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Immunohistochemical characterization of the crypt neurons in the olfactory epithelium of adult zebrafish

Valentina Parisi[a], Maria C. Guerrera[a], Francesco Abbate[a], Olivia Garcia-Suarez[b], Eliseo Viña[b,c], Jose A. Vega[b], Antonino Germanà[a,*]

[a] Dipartimento di Scienze Veterinarie (Sezione di Morfologia), Università di Messina, Italy
[b] Departamento de Morfología y Biología Celular, Grupo SINPOS, Universidad de Oviedo, Spain
[c] Servicio de Cuidados Intensivos, Hospital de Cabueñes, Gijón, Spain

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SUMMARY

The fish sensory epithelium contains three types of sensory cells denominated ciliated, microvillous, and crypt neurons. Each one differs from the other in its morphological, ultrastructural and molecular features, as well as in their projections to the central nervous system. Crypt neurons are present in both bony and cartilaginous fish and can be identified on the basis of their morphology and the expression of some specific proteins and genes. In this study we have investigated the morphology of crypt neurons, as well as the occurrence and co-localization of S100 protein, calretinin and TRPV4, three proposed markers for crypt cells, in the olfactory epithelium of adult zebrafish (*Danio rerio*) using double immunofluorescence associated to laser confocal microscopy. A sparse population of superficial S100 protein positive cells was detected being identified as crypt neurons. The calretinin immunoreactive cells were more abundant, occasionally resembling the morphology of the crypt cells but never displaying co-localization of both proteins. The TRPV4 positive cells differed in morphology from crypt cells, thus excluding the occurrence of TRPV4 in those cells. These results demonstrate that only S100 protein immunoreactivity can be used to identify crypt cells. Because some calretinin positive cells showed localization and morphology similar to the crypt cells of the sensory epithelium, the occurrence of two subtypes of crypt cells, S100 protein and calretinin positive, cannot be excluded. The significance of these findings remains to be elucidated.

1. Introduction

Fish have developed various sensory mechanisms to capture aqueous environmental signals. Detecting and processing this information is essential for survival, feeding and reproduction as well as intra- and inter-specific interactions. Olfaction is one of the sensory systems able to perform these fundamental tasks (Hamdani and Døving, 2007).

Despite the anatomical differences in the anatomy of the olfactory system of vertebrates, the neural basis of odor sensing is highly conserved (see Taniguchi et al., 2011; Cande et al., 2013). Teleosts own paired peripheral olfactory organs located in the dorsal part of the snout within an olfactory cavity connected with the external aquatic environment. Inside the olfactory cavity, the olfactory epithelium is organized in lamellae converging in a central raphe forming a particularly shaped structure called an olfactory rosette (Laberge and Hara, 2001). Each lamella contains both non-sensory and sensory regions, the sensory epithelium being found in the central portion, which includes the medial extent of each lamella (Byrd and Brunjes, 1995; Weth et al., 1996; Hansen and Zeiske, 1998). Three types of olfactory receptor neurons have been identified in the olfactory epithelium of teleosts: ciliated, microvillous and cryptic. Each one differs from the other in its morphological, ultrastructural and molecular features, as well as in their projections to the central nervous system (Hansen et al., 2004; Hansen and Zielinski, 2005; Hamdani and Døving, 2006; Yoshihara, 2009; Oka and Korsching, 2011; Oka et al., 2012; Gayoso et al., 2012).

Crypt neurons were the last ones identified, and are present in both bony and cartilaginous fish (Hansen and Finger, 2000; Ferrando et al., 2006) and have now been morphologically well characterized (Hansen and Zeiske, 1998; Hansen and Finger, 2000). They can also be distinguished on the basis of the expression of a TrkA-like protein (Catania et al., 2003; Ahuja et al., 2013), the Ca^{2+}-binding proteins calretinin and S100 protein (Germanà et al., 2004; Germanà et al., 2007), the V1R-like ora4 gene (Oka et al., 2012), or the ion channel TRPV4 (Amato et al., 2012).

* Corresponding author.
E-mail address: agermana@unime.it (A. Germanà).

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Thanks to anatomical and molecular similarities to that found in the mammalian olfactory system, the small teleost zebrafish represents a valuable model for the investigation of chemosensory perception and the mechanisms of behavioral responses to olfaction (Braubach et al., 2009; Oka and Korsching, 2011). Nevertheless, the function of crypt neurons has not yet been fully elucidated (Sato et al., 2005; Hamdani el et al., 2008; Vielma et al., 2008), and it is conceivable that they participate in both olfaction and sex pheromones (Hamdani and Døving, 2006). Immunohistochemistry has been used to characterize crypt neurons but the results have been discordant (Germanà et al., 2004; Germanà et al., 2007; Gayoso et al., 2011, 2012). In this study we have investigated the occurrence and co-localization of S100 protein, calretinin and TRPV4, three proposed markers for crypt cells, in the olfactory epithelium of adult zebrafish (*Danio rerio*) using double immunofluorescence and laser confocal microscopy. The study was aimed to achieve a better understanding of the possible role of these intriguing neurons in olfaction and/or other sensory functions.

2. Materials and methods

2.1. Zebrafish breeding and tissue treatment

For this study, 15 adult, 6 month old zebrafish (*Danio rerio*) were used. Animals were obtained from CISS (Centro Ittiopatologia Sperimentale Sicilia), University of Messina, Italy, where they had been bred at a constant temperature of 28.5 °C and fed twice daily. The fish were anaesthetized with MS222 (ethyl-m-amino benzoate; 0.4 g L^{-1}) and sacrificed by decapitation. The heads of 5 animals were isolated, cleaned in cold saline solution, and processed for electron microscopy. The heads of another 5 animals were fixed in Bouin's fixative for 24 h and then routinely processed for double-immunofluorescence staining. The pieces were cut in 10 μm thick serial sections, and collected on gelatin-coated microscope slides. The heads of the remaining 5 animals were used for Western Blot as a whole or after dissecting out the brains ($n = 2$).

2.2. Transmission electron microscopy

Head samples with the olfactory rosetta were fixed in 0.1 M cacodylate buffer (pH 7.5) containing 4.5% paraformaldehyde, 2.2% glutaraldehyde, and 5% sucrose for 2 h in ice bath, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer with 5% sucrose for 1 at 48 °C. Pieces were then dehydrated and embedded in araldite, cut into 1 μm thin sections using a Reichert-Jung ultracut E and stained with toluidine blue. From selected fields, ultrathin sections (40 nm) were obtained, stained with uranyl acetate–lead citrate and examined in a Jeol Jem 100 SX transmission electron microscope at an accelerating voltage of 80 kV.

2.3. Double-label staining

Double immunostaining was performed on 10 μm thick deparaffinized and rehydrated sections. When necessary, antigen retrieval was carried out using a high pH buffer (Dako, Copenhagen, Denmark) at 70 °C for 20 min, followed by incubation for 20 min at room temperature in the same solution. Thereafter, the sections were incubated for 30 min in a solution of 1% bovine serum albumin in TRIS buffer solution (TBS) to avoid non-specific binding followed by incubation with the primary antibodies. The incubation was carried out overnight at 4 °C in a humid chamber, with a 1:1 mixture of rabbit anti-S100 antibody (Dako; diluted 1:100) and mouse anti-calretinin antibody (clone 6B3, Swant, Marly, Switzerland; diluted 1:100); or with a 1:1 mixture of rabbit anti-zebrafish TRPV4 antibody (Abcam, Cambridge, UK, catalog number ab69094; diluted 1:100) mouse anti-calretinin antibody. After rinsing in TBS, the sections were incubated for 1 h with Alexa fluor 488-conjugated goat anti-rabbit IgG (Serotec, Oxford, UK), diluted 1:1000 in TBS containing 5% mouse serum (Serotec), then rinsed again and incubated for another hour with CyTM3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch, Baltimore, MD, USA) diluted 1:50 in TBS. Both steps were performed at room temperature in a dark humid chamber. Finally, to ascertain structural details, sections were counterstained and mounted with DAPI diluted in glycerol medium (10 ng/ml) then washed, dehydrated and mounted with Entellan®. Triple fluorescence was detected using a Leica DMR-XA automatic fluorescence microscope (Servicio de Analisis de Imagenes, Universidad de Oviedo) coupled with a Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany) and the images captured were processed using the software Image J version 1.43 g Master Biophotonics Facility, Mac Master University Ontario (www.macbiophotonics.ca). To provide negative controls, representative sections were incubated with non-immune rabbit sera instead of the primary antibodies, or omitting the primaries antibodies, following the same procedure described above. Under these conditions no positive immunostaining was observed (data not shown).

2.4. Western blot

The procedure was as follows: the samples were pooled and homogenized (1:2, w/v) with a Potter homogenizer in TBS (0.1 M, pH 7.5) containing 1 μM leupeptin, 10 μM pepstatin and 2 mM phenylmethylsulfonyl fluoride. The homogenates were afterwards centrifuged at 25,000 rpm for 15 m at 4 °C, and the resulting pellet dissolved in 10 mM Tris HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, and 10% glycerol at 4 °C. The pellets were thawed and analyzed by electrophoresis in 15% discontinuous polyacrylamide SDS gels. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and blocked by immersion for 3 h in PBS containing 5% dry milk, and 0.1% Tween-20. The membranes were then incubated at 4 °C for 2 h with the primary antibodies against S100 protein. The antibody anti-S100 protein was raised in rabbit directed against bovine S100 protein (Dako, Glostrup, Denmark; code No. Z0311; diluted 1:1000) and is used to detect both S100A and S100B proteins (manufacturer's notice). After incubation, the membranes were washed with Tris buffered saline (pH 7.6) containing 20% Tween-20, and incubated again for 1 h with the goat anti-rabbit IgG (diluted 1:100) at room temperature. Membranes were washed again and incubated with the PAP complex diluted 1:100 for 1 h at room temperature. Finally, the reaction was developed using a chemiluminescent reagent (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to Hyperfilm. Marker proteins were visualized by staining with Brilliant Blue.

3. Results

We first attempted to identify crypt cells in adult zebrafish using transmission electron microscopy (Fig. 1). Crypt cells were found scattered throughout the olfactory epithelium, without apparent predilection for the different segments of the lamellae, and their presence was uncommon. Crypt cells were easily recognized on the basis of their morphology although they largely varied in depth within the olfactory epithelium and in the morphology of their luminal-apical pole. They are oval shaped, possess an eccentric nucleus, and showed an apical invagination filled with microvilli from which a submerged short cilium protrudes. Interestingly, the more superficial the cell, the more developed the cilium become (Fig. 1); and only in the opened crypts were fully mature cilia found

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