Electrophysiological Evidence for the Broad Distribution of Specific Odorant Receptor Molecules across the Olfactory Organ of the Channel Catfish

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Abstract

To determine if there is a spatial segregation of responsiveness to odorants within the olfactory epithelium, microelectrode recordings were obtained from small populations of olfactory receptor neurons located across different lamellar sensory regions of the olfactory organ of the channel catfish, *Ictalurus punctatus*. Stimuli included L-alanine, L-methionine, L-arginine hydrochloride, L-glutamic acid, ATP and a mixture of bile salts—odorants previously reported to stimulate independent receptor sites in aquatic species. The peak integrated olfactory receptor responses at each recording site were standardized to the response to L-alanine. The relative stimulatory effectiveness of the stimuli was preserved across the 10 olfactory lamellae recording sites. These data support previous molecular biological results of a broad distribution of receptor neurons that express specific receptor genes across the olfactory organ of the channel catfish. **Chem. Senses 21: 519–527, 1996.**

Introduction

The vertebrate olfactory system distinguishes among many different odorants (Shepherd, 1994; Mori and Yoshihara, 1995). Although the exact mechanism that accounts for such an exquisite ability to code information concerning odorant quality is unknown, knowledge of the specificity of the individual olfactory receptor neurons (ORNs) is fundamental to eventually understanding this process. Single ORNs in both vertebrates (Dionne, 1992; Ivanova and Caprio, 1993; Morales *et al.*, 1994; Kang and Caprio, 1995) and invertebrates (Akers, 1992; Michel and Ache, 1994) respond with excitation and suppression to odorants, and different ORNs show different patterns of responses to the same odorants. Individual ORNs thus possess different complements of receptors.

A number of previous studies have reported topographic differences in odorant-induced responses across the

olfactory epithelium of fishes (Thommesen, 1983), amphibians (Mustaparta, 1971; Mackay-Sim *et al.*, 1982; Mackay-Sim and Shaman, 1984; Kent and Mozell, 1992) and mammals (Thommesen and Döving, 1977; Edwards *et al.*, 1988; Mackay-Sim and Kesteven, 1994; Youngentob *et al.*, 1995), suggesting a spatial code for odorant quality discrimination at the level of the sensory epithelium. Although a differential distribution of ciliated and microvillous ORNs is evident in some fishes (Thommesen, 1982, 1983; Erickson and Caprio, 1984), the spatial distribution of receptor cell types has not been matched by a statistically significant differential distribution of odorant specificity. Recent reports in teleosts argue against a topographical map of receptor neurons and for a random (Ngai *et al.*, 1993) or at least broad dispersal (Riddle and Oakley, 1991; Riddle *et al.*, 1993; Baier *et al.*, 1994) of 'like' ORNs across the sensory epithelium. The apparent discrepancy between the former reports of inherent spatial patterning (Moulton, 1976) occurring in the olfactory epithelium of tetrapods and the recent evidence in fish, however, is reconcilable. A current hypothesis (Ngai *et al.*, 1993) is that the basic pattern established in fish to detect asmaller number of water-soluble components was duplicated evolutionarily in tetrapods to form multiple zones of ORNs. According to this hypothesis, different sets of odorant receptor genes in the different zones code for molecular receptors having different chemical specificities that are expressed randomly (Nef *et al.*, 1992; Ressler *et al.*, 1993; Vassar *et al.*, 1993; Strotmann *et al.*, 1994), possibly to cope with the increased number of volatile odorants that exist within the tetrapod environment.

Although the various experimental techniques used were complementary, the evidence against a topographical arrangement of ORNs in teleosts with similar odorant specificities remains circumstantial. Neither lectin binding (Riddle *et al.*, 1993), immunocytochemistry (Riddle and Oakley, 1992), *in situ* hybridization (Ngai *et al.*, 1993; Vogt *et al.*, 1995) nor nerve tracing (Riddle and Oakley, 1991; Baier *et al.*, 1994) studies have provided direct functional evidence for odorant specificity of the investigated ORNs. The present electrophysiological study investigates directly whether or not ORNs expressing specific odorant receptors to six odorants indicated to bind to relatively independent receptor sites in different aquatic species (Caprio and Byrd, 1984; Kalinoski *et al.*, 1987; Sorensen *et al.*, 1987; Bruch and Rulli, 1988; Michel *et al.*, 1988; Caprio *et al.*, 1989) are spatially localized within the olfactory organ of the channel catfish.

Methods and materials

Experimental animals

Channel catfish, *Ictalurus punctatus*, were maintained in floating cages in ponds and were fed commercial catfish chow. On a weekly basis, catfish were transported to LSU for experimentation. Prior to the experiments, the fish were maintained in a 250 l aquarium in aerated, charcoal-filtered, artesian tap water on a 12 h/12 h light/dark cycle at 25°C. Electrophysiological recordings were obtained from 28 catfish (23.0–58.9 g) that were held in the aquarium for <2 weeks.

Animal preparation

The animal preparation was the same as described by Kang and Caprio (1991). Briefly, each catfish was immobilized with an i.m. injection of the neuromuscular blocking agent, Flaxedil (gallamine thiethiodide; 0.05 mg/100 g body wt), wrapped in wet tissue paper and placed on a Styrofoam block held in a Plexiglas container. If necessary, supplemental Flaxidil was injected by a hypodermic needle imbedded in the flank musculature during the experiment. The gills were perfused with aerated, charcoal-filtered artesian water which contained the anesthetic, 0.005% (initial concentration) MS-222 (ethyl-*m*-aminobenzoate methane sulfonic acid), prior to surgery and throughout the experiment. Skin, connective tissue and small pieces of cartilage dorsal to the nasal capsule were removed to expose the olfactory organ (Caprio and Raderman-Little, 1978).

Stimuli and method of stimulation

The odorants L-alanine, L-methionine, L-arginine hydrochloride, ATP and each of the components in the mixture of bile salts (MBS; i.e. the sodium salts of cholic, taurocholic and taurolithocholic acids) were tested at 0.1 mM. L-Glutamic acid, a relatively poor olfactory stimulus to the channel catfish at 0.1 mM, was tested at 1 mM. With the exception of the daily preparation of ATP, 10^{-2} M stock solutions of all stimuli were prepared weekly in charcoal-filtered water and stored at 4°C. Charcoal-filtered artesian tap water was used both as the odorant solvent and as the control.

Stimulus delivery was similar to the method of Kang and Caprio (1991). Charcoal-filtered artesian tap water, 9–11 cm^3/min flow rate, constantly bathed the olfactory mucosa. Stimulus solutions were added to a 0.5 ml Teflon loop of a manual sample injection valve (model no. 1106; Omnifit

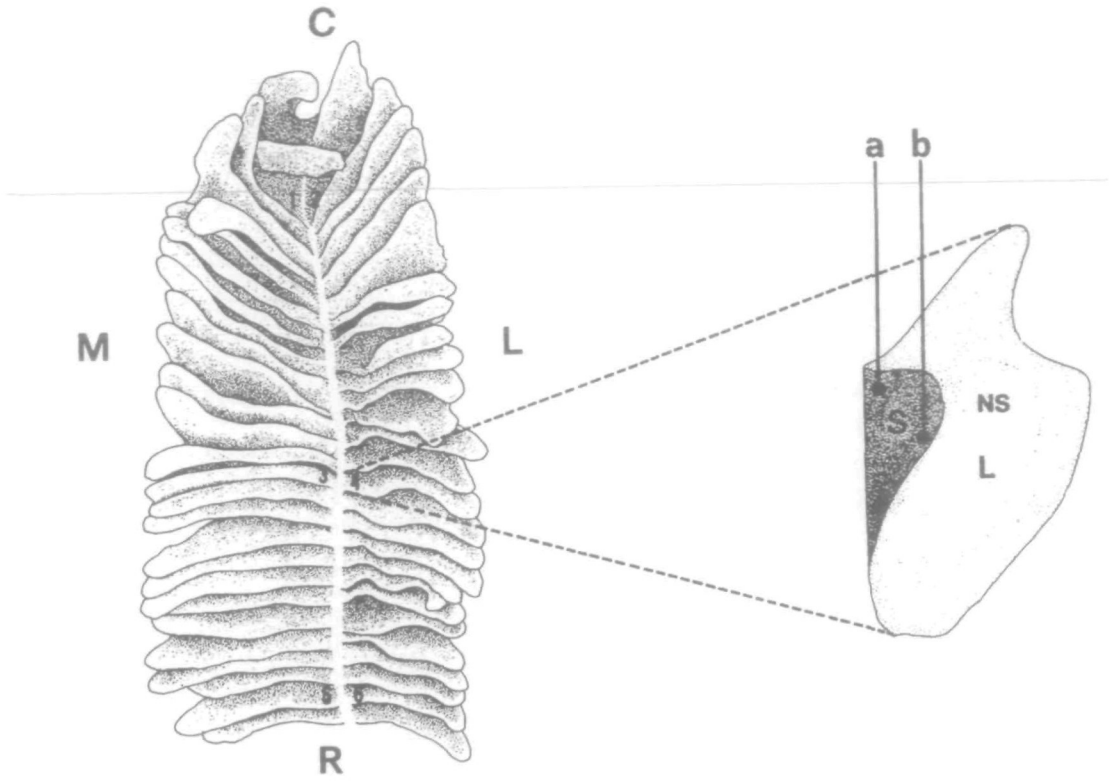

Figure 1 Illustrated is the left olfactory organ as viewed from directly above the fish (left) and a single olfactory lamella rotated caudally by 90° (right). The locations of the 10 recording sites are 1a, 2a and 2b, 3a and 3b, 4a and 4b, 5a and 5b, 6a. C, caudal; R, rostral, M, medial; L, lateral; a, dorsal recording site; b, ventral recording site S, sensory region; NS, non-sensory region.

USA, Atlantic Beach, NY) and injected into the water flow. The maximum stimulus concentration delivered to the olfactory mucosa was 75% of the concentration injected as determined by photodensitometry of dye solutions. The undiluted concentrations of the stimuli are reported in the text. Interstimulus intervals were 3 min. In order to reduce cross-contamination, the charcoal-filtered artesian tapwater rinsed the stimulus loop between odorant tests.

Olfactory electrophysiology

Multiunit olfactory neural activity was recorded *in vivo* with metal-filled glass capillary electrodes tip-plated with platinum (Pt) from the surface of sensory regions of selected olfactory lamellae (Figure 1) (Gesteland *et al.*, 1959; Caprio, 1995). The electrode (10–40 kΩ; Pt ball diameter ~15 μm) was r.c.-coupled (220 pF capacitor, 20 MΩ resistor) to one input grid of a high impedance probe. The other active input was grounded and connected to the reference electrode, a hypodermic needle embedded in the flank musculature. The multiunit neural activity was amplified (bandpass, 30–300 Hz), integrated (0.5 s rise time) and displayed on a pen recorder. The neural response magnitude was measured as the height in mm of the integrated phasic displacement from the baseline level. The neural activity was also recorded on one audio channel and a voice commentary of the experimental procedures was recorded on a second audio channel of a video recorder.

Experimental procedure

The center of the rostro-caudal axis of the olfactory organ was estimated by eye (Figure 1, labels 3 and 4); nine lamella both caudal (Figure 1, labels 1 and 2) and rostral (Figure 1, labels 5 and 6) from the medial lamella defined the other lamellar recording positions. Recordings to the odorants were obtained from dorsal sensory regions (Figure 1a) of two rostral, two intermediate and two caudal lamellae and from ventral sensory regions (Figure 1b) of one rostral, two intermediate and one caudal lamellae (Figure 1). The odorant responses were standardized by dividing each response by the average of the responses to replicates of 0.1mM L-alanine that bracketed each stimulus array (consisting of paired applications of 1–3 different odor-

ants). The responses to a particular stimulus array were not used in the analysis if the magnitude of the bracketing standards differed by ≥25%; with few exceptions, the data presented herein were derived from stimulus arrays whose bracketing standard responses did not differ by >10%. Each odorant was tested at least twice at each recording position tested per fish, and the averaged standardized response for each odorant at each site per fish was determined.

Responses were recorded from a total of 36–38 recording sites in 28 fish for each of six stimuli (i.e. three recording sites were sampled in one fish, two sites in eight fish and one site in 18 fish). The data obtained in the present study therefore comprise 224 averaged standardized responses to the six tested odorants (including the standard, L-alanine) across the specified 10 recording sites from a total of 18 fish. The order of the stimulus presentations varied. The experimental fish were divided into four groups (comprising 12, 10, 4 and 2 fish each); each group was tested with a different stimulus order. In addition, water controls were frequently applied, and responses to controls were generally baseline to ≤10% of the response to 0.1 mM L-alanine. At each lamellar location sampled, all responses to the test stimuli were greater than the control level. Any control response observed was subtracted from the responses to each of the test stimuli for that particular stimulus array.

Data analysis

A repeated measures ANOVA design (Moser *et al.*, 1990) was used to evaluate the data (SAS for mainframe, version 6.08; probability of type I error = 0.05).

Results

The magnitude of the integrated olfactory neural responses to the test odorants was significantly different (repeated measures ANOVA, $P < 0.0001$; Figure 2). In spite of the inherent differences in olfactory potencies of the odorant stimuli, the relative stimulatory effectiveness was similar at each of the 10 recording sites across the olfactory organ of the channel catfish (Table 2; repeated measures ANOVA, $P = 0.9173$). Irrespective of the recording site, olfactory receptor responses to 1 mM L-glutamic acid, 0.1 mM L-methionine and 0.1 mM L-alanine were consistently of greater magnitude than those to 0.1 mM L-arginine, which was of greater magnitude than those to 0.1 mM ATP and the trinary mixture of bile salts (each component at 0.1 mM; Table 1).

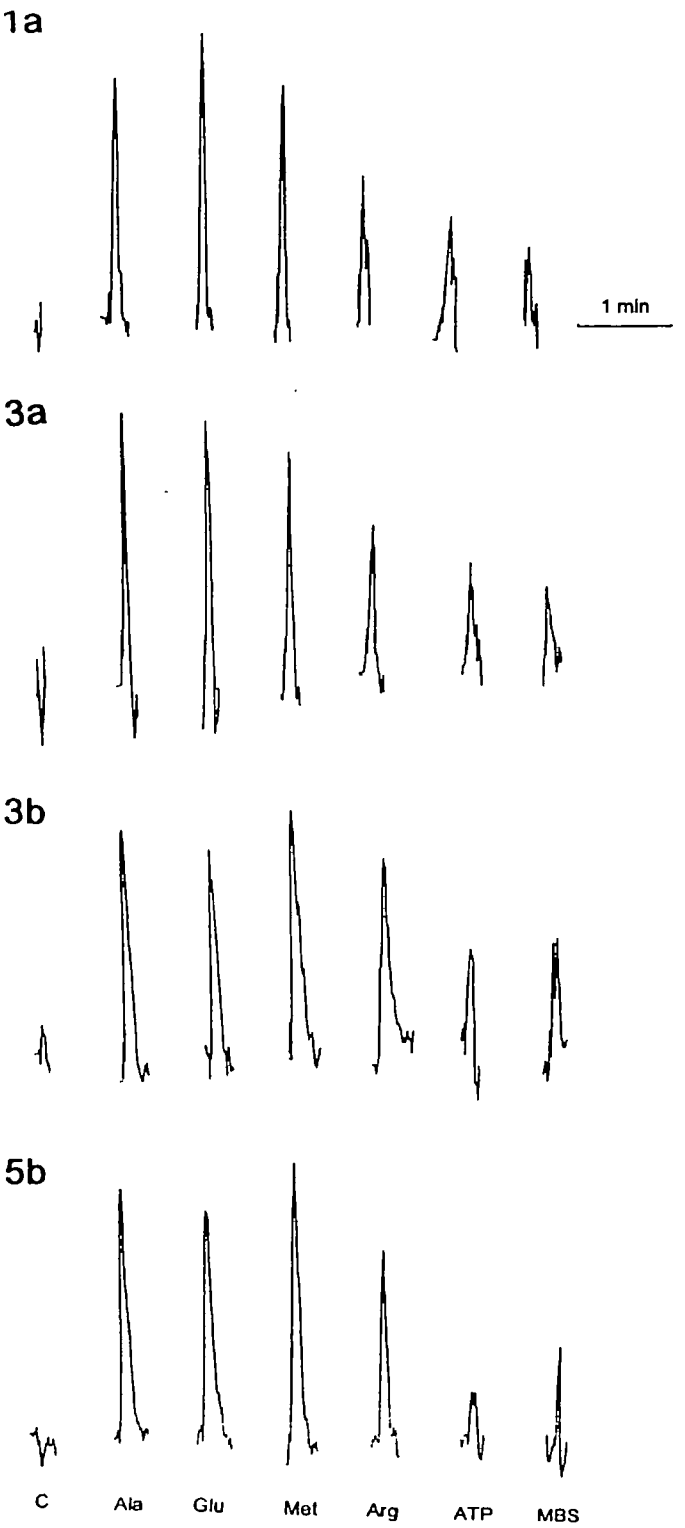

Figure 2 Representative records of integrated olfactory receptor responses to the respective stimuli at four of the ten different recording sites (Figure 1, 1a, 3a, 3b and 5b). With the exception of 1 mM Glu, all stimuli were tested at 0.1 mM.

Table 1 Relative stimulatory effectiveness of each odorant at each recording site

Recording site[a]	Glu 1 mM	Met 0.1 mM	Arg 0.1 mM	ATP 0.1 mM	MBS[b]
1a	108.1 ± 2.4[c] (3)	79.1 ± 11.1 (3)	58.1 ± 17.7 (2)	47.5 ± 6.3 (3)	50.6 ± 14.7 (3)
2a	104.9 ± 15.6 (4)	102.0 ± 26.0 (4)	73.3 ± 6.6 (4)	45.4 ± 4.3 (4)	42.0 ± 9.4 (4)
2b	83.9 ± 10.4 (4)	90.6 ± 4.6 (4)	66.8 ± 7.7 (4)	46.3 ± 6.0 (4)	39.3 ± 8.3 (4)
3a	101.3 ± 8.0 (4)	99.8 ± 22.6 (4)	42.6 ± 3.4 (4)	30.7 ± 6.2 (3)	34.7 ± 8.8 (4)
3b	94.8 ± 5.0 (4)	81.8 ± 6.1 (4)	59.8 ± 2.7 (3)	29.7 ± 6.2 (4)	30.7 ± 6.2 (4)
4a	111.4 ± 7.5 (5)	94.9 ± 9.3 (5)	66.7 ± 5.7 (5)	50.0 ± 9.8 (5)	33.8 ± 7.0 (5)
4b	83.4 ± 7.8 (4)	93.4 ± 10.2 (4)	61.3 ± 8.3 (4)	33.9 ± 8.7 (4)	45.3 ± 9.7 (4)
5a	101.1 ± 14.0 (3)	90.9 ± 4.1 (3)	58.6 ± 9.9 (3)	47.6 ± 0.8 (3)	46.3 ± 6.5 (3)
5b	90.3 ± 11.3 (3)	97.8 ± 8.8 (3)	63.0 ± 10.9 (3)	31.1 ± 4.7 (3)	43.8 ± 3.9 (2)
6a	93.1 ± 8.1 (4)	96.0 ± 7.6 (4)	64.3 ± 4.2 (4)	36.2 ± 5.5 (4)	39.5 ± 10.3 (4)
RSE[d]	97.4 ± 3.1 [38][e]	93.0 ± 3.9 [38]	61.9 ± 2.4 [36]	40.2 ± 2.3 [37]	39.8 ± 2.7 [37]

[a]See Figure 1.

[b]Mixture of bile salts: sodium salts of cholic, taurocholic and taurolithocholic, each at 0.1 mM.

[c]Standardized response (mean ± SEM) to 0.1 mM L-alanine at each recording site. The number in parentheses indicates the number of fish tested.

[d]Mean relative stimulatory effectiveness.

[e]Brackets indicate the total number of recording sites sampled from a total of 28 fish tested.

Discussion

The present investigation is the first quantitative electrophysiological study to indicate that molecular receptors for amino acids, bile salts and ATP—stimuli indicated to be behaviorally relevant natural odorants for different aquatic organisms (Döving *et al.*, 1980; Hara *et al.*, 1984; Caprio, 1988; Zimmer-Faust *et al.*, 1988; Cardwell *et al.*, 1992; Sola and Tosi, 1993; Li *et al.*, 1995)—are widely distributed across the sensory regions of the olfactory organ of the channel catfish. These results are consistent with both the ability to optimally detect chemicals that would themselves be randomly distributed in the incurrent water supply to the olfactory organ and with previous findings indicating that the sensitivity to amino acids and bile salts of the dorso-medial and ventro-lateral sensory regions of olfactory lamellae in the channel catfish are similar (Erickson and Caprio, 1984). All recordings in the study by Erickson and Caprio (1984), however, were obtained from only the central one-third of the olfactory organ, whereas the present study systematically investigated the specificity of ORNs located in rostral, intermediate and caudal lamellar sensory regions.

Electrophysiological evidence also indicates that amino acids, bile salts and ATP bind to different olfactory molecular receptors in aquatic organisms (Thommesen, 1982; Hara *et al.*, 1984; Carr *et al.*, 1986; Michel *et al.*, 1988). Further, a combination of receptor binding (Bruch and Rulli, 1988), lectin inhibition (Kalinoski *et al.*, 1987) and electrophysiological (Caprio and Byrd, 1984; Caprio *et al.*, 1989; Kang and Caprio, 1991, 1995) studies indicate that acidic, basic and neutral amino acids bind to relatively independent receptor sites on olfactory receptors of channel catfish. Different receptor sites for acidic, basic and neutral amino acids are common among different species of fishes (Rhein and Cagan, 1983; Rehnberg and Schreck, 1986; Novoselov *et al.*, 1988; Lo *et al.*, 1991). The independence of olfactory receptor molecules in the channel catfish for their specific ligands can be highly specific, as evidenced by receptor binding studies indicating that up to millimolar concentrations of acidic and basic amino acids do not compete effectively for the L-alanine binding site (Bruch and Rulli, 1988).

Previous evidence has indicated that the dissociation constants of the olfactory receptor sites in the channel catfish for at least the four different amino acids tested were similar and in the micromolar range (Bruch and Rulli, 1988;

Table 2 Comparison of responses to stimulus pairs

	Glu	Met	Arg	ATP	MBS
Glu					
Met	0.2170*				
	0.6391**				
Arg	0.0001	0.0001			
	0.2167	0.8245			
ATP	0.0001	0.0001	0.0001		
	0.6397	0.9392	0.4832		
MBS	0.0001	0.0001	0.0001	0.7931	
	0.4342	0.8859	0.8840	0.7532	

*The *P*-value (MANOVA) of the pairwise contrast of the responses to the stimuli.

**The *P*-value (MANOVA) of the effect of the recording position on the olfactory organ to the respective stimuli (a total of 38 recording sites from 28 animals were tested).

R.C. Bruch, personal communication). The present hypothesis, therefore, is that differences in the potencies of the tested odorants are most likely due to differences in the relative numbers of the different receptor sites; i.e. the more effective the stimulus, the greater the number of receptor sites. If the different receptor sites for the tested stimuli occur randomly, then both the relative proportion of the receptor sites and the relative ranking of the stimulatory effectiveness of the stimuli that bind to these sites and are measured electrophysiologically should be preserved. Unlike electro-olfactogram recordings, the use in the present study of the Pt-plated microelectrode made it possible to record from small populations of ORNs located anywhere across the sensory mucosa without being influenced by responses of ORNs at more distant locations (Erickson and Caprio, 1984).

Lectin-binding (Riddle *et al.*, 1993), immunocytochemical (Riddle and Oakley, 1992) and nerve tracing (Riddle and Oakley, 1991; Baier *et al.*, 1994) studies have suggested a broad distribution of olfactory receptor molecules across the olfactory epithelium of teleosts. However, the specific carbohydrate moieties to which the tested lectins bound on cilia/microvilli of ORNs of rainbow trout (Riddle *et al.*, 1993) may not be involved in odorant recognition. Similarly, the odorant specificities of the widely distributed ORNs determined by immunocytochemistry and nerve tracing that projected to the same glomerulus in the olfactory bulb of zebrafish (Baier *et al.*, 1994) and rainbow trout (Riddle and Oakley, 1991) are unknown. The present study, however, measured directly the responses of ORNs of the channel catfish and showed a similarity of the response specificity of the tested odorants across the olfactory organ, providing a direct electrophysiological correlate to the previous purely anatomical evidence.

Recent *in situ* hybridization evidence for the random distribution of mRNA in ORNs of the channel catfish (Ngai *et al.*, 1993) and zebrafish (Vogt *et al.*, 1995) of putative olfactory receptor molecules also lacks a functional correlate. Although the *in situ* hybridization results (Ngai *et al.*, 1993) and the present report are consistent in providing evidence for a broad distribution of olfactory receptor molecules across the sensory regions of the olfactory organ of the channel catfish, the proposed distribution of these receptor molecules within single ORNs is conflicting. The molecular biological results favor the expression of different molecular odorant receptors in nonoverlapping subsets of receptor neurons (Ngai *et al.*, 1993), whereas both *in vivo* (Kang and Caprio, 1995) and *in vitro* (Ivanova and Caprio, 1993) electrophysiological recordings from single channel catfish ORNs suggest that single ORNs contain heterogeneous assortments of multiple odorant receptor molecules. This apparent discrepancy may be explained by the fact that the probes utilized in the molecular biological study were designed to recognize only seven transmembrane receptor molecules. It is possible that odorants may also be detected through other receptive mechanisms, such as by

direct ligand-operated channels, as proposed for the L-isomers of arginine and proline in taste cells of channel catfish (Teeter *et al.*, 1992), and possibly by nonspecific receptor mechanisms (Kurihara *et al.*, 1989). The advantage of the present electrophysiological study is that it provides the first definitive evidence that olfactory receptor molecules for defined odorant molecules are broadly distributed across the sensory regions of the olfactory organ of a teleost. Whether the molecular receptors for the odorants tested in the present study are actually randomly distributed across the sensory regions of the olfactory organ of the channel catfish, as suggested from *in situ* hybridization studies (Ngai *et al.*, 1993; Vogt *et al.*, 1995), or whether there is an anomaly in their microdistribution, as observed in the zebrafish (Baier *et al.*, 1994), is currently unknown.

In channel catfish, microvillous ORNs outnumber ciliated ORNs by 2:1 in dorso-medial regions of the sensory region of a lamella, whereas ciliated ORNs occupy almost exclusively ventro-lateral portions of the sensory region of a lamella. Since in the present experiments there were no significant differences in the responses to the test stimuli between dorsal and ventral regions of the sensory portions of different lamellae, it is possible that all recordings were obtained from only ciliated receptor neurons since they are present in significant numbers in both regions. Another possibility is that responses were obtained from both ciliated and microvillous receptor neurons in which both receptor cell types randomly expressed the specific receptor molecules for the tested stimuli. A similar conclusion was offered by Erickson and Caprio (1984). With the sole exception of elasmobranchs, which possess ORNs with only microvilli (Theisen et al., 1986; Zeiske et al., 1986, 1987), no direct evidence of the odorant specificity of any microvillous ORN exists for any vertebrate.

ACKNOWLEDGEMENTS

Supported by ONR N00014-90-J-1583 and NSF IBN-9221891. We thank Drs R. Bruch, T. Gilbertson and two anonymous reviewers for their comments on an earlier draft of this manuscript. We also thank Dr M. Hollay for statistical help, Mr J. Tamplin for the Figure 1 illustration and Mr R. Bouchard for photographic assistance.

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Received on October 10, 1995; accepted on April 23, 1996