* left the remaining cell group unaffected (**Fig**. 7, **Movie 4**, n=6/6). In contrast the ablation of the *tg(tph1b:mCherry)* resulted in the local reorganisation of the remaining cells of the group and *tg(fgf8a.enhancer:gfp)* cell motility towards neighboring taste buds (**Fig**. 8, **Movie 5**, n=6/6).

In a complementary manner, we analysed the behaviour of *tg(fgf8a.enhancer:gfp)* cells in the *ascl1a-/-* zebrafish that are devoid of 5-HT cells ([108] **Fig. 9A**). The number of *tg(fgf8a.enhancer:gfp)* cells is not significantly different in *ascl1a-/-* larvae compared to the wild type siblings (**Fig**. **9D-E**). *tg(fgf8a.enhancer:gfp)* cells expressing variable levels of GFP contact each other and form groups in *ascl1a-/-* larvae (**Movie 6**). However, in contrast to the wild-type (n=0/16 cell groups, 5 embryos), *ascl1a-/- tg(fgf8a.enhancer:gfp)* cell groups can split into two approximately half–sized cell groups (**Movie 6,** 19th sec onwards, central group, **Fig. 10,** n=5/12 groups, 3 *ascl1a-/-* embryos, minimal and maximal distance between cell groups, 30.19µm and 150.1µm, respectively). Occasionally, cell groups contact each other (**Fig 10.** n=3/12, 3 embryos, **Movie 6,** 37th-39th sec). To examine whether the *tg(fgf8a.enhancer:gfp)* cell group fragmentation has any consequence in taste bud organ formation in *ascl1a-/-* larvae, we analysed taste bud marker expression at 5dpf. Although the number of *tg(fgf8a.enhancer:gfp)* cells is not significantly different between *ascl1a-/-* and wild-type siblings at 5dpf, there is a relatively small but significant increase of the number of taste buds in the ascl1a-/- mutant compared to the wild-type siblings (F**ig. 9A-E**).

Finally, we measured distance and contact time periods between adjacent cells in *ascl1a-/-* larvae. In the wild-type, the *tph1b* cell is the one at the shortest distance from the center of mass of the group (n=12/12 groups). Therefore, we used the *tg(fgf8a.enhancer:gfp)* cell in the corresponding position as a reference for each cell group in ascl1a-/- forming taste buds. First, as in the wild-type, we found adjacent *tg(fgf8a.enhancer:gfp)* cells that remain in stable contact over time (distance≤29.1µm, **Fig. 11a**), quit or join the group (**Fig. 11b**). However, in contrast to the wild-type, we also observed that neighbouring *tg(fgf8a.enhancer:gfp)* cells stay in proximity to each other without keeping a permanent contact between them overtime and without quitting the group (**Fig. 11c**). In the latter case, the time period that *tg(fgf8a.enhancer:gfp)* cells remain in contact to each other is comparable to the wild-type (**Fig. 5**).

In conclusion, altogether the results show that a) *tg(fgf8a.enhancer:gfp)* cells form taste bud organs, b) can differentiate into 5-HT cells c) developing taste bud cells are motile and can be displaced from one organ to another d) *tg(fgf8a.enhancer:gfp)*-5-HT cell contact results in the maintenance of *tg(fgf8a.enhancer:gfp)* cells into groups for longer time periods.

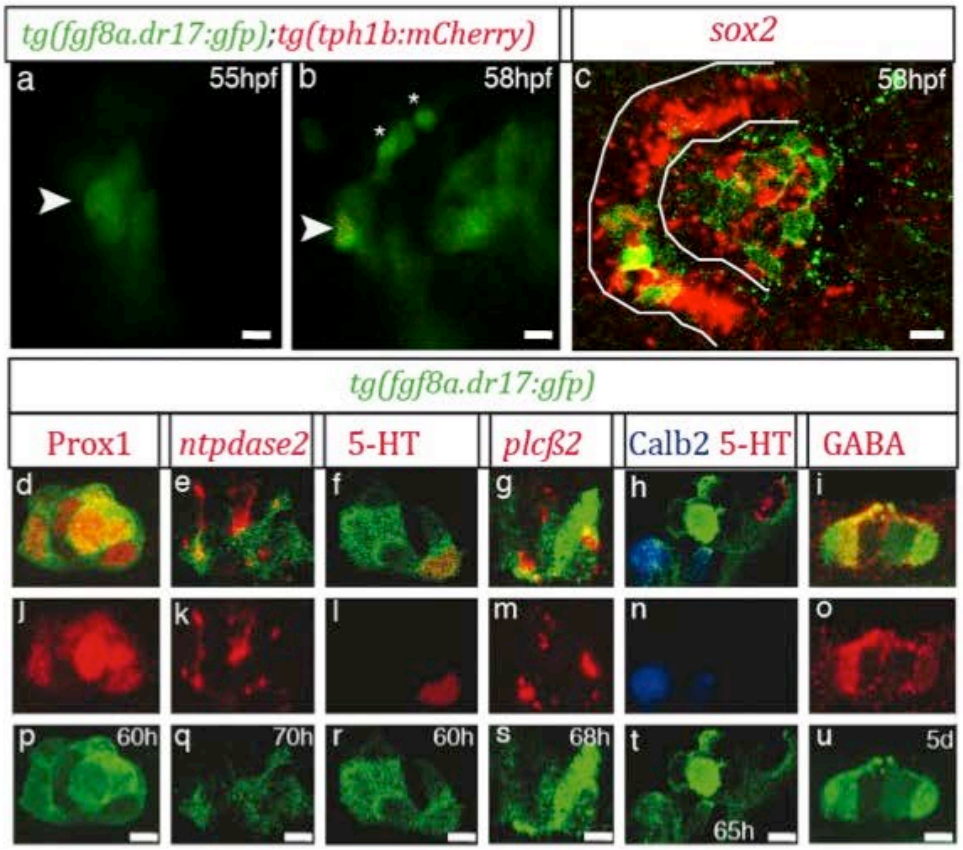


Figure 1. *Tg(fgf8a.enhancer:gfp)* is expressed in the developing taste buds. In all figures and movies unless otherwise mentioned, anterior is on the left.

A,B. 1µm optic sections of the developing mouth epithelium (anterior part) expressing only GFP (A) and GFP and mCherry (B) in a live *tg(fgf8a.enhancer:gfp); tg(tph1b:mCherry)* larva. Arrowheads point to the median first appearing taste bud and asterisks in additional strongly expressing GFP cells.

C. *sox2* is expressed in the mouth epithelium (red). Taste buds develop only in the zone delimited by white lines and filmed in the movies.

D-U. Expression analysis of taste bud cell specific markers. The first row is the merge of single or double channels shown in second and third rows. Optical sections 1.5µm.

Scale bar=5µm

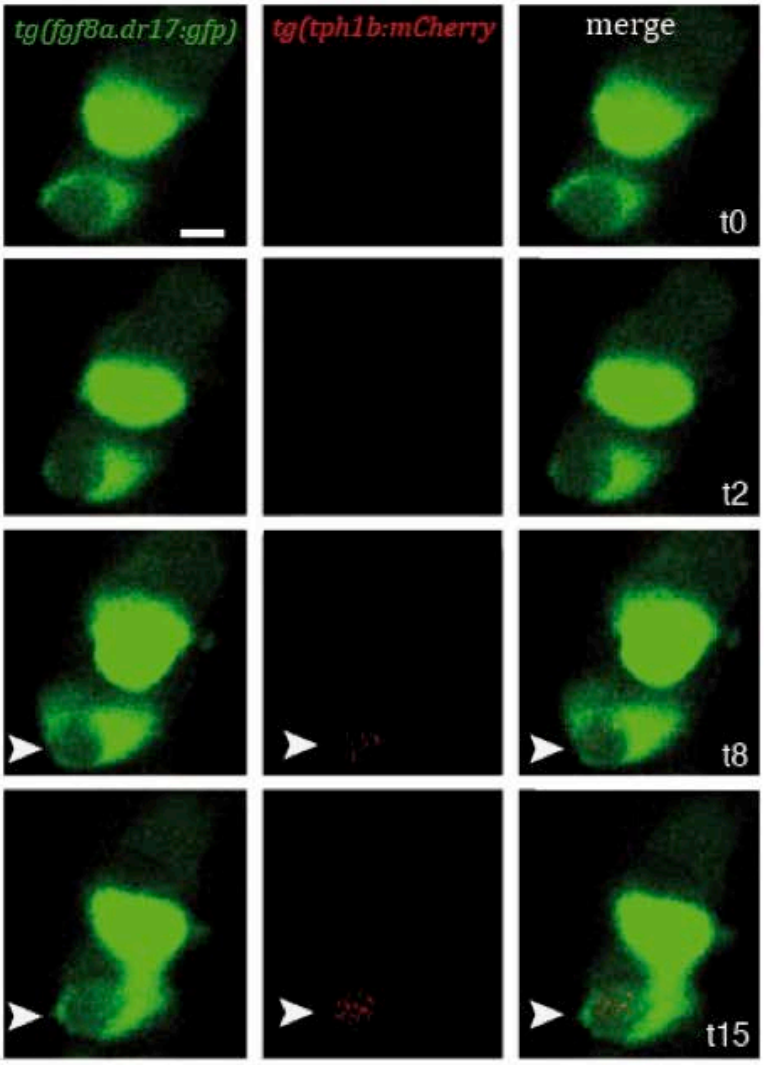


Figure 2. Optic sections (1µm) from a timelapse of developing *tg(fgf8a.enahncer:gfp)* cells (time points indicated bottom right, corresponing to 8 minutes each). One cell (arrowhead) starts expressing *tg(tph1b:mCherry)* at t8. Scale bar=4µm.

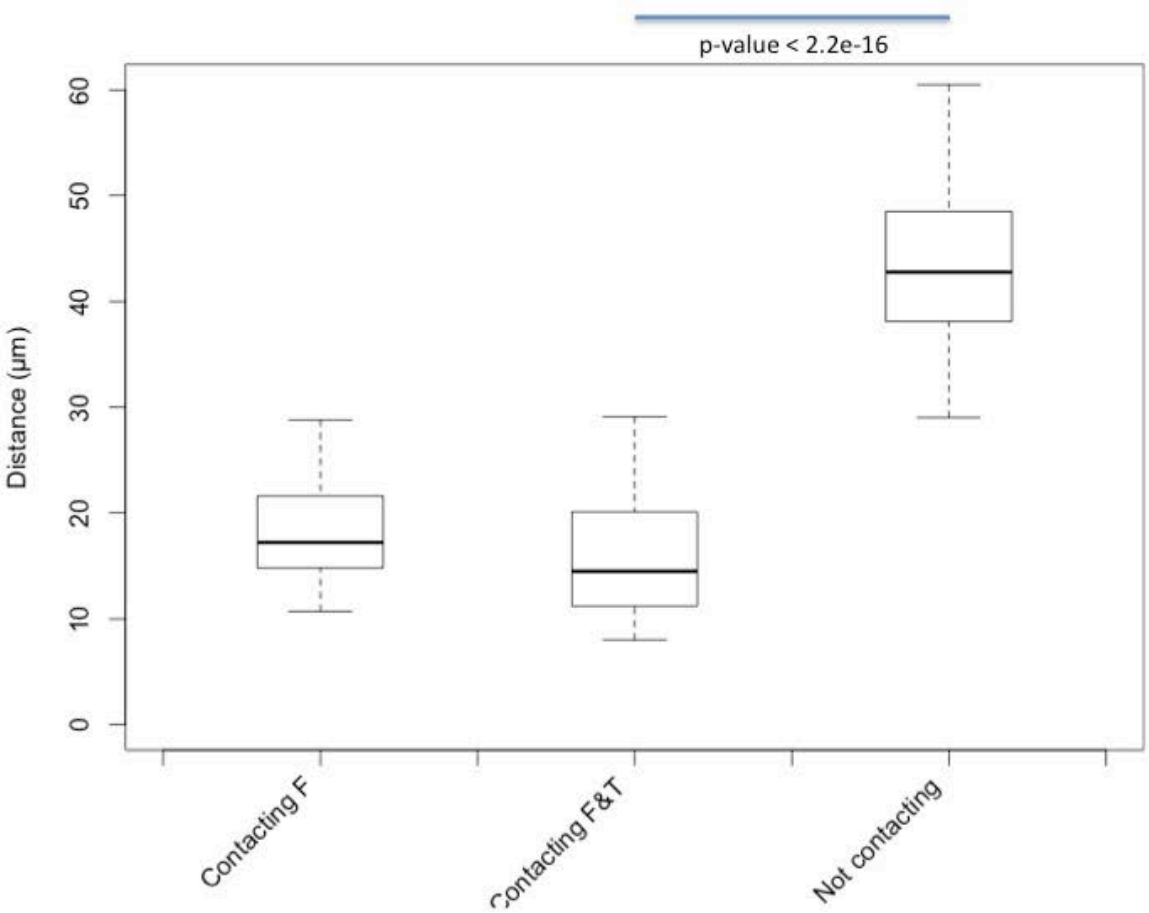


Figure 3. Distance (µm) between cells that contact each other.
F=*tg(fgf8a.enhancer:gfp)*, T=*tg(tph1b:mCherry)* expressing cells.
(Contacting F: n=42,
Contacting F&T, Not contacting: n=131, Wilcoxon paired)

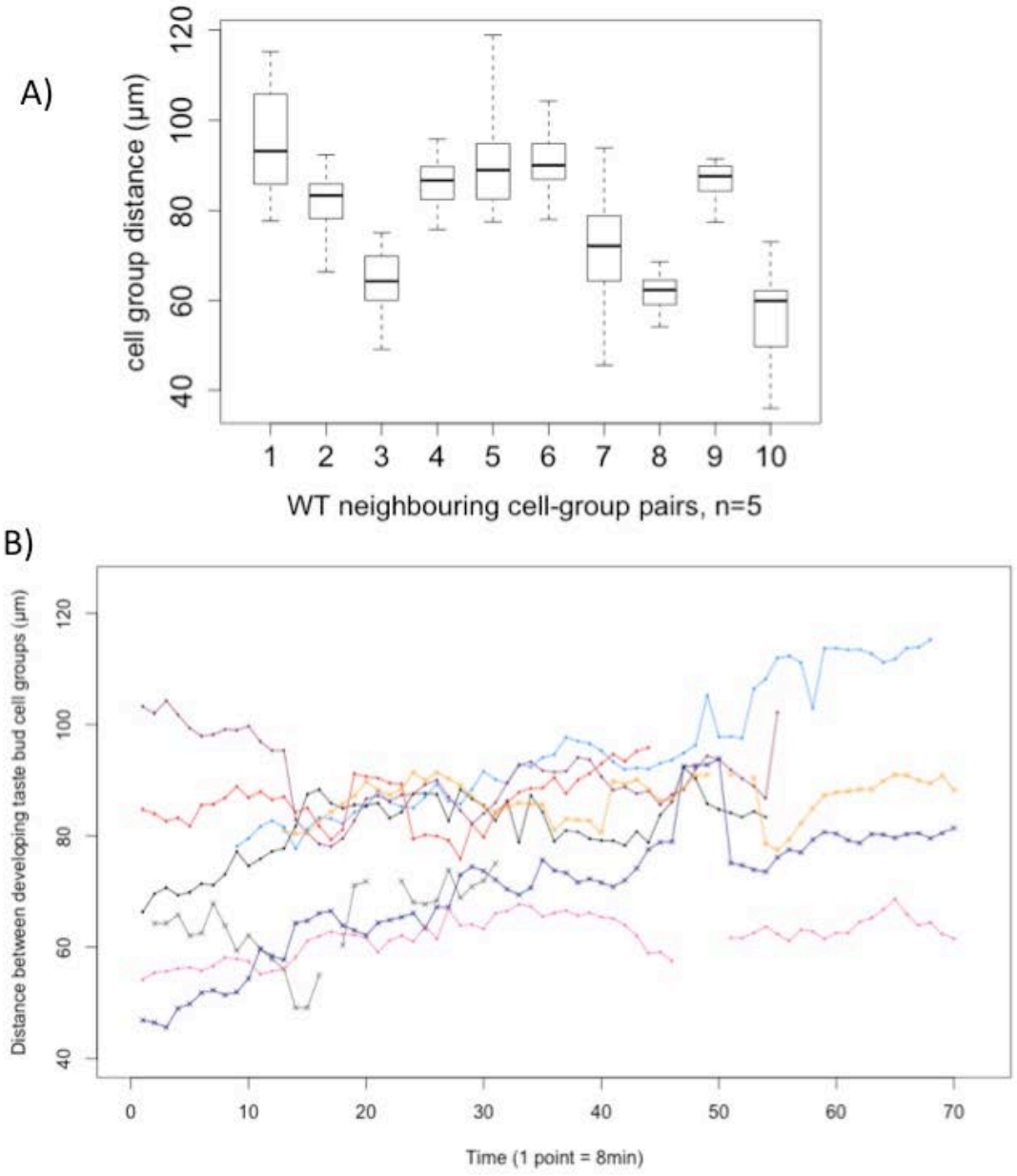


Figure 4. Distance between developing taste-bud cell groups expressing *tg(fgf8a.enhancer:gfp)* in the wild type embryo. The 10 pairs presented in A are located in the medial and lateral parts of the anterior mouth epithelium of 5 embryos staged between 60-69hpf. The cell groups do not form simultaneously; they appear laterally later than medially. The tracts of 8 pairs during time are represented in B. The time in all figures: to be used only as a reference for a single group; it does not reflect similar developmental stages between the different embryos.

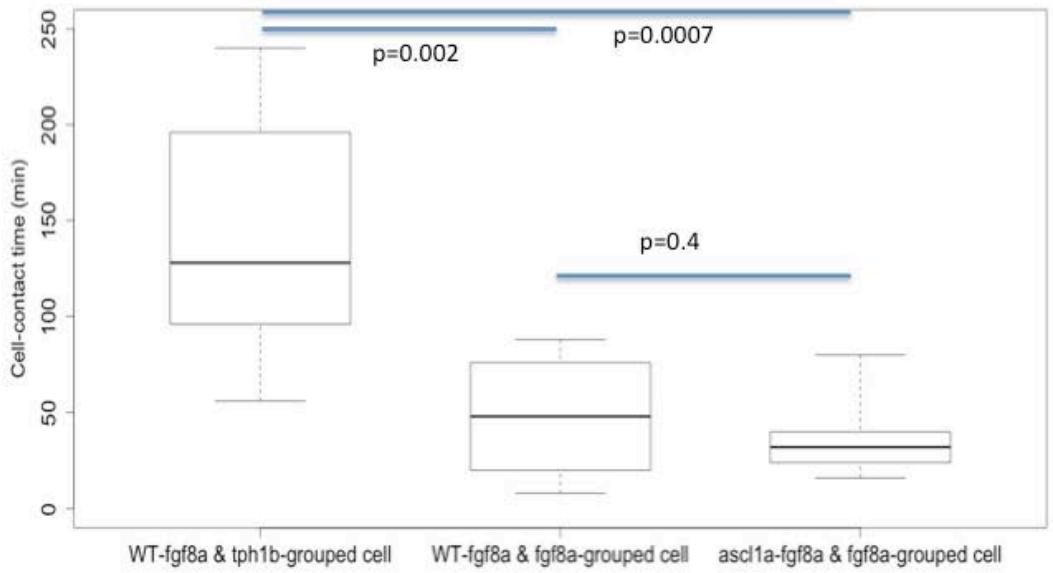
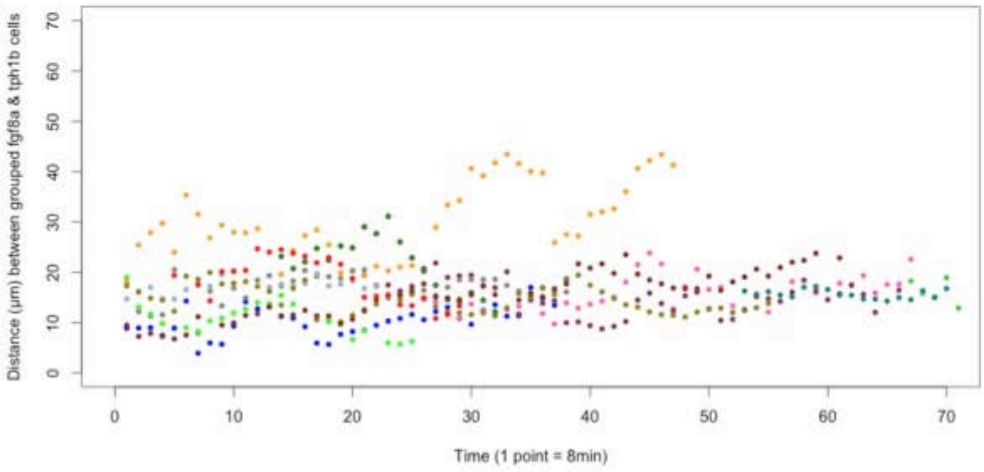


Figure 5. Contact time (n=15) between cells:
- wild-type motile *tg(fgf8a.enhancer:gfp)* - taste bud *tg(tph1b:mCherry)*
- wild-type motile *tg(fgf8a.enhancer:gfp)* - taste bud *tg(fgf8a.enhancer:gfp)*
- ascl1a-/- motile *tg(fgf8a.enhancer:gfp)* - taste bud(closest to cwm) *tg(fgf8a.enhancer:gfp)*
- (n=15, p-values, paired Wilkoxon).

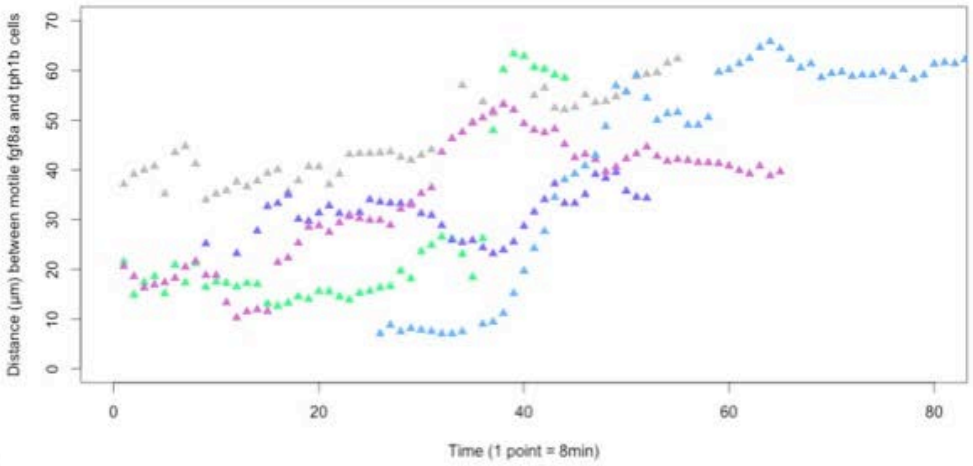
Figure 6 (next page). Profiles of distance between *tg(fgf8a.enhancer:gfp)* cells and the *tg(tph1b:mCherry)* cell from the proximal taste bud.
tg(fgf8a.enhancer:gfp) - *tg(tph1b:mCherry)* cell distance is shown for single cells (dot or triangle of the same colour) along time. The data here are collected from 7 taste buds of 3 wild type embryos. *tg(fgf8a.enhancer:gfp)* cells can be divided in three categories : A) those with constant contact with the *tg(tph1b:mCherry)* cell (distance ≤29.1µm, 15cells, shown only 10 for clarity of the graph). Exception to this rule is the yellow cell (1/15). B) those that lose contact with the *tg(tph1b:mCherry)* cell (distance ≥29.1µm) and quit the taste bud (n=4 cells). The grey cell does not belong to these taste buds and has been added for comparison). C) those that join the taste bud and make contact with the *tg(tph1b:mCherry)* cell (n=3, distance after contact ≤29.1µm).

Figure 6 (legend in the previous page)

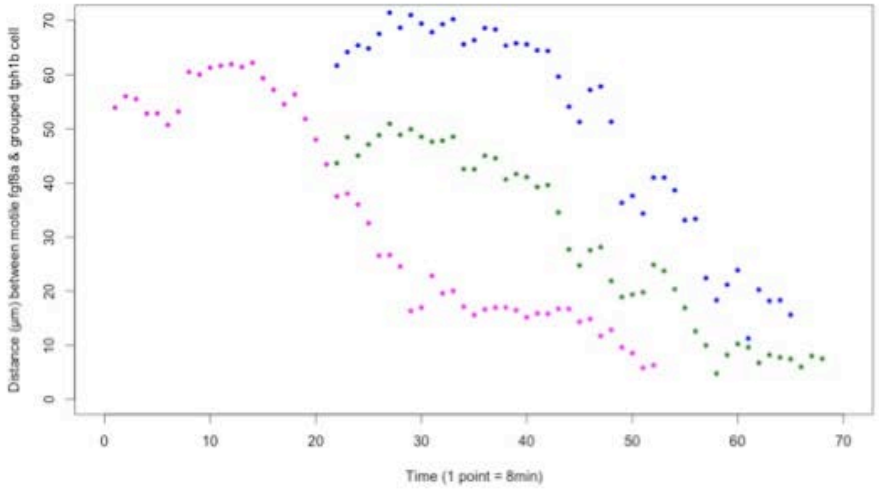
A)



B)



C)



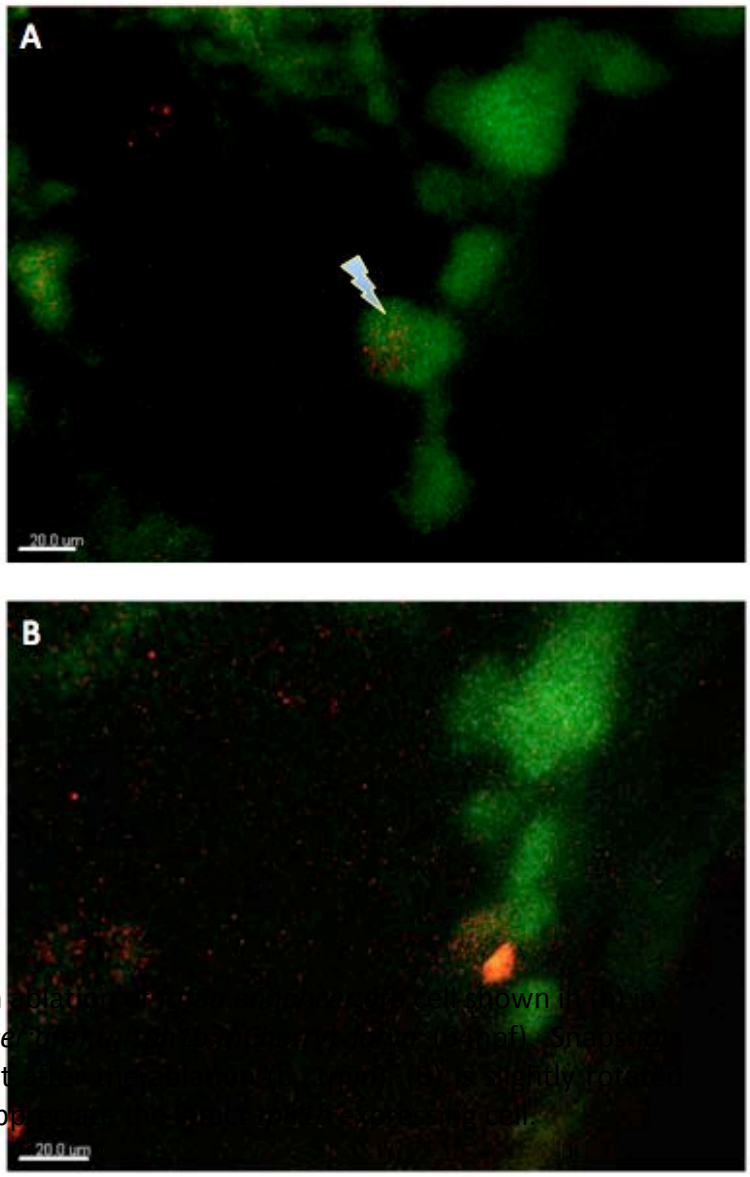


Figure 7. 2-photon [illegible] cell shown in [illegible] a *tg*(*fgf8a.enhance* [illegible] (24hpf). Snapshots before (a) and just [illegible] slightly rotated (30°) in order to ap[illegible] expressing cell.

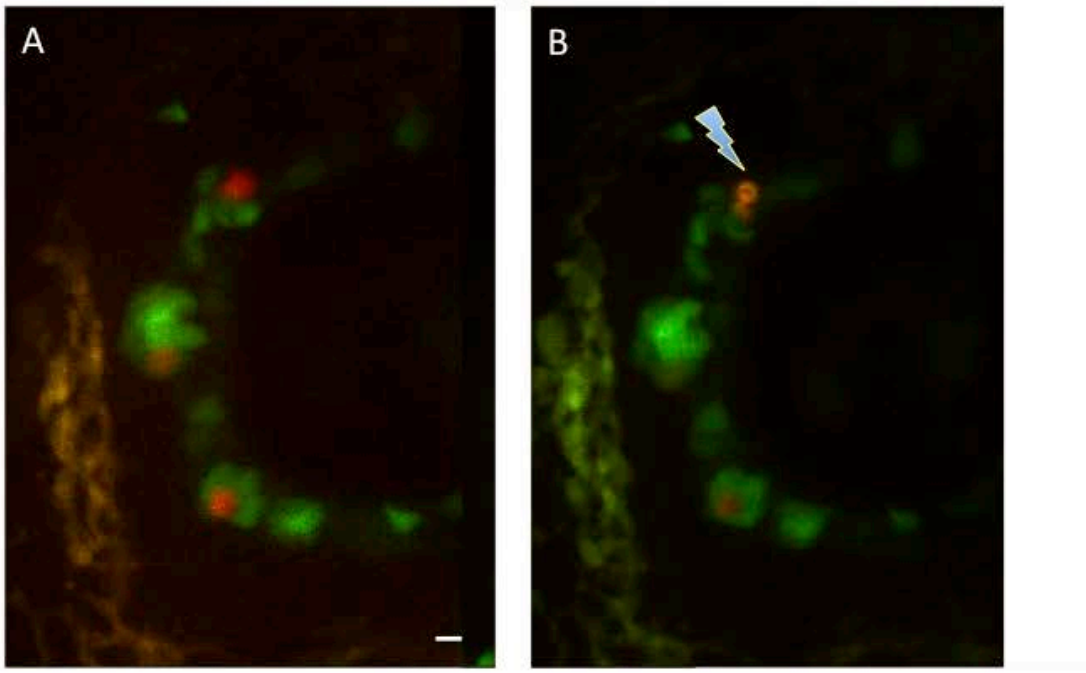


Figure 8. 2-photon ablation of the *tph1b:mCherry* cell shown in (b) in a *tg(fgf8.enahncer:gfp);tg(tph1b:mCherry) larva* (65hpf). Snapshots before (a) and just after the ablation (b, 1min). The fluorescence('network-like') on the left of the image is background emitted from the skin of the embryo. Scale bar 10µm

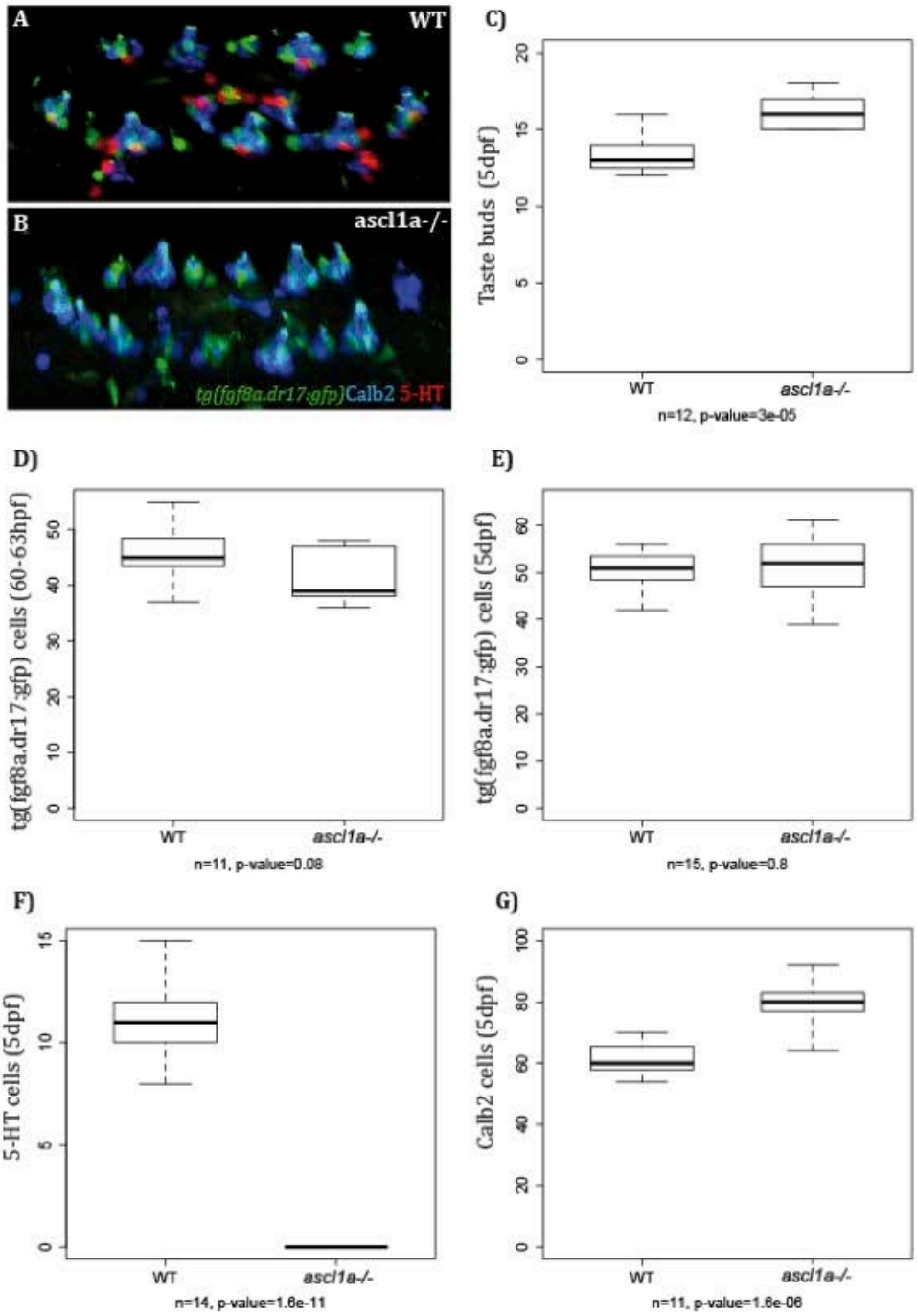


Figure 9. Taste bud cell analysis in the *ascl1a-/-* mutant. A,B confocal projections of the taste bud organs in the mouth . The number of taste buds, *tg(fgf8a.enhancer.gfp)*, 5-HT and Calb2 cells is shown in C-G, in wild type and ascl1a-/- embryos.

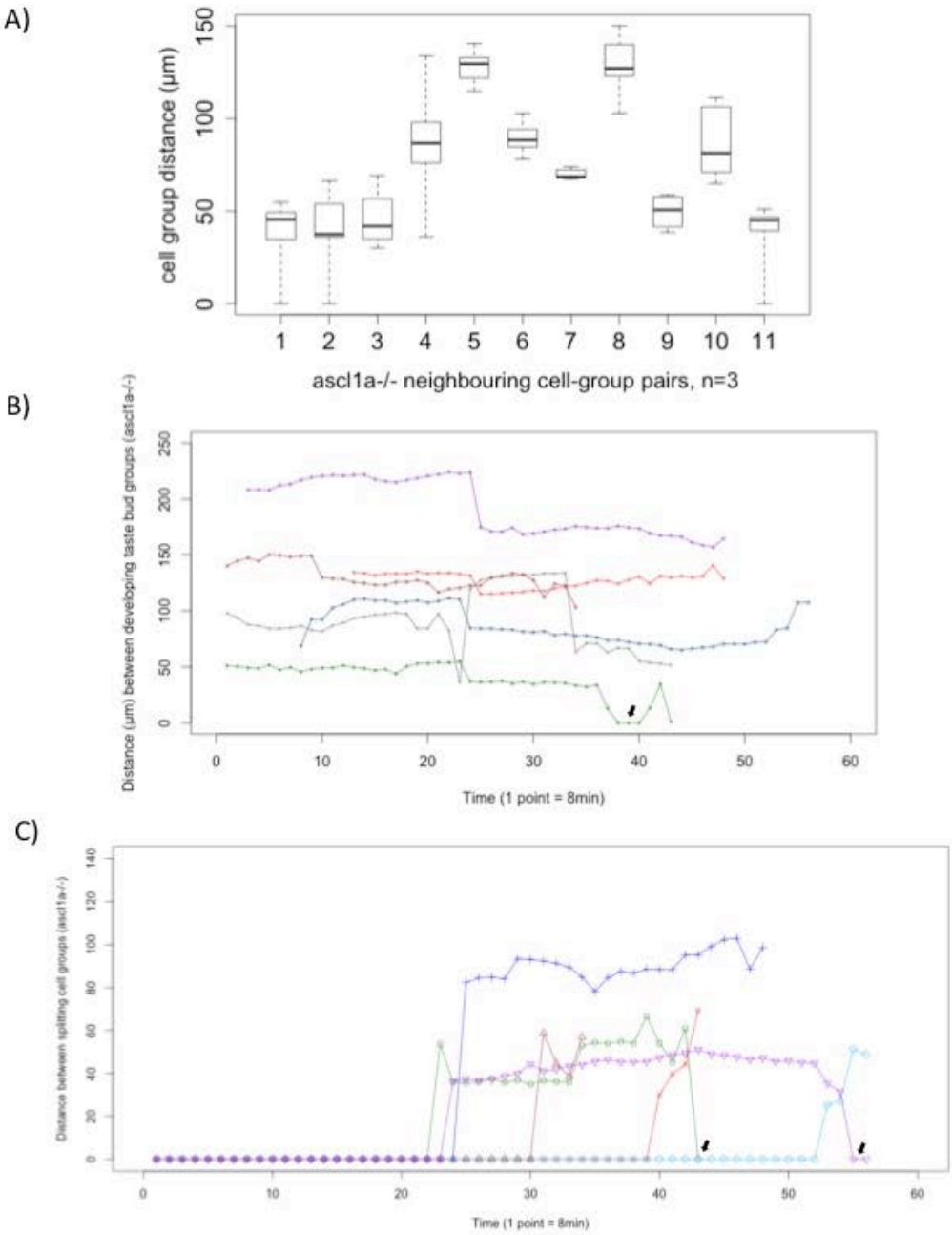


Figure 10. Distance between developing taste-bud cell groups expressing *tg(fgf8a.enhancer:gfp)* in the ascl1a-/- embryo (n=11, 3 embryos). From the 11 cases shown in A, 6 groups remain intact and the distance between them over time shown in B. Five taste bud groups will split into half and they are shown in C. In this case, the initial distance between them is considered 0µm and omitted from the boxplots. In B and C, 3 pairs of taste buds regroup (distance =0µm, arrows).

Figure 11 (figure legend next page)

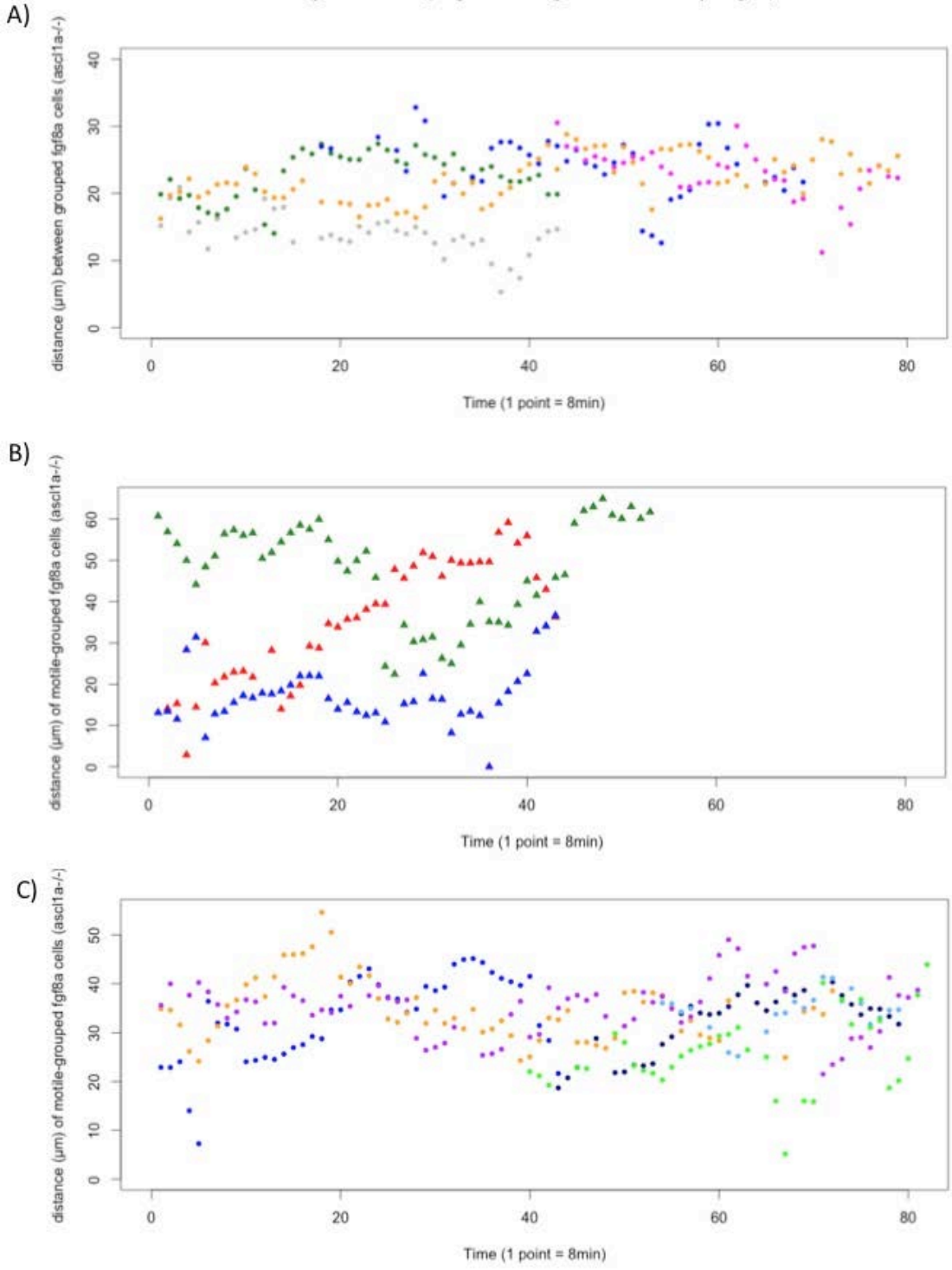
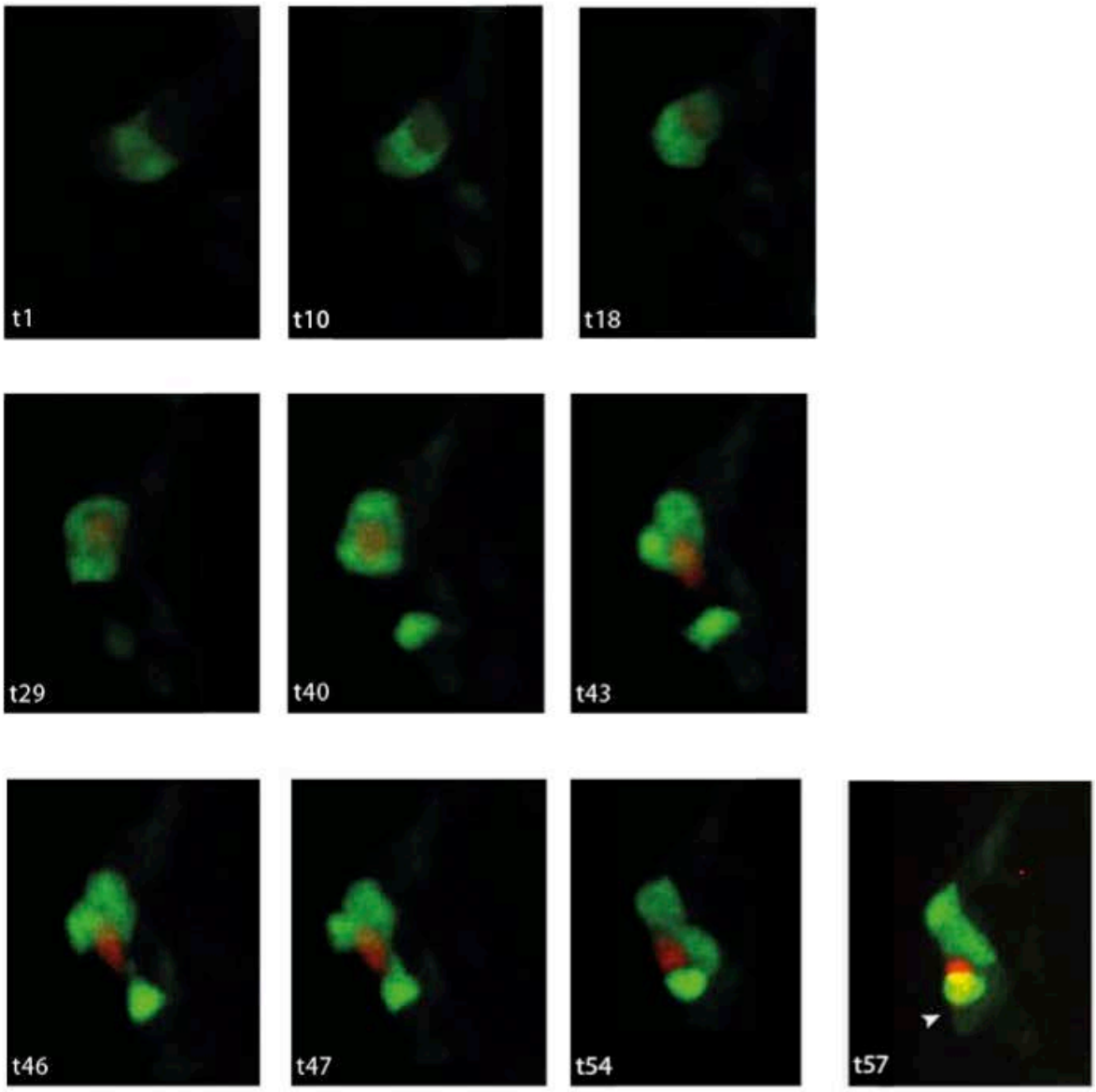
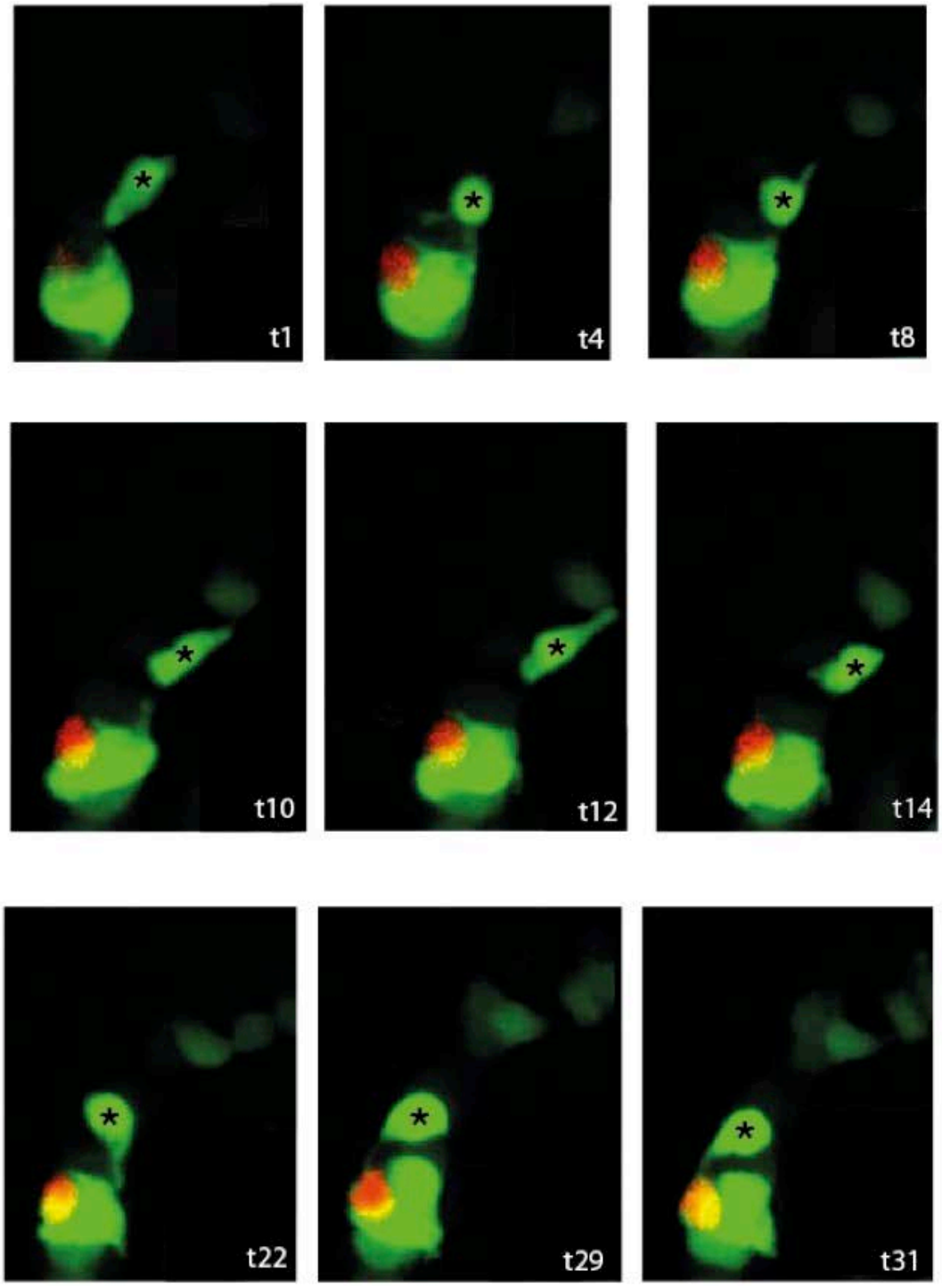


Figure 11 (previous page). Distance profiles of grouped and motile *tg(fgf8a.enhancer:gfp)* cells in the ascl1a-/- mutant (8 taste buds, 3 embryos). Distance is shown for single cells (dot or triangle of the same colour) along time. *tg(fgf8a.enhancer:gfp)* cells can be divided in three categories : A) those with constant contact with the *tg(fgf8a.enhancer:gfp)* cell closest to the center of the mass of the group (cm) (distance ≤29.1µm, 8cells, shown only 5 for clarity of the graph). B) those that lose contact with the 'cm'- *tg(fgf8a.enhancer:gfp)* cell (distance ≥29.1µm) and quit the taste bud (n=3 cells). C) those that make and loose contact with the 'cm'- *tg(fgf8a.enhancer:gfp)* cell (n=8, 6 shown here for clarity, contact distance ≤29.1µm).

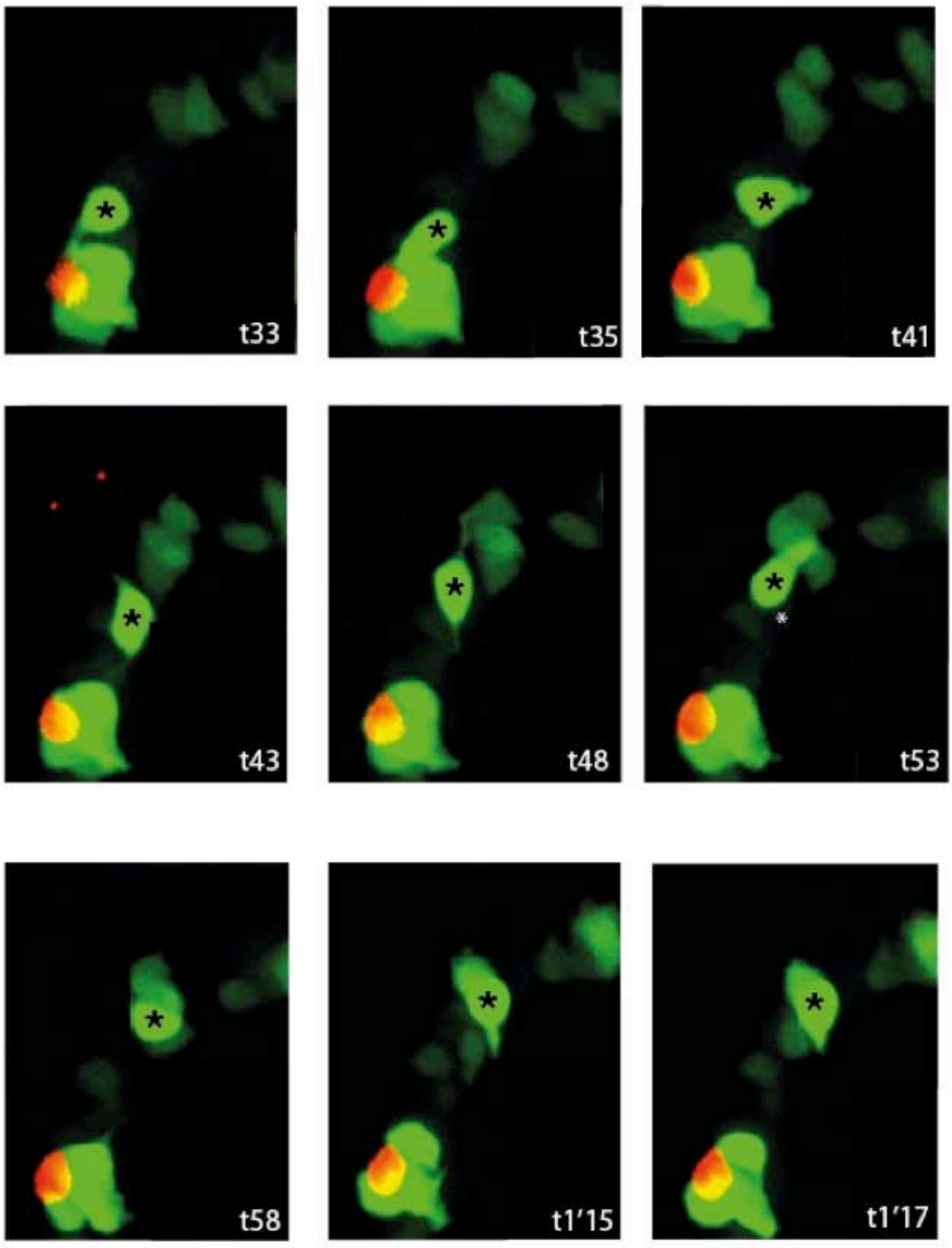


WT-Movie 1

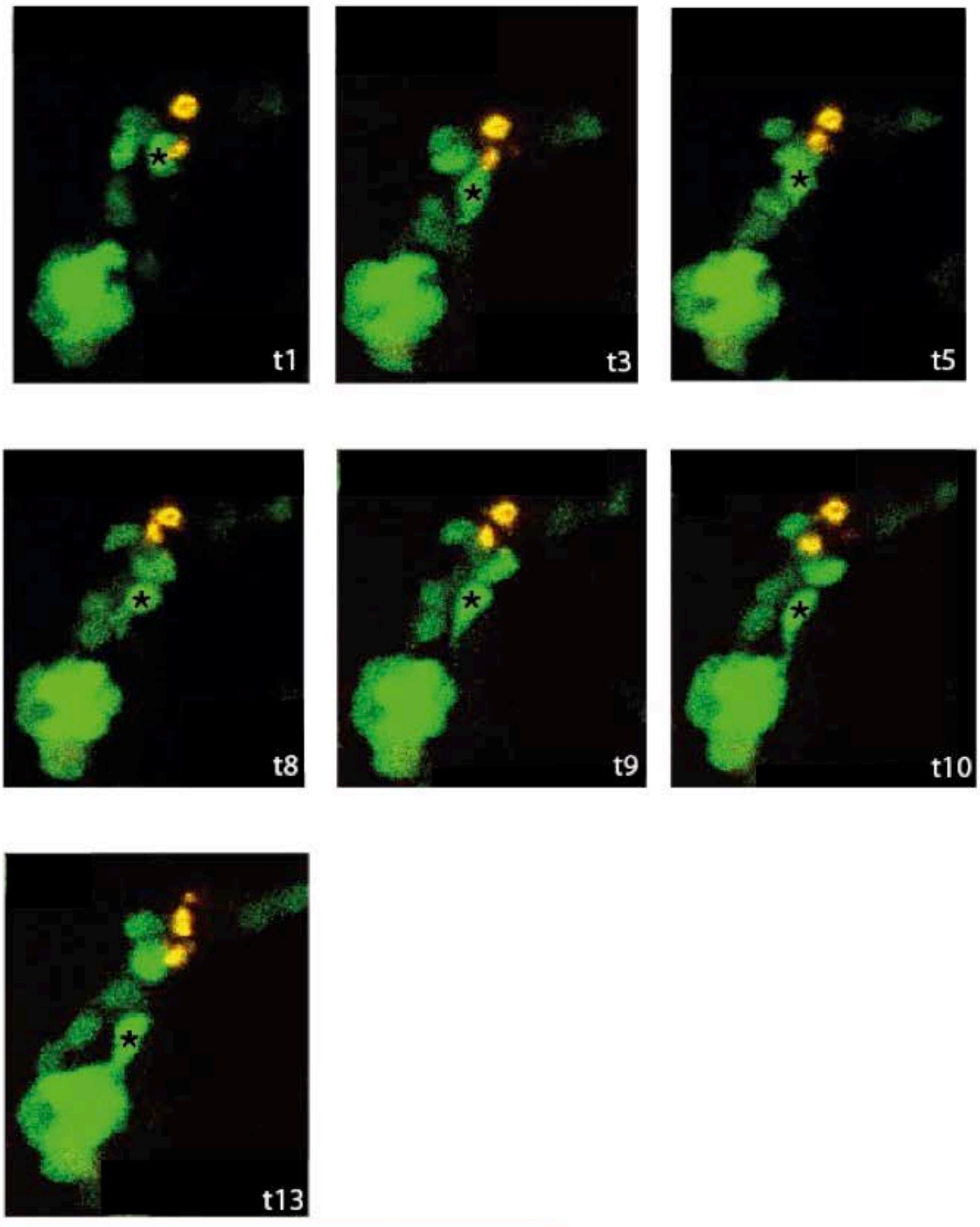
In this and following pages, selected snaphsots of the timelapse movies are provided. Asterisks or arrowheads point to the cells or events of interest. See Results text for details.



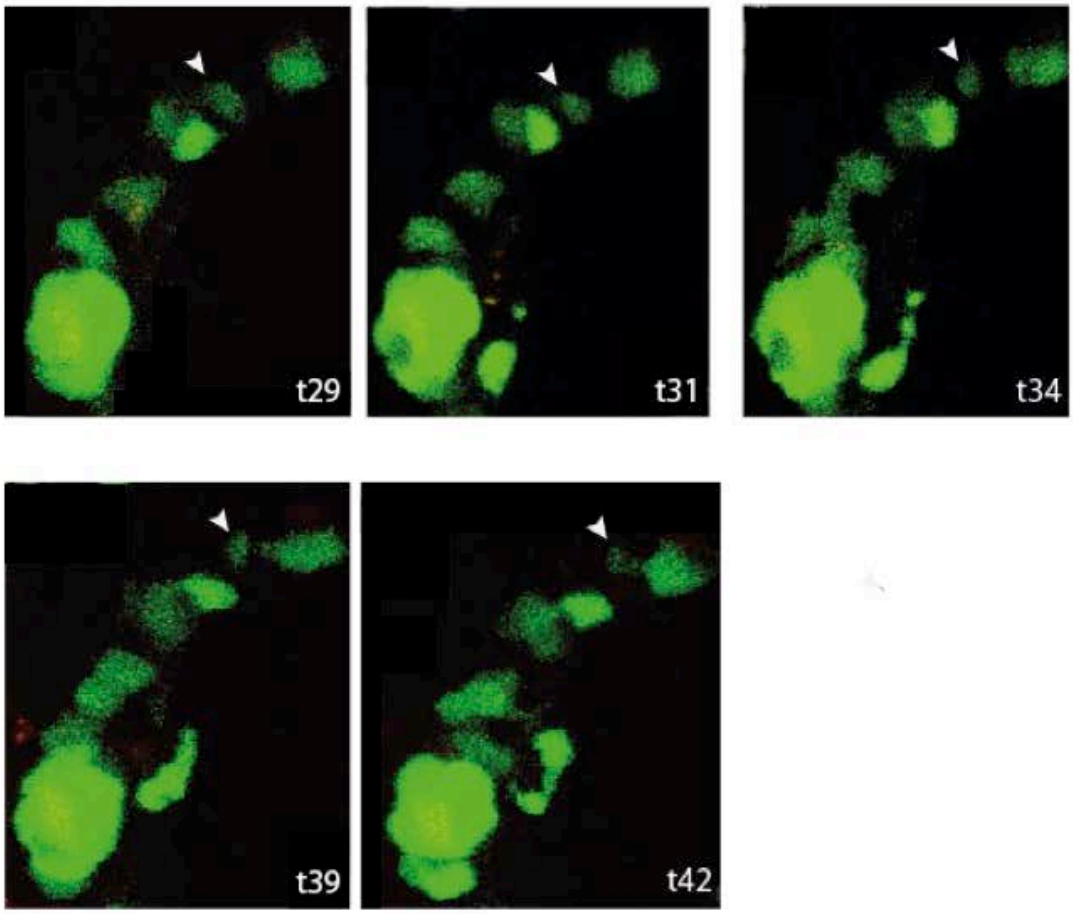
WT- Movie 2



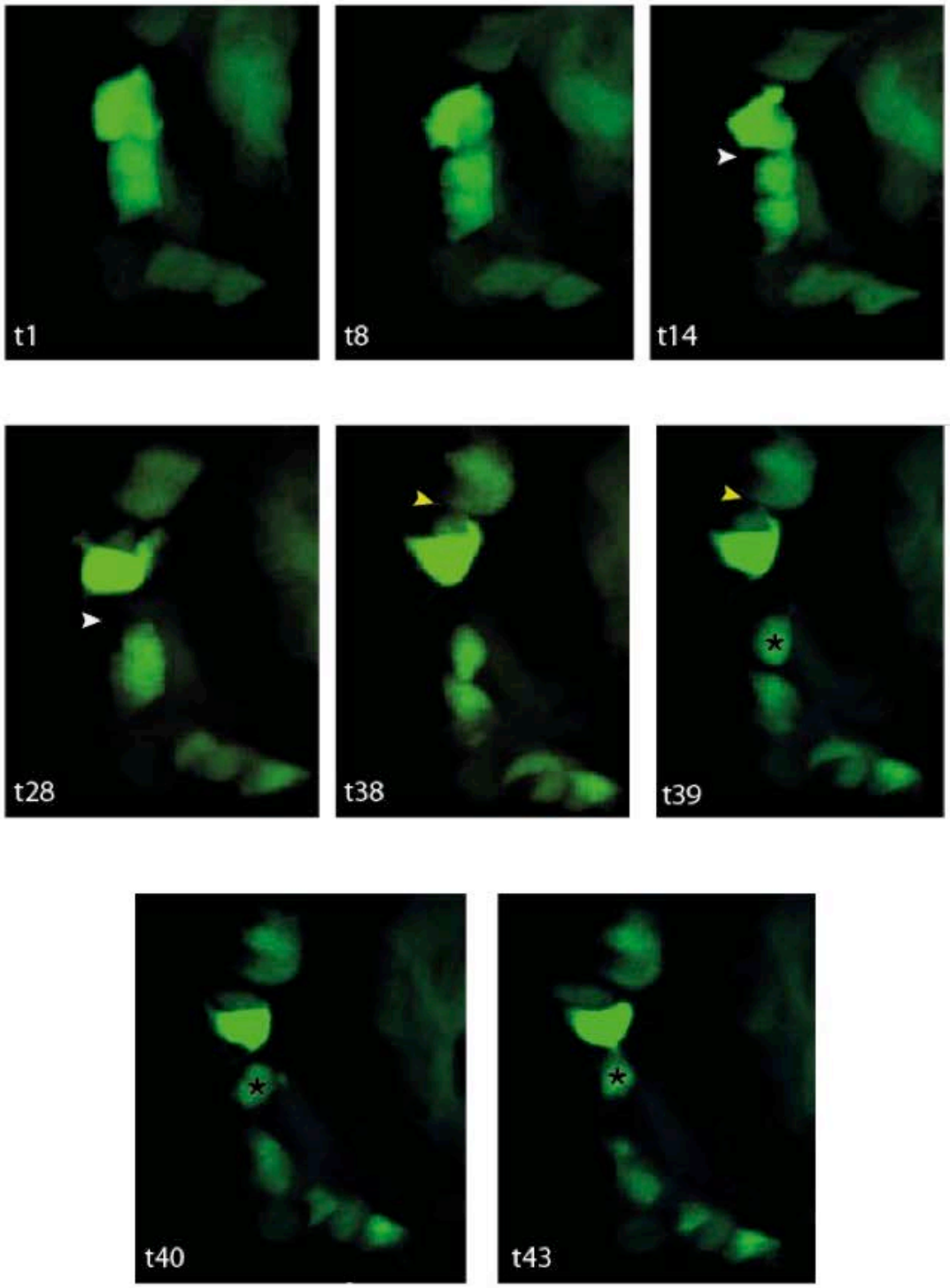
WT- Movie 2 (continued)



tg(tph1b:mCherry) cell ablation-
Movie 5



tg(tph1b:mCherry) cell ablation-
Movie 5 (continued)



ascl1a-/- Movie 6

DISCUSSION

For the first time the question of assembly of epithelial differentiating cells into a taste bud organ is addressed in this study. Using zebrafish fluorescent transgenic and mutant lines and two-photon laser ablation, we found that developing taste bud cells are motile and move from one developing organ to another. One of the factors regulating motility is cell-to-cell interaction within the organ. In particular, the cell contact with the *tph1b*/5-HT cell is required for the long-term maintenance of the developing cells into the forming taste bud.

One of the issues of this study is the quantification analysis of this phenomenon. Because of its complexity and the unexpected non-stereotyped early taste bud development in fish, the tracking of the cell position has to be verified and adjusted manually. For this reason, here are provided quantitative data for cell and taste bud distance from 5 out of 10 wild-type *tg(fgf8a.enhancer:gfp); tg(tph1b:mCherry)* and 3 out of 6 mutant *ascl1a-/-;tg(fgf8a.enhancer:gfp); tg(tph1b:mCherry)* larvae. In the same respect, the statistical analysis of cell speed is in progress. Nevertheless, the qualitative observation of all the samples leaves no doubt about the reproducibility of the phenomenon and once all samples analysed, the statistical significance of key parameters should be reinforced.

IV.1) *tg(fgf8a.enhancer:gfp); tg(tph1b:mCherry)* : an appropriate tool to analyse taste bud development in vivo

The assembly of differentiating cells into intact taste bud organs has been unexplored mainly due to the inaccessibility of the oropharynx during mouse embryogenesis. The zebrafish mouth provides a good model to address this question due to its accessibility and optical clarity. Here, two transgenic lines *tg(fgf8a.enhancer:gfp)* and *tg(tph1b:mCherry)* are analysed in respect to taste bud organ formation. We have also constructed and tried a *tg(plcß2:GFP)* line (*unpublished results*) but it is expressed later than *tg(fgf8a.enhancer:gfp)* and *tg(tph1b:mCherry)* and therefore key observations are missed. The *tg(fgf8a.enhancer:gfp)* seems to be an ideal tool since it is expressed early, widely and dynamically in the developing epithelium and more intensely in the developing taste bud organs. This differential *tg(fgf8a.enhancer:gfp)* expression profile between epithelium and taste buds is reminiscent of *sox2* [167]. However, it is uncertain until which step of the taste-bud differentiation process, *sox2* is expressed [108]. *tg(fgf8a.enhancer:gfp)*

expressing cells can express markers of all taste bud cell types; these markers are also expressed in cells devoid of *tg(fgf8a.enhancer:gfp)* expression (i.e. Fig. 1e,h). The simplest explanation for this mixed expression profile is that as development proceeds, *tg(fgf8a.enhancer:gfp)* is downregulated in taste bud cells and oropharyngeal epithelium; this is obvious in the 5-HT differentiating cells (Movie 1). In addition, at 5dpf, *fgf8a mRNA* is expressed in taste bud cells and *tg(fgf8a.enhancer:gfp)* is expressed in GABA- or Calb2-expressing taste bud cells but not in the surrounding epithelium (Fig. 1, data not shown). Therefore, *fgf8a* expression likely persists in other than the 5-HT differentiat-ing/ed taste bud cell types and allows following their behaviour and potentially function. To assess if *tg(fgf8a.enhancer:gfp)* cells differentiate into Type II cells, a fate mapping experiment has been undertaken that combines the mosaic photoconversion of protein psmOrange in *tg(fgf8a.enhancer:gfp)* cells with postfixation and immunohistochemistry with a Calb2 antibody.

IV.2) What is the functional significance of cell motility during taste bud organ formation?

IV2.1) Are the forming taste bud organs patterned during early development?

To our surprise, the epithelial sites where taste bud organs are initially induced are not stereotyped in zebrafish. The first organ does appear in the most median part of the rostral mouth epithelium but the distance between this and more lateral organs in the mouth epithelium as well as between lateral organs themselves is variable from one embryo to another. One caveat here is that precise staging of the taste bud and zebrafish development that is not possible with the presently available tools and embryonic maps [168]. We are uncertain for the situation in mouse (i.e E12-E15 fig.IV-1); although postnatal and adult taste buds are patterned to a large extent, it is also difficult to verify if this is the case during their early ontogeny. Nevertheless, in the wild type zebrafish, new organs do not develop adjacent to each other but at some although variable distance (Fig. 4). This is one of the key differences with the *ascl1a-/-* mutant, where taste bud organs not only form de novo within the epithelium, but also arise from the splitting of pre-existing organs.

Perhaps one of the roles of cell motility is indeed the patterning of taste bud organs; a newly forming epithelial ‘taste-bud-to be’ cell has two choices, either to form a

new organ around it or to join one of the pre-existing organs. The analysis of the molecular mechanism underlying the motility and eventual attraction of developing epithelial cells into the forming taste buds could perhaps give hints about this hypothesis.

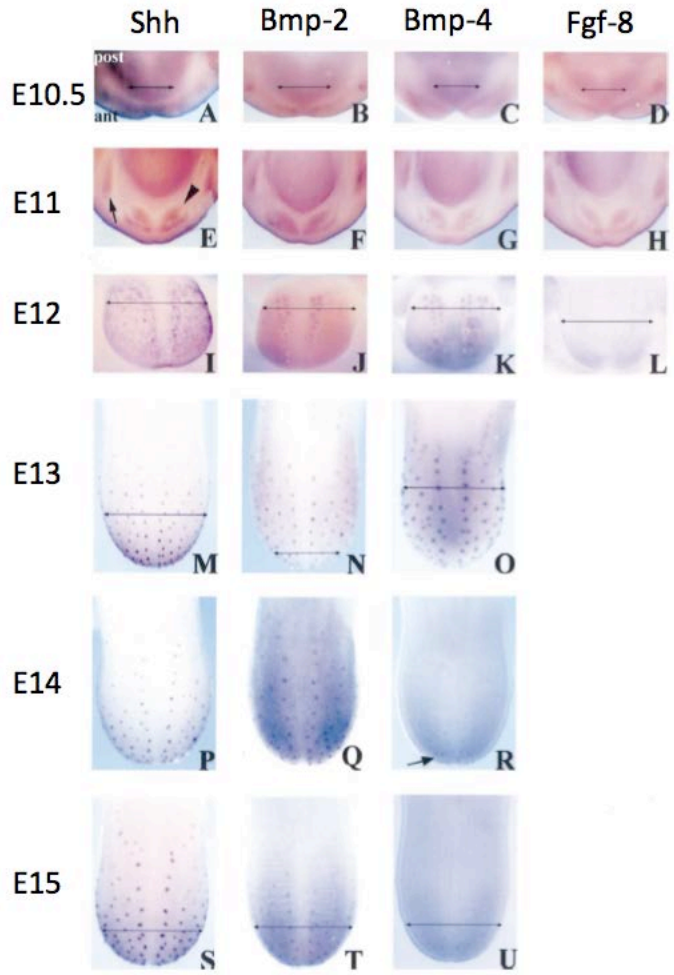


Figure IV-1. Expression patterns of sonic hedgehog (*Shh*), bone morphogenetic proteins -2 and -4 (*Bmp-2*), (*Bmp-4*) and fibroblast growth factor -8 (*Fgf-8*) from E10.5 to E15 of mouse tongue development with whole mount in situ hybridization (ant, anterior; post, posterior). All four genes are expressed uniformly in the tongue epithelium between E10.5 and 11. At E13, before morphologically detectable gustatory papillae initiation, Shh, Bmp-2 and Bmp-4 expression segregates into discrete spots, whereas, Fgf-8 is downregulated. At E14, small eminences in the anterior part of the tongue are the first morphological indications of fungiform papillae, and they express Shh and Bmp-2, whereas, Bmp-4 is almost absent in the tongue.

IV.2.2) Is the cell motility negatively correlated with the functional activity of the taste bud cell?

Taste bud cells are excitable and upon stimulation, release excitatory or inhibitory neurotransmitters to communicate with each other and the taste sensory ganglia. In fact, each taste bud comprises a mini-circuit corresponding to the first processing station of taste sensory information. Cell communication mediated through maintained cell contact can be crucial in this context. Here, we revealed that *tg(fgf8a.enhancer:gfp)* cell contact with the 5-HT cell -that likely corresponds to the presynaptic Type III mammalian cell-

enhances the maintenance of the motile cell into the group, in contrast to the contact between *tg(fgf8a.enhancer:gfp)* cells (Fig. 5). One hypothesis could be that *tg(fgf8a.enhancer:gfp)* cells get incorporated into the taste bud once their activity is correlated with other taste bud cells such as the 5-HT cell. This mechanism is common for newly born cells in the CNS [169]. Description of the spontaneous activity of the migrating cells and optogenetic manipulation could shed light in the functional mechanism leading to the incorporation of *tg(fgf8a.enhancer:gfp)* cells into the taste bud.

IV.2.3) What is the molecular mechanism underlying cell contact and adhesion during taste bud formation?

The cell motility observed during taste bud formation is reminiscent of the process of epithelial-mesenchymal transition (EMT). During EMT, epithelial cells downregulate epithelial characteristics, lose junctions and apico-basal polarity, their cytoskeleton is reorganised and start migrating with increased cell protrusions, motility and in many cases, an ability to degrade extracellular matrix (ECM) proteins to enable invasive behaviour [162]. A new transcriptional program is induced to maintain the new mesenchymal phenotype. The ability of epithelial cells to transition into mesenchymal cells and back, either partially or fully, illustrates an inherent plasticity of the epithelial phenotype.

Two key molecules independently involved in EMT and expressed in taste buds are miR-200 and Neural Cell Adhesion Molecule (NCAM) [108, 170]. miR-200 are co-expressed with Calb2, a differentiation marker of Type II cells and required for the formation of these cells [108]. Preliminary results show that miR-200 are expressed in *tg(fgf8a.enhancer:gfp).* It will be of interest to examine with a conditional approach whether compromised miR-200 activity results in any alteration of cell motility during taste bud formation. In this respect, NCAM is one of miR-200 direct targets [171] and it is expressed in the Type III cell in mouse but also in *tg(fgf8a.enhancer:gfp)* cells in zebrafish (*preliminary results*). Upregulation of NCAM expression accompanies the loss of expression of E-cadherin, the hallmark event of initiation of EMT [170].

Therefore miR-200 and NCAM are good candidate molecules to be involved in the process of taste bud cell motility and organ formation.

IV.2.4) Is cell motility a common phenomenon during sensory organ formation?

Perhaps the most appropriate sensory organs to compare with the taste buds are the Drosophila ommatidia. The Drosophila compound eye is a remarkably precise cellular pattern. It derives from a monolayer epithelium called eye-antennal disc [172-174] and consists of approximately 800 unit eyes assembling into a hexagon with a remarkable precision. There are 32-34 vertical columns of unit eyes. The patterning of the core of the ommatidium in the adult contains eight photoreceptor neurons, four lens secreting cone cells and two pigment cells.

The patterning of the drosophila compound eye originates from the posterior margin of the eye disc and advances anteriorly where undifferentiated cells assemble into ommatidial clusters. In the first stages of embryonic development, there are 8-10 cells in the eye-antennal discs whose number greatly increases in the first two larval instars [175]. At the late second/third larval transition differentiated cells form the morphogenetic furrow which patterns the eye disc. Clusters that arise within the morphogenetic furrow (channel) form the ommatidium. Undifferentiated cells integrate the morphogenetic furrow form preclusters called rosettes. Subset of the preclustered cells form photoreceptor neurons [176]. The cells that do not integrate the preclusters undergo mitosis and the newly born cells generate mature ommatidia [176] [177] [178]. The lens secreting cells are the last to incorporate the ommatidium. The cells that are not specified neither as photoreceptors nor lens will become pigment cells. The eye field generates more cells than required, thus these extra cells are eliminated by apoptosis [179] [180-182].

From the above description it is evident that cell rearrangement does occur to some extent to generate the precise, stereotyped cell content and position within the ommatidia. However, in any case it has not been described cell movement from one organ to another as we found during taste bud formation. This is also the case for sensory organs such as the fish lateral line where primordium-cell migration achieves appropriate position of neuromasts along the antero-posterior axis of the embryo. Cell exchange between neighboring lateral line organs has not been described. Exploring the functional significance of taste bud cell motility and incorporation will be helpful in order to better compare it with other cases in the nervous system.

IV.3) ANNEXE

IV.3.1) Regeneration in adult taste buds

Taste buds are multicellular organs and act as the mediator of the external environment and the brain hence to regulate food perception and ingestion. This raw exposure to the external dangers that the environment disposes has resulted that nature equipped taste buds with a continuous turnover mechanism throughout life and principally during adulthood where the contact with taste bud-destructive elements is more probable than childhood (spice, alcohol, hot, acid, chemical compounds etc.).

Taste bud cells, like other cell types of the lingual epithelium, are continually renewed from basal keratinocytes which are mitotically active and cytokeratin (K) 14+ [183]. This K14+ cells generate postmitotic cells (Type IV) that enter the taste bud and replace the damaged or apoptotic cell by differentiating into taste receptor cells [129, 130, 183-185]. Miura et al have shown in a recent study that Type IV cells are ovoid and situated basally in each taste bud, and many if not all of these basal cells express the morphogen sonic hedgehog (Shh) [96]. By using genetic lineage tracing, they demonstrated that Shh+ basal cells are immediate precursors for all taste cell types within taste buds [186]. In cultured embryonic tongues, they showed that exogenous SHH represses taste placode development [148], whereas pharmacological block of SHH signaling results in larger taste placodes in size and number, and also formed in regions of the tongue typically absent of placodes [143, 147]. It is already known that Shh is expressed by basal cells *inside* taste buds while the SHH target genes Ptch1 and Gli1 are expressed by K14+ progenitor cells adjacent to buds [187, 188] suggesting that SHH has a principal role in the regulation of taste bud regeneration. Previous studies have shown that Shh, by cell proliferation, regulates hair growth, feather formation and skin homeostasis [189, 190] structures that are ectoderm derivatives like lingual taste buds. Miura et al found that ectopic taste buds arise from K14+ progenitors and express genes required for the differentiation of endogenous taste cells, comprise all three differentiated taste cell types, increase in number and become positioned progressively more superficially in the lingual epithelium, and SHH induction of ectopic taste buds is independent of taste and somatosensory innervation [191].

In human, taste bud regeneration is extremely important in patients with head and neck cancer, who receive radiotherapy daily for at least 6-7 weeks. This therapy targets proliferating population of tumor cells, however, affects dramatically the food intake resulting in weight loss. Repeated irradiation affects progenitor cells which are progressively lost. Furthermore, patients that receive treatment for non- head and neck cancers report taste dysfunction. Once the drugs target the dividing cells, progenitor cells are irreversibly affected.

Bacterial and viral infections have been reported to affect taste probably due to inflammation provoked by these pathogens. Wang et al showed that these types of infections cause a response of interferon signalling pathways, which leads to taste cell death [192, 193].

IV.3.2) The *Drosophila* taste system

Concerning the organisation of the taste system, taste organs in flies and in many other insects are distributed in several body parts whereas in mammals are located in restricted regions of the mouth and pharynx. Flies are smaller than mammals, so they can walk and ramble on the surface of their food; it's for this reason why flies possess taste receptor cells not only in their mouth region but also on their proboscis, legs, wings and ovipostor.

To ingest their food, flies use proboscis, a structure that could be a comparable equivalent to the mammalian tongue. Proboscis, set at the base of the head, is a muscular tube connected to the pharynx and comprised of external and internal taste organs (Figure 1D). The external taste organ consists of two labella (or labial palps) that are fused at the end of the proboscis (Figures 1D and 1E). Each labellum displays 31 hair-like taste bristles (sensilla) on outer surface and 30 in inner taste pegs, which are exposed when flies start active feeding. Drosophila larvae also possess sensilla in dorsal terminal and ventral organs of the head region and pharynx.

All over the proboscis, legs and anterior wings there are gustatory receptor neurons (GRNs) housed within 200-300 sensory organs called sensilla and serve as gatekeepers allowing or not the food to proceed to the digestive tube. When stimulated, even by leg- or

labial taste neurons, the proboscis is extended and perceives nutrients or retracted to avoid repulsive substances. Fly GRNs are bipolar neurons that extend dendrites into the bristles and project axons to the central taste center in the subesophageal ganglion (SOG).

Based on their length, the sensilla are categorized in long (L), intermediate (I) and short (S). Each of the L- and S- contain four gustatory receptor neurons and the I- two. The taste bristles on legs and proboscis contain likewise long, intermediate and short GRNs. Each bristle possesses one mechanosensory and either two (intermediate) or four (short and long) gustatory neurons, which extend a dendrite at the tip of the bristle as well as an axonal process that, ends in the subesophageal ganglion [194].

The *Drosophila* gustatory bristles respond to extracellular signals and have the capacity to discriminate the different responses. The short and long bristles contain a single neuron that responds to sugars, one to water, one to salt and one to bitter and high salt-concentrated substances. The intermediate bristles have two chemosensory cells, one responds to both low salt and sugars and the other to high salt and bitter tastants. As a consequence the same cell and single neurons sense different taste qualities that have though the same valence so we could suggest that the guideline of taste in flies is classified in a « valence » labelled line model of coding. It has been shown that some GRNs are activated by sugars and fatty acids and thus promote feeding while another set of GRNs for bitter and high concentrations of salt lead to an aversive behaviour [195].

Number and distribution of sensilla and GRNs that function in contact chemosensation

Body location	Organ name	# gustatory sensilla/pegs	Sensilla type	GRNs	MSNs
External head	Labella palps (or labella)	31 sensilla total			
		9 sensilla	Long (l)	4	1
		10 sensilla	Intermediate (i)	2	1
		12 sensilla	Short (s)	4	1
		~30 pegs		1	
Internal head: pharynx	LSO (labial sense organs)	3 sensilla	7	8	
			8	1	1
			9	1	1
	VCSO (ventral cibarial sense organs)	3 sensilla	Proximal	2	
			Middle	2	
			Distal	4	
	DCSO (dorsal cibarial sense organs)	2 sensilla	Posterior	3	
			Anterior	3	
Leg tarsi					
Male — first leg		~50 sensilla		2–4	1
Female — first leg		~37 sensilla		2–4	1
Second leg		30 sensilla		2–4	1
Third leg		32 sensilla		2–4	1
Wing margins		~40 sensilla		4	1
Oviposiiter		~10 sensilla		?	?

Table1. Number and distribution of sensilla and GRNs that function in contact chemosensation [196], [197]

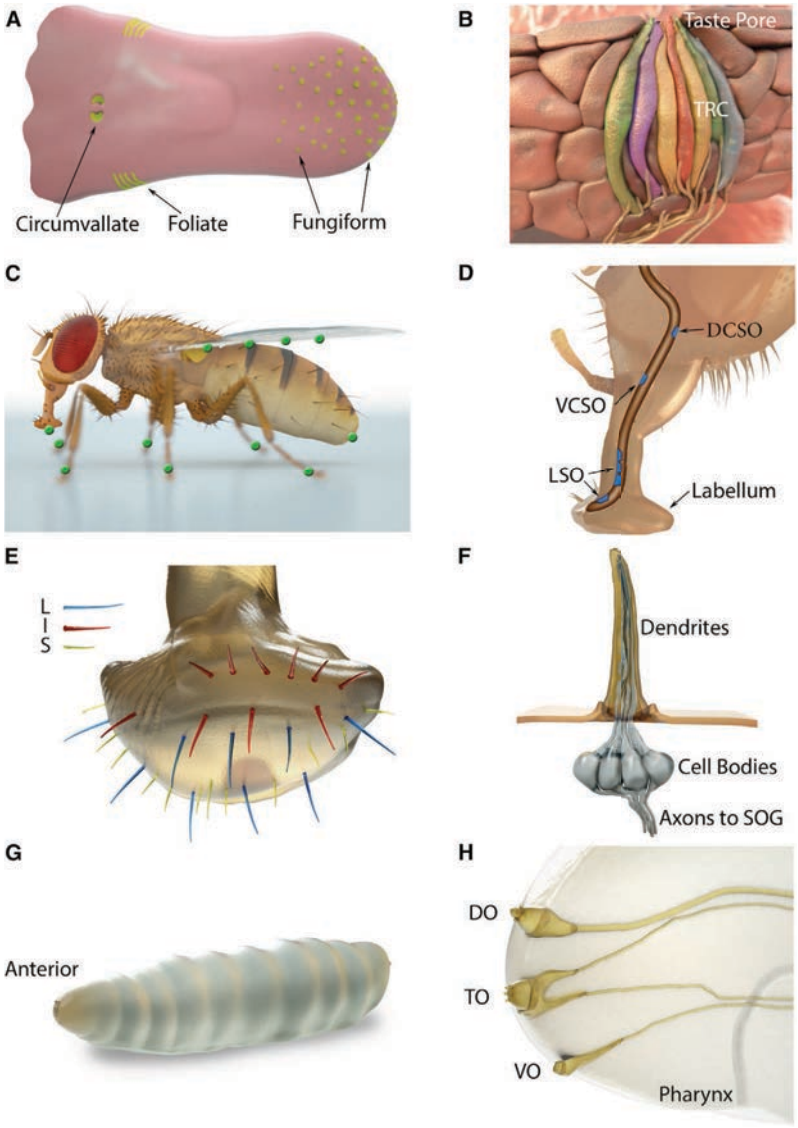


Figure IV-2. The Taste Organs in Mammals, Such as Mice and Humans, and in Flies
(A) The rodent tongue contains taste buds located in three distinct regions. (B) The taste bud is composed of 50–100 modified epithelial cells that extend a process to the taste pore, where they come into contact with ingested chemicals. At least five types of sensory cells (depicted in different colors) are found in the taste bud, corresponding to the five canonical tastes. (C) Green circles indicate locations of external gustatory organs distributed on an adult *Drosophila* female. (D) The Drosophila proboscis. Shown here the labellum and three internal taste organs indicated in blue: the labral sense organs (LSOs), the dorsal cibarial sense organ (DCSO), and the ventral cibarial sense organ (VCSO). (E) Distribution of the L-,I-,and S-type sensilla on a fly labellum. (F) An S- or L-type sensillum containing four GRNs. (G) A *Drosophila* larva. The external chemosensory organs are located at the anterior. (H) Anterior end of a larva. The locations of the dorsal organ (DO), the terminal organ (TO), and the ventral organ (VO) are indicated [198].

IV.3.2.1) Sweet bitter symphony in flies

Fruit flies like mammals are attracted by sweet-tasting compounds, low concentrated salient substances and averse bitter compounds but are indifferent to L-amino acids (umami) or low pH (sour) diets which could explain their preference in spoiled fruits. Gustatory receptors in flies is a group of 68 distantly related genes that encode putative heptahelical transmembrane proteins, originally thought to function as GPCRs but still it is not clear if the signal transmission is G protein dependent or ligand-gated ion channels.

IV.1.3.2.2) Bitter transduction: In flies different sets of bitter-sensitive gustarory receptor neurons have different responsiveness in bitter compounds. Specific populations of

gustatory receptor neurons express certain receptors for example trehalose (Gr5a) is a sweet taste receptor expressed in a population of gustatory neurons either than the bitter taste receptor (Gr66a) [199] [200] [201]. Each of these receptors is present in only one GRN in almost all bristles. Projections of the GRN end in different areas. In flies, as in mammals distinct populations are devoted to specific feeding behaviour of attraction or aversion. Gr5a labelled cells represent a labelled line for sweet tastants whereas Gr66a is a receptor that corresponds to a labelled line for bitter [200-202]. Gr32a, Gr33a and Gr66a are receptors necessary for the detection of bitter chemicals and they are expressed in all bitter responsive GRNs. Different combinations of these small template of receptors could possibly explain their capacity to detect a wide range of structurally diverse bitter substances.

Apart the GRs, three transient receptor potential channels (TRP) contribute to the detection of aversive compounds using a detection mechanism other than that of GRs. For instance TRPA1 is expressed in a subset of bitter GRNs and is involved in the detection of aristolochic acid. This channel increases the aristolochic detection when the temperature is increased, meaning that the taste responses are temperature dependent. TRP-like (TRPL) is another channel also expressed in GRs with sensitivity to camphor, a compound that is not toxic but provokes aversion in flies. When flies are exposed for a long period of time to camphor-containing food, they adapt to this primarily aversive substance.

IV.1.3.2.3) Sweet transduction: Flies, like humans, are attracted by sweet tastants, especially by disaccharides and oligosaccharides. The trehalose receptor (GPCR protein with a short N-terminal domain) of *Drosophila* is the first successful attempt to isolate a receptor corresponding to sweet tastants. Other receptors involved in this transduction are the GR5a, GR64a, GR64f [203, 204], [205-207]. More precisely, GR64a detects sucrose and maltose while GR5a detects trehalose and melezitose and GR43a (a cation channel) detects fructose. In drosophila, it has been observed an inhibition of sweet taste by bitter compounds, which suppress feeding by activating bitter-sensing GRNs and simultaneously inhibiting sugar-sensing GRNs.

IV.1.3.2.4) Umami transduction: The response on amino acids in flies depends on their sex; females prefer phenylalanine, cysteine, tyrosine and threonine rich food though males show stronger preference for leucine and histidine rich compounds. The receptors

known for sweet-taste transduction are not involved in umami transduction raising the possibility that taste pegs detect amino acids.

IV.1.3.2.5) Salty transduction

Flies respond to salty sensing compounds similar to human, for example they respond with pleasure to a low-salt food and averse compounds containing a high concentration of salt. Larvae detect salt via ENaC channel members *ppk11* and *ppk19* but these channels do not appear in adult flies. An ionotropic glutamate receptor (IR) is required for low-salt sensing and they are expressed in GRNs. More precisely IR76b is expressed in GRNs- different than the ones that respond to sweet and bitter- and extend their projections into a single region in the SOG. Adult flies detect low-salt compounds with GRNs in L-type sensilla and aversively the high-salt compounds with S-type sensilla, which consequently suppress feeding. Therefore, the salt concentration is in a competition between these two sensilla. When the Na^+ concentration is really increased, the Na^+ flux through ENaC and IR76b depolarise the taste receptor cells, so flies manipulate these Na^+ leak channels and better respond to salt's quantity.

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Abstract

Taste buds are the sensory organs of taste, located within the oropharyngeal cavity and conserved in jawed vertebrates. They derive from two epithelia, ectodermal rostrally and endodermal caudally, without showing any major physiological differences. They are multicellular organs, composed of three distinct cell types that detect different taste stimuli. Several signaling pathways, including Wnt/b-catenin, Shh, Bmp, Notch and Fgf are required in the induction and differentiation of taste bud cells.

The current work aims at understanding how oropharyngeal epithelial cells that differentiate to taste bud cell types, do behave in vivo to form functional sensory organs. The zebrafish larva is used as model because of its genetics, optical clarity and accessibility of the oropharynx. Firstly, a new transgenic line *tg(fgf8a.enhancer:GFP)* is characterized with respect to taste bud development. It is expressed in epithelial and all differentiating taste bud cell types. Second, spinning disk timelapse shows that *gfp+* cells form groups, one of them differentiates to the 5-HT cell and altogether correspond to a developing taste bud organ. Third, surprisingly, developing taste bud cells are motile between neighbouring taste bud organs. Finally, focal two-photon laser ablation of the 5-HT cell and analysis of ascl1a-/- that is devoid of 5-HT cells show that the 5-HT cell is required for the maintenance of *gfp+* cells into the developing taste bud. This is the first evidence that taste bud formation is dynamic, developing cells are motile between taste buds and require one taste bud cell type (5-HT) for their maintenance into the organ.

Resumé

Les bourgeons gustatifs, les organes sensoriels du goût, sont localisés dans la cavité oropharyngéenne et ils sont conservés chez les vertébrés à mâchoire. Selon leur localisation, ils ont une origine épithéliale différente (rostralement, ectodermique et caudalement endodermique). Ils sont composés de trois types cellulaires distincts qui détectent les différentes qualités gustatives. Bien que l'induction et la différenciation cellulaire de ces organes sont bien étudiées, les mécanismes de la formation des organes intactes et fonctionnelles à partir de ces cellules restent inconnus.

L'objectif de ce travail est de comprendre comment les cellules épithéliales se comportent *in vivo* afin de former des organes sensoriels gustatifs fonctionnels chez le poisson zèbre. En premier, une nouvelle lignée transgénique *tg(fgf8a.enhancer:GFP)* (*gfp+*) a été caractérisée. Elle est exprimée dans tous les types cellulaires du bourgeon au cours du développement. En deuxième, la vidéomicroscopie in vivo montre que les cellules *gfp+* peuvent se différencier à des cellules sérotoninergiques (*tph1b*/5-HT) et elles se regroupent autour de ces dernières. En troisième, de façon surprenant, les cellules *gfp+* sont motiles et elles peuvent se déplacer d'un bourgeon à l'autre. Enfin, l'ablation au laser multi-photon et l'analyse du mutant *ascl1a-/-* montrent que la cellule 5-HT est nécessaire au maintien des cellules *gfp+* dans le bourgeon. Ce travail démontre pour la première fois que la formation des bourgeons gustatifs est un processus dynamique et que les cellules de cet organe sensoriel sont motiles.