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Projection of Sensory Neurons with Microvilli to the Lateral Olfactory Tract Indicates their Participation in Feeding Behaviour in Crucian Carp

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Abstract

In the olfactory system of vertebrates, a large number of primary sensory neurons terminate in glomeruli in the olfactory bulb, where they make synapses with a significantly smaller number of secondary neurons. We applied small amounts of a lipophilic neural tracer (DiI) in the glomerular regions of the lateral olfactory bulb in crucian carp, and investigated the centrifugal migration of this stain through the secondary neurons towards the brain and peripherally to the sensory neurons of the olfactory epithelium. In preparations where only the secondary neurons of the lateral olfactory tract (LOT) were stained, the majority (76%) of sensory neurons had cell bodies in the intermediate layer of the olfactory epithelium. Scanning electron microscopy revealed that most of the sensory neurons with cell bodies in the intermediate layers of the olfactory epithelium feature microvilli. Based on observations that the secondary neurons of the LOT mediate feeding behaviour, we feel that there is strong evidence to indicate that the sensory neurons that exhibit microvilli are responsible for mediating the behavioural patterns related to feeding. These results are discussed in relation to physiological experiments on the properties of the sensory neurons and to studies of the innervation pattern of sensory neurons.

Introduction

In the vertebrate olfactory system, a large number of sensory neurons located in the olfactory epithelium terminate in specific areas in the olfactory bulb known as glomeruli. In these neuropiles, the sensory neurons form synapses with secondary neurons (Retzius, 1894; Cajal, 1911). In the olfactory system of crucian carp, the axons of secondary neurons exit the olfactory bulb via three distinct bundles of the olfactory tracts and terminate in different regions of the brain.

In teleost fishes, there are different types of olfactory receptor neurons (ORNs): those equipped with cilia and those equipped with microvilli (Ichikawa and Ueda, 1977; Thommesen, 1983). Recently, a third type of ORN, crypt cells, has been discovered (Hansen *et al.*, 1997; Hansen and Finger, 2000). Any of these receptor cell types appear to be randomly distributed within each lamella, i.e. any given restricted site in the glomerular layer receives axons from ORNs which appear to be widely scattered throughout the epithelium (Oakley and Riddle, 1992). Studies of the responses of bulbar regions to specific odorants, utilizing electrophysiological (Døving *et al.*, 1980) and voltage-sensitive dye (Friedrich and Korsching, 1997) techniques, demonstrate localized activity within the bulb. In mammals, it has been shown that sensory neurons that express a particular odorant receptor project to one or two glomeruli within the olfactory bulb (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996). In teleosts, such as catfish, application of the lipophilic neural tracer DiI to restricted regions of the olfactory bulb has shown that the sensory neurons can be morphologically divided into different categories, depending on the localization of the soma and the length of the dendrite (Morita and Finger, 1998). Three types of ORNs have been recently described: those with tall, intermediate and short dendrites. The appearance of each type of ORN within the olfactory epithelium depends on which part of the olfactory bulb is labelled.

The olfactory sensory system in teleosts is organized such that each of the olfactory tract bundles mediates a specific and distinct class of behaviours. The medial part of the medial olfactory tract mediates alarm behaviour (Døving and Selset, 1980; Hamdani *et al.*, 2000), the lateral part of the medial olfactory tract mediates reproductive behaviour (Døving and Selset, 1980; Stacey and Kyle, 1983; Demski and Dulka, 1984; Kyle *et al.*, 1987), and the lateral olfactory tract (LOT) mediates feeding behaviour (Døving and Selset, 1980; Hamdani *et al.*, 2001). Although these findings suggest that a spatial organization exists in the olfactory system,

whether this organization extends to the sensory neurons in the olfactory epithelium has not yet been established.

In the present study we applied DiI to the synaptic region in the lateral olfactory bulb of the crucian carp. We observed stain in both the peripheral olfactory epithelium, and also in the axons of the secondary neurons, which lead to the brain. If only axons in one bundle of the olfactory tract are labeled, then presumably the corresponding sensory neurons, which are labelled, would be those that terminate on the corresponding secondary neurons. This would indicate which type of ORN is responsible for mediating feeding behaviour.

Materials and Methods

Crucian carp, *Carassius carassius* L., were caught in a small lake in the outskirts of Oslo, Norway. They were transported to the aquarium facilities at the Department of Biology. The aquaria was provided with free-flowing dechlorinated city water and the fish were fed *ad libitum* three times a week.

Nine fish (20–35 g) were netted from the aquaria and anaesthetized with benzocaine (45 mg/l). After exposure sufficient for lethality, each fish was placed in a holding apparatus and were perfused through the heart with 4% buffered paraformaldehyde (phosphate buffer 0.1 M, pH 7.4). The cranial bones just above the olfactory bulbs and tracts were removed and the mesenchymal tissue in the brain case was aspirated and the meninges around the olfactory bulbs were removed by fine forceps. The heads were then cut at a level corresponding to the most anterior portion of the gill covers and were placed in fixative (paraformaldehyde). After 2 days, the skull preparations were placed under a dissection microscope. Small crystals of DiI (1,1-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR, USA) were inserted by a sharp needle into discrete posterior areas in the lateral part of the olfactory bulbs *in situ* (Figure lA). The olfactory systems at both sides were used in all fishes, giving 18 preparations. The brain cavity was filled by a 2% agar–agar solution to prevent migration of the crystal away from the site of application. These preparations were placed into buffered paraformaldehyde and kept at room temperature for 8 weeks to permit diffusion of the dye. After this time period, the olfactory rosette, the olfactory nerve, the olfactory bulb and a part of the olfactory tract on each side was dissected out as a single unit. These preparations where then embedded in 12% gelatin solution and placed into separate casting moulds. The blocks were fixed in 4% paraformaldehyde at 4°C for a minimum of 2 days and cut at 50 μm section on a Vibratome. Sections obtained were inspected with fluorescence (550 nm excitation, 565 nm emission) on an Olympus microscope (BX50WI) and photographed by an Olympus digital camera (DP50) to show the distribution of the labelled neurons within the lamella.

To colour all sensory neurons, two preparations were made by applying DiI to the olfactory nerve close to the epithelium. To colour the soma of all cells in the sensory epithelium a DNA probe, the nuclear stain Bisbenzimid (H33258; Reidel de Haen AG, Sultze, Hanover, Germany), was applied to two slices of these preparations. In a segment of a lamella, all nuclei in the different layers were counted.

For all Vibratome sections of the olfactory rosette, the position of the cell body of each stained sensory neuron was categorized by the location of its nucleus within the epithelium. The sensory epithelium was divided in five equal layers from the surface to the basal lamina, layer 1 being the uppermost layer and the layer 5 closest to the basal membrane (Figure 1E). Thus, the position off each cell soma was assigned to a particular layer.

Scanning electron microscopy (SEM)

To investigate the morphology of the different ORNs, three crucian carp (21–230g) that were exposed to a lethal dose of benzocaine were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The intact olfactory epithelia were isolated and placed in a fixative containing 0.4% glutaraldehyde and 2.5% paraformaldehyde in sodium phosphate buffer, pH 7.4. These preparations were dried, cut in transverse sections to observe the shape and position of the receptor neurons *in situ*, sputtered with a Pt/Ir mixture and observed in a scanning electron microscope (Jeol JSM 6400). Sensory epithelia were photographed both at high magnification (10 000×) to examine the apical part of a particular sensory neuron and at low magnification (1500×) to examine the position of the cell soma within the lamella. The sensory neurons thus identified as having cilia or microvilli were counted.

Results

In the crucian carp used in the present study, the olfactory rosette generally consisted of seven symmetrical pairs of lamellae situated around a central raphe (Figure 1A). The sensory epithelium is pseudostratified and cell somas are found in all depths from the surface to the basal lamina. The distribution of soma of all cells was visualized by staining with a fluorescent probe for DNA that would stain all types of cells present in the sensory epithelium, namely sensory neurons, supporting cells and basal cells. Following the application of DNA probe to a preparation, labelled somas were found at various depths. The distribution of the cell bodies was carried out by assigning the position of the nuclei into five layers, using the same criteria as for the sensory cell soma stained with DiI. Counting all nuclei in a limited region of a lamella revealed 426 stained cell soma. The distribution of these nuclei in relation to the depth in the epithelium showed that about one-fifth of the cells appeared in each layer (Figure 2). Following application of DiI to the olfactory nerve, which would presumably

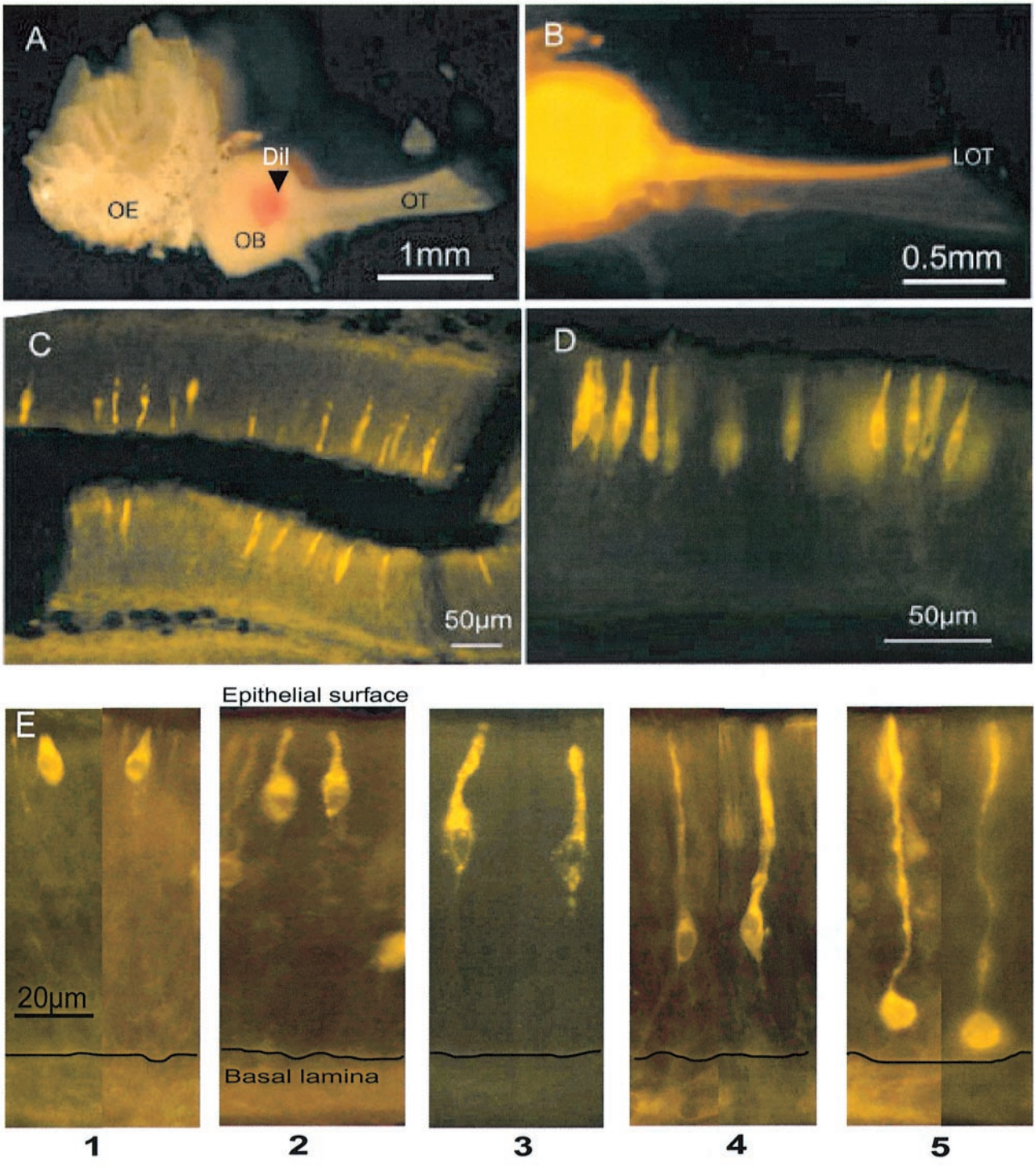

Figure 1. Olfactory system in crucian carp. **(A)** photograph of the olfactory system in white incident light showing the position of DiI application (arrowhead) in the olfactory bulb. OE, olfactory epithelium; OB, olfactory bulb; OT, olfactory tract. **(B)** same preparation as in (A) in fluorescent light showing the selective staining of the axons in the LOT. **(C,D)** Photographs of the sections of the olfactory epithelium from the preparation shown in (A) demonstrating the abundance of ORNs with intermediate dendrites, and cell somas in layer 3. **(E)** Sensory neurons stained by applying DiI to the olfactory nerve showing the position of cell somas at five different layers of the sensory epithelium.

stain all types of sensory axons, we found 542 retrogradely labelled ORNs (Figure 1E). The cell somas of these ORNs were evenly distributed throughout the depth of the sensory epithelium. There were no significant differences in the distribution of the somas between the two staining techniques.

As seen from Figure 1E, it is noteworthy that the morpho-

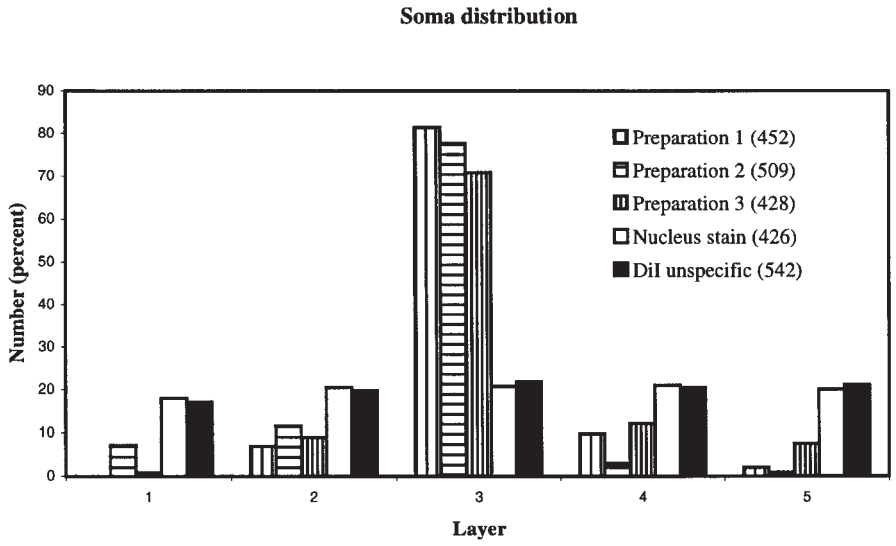

Figure 2 Layer distribution of ORNs. The histogram shows the distribution of ORN according to which layer the cell soma was found in preparations where DiI was applied to the lateral olfactory bulb. Data from three different preparations where only axons of the LOT were stained. The open bars are the position of all cell nuclei stained with a DNA probe. The black bars show the distribution of the ORN somas when DiI was applied to the olfactory nerve. The numbers in parenthesis show the total number of cell somas counted. See text.

logical appearance of the sensory neurons, which had soma in a particular layer, was similar. The cells in layer 1 had short dendrites that formed a cone to the apex. The cells in layer 2 and 3 had slender dendrites but often an enlargement of the dendrite towards the cell soma. Cells with soma in layers 4 and 5 had thin dendrites ending in a distinct olfactory vesicle. Similar distinctions between sensory neurons could be observed from photographs of the olfactory epithelium of the catfish (Morita and Finger, 1998).

To investigate if a particular subset of olfactory neurons was connected to the lateral part of the olfactory bulb, DiI was applied to the glomerular region of the bulb. In the first 13 series of experiments the amount of DiI applied to the bulb was so extensive that axons of all bundles of the olfactory tracts were stained. Subsequent experiments employed sufficiently sparing applications of DiI in order to visualize discrete axons. We obtained three preparations where only the axons in the LOT were stained following an application of DiI in the lateral part of the olfactory bulb (Figure 1A,B), displaying a relatively small number of sensory neurons in each lamella. In these three preparations (1, 2, 3), where selective staining was evident in the olfactory tract, the total number of ORNs observed in the epithelium was 452, 509 and 428. Inspection of the olfactory epithelium in these preparations showed that the majority of the cell somas of ORNs occurred in layer 3 of the olfactory epithelium. These sensory neurons had dendrites with intermediate lengths. As seen from the histogram in Figure 2 between 71 and 81% (mean 76%) of the somas of the sensory neurons were found in layer 3.

Application of DiI to the olfactory bulb led to staining of sensory neurons in all lamellae. There was no indication that particular lamellae had more ORN than others, and there was no apparent aggregation of ORN in any particular region of a lamella.

The difficulty in identifying the peripheral extension of the dendrites in the DiI preparations may be attributed to poor staining of these structures or due to the embedding and sectioning technique. Apical cilia were seen in sensory neurons in other preparations where ORNs with long dendrites were stained.

SEM

We wanted to observe if there was a correlation between the length of the dendrite and the appearance of cilia or microvilli at the apical dendrite. We made scanning micrographs that showed the position of the cell soma of the sensory neurons and the corresponding apical end of the dendrite. These scanning micrographs revealed that 30 of the dendrites of the intermediate neurons, i.e. with cell soma in layer 3, ended in a tuft of microvilli, while only four of these intermediate cells had cilia (Figure 3). Conversely, of the sensory neurons with long dendrites, i.e. with cell soma in layers 4 or 5, we could identify only three that had microvilli, whilst 24 had cilia.

To summarize, our results indicate that the olfactory receptor neurons with intermediate dendrites, i.e. cell somas in layer 3, and equipped with microvilli terminate on secondary neurones that have projections to the brain via the LOT.

Discussion

The present study indicates that, in those cases where staining was restricted to the axons of the secondary neurons in the LOT, the corresponding sensory neurons had cell bodies in the intermediate layer of the olfactory epithelium. Addi-

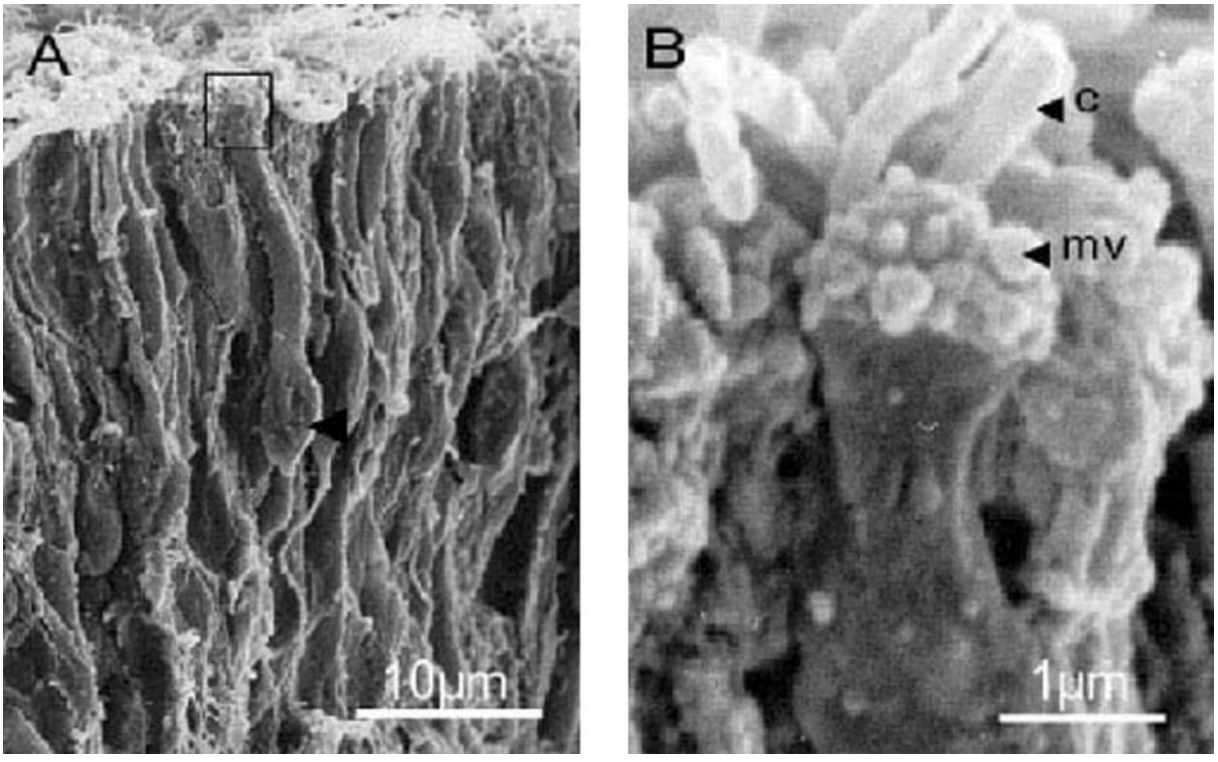

Figure 3. SEM of the sensory epithelium. **(A)** Scanning micrograph of the olfactory epithelium of the crucian carp. The arrow indicates the cell soma of a sensory neuron with microvilli. **(B)** enlargement of the insert region marked in (A) demonstrating a neuron that had microvilli at the dendritic end. c, cilia; mv, microvilli.

tionally, most of these olfactory neurons were equipped with microvilli. Because the LOT mediates olfactory-induced feeding behaviour it is probable that microvillous sensory neurons participate in the feeding behaviour in crucian carp (Hamdani *et al.*, 2001).

One must exersize caution when using DiI in the olfactory system, as there are a large number of unmyelinated axons of sensory neurons of the olfactory nerve, which can be found within a single mesaxon. This feature provides the opportunity for a lipophilic tracer like DiI to diffuse from the membrane of one axon to neighbouring axons. In several of our preparations, where the DiI was permitted to diffuse for a long period of time, we did observe a number of examples of staining of all types of sensory neurons. Additionally, in other preparations, we noted the staining of axons in several of the olfactory tracts, indicating that the initial application of DiI was not optimally placed in the olfactory bulb. We made a sufficient number of preparations with varying stain-incubation times to be able to visualize preparations with DiI staining sufficient to follow all the neurons without encountering overstaining problems. On those preparations, which were not incubated for an extended period of time and showed discrete staining of particular olfactory tracts, we did observe consistent staining of receptor neurons, which were also consistently correlated with the particular tract stained.

The first experimental evidence that a specific morphological class of receptor neurons in fish respond to a particular class of odorants were made by Thommesen (Thommesen, 1982, 1983), whose investigation in salmonids suggested that ciliated ORN responded to bile salts, while microvillous ORN responded to amino acids. This finding was not, however, supported by similar studies in catfish as the spatial distribution of ciliated and microvillous olfactory receptor neurons were not matched by a differential specificity to amino acids and bile salt stimuli (Erickson and Caprio, 1984). More recent studies on the goldfish olfactory system by Zippel and co-workers (Zippel *et al.*, 1997), which combined electrophysiological, behavioural and histological methods, indicated that sensitivity to steroid pheromones correlated with the presence of microvillous ORNs and the sensitivity to amino acids with the presence of ciliated ORNs. Subsequent studies on ORNs of the rainbow trout, however, indicates that ciliated ORNs are generalist which respond to both amino acids and pheromones whereas the microvillous sensory neurons respond to amino acids Suzuki and Sato (Suzuki and Sato, 2001). In the present study, none of the ORNs that stained upon application of DiI in the olfactory bulb had conspicuous extensions at the epithelial surface, which could be interpreted as cilia. We observed in SEM that the majority of sensory neurons with cell bodies in the intermediate position in the olfactory epithelium had microvilli at the end of the dendrite. It should, however, be

noted that SEM experiments also showed instances where these intermediate ORNs were equipped with cilia.

It is interesting to note that in a study in catfish, ventral and dorsal injection of DiI in the olfactory bulb stained 73% of the ORNs with tall dendrites and 82% of the ORNs with short dendrites respectively (Morita and Finger, 1998). These numbers are similar to those seen in our study. In the study in catfish it was observed that the intermediate ORN had cilia and, since our SEM observations indicate that the ORNs with intermediate dendrites could have either cilia or microvilli, it is possible that there are species differences or that there are different types of ORNs with intermediate dendrites.

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