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Distribution of calretinin during development of the olfactory system in the brown trout, *Salmo trutta fario*: Comparison with other immunohistochemical markers

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ABSTRACT

Immunocytochemical techniques were used to investigate the appearance and distribution of calretinin in the olfactory system of developing and adult brown trout (*Salmo trutta fario* L.). The earliest calretinin-immunoreactive (CR-ir) cells were detected in the olfactory placode of 5-mm embryos. In 8-mm embryos, a CR-ir olfactory nerve was observed. The number of CR-ir olfactory receptor cells increased rapidly, and in fry and adults they were characterized by light and electron microscopy as pertaining to three morphological types of receptor cell, called microvillous, ciliated and rod-like cells or crypt cells. Comparisons of the cells labeled with CR and with more general olfactory markers (acetylated tubulin and keyhole limpet haemocyanin) in alevins and fry revealed that CR-ir cells represent only a subpopulation of olfactory receptor cells. Large cells located in the primordial mitral cell layer were the first CR-ir neuronal population of the olfactory bulbs and were observed in 7-mm embryos. These cells express high HuC/D immunoreactivity and were negative for glutamic acid decarboxylase and tyrosine hydroxylase. CR immunoreactivity diminished with development and most large cells of the mitral cell layer were CR-negative in fry. In later embryos and in alevins, CR-ir granule-like cells were observed in the olfactory bulbs. Comparisons of the terminal fields of primary olfactory fibers labeled with CR and with a more general olfactory marker in the olfactory bulbs of fry and adults revealed significant differences, with most glomeruli of the dorsomedial field receiving CR-negative olfactory fibers. These results suggest new criteria for understanding the organization of the olfactory system of the trout, and hence of teleosts. Our results also suggest that CR is involved in specific functions in the olfactory system during development.

1. Introduction

Calretinin (CR) is a 29-kDa calcium-binding protein of the EF-hand family, which was first identified by gene cloning from chick retina (Rogers, 1987). The EF-hand family of calcium-binding proteins also includes parvalbumin and calbindin D-28k, which share 50–60% of the amino acid sequence with CR. Calcium-binding proteins contribute to calcium homeostasis by buffering the intracellular free calcium concentration (Miller, 1991; Baimbridge et al., 1992; Lledo et al., 1992). Calcium-binding proteins are expressed mainly by distinct groups of neurons in both the central and peripheral nervous system of vertebrates, having been employed as selective neuronal markers (Arai et al., 1991; Baimbridge et al., 1992; Résibois and Rogers, 1992).

In teleosts, as in other vertebrates, the olfactory system comprises an olfactory organ, attached to the olfactory bulbs through the olfactory nerve (for a comprehensive revision, see Eisthen and Polese, 2007). The olfactory organ of teleosts contains two major types of olfactory receptor neurons (ORNs): ciliated and microvillous (Zeiske et al., 1976; Yamamoto, 1982; Muller and Marc, 1984; Zielinski and Hara, 1988; Hansen and Finger, 2000; Hansen et al., 2004; Hansen and Zielinski, 2005). Besides the two major cell types, a further ORN type, the crypt cells, has been recognized in some Osteichthyes (Hansen and Zeiske, 1998; Hansen and Finger, 2000; Zeiske et al., 2003; Hansen et al., 2004). Different ORN types possess specialized odorant receptors and G-proteins, suggesting distinct signal transduction mechanisms (Hansen et al., 2004). The morphological development and functional maturation of the rainbow trout olfactory organ has been studied by Zielinski and Hara (1988).

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The teleost olfactory bulb is composed of four layers: olfactory nerve layer (ONL), glomerular layer (GL), mitral cell layer (MCL), and granule cell layer (GCL) (Catois, 1901; Kosaka and Hama, 1982–1983). In trout, as in other teleosts, primary olfactory fibers reach the various glomerular fields of the GL (Riddle and Oakley, 1992; Riddle et al., 1993; Becerra et al., 1994; Porteros et al., 1997), where they contact mitral cells, which in turn project to various telencephalic and extratelencephalic targets (see Folgueira et al., 2004). Besides, extrabulbar primary olfactory projections have also been reported in the trout (Riddle and Oakley, 1992; Becerra et al., 1994; Anadón et al., 1995; Folgueira et al., 2004), connecting mainly to the basal telencephalon, preoptic area, hypothalamus and posterior tubercle. Among calcium-binding proteins, CR has proved to be a valuable marker of some developing neuronal populations in the olfactory system of mammals (Bastianelli and Pochet, 1994, 1995; Wouterlood and Härtig, 1995; Malz et al., 2000, 2002; Alonso et al., 2001; Briñón et al., 2001; Kakuta et al., 2001). CR-ir neurons have been reported in the olfactory organ and/or the olfactory bulbs of a few developing (Porteros et al., 1997) or adult teleosts (Porteros et al., 1997; Díaz-Regueira and Anadón, 2000; Castro et al., 2006), as well as in the adult river lamprey (Pombal et al., 2002).

Here, immunocytochemical techniques were used with the aim of characterizing the expression of CR in the olfactory organ (olfactory rosette) and olfactory bulb of the brown trout from embryos to adults. We also aimed to characterize cytologically the CR-ir olfactory receptor neurons (ORNs) in order to see if CR is selectively expressed in ORN morphological subtypes, and to characterize the CR-ir terminal fields and cells in the olfactory bulbs. For these goals we also used single and double immunohistochemistry for a number of additional markers of olfactory system cell populations. Further knowledge of these populations may advance understanding of the organization of the olfactory system in teleosts.

2. Materials and methods

2.1. Subjects

Specimens of brown trout *Salmo trutta fario* (Salmonidae, Teleostei) were obtained from a local fish farm (El Veral, Lugo, Spain). They were staged by total body length. Vernier's embryo stages (VS) (Vernier, 1969), and the hatching time index (HTI: percentage of time to hatching; see Castro et al., 1999) were also used. The following animals ($n = 96$) were used: embryos of 4 mm (VS 18; HTI 27), 5 mm (VS 19; HTI 32), 7 mm (VS 21; HTI 42), 8–8.5 mm (VS 25; HTI 57), 10 mm (VS 26; HTI 63), 11 mm (VS 27; HTI 74), 12 mm (VS 28; HTI 82), 14 mm (VS 29; HTI 95) in length; alevins of 15 mm (hatchlings: VS 30; HTI 100), 18, 20, 22, and 24 mm in length; fry of 25, 28, 30, 32, 35, 40, 50 and 60 mm in standard length; and adults (125–360 mm in standard length). The developing fish were anesthetized with 0.1% 3-aminobenzoic acid ethyl ester methane sulfonate salt (MS-222; Sigma, St. Louis, MO) in fresh water and fixed by immersion. Adult trout were fixed after deep anesthesia with MS-222 by transcardial perfusion with teleost Ringer's solution followed by fixative; their brains were then dissected out and left in fixative for 24 h. All experiments conformed to the European Community's guidelines on animal care and experimentation.

Embryos, alevins, and fry were fixed either with Bouin's fluid without acetic acid (BF) or with 4% paraformaldehyde in 0.1 M pH 7.4 phosphate buffer (PB). Heads (embryos, alevins, and fry) or brains (adults) fixed in BF were then embedded in paraffin wax and sectioned on a rotary microtome at a thickness of 8–12 μm. Most specimens fixed in 4% paraformaldehyde in PB were cryoprotected in 30% sucrose in PB, and frozen with liquid nitrogen. Transverse and sagittal sections through the whole heads of embryos, alevins and fry, and through the brains of adults, were cut on a cryostat (10–25 μm thick), mounted on gelatinized slides and processed for immunocytochemistry. Two paraformaldehyde-fixed adult brains were embedded in 3% agarose in PB and cut on a vibratome (100 μm in thickness).

2.2. Immunocytochemistry

The paraffin sections were dewaxed and rehydrated before immunocytochemistry. Sections (either paraffin, cryostat or vibratome sections) were processed for CR immunocytochemistry as follows: (1) rinsed several times in 0.01 M phosphate-buffered saline (PBS), pH 7.4; (2) treated with 3% H_2O_2 in PBS for 30 min to block endogenous peroxidase activity; (3) rinsed twice in PBS (10 min each); (4) treated with normal goat serum (Dako, Glostrup, Denmark) diluted 1:10 in PBS with 0.1% Triton X-100 (PBS-T) for 1 h; (5) incubated with rabbit polyclonal CR antiserum (SWant, Bellinzona, Switzerland; Code 7699/4, Lot 18299; dilution 1:1000 in PBS) for 16–20 h at 4 °C in a moist chamber; (6) rinsed twice in PBS (10 min each); (7) incubated with goat anti-rabbit immunoglobulin (Dako; diluted 1:50 in PBS-T) for 1 h; (8) rinsed twice in PBS (10 min each); (9) incubated with peroxidase–antiperoxidase complex (PAP; Dako; diluted 1:200 in PBS-T) for 1 h; and (10) rinsed twice in PBS (10 min each). Finally, the immune complex was visualized by incubation with 0.06% 3,3′-diaminobenzidine (DAB, Sigma) and 0.005% H_2O_2 in PBS for 10 min. The sections were then rinsed in PBS, dehydrated and coverslipped. Free-floating vibratome sections were processed as above, although the times of incubation in each antiserum and the concentration of Triton X-100 were doubled; finally, they were mounted on slides and dried at room temperature for 20 h before coverslipping. Controls were run by incubating sections similarly but without the CR antiserum at step 5: no immunoreactivity was observed in these sections.

As a more general "olfactory marker" we used parallel series of transverse paraffin sections (8–12 μm thick) of fry heads (50 mm in length) and adult trout brain immunostained with rabbit polyclonal antisera to keyhole limpet hemocyanin (Sigma, code H0892, batch 096K4851; dilution 1:1000). This antibody reproduced the staining pattern of olfactory projections observed previously with some antibodies raised against peptides coupled to keyhole limpet hemocyanin (see below; Becerra et al., 1994). Alternate parallel series of the same individuals were stained for CR in order to compare labeled structures in adjacent sections. We also used our series of transverse paraffin sections (8–12 μm thick) of developing and adult trout brain immunostained with rabbit polyclonal antisera to substance P (SP; Chemicon; dilution 1:1000) and α-melanocyte stimulating hormone (α-MSH; Dako, dilution 1:1000) (for further details see Becerra et al., 1994). These antibodies were raised against the peptide coupled to keyhole limpet hemocyanin and intensely stained primary olfactory fibers of trout even after liquid phase preabsorption with the corresponding peptide, as also described for other similar antibodies in fish (see Riddle and Oakley, 1992).

2.3. Immunoelectron microscopy

In order to assess the types of olfactory neurons immunoreactive to CR, the olfactory rosettes of three adult trout fixed by vascular perfusion in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M PB were removed, postfixed in the same solution at 4 °C for 20 h and embedded in 3% agarose. Agarose blocks were cut at 50 μm on a vibratome, and the sections were processed for immunocytochemistry as above, although the incubation was done with mild shaker and the times of incubation with the different antisera were doubled. Sections stained for CR were postfixed in cold 1% osmium tetroxide in 0.1 M PB (pH 7.4) for 1 h. Sections were then dehydrated in acetone, embedded in Spurr's resin (Taab, Berkshire, UK) and cut on an ultramicrotome (60–70 nm). Ultrathin sections were counterstained with lead citrate and 1% uranyl acetate, before observation in a Philips CM12 electron microscope.

2.4. Additional markers used for identification of olfactory organ and bulb populations: a double immunofluorescence study

In order to further investigate the CR-immunoreactive (CR-ir) neurons observed in the olfactory system (olfactory mucosa and bulb), we used different additional immunocytochemical markers. For labeling the ORNs in the olfactory mucosa and their bulbar and extrabulbar projections we employed a polyclonal antiserum developed in rabbit against keyhole limpet hemocyanin (KLH, Sigma). In addition, we used a monoclonal antibody raised in mouse against the human neuronal protein HuC/D (Invitrogen, Molecular Probes, Eugene, Oregon, USA; Code A21271, Lot. 53877A) as a marker of neuronal cells of the olfactory system and which has previously been employed for distinguishing mitral cells in zebrafish (Li et al., 2005), and that also labeled specifically neurons in other teleosts (HuC/D: Lamas et al., 2007). Bulbar catecholaminergic cells were identified by means of polyclonal (Cat. no. AB152, Lot. no. LV1375881) and monoclonal (MAB 318, Lot. 21082054) antibodies raised against the enzyme tyrosine hydroxylase (TH; Chemicon, Temecula, CA). Both antibodies are well characterized and stained selectively the catecholaminergic system in trout and/or other teleosts (Manso et al., 1993; Castro et al., 2006). We also used two other antisera in this study: a monoclonal anti-acetylated tubulin antiserum (T, Clone 6-11B-1; Sigma; T-6793, Lot. 103K4819), employed here as a neuronal marker, and a monoclonal antiserum raised against the proliferating cell nuclear antigen (PCNA, Clone PC10; Sigma; P 8825, Lot. 036K4787), which has been used to demonstrate trout proliferating cells in other studies of our group (Candal et al., 2005a,b, 2008). To assess the location of GABAergic cells in the trout olfactory bulb, some series of developing trout immunostained with polyclonal anti-glutamic acid decarboxylase (GAD) antibodies were also at our disposal (see Yáñez et al., 1997; Candal et al., 2008).

For studying possible co-localization of these additional markers and CR, some sections of the olfactory mucosa and bulb were processed by a standard indirect immunofluorescence method. Some cryostat sections (10–12 μm in thickness) were incubated for 20 h at room temperature with a cocktail of the polyclonal rabbit