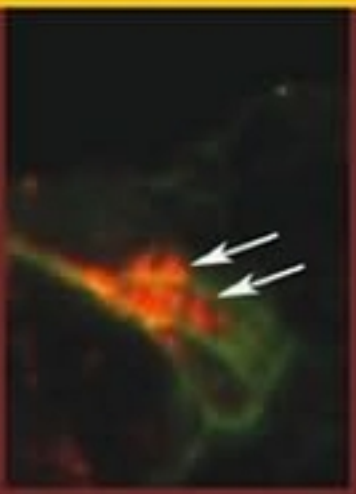
# AIRWAY CHEMORECEPTORS IN THE VERTEBRATES

## Structure, Evolution and Function




Editors

Giacomo Zaccone

Ernest Cutz

Dirk Adriaensen

Colin A. Nurse

Angela Mauceri

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## Cover Illustrations

*Left-hand side figure*

Immunocytochemical double staining for acetylcholinesterase (AchE) and neuronal nitric oxide synthase (nNOS) of two neuroepithelial cells (NECs) in the lung of the ray-finned fish, bichir Polypterus bichir bichir. Nitrergic nerve terminals (arrowed) are seen running between these cells (green). Micrograph courtesy of G. Zaccone.

*Right-hand side figure*

Immunocytochemical triple staining for vesicular glutamate transporter 2 (VGLUT2; red fluorescence), calbindin D28k (CB; blue fluorescence) and myelin basic protein (MBP; green fluorescence) of a neuroepithelial body (NEB) in a rat intrapulmonary airway. The CB-immunoreactive (ir) NEB is contacted by a CB- and VGLUT2-ir vagal nodose sensory nerve fiber, which is wrapped in an MBP-ir myelin sheath that ends in the immediate neighborhood of the NEB. VGLUT2 expression is seen in extensively branching nerve terminals between the NEB cells. The image shows a combination of the three color channels of a maximum value projection of confocal optical sections (PerkinElmer confocal UltraVIEW ERS). Also see Brouns et al., Chapter 11, Figure 2. Reproduced by kind permission of Dirk Adriaensen.

# Airway Chemoreceptors in the Vertebrates

## Structure, Evolution and Function


**EDITORS**

**Giacomo Zaccone**
Deparment of Animal Biology and Marine Ecology
Messina University, Messina, Italy

**Ernest Cutz**
Department of Paediatric Laboratory Medicine
University of Toronto, Toronto, Canada

**Dirk Adriaensen**
Department of Veterinary Sciences
University of Antwerp, Antwerp, Belgium

**Colin A. Nurse**
Department of Biology
Mc Master University, Hamilton, Canada

**Angela Mauceri**
Department of Animal Biology and Marine Ecology
Messina University, Messina, Italy



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Science Publishers
234 May Street
Post Office Box 699
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General enquiries : info@scipub.net
Editorial enquiries : editor@scipub.net
Sales enquiries : sales@scipub.net

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*"What is the Genius if not that productive force generating things that are worthy showing themselves in the presence of God and the Nature, and that therefore have followed in the time? All the Mozart's works are like this. There is a creative force that continues to act from generation to a generation, and that never it would have to be get exhausted"*

— Wolfgang Goethe to Eckermann

# Preface

It is both an honor and a privilege for me to write this Preface to "Structure, evolution and function of the airway chemoreceptors in the vertebrates". The full credit for realizing this book is due to Professor Zaccone who had initiated this undertaking and solicited contributions from experts from diverse fields of vertebrate biology with shared interest in the investigation of the structure, function and evolution of airway chemoreceptors. The end result is a one of a kind publication reflecting a truly global effort from five continents encompassing expertise in marine biology, zoology, animal physiology, cellular and molecular biology as well as pathobiology. The book provides a comprehensive up to date account of the information available on the morphological, physiological and evolutionary aspects of specialized cells distributed within the epithelia lining the air conducting structures of air breathing species or in the gills of fishes. Where available, recent advances in cellular and molecular aspects of oxygen and carbon dioxide sensing, relevant to the function of the chemoreceptor cells are highlighted. These cells, by virtue of their role as sensors that detect and signal changes in the external and internal environments, likely played an important role in the survival of various species. Consequently, these cells were conserved throughout evolution as evidenced by their occurrence in ancient fish to higher vertebrates including humans. These cells are commonly referred to as neuroendocrine/neurosecretory (NE) or pulmonary neuroendocrine cells (PNECs) in mammals, owing to the expression of a variety of neural and endocrine cell markers as well as prominent innervation that carries the signals to the central nervous system (CNS). While in primitive species solitary NE cells predominate, innervated clusters of NE cells, referred to as neuroepithelial bodies (NEBs), are observed only in the lungs of amphibia and higher vertebrates, perhaps reflecting adaptation from an aqueous to a terrestrial environment.

The book includes 16 contributions divided into seven chapters based on the evolution from primitive to more complex species. Accordingly, the first chapter covers NE cells in the airways and carotid labyrinth of aquatic vertebrates. Recent studies on oxygen-sensitive NE cells in the gills of fish and larval amphibians are reviewed. Of interest are electrophysiological studies using the patch-clamp technique demonstrating remarkable similarities with the $O_2$ sensing mechanism in mammals that depends on reversible inhibition of an $O_2$-sensitive $K^+$ current. Further similarity with mammals is the expression of serotonin (5-HT) in these cells, which may be acting as a neurotransmitter of the hypoxia stimulus as well as a mediator of vascular responses. Because of the relatively low solubility of $O_2$ in water as compared to ambient air, the physiological consequences of reduced $pO_2$ have more profound effects in aquatic vertebrates as opposed to air breathing animals. In addition, in fish and larval amphibians there is rapid elimination of $CO_2$, implicating that in the aquatic environment it is hypoxia rather than hypercapnia that plays a dominant role in driving cardiorespiratory responses.

The carotid labyrinth (CL) in fish and amphibians is analogous to the carotid body (CB) of higher vertebrates. It is a paired structure consisting of a dense capillary-like plexus located at the site of bifurcation of the common carotid artery. As in CB, glomus cells of CL are distributed within the intervascular stroma as single cells or in clusters and are innervated by an extensive network of afferent, efferent and reciprocal synapses. The glomus cells and related neural elements express a range of amines and neuropeptides likely acting as neurotransmitters or neuromodulators. The function of CL is multimodal and includes chemoreception, regulation of vascular tone to maintain blood supply to the eyes and brain, baroreception, and maintenance of brain and intraocular temperature.

The chapter on NE cells in the lung of amphibians and in the respiratory organs of air-breathing fish reviews the morphology and postulated functions of these cells.

An interesting observation from the phylogenetic perspective is the pattern of NE cell evolution in the respiratory organs. In the simpler species, such as air breathing fish (*Polypterus* and *Amia*), solitary non-innervated NE cells of the open type (reaching the airway lumen) and closed type (without luminal contact) are found distributed within the ciliated airway epithelium. A more complex structural arrangement with innervated solitary NE cells of closed type are observed in *Triturus*, whereas NE cells of open type are found in lungfish *Protopterus*. Interestingly, clusters of innervated NE cells resembling mammalian NEB are observed only in amphibians and higher vertebrates. Another striking similarity between NE cells in amphibians and mammals is the expression of 5-HT and neuropeptides, including bombesin, gastrin-releasing peptide (GRP), calcitonin and others. Although at present there are no direct physiological studies on amphibian NE cells or NEBs, by analogy with mammalian counterparts they are presumed to function as airway $O_2$ sensors involved in the control of breathing or alternatively in local paracrine regulation of airway and/or vascular responses.

Of particular interest are studies on "bimodal breathers" that include various amphibian vertebrates that use different anatomical structures (i.e., skin, gills, lungs) to achieve gas exchange. Many amphibious vertebrates, at some stage of their development use a combination of skin and gills plus lungs to breath both water (skin and/or gills) and air (skin and/or lungs), thus actually representing "trimodal" breathers. The respiratory control of amphibious vertebrates, using multiple modes of gas exchange, is complex and therefore requires sophisticated sensory systems for regulation, including various chemoreceptor structures. For example, pulmonary stretch receptors in amphibians not only respond to pulmonary volume changes but also to increasing intrapulmonary $CO_2$ levels by decreasing their firing rate. Additional receptor systems include olfactory $CO_2$ receptors, arterial chemoreceptors represented by the CL, arterial $O_2$-sensitive chemoreceptors located in the aortic arch, and chemoreceptors within the pulmonary vasculature and the airways represented by NEBs. Air breathing fish and amphibians illustrate a fascinating functional transition stage during evolution of terrestrial tetrapods from their aquatic fish-like ancestors. It has been pointed out that animals that are more aquatic in nature possess sophisticated receptors and respond primarily to changes in $pO_2$ levels in the internal environment. Transition to air-breathing and terrestrial life necessitated $CO_2$/pH sensitive receptors that are involved in respiratory control. Finally it is noted that during evolution, $CO_2$ chemoreception translocates centrally into the brain stem, while $O_2$ chemoreception remains peripherally located.

The NE cell system in the reptilian respiratory tract includes solitary cells that are present in both extra and intrapulmonary airways and NEBs that are found mostly in an intrapulmonary location. As in other species, NE cells in the airway mucosa of tortoises, lizards and snakes express 5-HT and peptides such as calcitonin, calcitonin gene-related peptide (CGRP), and in some species leu-enkephalin. Peptidergic innervation, expressing VIP, SP, NPY and PYY, has also been described. Although the precise function of NE cells in reptilian lung remains unknown, based on morphological observations the possible role as mechanoreceptors has been suggested. The latter, however, remains speculative as there are no direct functional studies on NE cells in the reptilian respiratory system.

In contrast, more physiological data is available on airway receptors in birds. The avian respiratory system differs from other species in that it consists of non-expandable lungs and series of air sacs providing a unique unidirectional system of airflow. In the avian respiratory system, both chemoreceptors and mechanoreceptors appear to play a role in feedback mechanisms of respiratory control. In birds, as in mammals, respiratory structures are innervated by the vagus nerve. Based on physiological studies, two main types of receptors have been identified in birds: an inspiratory-inhibitory $CO_2$-sensitive receptor located in the lung, corresponding to vagally innervated NEBs described in several avian species, and a slowly adapting mechanoreceptor presumed to be located in the walls of the air sacs or subpleural membrane since birds lack a diaphragm and have non-expanding lungs. The intrapulmonary chemoreceptors (IPC) show sensitivity to

$pCO_2$ but not $pO_2$. During the last 40 years a great deal of progress has been made in our understanding of the $CO_2$ signal transduction mechanism in avian lungs. Recent evidence indicates that IPC sense changes in intracellular pH ($pH_i$), resulting from the activity of carbonic anhydrase that catalyses hydration/dehydration of $CO_2$ rather than from direct $CO_2$ sensing. Other recent data shows that pH sensing in IPC also involves additional mechanisms including membrane based acid–base transporters, modulation by $Ca^{2+}$ influx, and the expression of a pH-sensitive TREK tandem pore domain $K^+$ leak channel. Due to the relative ease with which the air sac cavities and membranes of the avian respiratory system can be accessed for experimental purposes, they provide a useful model for further studies of both the peripheral and central components of respiratory control.

The Chapter on PNECs in mammalian lungs reviews recent findings on the structure, molecular markers, ontogeny and postulated functions. The general morphologic features including the ultrastructure and immunohistochemical/molecular markers of NE cells and NEBs in mammalian lung have been previously well defined. However the complexity of NEB innervation became apparent only recently. Thus far, only postnatal rat had been studied extensively in terms of the origin of NEB cell innervation and its neurochemical coding, which was extended to mice in a present contribution. Due to known species variations in lung innervation, the findings in the rat and mouse lungs would need to be verified in other species including human to better define their functional significance. Experimental denervation and neural tracing studies combined with multilabel immunohistochemistry and confocal imaging have revealed a complex neural network in rodent NEBs, consisting of vagus nerve derived afferent nerve fibers originating in nodose ganglion neurons, a CGRP expressing component originating in spinal ganglia, and a nitrergic component derived from intrinsic airway ganglia. Thus a complex picture is emerging where different potential signals that may include changes in airway $pO_2$ and possibly $pCO_2$ as well as mechanical forces could be transduced by NEB cells and modulated by their complex innervation. Based on observations of a large number of myelinated vagal nodose afferents in rodent intrapulmonary airways that selectively innervate NEBs, it has been proposed that discharges from NEB-related myelinated vagal afferent fibers may be part of the already characterized vagal myelinated receptors in lower airways.

Therefore, NEBs in mammals can be viewed as multimodal airway receptors that may serve different functions during ontogeny, during transition from the euoxic (relative fetal hypoxia) aqueous intrauterine environment to an extrauterine life dependent on air breathing, and subsequently in the postnatal and adult lung.

Recent electrophysiological studies, using the patch-clamp technique combined with molecular approaches, have provided evidence indicating that NEB cells are transducers of hypoxic stimuli via a membrane bound molecular complex ("oxygen sensor"), characteristic of specialized cells that monitor and signal hypoxia in different parts of the body to maintain homeostasis. The $O_2$ sensor in NEB cells of mammalian

lungs and of the related human small cell lung carcinoma cell line (H146) has been partially characterized and consists of a hydrogen peroxide ($H_2O_2$) generating, multicomponent NADPH oxidase coupled to $O_2$ sensitive $K^+$ channels. Under normoxia, the oxidase tonically generates $H_2O_2$ that, used as a second messenger, modulates the $O_2$-sensitive $K^+$ channel activity via a redox mechanism involving cystein residues in critical components of the $K^+$ channel gating mechanism. Hypoxia leads to decreased generation of $H_2O_2$ resulting in $K^+$ channel closure that triggers downstream events leading to neurotransmitter release. Studies using oxidase (gp91phox/Nox2) deficient neonatal mice confirmed the critical role of gp91phox/Nox2 protein in NEB $O_2$ sensing, as these mice failed to respond to hypoxia in both in vitro and in vivo studies. Thus the predominant $O_2$ sensor in NEBs appears to be a complex of Nox2/$K_v$3.3 (and possibly combinations with other $K_v$ alpha subunits). Future studies will determine the role of recently described Nox homologues and their potential $O_2$ sensitive $K^+$ channel partners (i.e., acid sensitive two pore domain TASK channels). The membrane based $O_2$ sensor used by pulmonary NEBs appears to be unique since other $O_2$ sensing cells (i.e., CB glomus cells, adrenal medullary cells and pulmonary artery smooth muscle cells) use mitochondria as the primary source of $H_2O_2$ that modulates $K^+$ channel activity, suggesting cell and organ specific $O_2$ sensing mechanisms rather than a monolithic system. However in spite of progress in the molecular characterization of $O_2$ sensing, the precise role(s) for NEBs in the lung remain(s) undefined. Postulated functions include that of airway $O_2$ sensors involved in respiratory control, particularly during the perinatal period. There is accumulating evidence that NEB cells could also be sensing $pCO_2$/pH changes in the airways and thus may represent bi-modal receptors similar to the CB. Clearly many more studies, using physiological approaches are required to establish the role and function of NEBs in normal and diseased lungs.

The observation of large relative numbers of PNECs/NEBs in fetal lung compared to the adult focused attention on a possible role of these cells during lung development. Since PNEC are the first cell type to differentiate within the primitive airway epithelium, prior to the emergence of other airway epithelial cell types, a role in lung growth and epithelial differentiation has been suggested. The question of the origin, ontogeny and molecular regulation of PNEC differentiation has been a subject of recent investigations. Although the precise origin of PNECs is at present unclear, current evidence suggests derivation from the foregut endoderm that gives rise to pluripotent epithelial progenitors, analogous to endocrine cells in the gastrointestinal tract and pancreas. Putative PNEC progenitors have been identified using antibodies against early neuronal developmental markers, such as FORSE-1 that recognizes the "forebrain surface embryonic" antigen expressed in the developing CNS. During early stages of fetal lung development, FORSE-1 antibody labeled all the cells of the primitive airway epithelium and later became restricted to 5-HT positive PNEC/NEB. At later stages of lung development there was gradual loss of the FORSE-1 epitope with

further PNEC differentiation. The presence of a few FORSE-1/5-HT expressing cells in postnatal lung is consistent with the retention of progenitors in mature lungs.

At the molecular level, it is now evident that genes involved in neuronal fate determination belonging to the family of basic-loop-helix (bHLH) transcription factors, such as achaete-scute-homolog-1 (Mash 1 in rodents and hASH 1 in humans) and hairy-enhancer of split (Hes1), play related but opposite roles in governing PNEC lineage fate in the lung. Mash1 k/o mice lack PNECs and die of respiratory failure soon after birth. It is of interest that the lungs of heterozygote (Mash+/-) mice show 50% reduction in the number of PNEC/NEB accompanied by an abnormal breathing pattern, supporting the role of these cells as airway sensors. There is also accumulating evidence that NEBs may serve as a pulmonary stem cell niche, providing a cell microenvironment for toxin-resistant stem cells as demonstrated in the naphthalene mouse model of acute airway injury.

Based on these recent studies, an overall picture is emerging where PNEC/NEB can be viewed as a multifunctional cell system with roles that are developmentally regulated. Thus during the early stages of lung development, PNECs could be modulators of fetal airway growth and differentiation, at the time of birth as airway $O_2$ sensors involved in neonatal adaptation, and postnatally and beyond as providers of a lung stem cell niche that is important for airway epithelial regeneration and in lung carcinogenesis.

An additional role for NEBs may involve modulation of immune responses in the airways via production of a variety of peptide mediators such as CGRP that, in addition to vasodilatory effects, exhibits chemotactic properties, and bombesin/GRP that appears to be involved in mast cell recruitment and activation. In an experimental mouse model of asthma, increased exocytosis of dense-cored granules (the storage site for amine and peptide mediators) from NEB cells was observed after allergen challenge, suggesting a potential role for these cells in the pathophysiology of asthma.

When comparing morphologic, physiological and developmental aspects of pulmonary NEBs, the presumed airway $O_2$ sensors, with that of CBs, well established and extensively studied arterial chemoreceptors, a number of similarities and fundamental differences emerge. At the anatomical level, CBs form discrete paired organs composed of densely vasularized clusters of glomus cells that produce and secrete amine and peptide neurotransmitters and are innervated by the glossopharyngeal nerve. These anatomical features have facilitated physiological and other experimental studies on CBs that defined their role as principal arterial chemoreceptors monitoring arterial $pO_2$, $pCO_2$ and pH. In contrast NEBs monitor $pO_2$ in inhaled air, are composed of small cell clusters widely dispersed within the epithelium of intrapulmonary airways, and are innervated mainly by vagal afferents. The relatively small number of NEB, representing <1% of all airway epithelial cells, together with a difficult access, complicates direct experimental studies. This obstacle has been partially overcome by developing methods to study NEBs *in vitro* and by the use of NEB cell related tumor cell lines to investigate their biochemical and molecular aspects. Using the patch-clamp

and carbon fiber amperometric methods, it has been shown that NEB cells, as their CB glomus cell counterparts, exhibit features of neurosecretory cells with membrane properties of excitable cells. In both cell types the response to hypoxia is modulated by $O_2$ sensitive $K^+$ channels that trigger downstream events resulting in neurotransmitter release.

Possible interactions and complementary activity between the two chemoreceptor systems, i.e., the airway based NEB and arterial CB is evidenced by an observation of differences in the time course of structural and functional maturation. During the early perinatal period, NEBs appear to mature in advance of the CB, suggesting a critical function in neonatal adaptation. Theoretically, detection of changes in $pO_2$ directly in the airway facilitates a more rapid response to hypoxia that may be of importance to the neonate.

The chapter on solitary chemosensory cells (SCCs) in the airways reviews data on the distribution, immunocytochemistry, fine structure, ontogeny and function of these cells in different species from aquatic vertebrates to mammals. Studies in primary aquatic vertebrates have identified SCCs in the epidermis, oropharynx and gills. These diffusely distributed chemoreceptor cells are related to but distinct from the taste buds. SCCs constitute a third chemosensory modality in addition to the better known senses of taste and smell. The elementary unit of SCCs consists of a single bipolar cell contacted by an afferent nerve fiber. The overall shape of the cell is determined by its location within a particular epithelium, usually with their apical surface contacting the lumen to receive sensory stimuli. Their presumed function is to monitor the incoming water or air stream for possible irritants or toxins, and for predator avoidance. Phylogenetically, SCCs appear prior to the development of taste buds.

In mammals SCCs have been described at specific sites in the digestive and respiratory systems. For example, in the rat SCCs were found in the nasal cavity, in the vallate papillae of the tongue, in the larynx, trachea and bronchi. In humans, they have so far been described in the nasal cavity. The so-called "brush cells", which may be related to SCCs, are characterized by distinct apical microvilli and pleiomorphic cytoplasmic granules. These cells have been described in the epithelia of trachea, bronchi and even in the alveolar region of the rat and human respiratory system. Immunohistochemical studies have identified in SCCs expression of alpha-gustducine and phospholipase C beta 2, both markers of gustatory sensory receptor cells. The role of these enzymes in the function of SCCs is at present unknown but could include local antimicrobial defense via G-protein mediated secretory responses.

The tracheal epithelium of mammalian the respiratory system harbors solitary PNECs that express 5-HT as well as CGRP and bombesin (in human).These cells, as their NEB counterparts in the intrapulmonary airways are extensively innervated as shown by recent studies using confocal microscopy. Tracheal PNECs also express $O_2$ sensing properties and may effect bronchomotor tone as demonstrated in experiments using isolated tracheal preparations with intact and denuded epithelium. It has been

proposed that, at least in the guinea pig, solitary PNECs may control spontaneous airway contraction, and may potentially be involved in the pathophysiology of asthma.

The last chapter discusses the evolutionary trends in $CO_2/H^+$ chemoreception. In most vertebrates, $CO_2$/pH-sensitive chemoreceptors of the airway passages play an important role in regulating ventilation. In strictly water-breathing fish, cardiorespiratory reflexes are responsive primarily to changes in $O_2$ concentration, but a modulatory role is played by water-sensing, $CO_2$-sensitive, branchial chemoreceptors that are activated by hypercarbia. The transition to air breathing has resulted in the emergence of central $CO_2$/pH chemosensitivity, while the peripheral airway $CO_2$/pH chemoreceptors play a modulatory role in ventilatory control. In bimodal breathers, current evidence supports the existence of both water-sensing $CO_2$/pH chemoreceptors located in the gills or orobranchial cavity, and $CO_2$-sensitive pulmonary stretch receptors. In air-breathing vertebrates, several types of $CO_2$/pH-sensitive receptor groups have been identified, particularly in the nasal epithelium. These olfactory $CO_2$-sensitive chemoreceptors are inhibitory, but their precise role is unclear. In birds and reptiles, intrapulmonary $CO_2$/pH-sensitive chemoreceptors are linked to pulmonary stretch receptors in which the pattern of response to the primary stimuli of changes in lung volume, pressure or wall tension is modulated by $CO_2$. The function of these receptors is to reduce dead space ventilation and enhance the efficiency of $CO_2$ elimination during hypercarbia. Thus, the $CO_2$/pH-sensitive chemoreceptors of the respiratory tract share a common purpose of modulating breathing pattern.

It is hoped that the readers of this book will be inspired by the enthusiasm and fascination of the authors with the complexity and diversity of airway chemoreceptors, which at a structural and functional level share many fundamental similarities across the various species. Opportunities abound for further investigations of airway sensory systems and their roles in normal physiology and in disease. As the future of the planet earth is being threatened by global warming and climate change, a better understanding of the sensory systems that monitor and respond to changes in the oxygen and carbon dioxide concentrations in the environment may be of vital importance for the survival of all species.

**ERNEST CUTZ,** MD, FRCPC
Professor of Pathology
The Hospital for Sick Children
*and*
University of Toronto
Toronto, Ontario, Canada

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**Color Plate Section**

# List of Contributors

**Adriaensen, D.**
Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171 BE-2020, Antwerp, Belgium

**Bailly, Y.J.R.**
Cytologie et Cytopathologie Neuronales, Département Neurotransmission et Sécrétion Neuroendocrine, Institut de Neurosciences Cellulaires et Intégratives CNRS UMR 7168, 5, rue Blaise Pascal, 67084 Strasbourg, France

**Beltrán-Frutos, E.**
Department of Cellular Biology and Histology, Medical School, University of Murcia, Campus de Espinardo, 30100, Murcia, Spain

**Benati, D.**
Department of Morphological-Biomedical Sciences, Anatomy and Histology Section, University of Verona, Medical Faculty, Italy

**Bollé, T.**
Leuven Catholic University, Medical Faculty, Department of Morphology and Molecular Pathology, Minderbroedersstraat 12

**Brouns, I.**
Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171 BE-2020, Antwerp, Belgium

**Burggren, W.**
Department of Biological Sciences, University of North Texas,, Denton, TX 76205, USA

**Cecchini, M.P.**
Department of Morphological-Biomedical Sciences, Anatomy and Histology Section, University of Verona, Medical Faculty, Italy

**Crescimanno, C.**
Faculty of Exercise and Sport Science, Kore University, Enna, Italy

**Cutz, E.**
CIHR Group on Lung Development Division of Pathology and Department of Paediatric Laboratory Medicine, The Hospital for Sick Children, and Department of Laboratory Medicine & Pathobiology, University of Toronto, Canada

**De Proost, I.**
Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171 BE-2020, Antwerp, Belgium

**Finger, T.E.**
Department of Cell and Developmental Biology, University of Colorado, School of Medicine, Mail Stop 8108, PO Box 6511 Aurora, CO 80045

**Fu, W.X.**
Division of Neuroscience, Oregon Health Science University, Beaverton, Oregon, USA

**Gilmour, K.M.**
Department of Biology, University of Ottawa, Ottawa, ON, Canada

**Goniakowska-Witalińska, L.**
Jagiellonian University, Department of Comparative Anatomy, Institute of Zoology, Krakow, Poland

**Gopesh, A.**
Department of Zoology, University of Allahabad, Allahabad 211002, India

**Hansen, A.**
Department of Cell and Developmental Biology, University of Colorado, School of Medicine, Mail Stop 8108, PO Box 6511 Aurora, CO 80045

**Hempleman, S.C.**
Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011-5640, USA

**Jonz, M.G.**
Department of Biology, University of Ottawa, Ottawa, ON, Canada

**Kubke, M.F.**
Department of Anatomy with Radiology, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

**Kusakabe, T.**
Laboratory for Anatomy and Physiology, Department of Sport and Medical Science, Kokushikan University, Tokyo, Japan

**Merigo, F.**
Department of Morphological-Biomedical Sciences, Anatomy and Histology Section, University of Verona, Medical Faculty, Italy

**Milsom, W.K.**
Department of Zoology, University of British Columbia, Vancouver, BC, Canada.

**Nurse, C.A.**
Department of Biology, McMaster University, Hamilton, ON, Canada

**Osculati, F.**
IRCCS Centro Neurolesi "Bonino-Pulejo" Messina, Italy

**Pan, J.**
CIHR Group on Lung Development, Division of Pathology and Department of Paediatric Laboratory Medicine, The Hospital for Sick Children, and Department of Laboratory Medicine & Pathobiology, University of Toronto, Canada

**Pan, T.-C.**
Department of Biological Sciences, University of North Texas, Denton, TX 76205, USA

**Pastor, L.M.**
Department of Cellular Biology and Histology, Medical School, University of Murcia, Campus de Espinardo, 30100, Murcia, Spain

**Pecio, A.**
Jagiellonian University, Institute of Zoology, Department of Comparative Anatomy, R. Ingardena 6, 30-060 Krakow, Poland

**Pilarski, J.Q.**
Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011-5640, USA

**Pintelon, I.**
Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171, BE-2020 Antwerp, Belgium

**Podkowa, D.**
Jagiellonian University, Institute of Zoology, Department of Comparative Anatomy, R. Ingardena 6, 30-060 Krakow, Poland

**Sbarbati, A.**
Department of Morphological-Biomedical Sciences, Anatomy and Histology Section, University of Verona, Medical Faculty, Italy

**Skogvall, S.**
PharmaLundensis AB, Bio Medical Center D10, 221 84 Lund, Sweden

**Suthers, R.A.**
Medical Sciences, Indiana University, Bloomington IN 47405, USA

**Timmermans, J.-P.**
Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171, BE-2020 Antwerp, Belgium

**Tizzano, M.**
Rocky Mountain Taste and Smell Center, Department of Cell and Developmental Biology, University of Colorado, Denver, Aurora, USA

**Van Lommel, A.T.L.**
Leuven Catholic University, Medical Faculty, Department of Morphology and Molecular Pathology, Minderbroedersstraat 12, 3000 Leuven, Belgium

**Wild, J.M.**
Department of Anatomy with Radiology, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

**Yeger, H.**
Division of Pathology, Department of Paediatric Laboratory Medicine, The Research Institute, CIHR Lung Development Group, The Hospital for Sick Children, Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

**Zaccone, G.**
Department of Animal Biology and Marine Ecology, Faculty of Science, University of Messina, Salita Sperone 31, Messina, S. Agata I-98 166, Italy

## Neurosecretory Epithelial Cells (NEC's) in the Airways and Carotid Labyrinth of Aquatic Vertebrates: Morphology, Distribution, Innervation and Function