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Ligand-specific induction of endocytosis in taste receptor cells

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SUMMARY

We demonstrate a ligand-specific induction of endocytosis in cells of juvenile brown trout taste buds. The process is fast, massive and selective, as only a few cells in each taste buds are stained by exposure of the oral cavity to the taste stimulant L-cysteine together with a dye at 20°C. Low temperature (+2°C) and disruption of microtubules with nocodazole caused a substantial reduction in the number of taste cells stained, indicating endocytic uptake of dye and transport towards the cell soma in vesicles. As endocytosis is evoked by the presence of ligands, it is most likely that the stained cells are the so-called receptor cells, which have taste receptors and the molecular machinery for downstream processing. The number of stained taste cells and taste buds containing stained taste cells increased with the concentration of L-cysteine. Control experiments with different dyes revealed great variability in the ability to induce staining on their own. In particular, Texas Red dextran was efficient and stained many cells within each taste bud. Behavioural experiments demonstrated that Texas Red dextran is a deterrent taste substance for brown trout. In fish first exposed to the stimulant L-cysteine plus a dye and subsequently to a deterrent, either Texas Red, or glycine, the majority of stained cells were found in separate taste receptor cells, indicating that the majority of taste receptors for stimulants and deterrents are expressed in separate taste buds. These results also strengthen the assumption that the stained cells take part in the initiation of taste processes that are related to perception. The functional implication of the induced endocytosis is discussed.

Key words: fish, endocytosis, taste cells, stimulants, deterrents.

INTRODUCTION

The taste system recognizes and discriminates a large number of taste substances; and in fish, these tastants give rise to acceptance or rejection (Kasumyan and Døving, 2003). In mammalian taste buds, three different subtypes of elongated epithelial cells and proliferative basal cells have been described (Murray, 1974). One of these types of taste cells possesses synapses (type III cells, presynaptic cells), another expresses chemosensory transduction proteins (type II cells, light cells, taste receptor cells), and the third class expresses none of these properties (type I cells, dark cells) (Clapp et al., 2006; DeFazio et al., 2006; Tomchik et al., 2007; Yee et al., 2001). It has been stated that cells of the fish taste buds have morphological similarities to those of taste buds of rodent fungiform papillae (Murray, 1974); and a similar classification might be valid for fish taste buds (Reutter et al., 2000; Reutter and Hansen, 2005).

Numerous electron microscopy studies have described vesicles, microtubules and filaments in the apical parts of taste bud cells of mammals and fishes (Farbman, 1965; Ganchrow et al., 1993; Reutter and Hansen, 2005; Reutter and Witt, 1993). These elements are essential for endocytosis and transport of endocytic vesicles. Endocytosis was studied in the sensory cells of frog taste disks with multi-photon microscopy (Li and Lindemann, 2003), and the authors found a staining of sensory cells after 24 h exposure of taste buds to the styryl dye FM1-43. It has also been reported that FM1-43 rapidly and specifically labels taste buds by a channel permeation mechanism in mice after subcutaneous injection (Meyers et al., 2003).

In the present study, we demonstrate a ligand-specific induction of endocytosis in taste cells. Such a feature has, to our knowledge, not been demonstrated before. We used brown trout because of the abundance of taste buds in the oral cavity (Hara et al., 1994; Marui et al., 1983) and because of knowledge of substances accepted (stimulants) or rejected (deterrents) when presented to brown trout in food pellets (Kasumyan and Sidorov, 2005). The oral cavity was exposed to taste substances in combination with dyes or to dyes alone. Thus, we stimulated the taste buds in a way that is normal for taste perception. As we see a ligand-specific induction of endocytosis, it seems probable that the stained cells were of the ‘taste receptor cell’ type. Fluorescent styryl dyes (FM dyes) have been frequently used as probes for membrane trafficking and endocytic and exocytic activity because of their physical properties giving an enhanced fluorescence in lipids (Betz et al., 1992a; Betz et al., 1992b; Betz et al., 1996; Brumback et al., 2004; Cochilla et al., 1999). There are several forms of endocytosis and although clathrin-dependent uptake of receptor–ligand complexes has received much attention clathrin-independent forms also exist (Mukherjee et al., 1997; Mayor and Pagano, 2007; Sandvig et al., 2008).

In our experiments, dye reached all parts of taste receptor cells rapidly after apparent endocytic uptake and vesicular transport, since low temperature and disruption of microtubules inhibit ligand-induced staining of the taste receptor cells. An additional finding is that taste receptors for a stimulant and a deterrent seem to be expressed mainly in separate taste receptor cells.

MATERIALS AND METHODS

Biological specimens

Experimental procedures were approved by the Norwegian Animal Research Authority and were conducted in accordance with the Norwegian Animal Welfare Act of 1974, and the Regulation of Animal Experimentation of 1996. Juvenile brown trout *Salmo trutta* L. (6–10 g, 5.5–10 cm body length) were obtained from a local fish farm (Oslomarkas fiskeriadministrasjon, OFA, Sørkedalen, Oslo, Norway). The fish were kept in the aquaria facilities at the Department of Molecular Biosciences, University of Oslo at a water temperature of 8°C and at 12 h:12 h L:D photoperiod. The fish were fed twice a week with commercial food pellets. Fish were given an intraperitoneal injection of Hypnodil (metomidatum hydrochloridium; Janssen, Pharmaceutica Beerge, Belgium; 60 mg kg^{-1}). Anesthetized fish were wrapped in wet paper, adjusted in a cradle belly up. For experiments lasting more than 5 min, two catheters supplied tap water irrigation of the gills. In total 96 fish were used for the physiological studies.

Staining procedure

Fluorescent dye uptake by taste receptor cells was examined at room temperature (20°C), unless otherwise indicated. Dye solutions were applied to the oral cavity of fish at a rate 1 ml min^{-1}. To examine the rate of styryl dye entry into taste cells, we bathed the oral cavity for ~10 s, 30 s, 1, 2, 5 and 10 min. In all experiments the test substances were dissolved in distilled water. Immediately after the end of application, the oral cavity was rinsed in tap water, the fish was decapitated and the head fixed in paraformaldehyde (4% in 0.1 mol l^{-1} phosphate buffer, pH 7.1). To study the temperature dependence of dye uptake, experiments were repeated at +2°C. Long-range transport of endocytic vesicles are often dependent on molecular motors and microtubules which can be disrupted by nocodazole (Schliwa and Woehlke, 2003) so the effect of nocodazole was studied with a pre-exposure for 20 min at 35 μmol l^{-1} at room temperature before exposing the oral cavity to a mixture of FM dye and L-cysteine for 5 min. The dextrans were applied at 10 or 20 μmol l^{-1}.

Reagents

FM 1-43 and FM4-64 FX were purchased from Molecular Probes (Eugene, OR, USA). L-cysteine, glycine and nocodazole M1404 were purchased from Sigma-Aldrich (St Louis, MO, USA). AM1-43, the fixable analogue to FM1-43 was purchased from Biotium (Hayward, CA, USA). The following fluorescent dextrans were purchased from Invitrogen (Carlsbad, CA, USA): 3 kDa, Texas Red, lysine fixable D3328; 3 kDa Alexa Fluor 488; anionic D-34682; 3 kDa Alexa Fluor 688, D34681, and 40 kDa fluorescein, anionic lysine fixable D1845.

Fluorescence and confocal examination

The upper and lower jaws were mounted in Petri dishes to view taste buds on the epithelium. All photographs were made of taste buds *in situ*. We standardized our examination to the following regions of the oral cavity: the upper and lower lips, 3 mm from the midline to the left and to the right, the anterior 3 mm of the vomer and the anterior 3 mm of the tongue. Taste buds with stained taste receptor cells were counted using a fluorescence microscope (BX50WI, with a 20× water immersion lens; Olympus, Tokyo, Japan) equipped with a digital camera (ProgRes, Jena, Germany). The software program ImageJ (1.37v, Wayne Rasband, NIH, USA) was used to extract information from photographs taken at different depths, usually 2.5 μm. Images were imported and the stacks added in a z-projection with the max intensity parameter. A confocal microscope (Olympus FluoView 1000, BX61W1) was used to observe the details of the taste receptor cells in the taste buds and the possible overlap or colocalization between taste receptor cells in double staining experiments. Images were taken in planes, separated by z-axis steps varying between 0.4 and 2 μm.

Behaviour

Eight brown trout juveniles were placed in individual aerated aquaria (18°C) and trained to take 2% agar pellets, 3.0 mm in length and 1.3 mm in diameter, prepared with Ponceau 4R (red; 5 μmol l^{-1}). In trial sessions, test pellets with dextran Texas Red (0.5 mmol l^{-1}), L-cysteine (100 mmol l^{-1}) or Chironomidae larvae water extract (75 g l^{-1}) were offered to the fish at random, one by one at intervals of more than 20 min. In total, 141 taste trials were performed. The acceptance ratio was calculated as the number of pellets accepted over the number of pellets taken into the mouth.

RESULTS

The taste structures examined were from the anterior 3 mm of the upper and lower lips (left and right side), the vomer, and the tongue in juvenile brown trout. In these regions, there are a total of about 600 taste buds. The tongue and vomer in brown trout have lobes that make it difficult to see all taste buds. We did not attempt to estimate the number of cells in the different taste buds.

Staining of taste cells by different dyes

The different dyes when they were applied alone varied greatly in their ability to stain cells. Table 1 give the dyes used, the number of cells stained, and the number of taste buds with stained cells. From the table it is evident that fluorescein dextran, Alexa Fluor 488 dextran and Alexa Fluor 680 dextran, stained only a few taste cells. FM1-43 resulted in a moderate number of stained taste cells, whereas the fixable analogue AM1-43 stained more receptor cells than FM1-43. The staining efficiency with the dextrans was dependent upon the conjugated dye. The efficiency of Texas Red and tetramethylrhodamine-conjugated 3 kDa dextran was particularly high, and it is worth noting that more than two taste cells per taste bud were stained.

FM dye and stimulant

Exposure of the oral cavity to FM1-43 in distilled water (1 μmol l^{-1}; 2 min) stained only 40 taste cells in 32 taste buds (Table 1). By

Table 1. Staining efficiency obtained with different fluorescent dyes

Dye	Concentration (μmol l^{-1})	Taste buds	Receptor cells	Ratio RC:TB
3 kDa dextran-fluorescein	20	1	1	1
3 kDa dextran-Alexa Fluor 488	10	4	5	1.3
3 kDa dextran-Alexa Fluor 680	10	9	11	1.2
FM1-43	1	32	40	1.3
3 kDa dextran-tetramethylrhodamine	20	61	143	2.3
AM1-43	1	143	192	1.3
3 kDa dextran-Texas Red	20	145	419	2.9

Values are the mean number of taste buds (TB) and receptor cells (RC) stained in the lips, tongue and vomer. Mean of two fish for each dye; see Materials and methods for details.

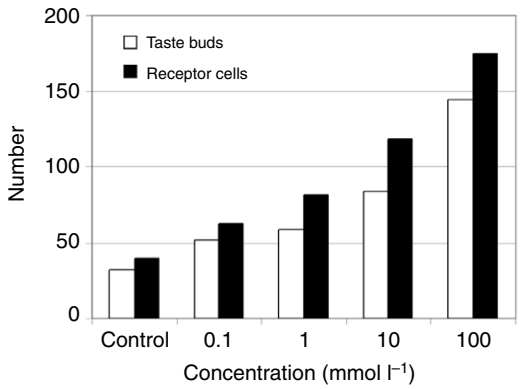

Fig. 1. Staining of taste receptor cells is concentration dependent. The histograms demonstrate the increase in number of stained taste receptor cells and of taste buds containing stained receptor cells with increasing concentration of the stimulant amino acid L-cysteine together with 1 μmol l^{-1} FM1-43, over a 2 min exposure. Means of two fish. The control is 1 μmol l^{-1} FM1-43 alone. Cells were counted in the anterior region of the lips, tongue and vomer.

contrast, exposing the oral cavity to FM1-43 (1 μmol l^{-1}) together with the taste stimulant L-cysteine (100 mmol l^{-1}), stained 175 taste cells in 144 taste buds distributed as follows: the upper lip 31, vomer 51, lower lip 49, and tongue 44.

The number of stained taste cells and taste buds containing stained taste cells increased with the concentration of L-cysteine in the range 0.1–100 mmol l^{-1} applied together with 1 μmol l^{-1} FM1-43 (Fig. 1). The ratio between taste cells and taste buds in the regions examined varied between 1.2 and 1.4, demonstrating that most taste buds contained only one stained taste cell. The maximum number of stained cells in a single taste bud was 3.

Threshold

The behavioural threshold for L-cysteine in taste preference tests with agar pellets is about 10 mmol l^{-1} (Kasumyan and Sidorov, 2005). At this concentration we found 84 taste buds with stained cells. Given the background staining with FM1-43 alone (32 taste buds), it indicates that about 52 taste buds (9%) have to be activated to evoke an appropriate behavioural response to a taste stimulant. At the subthreshold concentration of 1 mmol l^{-1}, around 27 additional taste buds (5%) were stained, suggesting that these numbers are insufficient for the fish to assess the palatability of the food object and make the decision to swallow it.

Morphology of stained cells

Application with a short duration of ~10 s gave a weak staining of about 20 μm depth of the apical part of taste cells. A 30 s exposure was sufficient to stain most parts of a taste cell (Fig. 2A). It should, however, be noted that it may take some time before the fixative penetrates into the taste bud and reaches the transport processes along the microtubules; thus allowing movements of vesicles for somewhat longer time. With 1, 2, 5 and 10 min exposure before application of fixative, the whole taste cell was stained (Fig. 2).

Fluorescence was distributed throughout the cytosol, and the cell soma was clearly visible. The staining was punctate, i.e. bright spots on a diffuse background (Fig. 2A). The staining made it easy to see the pyriform shape of the taste cells with the apical part being long, slender and curved, and often more intensely staining than the basal part. The cytoplasm around the nucleus was thin. Often the basal part of the cell consisted of a thin protrusion that ended in a small foot (Fig. 3A).

In a single taste bud, with two or more stained cells, these were seldom found close to one another at the nuclear level. The distance between nearest neighbour cell nuclei was around 10 μm.

Dye entry is *via* endocytosis

There are a variety of endocytic mechanisms which have been determined in a number of different tissues (Sandvig and van Deurs, 2005). In mammals, temperatures near 0°C effectively stop all types of endocytosis. In rainbow trout, *Oncorhynchus mykiss*, and tilapia, *Oreochromis nilotica*, endocytic activity is strongly reduced at +5°C during the initial lowering the temperature (Padron et al., 2000). We placed the anesthetized fish in iced water, irrigated the gills and exposed the oral cavity to ice cold solutions of 100 mmol l^{-1} L-cysteine together with 1 μmol l^{-1} AM1-43. The temperatures

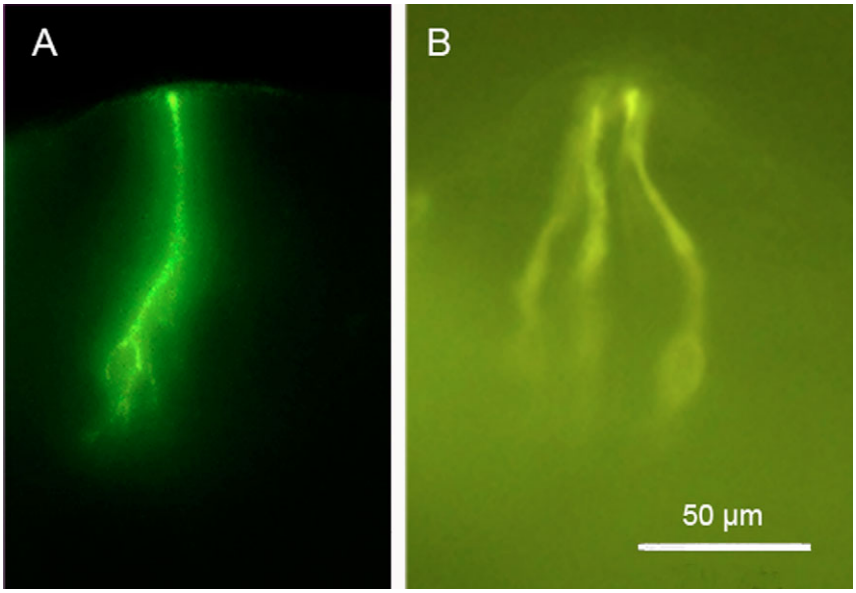

Fig. 2. Receptor cells in taste buds of juvenile trout. (A) A receptor cell from a taste bud in the vomer, stained with L-cysteine (100 mmol l^{-1}) + FM1-43 (1 μmol l^{-1}). The taste bud was exposed for 30 s and fixed with paraformaldehyde in 0.1 mol l^{-1} phosphate buffer. (B) A taste bud on the tongue with three sensory cells stained with L-cysteine (100 mmol l^{-1}) + FM1-43 (1 μmol l^{-1}; 5 min). The images are z-projections taken using a fluorescence microscope with 2.5 μm distance, stack parameter 'max intensity' and processed with ImageJ. The scale bar is for both images.

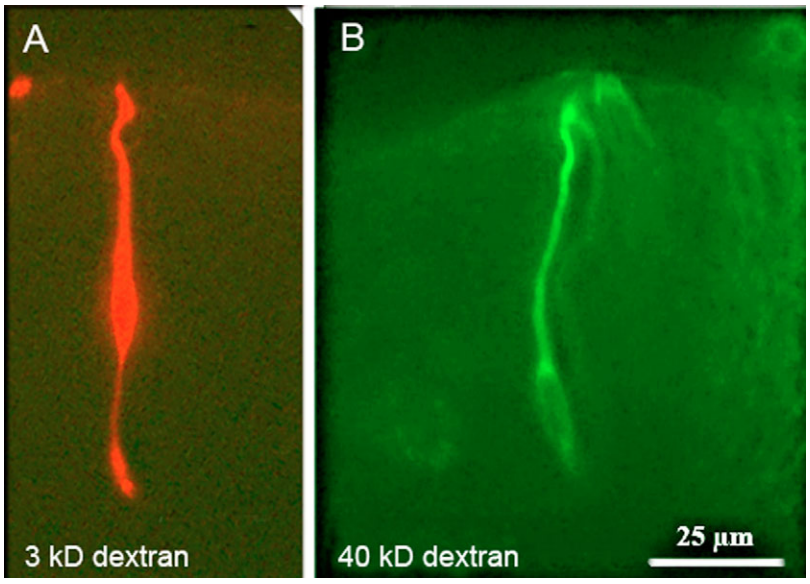

Fig. 3. Taste receptor cells stained with dextrans. (A) Taste receptor cell stained with L-cysteine (100 mmol l^{-1}) + 3 kDa dextran Texas Red (20 µmol l^{-1}; 5 min). (B) A taste receptor cell stained with L-cysteine (100 mmol l^{-1}) + 40 kDa dextran fluorescein (20 µmol l^{-1}, 5 min). The images are z-projections taken using a fluorescence microscope with 2.5 µm distance. Processed by ImageJ with the stack parameter 'max intensity'.

measured in the solution and mouth cavity was +2°C. We observed on the upper jaw (lip and vomer) a considerable reduction from 431 taste cells in 248 taste buds at room temperature to 15 stained taste cells in 14 taste buds at +2°C (Table 2).

Application of nocodazole (see Materials and methods) reduced substantially the number of stained taste cells on the upper lip and vomer from 431 to 111 (Table 2). The reduction in the number of stained taste cells in the vomer was less efficient, probably because irrigation of the gills with tap water caused some backflow and rinsing during application of nocodazole. In sum, these results indicate a taste-specific induction of endocytosis, and furthermore that the endocytic vesicles are transported *via* microtubules toward the taste receptor cell soma.

A single subcutaneous injection of FMl-43 in mice brightly labels hair cells, Merkel cells, muscle spindles, taste buds, enteric neurons and primary sensory neurons within the cranial and dorsal root ganglia (Meyers et al., 2003). The authors provided evidence that this cationic dye is able to pass through a number of different ion channels. Given these studies, we applied dextrans with high molecular mass, which are unlikely to pass through ion channels: Texas Red 3 kDa dextran and FITC 40 kDa dextran, together with 100 mmol l^{-1} L-cysteine. Both types of dextran stained a substantial number of taste receptor cells (Fig. 3). It is unlikely that these large conjugated dextrans would pass through ion channels. Our data suggest that they are internalized by endocytosis.

Texas Red is a taste deterrent

The basic knowledge of which substances are stimulants and which deterrents in fish is surprisingly fragmentary (Kasumyan and Døving, 2003). As seen from Table 1, Texas Red 3 kDa dextran alone was remarkably efficient in staining taste receptor cells in brown trout. These findings provoked a series of taste preference studies with Texas Red dextran to verify that this substance, which stains taste receptor cells, also evoked a taste sensation in brown trout. The behavioural taste experiments demonstrated that the Texas Red-conjugated dextran at a concentration of 0.5 mmol l^{-1} evoked an acceptance ratio of 6%. In comparison, control pellets, and pellets with L-cysteine or water extract of Chironomidae larvae (75 g l^{-1}) gave acceptance ratios of 29, 91 and 94%, respectively (Table 3). These findings demonstrated that pellets with Texas Red dextran (0.5 mmol l^{-1}) were spat out, and this dye is a deterrent for brown trout at a much lower concentration than is effective for the stimulant, L-cysteine (100 mmol l^{-1}). It is probable that the dye Texas Red alone is responsible for the aversive effect, since dextrans conjugated with other dyes induced a small number of stained taste receptor cells.

Application of stimulant and deterrent substances

Taste substances that induce different behavioural effects ought to stain different taste cells and/or different taste buds if the staining that we see are cells giving rise to taste perception. We carried out several experiments to investigate this assumption.

From previous behavioural experiments it is known that glycine is a deterrent taste substance for brown trout (Kasumyan and Sidorov, 2005). We applied this substance at a concentration of 100 mmol l^{-1} together with 1 µmol l^{-1} AM1-43 to three fish (for 5 min). In three other fish we applied 100 mmol l^{-1} L-cysteine together with 1 µmol l^{-1} AM1-43 (5 min). In the anterior 3 mm of the lips, tongue and vomer we counted the number of taste buds with stained cells, and how many stained cells there were in each taste bud (Table 4). There were no great differences in distribution

Table 2. Effect of low temperature and nocodazole on taste receptor cells

	+20°C		+2°C		Nocodazole	
Number of RC in TB	TB	RC	TB	RC	TB	RC
1	138	138	13	13	47	47
2	65	130	1	2	20	40
3 or more	44	163	0	0	8	24
Total	247	431	14	15	75	111

Values are the number of taste buds (TB) with stained receptor cells (RC) observed on the lip and vomer of the upper jaw after application of 100 mmol l^{-1} L-cysteine together with 1 µmol l^{-1} AM1-43 for 5 min, at +20°C, at +2°C, and after pre-exposure to 37 µmol l^{-1} nocodazole for 20 min (20°C). Mean from two fish for each experiment. The numbers have been rounded up to the nearest integer.

Table 3. Taste acceptance of pellets by brown trout

Stimulus	Pellets sampled	Pellets accepted	Acceptance ratio (%)
Chironomidae water extract, 75 g l^{-1}	54	51	94
L-Cysteine, 100 mmol l^{-1}	21	19	90
Control	35	10	29
Texas Red dextran, 0.5 mmol l^{-1}	31	2	6

The pellets were made of 2% agar with red dye Ponceau 4R, 5 μmol l^{-1} (control). Pellets were 3 mm long and 1.3 mm in diameter. The different stimuli were added at the concentrations indicated (*N*=8).

in the regions investigated, or in the number of stained cells per taste bud.

Given that there are no gross differences in distribution in the regions investigated, one could ask if there are differences in the small scale. Could it be that stimulant and deterrent substances stained taste cells in the same or in separate taste buds? In order to answer this question, we did several experiments with stimulants and deterrents. First, we exposed the oral cavities of two fish first to 100 mmol l^{-1} glycine + 1 μmol l^{-1} AM1-43, then to 100 mmol l^{-1} L-cysteine + 20 μmol l^{-1} Alexa Fluor 680 dextran for 3 min each solution. Inspection under the fluorescence microscope revealed that in these fish, a total of 324 taste buds contained stained cells. Of these taste buds, 164 were stained yellow with 100 mmol l^{-1} glycine (deterrent) + 1 μmol l^{-1} AM1-43, 98 were stained red with 100 mmol l^{-1} L-cysteine (stimulant) + 20 μmol l^{-1} Alexa Fluor 680 dextran, and 62 taste buds had both yellow and red cells. Thus, the majority of taste buds responded either to glycine or to L-cysteine. However, 19.1% of the taste buds had taste cells that stained either yellow for glycine (deterrent) or red for L-cysteine (stimulant) at these concentrations of the applied substances.

Second, the oral cavity of three fish were exposed, first to 100 mmol l^{-1} L-cysteine + 1 μmol l^{-1} AM1-43, then to 100 mmol l^{-1} glycine + 2 μmol l^{-1} FM4-64 FX each for 3 min. Inspection of the vomer in these fish showed that in total 99 (46.3%) taste buds had only yellow stained cells, 78 (36.4%) had only red stained cells, and 37 (17.3%) had both yellow and red stained cells. Confocal microscopy of some of these taste buds with both yellow and red stained cells demonstrated colocalization of the two dyes in the same cell (Fig. 4).

Third, as the dye Texas Red was found to be a deterrent substance by itself, we also exposed fish first to 20 μmol l^{-1} Texas Red, and then to a mixture of the stimulant 100 mmol l^{-1} L-cysteine together with 20 μmol l^{-1} fluorecein 3 kDa dextran for 2 min. Inspection under the fluorescence microscope revealed that in three fish, a total of 281 taste buds contained stained cells. Of these taste buds, 210 had only Texas Red staining, 62 had only fluorescein staining, and nine taste buds had both yellow and red cells. Thus, only 3.2% of the taste buds had taste receptor cells that stained both for L-cysteine (stimulant) and Texas Red dextran (deterrent) at these concentrations of the applied substances (Table 5).

DISCUSSION

The most important finding in the present study is that there is prominent ligand-specific induced endocytic activity in taste cells of brown trout. The endocytosis is dependent upon the taste substance being present at the taste bud pore. Since the endocytic activity can be identified with styryl dyes that have a more efficient fluorescence in lipid than in water solution, the procedure of including the taste substance together with a dye is an efficient way of labelling activated taste cells. However, all substances present at the taste bud pore could be included in the endocytic vesicles as high molecular mass dextrans with dyes were found to stain the cells. Use of stimulant and deterrent substances revealed that the majority of taste buds had taste cells that stained for either one or the other of the two substances. However, some taste buds had cells that stained for both stimulant and deterrent substances.

What type of cell is stained?

In the vertebrate taste bud there are three types of epithelial cells. Several arguments suggest that the cells that stained in our experiments were the so-called receptor cells, or type II cells, following the nomenclature of Murray (Murray, 1974). These cells have the taste receptors and the molecular machinery for downstream processing and thus express the elements of the taste transduction cascade.

There is a vast literature on electron microscopy studies of taste buds in vertebrates, and also taste buds in fishes have been described in great detail (Reutter and Witt, 1993). These electron microscopic examinations indicate that the taste cells contain elements such as vesicles and microtubules that could indicate endocytic processes (Ezeasor, 1982; Fujimoto et al., 1993). Also different types of filaments or filament-associated structures have been found in taste cells, such as actin, espin and keratin (Hofer and Drenckhahn, 1999; Sekerkova et al., 2004; Takeda et al., 1990). It seems reasonable to assume that a similar division might apply also to taste buds in fish (Finger, 2005). Also, the taste receptor cells have a large, round nucleus (Finger, 2008), a characteristic feature of the cells that stained in the present study.

Our data demonstrate that ligands are essential for staining of cells, and one dye (Texas Red) that was particular efficient when tested as control, turned out to be a taste deterrent. The number of stained cells increased with increasing concentration of ligand. These

Table 4. Staining of receptor cells and taste buds to stimulant and deterrent taste substances

	100 mmol l^{-1} L-cysteine + 1 μmol l^{-1} AM1-43					100 mmol l^{-1} glycine + 1 μmol l^{-1} AM1-43				
RC/TB	Upper lip	Lower lip	Vomer	Tongue	Total	Upper lip	Lower lip	Vomer	Tongue	Total
1	43	44	27	24	138	15	24	55	29	123
2	18	18	18	11	65	6	4	20	11	41
3	9	7	13	1	30	3	1	2	2	8
4	2	1	5	0	8	1	0	1	0	2
5	0	2	4	0	6	0	0	1	0	1
TB	72	72	67	36	247	25	29	79	42	175
RC	114	115	153	49	431	40	35	110	57	242

Values are the mean number of stained receptor cells (RC) per taste bud (TB) for the stimulant L-cysteine (*N*=3) and the deterrent glycine (*N*=3) in four regions of the mouth. Exposure time was 5 min for both substances. The numbers have been rounded up to the nearest integer. See text for details.

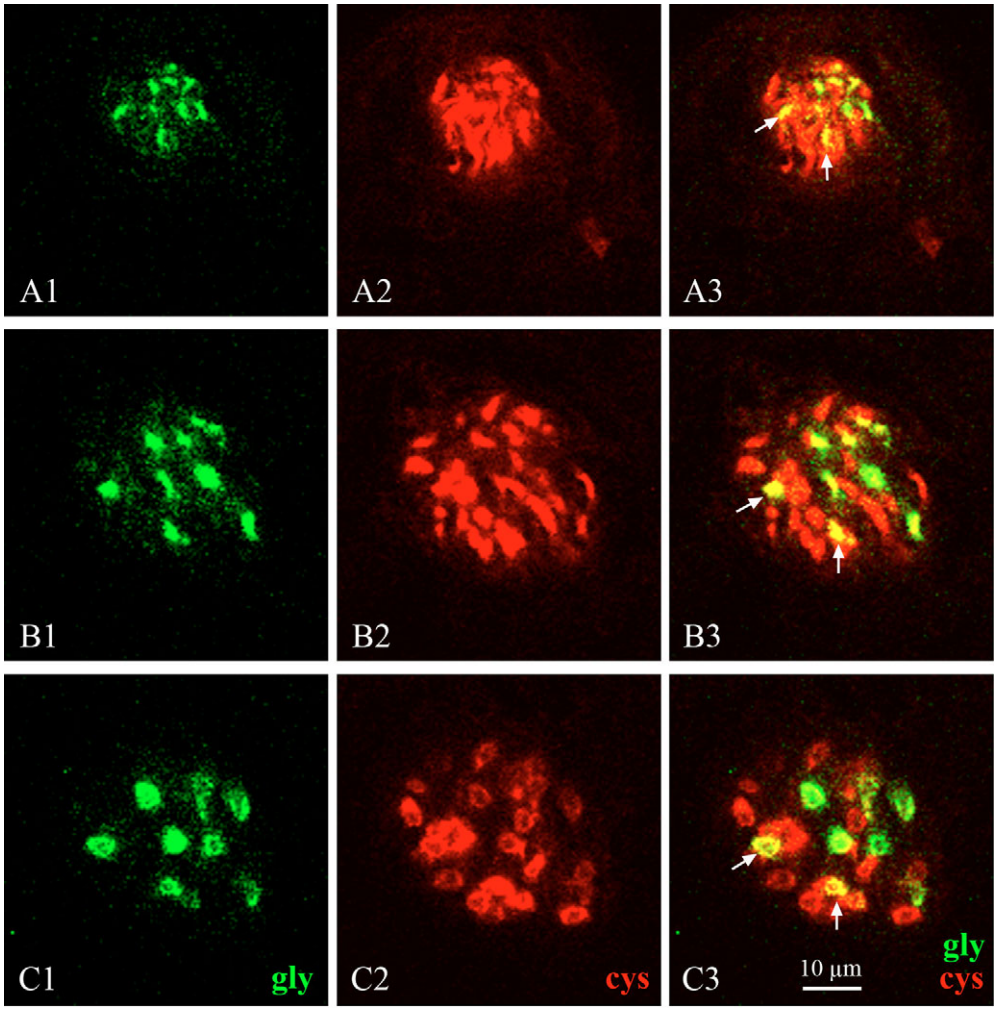

Fig. 4. Cells in a taste bud stained by stimulant and deterrent taste substances. Confocal micrographs showing cross sections of a taste bud taken at the taste bud pore (A), 20 μm below (B), and 40 μm below (C) that level. The oral cavity was first exposed for 5 min to glycine (gly), 100 mmol l^{-1} + AM1-43, 1 μmol l^{-1} (green, left column) and then for 5 min to L-cysteine (cys), 100 mmol l^{-1} + FM4-64FX, 20 μmol l^{-1} (red, middle column). The right column is the merged pictures. The yellow colour in some cells (arrows) indicates colocalization of green and red dye in the same cell. Note that two cells are yellow at all levels. Scale bar is for all images. See text for details.

results also support the assumption that the stained cells are related to taste perception.

Finally, our results demonstrate that the majority of taste buds had cells that respond to either stimulants or deterrents, and both types of taste buds are evenly distributed throughout the fish mouth. Thus, the majority of taste cells either expressed receptors for taste stimulants or deterrents, which is a prerequisite if the substances should induce different behavioural effects, i.e. acceptance and rejection, respectively. The fact that the majority of these cells were found in different taste buds, might be related to the species and sets of substances that we used. Ishimaru et al. and Oike et al. examined the types of taste receptors (T1R and T2R) expressed in the taste buds of zebrafish and medaka (Ishimaru et al., 2005; Oike et al., 2007). They suggested that, among vertebrates, attractive tastants are recognized by T1Rs and aversive tastants by T2Rs. They showed that although the T1Rs and T2Rs are expressed in different taste cells, they generally are co-expressed in the same taste bud. These results are in contrast to the present findings in brown trout, showing that stimulants and deterrents, in general, stained receptors cells in different taste buds. By combining the ligand-specific endocytosis with *in situ* hybridization techniques in the same species, it should be possible to verify these hypotheses.

Table 5. Combined staining with a stimulant and a deterrent

	L-Cysteine, 100 mmol l^{-1} + fluorecein dextran, 20 μmol l^{-1}	Texas Red 20 μmol l^{-1}	Combined
Fish 1	11	67	2
Fish 2	20	69	3
Fish 3	31	74	4
Total	62	210	9
Mean (%)	22.1	74.7	3.2

Values are the number of taste buds on the lips, tongue and vomer that had green, red or both green and red stained receptor cells. The oral cavity was first exposed to 100 mmol l^{-1} L-cysteine + fluorescein dextran (green), followed by 20 μmol l^{-1} Texas Red dextran (red). Exposure time was 2 min for both solutions.

Uptake of dye by endocytosis

The results described here support the surprising finding that there is a massive taste-specific induction of endocytosis in receptor cells of taste buds: there is a substantial reduction of dye uptake at low temperature similar to the reduction of endocytosis in other cell types (Sandvig and Olsnes, 1979). There is also a reduction of dye uptake and staining of taste receptor cells after exposure to nocodazole, which disrupts the microtubules needed for transport of endocytic vesicles. There is a rapid, nocodazole-sensitive transport of vesicles with dye into the basal part of the taste receptor cells, and the uptake is concentration dependent.

Li and Lindemann (Li and Lindemann, 2003) showed that in the taste receptor cells of the frog taste disks there is an uptake of FM dyes by endocytosis after 24 h exposure; although without the use of taste substances. In mice, a single subcutaneous injection of FM1-43 brightly labels hair cells, Merkel cells, muscle spindles, taste buds, enteric neurons and primary sensory neurons within the cranial and dorsal root ganglia (Meyers et al., 2003). The authors presented evidence that entry of dye into the hair cells was *via* transduction channels. However, the possibility that the entry into taste cells was by endocytosis was not discussed. In the present experiments, 3 and 40 kDa dextrans together with L-cysteine were used and extensive staining of the taste receptor cells was found. As the 40 kDa dextran in particular is so large that it would hardly enter the cells through non-selective ion channels, it supports our conclusion that the internalization of stain in taste receptor cells of taste buds is *via* endocytosis. The specific induction of endocytosis we describe is initiated by the taste substance presented in the medium applied on the taste bud pore. The use of FM1-43 alone stained a modest number of taste receptor cells. We propose the following scenario as a possible mechanism: the ligand binds to a taste receptor and induces a series of transduction events that finally results in exocytosis of transmitter substance at the basal part of the cell. This process is accompanied by internalization apically, probably of the receptor with ligand. Whether also vesicles without receptors are formed is an open question. There could be induction of different types of endocytosis similar to those previously described at the apical pole of polarized MDCK cells (Sandvig and van Deurs, 2005). Vesicles formed are transported *via* microtubules towards the cell soma. The fate of the internalized membrane will be investigated in future studies.

Specificity of taste cells

The successive application of stimulants and deterrents, substances that evoke different feeding behaviours in brown trout, demonstrated selective staining of taste receptor cells in the taste buds. It is important to note that the majority of stained taste receptor cells were in separate taste buds. We seldom observed doubled labelled taste buds, but in some cases confocal images showed co-staining in single receptor cells. A possible conclusion from these observations would be that most taste receptor cells have taste receptors either for a stimulant or a deterrent.

Why endocytosis?

The prominent endocytosis in taste cells is a new observation and there are a multitude of questions related to this process. The massive endocytosis in the peripheral part of the taste cells would require a recycling of the membrane. At present, we do not know how this is achieved. What could be the physiological role of this endocytosis? Could it be that receptor proteins are internalized to reduce that taste perception? Are the receptors recycled at the apical part of the cell? What is the fate of ligands? Is the endocytosis related to adaptation of the physiological response from taste nerve fibres? To what extent will the internalization of activated receptor proteins regulate the homeostasis of this sensory system? These questions will be addressed in future studies.

Ligand-specific induction of endocytosis; a common process in chemosensory organs?

In other chemosensory cells, as for example the sensory neurons in the olfactory and vomeronasal organs of mice, the structure of the endocytic machinery has been studied and demonstrated to be an uptake of ferritin molecules (Bannister and Dodson, 1992). In isolated olfactory sensory neurons of catfish, clathrin-dependent endocytosis was reported to occur after perfusion with L-glutamate together with FM1-43, but not with a mixture of L-alanine, L-arginine, L-cysteine and FM1-43 (Rankin et al., 1999). It seems, however, probable that ligand-specific processes similar to those shown by the present *in situ* study, is common for all chemosensory structures in vertebrates.

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REFERENCES

Bannister, L. H. and Dodson, H. C. (1992). Endocytic pathways in the olfactory and vomeronasal epithelia of the mouse: ultrastructure and uptake of tracers. *Microsc. Res. Tech.* **23**, 128-141.

Betz, W. J., Bewick, G. S. and Ridge, R. M. (1992a). Intracellular movements of fluorescently labeled synaptic vesicles in frog motor nerve terminals during nerve stimulation. *Neuron* **9**, 805-813.

Betz, W. J., Mao, F. and Bewick, G. S. (1992b). Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *J. Neurosci.* **12**, 363-375.

Betz, W. J., Mao, F. and Smith, C. B. (1996). Imaging exocytosis and endocytosis. *Curr. Opin. Neurobiol.* **6**, 365-371.

Brumback, A. C., Lieber, J. L., Angleson, J. K. and Betz, W. J. (2004). Using FM1-43 to study neuropeptide granule dynamics and exocytosis. *Methods* **33**, 287-294.

Clapp, T. R., Medler, K. F., Damak, S., Margolskee, R. F. and Kinnamon, S. C. (2006). Mouse taste cells with G protein-coupled taste receptors lack voltage-gated calcium channels and SNAP-25. *BMC Biol.* **4**, 7.

Cochilla, A. J., Angleson, J. K. and Betz, W. J. (1999). Monitoring secretory membrane with FM1-43 fluorescence. *Annu. Rev. Neurosci.* **22**, 1-10.

DeFazio, R. A., Dvoryanchikov, G., Maruyama, Y., Kim, J. W., Pereira, E., Roper, S. D. and Chaudhari, N. (2006). Separate populations of receptor cells and presynaptic cells in mouse taste buds. *J. Neurosci.* **26**, 3971-3980.

Ezeasor, D. N. (1982). Distribution and ultrastructure of taste buds in the oropharyngeal cavity of the rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* **20**, 53-68.

Farbman, A. I. (1965). Fine structure of the taste bud. *J. Ultrastruct. Res.* **12**, 328-350.

Finger, T. (2005). Cell types and lineages in taste buds. *Chem. Senses* **30**, i54-i55.

Finger, T. E. (2008). Sorting food from stones: the vagal taste system in Goldfish, *Carassius auratus*. *J. Comp. Physiol. A* **194**, 135-143.

Fujimoto, S., Yamamoto, K., Yoshizuka, M. and Yokoyama, M. (1993). Pre- and postnatal development of rabbit foliate papillae with special reference to foliate gutter formation and taste bud and serous gland differentiation. *Microsc. Res. Tech.* **26**, 120-132.

Ganchrow, J. R., Ganchrow, D., Royer, S. M. and Kinnamon, J. C. (1993). Aspects of vertebrate gustatory phylogeny: morphology and turnover of chick taste bud cells. *Microsc. Res. Tech.* **26**, 106-119.

Hara, T. J., Kitada, Y. and Evans, R. E. (1994). Distribution patterns of palatal taste buds and their responses to amino acids in salmonids. *J. Fish Biol.* **45**, 453-465.

Hofer, D. and Drenckhahn, D. (1999). Localisation of actin, villin, fimbrin, ezrin and ankyrin in rat taste receptor cells. *Histochem. Cell Biol.* **112**, 79-86.

Ishimaru, Y., Okada, S., Naito, H., Nagai, T., Yasuoka, A., Matsumoto, I. and Abe, K. (2005). Two families of candidate taste receptors in fishes. *Mech. Dev.* **122**, 1310-1321.

Kasumyan, A. and Døving, K. B. (2003). Taste preferences in fish. *Fish and Fisheries* **4**, 289-347.

Kasumyan, A. O. and Sidorov, S. S. (2005). Taste preferences of the brown trout Salmo trutta from three geographically isolated populations. *J. Ichthyol.* **45**, 111-123.

Li, J. H. and Lindemann, B. (2003). Multi-photon microscopy of cell types in the viable taste disk of the frog. *Cell Tissue Res.* **313**, 11-27.

Marui, T., Evans, R. E., Zielinski, B. and Hara, T. J. (1983). Gustatory responses of the rainbow trout (*Salmo gairdneri*) palate to amino acids and derivates. *J. Comp. Physiol.* **153A**, 423-433.

Mayor, S. and Pagano, R. E. (2007). Pathways of clathrin-independent endocytosis. *Nat. Rev. Mol. Cell. Biol.* **8**, 603-612.

Meyers, J. R., MacDonald, R. B., Duggan, A., Lenzi, D., Standaert, D. G., Corwin, J. T. and Corey, D. P. (2003). Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. *J. Neurosci.* **23**, 4054-4065.

Mukherjee, S., Ghosh, R. N. and Maxfield, F. R. (1997). Endocytosis. *Physiol. Rev.* **77**, 759-803.

Murray, R. G. (1974). The ultrastructure of taste buds. In *The Ultrastructure of Sensory Organs* (ed. I. Friedmann), pp. 1-81. Amsterdam: North-Holland.

Oike, H., Nagai, T., Furuyama, A., Okada, S., Aihara, Y., Ishimaru, Y., Marui, T., Matsumoto, I., Misaka, T. and Abe, K. (2007). Characterization of ligands for fish taste receptors. *J. Neurosci.* **27**, 5584-5592.

Padron, D., Bizeau, M. E. and Hazel, J. R. (2000). Is fluid-phase endocytosis conserved in hepatocytes of species acclimated and adapted to different temperatures? *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278**, R529-R536.

Rankin, M. L., Alvania, R. S., Gleason, E. L. and Bruch, R. C. (1999). Internalization of G protein-coupled receptors in single olfactory receptor neurons. *J. Neurochem.* **72**, 541-548.

Reutter, K. and Hansen, A. (2005). Subtypes of light and dark elongated taste bud cells in fish. In *Fish Chemosenses* (ed. K. Reutter and B. G. Kapoor), pp. 211-230. Enfield, NH: Science Publishers.
Reutter, K. and Witt, M. (1993). Morphology of vertebrate taste organs and their nerve supply. In *Mechanisms of Taste Transduction* (ed. S. A. Simon and S. D. Roper), pp. 29-82. Boca Raton, FL: CRC Press.
Reutter, K., Boudriot, F. and Witt, M. (2000). Heterogeneity of fish taste bud ultrastructure as demonstrated in the holosteans *Amia calva* and *Lepisosteus oculatus*. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **355**, 1225-1228.
Sandvig, K. and Olsnes, S. (1979). Effect of temperature on the uptake, excretion and degradation of abrin and ricin by HeLa cells. *Exp. Cell Res.* **121**, 15-25.
Sandvig, K. and van Deurs, B. (2005). Delivery into cells: lessons learned from plant and bacterial toxins. *Gene Ther.* **12**, 865-872.
Sandvig, K., Torgersen, M. L., Raa, H. A. and van Deurs, B. (2008). Clathrin-independent endocytosis: from nonexisting to an extreme degree of complexity. *Histochem. Cell Biol.* **129**, 267-276.
Schliwa, M. and Woehlke, G. (2003). Molecular motors. *Nature* **422**, 759-765.
Sekerkova, G., Zheng, L., Loomis, P. A., Changyaleket, B., Whitlon, D. S., Mugnaini, E. and Bartles, J. R. (2004). Espins are multifunctional actin cytoskeletal regulatory proteins in the microvilli of chemosensory and mechanosensory cells. *J. Neurosci.* **24**, 5445-5456.
Takeda, M., Obara, N. and Suzuki, Y. (1990). Keratin filaments of epithelial and taste-bud cells in the circumvallate papillae of adult and developing mice. *Cell Tissue Res.* **260**, 41-48.
Tomchik, S. M., Berg, S., Kim, J. W., Chaudhari, N. and Roper, S. D. (2007). Breadth of tuning and taste coding in mammalian taste buds. *J. Neurosci.* **27**, 10840-10848.
Yee, C. L., Yang, R., Bottger, B., Finger, T. E. and Kinnamon, J. C. (2001). "Type III" cells of rat taste buds: immunohistochemical and ultrastructural studies of neuron-specific enolase, protein gene product 9.5, and serotonin. *J. Comp. Neurol.* **440**, 97-108.