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First detection of neuropeptide Y (NPY)-like immunoreactivity in the lateral line: Presence and distribution in the neuromasts of the Antarctic notothenioid fish *Trematomus bernacchii*

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ABSTRACT

The mechanosensory lateral line (LL) is involved in many fish and amphibian behaviors, however little is known about the molecules involved in the signal transmission. Neuropeptide Y (NPY) has a number of functions in vertebrate physiology and also plays important roles in different sensory systems. The Antarctic nototheniods are a monophyletic radiation of fishes that have evolved under the extreme environmental conditions of low light and cold, where non-visual sensory structures, such as LL, are of importance. In this study we describe the presence of NPY-like immunoreactivity (IR) in LL of the Antarctic notothenioid fish, *Trematomus bernacchii* Boulenger. Differences in size and cellular composition between the two neuromasts were in compliance with previous descriptions of these sensory organs. Despite structural and functional differences between canal and superficial neuromasts, the distribution of NPY-like IR was similar within both the receptors classes. In particular, NPY IR was observed in all three cell types which constitute these sensory organs, allowing us to hypothesize the involvement of this molecule in the processing of the sensory information.

Fishes of the suborder Notothenioidei have radiated in the cold and low light waters of the high latitude shelf [15,24,25], forming a special type of adaptive radiation known as a species flock [26–28]. Although the Antarctic waters are characterized by low light levels for the majority of the year [38], the eye of the notothenioids does not evince any specialization [23,52,53]. Thus, non-visual senses, like the mechanosensory lateral line system (LL), are most likely to be important for these organisms [13], and so their investigation will highlight clear advantages within monophyletic radiation [25,45,51].

The lateral line is present in all fishes and in aquatic amphibians and consists of two receptor classes; those on the epidermis (superficial neuromasts) and those in sub-epidermal canals (canal neuromasts) that open to the exterior via small pores [48]. The lateral line is on the head region and trunk of fishes [63], which use this mechosensory system to detect water movements. It plays an important role in a number of diverse behaviors, such as prey detection [36], intraspecific communication [56] and rheotactic behaviour [47].

Although many studies have revealed different aspects of the morphology, physiology and development of LL sensory system [19,34,46] little is known about the molecules involved in signal transmission and few neuropeptides have been detected [4,5,9,32,62,67].

Neuropeptide Y (NPY) is a 36-amino-acid high conserved neuropeptide that is broadly expressed in the central and peripheral nervous system of vertebrates [1,10,21,40], where it generally regulates feeding behaviour, gastrointestinal activity and vascular function [60]. NPY has been localized in different sensory districts with different and peculiar roles; in the vestibular system it serves as a vasoconstrictor [57], in the ear it seem to be an effector molecule of the calcium and cAMP signaling system [41], in the taste buds it probably contributes to the transmission of multiple messages upon taste stimulation [68], whilst in the olfactory organ and in the retina it plays a double role as neurotransmitter and neuroproliferative factor [2,33,35]. Among the Antarctic nothotenioid fishes, NPY was detected in the brain and in nerve elements and endocrine cells of the gut, evincing particular distribution patterns [43,61].

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In this study, we describe the presence of NPY-like immunoreactivity in the trunk neuromasts of the Antarctic nototheniod fish, *Trematomus bernacchii* Boulenger, a species that has been the subject of several investigations and has one of the best physiologically known LL systems of the notothenioid suborder [13,17,18,37,44].

Adult specimens of *T. bernacchii* were collected between late October and November 2005 at Terra Nova Bay, Ross Sea, Antarctica. Sampling was performed using handlines and trammel-nets from the under-surface of the sea ice by drilling ice-holes. Fish ranged in size from 192 to 206 mm standard length. After catching, the fishes were rapidly stored alive in insulated boxes until return to the aquarium of the Italian base Mario Zucchelli Station. After catching, the fish were rapidly anesthetized with 0.01% MS Sandoz 222 (tricainemethanesulfonate; Argent, Redmond, USA; dilution 1:1000 in sea water) and quickly sacrificed by decapitation few seconds after recording the absence of movement in the fishes, in order to avoid potential damage on the neuromasts by a too long contact with the anesthetic. Then the specimens were dissected to collect the trunk portion of LL system for histological and immunohistological analyses. These samples were rapidly fixed in 4% p-formaldehyde solution in phosphate buffer saline, pH 7.4 (PBS). Samples were then washed in phosphate buffered saline, pH 7.4 (PBS) and stored by immersion in 70% ethanol. Alternately some individuals were sacrificed by a lethal overdose of MS Sandoz 222 and then immediately whole frozen at −80° C for Western blot analyses. Ethanol stored samples were dehydrated, Paraplast embedded (Bioptica, Italy) and 5 μm sectioned. Dewaxed and rehydrated sections were Haematoxylin-Eosin (Bioptica, Italy) stained for histological observations. Immunohistochemical reactions were carried out using a primary polyclonal anti-NPY antiserum raised in rabbit against human NPY (1:200 in PBS, Santa Cruz, USA, Cat no. sc-28943). Dewaxed and rehydrated sections were pre-treated with 4% H_2O_2 in PBS to eliminate endogenous peroxidase activity when needed, treated with 0.3% Triton-X and 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature and incubated overnight in a moist chamber at 4 °C with NPY polyclonal antiserum. After rinsing in PBS, sections were incubated for 1 h with a goat anti-rabbit antiserum conjugated with Alexa Fluor 488 (Molecular Probes, USA, 1:800 in PBS). Alternatively the sections were incubated for 1 h in a moist chamber at 4 °C with NPY polyclonal antiserum, followed by the anti-rabbit secondary detection system using diaminobenzidine (DAB) with the EnVision system (Dako, Denmark) and then counterstained with Haematoxylin (Bioptica, Italy). Controls included omission of the primary antiserum and the use of sections of mouse brain as known positive tissue. All the sections were examined with an Olympus BX 60 Microscope (light and epi-fluorescence microscope). Images were visualized through a Color-View Camera (Olympus, Japan) and acquired using AnalySIS (Soft Imaging System, USA) software. All operations to obtain lateral line homogenate were carried out at 4 °C. LL 10 g was homogenated by a Politron system in 30 ml of homogenizing medium containing 0.40 M sucrose, 10 mM Tris–HCl pH 7.4 and a protease inhibitors cocktail. The homogenate was stored at −80 °C and the protein concentration was determined by Bradford methods [11]. Denaturing electrophoresis was performed using a Laemmli protocol [39,40] with minor modifications. In these experiments 0.025 mg of lateral line homogenate was loaded in the gel. Electrophoresis was carried out using a Mini Protean III (BioRad, Hercules, USA) apparatus (60 × 80 × 1.5 mm), in which both faces of the gel sandwich were immersed in the buffer. Separating gel was a gradient from 8% to 19% (w/v) of polyacrylamide and 0.1% SDS, pH 8.8. Stacking gel contained 5% w/v polyacrylamide and 0.1% SDS. Samples were boiled for 5 min with one-fourth the total volume of 4× the sample buffer (40%, w/v sucrose, 8% SDS w/v in 125 mM Tris–HCl (pH 6.8), 1.25% v/v b-mercaptoethanol). Bromophenol blue (0.008%, w/v) was the tracking dye, which diffused out of the gels before the run was stopped. A run was performed at 4 °C, at 20 mA for each gel, for 120–150 min with running buffer (0.05 M Tris (pH 8.0); 0.4 M glycine; 1.8 mM EDTA, and 0.1% SDS). Protein Molecular Weight (MW) markers were purchased from Bio Rad (BioRad, Hercules, USA). After the run, proteins were transferred onto nitrocellulose (NC) membranes (Hybond ECL; GE Healthcare, Sweden) in a mini transblot device (Bio-Rad Laboratories, Hercules, USA) at 400 mA for 1 h. Sheets were blocked over night at 4 °C, in Tris Buffered Saline (TBS; 25 mM Tris–HCl pH 7.4, 150 mM NaCl) plus 5% BSA. After washing with TBS, NC was incubated and stirred with the antibody against NPY (Santa Cruz, USA, Cat no. sc-28943) diluted 1:100 in TBS plus 3% BSA, for 1 h at 20 °C. After extensive washing with TBS plus 0.15% Tween, binding of Ab was revealed by the ECL detection system (Roche, Germany) using HRP-conjugated Protein A (Amersham Pharmacia Biothech, Sweden) diluted 1:2000 in TBS plus 1% BSA. Blots were then autoradiographed onto Hyperfilm ECL (Amersham Pharmacia Biothech, Sweden) and films acquired.

Scales and canals are the seats of superficial and canal neuromasts, respectively (Fig 1A, B).

Superficial neuromasts were located in the epidermis covering the scales and their apical portion is almost circular with a diameter of approximately 65 μm. (Fig. 1C). Superficial neuromasts consist of a central portion with few bottle shaped sensory hair cells, surrounded by many support cells and a peripheral zone made of numerous layers of mantle cells (Fig. 1C). The sensory hair cells of the superficial neuromasts are approximately 77 μm long and 6 μm wide at the level of the nuclei and they extend from their cell body, localized in the middle zone of the neuromasts, to the apex of the sensory receptor (Fig. 1C). The body cell of numerous support cells are observed in the central area of the neuromast bottom: they are thin, long cells with prominent nuclei departing from the basement membrane to the neuromast apex (Fig. 1C). Mantle cells are located in the peripheral zone of the neuromast. They are large cells with big nuclei and depart from the basement membrane to the apex of the sensory organ, as the support cells do (Fig. 1C). Canal neuromasts are located at regular intervals in the ventral side of the subdermal canal system and their apical surfaces are ellipsoidal, with the longitudinal axis parallel to that of the canals and with a maximum diameter of approximately 230 μm (Fig. 1D). Canal neuromasts are composed of numerous flask-like sensory hair cells, in association with numerous support cells and relatively few layers of mantle cells (Fig. 1D). The sensory cells depart from the upper third of the neuromasts and are shorter than those in the superficial neuromasts, measuring 40 μm long and approximately 6 μm wide at the level of the nuclei (Fig. 1D). As in superficial neuromasts, support cells and mantle cells depart from the central bottom side and the peripheral areas of the neuromasts, respectively and are characterized by the presence of large nuclei (Fig. 1D). Nerve fibers reach the basal mid portion of both the neuromasts through connective tissue (Fig. 1E).

NPY immunoreactivity (IR) was observed in the superficial and canal neuromasts, including all three cell types comprising them. In particular, NPY IR was evident at the periphery and central area of the two neuromasts, whilst their apical portion was not positive (only immunopositivity in both the immunoreactions were considered specific) (Fig. 2A,B,C,D). Although the immunopositivity presented a similar distribution in both receptor classes, different NPY IR patterns could be described within the different cell types. Some peripheral mantle cells had a strong NPY IR in the perinuclear region, while others were faintly or not positive (Fig. 2E). In positive mantle cells the IR was largely localized around the nucleus and in part of the cytoplasm of the cell body (Fig. 2E). On the contrary most of the sensory hair cells and support cells showed a faint NPY IR, although the first were localized in a brighter narrow line upon the nucleus only, and the second were regularly observed from the base to the two third of their body cell (Fig. 2F,G). To obtain conclusive evidence of the presence of NPY in lateral line of *T. bernachii*, we