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SHORT COMMUNICATION

The Alarm Reaction in Crucian Carp is Mediated by Olfactory Neurons with Long Dendrites

El Hassan Hamdani and Kjell B. Døving

Division of General Physiology, Department of Biology, PO Box 1051, University of Oslo, N-0316 Oslo, Norway

Correspondence to be sent to: Kjell B. Døving, Division of General Physiology, Department of Biology, PO Box 1051, University of Oslo, N-0316 Oslo, Norway. e-mail: kjelld@bio.uio.no

Abstract

In the present study, we applied a lipophilic tracer, DiI (1,1-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), to the synaptic region of the medial olfactory bulb in formaldehyde-fixed preparations from the crucian carp. We observed staining both in the axons of secondary neurons leading to the brain and in the olfactory receptor neurons (ORNs) of the olfactory epithelium. In those preparations, where staining of the tract was restricted to axons of the medial part of the medial olfactory tract, the majority (86–98%) of the somata of the sensory neurons were found in the deep layers of olfactory epithelium. Since the medial bundle of the medial olfactory tract mediates alarm behaviour in the crucian carp, we conclude that the sensory neurons with long dendrites participate in the reception of alarm pheromones.

Introduction

To date, three morphological types of olfactory receptor neurons (ORNs) have been described in the fish olfactory epithelium: those with tall, intermediate and short dendrites (Ichikawa and Ueda, 1977; Thommesen, 1983; Hansen *et al.*, 1997; Hansen and Finger, 2000). In the catfish, *Ictalurus punctatus*, application of the lipophilic neural tracer DiI to restricted regions of the olfactory bulb has shown that ORNs can be morphologically divided into different categories, depending on the localization of the soma and the length of the dendrite (Morita and Finger, 1998). The appearance of ORNs of these categories in the olfactory epithelium depends on which specific part of the olfactory bulb is labelled. In a previous study (Hamdani *et al.*, 2001a), we observed that the sensory neurons with intermediate dendrites and microvilli were labelled concomitantly with the labelling of the lateral olfactory tract (LOT) axons. As we also observed that the LOT mediates feeding behaviour (Hamdani *et al.*, 2001b), we concluded that ORNs with intermediate dendrites and microvilli mediate feeding behaviour in the crucian carp.

Given the exquisite relationship between morphology and behaviour in the feeding behaviour neurons, we undertook the present study to explore the possibility that a particular morphological type of sensory neuron connects to the medial bundle of the medial olfactory tract (mMOT) and participates in the alarm reaction (Hamdani *et al.*, 2000). The lipophilic neural tracer DiI (1,1-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) was applied to the synaptic region in the medial olfactory bulb of the crucian carp. In the cases where we observed a staining only of the axons of the mMOT and not in other parts of the tract, the majority of sensory neurons stained had long dendrites.

Materials and methods

The procedures followed in this study were the same as those used in our previous study (Hamdani *et al.*, 2001a). 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 had free-flowing de-chlorinated city water provisions and the fish were fed *ad libitum* three times a week.

Six fish (21–32 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 transcardially 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 opercula and were placed in fixative (paraformaldehyde). After 2 days, the skull preparations were placed under a dissection microscope. Small crystals of DiI (Molecular Probes, Eugene, OR, USA) were inserted by a sharp needle into discrete caudal areas in the medial part of the olfactory bulb *in situ*. The olfactory systems from both sides of each fish were used, giving a total of 12 preparations. After application of the dye, 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 6 weeks to permit diffusion of the dye. After this time period, the olfactory epithelium, the olfactory nerve, the olfactory bulb and a part of the olfactory tract on each side was dissected out as a single unit. The 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 sections 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 visualize the distribution of soma of all cells in the olfactory epithelium, a DNA probe, the nuclear stain Bisbenzimid (H33258, Reidel de Haen AG, Sultze, Hanover, Germany), was applied to two slices of the histological preparations used for DiI. In a segment of a lamella, all nuclei in the different layers were counted. We should note that for this part of the present study, we used data from our previous study (Hamdani *et al.*, 2001a).

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

Results

Fluorescent probe for DNA

The sensory epithelium is pseudostratified (Farbman, 1992) and cell somas are found in all depths from the surface to the basal lamina. To observe the distribution of all cell bodies, a DNA probe was applied to two preparations of lamellae and cell somas were apparent at various depths. Counting all nuclei in a limited region of a lamella revealed 426 stained cell somas. The position of each soma was carried out by coarsely dividing the epithelium in five layers (Figure 1). As described, layer 1 is localized at the epithelium surface and layer 5 is localized at the basal lamina.

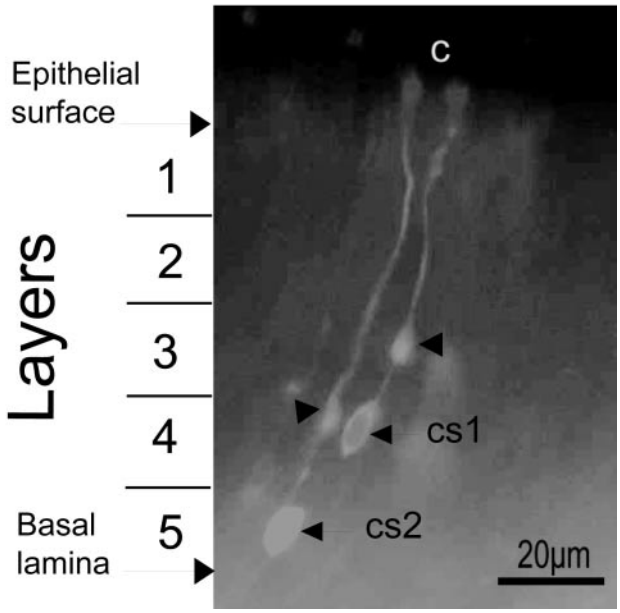

Figure 1 Photograph of a section of the olfactory epithelium from preparation 3, demonstrating two ciliated ORNs with thin and long dendrites. The numbers 1–5 correspond to the hypothetical layers of the epithelium. c, cilia; cs1 and sc2, cell somas of ORNs situated in layers 4 and 5, respectively. Note the thickenings of the dendrites (arrow heads).

The distribution revealed ~20% of the cells in each of the five layers (Figure 3).

DiI injection into the medial part of the olfactory bulb

The olfactory bulb has an ellipsoid shape ~1.6 mm long and 1.3 mm in diameter. Of the 12 preparations where the DiI was applied in the medial part of the bulb, there were only three preparations that showed a selective staining of the axons in the mMOT. It is important to realize that only in the three preparations where there was a selective staining of the axons of the mMOT, was there a selective staining of the ORNs in the olfactory epithelium. In the other preparations where there was a staining of other bundles of the olfactory tract, all types of sensory neurons were stained. Figure 1 shows two typical sensory neurons with cell bodies in layers 4 and 5. These neurons have long dendrites and can be further characterized by a thickening of the dendrite a short distance from the soma. These sensory neurons end in a distinct olfactory vesicle. Although we could reveal cilia at the olfactory vesicle in some cases, further studies are needed to ascertain the kind of appendages associated with these sensory neurons.

In nine preparations, the DiI application was placed so that axons of all three bundles in the olfactory tract were stained and in the olfactory epithelium one could observe all three types of sensory neurons at various depths. These observations strengthen our results because placing the DiI crystals outside the synaptic region would tend to increase

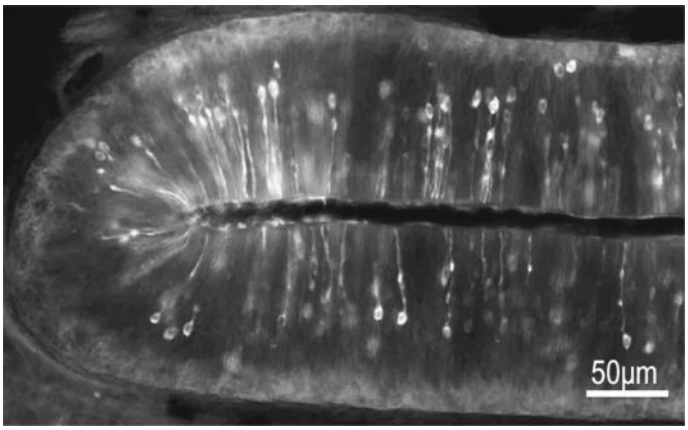

Figure 2 Photograph of a section of the olfactory epithelium from preparation 2, demonstrating the abundance of the ORNs with long dendrites, and cell somas in layers 4 and 5.

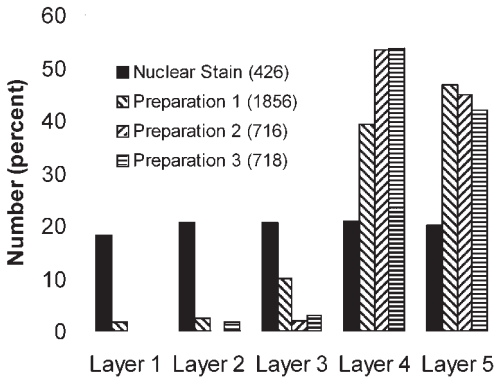

Figure 3 Layer distribution of the ORNs. The histogram shows the distribution of ORNs according to which layer the cell soma was found in preparations where DiI was applied to the posterior part of medial olfactory bulb. Data are from three different preparations where only axons of the mMOT were stained. The black bars show the position of cell nuclei stained with a DNA probe. The numbers in parentheses show the total number of cell somas counted. See text.

the possibilities of staining different types of primary as well as secondary neurons.

Counting the ORNs labelled in the epithelium revealed 1856 in preparation 1, 716 in preparation 2 and 718 in preparation 3 (Figure 3). The stained sensory neurons were found in all lamellae of the olfactory rosette. There was no indication that a particular lamella had more ORNs than others and there was no apparent aggregation of ORNs in any particular region of a lamella. Concomitant with the selective staining of the mMOT, when DiI was applied to the medial region of the bulb, the majority of the cell somas of ORNs occurred in layers 4 and 5 of the olfactory epithelium (Figure 2). These were sensory neurons with long dendrite. As seen from the histogram in Figure 3, between 86 and 98% of the cell somas of the sensory neurons stained were found in layers 4 and 5.

In summary, our results indicate that ORNs with long dendrites terminate on secondary neurons that have projections to the brain via the medial part of the medial olfactory tract.

Discussion

It seems to be a general feature of the olfactory system that primary olfactory neurons expressing a particular receptor, although randomly distributed in domains of the epithelium, project their axons to one or a small number of glomeruli (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996). The anterograde staining of the sensory neurons by application at their terminals in the olfactory bulb in fish demonstrates that a discrete set of neurons can be visualized (Morita and Finger, 1998). In the present study on crucian carp, we have associated the sensory neurons, which have their cell bodies close to the basal lamina, with the secondary neurons that form the mMOT. Since this bundle is known to mediate the alarm reaction, it is conceivable that this type of sensory neuron participates in the reception of alarm substance.

Morphology and function

Our investigations have demonstrated a previously overlooked feature of the pattern of connections of sensory neurons to the olfactory bulb, permitting us to suggest that a particular morphological type of sensory neuron may be allotted to a specific behaviour. In the present study, we show that ORNs with somas in the deep layer of the olfactory epithelium and possibly equipped with cilia project to the medial part of the bulb, suggesting that they participate in an alarm reaction elicited by pheromones. ORNs with somas in the middle layer of the olfactory epithelium equipped with microvilli project to the lateral part of the bulb and participate in feeding behaviour. Our findings are in accordance with physiological data from isolated ORNs of the rainbow trout showing that ciliated sensory neurons respond to pheromones, and that microvillous sensory neurons respond to amino acids (Sato and Suzuki, 2001). We can, therefore, speculate on the third type of ORNs, i.e. the crypt cells with short dendrites forming the apical layer. If these crypt cells project to the lateral bundle of the medial olfactory tract (lMOT) it is possible that they participate in the reception of sexual pheromones, as this part of the tract mediates the behavioural patterns related to courtship in goldfish (Stacey and Kyle, 1983) and cod (Døving and Selset, 1980). This statement is supported by the presence of all three types of sensory neurons in our preparations where staining was not confined to axons of a single olfactory tract.

Sensory neurons with long dendrites and alarm

In previous experiments, we found that the mMOT mediates the alarm reaction. This finding does not, however, imply that this is the only type of behaviour reactions mediated by the mMOT. Consequently, the sensory neurons that terminate on the secondary neurons making up the mMOT

may also be devoted to other types of behaviour induced by pheromones. Interestingly, recent studies indicate that alarm reaction might not only be confined to ostariophysi (Schutz, 1956), but also to other groups of fishes (non-ostariophysian), including gobies (Smith, 1989; Smith *et al.*, 1991), poeciliids (Reed, 1969; García *et al.*, 1992; Brown and Godin, 1999), cichlids (Wisenden and Sargent, 1997) and salmonids (Brown and Smith, 1997; Mirza and Chivers, 2000, 2001). Consequently, it is plausible that the alarm reaction is more common than hitherto believed. Thus, the present study may imply that the sensory neurons with long dendrites respond to alarm pheromones.

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