Neuroepithelial cells (NECs) and mucous cells express a variety of neurotransmitters and neurotransmitter receptors in the gill and respiratory air-sac of the catfish Heteropneustes fossilis (Siluriformes, Heteropneustidae): a possible role in local immune defence

Lauriano, E. R., Capillo, G., Icardo, J. M., Fernandes, J. M. O., Kiron, V., Kuciel, M., Zuwala, K., Guerrera, M. C., Aragona, M., Germana, A. & Zaccone, G.

Published in: Zoology
DOI: 10.1016/j.zool.2021.125958

Available online: 24 Jul 2021

Citation:
Lauriano, E. R., Capillo, G., Icardo, J. M., Fernandes, J. M. O., Kiron, V., Kuciel, M., Zuwala, K., Guerrera, M. C., Aragona, M., Germana, A. & Zaccone, G. (2021). Neuroepithelial cells (NECs) and mucous cells express a variety of neurotransmitters and neurotransmitter receptors in the gill and respiratory air-sac of the catfish Heteropneustes fossilis (Siluriformes, Heteropneustidae): a possible role in local immune defence. Zoology, 148: 125958. doi: 10.1016/j.zool.2021.125958

Neuroepithelial cells (NECs) and mucous cells express a variety of neurotransmitters and neurotransmitter receptors in the gill and respiratory air-sac of the catfish *Heteropneustes fossilis* (Siluriformes, Heteropneustidae): a possible role in local immune defence

Eugenia Rita Lauriano (1), Capillo Gioele (2,3) *, Jose Manuel Icardo (4), Jorge Oliveira Fernandes (5), Kiron Viswanath (5), Michal Kuciel (6), Krystyna Zuwala (7), Maria Cristina Guerrera (2), Marialuisa Aragona (2), Antonino Germana' (2) and Giacomo Zaccone (2)

(1) Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale F. Stagno d'Alcontres 31, I-98166 Messina, Italy

(2) Department of Veterinary Sciences, University of Messina, Polo Universitario dell'Annunziata, I-98168 Messina, Italy

(3) Institute of Marine Biological Resources and Biotechnology - National Research Council (IRBIM, CNR), Spianata S. Raineri, 86 98122 Messina, Italy

(4) Department of Anatomy and Cell Biology, Poligono de Cazona, Faculty of Medicine, University of Cantabria, Santander, Spain

(5) Faculty of Biosciences and Aquaculture, University of Nordland,8049 Bodo, Norway

(6) Poison Information Centre, Department of Toxicology and Environmental Disease, Faculty of Medicine, Jagellonian University, Kopernika 15, 30-501, Cracow, Poland

(7) Department of Comparative Anatomy, Faculty of Biology, Institute of Zoology and Biomedical Research, Jagiellonian University, Cracow, Poland

*Corresponding Author gcapillo@unime.it, Department of Veterinary Sciences, University of Messina, Polo Universitario dell'Annunziata, I-98168 Messina, Italy

Abstract

Heteropneustes fossilis is an air-breathing teleost inhabiting environments with very poor O_2 conditions, and so it has evolved to cope with hypoxia. In the gills and respiratory air-sac, the sites for O_2 sensing and the response to hypoxia rely on the expression of acetylcholine (Ach) acting via its nicotinic receptor (nAChR). This study examined the expression patterns of neuronal markers and some compounds in the NECs of the gills and respiratory air sac having an immunomodulatory function in mammalian lungs.

Mucous cells, epithelial cells and neuroepithelial cells (NECs) were immunopositive to a variety of both neuronal markers (VAChT, nAChR, GABA-B-R1 receptor, GAD679) and the antimicrobial peptide piscidin, an evolutionary conserved humoral component of the mucosal immune system in fish.

We speculate that Ach release via nAChR from mucous cells may be modulated by GABA production in the NECs and it is required for the induction of mucus production in both normoxic and hypoxic conditions. The presence of piscidin in mucous cells may act in synergy with the autocrine/paracrine signals of Ach and GABA binding to GABA B R1B receptor that may play a local immunomodulatory function in the mucous epithelia of the gills and the respiratory air sac.

The potential role of the NECs in the immunobiological behaviour of the gill/air-sac is at moment a matter of speculation.The extent to which the NECs as such may participate is elusive at this stage and waits investigation.

Key Words: Cholinergic Mucous cells, NECs, Ach, nAChR, GAD67/GABA, Gill, Air sac, *Heteropneustes fossilis*

1. Introduction

The Asian catfish *Heteropneustes fossilis* lives in different trophic zones of the swamps and stagnant ponds. In general, these swamps have very poor O_2 conditions, and the lower layers are often deoxygenated. The ABO of the catfish *H.fossilis* is similar in many ways to that of the clariids with the major differences being the absence of respiratory trees and the presence of paired tubular, sac-like chambers extending from the suprabranchial chamber back into the body. These tubes are embedded in the myotomes and extend to as much as one-half body length (see for review Graham, (1997)). More detailed accounts regarding combined aspects of the morphology and physiology of air-breathing organs (ABOs) were reviewed in Munshi (Munshi, 1993). The configuration of the respiratory epithelium covering the respiratory tubes confirms its derivation from the gill tissue. The main feature of the respiratory epithelium is the presence of islet lamellae, discontinuous blood spaces and mucous cells distributed in the lining surface that function to prevent desiccation(Graham, 1997). In the respiratory sac of *H. fossilis*, which is flooded with water no surfactant has been found on the islets.

Acetylcholine (ACh) is regarded as a classical neurotransmitter. The ACh receptors, namely nicotinic ACh receptors (nAChRs) and muscarinic receptors (mAChRs) belong to the ligand-gated ion channel and the G-protein-coupled receptor superfamilies respectively that mediate chemical neurotransmission at neurons, ganglia and effector organs such as heart, smooth muscle and glands (see for review Wessler and Kirkpatrick, (2008)). In the last decades several reviews focused on the synthesis of ACh in non-neuronal cells (Kurzen et al., 2007; Wessler and Kirkpatrick, 2008). The key role of ACh is to play a role in the interactions of non-neuronal cells with the external environments, hormones, growth factors, cytokines and also the nervous system.

Recently studies by Zachar et al.(2016) on zebrafish gills demonstrated VAChT and ChAT expression in unclassified apical epithelial cells using antibodies to SV2 and VAChT antibodies the latter reported in this study. The zebrafish epithelial cells were closely located next to 5-HT positive NECs but these cells did not express SV2 (Zachar et al., 2016). Very recently Zaccone et al (2020) reported

the occurrence of immunoreactivity for ACh and its muscarinic receptor (mAChR) in the neuroepithelial cells(NECs) of the gills and the glottis of *Arapaima gigas*.

The synthesis of ACh beyond the nervous system has changed the paradigm of ACh acting as a merely neurotransmitter, but its presence and function in non-neuronal cells in fish is not sufficiently investigated and clarified except for its specific role in the mucous cells in the mammalian airway epithelium. Also, unlike the neuronal ACh that is released by exocytosis, most non-neuronal cells expressing ACh are not innervated by cholinergic neurons and have ACh receptors that are a part of the local regulatory loops of non-neuronal ACh released by these cells. The evidence of a non-neuronal cholinergic signalling in fish is recently reported in the gill of Pantodon bucholzi(Capillo et al., 2021).

Very recently Kobayashi et al (2018) pointed to the neuroimmunomodulator functions of pulmonary neuroendocrine cells (PNEC) due to the production of the GABA by these cells that induce mucous metaplasia through the interaction of immune cells. Fu and Spindle (2009) reported the presence of GABA receptor subunits, GAD (Glutamate decarboxylase) and alpha 7 nicotinic receptor (nAChR) subunits in the NEB cells of monkey lung. Barrios et al (2019) have also demonstrated more recently the occurrence of GABA in the PNEC using antibodies to the biosynthetic enzyme GAD67. They found that a nicotine GABA production and GABA alpha receptor expression in the mammalian airway epithelium leads to mucus overproduction. Previous investigation by Lauweryns et al (1973a; 1973b) and Van Lommel and Lauweryns (1995) have pointed to the role of several neurotransmitter substances in the neuroepithelial bodies(NEBs) that exert a chemoattractant role on immune cells

(mast cells and eosinophil granulocytes) and the regulation of the mucous secretion, demonstrating a contribution to local immune defense. Recent investigations coming from our laboratory indicated the presence of the antimicrobial peptide Piscidin 1 in the mucous secretions of the fish gills (Capillo et al., 2021).

Our aims are to investigate the distribution of acetylcholine and its alpha 7 subunit of the nicotinic receptor (nAChRs), gamma amino butyric acid (GABA) by the use of the antibody against its biosynthetic enzyme GAD67, as potential targets to control mucus formation and their involvement in immune defence and the antimicrobial peptide Piscidin 1 in the gills and the respiratory air sac of the catfish *H. fossilis*. Evidence of mucus overproduction in the gill epithelium of fishes is considered a response to environmental stressors or hypoxia (Salinas, 2015).

This paper is a report of some new findings in support of the theory that NECs of the fish may function as immunomodulator-like substances that have been previously reported in the neuroepithelial bodies(NEBs) of the mammalian lungs where their contribution to local immune responses and defenses and their participation in the most diverse aspects of inflammation and allergy were documented(Van Lommel et al., 2009).It is clear that here is a new, vast and largely uncharted area of future research.

2. Materials and Methods

2.1 Ethical Statement

Handling and care of animals were conducted in accordance with the ethical principles indicated by the European Union Directive (63/2010/EU) on the use of animals for scientific purposes.

2.2. Animals and Tissue preparation

Adult catfish (n= 10, 5 female and 5 male) of *H. fossilis*, body weight 30-40 g, were collected in the local ponds around the Allahabad University (India) and kept at the laboratory of Zoology, University of Allahabad in aerated fresh water aquaria at 26-28 °C on a 12/12 h photoperiod. Fish were fed with commercial aquarium fish food twice a day. Dissolved oxygen (DO) ranged from 8.3 (mg/ml) to 5.4 (ml/l) in collection locations. Catfish were euthanized by an overdose via immersion MS 222, and the entire gill arches were dissected out. The paired air-sacs were also removed with myotomes. The samples from the different regions of the gills and respiratory air-sacs were sampled for routinely histology and immunohistochemistry. The tissues were immersed in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) pH=7.4, for 6-8 h, dehydrated in graded ethanol, cleared in xylene and embedded in Paraplast (Mc Cormick-Scientific, St Louis, MO, USA) and cut into 5-10 um sections using a rotary microtome (Leica, RM2135, Nussloch, Germany) and collected on gelatine-coated microscope slides.

2.3 Immunohistochemistry and Confocal Immunofluorescence Microscopy and Transmission Electron Microscopy

Techniques for immunolabeling were similar to those previously described for a wide variety of fish tissues (Lauriano et al., 2016; Zaccone et al., 2020; Zaccone et al., 2015a; Zaccone et al., 2018; Zaccone et al., 2015b; Zaccone et al., 2019). Double immunostainings were carried out at room temperature. Samples were rinsed in freshly prepared phosphate buffered saline (PBS, pH = 7.4) between each incubation step unless indicated otherwise. The primary and secondary antibodies used in this study, as well as the dilutions, are listed in Table 1. The antigens in the double-labelling experiments with primary antisera raised in different species, were simultaneously detected by means the indirect immunofluorescence. 5-10 μm deparaffinized sections were rinsed three times with double distilled water and transferred for 1 h to blocking solution with PBS containing 0.5% (v/v) Triton-100 (Sigma-Aldrich), 0.2% (w/v) bovine serum albumin (BSA, Jackson Immunoresearch, West Grove, PA, USA), 1% (v/v) dimetyl-sulphoxide, 0.02% (w/v) sodium azide and 5% (v/v)

normal horse serum (NHS, Jackson Immunoresearch). Next, the permeabilized tissue sections were incubated with the primary antibodies for 24 h at 4°C, and with secondary antibodies at room temperature for 1 h in darkness. Preparations were then placed on glass microscope slides in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA), to reduce photobleaching during confocal scanning. Sections were analyzed and images acquired using a Zeiss LSMDUO confocal laser scanning microscope with META module (Carl Zeiss, Micro Imaging, GmbH, Germany). Small tissue samples fixed in 3% glutaraldehyde were postfixed in 1% osmium tetroxide, dehydrated in graded acetone and embedded in Araldite (Fluka, Buchs, Switzerland), following routine procedures. Ultrathin sections cut with a Leica ultracut UCT were stained with uranyl acetate and lead citrate and examined with a Jeol-JEM-1011 working at 80 KV. The microscope was equipped with a Gatan ORISUS SC 1000 CCD camera.

2.4 Figure analysis and preparation

Zen 2011(LSM 700 Zeiss software) built in “colocalization view” was used to highlight the expression of both antibody signals in order to produce a “colabelling” signal. Digital images were cropped, and the figure montage prepared using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

2.5 Primary Antibodies and Antibody Specificity

All primary antibodies were chosen based on previous morphological studies performed in zebrafish and various teleost fish species. The characterization, specificity, and reliability of the antibodies and their application in morphological studies of the skin, gills and the air-breathing organs (ABOs) in fish have already been described by previous authors (Jonz et al., 2016; Zaccone et al., 2017a; Zaccone et al., 2020; Zaccone et al., 2015a; Zaccone et al., 2018; Zaccone et al., 2017b; Zaccone et al., 2015b; Zaccone et al., 2019; Zachar et al., 2016; Zaccone et al., 2020; Capillo et al., 2021).).

Table 1. Primary antibodies, peptides and secondary antibodies used in this study

Primary antibody	**Supplier**	**Catalogue number**	**Dilution**
Monoclonal Mouse anti-human Serotonin, clone 5HT-H209	Dako	M0758	1:50
Anti-VAChT	Sigma Aldrich	SAB4200559	1:500
Anti-nicotinic Acetylcholine Receptor Alpha 7 Antibody, CHRNA7, Polyclonal	Alomone Labs	ANC= 007	1:100
CHRNA7(negative control antigen)	Alomone Labs		
GAD67, Polyclonal Antibody	Invitrogen	PA5-21397	1:100

GAD67 (synthetic peptide)	Invitrogen		
GABA B R1(D-2)	Santa Cruz Biotechnology	sc= 166408	1:50
GABA B R1(D-2) (blocking peptide)	Santa Cruz Biotechnology	sc= 166408P	
Anti-Piscidin 1(Pis1) antibody Polyclonal	GeneScript	Produced on demand	1:100
Secondary antibodies	**Supplier**	**Catalog number**	**Dilution**
Alexa fluor 488 Donkey anti-mouse	Invitrogen	A 21202	1:300
Alexa fluor Donkey anti-rabbit	Invitrogen	A 21207	1:300

Table 2. Primary antibodies and protein isoforms reported in fish morphological and molecular studies

Primary antibodies and protein isoforms	References
5HT	(Jonz et al., 2016; Jonz Michael G, 2003; Rahbar et al., 2016; Saltys et al., 2006; Shakarchi et al., 2013; Zaccone et al., 2020; Zaccone et al., 2018; Zaccone et al., 2017b; Zaccone et al., 2019)
VAChT	(Porteus et al., 2013; Regan et al., 2011; Shakarchi et al., 2013; Stoyek et al., 2015; Zaccone et al., 2017a; Zaccone et al.,

	2020; Zaccone et al., 2015a; Zaccone et al., 2018; Zaccone et al., 2019)
mAChR	(Zaccone et al., 2020)
nAChRs	(Ackerman and Boyd, 2016)
GAD67	(Bosma et al., 1999)
Piscidin 1	(Mulero et al., 2008; Ruangsri et al., 2012b; Silphaduang et al., 2006b)

3. Results and Discussion

3.1 The gills and respiratory air sac. Neurochemical markers and ultrastructural features

The gills of *H. fossilis* exhibited filaments and secondary lamellae. Previous immunohistochemical studies have localized the NECs along the efferent arteries that course through the leading edge of the filament [14]. Most NECs were located in the distal half of the filament, appeared either as single cells or in small groups and were positive to both 5-hydroxytryptamine (5-HT) and neuronal nitric oxide synthase (nNOS) (Zaccone et al., 2018). Under TEM, we identified NECs by the presence of a clear cytoplasm, a few mitochondria and a variable number of small, rounded granules filled with a dense, uniform content (Fig. 1a). This cell phenotype is compatible with NEC morphology (Lauriano et al., 2015) although NECs in the gill epithelium may show different phenotypes (Porteus et al., 2012; Zaccone et al., 2006). in addition to NECs, rounded and dense cytoplasmic granules were only observed in the vascular endothelium (not shown). Mucous cells decorated the leading and trailing edges of the filaments.

The respiratory air sac contained respiratory epithelium derived from gill tissue (Munshi, 1993). The respiratory epithelium was formed by respiratory islets (lamellar areas) that protruded into the sac lumen and were separated by lanes covered by cells with surface microridges. These results confirmed previous observations (Olson et al., 1990) and are not included here. The air sac wall also

contained NECs (Fig. 1b) that showed the same ultrastructural characteristics than those observed in the gills. NECs were also positive to 5HT (Zaccone et al., 2018). Similarly, vascular endothelial cells exhibited small, dense granules. We also detected the presence of eosinophils. These cells showed a bi-lobed nucleus and numerous cytoplasmic granules containing low-density tubules embedded in a dense matrix (Fig. 1c). Eosinophils were observed both within vessels and as free cells in the extracellular space and could be seen in close association with NECs (Fig. 1c). Mucous cells are present throughout the respiratory epithelium being very numerous in the interlamellar areas where they appear interspersed with the microridged cells (Fig. 1d). A thin layer of smooth muscle, and a cucullaris muscle innervated by cholinergic nerve fibers (Zaccone et al., 2018), ensheathed the respiratory sac.

3.2 Structural morphology of the gill / respiratory air sac and neurochemical markers

As previously reported by Zaccone et al (2018), the NECs in the gill epithelium of the catfish *H. fossilis* are located on the sides of efferent filament arteries that are seen on the leading edge of the filament. A largest number of these cells is generally found in the distal half of the filament both isolated and sometimes in cell clusters. Mucous cells were mainly located on the leading and trailing edges of the filaments. The respiratory air sac is modified into distinct zones, a basal non-respiratory and a peripheral respiratory zone. Mucous cells were seen in the respiratory interlamellar areas interspersed among the polygonal epithelial cells (Munshi et al., 1986) and showed VAChT immunostaining(Zaccone et al., 2018). A cucullaris muscle innervated by cholinergic nerve fibers encircled the respiratory air sac as well as NECs were also ascertained in a few numbers in the lamellae using antibodies to 5HT (Zaccone et al., 2018). The different distribution patterns of the NECs in the air sac with regard to that of the gill should be associated with a reduction of O_2 availability due to the alternative delivery route of oxygenated blood from the efferent air sac artery to the dorsal aorta (Munshi, 2003).

3.2.1 Gill

3.2.1.1 Double immunolabeling for 5HT and VAChT

Experiments using the combination of the antibodies to 5-HT and VAChT have verified that at least two NECs populations were determined: a serotonergic and a cholinergic serotonergic population in that two distinct type of cells exhibit 5-HT immunoreactivity alone and the coexistence of 5-HT and VAChT respectively (Fig. 2 A, B, C). These populations are located in the filament epithelium of the leading edge. In the secondary epithelium of the respiratory lamellae the same NEC cell types occur. The majority of NECs are exposed directly to the external environments and some of the immunolabelled cells show their basal part lying near fenestrated capillaries (see also below NECs in the leading edge of the filament immunopositive to 5HT and nAChR antibodies). Mucous cells in the leading edge of the filament were labelled by the VAChT antibodies (Fig. 2 D, E), but are not colabelled with 5-HT. The VAChT signal found in the perimeter of mucous cells suggests that vesicular Ach is released on cell surface thus suggesting the capacity of mucous cells for both Ach and mucus secretion. This is also observed in the apical domed surface of the epithelial cells on the gill lamellae of *P. buchholzi* (Capillo et al.,2021)).

3.2.1.2 Double immunolabeling for 5HT and nAChR alpha 7 subunit

Simultaneous detection of 5HT alpha 7 subunit of nAChR revealed the coexpression of the two neurochemical markers in the NECs found in the distal regions of the gill filaments and the secondary epithelium of respiratory lamellae (Fig. 3 A, B, C, D). However, a relatively small proportion of NECs was labelled for antibodies to 5HT and were negative to the antibodies to nAChR alpha 7 subunit (Fig. 3 A, B). This would suggest that two NEC populations were determined: a serotonergic and a cholinergic nicotinic (Fig. 3 B) receptor population. It is also in line with other studies that have indicated the presence, in addition of 5HT, of other neurochemical candidates such as nitric oxide and tyrosine hydroxylase playing a role in afferent neurotransmission between O_2 chemoreception

and sensory nerve fibers (Burleson et al., 2006; Zaccone et al., 2018). Immunohistochemical evidence of the 5HT positive and 5HT/alpha 7 nAChR alpha 7 subunit positive NECs suggests that the neurochemical basis of O_2 chemoreception in the gill of the species studied involves different populations of NECs with multiple neurotransmitter substances having a diversity of excitatory, inhibitory and modulatory mechanisms (Zaccone et al., 2020; Zaccone et al., 2018). Mucous cells found in the leading edge of the filaments are labelled by antibodies to nAChR alpha 7 subunit (Fig. 3 C, D). Surface binding of nAChR is demonstrated on the mucous cells (Fig. 3 F). This is in agreement with the localization of the nAChR subunits consisting of transmembrane and intracellular domains in the cholinergic cell systems (und Halbach and Dermietzel, 2006). A circular labeling of nerve fibers for nAChR alpha 7 subunit and 5HT is also found on the apical dome surface of the mucous cells (Fig. 3 C, D, E) thus indicating innervation and identification of targeted expression of nAChR to plasma membrane for the release of Ach from cholinergic mucous cells by paracrine/ autocrine signalling in addition to crosstalk between the cholinergic and serotoninergic systems.

3.2.1.3 Double immunolabeling for 5HT - GAD67 and GABA B R1-5HT

Pulmonary neuroepithelial cells (PNECs) have been shown to be the only source of GABA in the lung of primates and humans through the activity of biosynthetic enzyme glutamate decarboxylase 67 (GAD67) (Barrios et al., 2019). The NECs in the species studied have been shown in clusters and in close association with mucous cells in the leading edge of the multi-layered filament epithelium and along the filament epithelium using both the antibody to GAD67 and GABAB R1 used in colocalization procedures with 5HT (Figs. 4 A-F; Fig. 5 A, B, C, D). We found that both 5HT and GAD 67 as well as 5HT and GABA B R1 are coexpressed in the NECs (Fig. 4 A, B; Fig. 5 C, D). With the colocalization procedure using both 5HT and GABA B R1 the two neuronal markers are not coexpressed in some NECs, and at least two NEC supopulations are determined (Fig. 5 A, B) involved in a possible paracrine pathway in the gill. Notably some NECs are seen contouring the mucous cell and display neuron-like fluorescent processes (Fig. 5 B). GABA B R1 immunoreactivity is noticed

in subepithelial 5HT positive filament gill neurons (Fig. 5 C, D). GABA B R1 immunoreactivity is noticed in mucus droplets od the mucous goblet cells of the filament epithelium. Both GABA signalling and GABA B R1 receptor are regarded as an evolutionary component of mucus production (Barrios et al., 2019). Whether GABA B R1 receptor contributes to mucus secretion warrants future investigations. We speculate that both production by the NECs and a possible GABA receptor signalling by mucous cells may serve as a target to direct mucus secretion by paracrine mechanisms to affect mucous cells in fish gill.

3.2.1.4 Double Immunolabeling for 5HT and Pis1

The presence of Pis1 and 5HT immunoreactivity was evident in the clusters of NECs in the leading edge of the filament and isolated cells along the gill filament. Colabelling with the two antibodies revealed an overlay of both 5HT and Pis1 immunoreactivity in most cells (Fig. 6 A, B, C, D) besides a NEC population expressing 5HT alone. Pis 1 immunoreactivity is also found in the nerve fibers innervating the vascular walls of the efferent filament arteries (Fig. 6 C, D). To the best of our knowledge, this is the first report of *piscidin* expression in nerve fibers in this species and the nerve plexuses in the gill filament arteries of the butterfly fish (Capillo et al., 2021) and its physiological relevance warrants further studies.

3.2.2 Respiratory air sac

3.2.2.1 Double immunolabeling for 5HT and VAChT

Simultaneous detection of 5HT and VAChT revealed the occurrence of a few numbers of NECs that appeared in cell clusters and closely associated to mucous cells. A coexistence of these neuronal markers was noticed in these cells (Fig. 7 A, B, C) as well as a subpopulation of NECs displaying VAChT immunoreactivity. High magnification of the apical portion of the NECs revealed puncta of

both 5HT (green) and VAChT (red) indicating innervation and colocalization of the two markers. The respiratory cells in the apical surface expressed VAChT at their cell perimeter thus suggesting that these cells had secretory vesicles bearing the vesicular choline transporter protein. VAChT antibodies labelled the mucous cells with nearby VAChT positive nerve fibers and nerve bundles (Fig. 8 A, B; Fig. 9 A, B, C). In most of these cells VAChT immunostaining labelled both the domed membranes at their apical sides as well as the base perimeters. Focal planes beneath the NECs revealed the VAChT labelling of the mucous cells, thus suggesting that both cell types belong to a cholinergic circuit sensing response to hypoxia (Milsom, 2012).

3.2.2.2 Double immunolabeling for 5HT and nAChR alpha 7 subunit

The cholinergic mucous cells were found in the interlamellar areas and binding of alpha 7 subunit of nAChR was demonstrated on the entire cell surfaces (Fig. 10 A, B). No co-labelling of VAChT antibody with nAChR one was done, but the VAChT probe (see above) was concentrated at the cell perimeter of the mucous cells, thus indicating that the Ach transporter coincided with the distribution pattern of alpha 7 subunit of nAChR and suggested the Ach release from the mucous cells. Notably puncta showing labeling for both nAChR and 5HT markers were found in the apical dome and lateral surfaces of the mucous cells thus indicating innervation.

3.2.2.3 Double Immunolabeling for 5HT - GAD67, 5HT-GAB B R1 and 5HT- Piscidin 1(Pis1)

GABA B R1 immunoreactivity was noticed in the epithelial polygonal cells (Fig. 10 A) or near the mucous cells, the surface epithelium and sometimes in the mucus droplets of the large mucous cells (Fig. 11 D, E), but the use of the GAD67 antibody yielded no positive results. GABA , GABA B receptor and GAD67 have been recently identified in the airway epithelium (Barrios et al., 2019) and immune system such as T lymphocyte and monocytes (Vargas, 2018). GABA can modulate the activity of the immune system by activating or inhibiting cytokine production and modifying the

migration of the defence cells. Immunostaining with the antibodies to 5HT and GABA B R1 revealed that the two neurochemical markers completely localized in some NECs found in close vicinity to the large mucous cells (Fig. 11 B, C, D, E, F). The mucous cells were immunoreactive for piscidin 1 (Fig. 11 B, C), an antimicrobial peptide found in the immune cells of fish gills (Raju et al., 2020a; Rakers et al., 2013). Double immunostaining with antibodies to 5HT and Pis 1 evidenced a population of NECs in the lamellae of the respiratory islets of the air sac, and a colocalization of the two neuronal markers (Fig. 11 A). Pis 1 immunoreactivity is also found in extensive nerve bundles that approach the efferent air sac vessel (Fig. 12 A, C, D). Mast cells immunopositive to Pis 1 antibody with a higher signal intensity were observed in the gill filament epithelium and near to mucous cells and also in the cucullaris muscle of the respiratory air sac (Fig. 12 E). The Pis1 immunoreactivity in the mucous cells in the species studied is reported for the first time in fish mucosal epithelia, although an ubiquitous expression of this peptide was ascertained in several visceral organs of Atlantic cod (Ruangsri et al., 2012a). The expression of GABA B R1 in the polygonal epithelial cells and the NECs near the mucous cells in the air sac should be correlated with a profuse mucous production in both hypoxic or homeostatic conditions or in adverse environments not investigated in this study. GABA has an important role in inhibiting bronchoconstriction stimulated by acetylcholine and cytokines and contributing to an overproduction of mucus (Vargas, 2018). The presence of the piscidin 1 in mucous cells and the modulation of the mucous secretion by GABA as well as the release of Ach from the mucous cells should be correlated with the multifunctional and immunomodulatory aspects of the above neurotransmitters and piscidin (Zanetti et al., 2006) but more studies are required to confirm these hypotheses. In addition the production of GABA by the NEC as demonstrated by the antibody to GAD67 (the biosynthetic enzyme glutamic acid decarboxylase) in the gills, and the possible existence of a local GABAergic cell system in non-neural tissues such as the gill and air sac in the species studied, with autocrine/paracrine mechanisms is suggested.

3.2.2.4 Cholinergic cells, Ach nicotinic receptor, GABAergic cell system and Pis1

The Asian stinging catfish, *H. fossilis* is an air-breathing fish that represents one of the most highly adapted and diverse group of vertebrates living is swampy environments. The transition from aquatic to aerial respiration requires structural modification of respiratory organs and their vascular supply to accommodate the combined activities of the gills and the accessory respiratory organs (ABO) (Munshi, 2003). In the gills and ABO of the species studied- a possible site for O_2 sensing -the expression of acetylcholine via its nicotinic and muscarinic receptors (Zaccone et al., 2020), could be strictly correlated with a response to hypoxia by the stimulatory effects on sensory nerve fibers and cardiorespiratory reflexes (Burleson and Milsom, 1995a, b). We found the expression of acetylcholine in both NECs and mucous cells in the gill filaments and the lamellae of the respiratory air sac. VAChT immunopositive NECs via nicotinic and muscarinic receptors (Zaccone et al., 2020) and cholinergic mucous cells suggest a cholinergic pathway in the gill and ABO of *H. fossilis*, that may contribute to hyperventilatory response to hypoxia (Burleson and Milsom, 1995a, b; Regan et al., 2011). Cholinergic neurons were previously described in the filament core of the trout and the mudskipper (Porteus et al., 2013; Zaccone et al., 2018; Zaccone et al., 2017b) and also reported GABA R1 in the gill neurons of the species studied, and the presence of acetylcholine is also evidenced in the dome apical caps in the epithelial cells of the gill lamellae of *P. bucholzi* (Capillo et al., 2021). To our knowledge the localization of acetylcholine and the antimicrobial peptide Piscidin 1 in fish mucous cells are reported for the first time in this species. A large body of evidence indicates that mucous goblet cells in mammalian counterpart have multiple contribution to innate and adaptative immune response at mucosal surface (Knoop and Newberry, 2018). Recent investigations point to the important role of the submucous glands in the lung respiratory host defense due to the production of antimicrobials and mucins (Whitsett, 2018; Widdicombe and Wine, 2015). Mucous cells secrete mucus in response to a number of mediators including acetylcholine and prostaglandins (see for review, Knoop and Newberry (2018)). Sentinel mucous goblet cell functions have only recently been appreciated in the intestinal tract. Similarities between intestinal mucous goblet cells and those in other mucosal surfaces, such as the lung epithelia, suggest that these mucous goblet cell functions

may exist at other sites. Further investigations will be required to determine if the release of the acetylcholine from the goblet cells of the gill/ABO in the species studied, their interaction with the nervous system and the significance of conversation (if any) with the local immune system are regulated, and if the proposed functions are a characteristic of a subset of mucous goblet cells of the airways epithelia of air-breathing fish or teleosts coping with hypoxic environments. NECs and mucous cells secrete acetylcholine and are cholinergic.

Besides, a serotonergic NEC population and a cholinergic NEC population already reported in the gill of *Kryptolebias* (Regan et al., 2011), we found a NEC population where GAD67 and 5HT are coexpressed. Whether this population plays a role in the maintenance of both mucus production or overproduction by GABA secretion is not known. Recent evidence demonstrated that secretion of GABA induces mucous secretion to relay signals for stimulating immune cells (Barrios et al., 2019; Kobayashi and Tata, 2018). Clearly our study may show that NEC secretion may be regulated by a neurocircuitry that innervates the NECs in the gill and ABO epithelia. These cells in mammalian lung are regarded primarily as O_2 sensors, but also involved in the regulation of goblet cell hyperplasia (Kobayashi and Tata, 2018). It is becoming increasingly clear that the contribution of mucous cells to mucosal immunity extends well beyond mucus secretion.

The immunohistochemical localization of piscidin 1 in the gill and ABO mucous cells is in agreement with reports in mast cells, rodlet cells and other not morphologically identified epithelial cell types both in the gill filament and lamellae of teleosts from a wide taxonomic range of fish species that express piscidin (Silphaduang et al., 2006b). Also Pis 3 immunoreactivity was reported in the mast cells associated with the mucous cells in both gill filament and lamellae of sea bream (Zaccone et al., 2001). In Atlantic cod (Ruangsri et al., 2012b), we reported the presence of Pis1 immunoreactivity in the gas gland cells and parasympathetic ganglia of the wall in the swim bladder, the mucosal epithelium of the proximal intestine and the cartilaginous cells in the cartilage rod of the gill filament .We also observed a strong Pis immunoreactivity in the nerve plexuses around the vascular walls in the gill efferent filament arteries and in the nerve bundles in the submucosal layer of the air sac. The

immunoreactivity of Pis1 in the NECs of the gill and air sac and nerve bundles parallel to the efferent filament arteries is reported for the first time in the species studied. Pis1 could be regarded as a neurogenic peptide since its immunoreactivity is observed in the neural tissues of the Atlantic cod (Ruangsri et al., 2012b), suggesting a crosstalk between nervous and immune systems, including the close interrelationship between the neuroepithelial cells and the immune cells (Van Lommel et al., 1995). This also suggests that this peptide expressed in NECs could be associated with an immune function as these cells producing GABA could be involved in an mutual and dynamic immune defence network with other cell types, namely the Pis1-containing mucous cells . Another possible role for this ubiquitous peptide is related to its wide distribution in fish epithelia in both immune-related and non-immune tissues as confirmed at molecular level (Ruangsri et al., 2012a), and its involvement as a cytoprotective molecule to maintain tissue homeostasis (Ruangsri et al., 2012a). The specificity of the anti-Pis1 antibody in *H. fossilis* was confirmed by pre-incubating the antibody with piscidin 1. The antibody to Pis 1 is a rabbit polyclonal antibody raised against the N-terminus fragment of piscidin 1 from Atlantic cod. Fish mucous cells are structurally similar to their mammalian counterparts and their mucus contains neutral and acid sulfated/carboxylated glycoconjugates that are accumulated in the extracellular regions. The highly effective mucus production includes most of the known piscine anti-infection antimicrobial peptides AMPS (Fernandes et al., 2010), that vary with the environmental conditions (Zaccone et al., 2001). Fish AMPS (e.g. piscidins) as above reported contribute to innate immune system in fish skin and a variety of organs and are species specific (see for review, (Raju et al., 2020b).

Besides their action as hypoxia-sensitive chemoreceptors, NECs may be candidates for hypothetical immunomodulatory effects exerted by the various known molecules that are responsible to induce mucous secretion and activation of the mucosal immune system. This is in agreement with the multiple functions of the mucous goblet cells that can regulate and the shape mucosal immune response to environmental factors.

4 Conclusion

The present study demonstrated the presence of acetylcholine and the expression of alpha 7 subunit of nicotinic receptor in the NECs and mucous cells in the gill and respiratory air sac of the catfish *H. fossilis*. The wide expression of non-neuronal acetylcholine and acetylcholine sensitive receptors has been found in the airways epithelia and immune cells of vertebrates. It is believed that the nicotinic receptors represent the target for the non-neuronal acetylcholine expressed in the NECs and mucous cells to mediate auto-paracrine effects and therewith to control cell and organ homeostasis. The present findings also demonstrated that the NECS and epithelial cells in the gill and air sac have a GABA production system and indicated that GABA may play functional roles through the GABA B R1 receptor, like acetylcholine, in the regulation of the secretion of mucus and antimicrobial peptides. It can be concluded that in the course of evolution the gill materials in *H. fossilis* were involved in the formation of the gill epithelium (Singh, 1993). The respiratory air-sac originates from the conversion of the gill lamellae. The NECs occurring in the air-sac represent the most primitive stage in the evolution of the O_2 sensors and a rich storehouse of immunomodulators in this fish that used a neo-morphic air-breathing organ comprising a respiratory epithelium suitable for gas exchange with air.

A potential contribution of the NECs as hypothetical immunomodulatory-like cells is emerging as a consequence of the fundamentally endocrine nature of these cells e.g. contact with exterior environment with antigens or toxicants , or at least synthesis and storage of immunomodulatory substances that are released by the NECs in the mammalian lung upon antigenic stimulation. At moment, there is a need of quite substantial experimental evidence for NEC-secretory products to be involved in these mechanisms.

References

Ackerman, K.M., Boyd, R.T., 2016. Analysis of Nicotinic Acetylcholine Receptor (nAChR) Gene Expression in Zebrafish (Danio rerio) by In Situ Hybridization and PCR, in: Li, M.D. (Ed.), Nicotinic Acetylcholine Receptor Technologies. Springer New York, New York, NY, pp. 1-31.

Baghdadwala, M.I., Duchcherer, M., Trask, W.M., Gray, P.A., Wilson, R.J., 2016. Diving into the mammalian swamp of respiratory rhythm generation with the bullfrog. Respiratory Physiology Neurobiology 224, 37-51.

Barrios, J., Kho, A.T., Aven, L., Mitchel, J.A., Park, J.-A., Randell, S.H., Miller, L.A., Tantisira, K.G., Ai, X., 2019. Pulmonary neuroendocrine cells secrete γ-aminobutyric acid to induce goblet cell hyperplasia in primate models. American Journal of Respiratory Cell Molecular Biology 60, 687-694.

Bosma, P.T., Blázquez, M., Collins, M.A., Bishop, J., Drouin, G., Priede, I.G., Docherty, K., Trudeau, V.L., 1999. Multiplicity of glutamic acid decarboxylases (GAD) in vertebrates: molecular phylogeny and evidence for a new GAD paralog. Molecular Biology 16, 397-404.

Burleson, M.L., Mercer, S.E., Wilk-Blaszczak, M.A., 2006. Isolation and characterization of putative O2 chemoreceptor cells from the gills of channel catfish (Ictalurus punctatus). Brain Research 1092, 100-107.

Burleson, M.L., Milsom, W.K., 1995a. Cardio-ventilatory control in rainbow trout: I. Pharmacology of branchial, oxygen-sensitive chemoreceptors. Respiration Physiology 100, 231-238.

Burleson, M.L., Milsom, W.K., 1995b. Cardio-ventilatory control in rainbow trout: II. Reflex effects of exogenous neurochemicals. Respiration Physiology 101, 289-299.

Capillo, G., Zaccone, G., Cupello, C., Fernandes, J.M.O., Viswanath, K., Kuciel, M., Zuwala, K., Guerrera, M.C., Aragona, M., Icardo, J.M., Lauriano, E.R., 2021. Expression of acetylcholine, its contribution to regulation of immune function and O2 sensing and phylogenetic interpretations of the African butterfly fish Pantodon buchholzi (Osteoglossiformes, Pantodontidae). Fish & Shellfish Immunology 111, 189-200.

Fernandes, J.M., Ruangsri, J., Kiron, V., 2010. Atlantic cod piscidin and its diversification through positive selection. PloS ONE 5, e9501.

Fu, X., Spindel, E., 2009. Recruitment of GABA A Receptors in Chemoreceptor Pulmonary Neuroepithelial Bodies by Prenatal Nicotine Exposure in Monkey Lung, Arterial Chemoreceptors. Springer, pp. 439-445.

Gargaglioni, L., Branco, L., 2009. Midbrain structures and control of ventilation in amphibians, Cardio-Respiratory Control in Vertebrates. Springer, pp. 241-261.

Graham, J.B., 1997. Air-breathing fishes: evolution, diversity, and adaptation. Academic Press.

Grando, S.A., 1997. Biological functions of keratinocyte cholinergic receptors, Journal of Investigative Dermatology Symposium Proceedings. Elsevier, pp. 41-48.

Jonz, M.G., Buck, L.T., Perry, S.F., Schwerte, T., Zaccone, G., 2016. Sensing and surviving hypoxia in vertebrates. Annals og the New York Academy of Sciences 1365, 43–58.

Jonz Michael G, N.C.A., 2003. Neuroepithelial Cells and AssociatedInnervation of the Zebrafish Gill: AConfocal Immunofluorescence Study. The Journal of Comparative Neurology 461, 1–17

Knoop, K.A., Newberry, R.D., 2018. Goblet cells: multifaceted players in immunity at mucosal surfaces. Mucosal Immunology 11, 1551-1557.

Kobayashi, Y., Tata, P.R., 2018. Pulmonary Neuroendocrine Cells: Sensors and Sentinels of the Lung. Developmental Cell 45, 425-426.

Kurzen, H., Wessler, I., Kirkpatrick, C., Kawashima, K., Grando, S., 2007. The non-neuronal cholinergic system of human skin. Hormone Metabolic Research 39, 125-135.

Lauriano, E., Pergolizzi, S., Capillo, G., Kuciel, M., Alesci, A., Faggio, C., 2016. Immunohistochemical characterization of Toll-like receptor 2 in gut epithelial cells and macrophages of goldfish Carassius auratus fed with a high-cholesterol diet. Fish & Shellfish Immunology 59, 250-255.

Lauriano, E.R., Icardo, J.M., Zaccone, D., Kuciel, M., Satora, L., Alesci, A., Alfa, M., Zaccone, G., 2015. Expression patterns and quantitative assessment of neurochemical markers in the lung of the gray bichir, Polypterus senegalus (Cuvier, 1829). Acta histochemica 117, 738-746.

Lauweryns, J., Cokelaere, M., 1973a. Intrapulmonary neuro-epithelial bodies: hypoxia-sensitive neuro (chemo-) receptors. Experientia 29, 1384-1386.

Lauweryns, J.M., Cokelaere, M., 1973b. Hypoxia-sensitive neuro-epithelial bodies intrapulmonary secretory neuroreceptors, modulated by the CNS. Zeitschrift für Zellforschung und Mikroskopische Anatomie 145, 521-540.

Magadan, S., Sunyer, O.J., Boudinot, P., 2015. Unique features of fish immune repertoires: particularities of adaptive immunity within the largest group of vertebrates, Pathogen-Host Interactions: Antigenic Variation v. Somatic Adaptations. Springer, pp. 235-264.

Milsom, W.K., 2012. New insights into gill chemoreception: receptor distribution and roles in water and air breathing fish. Respiratory Physiology Neurobiology 184, 326-339.

Mulero, I., Noga, E.J., Meseguer, J., García-Ayala, A., Mulero, V., 2008. The antimicrobial peptides piscidins are stored in the granules of professional phagocytic granulocytes of fish and are delivered to the bacteria-containing phagosome upon phagocytosis. Developmental Comparative Immunology 32, 1531-1538.

Munshi, J., 1993. Structure and function of the air breathing organs of Heteropneustes fossilis. Advances in fish research I, 99-138.

Munshi, J.D., 2003. Sensory Biology of Jawed Fishes: New Insights. JSTOR.

Munshi, J.D., Weibel, E., Gehr, P., Hughes, G., 1986. Structure of the respiratory air sac of Heteropneustes fossilis (Bloch)(Heteropneustidae, Pisces). An electron microscope study, Proc Indian Nat Sci Acad B, pp. 703-713.

Olson, K., Munshi, J., Ghosh, T., Ojha, J., 1990. Vascular organization of the head and respiratory organs of the air-breathing catfish, Heteropneustes fossilis. Journal of Morphology 203, 165-179.

Olsson, C., Holmberg, A., Holmgren, S., 2008. Development of enteric and vagal innervation of the zebrafish (Danio rerio) gut. Journal of Comparative Neurology 508, 756-770.

Perry, E., Piggott, M., 2000. Neurotransmitter mechanisms of dreaming: implication of modulatory systems based on dream intensity. Behavioral and Brain Sciences 23, 990-992.

Porteus, C.S., Brink, D.L., Coolidge, E.H., Fong, A.Y., Milsom, W.K., 2013. Distribution of acetylcholine and catecholamines in fish gills and their potential roles in the hypoxic ventilatory response. Acta Histochemica 115, 158-169.

Porteus, C.S., Brink, D.L., Milsom, W.K., 2012. Neurotransmitter profiles in fish gills: putative gill oxygen chemoreceptors. Respiratory Physiology Neurobiology 184, 316-325.

Profita, M., Bonanno, A., Siena, L., Ferraro, M., Montalbano, A.M., Pompeo, F., Riccobono, L., Pieper, M.P., Gjomarkaj, M., 2008. Acetylcholine mediates the release of IL-8 in human bronchial epithelial cells by a NFkB/ERK-dependent mechanism. European Journal of Pharmacology 582, 145-153.

Rahbar, S., Pan, W., Jonz, M., 2016. Purinergic and Cholinergic Drugs Mediate Hyperventilation in Zebrafish: Evidence from a Novel Chemical Screen PLoS ONE 11, 4.

Raju, S., Sarkar, P., Kumar, P., Arockiaraj, J., 2020a. Piscidin, Fish Antimicrobial Peptide: Structure, Classification, Properties, Mechanism, Gene Regulation and Therapeutical Importance. International Journal of Peotide Research Therapeutics.

Raju, S.V., Sarkar, P., Kumar, P., Arockiaraj, J., 2020b. Piscidin, fish antimicrobial peptide: Structure, classification, properties, mechanism, gene regulation and therapeutical importance. International Journal of Peptide Research Therapeutics, 1-17.

Rakers, S., Niklasson, L., Steinhagen, D., Kruse, C., Schauber, J., Sundell, K., Paus, R., 2013. Antimicrobial Peptides (AMPs) from Fish Epidermis: Perspectives for Investigative Dermatology,. Journal of Investigative Dermatology 133 Pages 1140-1149.
Regan, K.S., Jonz, M.G., Wright, P.A., 2011. Neuroepithelial cells and the hypoxia emersion response in the amphibious fish Kryptolebias marmoratus. Journal of Experimental Biology 214, 2560-2568.

Ruangsri, J., Fernandes, J.M., Rombout, J.H., Brinchmann, M.F., Kiron, V., 2012a. Ubiquitous presence of piscidin-1 in Atlantic cod as evidenced by immunolocalisation. BMC Veterinary Research 8, 46.

Ruangsri, J., Salger, S.A., Caipang, C.M., Kiron, V., Fernandes, J.M., 2012b. Differential expression and biological activity of two piscidin paralogues and a novel splice variant in Atlantic cod (Gadus morhua L.). Fish Shellfish Immunology 32, 396-406.

Salinas, I., 2015. The mucosal immune system of teleost fish. Biology 4, 525-539.

Saltys, H.A., Jonz, M.G., Nurse, C.A., 2006. Comparative study of gill neuroepithelial cells and their innervation in teleosts and Xenopus tadpoles. Cell and Tissue Research 323, 1-10.

Saracino, L., Zorzetto, M., Inghilleri, S., Pozzi, E., Stella, G.M., 2013. Non-neuronal cholinergic system in airways and lung cancer susceptibility. Translational Lung Cancer Research 2, 284.

Shakarchi, K., Zachar, P.C., Jonz, M.G., 2013. Serotonergic and cholinergic elements of the hypoxic ventilatory response in developing zebrafish. Journal of Experimental Biology 216, 869-880.

Silphaduang, U., Colorni, A., Noga, E., 2006a. Evidence for widespread distribution of piscidin antimicrobial peptides in teleost fish. Diseases of Aquatic Organisms 72, 241-252.

Silphaduang, U., Colorni, A., Noga, E.J., 2006b. Evidence for widespread distribution of piscidin antimicrobial peptides in teleost fish. Diseases of Aquatic Organisms 72, 241-252.

Singh, B., 1993. Development, origin and evolution of the neomorphic air breathing organs in teleosts. Advances in Fish Research 1, 1-16.

Stoyek, M.R., Croll, R.P., Smith, F.M., 2015. Intrinsic and Extrinsic Innervation of the Heartin Zebrafish (Danio rerio). The Journal of Comparative Neurology 523, 1683–1700.
und Halbach, O.v.B., Dermietzel, R., 2006. Neurotransmitters and neuromodulators: Handbook of receptors and biological effects. John Wiley & Sons.

Uyttebroek, L., Shepherd, I.T., Harrisson, F., Hubens, G., Blust, R., Timmermans, J.P., Van Nassauw, L., 2010. Neurochemical coding of enteric neurons in adult and embryonic zebrafish (Danio rerio). Journal of Comparative Neurology 518, 4419-4438.

Van Lommel, A., Lauweryns, J., De Leyn, P., Wouters, P., Schreinemakers, H., Lerut, T., 1995. Pulmonary neuroepithelial bodies in neonatal and adult dogs: histochemistry, ultrastructure, and effects of unilateral hilar lung denervation. Lung 173, 13-23.

Vargas, R.A., 2018. The GABAergic System: An Overview of Physiology, Physiopathology and Therapeutics. International Journal of Clinical Pharmacology Pharmacotherapy 3

Wessler, I., Kirkpatrick, C., 2008. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. British Journal of Pharmacology 154, 1558-1571.
Whitsett, J.A., 2018. Airway Epithelial Differentiation and Mucociliary Clearance. Transatlantic Airway Conference 15, 143-148.
Widdicombe, J.H., Wine, J.J., 2015. Airway gland structure and fuction. Physiological Reviews 95, 1241–1319.

Yabumoto, Y., Watanabe, M., Ito, Y., Maemura, K., Otsuki, Y., Nakamura, Y., Yanagawa, Y., Obata, K., Watanabe, K., 2008. Expression of GABAergic system in pulmonary neuroendocrine cells and airway epithelial cells in GAD67-GFP knock-in mice. Medical molecular morphology 41, 20-27.

Zaccone, D., Icardo, J.M., Kuciel, M., Alesci, A., Pergolizzi, S., Satora, L., Lauriano, E.R.,

Zaccone, G., 2017a. Polymorphous granular cells in the lung of the primitive fish, the bichir Polypterus senegalus. Acta Zoologica 98, 13-19.

Zaccone, G., Cupello, C., Capillo, G., Kuciel, M., Nascimento, A.L., Gopesh, A., Germanà, G.P., Spanò, N., Guerrera, M.C., Aragona, M., 2020. Expression of Acetylcholine-and G protein coupled Muscarinic receptor in the Neuroepithelial cells (NECs) of the obligated air-breathing fish, Arapaima gigas (Arapaimatidae: Teleostei). Zoology 139, 125755.

Zaccone, G., Fudge, D.S., Winegard, T.M., Capillo, G., Kuciel, M., Funakoshi, K., Lauriano, E.R., 2015a. Confocal imaging and phylogenetic considerations of the subcutaneous neurons in the Atlantic hagfish Myxine glutinosa. Acta Zoologica 96, 209-217.

Zaccone, G., Kapoor, B., Fasulo, S., Ainis, L., 2001. Structural, histochemical and functional aspects of the epidermis of fishes. Advances in Marine Biology 40, 253-348.

Zaccone, G., Lauriano, E.R., Capillo, G., Kuciel, M., 2018. Air-breathing in fish: Air-breathing organs and control of respiration: Nerves and neurotransmitters in the air-breathing organs and the skin. Acta Histochemica 120, 630-641.

Zaccone, G., Lauriano, E.R., Kuciel, M., Capillo, G., Pergolizzi, S., Alesci, A., Ishimatsu, A., Ip, Y.K., Icardo, J.M., 2017b. Identification and distribution of neuronal nitric oxide synthase and neurochemical markers in the neuroepithelial cells of the gill and the skin in the giant mudskipper, Periophthalmodon schlosseri. Zoology.

Zaccone, G., Lauriano, E.R., Silvestri, G., Kenaley, C., Icardo, J.M., Pergolizzi, S., Alesci, A., Sengar, M., Kuciel, M., Gopesh, A., 2015b. Comparative neurochemical features of the innervation patterns of the gut of the basal actinopterygian, Lepisosteus oculatus, and the euteleost, Clarias batrachus. Acta Zoologica 96, 127-139.

Zaccone, G., Maina, J., Germanà, A., Montalbano, G., Capillo, G., Aragona, L., Kuciel, M.J., Lauriano, E.R., Icardo, J.M., 2019. First demonstration of the neuroepithelial cells and their chemical code in the accessory respiratory organ and the gill of the sharptooth catfish, Clarias gariepinus: A preliminary study. Acta Zoologica 100, 160-166.

Zaccone, G., Mauceri, A., Fasulo, S., 2006. Neuropeptides and nitric oxide synthase in the gill and the air-breathing organs of fishes. Journal of Experimental Zoology Part A: Comparative Experimental Biology 305, 428-439.

Zachar, L., Bačenková, D., Rosocha, J., 2016. Activation, homing, and role of the mesenchymal stem cells in the inflammatory environment. Journal of Inflammation Research 9, 231–240.
Zanetti, L., de Kerchove D'Exaerde, A., Zanardi , A., Changeux, J.-P., Picciotto, M.R., Zoli, M., 2006. Inhibition of both α7* and β2* nicotinic acetylcholine receptors is necessary to prevent development of sensitization to cocaine-elicited increases in extracellular dopamine levels in the ventral striatum. Psychopharmacology 187, 181–188.

Captions to figures

TEM

Fig. 1.- Ultrastructural details of gills and air sac. a: Gill filament. Group of three NECs. These cells show a variable number of small, dense granules (arrows). Arrowheads, mitochondriae. b: Air sac. Three NECs with cytoplasmic granules (arrows) appear in the centre of the panel. Arrowheads indicate the presence of desmosomes between mucosal cells. Note that NECs are free cells. c: An eosinophil with granules appears closely associated to a NEC. E, circulating erythrocytes. Arrowheads, desmosomes. d: Air sac. Two mucous cells are filled with secretory granules. Epithelial cells show numerous microridges (arrowheads). Scale bars: a-d, 2 μm.

CONFOCAL LASER SCANNING MICROSCOPY

Gill

Figs. 2 A-E. Clustered NECs within the multilayered epithelium of the leading edge of the gill filament of *Heteropneustes fossilis* immunostained with antibodies against 5HT (green) and VAChT (red). A, B. Confocal images show two separate populations of NECs (arrows) labelled simultaneously with antibodies to 5HT (green) and VAChT (red), and VAChT alone. C Merged image showing NECs positive for both 5HT and VAChT are labelled orange (arrows). Large mucous cells (MC) also reveal a strong VAChT immunoreactivity. eFA, Efferent filament artery. D, E Longitudinal section of the gill filament. The leading edge. Within the filament mucous cells (MC) are seen on the external side showing a strong labelling with VAChT antibody as revealed by the immunostaining of the mucous droplets (E). 5HT NECs are seen in association with mucous cells and also lie on the basal lamina (bl). Scale bars: 20 μm

Figs. 3 A-F. A Longitudinal section of two gill filaments of *H. fossilis* labelled with antibodies against 5HT and alpha 7 subunit of acetylcholine nicotinic receptor (alpha 7 nAChR) showing the co-expression of the two neurochemical markers in the NECs that are positive for both 5HT and nAChR alpha 7 subunit (long arrows) and are located within the multilayered epithelium of the

filament and gill lamella. Please also note more NECs that are positive for 5HT but nAChR alpha 7 subunit negative and are labelled in green (arrowheads). Nerve fibers (green) are labelled with 5HT antibodies and seen in subepithelial localization (N). Scale bar: 20 μm. B NECs in the gill filament(gf) and gill lamella (gl) are labelled by antibodies against nAChR alpha 7 subunit. Scale bar: 20 μm. C Expression of nAChR alpha 7 subunit in the NECs in the gill filament and gill lamellae (arrows) and nerve fibers around a mucous cell (Arrowhead and in inset). Scale bar: 20 μm. D The region of the image in C revealing colocalization of 5HT and nAChR alpha 7 subunit in the NECs and nerve fibers encircling a mucous cell (MC). Scale bar: 20 μm. E Mucous cells in the leading edge of the gill filament revealing their close association with nerve fibers (arrowheads) as evidenced by colocalization of 5HT and nAChR alpha 7 subunit. Scale bar: 20 μm. F. The leading edge of filament. Mucous cells display nAChR alpha 7 subunit immunoreactivity in the peripheral cytoplasm. Scale bar: 20 μm.

Figs. 4 A-F. A, B. The leading edge of the gill filament of *H. fossilis*. Within the filament epithelium (A), on the left, mucous cells (MC) and neuroepithelial cells (arrows) lying on the basal lamina, and in close vicinity to mucous cells (B) are seen. NECs show 5HT (green) and GAD67 (red) immunoreactivity in the single-color channels respectively. E, F Merged channel figures present co-labeled by 5HT and GAD67 NECs (arrows). Scale bars: 20 μm.

Figs. 5 A-D. A, B Leading edge of the gill filament of *H. fossilis*. A clustering of NECs immunostained in green with antibodies against GABA B R1 is seen. NECs lie in close proximity to mucous cells (MC) and some of these cells have neuron-like fluorescent processes (arrowheads) in close contact with the mucous cells. A 5HT positive NEC is also visible and stained in red(arrow). Scale bar: 20 μm. C, D. C NECs and a filament neuron (N) in the apical region of the gill filament immunostained with antibodies against GABA B R1. eFA, Efferent filament artery. Scale bars: 20 μm. D. A region of the image in C showing the NECs positive for both 5HT and GABA B R1 are labelled green/yellow lying on the basal lamina. Scale bar: 20 μm.

Figs. 6 A-D. A, B. Longitudinal section of the gill filament of *H. fossilis*. NECs (arrows) within the filament epithelium (A) and the leading edge of the filament (B) are double immunostained for Pis1 and 5HT. C, D. The same cells from the images A, B in the single-color channels display Pis1 immunoreactivity. Note also the presence of extensive bundles (NB) of Pis1 immunoreactive nerve fibers that approach the efferent filament artery (efa). MC, Mucous cells. Scale bars: 20 μm.

Respiratory Air-sac

Figs. 7 A-C. Transverse section of the lamellae in the respiratory islets of the air-sac of *H. fossilis* double immunostained with the antibodies against 5HT and VAChT. Most of the NECs positive for both 5HT (A) and VAChT (B) are labelled yellow/orange (C, merge) (arrows). Scale bars: 20 μm.

Fig. 8 B, C. Confocal images of the respiratory air sac of *H. fossilis* double immunolabelled with antibodies to 5HT and VAChT. A. Mucous cells (MC) expressing VAChT are innervated by VAChT positive nerve fibers (arrows). B. NECs (arrows) are labelled with antibodies to 5HT (green) and VAChT (red). Mucous cells (MC) are labelled by antibodies to VAChT. Note the location of 5HT positive NEC (green) and puncta (arrowhead) on these cells indicating innervation. Scale bars: 20 μm. Reproduced from Zaccone et al., 2018 with permission. ELSEVIER LICENSE N◦ 4952981202820.

Figs. 9 A-C. A, B, C. Section of a lamella of the respiratory air sac of *H. fossilis* double immunostained with antibodies against 5HT (green) and VAChT (red). Note the immunolabelling of the mucous cells (MC) by the VAChT antibody and associated nerve bundles (arrows and in inset). Scale bars: 20 μm.

Figs. 10 A-B. Section of lamellae of the respiratory air sac double immunostained with antibodies to 5HT (green) and nAChR alpha 7 subunit (red). Note the lacking 5HT immunostaining of mucous

cells (asterisks) in A and the strong nAChR alpha 7 labelling of these cells and its confinement to the cell periphery (arrows) in B. EASV, Efferent Air sac Vessel. Scale bars: 20 μm.

Figs. 11 A-F. A Transverse section of a lamella of respiratory islet double immunostained by antibodies to GABA B R1 and 5HT showing the outer respiratory region containing autofluoresceing red blood cells (asterisks) and mucous cells (MC) in the non-respiratory interlamellar areas. The polygonal epithelial cells amongst which are found mucous cells (MC) are strong labelled by the GABA B R1 antibody. Scale bar: 20 μm. B, C. Section of lamellae of the respiratory islet double immunostained by the antibodies to GABA B R1 and 5HT antibodies. A NEC (arrow) positive to GABA B R1 antibody(green) is seen in close proximity to a mucous cell (MC). The same cell is stained in red by the anti-5Ht antibody in C. In the tip of lamella are seen many autofluorescein g red blood cells. EASU, Efferent Air sac Vessel. Scale bars: 20 μm. D, E. Transverse section of the lamellae of respiratory air sac showing the presence of GABA B R1 positive NECs (arrows) in close association with mucous cells (MC). On the right a mucous cell (MC) is seen containing GABA B R1 immunoreactivity. The NECs are stained in red by the 5HT antibody in E and are seen in the single-color channel. Scale bars: 20 μm. F. A cluster of GABA B R1 positive NECs in the lamellae of the air sac near to mucous cells (asterisks). Scale bars: 20 μm.

Figs. 12 A-E. A tangential section of lamellae double immunostained with antibodies to 5HT (green) and Pis1 (red). Note the presence of NECs (arrowheads) are stained for both the two markers orange and the presence of distal respiratory part containing autofluoresceing blood cells (arrows). Extensive bundles of nerve fibers contain Pis 1 immunoreactivity and approach the efferent air sac artery (EASV). Scale bar: 20 μm. B, C Mucous cells (MC) in the non-respiratory interlamellar areas are strong labelled by the Pis 1 antibody. Arrows in B point to Pis 1 positive nerve varicosities close to basal lamina of the epithelium (arrows). Scale bars: 20 μm. D Section of lamella showing the outer respiratory region containing blood cells (arrows) and the double occurrence of both efferent primary air sac artery (EASV) innervated by Pis 1 positive nerve fibers

(arrows) and the afferent primary air sac artery (AASV). Scale bar: 20 μm. E. Section of lamella showing the outer respiratory part containing red blood cells (arrows) and the dorsal aspect of cucullaris muscle (CM) with embedded mast cells (arrows) containing 5HT-Pis1 immunoreactivity. Scale bar: 20 μm.