

Specific Projection of the Sensory Crypt Cells in the Olfactory System in Crucian Carp, *Carassius carassius*

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Abstract

To study the projection of a special type of sensory neuron called crypt cells in the olfactory system in crucian carp, *Carassius carassius*, we applied the neural tracer 1,1-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) in the olfactory bulb (OB). Small crystals of DiI were applied in a small area at the synaptic region at the ventral part of the OB, where a population of secondary neurons specific for sex pheromones has been identified. In those samples (4 out of 24) where only axons in the lateral bundle of the medial olfactory tract were stained, the majority (50–66%) of olfactory sensory neurons stained were crypt cells situated in the peripheral layer of the olfactory epithelium. Because this bundle of the tract mediates reproductive behavior, it is conceivable that crypt cells express olfactory receptors for sex pheromones.

Key words: convergence, fish, olfaction, projection, receptor neurons, sex

Introduction

The projection pattern of the axons of olfactory receptor neurons (ORNs) into the olfactory bulb (OB) is a basic key for understanding the ability of animals to discriminate between a vast number of odorants (for reviews, see Mori *et al.*, 1999; Buck, 2000, 2004; Mombaerts, 2004). In mammals, the ORNs that express a particular odorant receptor project to one or two glomeruli in the OB (Ressler *et al.*, 1994; Vassar *et al.*, 1994).

The concept of odotopic representation in the teleost OB was discovered by Thommesen (1978) and has been a subject of many studies (Døving *et al.*, 1980; Friedrich and Korsching, 1997; Nikonov and Caprio, 2001, 2004; Hamdani and Døving, 2003). In an adjacent study, we demonstrate that a population of secondary neurons specific for sex pheromones is located in the ventral part of the OB (Lastein *et al.*, 2005). In fish, three different types of ORNs are recognized within the same olfactory epithelium: ciliated, microvillous (Ichikawa and Ueda, 1977; Thommesen, 1983), and crypt neurons (Hansen *et al.*, 1997, 2003, 2004; Hansen and Finger, 2000; Zeiske *et al.*, 2003). Previous studies on the crucian carp have shown that axons of ciliated ORNs project to the medial part of the medial olfactory tract and mediate alarm reaction. The axons of the microvillous ORNs project

to the lateral olfactory tract and mediate feeding behavior (Hamdani *et al.*, 2001; Hamdani and Døving, 2002). These topographical projections were recently shown to be similar in channel catfish (Hansen *et al.*, 2003) and zebra fish (Sato *et al.*, 2005). The axons of crypt cells expressing $G\alpha_o$ are found to project to the ventral midline of the OB in catfish (Hansen *et al.*, 2003). These cells are found in the most superficial layer of the olfactory epithelium.

In the present study, we examine the projections of the axons of the sensory neurons by the application of the neural tracer 1,1-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) to the ventral region of the OB in crucian carp. As in previous studies, we specifically looked for the type of sensory neurons that was stained when only one of the bundles of the olfactory tract was stained. In a few preparations with specific staining of the lateral part of the medial olfactory tract (IMOT), the majority of sensory neurons were found in the superficial layer. Due to their position and shape, they are most likely crypt cells. The IMOT mediates reproductive behavior (Weltzien *et al.*, 2003). We discuss the possibility that crypt cells in the superficial layer of the olfactory epithelium participate in fish reproductive behavior.

Materials and methods

Animal and tissue preparation

Crucian carps, *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 dechlorinated city water provisions, and the fish were fed three times a week.

Thirteen fish (25–35 g) were netted from the aquaria at different periods of the year from February to September and anesthetized with benzocaine (45 mg/l). After exposure sufficient for lethality, each fish was placed in a holding apparatus and perfused through the heart with 4% buffered paraformaldehyde (phosphate buffer 0.1 M, pH 7.4). The cranial bones just above the OBs and tracts were removed, the mesenchymal tissue in the brain case was aspirated, and the meninges around the OBs were removed by fine forceps. The heads were then cut at a level corresponding to the most anterior portion of the gill covers and placed in the fixative (paraformaldehyde). After 2 days, the olfactory rosette, the olfactory nerve, the OB, and a part of the olfactory tract on each side were dissected out as a single unit. The olfactory systems at both sides were used in 13 fish giving 26 preparations.

Dil application

In 24 preparations, small crystals of DiI (Molecular Probes, Eugene, OR) were inserted by a sharp needle into discrete ventral areas in the OB, where a population of neurons specific for sex pheromones has been identified (Lastein *et al.*,

2005) (Figure 1A). In two preparations, DiI was applied to the olfactory nerve close to the epithelium in order to color all types of sensory neurons. Each preparation was then covered by a 2% agar-agar solution in fixative to prevent migration of the crystal away from the site of application. These preparations were placed in buffered paraformaldehyde and kept in dark at room temperature for 5 weeks to permit diffusion of the dye.

Sectioning and photography

An initial examination of the preparation was made in a fluorescent dissecting microscope to ascertain the staining of the IMOT only. The preparations were embedded in 12% gelatine solution and placed into separate casting molds. The blocks were fixed in 4% paraformaldehyde at 4°C for a minimum of 2 days and cut at 50- μ m sections on a Vibratome. Sections obtained were inspected with fluorescence (550-nm excitation, 565-nm emission) in a fluorescent microscope (Olympus BX50WI) and photographed using an Olympus digital camera (DP50) to show the distribution of the labeled neurons within the lamella. Some preparations were also examined with a confocal microscope (Olympus FluoView 1000, BX61WI).

For all sections of the olfactory rosette, the position of each stained sensory neuron was categorized by the location of its cell body 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 being closest to the basal membrane (Figure 2). Thus, the position

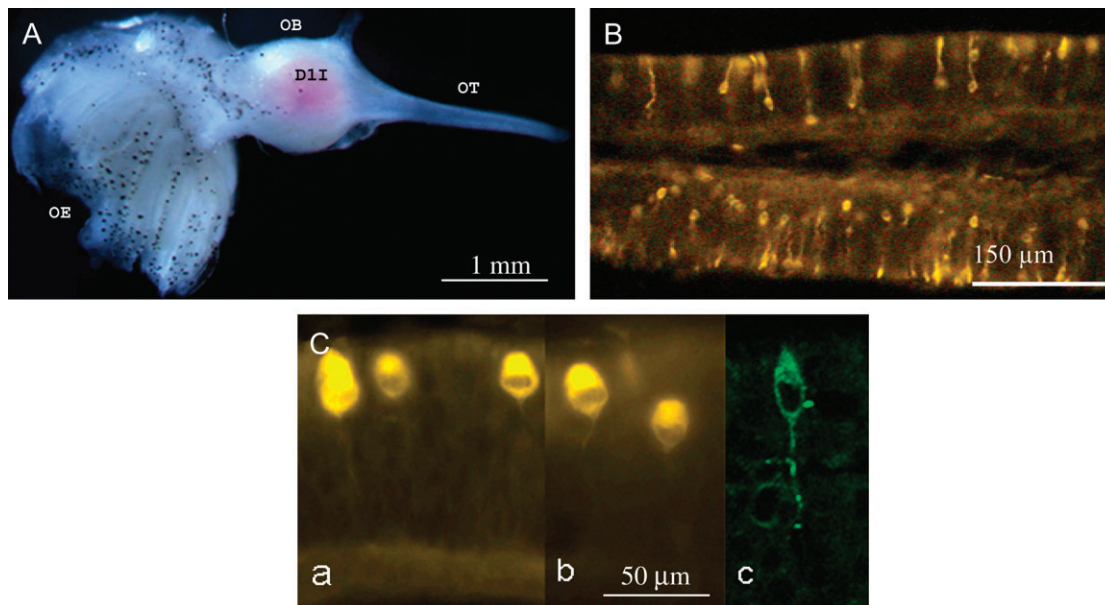


Figure 1 Retrograde staining of the olfactory sensory neurons. **(A)** The olfactory epithelium, nerve, and bulb of a crucian carp, ventral view. The application site of DiI in the bulb is seen as a red region. **(B)** Different olfactory neurons stained upon an unspecific staining of the olfactory nerve. **(C)** The appearance of the crypt cells in a preparation where only the IMOT was stained. (a) Crypt cells reaching the surface. Note that the position of the crypt cell to the right in (b) is below the surface of the epithelium, indicating that it is not in direct contact with the epithelial surface. (c) A crypt cell presenting a distinct sharp ending. OB = olfactory bulb; OE = olfactory epithelium; OT = olfactory tract.

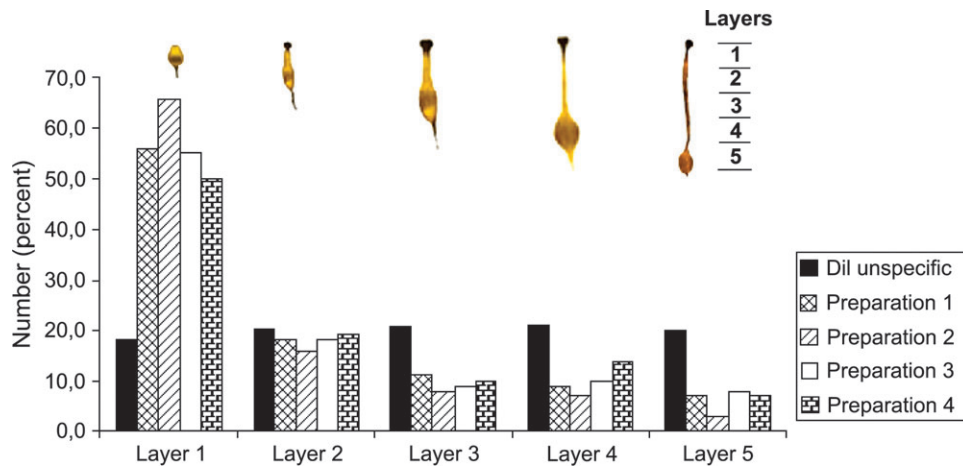


Figure 2 Histogram of the position of cell somas of the sensory neurons in the olfactory epithelium of the crucian carp. Black bars show the distribution of all types of sensory neurons for an application of DiI to the olfactory nerve. The other bars show the distribution of the cell bodies of the sensory neurons for the four preparations where the application was made in a small region of the ventral aspect of the bulb. The photographs above the histogram show the different types of sensory neurons in the olfactory epithelium in relation to the layers where their somas are positioned.

of each cell soma was assigned to a particular layer. Efforts were made to count all cells stained in each preparation. Thus, we were counting all sections and all cells in the sections.

Results

All types of ORNs, located at different depths in the olfactory epithelium, could be visualized by an unspecific DiI injection in the olfactory nerve (Figure 1B). To observe the distribution of each of these ORNs, we divided the olfactory epithelium into five layers as described earlier and in previous studies (Hamdani *et al.*, 2001; Hamdani and Døving, 2002). Counting of sensory neurons in preparations where the staining was unspecific revealed ~20% of cell bodies in each of the five layers (Figure 2). Thus, sensory neurons were equally distributed with respect to depth in the sensory epithelium. Sensory neurons located at the uppermost layers are assumed to be crypt neurons, those located in the middle layers are microvillous neurons, and those located in the deep layers of the epithelium are ciliated neurons (see also Hansen *et al.*, 2004).

DiI injection into the ventral part of the OB

Of the 24 preparations where DiI was applied in a discrete area in the ventral region of the OB (Figure 1A), only four showed specific staining of the axons in the IMOT, as observed in a fluorescent dissecting microscope and in the ordinary fluorescent microscope. The majority of cells stained in these four preparations are found in the upper layer, called layer 1. Presumably, the cells in this layer are crypt cells. The crypt cells are conspicuous because of their ovoid form and their location near the epithelial surface. We cannot state that they are all crypt cells, but from the works of Hansen

et al. (1997, 2003, 2004), Zeiske *et al.* (2003), and our own studies, this seems to be the case. The cells in layer 1 that we see in our preparations are also similar in form to those seen by staining with the S100 antibody (Germana *et al.*, 2004). In the four preparations where only axons in the IMOT were stained, we found that the total numbers of cells counted were 340, 318, 296, and 105, and the corresponding percentages of cells in layer 1 were 56%, 66%, 55%, and 50%. Examples of these cells are shown in Figures 1C and 2.

Crypt cell variations over the year

The four preparations showing specific staining of the axons in the IMOT were from four individuals taken in April, May, and August. In the other preparations from the same period, the number of crypt cells in the epithelium was high. In fish taken in February, March, and September, the number of crypt cells was very small. This observation was true also for preparations where staining was not specific to the IMOT. In many preparations, we could not observe any crypt cells, even though we observed fine staining of the axons in the olfactory nerve under the ordinary fluorescence microscope. Occasionally, in a confocal microscope, we could see faint traces of staining in the apical layer of the epithelium that resembled the outline of a crypt cell (data not shown). Finally, some sensory neurons that stained, categorized as crypt cells, did not seem to reach the epithelial surface (Figure 1C, part b). Other crypt cells from preparations in April, May, and August had a distinct sharp ending protruding to the surface (Figure 1C, part c).

Discussion

The present study demonstrates that crypt cells send axons to the ventral part of the OB where they make synapses with

mitral cells whose axons make up the IMOT. This finding is in accordance with our previous studies showing that microvillous ORNs project to the lateral olfactory tract, which mediates feeding behavior, and that ciliated ORNs project to the medial bundle of the medial olfactory tract mediating alarm reaction (Hamdani *et al.*, 2001; Hamdani and Døving, 2002). Congruently, crypt neurons were labeled only by DiI injections into two specific ventral sites in catfish (Hansen *et al.*, 2003). These findings, further, indicate that in the fish olfactory system, the three types of ORNs are organized in three parallel pathways projecting to different regions in the OB. From the bulb the information is transmitted to the telencephalon in three separate tract bundles.

Because the IMOT mediates reproductive behavior in crucian carp (Weltzien *et al.*, 2003), it is conceivable that crypt cells express olfactory receptors for sex pheromones. Crypt cells make up a distinct morphological type of ORNs in fish. They were discovered using transmission electron microscopy (TEM) (Hansen *et al.*, 1997; Hansen and Finger, 2000). Their ovoid shape and location within the apical layer of the olfactory epithelium make them easily distinguishable in both retrograde tracing experiments and TEM. Such cells can also be seen in scanning electron microscopy (SEM), in preparations of which lamellae are broken. However, we were not able to see them at the lamella surface using SEM. This feature is probably due to their emplacement and the fact that they are enwrapped in supporting cells. Recently, these neurons were also discovered in zebra fish using S100 proteinlike antibody (Germana *et al.*, 2004). The fact that only four out of 24 preparations did demonstrate a specific staining of the IMOT indicates that there are few glomeruli that receive synaptic contact with the crypt cells. This is a finding that is in accordance with our electrophysiological studies (Lastein *et al.*, 2005) and the studies by Friedrich and Korsching (1997, 1998).

In the preparations made in April, May, and August, the sensory neurons stained concomitantly with the staining of the IMOT were located mostly in layer 1 and displayed distinct endings protruding to the surface (Figure 1C). In these preparations, the cells in layer 1 were visible in ordinary fluorescence microscopy. In the preparations made in February, March, and September, stained axons were observed in the epithelium, but cells in layer 1 were visible only in a few cases and with confocal microscopy. One additional feature attracting our attention while we analyzed our samples is that many of the stained cells in layer 1 did not seem to reach the epithelial surface. Moreover, the ones that did reach the epithelial surface had a distinct sharp ending, close to the surface. These findings might be relevant in view of the possibility that the cells in layer 1 are related to the reproductive status of the crucian carps. Crucian carps, like their close relative, goldfish, undergo gonadal growth during winter and spawn during spring and summer. The role of the olfactory system in spawning behavior is documented in several works (for review, see Stacey *et al.*, 2003). It is interesting

that crucian carp mature males and regressed males, that is, males that had previously been spermiating but showed no expressible milt or pectoral fin tubercles, had higher sensitivity to prostaglandin $F_{2\alpha}$ than immature males (Bjerselius and Olsén, 1993). This could be related to the appearance or position of the crypt cells in the epithelium. It is possible that crypt cell appearance is related to the seasonal variation of reproduction and to gender (Lastein *et al.*, 2005). Studies of such relations require examinations of the variability in appearance of the cells in layer 1 with sex and season, made by sampling throughout the year from the natural habitat. Such studies are under way.

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