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Amino Acid Odorants Stimulate Microvillar Sensory Neurons

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Abstract

The olfactory epithelium (OE) of zebrafish is populated with ciliated and microvillar olfactory sensory neurons (OSNs). Whether distinct classes of odorants specifically activate either of these unique populations of OSNs is unknown. Previously we demonstrated that zebrafish OSNs could be labeled in an activity-dependent fashion by amino acid but not bile acid odorants. To determine which sensory neuron type was stimulated by amino acid odorants, we labeled OSNs using the ion channel permeant probe agmatine (AGB) and analyzed its distribution with conventional light- and electron-microscope immunocytochemical techniques. Approximately 7% of the sensory epithelium was labeled by AGB exposure alone. Following stimulation with one of the eight amino acids tested, the proportion of labeled epithelium increased from 9% for histidine to 19% for alanine; amino acid stimulated increases in labeling of 2–12% over control labeling. Only histidine failed to stimulate a significant increase in the proportion of labeled OSNs compared to control preparations. Most amino acid sensitive OSNs were located superficially in the epithelium and immuno-electron microscopy demonstrated that the labeled OSNs were predominately microvillar. Large numbers of nanogold particles (20–60 per 1.5 μm^2) were associated with microvillar olfactory sensory neurons (MSNs), while few such particles (<15 per 1.5 μm^2) were observed over ciliated olfactory sensory neurons (CSNs), supporting cells (SCs) and areas without tissue, such as the lumen above the OE. Collectively, these findings indicate that microvillar sensory neurons are capable of detecting amino acid odorants.

Introduction

Fish detect olfactory signals such as amino acids, bile acids, prostaglandins and steroids with a mixed population of ciliated (CSNs), microvillar (MSNs) and crypt olfactory sensory neurons (Zeiske *et al.*, 1976, 1986; Theisen *et al.*, 1980; Moran *et al.*, 1992; Hansen and Zeiske, 1998; Hansen and Finger, 2000). Previous attempts to determine if any of these groups of receptor cells are sensitive to particular odorants have relied on indirect evidence. In char, for example, the largest responses to general feeding stimuli (amino acids) were recorded from areas of olfactory epithelium (OE) enriched in MSNs, while areas enriched in CSNs had larger responses to social stimuli—bile acids (Thommesen, 1982, 1983). In the channel catfish, no correlation was found between the distribution of MSNs and CSNs and sensitivity to amino acid and bile acid stimuli (Erickson and Caprio, 1984). In the goldfish, MSNs appear to respond both to amino acids and bile acids. It has been reported that sensitivity to bile acid and amino acid odorants following olfactory nerve transection recovers with time courses similar to the epithelial regeneration of MSNs and CSNs, respectively (Zippel *et al.*, 1997). Another study (Speca *et al.*, 1999) concluded on the basis of the superficial location of the cells expressing an arginine sensitive V2R receptor that the MSNs were amino acid sensitive. However, location of the cell soma in the epithelium may not unambiguously distinguish MSNs from CSNs. Morita and Finger identified short (presumably MSNs) and long (presumably CSNs) OSNs and a population of intermediate length OSNs that were morphologically similar to the other cell types (Morita and Finger, 1998). A more direct approach to the identification of odorant stimulated OSNs is required to determine conclusively if odorants stimulate specific types of OSNs.

We have previously shown that a cation channel permeant guanidinium analog, agmatine (AGB), labels amino acid stimulated OSNs in an activity-dependent fashion (Michel, 1999; Lipschitz and Michel, 1999; Michel *et al.*, 1999). The superficial location of the odor-stimulated OSNs and the inability of AGB to permeate through open CNG channels (Michel *et al.*, 1999) suggested the labeled cells were microvillar, but labeled knoblike dendritic endings was consistent with the labeling of ciliated OSNs. Light microscopy provided insufficient resolution to permit the discrimination of the ciliated and microvillar apical processes needed for unambiguous identification. Using electron microscopy, MSNs and CSNs can be distinguished by the presence of

numerous thin (0.04 μm diameter) microvilli, or thicker (0.2–0.25 μm diameter proximally) cilia covering the apical surface (Moran *et al.*, 1992; Hansen and Zeiske, 1998). In the current investigation, we used activity-dependent labeling techniques in combination with conventional immunocytochemical electron microscopy to confirm the identity of the amino acid sensitive OSNs. The OE of adult zebrafish was stimulated with amino acids previously shown to interact with the acidic, basic and neutral amino acid receptor sites of fish (Caprio and Byrd, 1984; Ohno *et al.*, 1984; Bruch and Rulli, 1988; Friedrich and Korsching, 1997; Michel and Derbidge, 1997). Results of the current study confirm that MSNs are stimulated by amino acid stimuli. A portion of this study has been previously published in abstract form (Lipschitz and Michel, 2000).

Materials and methods

Animal maintenance

Zebrafish (*Danio rerio*) were purchased from a commercial supplier, housed in recirculating 40–80 l aquaria (28.5°C) at the University of Utah and fed flake food (Tetramin) daily.

Activity-dependent labeling procedures

Zebrafish were stimulated *in vivo* after immobilization with an i.m. injection of Flaxedil (60 μg/g body wt) and positioning in a recording chamber. Immediately thereafter, the olfactory rosettes were provided with a continuous flow of fish Ringer's solution (FR, see solutions). The gills were irrigated with 3 ml/min of artificial freshwater (AFW) containing a general anesthetic (MS-222, 20 mg/l). The anesthetic was prevented from contacting the olfactory organs in order to minimize the loss of afferent sensory activity associated with topical application of the anesthetic (Spath and Schweickert, 1977). After a 5 min FR wash, both olfactory rosettes were stimulated for 10 s/min for 10 min with either 5 mM AGB (control) or 5 mM AGB plus amino acid odorant (100 μM). Thus, during the 10 min stimulation period, the OE received 10 discrete stimulations each consisting of 10 s of odor stimulation each minute followed by a 50 s FR rinse to minimize desensitization before the next odorant stimulation. After a 5 min FR wash, the fish was decapitated and the head immersed in cold fixative solution (see solutions) and stored at 4°C overnight to several days. Fixed tissue was washed in 0.1 M phosphate buffer (PB) for 30 min and dehydrated through a graded series of methanol and absolute acetone, then embedded in Eponate resin (Pella Inc.). The University of Utah Institutional Animal Care and Use Committee approved all experimental procedures used in the current study.

Tissue processing

Light microscopy

Semi-thin sections (250–500 nm) were prepared using a diamond knife and an American Optical Ultracut ultramicrotome. Individual sections were placed in a well of a teflon-coated spot slide (Erie Scientific, Portsmouth, NH), deplasticized using sodium ethoxide (NaEtOH, a saturated solution of sodium hydroxide in ethanol diluted 1:4 with ethanol before use), rinsed, dried and treated overnight in an anti-AGB IgG antibody. The rabbit anti-AGB primary antibody (1:100 dilution, Signature Immunologics, Salt Lake City, UT) was raised against a glutaraldehyde-conjugated AGB–albumin complex. Immunoreactive sites were visualized with a nanogold-conjugated goat anti-rabbit secondary antibody (1 nm, Amersham) and silver intensification using previously described methods (Marc, 1999a,b).

Electron microscopy

Single ultra-thin sections (50 nm) were collected on formvar-coated gold grids and the adjacent serial sections were individually mounted in wells of teflon-coated spot slides for light microscope immunocytochemistry (see above). Sections were treated with either 10% NaEtOH for 1–2 min or 10% H_2O_2 for 10 min, rinsed, dried, incubated with 3% goat serum in 0.1 M PB for 1 h to block non-specific labeling and then incubated overnight in anti-AGB antibody. After several washes in 0.1 M PB, the tissue was placed in nanogold-conjugated goat anti-rabbit secondary antibody for 1 h (1:10 dilution, 15 nm gold particle size, Amersham), washed, stained with uranyl acetate for 45 min, rinsed and dried. Viewing and photography employed a Hitachi Model No. H-7100 transmission electron microscope.

Quantification of AGB immunoreactivity

Light microscopy

Digital images (8-bit gray scale) were obtained using a Zeiss Axioplan 2 microscope equipped with bright field illumination and a CCD camera (Axiocam, Carl Zeiss Inc., Thornwood, NY) and Axiovision software. For each image, a region of interest (ROI) was drawn around a portion of the sensory epithelium that contained no labeled neurons. The mean pixel intensity and standard deviation of this ROI was used to calculate a background staining value with a 95% confidence interval (CI). The lower 95% confidence interval limit was used to discriminate labeled pixels from background staining in a second ROI of the entire sensory epithelium. The number of labeled pixels below the cutoff was divided by the total pixels in this ROI and multiplied by 100 to obtain the percentage of labeled sensory epithelium. For each olfactory rosette, the average proportion of labeled epithelium was calculated for four to six ROIs from each of four planes of section, each separated by a minimum of 10 μm.

Electron microscopy

Following electron-microscope immunocytochemistry, analysis of AGB labeling involved digitization of 7000× photo-

graphic negatives (resolution 600–1200 p.p.i., Scanmaker 5, Microtek, CA) and calculating the number of gold particles in a standard rectangular area (1.5 μm^2) placed at least 2 μm below the apical surface of identified cells. Levels of labeling were established from measurements taken in the lumen, the mucous and fluid filled region above the OE and between adjacent olfactory lamellae.

Determination of centroid length

An Excel (Microsoft) macro estimated each cell's centroid length as the shortest distance from the centroid of the cell body to a straight line overlaying the mucosal surface. For both light- and electron-microscope images, an ROI outline was drawn around each labeled area or cell to be measured and the coordinates of the cell centroid were applied to the macros. For light-microscope samples, measurements were carried out only on those cells with cell bodies that could be easily separated or individually distinguished. Measurements of cells in electron-microscope images were carried out only for cells in which both identifiable apical processes and cell bodies were visible.

Statistical analysis

For light-microscope immunocytochemistry, the percentage labeling of sensory regions under the control condition (AGB only) was statistically compared to labeling with a binary mixture of AGB plus an amino acid odorant. A one-way ANOVA for unbalanced data determined the overall significance for the odorant main effect, while a *post hoc* Tukey's multiple comparison of means test was used to determine individual differences between the control and each binary mixture. The mean cell centroid length for the control condition (AGB only) was compared to the mean cell centroid length for each binary mixture of AGB and odorant using a one-way ANOVA and a *post hoc* Tukey's multiple comparison of means test. For electron-microscope immunocytochemistry, the numbers of gold particles were compared across odorants for each cell type and the lumen using a two-way ANOVA. Individual comparisons within odorants and within cell types were made using a one-way ANOVA, while a Tukey's multiple comparison of means test was used to establish individual differences within each variable. Further analysis determined whether any significant differences existed among cell types in the percentages of cells with labeling greater than background staining in the lumen. A 99% CI was calculated for the lumen of each odorant condition and the upper limit was used as a cutoff to calculate the percentage of OSNs and SCs containing significantly greater numbers of nanogold particles than the lumen. A χ^2 Tukey-type multiple comparison test of proportions (Zar, 1984) was used to compare the percentages of labeling among cell types for each odorant condition.

Chemicals and solutions

The composition of artificial freshwater (AFW) was (in mM): NaCl, 3; KCl, 0.2; $CaCl_2$, 0.2; $MgCl_2$, 0.2; HEPES, 1; pH 7.2. The composition of fish Ringers was (in mM): NaCl, 140; KCl, 10; $CaCl_2$, 1.8; $MgCl_2$, 2; HEPES, 5; pH 7.2. AGB Ringers was prepared by substituting 5 mM NaCl in the fish Ringers with 5 mM AGB. Fixative consisted of 11.2 ml of 18.3% paraformaldehyde, 10 ml of 50% glutaraldehyde, 80 ml of 0.2 M PB, 12 ml of 0.02% $CaCl_2$, 6 g sucrose and sufficient distilled water to bring the final volume to 200 ml, yielding a final concentration of 2.5% glutaraldehyde and 1% paraformaldehyde. AGB and the eight L-amino-acid odorants, L-alanine (Ala), L-arginine (Arg), L-cysteine (Cys), L-glutamate (Glu), L-glutamine (Gln), L-histidine (His), L-lysine (Lys) and L-methionine (Met), were of the highest purity available from Sigma Chemical Co. (St Louis, MO). All amino acid odorants were tested at 100 μM.

Results

Throughout the results, the term 'OSNs' designates AGB labeled cells that have not been examined by transmission electron microscopy to identify definitively microvillar or ciliated processes. The terms 'MSNs' and 'CSNs' are reserved for cells definitively identified by electron microscopy.

Relative stimulatory effectiveness of amino acid odorants

A total of 18 adult zebrafish (two per odorant) were used to analyze responses to each of the amino acid odorants. One olfactory rosette from each fish was used to measure the percentage labeled OE and the lengths of the amino acid stimulated OSNs. As previously reported (Michel *et al.*, 1999), AGB immunoreactivity was observed in the sensory epithelium and olfactory nerve layer but not in the nonsensory epithelium (Figure 1). All of the amino acid stimuli (100 μM) increased the percentage of sensory epithelium labeled above that observed in the AGB control preparations of ~7% (Figure 2A). The percentage of labeled sensory epithelium ranged from 9% for L-histidine to 19% for L-alanine. The ANOVA indicated that these increases were significant (ANOVA, $F = 7.98$; d.f. = 8; $P < 0.0001$), while the Tukey's comparison of means showed that, with the exception of histidine, all the odorants tested significantly increased the percentage of labeled sensory epithelium compared to the AGB control.

Location of odor-stimulated OSNs

As shown in the light micrographs in Figure 1, the majority of cells labeled by either AGB alone or by AGB and amino acid stimulation were closer to the apical than the basal surface of the OE. The average length of labeled cells from the apical surface to the somal centroid for all measured OSNs was 6.5 ± 3.2 μm ($n = 6168$), while the average epithelial thickness at the same locations was 20.0 ± 3.6 μm ($n = 6168$). Frequency distributions for each of the odorants

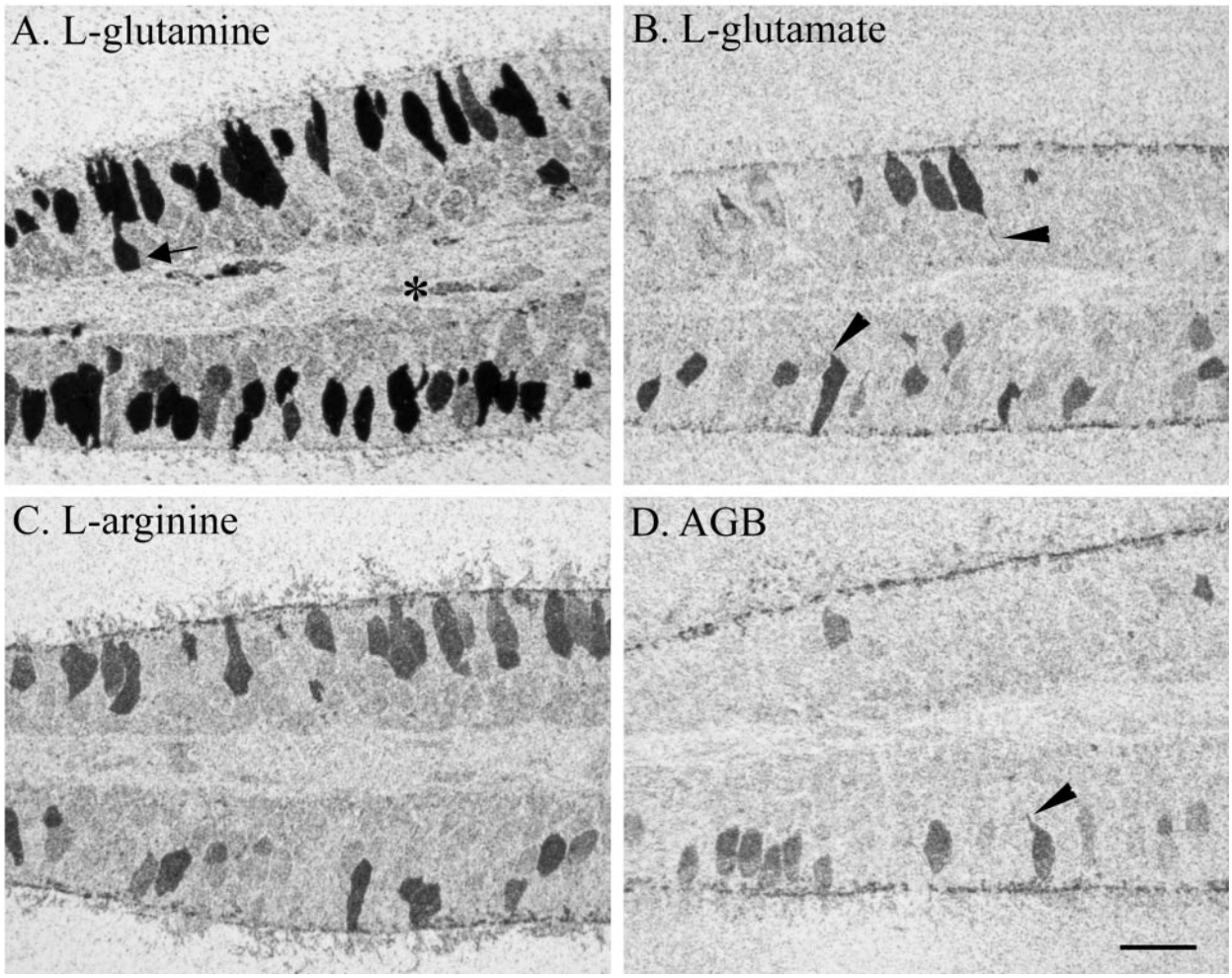

Figure 1 Amino acids stimulate activity-dependent labeling of the zebrafish OE. Each image illustrates the AGB labeling in olfactory lamellae in response to 5 mM AGB plus 100 μM L-glutamine **(A)**, L-glutamate **(B)**, L-arginine **(C)** and 5 mM AGB alone **(D)**. Note the location of most labeled OSNs in the apical portion of the OE. Arrow in (A) indicates a cell positioned at the basal surface of the OE. Asterisk demarks area of labeled axons cut in cross-section. Arrowheads in (B) and (D) indicate labeled axons. Image of L-glutamate stimulated labeling in (B) was selected to illustrate axonal labeling, but under-represents the average labeling observed in all regions of interest examined (see Figure 2). All images were captured with identical illumination and microscope settings and subsequently contrast-adjusted identically in Photoshop 5.5 (Adobe). Non-sensory epithelium not shown. Scale bar = 10 μm.

revealed a small population of labeled cells that were 15–20 μm long and whose somata were located near the basal surface (see Figure 2B). Although the cell centroid length distributions for the different odorant conditions overlapped considerably, mean cell centroid lengths for several amino acid combinations were significantly different (ANOVA, $F = 87.611$, d.f. = 8, $P < 0.001$). The Tukey's multiple comparison of means test indicated that the mean cell centroid length for AGB stimulated preparations was significantly longer than for histidine, arginine, glutamate, cysteine and alanine, but significantly shorter than for methionine.

Lengths of identified MSNs and CSNs

The lengths of identified CSNs and MSNs were measured in electron micrographs from five olfactory rosettes from five different adult zebrafish. Uranyl acetate staining alone permitted identification of cells on the basis of their apical surface morphology. Large differences in average diameter distinguished cilia (0.20 ± 0.03 μm, $n = 8$) from microvilli, (0.06 ± 0.01 μm, $n = 9$) and thus provided a reliable means of establishing the identity of MSNs and CSNs; ciliary bodies in the CSNs were also common (Figure 3; also see Figure 5). All measured cells were required to have an attached apical process and cell body to be included in the data set. The mean cell centroid length of the 36 MSNs was 4.9 ± 1.2 μm and is consistent with the hypothesis that MSNs occupy a superficial location in the sensory epithelium. The mean length of the 45 CSNs was 10.2 ± 2.5 μm, indicating a displacement at least one cell diameter below the MSNs. However, inspection of the lengths of the MSNs and CSNs reveals some overlap in the distribution of the two OSN types (Figure 4A). The average epithelial thickness in the regions used to measure OSN length was

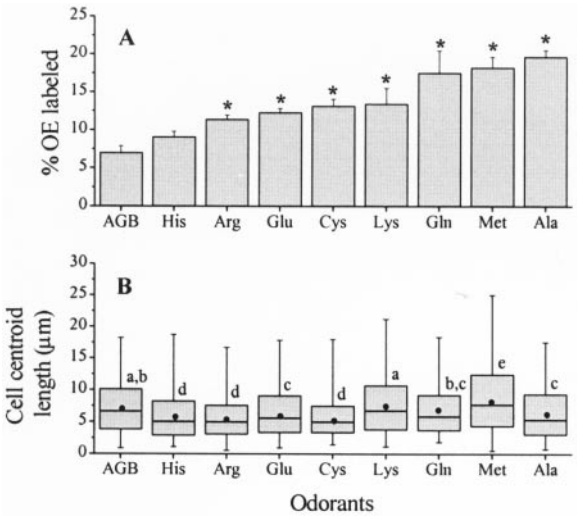

Figure 2 Quantification of activity-dependent labeling in the zebrafish OE. **(A)** Stimulation with the neutral amino acids (L-alanine, L-glutamine, L-methionine, L-cysteine, L-histidine) resulted in more labeling of the OE than did either acidic (L-glutamate) or basic (L-lysine, L-arginine) amino acids. All of the amino acid odorants except histidine stimulated a significant increase in the percentage of labeled sensory epithelium compared to the AGB control preparations (Tukey's *post hoc* comparison of means, $P < 0.05$). **(B)** Box and whisker plots of cell centroid length distributions of the labeled OSNs for each of the amino acids plus AGB and for AGB alone. The horizontal line within the box indicates the median cell centroid length, and the lower and upper lines comprising the box the 25 and 75% percentiles, respectively; whisker bars indicate minimum (lower) and maximum (upper) values; (●) mean cell centroid length. Although the distributions are largely overlapping, the ANOVA indicated significant differences among odorants ($F = 87.61$, d.f. = 8, $P < 0.001$). Abbreviations: agmatine, AGB; L-alanine, Ala; L-arginine, Arg; L-cysteine, Cys; L-glutamate, Glu; L-glutamine, Gln; L-histidine, His; L-lysine, Lys; L-methionine, Met. The same letters above the bars in (B) identify groups of odor-stimulated OSNs with statistically similar mean cell centroid lengths.

15.4 ± 3.2 μm (n = 81, range, 9.7–23.3 μm). Epithelial thickness was positively correlated with cell centroid lengths for both MSN (coefficient of correlation, R = 0.66, $P < 0.001$, slope = 0.24) and CSN ($R = 0.70$, $P < 0.001$, slope = 0.52) distributions (Figure 4B).

Direct AGB labeling of OSNs

For AGB electron-microscope immunocytochemistry, cells from one olfactory rosette were analyzed following either 5 mM AGB stimulation alone or exposure to a binary mixture of 5 mM AGB and 100 μM glutamine, arginine or glutamate (Figure 5, Table 1). Immunogold particles were quantified in all cell types and compared to their incidences in the lumen (Figure 6): 0–14 gold particles/1.5 μm^2 for lumen; 0–62 gold particles/1.5 μm^2 for MSNs; 0–10 gold particles/1.5 μm^2 for CSNs; and 0–15 gold particles/1.5 μm^2 for SCs. The labeling in MSNs was up to four to six times higher than in other cell types (Figure 6). A significant relationship between cell type and odorant variables was noted (ANOVA, $F = 7.1$, d.f. = 9, $P < 0.001$). Comparisons of mean numbers of gold particles within cell types and the

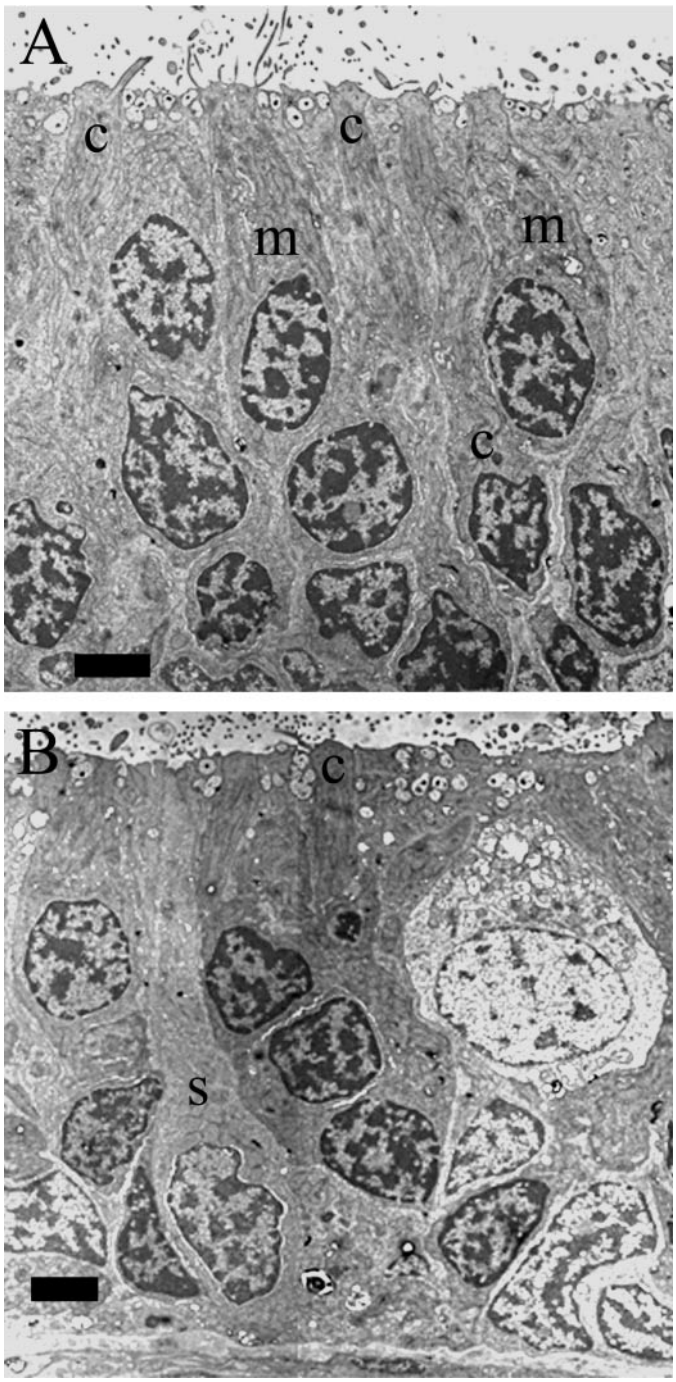

Figure 3 Classification of cell types in the OE using electron microscopy. **(A)** microvillar sensory neurons (m) and ciliated sensory neurons (c) can be distinguished on the basis of the diameter of the apical processes. Note that the cell body of the complete CSN is positioned below the two identified MSNs. **(B)** A second image of OE shows another CSN along with a SC (s). Note that the electron-lucent, SC nucleus is positioned near the basal lamina. The images are photomontages that were scanned from negatives and adjusted for brightness and contrast in Photoshop 5.5. Scale bars = 2 μm.

lumen were significant for lumen (ANOVA, $F = 19.22$, d.f. = 3, $P < 0.001$) and MSNs (ANOVA, $F = 3.27$, d.f. = 3, $P < 0.025$) only. Arginine stimulated labeling in the lumen

was significantly greater than the corresponding AGB, glutamine or glutamate stimulated labeling (Tukey's test, $P < 0.05$). Within all preparations, the mean number of gold particles in MSNs was significantly greater than in the lumen and in other cell types (AGB, $F = 13.38$, d.f. = 3, $P < 0.001$; glutamine, $F = 61.16$, d.f. = 3, $P < 0.001$; arginine, $F = 18.57$, d.f. = 3, $P < 0.001$; glutamate, $F = 49.90$, d.f. = 3, $P < 0.001$). Neither CSNs nor SCs had elevated gold particles relative to control values for the lumen.

The upper limit of the 99% CI for labeling in the lumen was used as a cutoff to determine the percentages of OSNs and SCs with significantly increased labeling for each of the odorants (Table 2). Comparisons among cell types were significant overall for each odorant condition (AGB, $\chi^2 = 65.5$, d.f. = 2, $P < 0.001$; Gln, $\chi^2 = 90.7$, d.f. = 2, $P < 0.001$; Arg, $\chi^2 = 64.5$, d.f. = 2, $P < 0.001$; Glu, $\chi^2 = 49.9$, d.f. = 2, $P < 0.001$). For pairwise comparisons, percentages of MSNs with gold particles above background were significantly elevated compared to both CSNs and SCs for all odor-stimulated conditions, while percentages of SCs were significantly greater than CSNs for glutamine and glutamate stimulation.

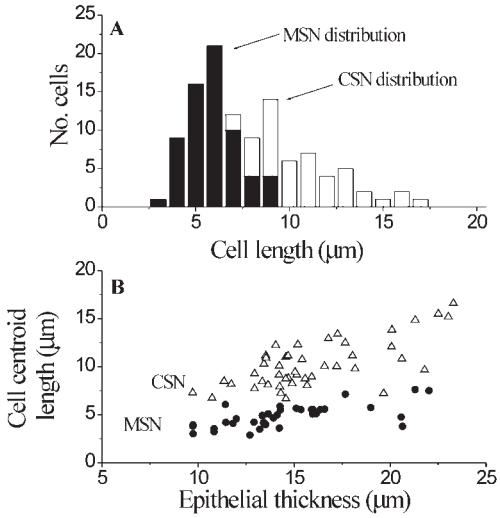

Figure 4 MSNs are generally shorter than CSNs. **(A)** Comparison of distributions of CSN and MSN centroid length reveals that the MSNs are shorter and, as a consequence, are located closer to the apical surface. MSN and CSN centroid lengths were rounded to the nearest micrometer and binned to generate frequency histograms. **(B)** Cell centroid length of both MSNs (●) and CSNs (△) are correlated with epithelial thickness. Linear regressions were significant for both MSNs ($R = 0.66$, $P < 0.001$, slope = 0.24) and CSNs ($R = 0.70$, $P < 0.001$, slope = 0.52).

Discussion

Sensory neurons in the OE of fishes detect a broad spectrum of chemically diverse olfactory stimuli, ranging from amino acids, serving as general feeding stimulants, to bile acids, acting as social stimuli, and prostaglandins and steroids, serving as specific pheromones. This investigation addressed the issue of whether particular OSN types mediate the detection of a specific class of stimuli, the amino acids. Light microscope activity-dependent immunocytochemistry demonstrated that all amino acid odorants (except histidine) elicited an increase in the proportion of labeled OE compared to the AGB control preparations, and it revealed that most labeled OSNs were superficially located. Immuno electron microscopy identified the majority of labeled cells as MSNs. Thus, stimulation with three amino acid odorants presumed to activate distinct odorant receptors (Michel and Derbidge, 1997) resulted in AGB labeling and a high incid-

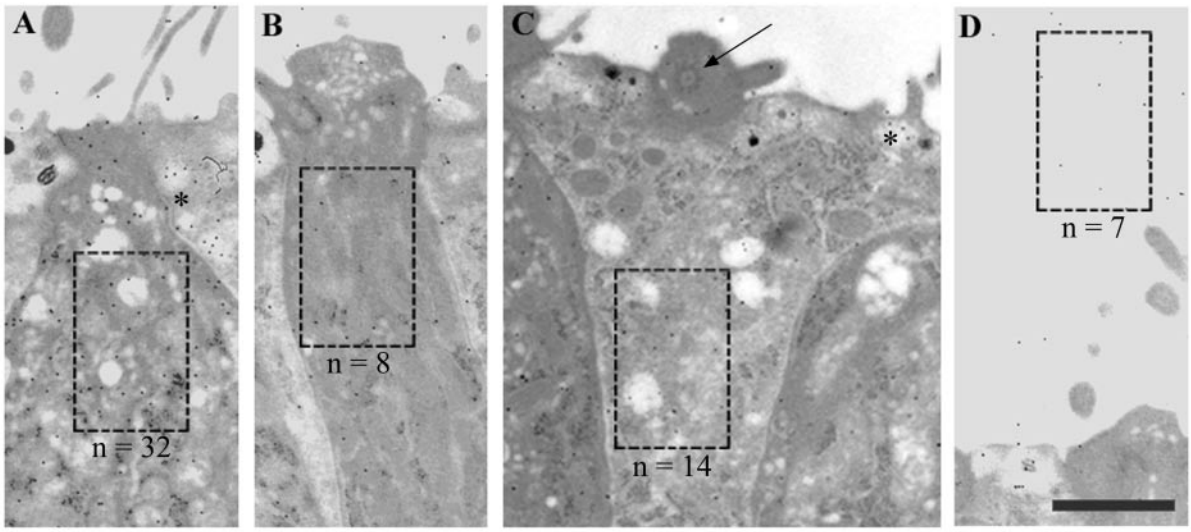

Figure 5 Distribution of AGB labeling in each of the cell types and lumen control following electron-microscope immunocytochemistry. **(A)** MSN, **(B)** CSN, **(C)** SC, **(D)** lumen. The number of nanogold particles (n) quantified within the 1.5 μm^2 areas are indicated. In (C), the arrow indicates the ciliary body in the dendrite knob of a CSN enveloped by the SC. Asterisks in (A) and (C) indicate an accumulation of gold particles over vesicles in SCs. The images were scanned from photographic negatives and adjusted for brightness and contrast in Photoshop 5.5. Scale bar = 1 μm.

Table 1 Mean (± SEM) gold particles per 1.5 μm^2 in identified cell types in the OE and the lumen following odor stimulation

Odorant	Lumen	Cell type		
		MSN	CSN	SC
AGB	3.1 ± 0.3 (49)	8.1 ± 1.4 (15)[b]	3.5 ± 0.3 (47)	4.5 ± 0.4 (45)
Glutamine	3.0 ± 0.2 (62)	11.9 ± 1.4 (41)[b]	3.3 ± 0.3 (56)	4.2 ± 0.5 (35)
Arginine	5.3 ± 0.4 (71)[a]	11.4 ± 2.3 (14)[b]	3.7 ± 0.3 (46)	4.4 ± 0.4 (47)
Glutamate	2.8 ± 0.2 (56)	18.8 ± 4.3 (18)[b]	3.1 ± 0.3 (48)	5.1 ± 0.5 (39)

Numbers of areas/cells examined are given in parentheses.

[a]Significant difference in mean number of gold particles in the lumen compared to the labeling in the lumen of the AGB control (Tukey's comparison of means, $P < 0.05$).

[b]Significant increase in labeling of cell types compared to the appropriate lumen control (Tukey's, $P < 0.05$).

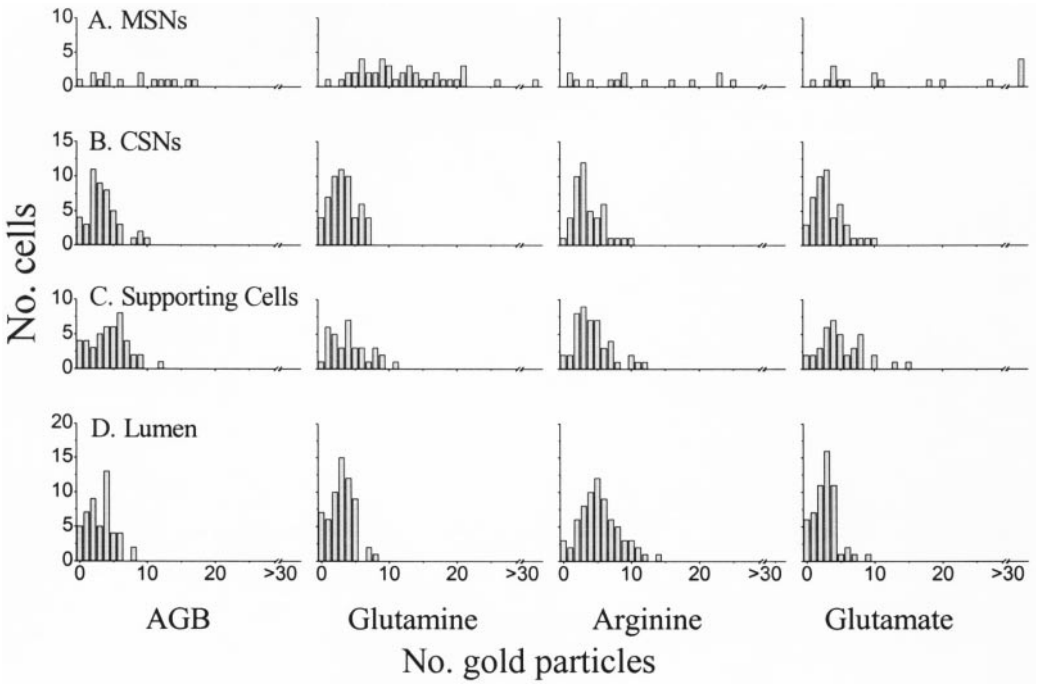

Figure 6 Distributions of nanogold particles within olfactory epithelial cells and lumen for each odorant condition. **(A)** MSNs, **(B)** CSNs, **(C)** SCs, **(D)** lumen. Note the break in the *x*-axes of the histograms to accommodate the higher numbers of gold particles in MSNs. A single glutamine-stimulated MSN had >30 gold particles/1.5 μm^2 (n = 32 gold particles/1.5 μm^2). Four glutamate-stimulated MSNs had >30 gold particles/1.5 μm^2 (n = 32, 40, 54 and 62 gold particles/1.5 μm^2). A 99% CI was generated from lumen distributions to establish whether identified cell types were significantly labeled (see Table 1).

ence of nanogold particles over many, but not all MSNs. We previously suggested that the amino acid stimulated labeling of cells with a prominent dendritic knob was consistent with amino acid sensitive ciliated sensory neurons (Michel *et al.*, 1999). In the current investigation, electron microscopy reveals knoblike terminations on both CSNs and MSNs, but only a few weakly labeled CSNs (and SCs). Too few crypt cells were observed to determine if amino acids stimulated these cells.

Activity-dependent permeation of AGB into weakly stimulated OSNs produced levels of electron-microscope labeling only slightly greater than background, while heavily labeled MSNs had signal-to-noise ratios comparable to, or better than, other nanogold-based immuno electron microscopy studies (Yang *et al.*, 2000). Given an average background labeling of three to five nanogold particles per 1.5 μm^2 (Table 1) and 62 nanogold particles per 1.5 μm^2 in the most heavily labeled MSN, the calculated signal-to-noise ratio ranges from 12:1 to 20:1. Though not systemically investigated, antigen exposure with either H_2O_2 (glutamine preparation) or NaEtOH (glutamate, arginine and AGB preparations) yielded similar patterns of immunostaining without obvious differences in either background or maximal labeling.

Table 2 Percentages of identified cell types with numbers of gold particles significantly greater than background levels

Odorant	Cutoff[a]	Cell Type					
		MSN		CSN		SC	
		Labeled/total	%	Labeled/total	%	Labeled/total	%
AGB	8.3	8/15	53^{A}	3/47	6^{B}	3/45	7^{B}
Glutamine	7.7	29/41	71^{A}	0/56	0^{B}	6/35	17^{C}
Arginine	12.9	5/14	36^{A}	0/46	0^{B}	0/47	0^{B}
Glutamate	7.3	10/17	59^{A}	3/48	6^{B}	9/39	23^{C}

[a]99% Upper confidence limit for the number of gold particles in the lumen (see Table 1 for mean and confidence intervals used to establish the cutoff values).

Different superscript capital letters indicate significance for comparisons of cell types within each odorant (χ^2 multiple comparisons of proportions test, $P < 0.05$).

The amino acids used in the current study were selected because they interact with at least partially independent odorant receptors (Friedrich and Korsching, 1997; Michel and Derbidge, 1997; Lipschitz and Michel, 1999). In general, the relative proportion of OE labeled by each of the amino acid stimuli is in good agreement with the relative stimulatory effectiveness established in earlier electrophysiological studies (Michel and Lubomudrov, 1995) and neutral amino acids were found to be more potent than basic and acidic amino acids. Labeling during AGB stimulation alone is also not unexpected, since we have previously shown AGB to be a relatively potent odorant (Lipschitz and Michel, 1999; Michel *et al.*, 1999). The proportions of electron-microscope-identified MSNs labeled by each of the amino acid odorants were substantially higher than the corresponding proportions of total OE labeled (glutamine, 71 versus 18%; glutamate, 59 versus 12%; arginine, 36 versus 11%; AGB, 51 versus 7%). In part, this discrepancy can be accounted for by our bias of selecting lamellae previously shown to have robust labeling for electron-microscope examination. If, however, we assume that MSNs occupy only 30–50% of the total area in the OE and that the majority of activity-dependent labeling is associated with this cell type, then a high proportion of the MSNs would have to be labeled to account for the proportions noted for the entire epithelium. The relatively low numbers of labeled CSNs and SCs (maximum 7 and 23%, respectively) are consistent with this interpretation; however, the labeling noted in these two cell groups warrants further comment.

Only a few, very weakly labeled CSNs were noted. Failure to label CSNs was anticipated in light of the inability of AGB to permeate through cyclic-nucleotide-gated channels (Michel *et al.*, 1999), the ion channels thought to mediate olfactory transduction in the CSNs of fish (Ngai *et al.*, 1993; Speca *et al.*, 1999). Many odorants, such as bile acids and polyamines, elicit robust electrophysiological responses yet fail to stimulate activity-dependent labeling, presumably due to their activation of the CNG pathway of CSNs (Ma and Michel, 1998; Michel, 1999). Considering our inability to stimulate robust labeling of CSNs with any stimulus, we are unable to conclude that amino acid odorants do not stimulate CSNs.

Available evidence suggests that amino acid odorants activate an IP_3-mediated transduction cascade rather than the cyclic-nucleotide-mediated transduction pathway (Bruch and Teeter, 1990; Restrepo *et al.*, 1993). In zebrafish, drugs thought to perturb IP_3-mediated transduction affect amino acid elicited responses to a greater extent than bile acid elicited responses (Ma and Michel, 1998) and eliminated AGB and amino acid stimulated labeling (Michel, 1999; Michel *et al.*, 1999). In goldfish, an L-arginine-sensitive V2R receptor couples to phospholipase C and IP_3 production when heterologously expressed and is superficially located in the OE in a position consistent with the MSNs (Speca *et al.*, 1999). The goldfish L-arginine-sensitive receptor was shown to be insensitive to AGB (Speca *et al.*, 1999), the arginine analog which served as the activity probe in the current investigation. Thus, if a zebrafish homolog of this receptor exists it is not likely to be mediating background AGB labeling. Consistent with this interpretation is the electrophysiological observation that AGB and L-arginine interact with largely independent odorant receptors in the peripheral olfactory system of zebrafish (Lipschitz and Michel, 1999).

An unexpected finding was the labeling of SCs above background levels in the lumen in three of four preparations. Much of the labeling appears to be very apically located and associated with intracellular vesicles (see Figures 3 and 5). The mechanism of AGB entry into SCs is unknown, but may be associated with the endocytosis of secretory vesicular membrane or the activity of an amino acid transporter. Electron-lucent SC nuclei are located deeper in the OE, near the basal lamina (Moran *et al.*, 1992; Byrd and Brunjes, 1995; Hansen and Zeiske, 1998) in areas corresponding to or deeper than CSNs. Thus, regardless of the mechanism of

labeling, we must conclude that SCs account for some of the labeling observed at the light-microscope level and probably account for more of the basally located labeling than the CSNs.

Differences in the epithelial thickness might contribute to differences we noted in the lengths of electron-microscope identified OSNs and activity-labeled OSNs from light-microscope measurements. The average length of the labeled OSNs in silver-intensified light-microscope images was 6.5 μm, while the average lengths of identified MSNs and CSNs from electron-microscope images were 4.9 and 10.2 μm, respectively. The average epithelial thicknesses for the measured OSNs and the identified MSNs and CSNs were 20 and 15.4 μm, respectively. As shown in Figure 4B, both CSN and MSN cell centroid length is correlated with epithelial thickness, thus the intermediate location of OSN lengths relative to the measured lengths of MSNs and CSNs may, in part, be a result of the epithelial thickness of each of the preparations. Normalizing average cell centroid length to the average epithelial thickness for both the light- and electron-microscope preparations eliminates this effect. Odor-stimulated light- and electron-microscope OSN cell centroid lengths normalized to epithelial thickness resulted in values of 0.325 μm for light-microscope OSNs, 0.318 μm for identified MSNs and 0.662 μm for the identified CSNs. Comparison of the cell centroid length data obtained from light-microscope immunocytochemistry in this study for five other amino acid odorants and for a series of arginine analogs tested in an earlier study (Lipschitz and Michel, 1999) suggests that the amino acid stimulated cell centroid lengths most closely match the distribution of identified MSNs. Nonetheless, a shoulder on each of the OSN length distributions indicates the presence of a small population of longer cells. These cells may correspond to the relatively long SC population shown to be labeled under three of four odor-stimulated conditions. Alternatively, we noted that the average cell centroid length of methionine stimulated OSNs was significantly longer than the AGB stimulated OSNs and the average lengths of histidine, arginine, glutamate, cysteine and alanine stimulated OSNs were significantly shorter than the AGB stimulated OSNs. Perhaps there are subclasses of OSNs of varying lengths that respond to specific stimuli. It has been reported (Morita and Finger, 1998) that focal DiI injections into the channel catfish olfactory bulb labeled short, intermediate and long OSNs in the OE.

Odorants (and drugs) eliciting electrophysiological responses from the OE of the zebrafish can be divided into two groups: those that stimulate activity-dependent labeling and those that do not. Most amino acids and several guanidinium analogs (Lipschitz and Michel, 1999) stimulate labeling of many of what we now assume to be MSNs. Other odors, such as polyamines and bile acids, failed to stimulate labeling of MSNs, CSNs or crypt receptor cells. Although we cannot exclude the possibility that MSNs support a second transduction cascade impermeant to AGB, it seems more likely that some of these odorants are specifically detected by the CSNs (or crypt cells). At this time, we cannot exclude the possibility that CSNs also serve a role in the detection of amino acids. Information from activity maps in the olfactory bulb suggests that there is a chemotopic separation of input in the OB (Friedrich and Korsching, 1997, 1998). Amino acids and nucleotides preferentially activate regions of the lateral OB, while bile acids activate the medial bulb. It will be interesting to determine if the two transduction pathways map specifically to MSNs and CSNs and whether there is a subsequent mapping to distinct areas in the OB.

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References

Bruch, R.C. and **Rulli, R.D.** (1988) *Ligand binding specificity of a neutral L-amino acid olfactory receptor*. Comp. Biochem. Physiol. B, 91, 535–540.

Bruch, R.C. and **Teeter, J.H.** (1990) *Cyclic AMP links amino acid chemoreceptors to ion channels in olfactory cilia*. Chem. Senses, 15, 419–430.

Byrd, C.A. and **Brunjes, P.C.** (1995) *Organization of the olfactory system in the adult zebrafish: histological, immunohistochemical, and quantitative analysis*. J. Comp Neurol., 358, 247–259.

Caprio, J. and **Byrd, R.P.** (1984) *Electrophysiological evidence for acidic, basic, and neutral amino acid olfactory receptor sites in the catfish*. J. Gen. Physiol, 84, 403–422.

Erickson, J.R. and **Caprio, J.** (1984) *The spatial distribution of ciliated and microvillous olfactory receptor neurons in the channel catfish is not matched by a differential specificity to amino acid and bile salt stimuli*. Chem. Senses, 9, 127–141.

Friedrich, R.W. and **Korsching, S.I.** (1997) *Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging*. Neuron, 18, 737–752.

Friedrich, R.W. and **Korsching, S.I.** (1998) *Chemotopic, combinatorial, and noncombinatorial odorant representations in the olfactory bulb revealed using a voltage-sensitive axon tracer*. J. Neurosci., 18, 9977–9988.

Hansen, A. and **Finger, T.E.** (2000) *Phyletic distribution of crypt-type olfactory receptor neurons in fishes*. Brain Behav. Evol., 55, 100–110.

Hansen, A. and **Zeiske, E.** (1998) *The peripheral olfactory organ of the zebrafish,* Danio rerio*: an ultrastructural study*. Chem. Senses, 23, 39–48.

Lipschitz, D.L. and **Michel, W.C.** (1999) *Physiological evidence for the discrimination of L-arginine from structural analogs by the zebrafish olfactory system*. J. Neurophysiol., 82, 3160–3167.

Lipschitz, D. L. and **Michel, W.C.** (2000) *Do amino acids stimulate ciliated or microvillar olfactory sensory neurons?* Chem. Senses, 25, 669–670.

Ma, L. and **Michel, W.C.** (1998) *Drugs affecting phospholipase C-mediated signal transduction block the olfactory cyclic nucleotide-gated current of adult zebrafish*. J. Neurophysiol., 79, 1183–1192.

Marc, R.E. (1999a) *Kainate activation of horizontal, bipolar, amacrine, and ganglion cells in the rabbit retina*. J. Comp. Neurol., 407, 65–76.

Marc, R.E. (1999b) *Mapping glutamatergic drive in the vertebrate retina with a channel- permeant organic cation*. J. Comp. Neurol., 407, 47–64.

Michel, W.C. (1999) *Cyclic nucleotide-gated channel activation is not required for activity dependent labeling of zebrafish olfactory receptor neurons by amino acids*. Biol. Sign. Recept., 8, 338–347.

Michel, W.C. and **Derbidge, D.S.** (1997) *Evidence of distinct amino acid and bile salt receptors in the olfactory system of the zebrafish,* Danio rerio. Brain Res., 764, 179–187.

Michel, W.C. and **Lubomudrov, L.M.** (1995) *Specificity and sensitivity of the olfactory organ of the zebrafish,* Danio rerio. J. Comp. Physiol. A, 177, 191–199.

Michel, W.C., Steullet, P., Cate, H.S., Burns, C.J., Zhainazarov, A.B. and **Derby, C.D.** (1999) *High-resolution functional labeling of vertebrate and invertebrate olfactory receptor neurons using agmatine, a channel permeant cation*. J. Neurosci. Methods, 90, 143–156.

Moran, D.T., Rowley, J.C., III, Aiken, G.R. and **Jafek, B.W.** (1992) *Ultrastructural neurobiology of the olfactory mucosa of the brown trout,* Salmo trutta. J. Microsc. Res. Tech., 23, 28–48.

Morita, Y. and **Finger, T.E.** (1998) *Differential projections of ciliated and microvillous olfactory receptor cells in the catfish,* Ictalurus punctatus. J. Comp. Neurol., 398, 539–550.

Ngai, J., Dowling, M.M., Buck, L., Axel, R. and **Chess, A.** (1993) *The family of genes encoding odorant receptors in the channel catfish*. Cell, 72, 657–666.

Ohno, T., Yoshii, K. and **Kurihara, K.** (1984) *Multiple receptor types for amino acids in the carp olfactory cells revealed by quantitative cross-adaption method*. Brain Res., 310, 13–21.

Restrepo, D., Boekhoff, I. and **Breer, H.** (1993) *Rapid kinetic measurements of second messenger formation in olfactory cilia from channel catfish*. Am. J. Physiol. Cell Physiol., 264, C906–C911.

Spath, M. and **Schweickert, W.** (1977) *The effect of metacaine (MS-222) on the activity of the efferent and afferent nerves in the teleost lateral-line system.* Naunyn-Schmiedeberg's Arch. Pharmacol., 297, 9–16.

Speca, D.J., Lin, D.M., Sorensen, P.W., Isacoff, E.Y., Ngai, J. and **Dittman, A.H.** (1999) *Functional identification of a goldfish odorant receptor.* Neuron, 23, 487–498.

Theisen, B., Breuker, H., Zeiske, E. and **Melinkat, R.** (1980) *Structure and development of the olfactory organ in the garfish* Belone belone *(L.) (Teleostei, Atherniformes)*. Acta Zool. (Stockh.), 61, 161–170.

Thommesen, G. (1982) *Specificity and distribution of receptor cells in the olfactory mucosa of char (*Salmo alpinus *L.)*. Acta Physiol. Scand., 115, 47–56.

Thommesen, G. (1983) *Morphology, distribution, and specificity of olfactory receptor cells in salmonid fishes*. Acta Physiol. Scand., 117, 241–250.

Yang, R., Crowley, H.H., Rock, M.E. and **Kinnamon, J.C.** (2000) *Taste cells with synapses in rat circumvallate papillae display SNAP-25-like immunoreactivity*. J. Comp. Neurol., 424, 205–215.

Zar, J.H. (1984) Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ.

Zeiske, E., Melinkat, R., Breucker, H. and **Kux, J.** (1976) *Ultrastructure studies on the epithelia of the olfactory organ of cyprinodonts (Teleostei, Cyprinodontoidea)*. Cell Tissue Res., 172, 245–267.

Zeiske, E., Caprio, J. and **Gruber, S.H.** (1986) *Morphological and electrophysiological studies on the olfactory organ of the lemon shark,* Negaprion brevirostris *(Poey)*. Indo-Pacific Fish Biology: Proceedings of the 2nd International Conference on Indo-Pacific Fishes, 2, 381–391.

Zippel, H.P., Sorensen, P.W. and **Hansen, A.** (1997) *High correlation between microvillous olfactory receptor cell abundance and sensitivity to pheromones in olfactory nerve-sectioned goldfish*. J. Comp. Physiol. A, 180, 39–52.

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