Chem. Senses 33: 119–123, 2008
doi:10.1093/chemse/bjm072
Advance Access publication October 24, 2007

Seasonal Variations in Olfactory Sensory Neurons—Fish Sensitivity to Sex Pheromones Explained?

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Abstract

Olfactory sensory neurons of vertebrates regenerate throughout the life of the animal. In fishes, crypt cells are a type of olfactory sensory neurons thought to respond to sex pheromones. Here, we demonstrate that the number of crypt cells in the olfactory epithelium of the crucian carp varies dramatically throughout the year. During winter, few crypt cells are observed at any location within the sensory epithelium. In spring, the majority of crypt cells are located deep in the epithelium not yet exposed to the environment. However, during the summer spawning season, crypt cells are positioned at the epithelial surface. These findings may explain previous studies demonstrating a relationship between circulating androgen and olfactory sensitivity to sex pheromones.

Key words: crypt cells, olfaction, spawning, turnover

Introduction

The present study brings together different aspects on the sense of smell in vertebrates. One aspect is the conservation of the olfactory system during evolution (Hildebrand and Shepherd 1997). Another aspect is that sensory neurons in the mammalian olfactory epithelium regenerate throughout the life of the animal (Moulton 1974; Graziadei and Graziadei 1979; Schwob 2002). It is also significant that the fish olfactory system is highly sensitive to sex pheromones (Moore and Scott 1992; Lastein et al. 2006) and that androgen injections increase the olfactory receptor response to a sex pheromone (Cardwell et al. 1995).

The basis for the present study was the growing evidence of chemotopography of the fish olfactory system (Døving and Selset 1980; Thommesen 1983; Nikonov and Caprio 2001; Hamdani and Døving 2007). The sensory neurons that make up the assembly of cells detecting odorants in fishes constitute 3 types with different morphology: 1) Ciliated sensory neurons, with long dendrites and cilia, and axons that terminate in the medial part of the olfactory bulb. 2) Microvillous sensory neurons with shorter dendrites than the ciliated cells and with microvillae extending from their apical surface (Yamamoto and Ueda 1979; Thommesen 1983). Their axons go to the lateral part of the bulb. 3) Crypt cells (Hansen et al. 1997; Hansen and Finger 2000), which are spherical or pear-shaped cells located close to the epithelial surface and equipped with a few cilia and microvilli. In the crucian carp *Carassius carassius*, the axons of the crypt cells extend to the ventral part of the bulb and connect to secondary neurons with axons in the lateral part of the medial olfactory tract (Hamdani and Døving 2006). As this part of the olfactory tract mediates reproductive behavior (Weltzien et al. 2003), the crypt cells have been implied in the detection of sex pheromones (Hamdani and Døving 2002; Lastein et al. 2006). Similarly, in goldfish, *Carassius auratus* L., crypt cells project to discrete areas in the ventral part of the olfactory bulb (Hansen et al. 2004, 2005). In a patch-clamp study of sensory neurons of fish, recordings were claimed to be from crypt cell and reported to respond to amino acids; however, sex pheromones were not tested (Schmachtenberg 2006).

The present study was initiated because commonly we observed crypt cells at the epithelial surface but occasionally crypt cells were seen deep in the sensory epithelium. These findings suggested that there might be changes in the appearance of the crypt cells related to the natural conditions throughout the year. Previous experiments have been

conducted with animals that have been bred in laboratories for a long time. Thus, crucian carps were placed in an outdoor pond exposed to normal light and temperature variations. Fish were sampled each month, and the olfactory sensory neurons were labeled anterogradely by inserting a lipophilic tracer, 1,1-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI), into the olfactory bulb and visualized by fluorescent microscopy. Crypt cells were counted, and their position within the olfactory epithelium was recorded. A preliminary report of these findings has been published (Hamdani et al. 2006).

Materials and methods

Crucian carps were caught in a small lake in the outskirts of Oslo, Norway, placed in an outdoor pond at the premises of the Department of Molecular Biosciences, University of Oslo, and thus exposed to the normal light and temperature variations. Three fishes (18–55 g body weight) were sampled each month regardless of the sex, anesthetized with benzocaine (45 mg/l), and fixed transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The cranial bones above the olfactory bulbs and tracts were removed, and the mesenchymal tissue in the brain case was aspirated. The retrograde labeling of the sensory neurons in the olfactory epithelium was performed by inserting crystals of DiI (DiI perchlorate, Molecular Probes, Eugene, OR) into the whole olfactory bulb. The brain cavity was filled by a 2% agar–agar solution to prevent migration of DiI crystals away from the site of application. The preparations were then kept in buffered paraformaldehyde at room temperature for 4 weeks to permit diffusion of the dye. To ascertain that the different areas of the bulb were stained, we inspected the labeling of the 3 bundles of the olfactory tract. The olfactory epithelium was, then, imbedded in 12% gelatine, sectioned with a vibratome into 50-μm slices, and the crypt cells were counted. About 20–30 sections per olfactory organ were inspected by fluorescence microscope (BX50WI, Olympus, Japan) combined to a digital camera (ProgRes, Jena, Germany) and a confocal microscope (FluoView 1000, BX61W1, Olympus, Japan). The relative position of the crypt cells was assigned to the surface layer or deeper in the epithelium. The particular form of the crypt cells makes them conspicuous with a pear shape and lacking dendrites, thus easy to recognize. Their relative low number in the olfactory organs of crucian carp made it, in addition, feasible to count all crypt cells. For each month, 1 olfactory organ from 3 crucian carps was investigated and the total number of crypt cells in the olfactory epithelium counted. The experiments were carried out according to protocols approved by the Norwegian law concerning use of animals in research.

Results

The application of the neurotracer DiI to the olfactory bulb reveals all 3 types of sensory neurons in the olfactory epithelium as the dye diffuses along the membrane of the sensory axons. The photos in Figure 1 illustrate the appearance of sensory neurons at different times of the year. In the winter months, few crypt cells were present in the olfactory epithelium. It is also noteworthy that the majority of crypt cells in the preparations from March to April were located deep in the sensory epithelium. In the olfactory organs from fish taken in January and February, the total number of crypt cells was less than 30 per organ. In March, the mean number of crypt cells was 174 (Figure 2). In the period from March through September, the mean number varied between 133 and 242 per organ. In fish taken in October, November, and December, the number of crypt cells was found to be less than 40 per organ. In fish sampled from March to September, the average number of crypt cells was 177.6 ± 53.5 per olfactory organ. In contrast, the average number of crypt cells per organ in the winter period October to February was 22.4 ± 14.9. The difference between the mean numbers of crypt cells per organ in these 2 periods is highly significant ($P < 0.001$, Student t-test).

The percentage of crypt cells found in the surface layer of the olfactory epithelium increased from March to July (Figure 2). In July, August, and September, the majority

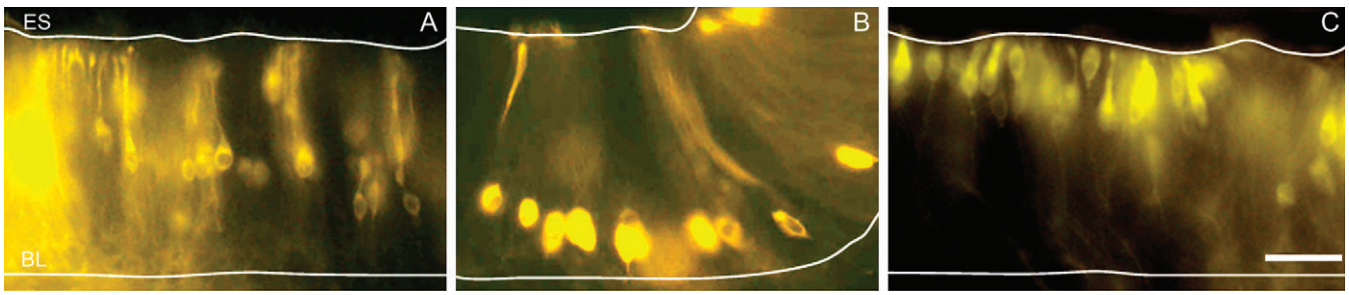

Figure 1 The appearance of crypt cells in the olfactory organ. Fluorescent microscopy micrographs showing variations in the number and position of crypt cells in the olfactory epithelium of crucian carp sampled in December **(A)**, March **(B)**, and August **(C)**. Note the absence of the crypt cells in (A), the location of the crypt cells deep in the epithelium in (B), and the large number of the crypt cells in contact with the epithelial surface in (C). In order to make the crypt cells visible, the photos (B) and (C) were taken with a focus on the crypt cells. The ciliated and microvillous neurons in the visual field were mostly out of focus. ES, epithelial surface; BL, basal lamina. Scale bar 50 μm.

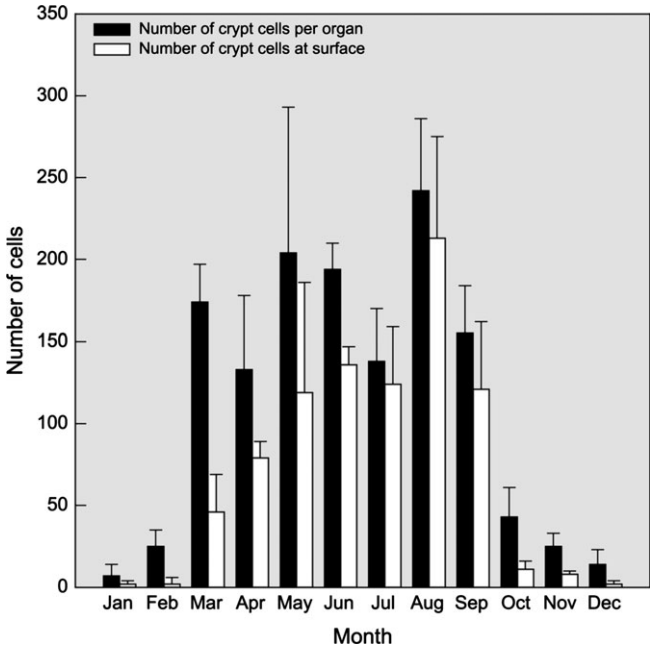

Figure 2 Histogram of the mean number of crypt cells throughout the year. The bars for each month are the average number of crypt cells of olfactory organs from 3 crucian carps (mean ± standard deviation). The open bars represent the number of crypt cells at the surface of the sensory epithelium.

of crypt cells were found in the surface layers. To make certain that the suspected sensory neurons observed in deep layers of the epithelium were crypt cells, preparations were inspected by confocal microscopy (Figure 3). The results showed that these cells are indeed pear shaped, lacking dendrites and their axons extend to the olfactory bulb.

Discussion

Olfactory sensitivity and crypt cells

The sensitivity to sex pheromones increases during the spawning season. In Atlantic salmon, *Salmo salar*, the olfactory sensitivity to prostaglandins increases during the reproductive season as measured by electro-olfactogram responses and the level of expressible milt (Moore and Waring 1996). In the tinfoil barb, *Puntius schwanenfeldi*, androgen treatment increases the olfactory responsiveness to 15-keto-prostaglandin-$F_{2\alpha}$, as measured both by electro-olfactograms and the frequency of courtship behavior (Cardwell et al. 1995). In salmon, the volume of the olfactory bulb, relative to that of the telencephalon, and the relative volume of the input layer of the bulb both undergo a marked, continuous increase (Jarrard 1997). These changes in the structure of the olfactory bulb may reflect increase in the number of sensory neurons that are mediating behavior related to homing and reproduction. The present study suggests that such changes in olfactory sensitivity to sex pheromones may depend upon the number of crypt cells exposed to the environment in the olfactory epithelium.

The interplay between sensory and endocrine systems

The crucian carp has an ovary of the asynchronous type (Aho and Holopainen 2000) and thus ovulates several times during the spawning period, which spans from late spring throughout summer. The hormonal changes in goldfish, a close relative to the crucian carp, have been studied extensively (Kobayashi et al. 1986; Pasmanik and Callard 1988). Females showed a peak in plasma content of testosterone in March and an estradiol peak in May. For the males, the estradiol peak was found in March, whereas testosterone and 11-ketotestosterone peaked in May.

There are few examples reported in vertebrates where a special sensory system shows seasonal variation in sensitivity during the life of the animal. Some species of temporary land-dwelling amphibians retain the lateral-line system during the terrestrial phases. The lateral-line organ in these species is partially dedifferentiated and overgrown by adjacent epidermal cells during the terrestrial phase, but this process with regression and regeneration is completely reversible (Noble 1931; Dawson 1936; Reno and Middleton 1973). The female midshipman fish *Porichthys notatus* use the auditory sense to detect and locate vocalizing males during the breeding season. Its auditory system shows a seasonal plasticity and an enhanced acquisition of auditory information needed for mate identification and localization during the breeding season (Sisneros and Tricas 2002). The visual spectral sensitivity in male 3-spined sticklebacks *Gasterosteus aculeatus* appears unchanged throughout the year. However, the spectral sensitivity in females increases at the red end of the spectrum during summer when reproductive activity is high. This difference between the sexes disappears during winter months when reproductive activity is at a minimum (Cronly-Dillon and Sharma 1968).

Regression and renewal in the olfactory system

In the original publications of Andres (1965), Graziadei et al. (1980), and Moulton (1974) on the mammalian olfactory organs, it was presumed that all types of olfactory sensory neurons undergo a continuous turnover. In the present study, we demonstrate that a specific type of sensory neuron disappears and is renewed in the spring. Because we used retrograde staining of the nerve terminals for identification of the sensory neurons, we could not tell if the crypt cells degenerated or if they were shed off the sensory epithelium. It remains speculative if such changes are signs of hormonal influence on the expression of odorant receptors related to reproduction, and we lack an understanding of the mechanisms that lead to these processes. If the crypt cells are the only type of cells undergoing such seasonal changes, it means that specific mechanisms are involved in the seasonal regression and neurogenesis. The results described in the present

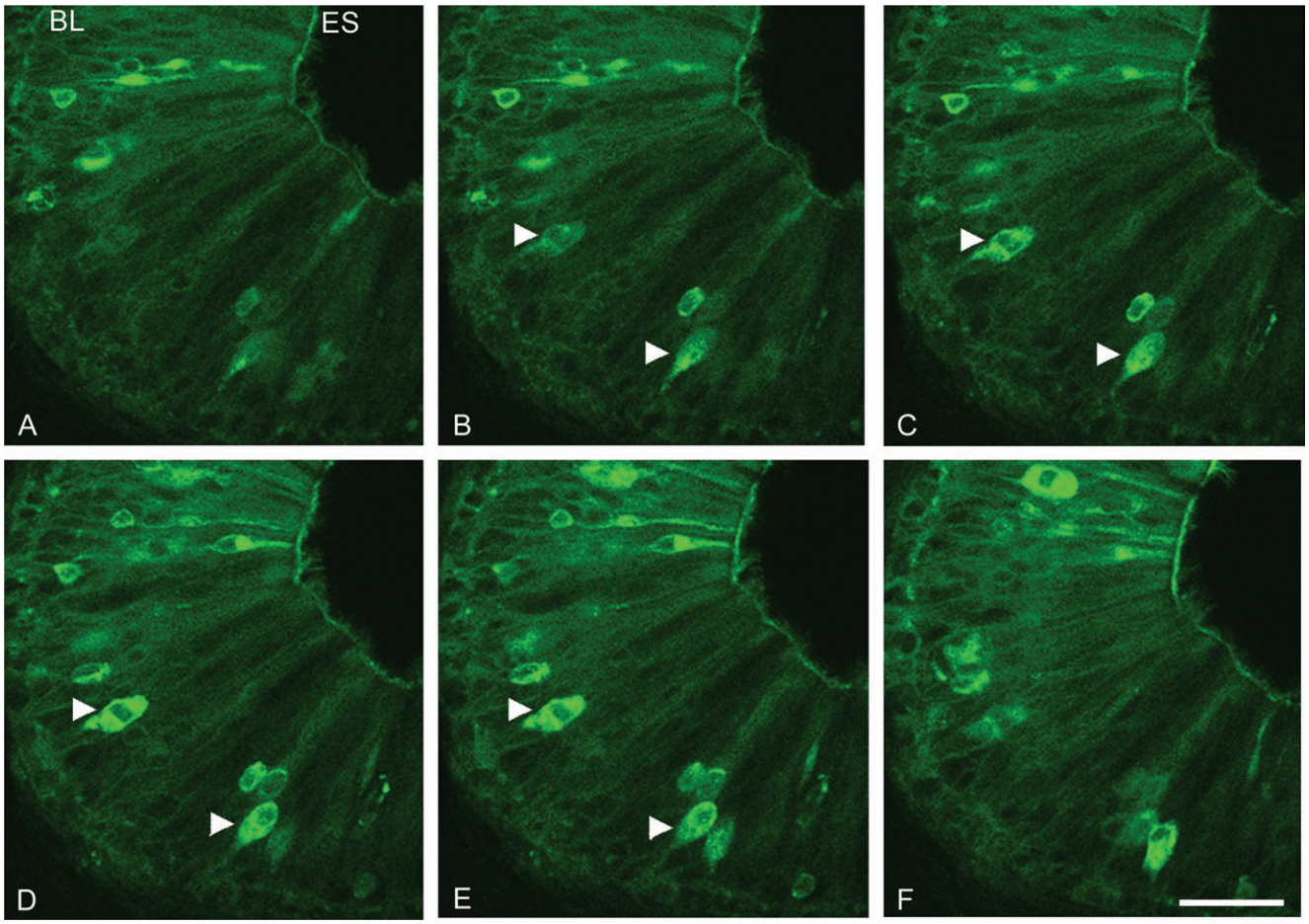

Figure 3 Confocal microscopy of crypt cells deep in the epithelium. *Z*-stack confocal micrographs taken by the confocal microscope showing 2 crypt cells in the middle of the olfactory epithelium (arrow heads) and demonstrating that these 2 cells do not have a long dendrite and is therefore not exposed to the epithelial surface. ES, epithelial surface; BL, basal lamina. Distance between the optical sections **(A—F)** is 3 μm. Scale bar 50 μm.

study indicate that fish might be a useful model organism for inquiries into the mechanisms underlying the regression and renewal of sensory neurons in the olfactory system.

What happens to the secondary neurons?

What happens to the target neurons in the olfactory bulb when the crypt cells and thus the presynaptic activity disappear? It has been known for more than a century that the mammalian olfactory bulb decreases in volume when the naris is occluded (von Gudden 1870). In zebra fish, the removal of the olfactory organ results in both nonneuronal and neuronal apoptosis in the olfactory bulb, which accounts for at least part of the deafferentation-induced olfactory bulb volume decrease (Byrd 2000; Vankirk and Byrd 2003). The loss of the primary sensory neurons demonstrated in the present study could thus lead to a similar dramatic change in the neurons connecting the olfactory bulb with the brain.

Funding

Research Council of Norway (159213/V40).

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Accepted September 19, 2007