MONOAMINE CONTAINING CELLS IN FISH GILL EPITHELIA

A QUANTITATIVE COMPARISON OF MONOAMINE CONTAINING CELLS IN FISH GILL EPITHELIA

By

ERIK DREIFELDS, B.Sc.H.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1997) McMaster University
(Biology) Hamilton, Ontario

TITLE:A Quantitative Comparison of Monoamine Containing Cells in Fish Gill Epithelia

AUTHOR: Erik Dreifelds, B.Sc.H. (Brock University)

SUPERVISOR: Professor C.A. Nurse

NUMBER OF PAGES: vi, 91

Abstract iii.

Serotonin positive ($5HT^+$) and tyrosine hydroxylase positive (TH^+) cells were identified using fluorescent immunocytochemical methods and quantified in the gill epithelium of six species of fish. $5HT^+$ cells were located in the filament epithelium in contact with the basal lamina on the efferent side, and in the lamellar epithelium where they were occasionally exposed to the external milieu. Thus, these cells appear to represent two populations of neuroepithelial cells (NEC) as proposed in other studies. In trout, bass and killi fish, NECs were revealed exclusively in the primary epithelium. In tilapia, NECs were located exclusively in the secondary epithelium, whereas in perch and zebrafish they occurred in both epithelial layers. The two types of NECs varied in number both within and among the species. Seasonal comparisons of NECs in perch revealed a decrease in cell density in the filament between July and November, though there was no significant difference in the density of NECs in the lamellae. TH^+ cells were identified in perch, zebrafish and killi fish. In zebrafish TH^+ cells occurred in similar numbers to $5HT^+$ cells, and were generally present in similar locations. It is likely that in this case, many of the labelled cells were positive for both markers. In two of the species, perch and killi fish, the density and distribution was such that the TH^+ cells and $5HT^+$ cells were unlikely to be the same. A quantitative comparison of total catecholamine (CA) stores, using high performance liquid chromatography (HPLC), revealed that gill tissues in general contained higher levels of epinephrine (EPI) than norepinephrine (NOR) and dopamine (DOP). Finally, attempts were made to determine whether NECs would survive in 2-4 day old cultures of dispersed gill cells from perch, using immunocytochemical labelling for 5HT. A few successful cases are presented.

Acknowledgements

I would like to thank Dr. Nurse for his supervision and giving me the opportunity to work in his lab.

I would also like to express my appreciation to Dr. MacDonald, who also allowed me to work in his lab and I am grateful for the technical advice and insights which brought new perspectives on difficult situations that both he and Dr. Wood gave generously. While working in Dr. Mac Donald's lab, Vandy Thomas gave her time and invaluable help which greatly facilitated my work, and for that I am very grateful.

I am glad to have had the opportunity to work as a teacher's assistant for Dr. Dingle and have gained an immense amount of knowledge about developmental biology. I have also learned a lot from our political and philosophical discussions, which I thoroughly enjoyed, they will be missed.

I am also grateful I had the opportunity to work with Dr. Adele Jackson. Her hard work and commitment to research was an inspiration to many around her. I would like to thank her not only for the invaluable assistance she gave me during the past two years but also for her friendship.

Cathy Vollmer puts her heart and soul into her work and generously gave her time to help whenever required, she also offered encouragement and laughter in difficult times for which I am truly grateful and I will always value our friendship.

To my wife, Nathalie, I would like to express my deep appreciation for her patience, kindness, support and love, through the good and bad times but particularly during the past months which I am sure have been very trying.

Table of Contents

Introduction

Pearse (1968) characterized a group of neuroendocrine cells and described the amine precursor uptake and decarboxylation (APUD) system. Specific characteristics of these cells include storage of amines and some bioactive peptides. They have a secretory function and act as either mechano- or chemo-receptors (Zaccone et al., 1994). A group of cells which belong to this family is found in the gills and lungs of lower vertebrates as well as mammalian pulmonary tissues called neuroepithelial cells (NEC). They are thought to have an O_2 sensitive chemoreceptive role (for review see Cutz, 1997). NECs have been found in numerous species and have been shown to contain many specific peptides. These markers are helpful in the identification and localization of these cells and include neuron specific enolase, calcitonin, enkephalins and substance P (for review see Polak et al. 1993). The most commonly employed indicator in the description of these cells, however, is serotonin (5-HT).

Neuroepithelial cells which have APUD properties were first characterized in mammalian lungs by Feyrter (1938) and later described in more detail, including the discovery of their innervation by Frohlich (1949). Since then, numerous studies have examined these cells in the respiratory lining of the lung and described their distribution as solitary cells, as well as in groups which are frequently exposed to the external environment, termed neuroepithelial bodies (NEB) by Lauweryns et al. (1972). This group also characterized the release of amines by NEBs in response to hypoxic conditions and hypothesised that they act as chemoreceptors. This was verified by Youngson et al.

(1993) who demonstrated that NEBs have an O_2-sensitive potassium channel coupled to an O_2 sensor protein, thereby implicating them in the detection of hypoxic conditions in the airway and possibly acting to regulate respiration .

With the aid of numerous markers, NECs have been described in the respiratory tissues of a wide variety of lower vertebrates including birds (Walsh and McLelland 1974), reptiles (Pastor et al. 1987), and amphibians (Goniakowska-Witalinska et al. 1992), suggesting it is a highly conserved cell type evolutionarily. The lungs of mammals which serve as a very large surface area for gas exchange between the blood and external environment, could be considered an "internalized gill" , an evolutionary precursor originating in lower vertebrates. In terms of evolutionary significance, an interesting observation was made in numerous air-breathing fishes which have gills and lungs. Goniakowska-Witalinska et al. (1995) revealed that the bowfin, *A. calva,* contains NECs in the epithelia of both respiratory tissues (Zaccone et al., 1997). NECs have also been described in numerous fish species including several teleosts.

Dunel-Erb et al. (1982) used immunohistochemistry and electron microscopy finding that the cells are located mostly in the primary or filament epithelial layer and are more numerous at the distal tips. The NECs were found to be 5-HT positive and in close proximity to both the external surface facing the water flow, and the vasculature containing arterial blood. These cells were located adjacent to the basal lamina of the epithelial layer and contained small dense core vesicles (DCV) which were highly concentrated in cytoplasmic areas adjacent to nerve terminals (Dunel-Erb et al., 1982).

After exposure to an anoxic environment the DCVs, thought to contain 5-HT, were degranulated, which suggests that NECs in fish gills may act as O_2-sensitive chemoreceptors. There are some studies which have conjectured that the NECs in the gills of fish are homologous to the NEBs in mammalian lungs and/or type I cells in the carotid body based on the evident similarities of these cells morphologically (Dunel-Erb et al. 1982), and similar physiological responses of the gills to the carotid body (Burleson and Milsom, 1993). Zaccone et al. (1992) have described NECs in fish gills and determined that these cells are located in both the filament and lamellar epithelia. Those located in the primary (filament) epithelium which never reach the external milieu have been termed "closed" type. Those cells touching the external surface found in the secondary lamellae and occasionally in the primary epithelial layer have been termed " open" type (Zaccone et al., 1992).

Quantification of the closed type NECs in the primary epithelium of trout using toluidine blue staining of semi-thin sections from resin imbedded tissues, determined there is a seasonal variation in cell numbers. NECs numbers decreased during the winter compared to spring (Dunel-Erb et al. 1994). The open type cells in the secondary epithelium were not examined.

Various groups have isolated NECs and NEBs from the pulmonary tissue of different mammals which is required in order to study cellular and membrane properties (Sonstegard et al., 1982, Cutz et al., 1985). The isolation of NECs from lower vertebrates may shed some light on the evolutionary questions related to this cell and its function.

This study has dispersed and cultured gill cells from the yellow perch and identified 5-HT positive cells in vitro using immunocytochemistry. This study also examined and quantitatively compares NECs, both closed and open types, in the gill epithelia of six species of fish including, rainbow trout (*Oncorhyncus mykiss*), Nile tilapia (*oreochromis niloticus*), killifish (*Fundulus heteroclitus*), bass (*Micropterus dolomieui*), Yellow perch (*Perca flavesens*) and zebrafish (*Danio rerio*) with special reference to possible seasonal effects on both populations of NECs in the perch. In addition, a population of tyrosine hydroxylase (TH) positive cells was found in the gills of three of these species. High performance liquid chromatography (HPLC) was used to separate the catecholamines (CA) stores in the gill tissue to determine if higher levels of CA could be detected in species with gill tissues rich in TH positive cells compared to those species lacking them.

Literature Review

Neuroendocrine Cells

Neuroendocrine cells characteristically contain small dense-cored granules and have the ability to take up and convert extracellular amine precursors such as tryptophan into serotonin and deoxyphenylalanine (L-dopa) into dopamine. Because of this unique capability, cells which have these characteristics are said to belong to a system called **A**mine **P**recursor **U**ptake and **D**ecarboxylation (**APUD**; Pearce, 1968). Cells belonging to this group have been found in numerous tissues including, the gastrointestinal tract, pancreas, thyroid gland, adenohypophysis, adrenal medulla, the carotid body and the respiratory lining of the lung (see Sorokin, 1988). Another organ known to contain neuroendocrine cells is the skin of fish and amphibians (Whitear, 1989).

Merkel Cells

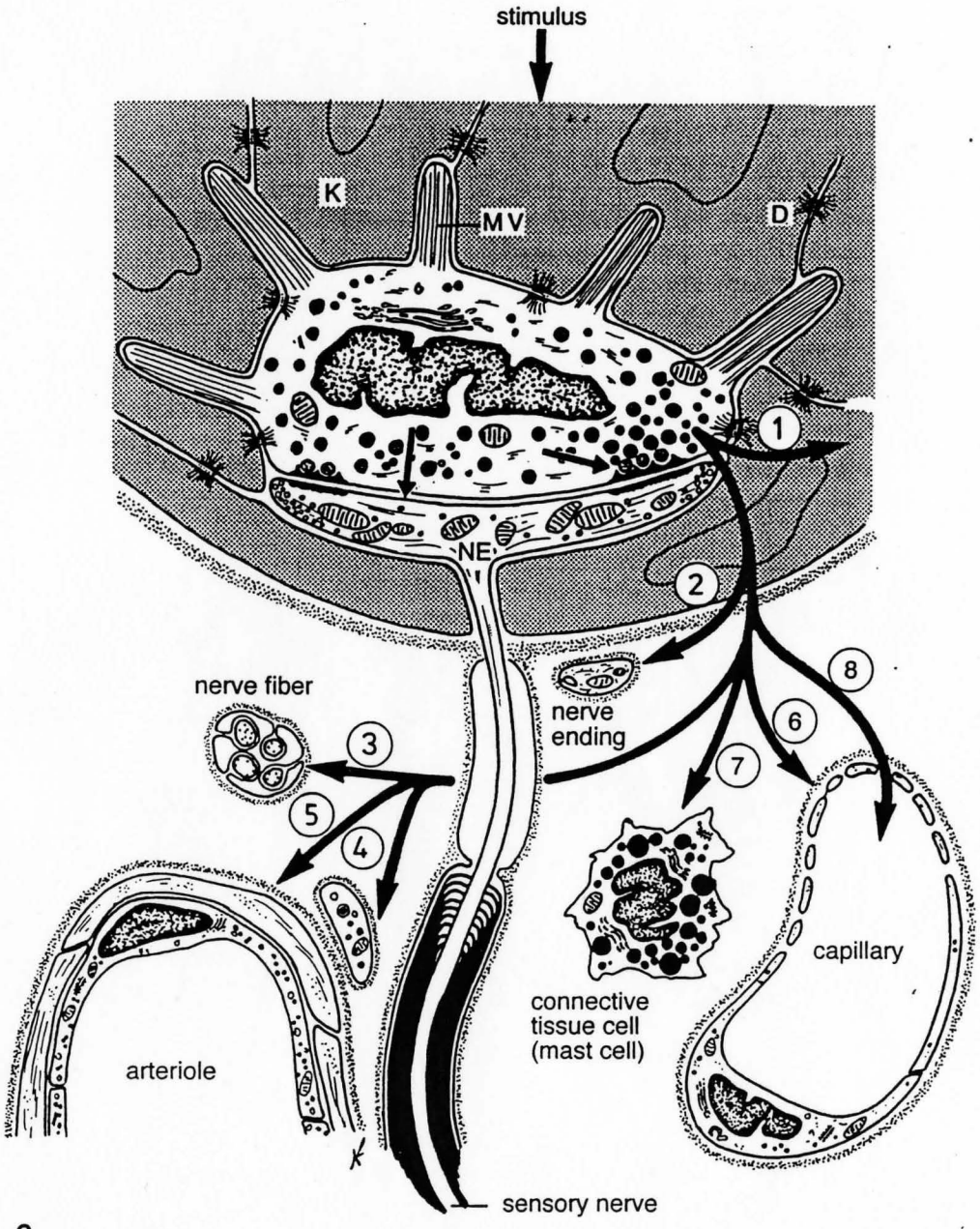
The skin in many lower vertebrates participates in osmoregulation and sensory reception as well as functions as a respiratory organ. The neuroendocrine cells of the skin have been named Merkel cells, and are found not only in the outer epidermis but also in the oral epithelium, the outer root sheath of vibrissae follicles in rats (Patrizi and Munger, 1966), and the skin of platypus bills (Manger and Hughes, 1992). The cells contain amines as well as bioactive peptides, and have been identified with numerous markers including neuron-specific enolase, chromogranin, substance P, peptide histidine isoleucine, calcitonin gene-related peptide, vasoactive intestinal polypeptide, met-enkephalin, leucine

aminopeptidase and serotonin (review Zaccone et al., 1994). These cells receive a sensory innervation, and are thought to display secretory function during mechanosensory (tactile) stimulation. The possible effects of a local secretion of these bioactive substances in the surrounding tissues are many. Figure 1 schematically illustrates potential effects on the tissues surrounding a Merkel cell (from Zaccone et al., 1994). These include release of growth factors, thought to play an important role in epidermal cell differentiation and replenishment (Moll et al., 1986). The secretions could also target local nerve endings, smooth muscles, blood cells and could even have effects on more distant targets by release of substances into the blood stream. Another cell found in many vertebrates which is morphologically similar to the Merkel cell and also belongs to the APUD cell family is the neuroepithelial cell (NEC).

Neuroepithelial Cells in Mammalia

NECs are located in the pulmonary lining of mammalian lungs sometimes referred to as pulmonary neuroendocrine cells or PNEC (Cutz, 1997). As discussed later, they are also found in the respiratory epithelium of gills and lungs of lower vertebrates. NECs were first described by Feyrter (1938) in mammalian tissues including urogenital, gastrointestinal and bronchial epithelial linings. The morphology of these cells was reported in more detail by Fröhlich (1949) who described their close association with nerve endings. The clustering of these cells in groups within pulmonary tissue was reported by Glorieux (1963) and the clusters were termed neuroepithelial bodies or NEB

Figure 1. A schematic representation of the structure and functions of a Merkel cell in mammalia skin. The cell has many dense core vesicles (DCV) and microvilli (MV) which contact keratinocytes (K). The Merkel cell is innervated by numerous nerve endings (NE). The release of DCV contents after stimulation act on sensory nerves via synaptic and non-synaptic contacts. Effects of release on neighbouring cells are exerted by (1) local release of growth factors on sensory nerve endings of the dermo-epidermal junction; (2) directly on nerve fibres; (3) on autonomic nerves during sensory development and regeneration; (4) on red blood cells; (5)and (6) on arteriole and capillary smooth muscle tone affecting permeability; (7) release of histamine from mast cells (8) and release into the blood stream which act on distant targets. Desmosomes (D). From Harschuh at al., (1986).


stimulus
K
MV
D
1
NE
2
nerve fiber
3
5
4
nerve
ending
8
6
7
capillary
connective
tissue cell
(mast cell)
arteriole
sensory nerve

6

by Lauweryns et al. (1972). The cells have been described as triangular with large round nuclei and are located adjacent to the basal lamina of the respiratory epithelium. The apex of the cells occasionally reached the lumen, thereby permitting contact with the external environment (Fröhlich, 1949). Ultrastructural studies in the electron microscope revealed the presence of characteristic small dense core vesicles (DCV) ranging from 60-200 nm in diameter. They were frequently concentrated in the cytoplasmic area, juxtaposed to the basal lamina (Scheuermann, 1997). Cutz et al. (1975) used formaldehyde-induced fluorescence (FIF) to reveal the presence of NEBs in tissue sections of the respiratory lining. These NEBs contained the indolamine, serotonin (5-HT), as revealed by the yellow fluorescence resulting from excitation of the fluorophore by U.V. light. It was also noted that the colour given off when the tissue was treated with FIF became greener after preincubation with dihydroxyphenylamine, suggesting that the NEBs also contained catecholamines (Scheuermann et al., 1988). In addition to the presence of monoamines there have been numerous bioactive peptides identified in PNECs. These peptides have also been found in other neuroendocrine cells such as the Merkel cell, mentioned above. Some of these markers include, neuron specific enolase, bombesin, chromogranins, calcitonin and leu-enkephalin and many have been located in the DCVs using immunocytochemical procedures combined with electron microscopy (reviewed by Scheuermann, 1997). NEB cells are also innervated and both afferent and efferent nerve profiles have been observed in contact with these cells. The presence of adrenergic, cholinergic, and non-adrenergic non-cholinergic, innervation have also been described and

there is evidence that the adrenergic innervation originates from sensory neurons of the nodose ganglion. NEB have long been hypothesised to act on O_2-sensitive chemoreceptors in the respiratory tissue. This theory was strengthened by the fact that the DCVs, which contain 5-HT and neuropeptides (see above), are depleted as the cells degranulate when stimulated by an hypoxic stimulus (Cutz et al., 1993). Direct evidence that NEBs were O_2 receptors was obtained in electrophysiological studies, where isolated NEB cells were found to have an O_2-sensitive potassium channel as well as a putative O_2 -sensitive protein, NADPH oxidase (Youngson et al., 1993). During hypoxia closure of these potassium channels increased membrane depolarization and/ or action potential frequency. This would be expected to stimulate neurotransmitter release and signal an increase in ventilation during hypoxia in the airways (Youngson et al., 1993).

NECs have been found to be highly conserved throughout evolution (Zaccone et al., 1997), and their characteristics will be described in birds, reptiles, amphibians, and fish (air and water breathers).

Neuroepithelial Cells in Aves

The pulmonary system of avians is unique relative to other vertebrates due to the air sac system. The lung tissue itself is, however, generally similar in structure to mammalian lungs. Adriaensen et al. (1994) investigated whether NECs or NEBs were present in the Quail respiratory lining. This study showed there were two populations of NECs, both of which were positive for 5-HT, but only one colocalized bombesin and

somatostatin. The cells could not be distinguished morphologically at an ultrastructural level. They were found in the primary and secondary bronchi of the lung, but not in any bronchioles. At the electron microscopic level, the NEC revealed many similarities with mammalian NEBs. They were found adjacent to the basal lamina in groups as well as single cells. Those that were innervated, contained characteristic DCVs concentrated in the basal portion of the cell adjacent to the nerve terminal. Adriaensen et al. (1994) argued that the grouped NECs should not be considered NEBs, since unlike mammals, these cells were never seen exposed to the external environment of the lumen. Other avian species such as pigeons (McLelland and MacFarlane, 1986) and chickens (Walsh and McLelland, 1974) do have mammalian-like NEC clusters which were exposed to the luminal surface . Among avian species NECs demonstrate differences in location as well as biogenic contents of the DCVs. In spite of these differences, in all the species examined the NECs where morphologically similar and contained monoamines and are classified as belonging to the APUD family.

Neuroepithelial Cells in Reptilia

Another group of lower vertebrates has been shown to contain NECs in the lung tissue. The pulmonary lining of the reptile, *Basiliscus vittatus,* commonly known as the Jesus-lizard, was found to contain cells which stained positively for 5-HT and calcitonin (Van den Steen, 1994). These cells were adjacent to the basal lamina and were never in contact with the luminal surface. In fact, a thick cell layer often separated them from the

external environment. NECs found in most species frequently extended cellular processes across the basal lamina and these processes contacted nerve profiles. The NECs in reptiles were found to be innervated in a unique manner. Nerve fibres in reptile respiratory epithelium frequently extended across the basement membrane and formed complex, branched structures which were observed in synaptic contact with NECs. This "basket formation" has been suggested to be prominent in NEBs which do not have luminal contact. Van den Steen (1994) suggested that the NECs may act as mechanoreceptors as opposed to chemoreceptors. This cellular morphology has been described in other lower vertebrates though it does not apply to all species. Scheuermann et al., (1983) have shown that NEBs open to the lumen in a reptilian species. Reptiles, much like birds, include species with NEC clusters which were exposed to the lumen surface and species with NECs which never reach the external environment. What was found to be unique in reptiles was the innervation pattern which had the "basket formation". Like the aves and the reptiles, some amphibian species also have NEBs which are exposed to the respiratory surface.

Neuroepithelial Cells in Amphibia

Amphibians have been shown to contain NECs and NEBs in the bronchial lining of the lungs (Goniakowska-Witalinska, 1997). Some amphibians such as newts have a relatively simple lung structure with a hollow sac-like appearance. The internal lining consists of one type of pneumocyte and ciliated cells which were observed along the

posterior side of the lung in close proximity to the pulmonary vein (Goniakowska-Witalinska, 1980). In this study NECs were strictly associated with the ciliated epithelium as single cells close to the lumen surface. They had an ovoid shape and contained a large lobed nucleus. The cells were in close association with the basal lamina and frequently, they were also adjacent to blood vessels located below the basal lamina. The NECs of most amphibians have small DCVs ranging from 30-200 nm depending on the species. Except for the lack of NEB structure, the cells are morphologically very similar to NECs found in other subclasses.

Goniakowska-Witalinska et al. (1992) examined NECs in the tiger salamander. The typical lung structure of salamanders is more complex than newts, consisting of three levels of septa located in the lumenal space. The first level of septa is the longest with the apex reaching towards the centre of the lumen; the second level is closer to the wall of the lung, while the third level is the shortest, with the apex furthest from the centre of the lumen. Single NECs were located at the apex of the second level of septa, separated from the external surface by a thin layer of mucous cells. NEBs on the other hand, were found in the apex of the primary septa where the cells adhered tightly to one another in clusters of 3-5. The NEBs were exposed to the lumen surface usually via one or two ciliated NECs, while the rest of the cells were covered by a layer of pneumocytes or mucous cells. Goniakowska-Witalinska et al. (1992) also reported for the first time in a vertebrate two types of NECs that were distinguished by the size of the DCVs. The first type of NEC (type 1) had many DCVs ranging in size from 70-140 nm and occasional

ones, 300-600 nm in diameter. The second type (type 2) had larger DCVs, mostly in the range 140-260 nm and occasional larger ones 320-700 nm in diameter. The significance of the subtypes is unclear, but it is known that type 1 NECs are found as single cells in the secondary septa, while both types of NECs are grouped in the primary septa as NEBs. Since the ciliated NECs which contact the lumen surface are type 2 NECs, it appears that the type 1 NECs do not normally contact the external environment. Immunocytochemistry revealed that the NEBs stained positively for enkephalins and 5-HT. The NEBs were also described as contacting both efferent and afferent nerve profiles.

Neuroepithelial Cells in Air breathing Fish (Palaeoniscoidea, Crossopterygii, and Holostei)

Thus far, the NECs and/or NEBs of numerous vertebrates have been described including the subclasses mammalia, aves, reptilia, and Amphibia. The next group which will be discussed are air breathing fish including *Polypterus, Protopterus, and Amia.* These bimodal breathers are seen as a key link in the evolutionary scale, since they appear to represent an intermediate step between the water breathing gills of lower vertebrates and the air breathing lungs of higher chordates. Zaccone et al. (1989) described NECs in the lung of *Polypterus* and these were found in the ciliated tracts of the respiratory epithelium. The cells were exposed to the lumen via small processes with microvilli. These NECs were not innervated but were adjacent to the basal lamina and contained

DCVs ranging in size from 80-165 nm in diameter. Another study by Adriaensen et al. (1990) revealed similar cells in the lungs of *Protopterus,* with the exception that they were innervated and had slightly larger DCVs.

In a third study NECs were found in the gills of the bowfin *Amia calva* and these appeared to lack an innervation (Goniakowska-Witalinska et al., 1995). They were usually found as single cells, but occasionally occurred in groups of two or three and they never reached the mucosal surface. The NECs were identified by immunocytochemistry and were found to colocalize 5-HT, neuron specific enolase, and enkephalins. In the lungs of the same species (reviewed by Zaccone et al., 1997), NECs were found in contact with the external environment, and were flanked by goblet cells. They were usually found as solitary cells or in small groups (2-3), and no innervation was observed.

NEC Phylogeny

The phylogeny of the NECs then, can be examined in steps relative to chordate subclasses. The bowfin demonstrated non-innervated NECs in both the gill and the lung; however, only in the latter were the cells exposed to the external surface. In this species the NEB structure appeared to be absent. The amphibians also have solitary NECs, though NEBs were present in some species. There were no NEBs present in newts, and though single NECs were present, they lacked an innervation. The cells of the NEB structure in salamanders occasionally reach the lumen surface and were found to be innervated. Reptiles, much like amphibians, demonstrate NEBs which are sometimes

exposed to the external environment depending on the species. For the most part, the reptilian cell clusters were innervated and, like all the NECs examined, were in close association with the basal lamina. Consistent with other subclasses, the NECs and NEBs in bird lungs varied in their degree of exposure to the outer surface but the majority of NEBs were richly innervated. Finally, in the mammals pulmonary NEBs were exposed to the external environment (ie. the airways) and were consistently innervated. The NEC demonstrates a highly conserved pattern through evolution in regards to morphology and though the cellular function has not been clearly defined in each case, the cells appear to be recepto-secretory. In order to better understand the origins and evolution of the neuroendocrine system with particular reference to the NEC, it is important to study early chordate subclasses, such as the Actinopterygii including Palaeoniscoidea (*Polypterus*), Holostei (*Amia*) and Teleostei (*Salmo*). The present study will concentrate on the third infraclass, teleosts or bony fish.

Gill Anatomy

Since a significant part of this thesis focuses on NECs in the gill epithelium, a detailed description of the gill structure is presented below.

The gill basket consists of four pairs of arches numbered one (closest to the oral opening) through four (furthest from the oral opening). Each arch consists of numerous filaments, the numbers of which vary depending on the species. The filaments are lined with lamellae which are thought to be the main tissue involved in gas exchange and are

oriented perpendicular to the filament axes (Fig. 2). The filaments grow or develop from undifferentiated cells at the apex, as opposed to the secondary lamellae which develop from the base and are derived from the undifferentiated cells of the primary epithelium (Morgan, 1974). A detailed description of gill vasculature was reviewed by Laurent (1984). In brief, the branchial afferent artery (in the gill arch) supplies the filamental afferent artery (see Fig. 3). This vessel runs along the base of the secondary lamellae and divides into the lamellar afferent arterioles. The blood travels up the filament through the lamellae and exits into the efferent filament artery before running back down the filament, where it joins the efferent branchial artery. A sphincter which controls blood flow is located in the efferent filamental artery before it joins with the efferent branchial artery. The venous system of the gill is not yet completely understood but it is made up of small nutrient arteries which branch from the efferent filament artery and intricately vascularize the local tissues. Eventually these arteries join the efferent arteriovenous anastomosis in the central venous sinus and empties into the branchial vein.

Figure 4 is a cross section of a trout filament demonstrating the afferent filamental artery at the bottom and the efferent filamental artery at the top. The lamellae are seen on the sides of the filament and the arrows indicate the direction of the blood flow from the afferent arteries through the lamellae and finally to the efferent arteries. Cartilaginous tissue is seen closer to the afferent artery. The gill has two main epithelial types, namely the filament or primary epithelium and the lamellar or secondary

Figure 2. Diagram of the structure of teleost gills showing the cartilaginous rod, afferent and efferent branchial arteries in the gill arch. The filaments are perpendicular to the arch in two hemibranches and the secondary lamellae, in turn are perpendicular to the filament. Large arrows indicate the direction of water flow over the gill and the small arrows represent the direction of blood flow. From Young (1981).

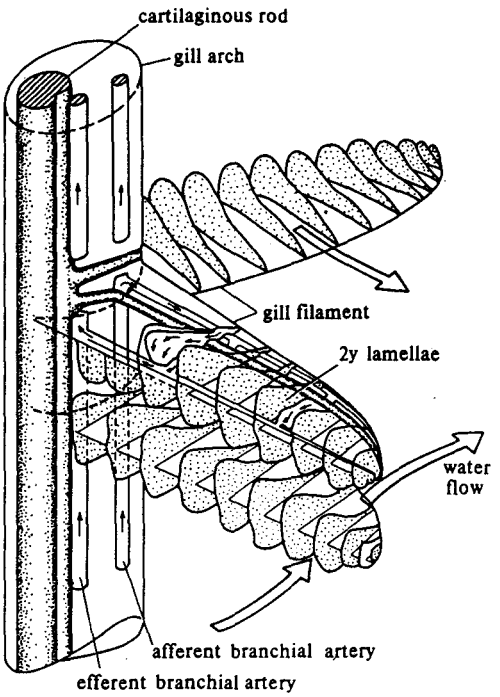

cartilaginous rod
gill arch
gill filament
2y lamellae
water flow
afferent branchial artery
efferent branchial artery

Figure 3. Schematic diagram representing trout gill filamental vasculature. Arrows indicate the direction of blood flow from the afferent branchial artery (af.BA), to the filament afferent artery (af.A) which diverts the blood to the lamellae (L). The oxygenated blood then exits through the efferent filament artery (ef.FA) past the efferent artery sphincter (sph) and enters the efferent branchial artery (ef.BA). The nutrient arteries (NA) branch from the ef.FA and supply the surrounding tissues. The venous blood then collects in the efferent arteriovenous anastomosis (ef.AVas) which empty into the central venous sinus (CVS) and eventually the blood exits via the branchial vein (BV). Cartilage © and the abductor muscle are also depicted (m).

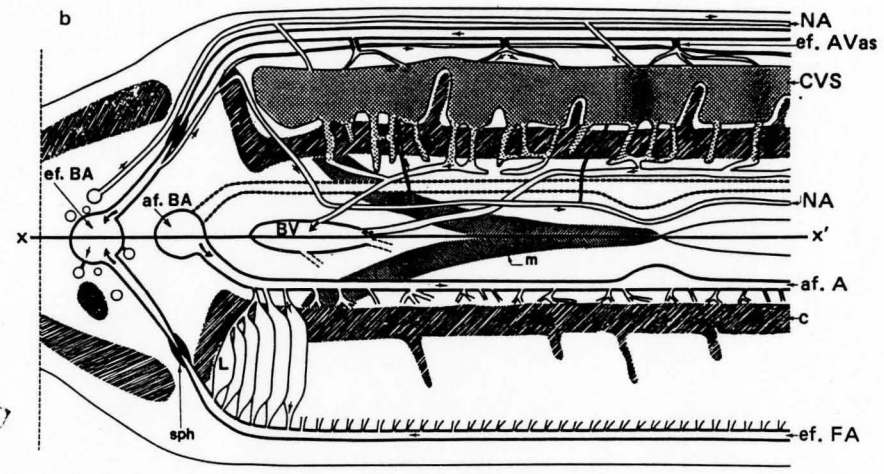
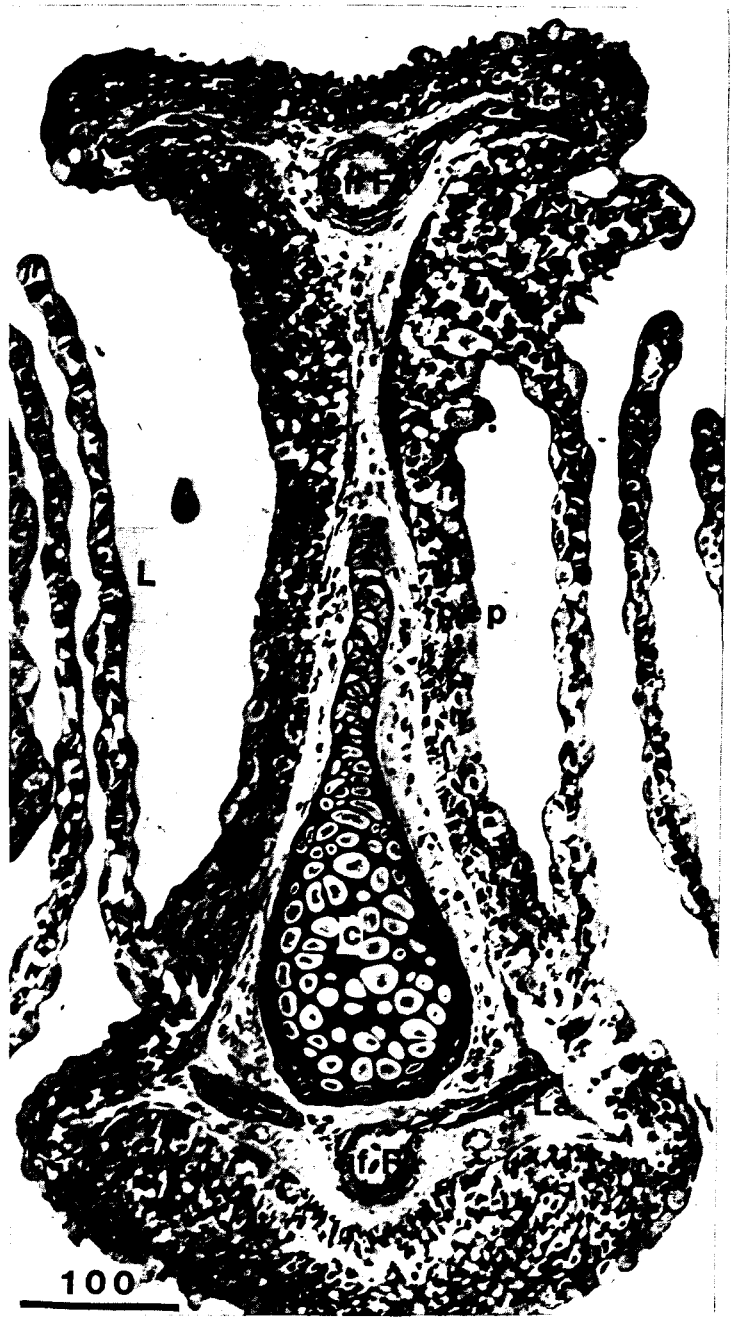

b
NA
ef. AVas
CVS
ef. BA
af. BA
NA
x
BV
x'
m
af. A
c
sph
ef. FA

Figure 4. Transverse section of trout filament with longitudinally cut lamellae (L). The efferent filament artery (ef.FA) is located at the top of the section joined by the efferent lamellae arterioles (ef.LA). Opposite is the afferent filament artery(af.FA) at the bottom joined to the afferent lamellar arterioles (af.LA). The primary epithelium (pep) lines the filament and is relatively thick compared to the secondary epithelium which lines the lamellae. Bar = 100 μm. Arrows indicate the direction of blood flow. Taken from Dunel-Erb et al., 1982).


L
p
100

epithelium. The primary epithelium lines the core of the filament and varies in thickness depending on the location. It is approximately 50 μm wide from the serosal side, which is lined by the basal lamina, to the mucosal surface which is exposed to the external environment. The epithelium is a multi layered structure made up of numerous cell types including mucous, squamous, chloride and neuroepithelial cells (Dunel-Erb et al., 1982). The secondary epithelium sometimes referred to as the respiratory epithelium, is thinner and is usually about two cell layers in thickness. It lines the lamellae, and the external surface is mostly made up of pavement cells (Laurent and Dunel, 1980). The serosal side, adjacent to the basal lamina, consists of undifferentiated cells. Zaccone et al. (1992) have also described NECs in the secondary epithelium of catfish. These are often exposed to the external surface on one side and juxtaposed to the basal lamina on the other.

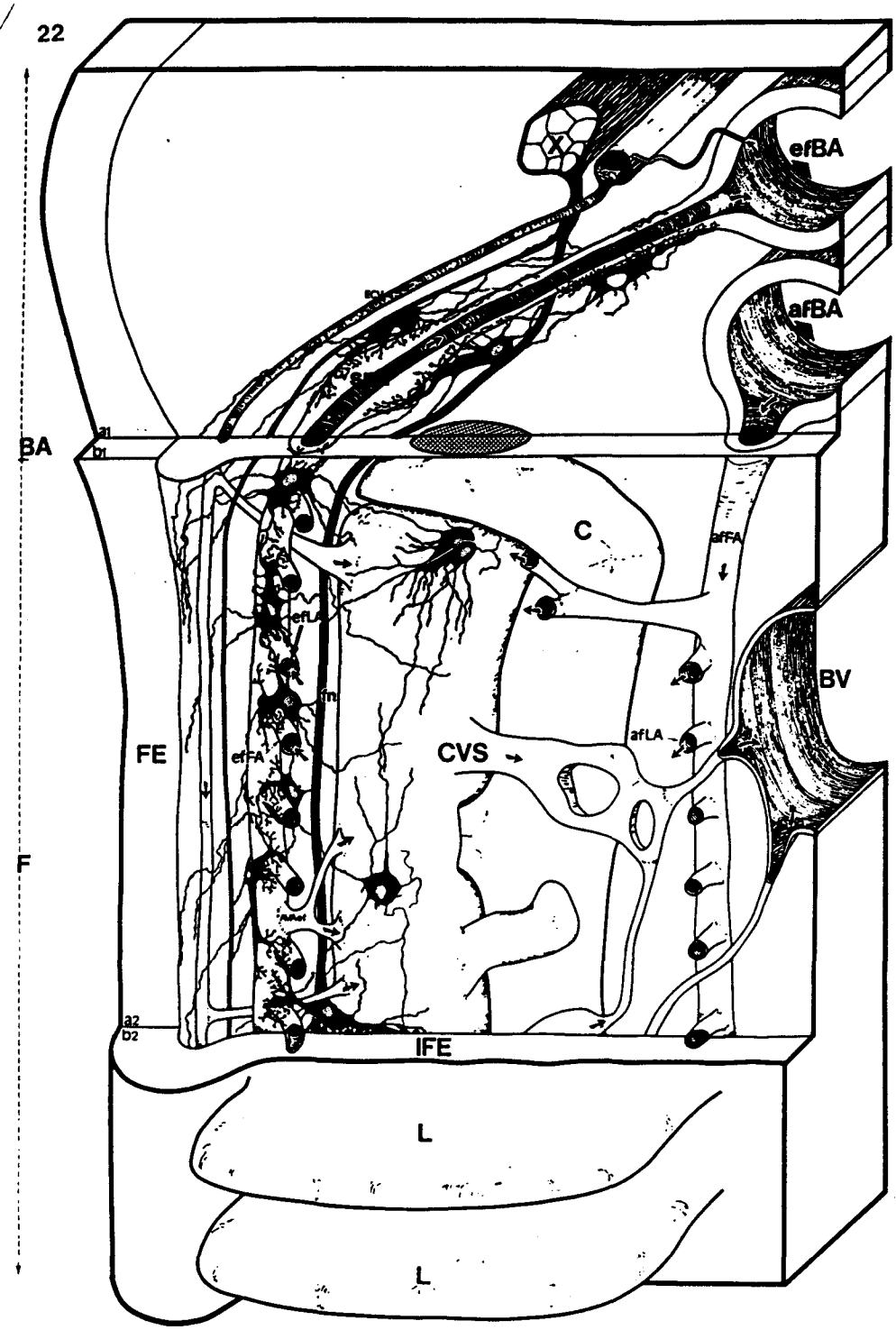
Gill innervation has been extensively reviewed by Nilsson (1984). All four arches of teleosts are innervated by cranial nerve X (Vagus), while only the first arch is additionally innervated by cranial nerve IX (Glossopharyngeal). Donald (1987) used formaldehyde-induced fluorescence to study the location of the adrenergic innervation of teleost gills. General observations among the species revealed that the lamellae did not show any adrenergic innervation at all, though nerve fibres were present on the afferent filamental arteries and on a few afferent lamellar arterioles. Some innervation was also noted on the nutrient arteries of the venous system and in the central venous sinus. Serotonergic innervation of the gill was observed to be concentrated at the proximal half of the filament, under the efferent filament artery, and appeared to originate locally from

the branchial artery (Bailly et al., 1989). In this region these nerves were also seen in contact with the efferent arterial sphincter and in close association with the efferent lamellar arterioles. Occasionally, the nerve fibres were observed running under the basal lamina sometimes coming very close to the primary epithelium and the NECs (Fig. 5). Serotonergic neurons were also identified in contact with catecholaminergic nerve fibres (Bailly et al., 1989). Acetylcholinesterase-positive and indoleaminergic neurons have also been described in the same areas as the serotonergic innervation (Bailly and Dunel-Erb, 1986).

Physiological Effects of Hypoxia in Fish

It is well known that hypoxia induces an increase in gill ventilation and a drop in heart rate in most species of fish (reviewed by Nilsson, 1984). This is accompanied by the release of catecholamines (CA) into the blood stream from chromaffin tissue and a compensatory increase in stroke volume of the heart to counteract the bradycardia . The predominant CA released during hypoxia are epinephrine (EPI) and norepinephrine (NOR)(Perry and Reid, 1994). Increases in CA plasma levels have numerous effects which help to compensate for hypoxia including, increased O_2 diffusion across the gill, expulsion of red blood cells from the spleen, and an increased O_2-haemoglobin binding affinity (see Perry and Reid, 1992). In order to discern the response characteristics of O_2 chemoreceptors in the gill, Milsom and Brill (1986) analysed the afferent impulses from

Figure 5. Schematic representation of the proximal innervation of trout (F) filament surrounding the efferent filament artery (efFA) and the efferent lamellar arterioles (efLA). Innervation of the central venous sinus (CVS)the filament epithelium (FE) and the inter filament epithelium (IFE) are also depicted. The neurons connect with the filament nerve (fn) which joins with cranial nerve X (glossopharyngeal). Other abbreviations: efferent branchial artery (efBA), afferent branchial artery (afBA), branchial arch (BA), branchial vein (BV), afferent filament artery (afFA) and afferent lamellar arterioles (afLA). Taken from Bailly et al. (1992).


efBA
afBA
BA
a1
b1
C
afFA
efL
fm
BV
FE
efFA
CVS
afLA
F
a2
b2
IFE
L
L

the vagus nerve which innervates the first gill arch of yellowfin tuna. The gill was isolated and perfused, the nerve was excised and prepared for single unit recording. The study demonstrated that the receptors in the arch yielded an increased discharge when exposed to either i) a lowered perfusion rate, ii) a hypoxic perfusate or iii) to a lower external PO_2. The authors concluded that the response from the gill was similar to responses by O_2-chemoreceptors in the mammalian carotid body. A similar study by Burleson and Milsom (1993) recorded output from the glossopharyngeal nerve in the isolated and perfused first gill arch of rainbow trout. This study characterized three receptors located in the arch including, proprioceptors, baroreceptors and O_2-sensitive receptors. A detailed investigation of O_2 receptors led to a characterization of three different types; one was sensitive to external hypoxia (in the surrounding water), one sensitive to internal hypoxia (in the blood), and a third was sensitive to both. Again the conclusion was reached that the O_2-chemoreceptors of the gill responded very similarly to mammalian carotid body receptors and that the gill receptors are likely precursors to the carotid body chemoreceptors. In a third study by this group (Burleson and Milsom, 1995a), using the same experimental protocol, the isolated first gill arch of trout was perfused with various neurochemicals and recordings were obtained from single units of the glossopharyngeal nerve. Units correlating to O_2-sensitive chemoreceptors were identified by their increased neural response to hypoxic perfusate and NaCN. The study revealed that perfused EPI and NOR had little effect, whereas dopamine (DOP) and 5-HT caused a small burst of activity followed by a weak inhibition. Muscarine had a small effect, but nicotine and

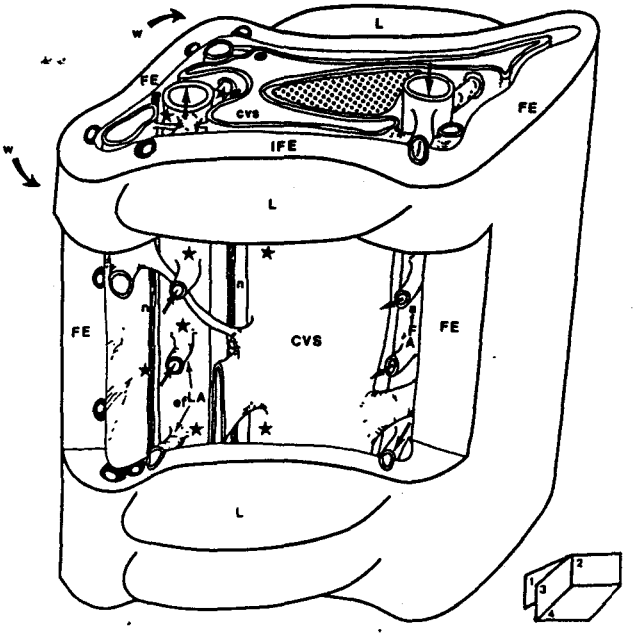
acetylcholine caused the largest stimulatory response. Burleson and Milsom (1995b) further showed that injection of these neurochemicals in the intact trout via a catheter in the dorsal aorta had variable effects on heart rate, ventilation, blood pressure and opercular pressure. The main conclusions from these studies are that O_2 chemoreceptors in the first gill arch are completely responsible for cardio-ventilatory effects of externally applied cyanide, and are partly responsible for the effects of serotonin and cholinergic drugs. However, they do not appear to play any role in mediating the effects due to exogenously-applied catecholamines.

NECs in Teleost Gills

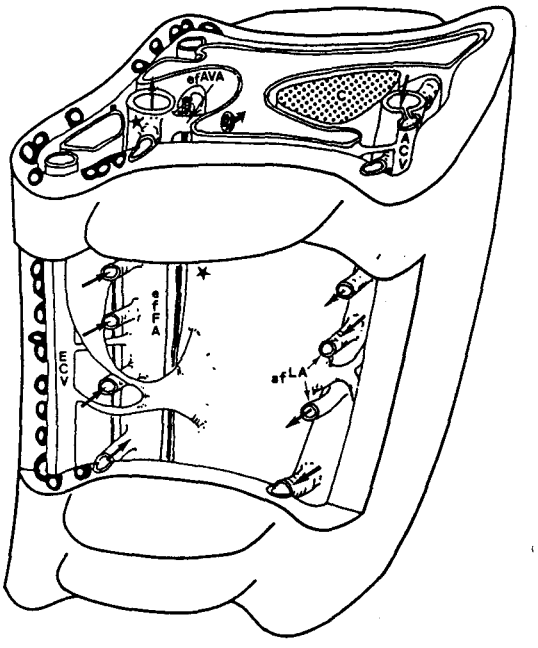
Since NECs were first described in teleost and elasmobranch fish gills by Dunel-Erb et al. (1982), many markers have been used to identify these cells. To date these include, neuron specific enolase, somatostatin, enkephalin, calbindin, calmodulin, S-100 protein, endothelin peptides, nitric oxide synthase and, primarily, 5-HT (reviewed by Zaccone et al., 1997). Dunel-Erb et al. (1982) first located NECs in fish gills using the formaldehyde induced fluorescent technique which yielded intense yellow and fast fading fluorescence indicative of 5-HT. The cells were located exclusively in the primary epithelium and occured mainly as single cells, though occasionally in groups of two or three. The NECs were on the serosal side adjacent to the basal lamina near the efferent filamental artery, and never reached the mucosal surface, ~50 μm away (Fig. 6). NECs were ~10 μm in diameter and electron microscopy revealed they contained DCVs

Figure 6. Diagram represents the relative location of neuroepithelial cells (NEC; black cells) in the proximal(a) and distal halves of trout gill structure. 5-HT neurons are represented by stars, note the low frequency of NECs on the afferent side both distally and proximally. Two filament nerve bundles run parallel to the efferent filament artery. Arrows represent the flow of water (w) and blood. Numbers are representative of longitudinal planes (1-3) and transverse (4). Abbreviations: afferent lamellar artery (afLA), afferent filament artery (afFA), cartilage (C), efferent companion vasculature (ECV), efferent arterio-venous anastomosis (efAVA), and lamellae (L). From Bailly et al. (1992).

1a


w
L
FE
CVS
IFE
FE
w
L
FE
CVS
FE
afA
afLA
L
1
2
3
4

b


afAVA
ACV
afFA
ECV
afLA

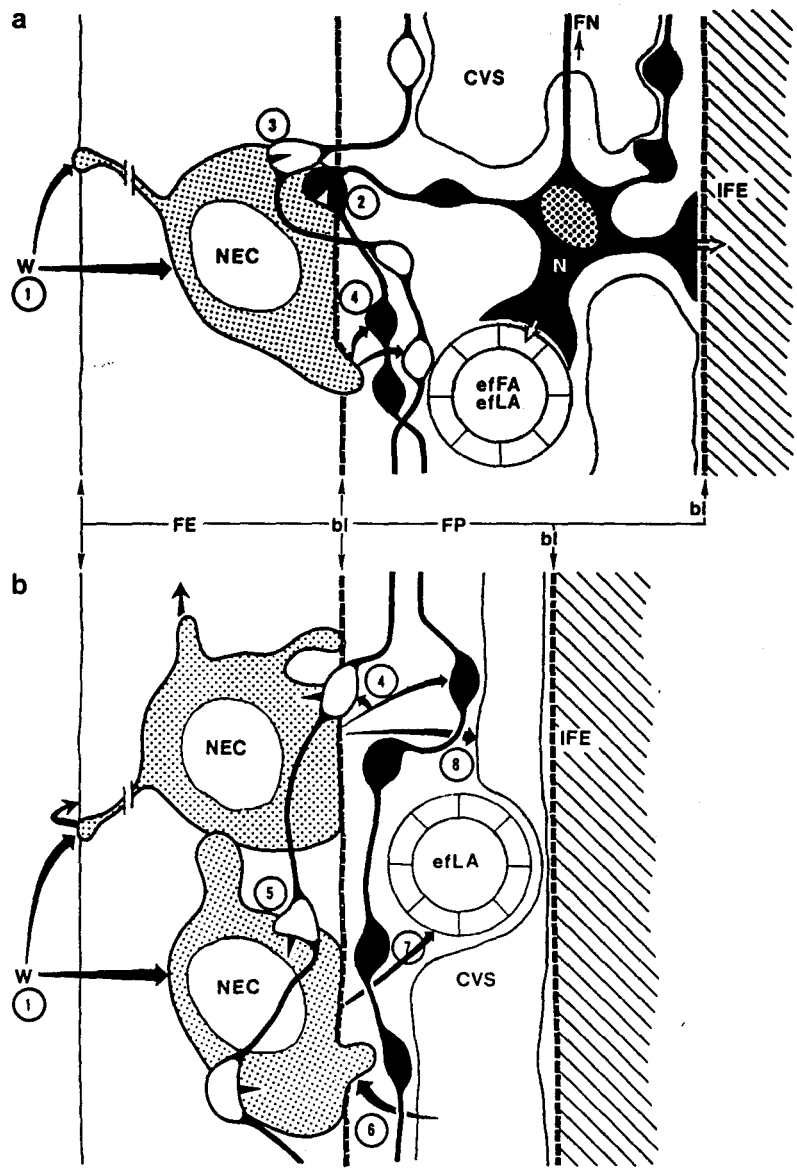
ranging in size from 80- 100 nm. Nerve fibres were identified running under the basal lamina and they eventually crossed over to establish contact with the NECs. The DCVs were scattered throughout the cytoplasm, but were often concentrated near the plasma membrane closest to the nerve profiles. Evidence that these NECs had a chemosensory function was obtained by exposing fish to hypoxic conditions before examination of the filament tissue in the electron microscope. It was found that numerous DCVs had degranulated in NECs, and this was suggestive of active exocytosis. These experiments provided evidence for an O_2-sensitive chemoreceptive role for these cells in fish gills.

Bailly et al. (1992) examined the innervation of NECs that were located in the primary epithelium of six species of teleosts. The single NECs found in the posterior half of the filament are frequently in contact or closely associated with nerve profiles. These profiles often cross the basal lamina and synapse with the NECs. Some of the nerve fibres are 5-HT positive and are usually located at the basal end of the cell, near areas with a high concentration of DCVs. NECs located in the distal half of the filament were shown to extend processes that reached through the primary epithelium and made contact with the external environment. Cells in both the distal and proximal ends of the filament made synaptic contacts with catecholaminergic nerve fibres. These fibres frequently surround blood vessels and innervate the endothelial layer of the central sinus venous. These studies suggest that NECs function may be regulated by the sympathetic nervous system which may help control 5-HT release from these cells (Lauweryns et al., 1982).

Summary of Possible Functions of NECs

Bailly et al. (1992) hypothesized various ways in which the NECs in the primary epithelial layer could possibly function (Fig. 7). The processes extending from the cells which are exposed to the external surface may act as O_2 detectors of the external milieu, and cells lacking processes could hypothetically sense the external O_2 through the epithelial layer. A chemoreceptor role of proximally-located NECs may function via chemical synapses with 5-HT afferent neurons, which join the filament nerve and could potentially relay signals to the vagus and/or glossopharyngeal nerve. Sympathetic CA nerves, which form synaptic connections with the NECs, may act to regulate local motor control of the efferent vasculature. The local release of peptides or 5-HT on the subepithelial layer could target vascular smooth muscle cells thereby affecting vasomotor control. The efferent CA innervation of the NECs could represent sympathetic control over the release of monoamines or bioactive peptides from the DCVs. NECs are located close to the central venous sinus and may be affected by chemical messengers from the venous blood. Finally, the NECs may play a role in osmoregulation. 5-HT neurons which innervate the interlamellar epithelium and the central venous sinus in the proximal half of the filament have a strong effect on the adenylate cyclase-dependent hyromineral exchangers (Laurent and Dunel, 1980), and could therefore affect water and ion movement. Since 5-HT innervation is lacking in the distal portion of the filament, it was hypothesized that the NECs could perform this osmoregulatory function. Zaccone et al., (1992) proposed that one possible function of the NECs located in the secondary

Figure 7. Schematic representation of possible effects of neuroepithelial cells (NEC) in the proximal (a) and distal filament of teleosts. 1) Effects of the environment, water (w) on NECs from direct contact with the processes and through the filament epithelium (FE); 2) afferent synaptic contact with the NECs which leads to the filament nerve (FN); 3) Afferent synaptic contact with catecholaminergic sympathetic nerves; 4) diffusion of secreted substances from the NEC (5-HT, peptides) on the subepithelial nerve plexus; 6) Substances reaching the NEC such as hormones from the central venous sinus (CVS); 7) effects of NECs on vascular smooth muscle of the efferent lamellar arteries (efLA) and 8) effects of NEC 5-HT on the CVS (osmoregulation). Abbreviations: filament parenchyma (FP), inter filament epithelium (IFE), basal lamina (bl). Taken from Bailly et al., (1992).


a
FN
CVS
IFE
3
2
W
1
NEC
4
N
efFA
efLA
FE
bl
FP
bl
bl
b
4
NEC
IFE
8
efLA
5
7
W
1
NEC
CVS
6

epithelium is that of a calcium modulator. Because the NECs appear to increase number in soft water acclimated catfish, and contain the calcium binding protein, calbindin, and are exposed to the external environment, these authors suggest that the cells may take up calcium and maintain calcium homeostasis.

Isolation of NECs in Culture

The study of pulmonary neuroepithelial cells (NEC) in the mammalian lung has led to an extensive morphological characterization via immunohistochemistry and electron microscopy (for review, see Scheuermann, 1997). Various groups have isolated NECs and NEBs from the pulmonary tissue of different mammals in order to study cellular and membrane properties (Sonstegard et al., 1982; Cutz et al., 1985).

The isolation and culture of cells have been employed for many reasons in cytological research. One benefit is that the cell's characteristics can be determined in a controlled environment, thereby eliminating unknown local effects of endogenous systems. However, since cells may change their properties *in vitro* caution must be exercised in attempts to correlate findings with the intact animal. Another positive aspect of cell isolation is to facilitate high resolution electrophysiological studies, which may directly lead to an understanding of cell function. However, there are numerous difficulties encountered when trying to examine a specific cell type. One particular problem is the relatively low number of NEBs in mammalian lung tissue, thought to be only 0.1% of the cell population (Speirs and Cutz, 1993). This paper also discusses "enriching" techniques

which have been implemented in order to overcome this obstacle. Similarly, the isolation of NECs from lower vertebrates may shed some light on the evolutionary questions related to this cell type. It is thought to be highly conserved, and it may well be that its origin in fish identifies the precursor to the NEBs found in mammalian lungs. Fish cell cultures have been used as a tool to examine cellular functions of various organs including gonad, spleen, liver and kidney. Stemming from these cultures there have also been numerous cell lines established from 74 fish species in the last thirty years (Fryer and Lannan, 1994). Verbost et al., (1994) have characterized a technique to isolate gill cells from freshwater and seawater fish. The focus of their work was to examine ionocytes or chloride cells from gills and to enrich the culture with this cell type. Therefore, the isolation of cells from fish tissues, has received much attention recently.

Goals of this Study

One goal of this study was to describe and compare the distribution of NECs in the gills of six fish species. The second goal was to quantify and compare the number of NECs both among the species and within the different epithelial layers. The third aim was to test for possible seasonal variations in the distribution of two populations of NECs in perch by comparing cell numbers between November and July. In addition, this study identified and quantified tyrosine hydroxylase (TH) positive cells in the gill epithelium of perch for the first time, and further, provided a quantitative comparison of the total CA stores in the filaments of the first gill arches of four species. Finally, this study has

modified the techniques of Verbost et al., (1994) and attempted to isolate and identify NECs in cultured gill epithelia of perch (*Perca flavesens*).

Materials and Methods

Rainbow trout (*Oncorhychus mykiss*; 15-20 cm), obtained from Harbor Springs, Ontario, Canada, were kindly donated by Dr. D. G. McDonald. The following fish were kindly provided by Dr. C.M. Wood and were obtained from the sources indicated: Nile tilapia (*Oreochromis niloticus*; 10-15 cm), Northern Tilapia, Ontario, Canada; killifish (*Fundulus heteroclitus*; ~5 cm), Nova Scotia, Canada; bass (*Micropterus dolomieui*; 25-30 cm), lakes in Ontario. Yellow perch (*Perca Flavesens*; 15 cm) were obtained from Hightide Farms, Ontario, Canada. All the above fish were maintained in 400L fiberglass tanks with flowing dechlorinated and aerated Hamilton tap water, and were fed twice weekly with Martin commercial trout pellets. Zebrafish (*Danio rerio*; 2-3 cm), were kept in aquaria with dechlorinated tap water at room temperature and fed twice weekly with commercial fish food. Zebrafish used in this study were a kind gift from Dr. A.D. Dingle.

Tissue Samples

Fish were stunned with a blow to the head and decapitated prior to tissue removal. The gill baskets were extracted and the first arches on either side were rinsed quickly in phosphate buffered saline (PBS, pH 7.6), followed by immersion in 4% paraformaldehyde/ 0.1M phosphate buffer (pH 7.2) for 4 hr at 4°C. The tissues were then rinsed 3 x 3 min in 0.1M phosphate buffer, pH 7.2 and left overnight in 20% sucrose/ 0.1M phosphate buffer at 4°C overnight. The following day the tissues were frozen in Tissue Tek and sectioned immediately, or stored at -80 °C and processed at a later date.

Cryomicrotomy and Immunohistochemistry

Tissues prepared as above, were sectioned at 18 μm thickness on an American Optical cryo-cut II microtome and mounted on slides coated with chromium potassium sulphate gelatin. The tissue samples were kept at -20 °C until all sectioning was completed. They were then thawed at room temperature for ~10 min and exposed to a solution containing 0.1M glycine, 500 mM NH_4Cl, 10% triton X-100, 0.2% gelatin and 20% fetal calf serum (FCS) in 0.1M phosphate buffer for 1 hr to minimize background fluorescence. Samples were then rinsed 3 x 3 min in 20% FCS/ 0.1M phosphate buffer and incubated overnight at 4°C with rabbit polyclonal antisera (IgG) against either serotonin (5-HT; Sigma, St. Louis, MO., U.S.A.) or tyrosine hydroxylase (TH; Chemicon, El Segundo, CA, U.S.A.), diluted 1:500 in 1% bovine serum albumin (BSA)/ 0.1 M phosphate buffer. The sections were rinsed 3 x 10 min with 20% FCS/ 0.1M phosphate buffer and incubated for 45 min at room temperature in the dark with a fluorescein (FITC)-conjugated goat anti rabbit IgG (Cappel, Malvern, PA, U.S.A.), diluted 1:50 in 1% BSA/ 0.1M phosphate buffer. After 3 x 10 min rinses in 20% FCS/ 0.1M phosphate buffer, tissues were mounted in Mowiol (1,4-diazabicyclo[2.2.2] octane, Lancaster Synthesis, Windham, NH, U.S.A.) prior to viewing under a Zeiss S16 microscope equipped with epifluorescence and phase optics. Photographs were taken with Kodak Elite II slide film (a.s.a. 100) using 20 sec manual exposures for fluorescent images and automatic exposures for phase contrast images. Specificity for 5-HT staining was tested

in control experiments where the antiserum was preincubated with 400 µg/ L 5-HT in 1% BSA/ 0.1M phosphate buffer for 36 hr prior to application to the tissue sections. In additional control experiments, the primary antiserum was omitted, but otherwise the tissue was processed in the usual way. No positive staining was detectable in any of these control experiments.

Cell Quantification

Gross measurements of filaments and lamellae were done on whole mounts. The area of sectioned primary epithelium was calculated after measuring the length from the base to the tip and the width between the outer edges of the epithelial layer. The area of the lamellae was calculated in the same manner; the length was measured from the base where the primary and secondary epithelial layers met to the tip. Measurements were obtained with the aid of a reticule in the eye piece of the microscope calibrated using a stage micrometer. The number of divisions that spanned the tissues or cells was recorded and the measurements were converted to the appropriate units (mm or µm). Fluorescent cells were counted in the primary and secondary epithelia separately. The cell numbers were averaged and expressed as the number of positive cells per mm^2 of sectioned tissue.

High Performance Liquid Chromatography

Fish were killed as above prior to the extraction of the primary gill arch. The filaments were removed and wet tissue weight was measured prior to homogenization on

ice in 500µl of 0.1M $HClO_4/Na_2EDTA$. The extract was spun at 10 000 rpm for 15 min in a Canlab. Biofuge A microcentrifuge. The supernatant was removed and spun at the same speed for another 15 min; this last step was repeated a total of three times. Catecholamines from the filament extract were separated by HPLC (Waters Model 510) with a Sherisorb-ODS2 column (10 x 0.46 cm, 3-µm particle size; Chromatography Sciences Company, Montreal, Quebec, Canada), coupled with an electrochemical detector (Coulochem II detector, Model 5200; ESA, Bedford, MA, U.S.A.) as described by Jackson and Nurse (1997). A conditioning cell (Model 5021) and an analytical cell (Model 5011) containing dual coulometric working electrodes made from porous graphite were used as the detection system. The first detector in the analytical cell was set at 0.05 V to reduce interference by contaminating electroactive compounds at the second detector, which was set at -0.3 V, the potential required for electroreduction of norepinephrine (NOR), epinephrine (EPI), dopamine (DOP) and the internal standard, di-3,4-hydroxybenzylamine hydrobromide (DHBA). Stock solutions of NOR, EPI, DOP, and DHBA (Sigma) were made up in 0.1M $HClO_4$ to a final concentration of 1.0 mM. Immediately prior to use, they were diluted in water to 25 nM catecholamine (CA) and used as standards. The mobile phase was made up in 5% methanol/ water and contained 250 mg/ L heptanesulfonic acid (Sigma), 6.9 g/ L NaH_2PO_4 (Sigma), and 80 mg/ L Na_2EDTA (BDH Chemicals, Toronto, Ontario, Canada). The pH was adjusted to 3.5 with H_3PO_4 followed by filtration (20 mm, 47 mm Nylon-66 disk filters; S.P.E., Concord, Ontario, Canada) and degassing prior to each assay. Water used to make up solutions was

distilled and deionized (NANOpure 11, D3700 series, cartridge deionization system, Barnstead, Newton, MA, U.S.A.). The flow rate was 1 ml/min at ~2,000 psi. The analysis of the chromatograms was done with a Waters 740 Data Module (Millipore, Milford, MA, U.S.A.) and quantified using the peak area ratio method. The peak area of a known amount of external CA standard was used to calculate the amount of CA in a sample from its integrated peak area. A known amount of internal standard, DHBA, was added to each sample to correct for differences in injection volume.

Cell Dispersion and Culture of Gill Cells

Procedures for cell dispersion and culture of dissociated fish gill cells (adapted from Verbost et al., 1994) were done in a laminar hood using sterile techniques. Fish were stunned with a blow to the head and quickly decapitated. The gill basket was removed and rinsed in phosphate buffered saline (PBS) at pH 7.6. The distal halves of the filaments were cut and immersed in sterile PBS containing 1% penicillin-streptomycin and 50μg/ ml gentamycin and were subjected to three washes of 10 min each with this solution on ice. After the final wash the filaments were rinsed quickly in 0.05% trypsin (Grand Island Biological Company, Grand Island, NY, U.S.A.) and 5 mM EDTA in PBS at room temperature. The tissues were then shaken by hand in fresh enzyme solution for 10 min followed by trituration (~100 times) with a 1 ml pipette. The solution containing the dispersed cells and undigested tissue was filtered through a 100 μm nylon filter and the filtrate mixed with a solution consisting of 10% fetal calf serum (FCS), 1% penicillin-

streptomycin and 50 µg/ml gentamycin in PBS and kept on ice. The undigested tissue left on the filter was transferred back into the original tube, and the dissociation procedure was repeated a total of four times to increase the yield of dispersed cells. The solution containing the dispersed cells was then spun for 5 min at 11 000 rpm in a benchtop centrifuge. The supernatant was discarded and the cells were resuspended in PBS containing 2.5% FCS, 1% penicillin-streptomycin and 50 µg/ml gentamycin. The cell suspension was spun again for 5 min at 11 000 rpm, and the pellet was resuspended in Leibovitz's L-15 culture medium containing 10% FCS, 1% penicillin-streptomycin, 50 µg/ml gentamycin, 5 mM dexamethasone, and 2 mM glutamine. The cells were plated in a central well formed by a fibronectin-coated (50 µg/ml) glass coverslip, that was sealed to the underside of a 35 mm tissue culture dish with an 8 mm hole drilled in the centre. These modified culture dishes were sterilized with U.V. light before the fibronectin was applied. Cultures were incubated at 17° C for 24 hr and then fed with 1.5 ml culture medium.

Immunocytochemical staining

After 48 hr *in vitro*, the medium was removed and the dishes were rinsed with PBS at pH 7.6. The cultures were fixed for 1 hr at room temperature with 4% paraformaldehyde/ PBS followed by three rinses of 10 min each in 0.1M phosphate buffer at pH 7.2. To reduce background fluorescence from endogenous sources, and especially free aldehyde groups, the cells were exposed for 1 hr to a solution containing 0.1 M

glycine, 500 mM NH_4Cl, 10% triton-X 100, 0.2% gelatin and 20% FCS in 0.1 M phosphate buffer. This was followed by 3 x 5 rinses with 20% FCS/ 0.1 M phosphate buffer. The cultures were then incubated overnight at 4°C with rabbit polyclonal antisera (IgG) against 5-HT, diluted 1:500 in 1% BSA/0.1M phosphate buffer. After 3 x 10 min rinses with 20% FCS/ 0.1 M phosphate buffer they were incubated for 45 min at room temperature in the dark with a Texas red-conjugated goat anti rabbit IgG diluted 1:50 in 1% BSA/ 0.1 M phosphate buffer. The cultures were rinsed (3 x 10 min each) in the buffer, before mounting in Mowoil and viewing with a Zeiss IM35 inverted microscope equipped with epifluorescence and phase optics. Photographs were taken on Kodak Elite II slide film (a.s.a. 100) using 60 sec manual exposures for fluorescent images and automatic exposures for phase contrast images. For non-specific staining controls, similar to those described above for cyrosections, were also done for cultured cells and no positive staining was observed.

Analysis

Graphs presented in the text represent the mean number of positive cells/mm^2 section ± SEM calculated for ns= number of sections; na= number of animals sampled. Statistical analyses were done using the Student's t test with level of significance set at $p < 0.05$.

Results

The gill basket in all species examined consisted of four pairs of gill arches. The first arch was used in all experiments. The primary epithelium of the filaments consisted of multiple layers of cells. This tissue is made up of numerous cell types including, mucous, squamous, chloride, and neuroepithelial cells (Conte, 1969 and Dunel-Erb et al., 1982)(see Fig. 8a). One side of the epithelium is lined by the basal lamina and the other faces the external surface. The secondary epithelium lines the lamellae and consists of two cell layers, made up mostly of pavement cells along the basal lamina, and organelle rich cells in contact with the external milieu (Laurent and Dunel, 1980; Zaccone et al, 1992)(Fig. 8c).

Species with 5-HT positive cell in both filament and lamellar epithelia

Perch and zebrafish were the only species examined which exhibited 5-HT positive NECs in both the primary and secondary epithelia. The distribution of these cells is considered for each species below.

Perch: Filaments measured approximately 3 mm in length from the base to the tip and 100 μm in width from one edge of the primary epithelium to the other. The lamellae were approximately 120 μm long from the base, where the primary and secondary epithelia meet, to the apex and approximately 35 μm wide. Data are expressed as the mean number of positive cells/mm^2 section ± SEM calculated for ns= number of sections; na= number of animals sampled. As shown in Fig. 9, there were

Figure 8. Perch (*Perca flavesens*) filament in phase (a), arrows point to outer edges of the primary epithelial layer. The same section under fluorescent light, reveals 5-HT positive cells in the lamellae (b). Scale bar for (a) and (b) represents 100 μm. Perch secondary lamellae under a higher magnification (c), arrow points to outer edges of secondary epithelium. The same section, exposed to fluorescence reveals NECs in the secondary lamellae (d), arrow indicates a cell exposed to the external milieu. Tilapia (*Oreochromis niloticus)* filament with short lamellae at top are perpendicular to the primary epithelium (e). The same section under fluorescent light reveals NECs in the secondary epithelium (f), arrow points to a cell which protrudes from the very tip of the lamellae. Trout (*Oncorhyncus mykiss*) primary filament epithelium is shown with phase optics (g), and fluorescent light (h). Arrow indicates processes between two 5-HT positive NECs. Scale bar for (c) through (h) represents 10 μm.

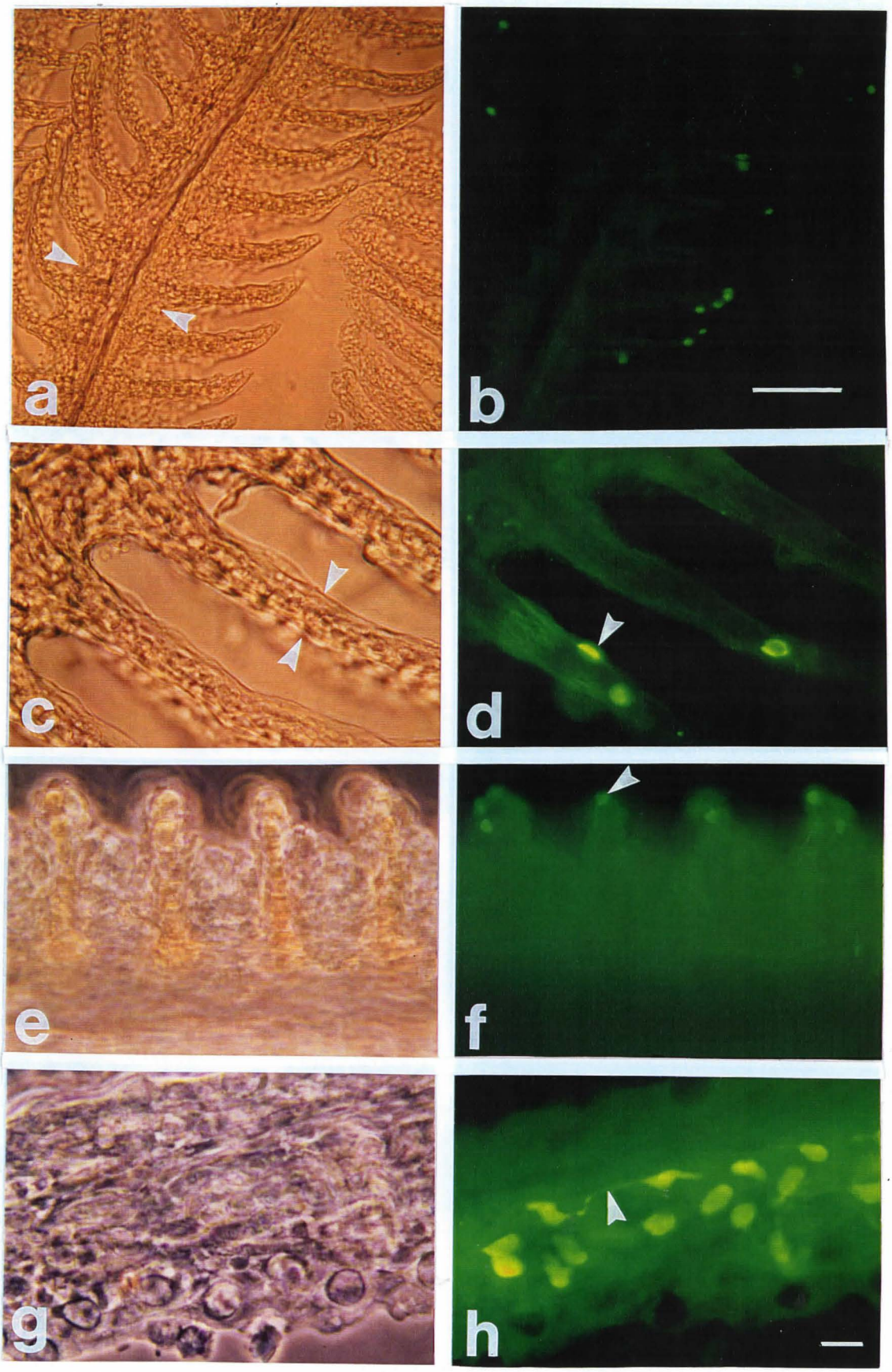
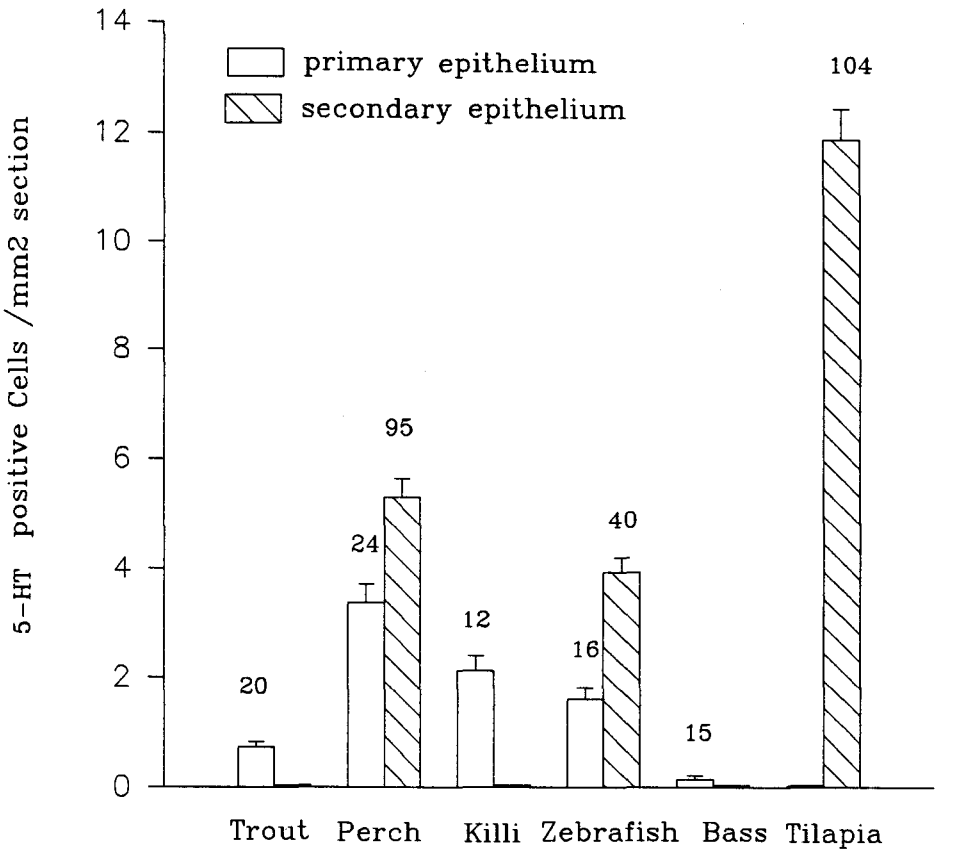
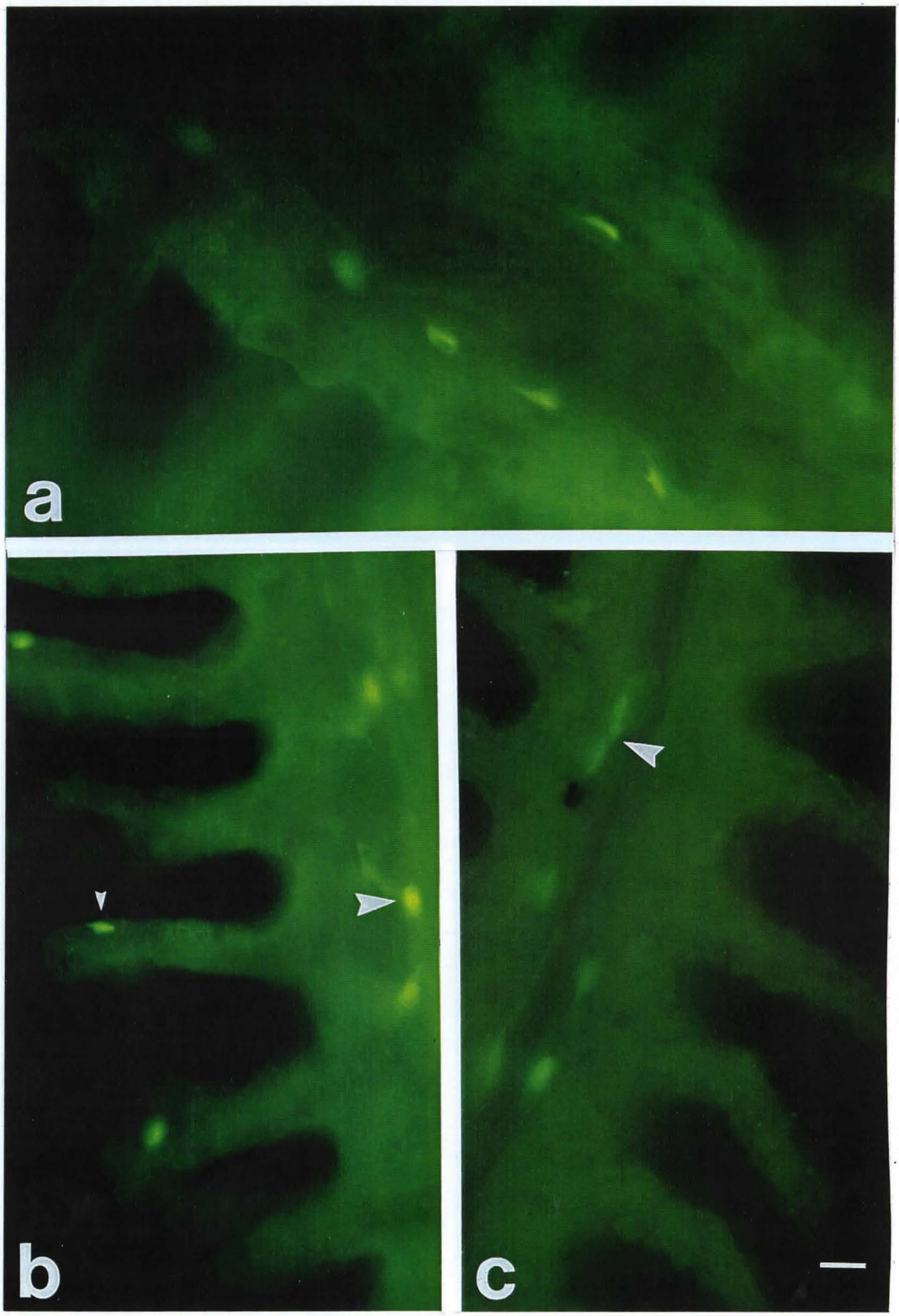

a
b
c
d
e
f
g
h

Figure 9. Histogram represents the mean number ±SEM of 5-HT positive cells per mm^2 section (na= number of animals) in primary and secondary gill epithelia of trout (na=3), perch (na=3), killifish (na=3), zebrafish (na=3), bass (na=2) and tilapia (na=3). Trout, killifish, and bass lack 5-HT positive NECs in the lamellae; and thus the corresponding bin is absent. Similarly, tilapia did not reveal any NECs in the primary epithelium and is represented by the number of positive cells in the secondary epithelium only. The numbers above each histogram represent the number of sections sampled (ns).


14
12
10
8
6
4
2
0
5-HT positive Cells /mm2 section
primary epithelium
secondary epithelium
20
24
95
12
16
40
15
104
Trout
Perch
Killi
Zebrafish
Bass
Tilapia

3.4 ± 0.34 (ns= 24; na=3) 5-HT positive cells/mm^2 section in the filament epithelium. These cells had an elongated shape and extended occasional short processes and their long axis varied between 8-13 μm. The 5-HT positive cells were found close to or resting on the basal lamina approximately 40 μm from the mucosal (external) surface all along the filament as shown in Fig. 10a. Some of these cells occurred singly-isolated, whereas others tended to occur in pairs. In the secondary lamellae 5-HT positive cells tended to be concentrated at the distal tips (Fig. 8a,b). The number of positive cells in each lamellae section was quite variable. Some lamellae were devoid of stained cells, whereas others had 1 to 5 5-HT positive cells (Fig. 8b). There was no obvious correlation between the number of cells and the location of the lamellae along the filament (Fig. 8b). Compared to the elongated cells found in the filament the ones in the lamellae were more oval and measured approximately 8 μm in size (range 6-10 μm). Lamellar 5-HT positive cells found in the secondary epithelial bilayer were in both the underlying layer, not exposed to the external milieu, and the outer mucosal layer exposed to the surface (Fig. 8d). Positive cells were found in the secondary lamellae at a frequency of 5.3 ± 3.2 cells/mm^2 of sectioned tissue (Fig. 9). Figure 10b demonstrates a section through both the filament and lamellar epithelia, in which 5-HT positive cells are present in each region. The "closed type" cells, which are completely surrounded by neighbouring cells, line the basal lamina of the primary epithelium. On the other hand, the "open type" cells, which are partially surrounded by undifferentiated cells, appear toward the tips of the lamellae and are occasionally exposed to the external environment.

Figure 10. Longitudinal section of perch filament demonstrates closed type 5-HT positive NECs in the primary epithelium lining the basal lamina (a). A perch filament (b) demonstrates both types of NECs (5-HT positive) in the primary (large arrow) and secondary epithelial (small arrow) layers. Note the cells in the lamellae which are exposed to the external milieu. Section of trout filament (c) shows 5-HT positive NECs (closed type) adjacent to the basal lamina on either side; single, and paired cells (arrow) can be seen, while the lamellae lack 5-HT positive cells. Scale bar = 10 μm.


a
b
c

Zebrafish: Filaments measured approximately 1.2 mm in length from the base to the tip and 100 μm in width. The lamellae were ~75 μm long and 40 μm in width and uniquely close together with little or no interlamellar space. Figure 9 illustrates that there were 1.6 ± 0.22 5-HT positive cells/mm^2 sectioned in the primary epithelium. These cells were observed lining both sides of the basal lamina all along the filament but appeared to be more numerous at the tips. The cells were located about 40 μm from the external surface and measured ~8 μm in diameter. They were spherical in shape with no evidence of processes. Some of these cells were singly-isolated and others were observed in pairs. Positive cells found in the secondary epithelium were similar in shape to those in the filament but were approximately 6 μm (range 4-7) in size and were concentrated in the distal half of the lamellae. Figure 9 demonstrates that they were found at a frequency of 4 cells/mm^2 section. The number of 5-HT positive cells in the lamellae varied. Some lacked stained cells completely and others had from 1-3 5-HT positive cells. These cells occasionally reached the external surface and there was no obvious indication of cell grouping as seen in the filament.

Species with 5-HT positive cells exclusively in the lamellar epithelium

Tilapia: Of the species investigated in this study tilapia was the only one which had 5-HT positive cells located solely in the lamellae. The primary filaments were approximately 2 mm long and about 110 μm wide. The lamellae were relatively short, approximately 20 μm in length and 17 μm wide as shown in Fig. 8e. As seen in Fig. 9, there were

12 ± 0.56 (ns= 104; na=3) cells/mm^2 section and the density of the cells was high because the cells were confined to the comparatively small area of the lamellae. These 5-HT positive cells did not appear to have processes and had a spherical shape. They ranged in size from 5-7 μm and frequently appeared to be exposed to the external environment occasionally protruding from the very tip of the lamellae as seen in Fig. 8f.

Species with 5-HT positive cells exclusively in the filament epithelium

Three species of fish were found to have 5-HT positive cells exclusively in the primary epithelium; the lamellae were devoid of such cells. Each is considered in turn below.

Trout: Filaments measured approximately 3 mm in length from the base to the tip and 120 μm in width. The lamellae were approximately 120 μm long and 40 μm wide. There were 0.7 ± 0.09 (ns= 20, na=3) 5-HT positive cells/mm^2 section in the filament epithelium as shown in Fig. 9. The appearance and distribution of these cells were reminiscent of 5-HT positive NECs previously described in this species by Dunel-Erb et al. (1982). The cells in both studies were found lining the basal lamina on both sides ~40 μm from the mucosal surface. They measured approximately 10 μm in diameter (range 8-11 μm), and were observed as solitary cells as well as in pairs as seen in Fig. 10c. The cells displayed an abundance of elaborate processes (Fig. 8g,h) which frequently extended towards and contacted other 5-HT positive cells. Similar processes were observed by Bailly et al. (1992) who observed them to "run tortuously" between epithelial cells, and occasionally

to extend to the basal lamina or to neighbouring NECs.

Bass: Filaments measured approximately 5 mm in length from the base to the tip and 120 μm wide. The lamellae were approximately 180 μm long and 40 μm in width. Figure 9 illustrates the cell density at 0.15 ± 0.07 (ns= 15; na=2) 5-HT positive cells/ mm^2 section in the filament epithelium. These cells were found in the primary epithelial layer exclusively, had an irregular shape, and ranged in size from 10-12 μm. Occasional short processes extending from the cells were observed. Like the cells described in the above species, they were located all along the basal lamina approximately 50 μm from the external surface and concentrated at the apex of the filament.

Killifish: 5-HT positive cells were observed in the primary epithelium of the killifish but unlike other species, they were found very rarely or completely absent in the proximal or mid region of the filament. The filaments of the killifish measured approximately 1.3 mm in length and 40 μm wide. The lamellae were approximately 40 μm long from the base where the primary and secondary epithelia meet to the apex and approximately 20 μm in width. As shown in Figure 9, there were 2.1 ± 0.27 (ns= 12; na= 3) cells/mm^2 section in the primary epithelium. These cells were approximately 9 μm (range 7-10 μm) in diameter and were closely associated with the basal lamina. The 5-HT positive cells were concentrated at the distal tips of the filament, frequently as single cells, but also occurred in a much closer arrangement in groups ranging from two to four cells. Processes

contacting the basal lamina and extending from one 5-HT positive cell to another were occasionally observed.

Distribution of Tyrosine Hydroxylase Immunoreactive Cells in Fish Gills

Tyrosine hydroxylase positive (TH^+) cells were detected in three of the species studied, killifish, zebrafish and perch. No TH^+ cells were detected in the gill epithelium of the remaining species, bass, trout or tilapia.

Killifish: TH^+ cells were located in the primary epithelium exclusively and measured 7-8 μm in diameter (Fig. 11a). These cells were located along the entirety of the filaments and did not appear to be concentrated in any particular area. Cells were frequently, though not exclusively, in contact with the basal lamina and in many cases they were found mid-way between the basement membrane and the external surface. In no case did the cells appear to be exposed to the external surface. There were approximately 12.7 ± 1.0 (ns= 70; na=3) TH^+ cells / mm^2 section and though the cells were numerous, no intercellular contact or processes were observed. When two adjacent sections were immunostained for 5-HT and TH respectively, it did not appear that the same cells were positive for both markers. The differences in the populations identified by the two markers such as location and numbers (discussed under *Quantitative Comparisons*) suggest that they are different cell types.

Zebrafish: TH^+ cells in the gill measured ~ 8 μm in both the primary and secondary epithelia (Fig. 11b). The cells in the filament were found along its entire length at a frequency of 2.5 ± 0.35 (ns= 12; na=3) TH^+ cells/mm^2 section (Fig. 12). The cells were more numerous at the tips, often in contact with each other (Fig. 11b). Those located in the

secondary epithelium were occasionally in contact with the external surface and though not all the lamellae contained positive cells, the others had between 1-3 TH^+ cells. The frequency of the cells in the lamellae as shown in Fig. 12 was 3.2 ± 0.3 (ns= 64; na=3) TH^+ cells/ mm^2 section. Processes were not evident in either population of cells. When adjacent sections stained with 5-HT and TH respectively, it appeared that the cells were in the same areas of both the filament and the lamellae. Secondly, the similarity in numbers of cells stained by both markers suggests they may represent at least an overlapping population.

Perch: Both the filament and lamellar epithelia contained TH^+ cells. The cells were approximately 8 μm in size and did not appear to have any processes. They were often located at the base of the lamellae where the primary and secondary layers meet and traverse into both types of epithelia (Fig. 11c). Only occasionally were the cells in contact with each other. Some of them in the lamellae were exposed to the external environment and cells in the primary epithelium did not reach the basal lamina. As shown in Fig. 12, there were 0.2 ± 0.02 cells/mm^2 section in the primary epithelium and

Figure 11. Sections of killifish (*Fundulus heteroclitus)* filaments immunostained against TH (a) show positive cells in the primary epithelium between the basal lamina and the outer surface. Stained zebrafish (*Danio rerio*) filaments reveal TH positive cells grouped in the primary epithelium and some cells extend into the lamellae (b). Perch filaments immunostained against TH is shown in (c), cells were found towards the base of the lamellae and in the area where the primary and secondary epithelia meet. An example of a 5-HT specificity control in perch, in which the primary antibody against 5-HT was preincubated with an excess of 5-HT prior to immunostaining is shown in (d). No positive staining in either the filament or the lamellae is seen. The base of a filament from perch shows 5-HT positive staining of nerve fibres (small arrow) in the primary epithelium in the area of the central venous sinus; large arrow indicates a cell body (e). Scale bar = 10 μm.

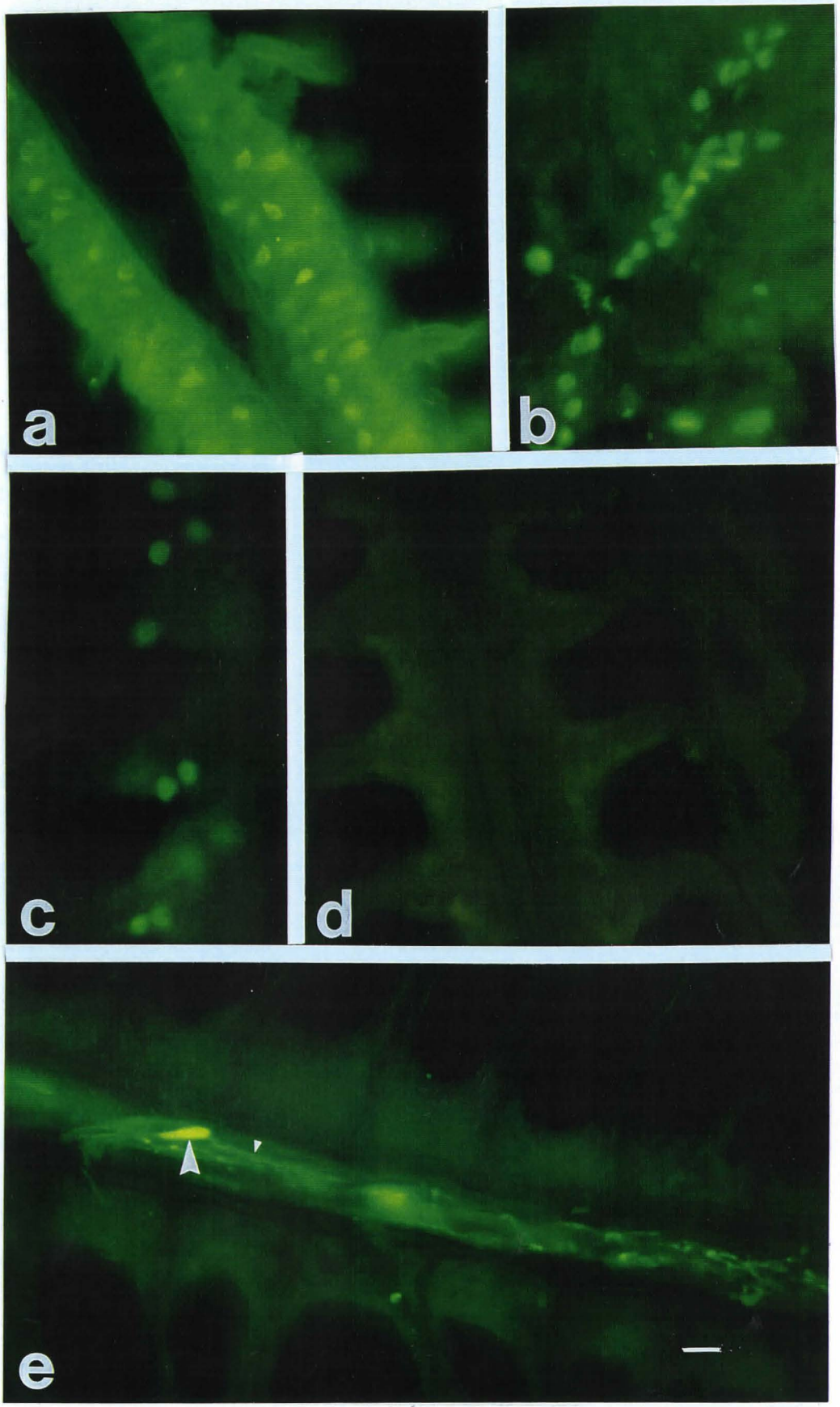
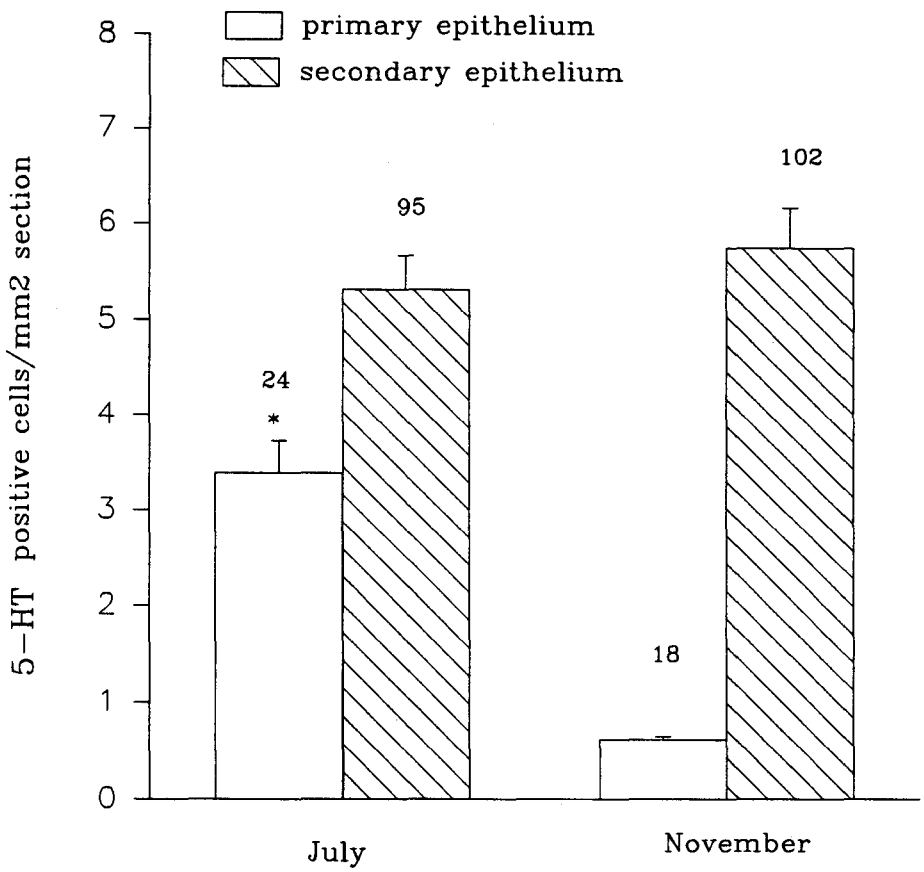

a
b
c
d
e

Figure 12. Histogram represents the mean number ± SEM of 5-HT positive cells per mm^2/ section (na= number of animals) in the primary and secondary epithelia in perch (na= 3) sampled during July and November. The values above the histograms represent the number of filaments or lamellae respectively, in which the cells were quantified . The asterisk indicates a significant difference from corresponding value in November.


primary epithelium
secondary epithelium
5-HT positive cells/mm2 section
0
1
2
3
4
5
6
7
8
24
*
95
18
102
July
November

0.3 ± 0.04 cells/mm^2 section in the secondary. The location of the TH$^+$ cells was unique compared to other species examined in the present study. They were found in defined areas, around the base of the lamellae. The positive cells were found in an area of approximately 10 consecutive lamellae and extended into the filament epithelium. The rest of the filament, including the lamellae, did not have any TH$^+$ cells and was confirmed by serial sectioning. The pattern in which they are found, explains their low frequency per mm^2 of the overall tissue. The location of the area in which the cells were found was not consistent. The cell "grouping" was usually located in the middle portion of the filament but did not appear in the same area from one filament to another. When consecutive serial sections were stained with 5-HT and TH respectively it did not appear that the same cells were positive for both markers. This is supported by the differences in their relative locations. 5-HT positive cells were usually found at the tips of the lamellae and along the basal lamina of the filament epithelium. The TH$^+$ cells on the other hand, were observed at the bases of the lamellae and were much closer to the external surface.

Control Experiments

Control experiments were carried out on gill sections from each species to test the specificity of the staining by the two antibodies. Preabsorption of the 5-HT antibody with excess 5-HT was used to confirm antibody specificity. For example, Fig. 11d shows a section from a perch gill filament exposed to the primary antibody against 5-HT which

was preincubated with 400 µg/ml 5-HT before exposure of the section to the FITC-conjugated secondary antibody. No specific staining was detectible in the primary or secondary epithelial layers in this section, or sections from the other species (trout, killifish, tilapia, bass, and zebrafish), treated in a similar manner. In control experiments for TH staining, sections incubated with secondary antibody, without prior exposure to the primary antibody, showed no positive staining above background.

Serotonergic Innervation

Dunel-Erb et al. (1989) described the innervation of the gill filament in five teleost species. This innervation is thought to regulate the vasculature and consists of at least three components revealed by the presence of indolaminergic, catecholaminergic and acetylcholinesterase-positive neurons and nerve fibres. The authors noted that the innervation is concentrated in the proximal third of the filament while the distal portions are poorly innervated. 5-HT positive cell bodies and nerve fibres however, were detected in the perch (Fig. 11e), and in agreement with the previous findings, they were only located in the proximal portion of the filament in the area of the central venous sinus.

Quantitative Comparisons

Figure 9 illustrates a quantitative comparison of the number of 5-HT positive cells/mm^2 in the primary and secondary epithelia of the six fish species examined. Perch and zebrafish demonstrated NEC populations in the primary and secondary epithelia. When examining

cell densities between the two species, it should be noted that perch with 3.4 cells/mm^2 section contained approximately twice the number of cells compared to zebrafish with 1.6 cells/mm^2 section in the filament. Likewise, the cell density in the perch secondary epithelium (5.3 cells/mm^2) is 25% more than zebrafish with 4 cells/mm^2 section. Tilapia, which only had NECs in the lamellae, had 12 5-HT positive cells/mm^2 section, approximately two to three fold higher than perch and zebrafish secondary epithelium. The three remaining species only had NECs in the primary epithelial layer. Of these species the killifish had the most number of cells with 2.1 cells/mm^2 section, three times higher than trout with 0.7 cells/mm^2 section, and 14 times more than bass which had the fewest number of NECs with 0.15 cells/mm^2 section in the primary epithelium.

Overall, the bass demonstrated the fewest number of NECs/mm^2 section in the primary epithelium and perch the most, while tilapia had the highest density of cells in the secondary lamellae.

Seasonal comparison of 5-HT positive cells in the perch gill

Figure 13 shows a quantitative comparison of 5-HT positive cell numbers in perch primary and secondary epithelia during summer and winter months. The cell density in the filaments increased about five fold between November (0.6 cells/mm^2 section) and July (3.4 cells/mm^2 section) while the secondary epithelial cell numbers were not significantly different between the two seasons (5.7 ±3.8 cells/mm^2 section in November vs 5.3 ±3.2 cells/mm^2 section in July).

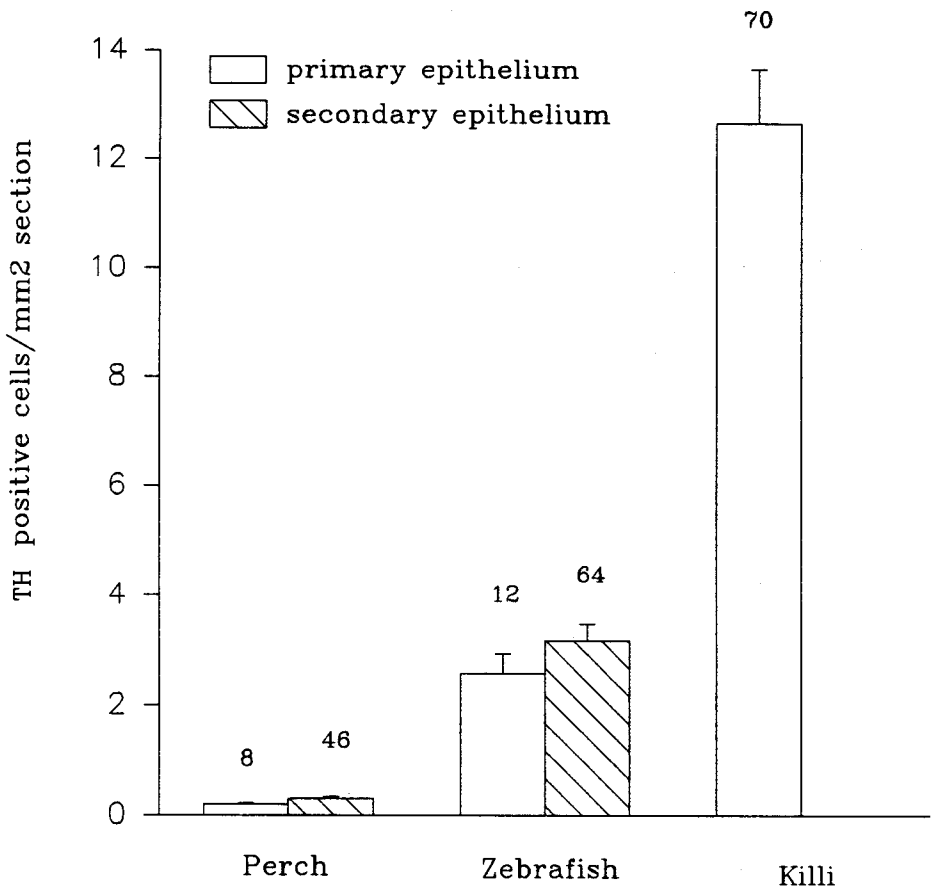
TH positive cells in the gill epithelia

TH positive cells were found in perch, zebrafish and killifish. Of these three species, perch had the lowest density with 0.2 and 0.3 cells/mm^2 section in the primary and secondary epithelia respectively. Zebrafish revealed a higher number of these cells in comparison with 2.5 cells/mm^2 section in the filament and 3.2 cells/mm^2 section in the lamellae. It is interesting to note that these data are relatively close in number to 5-HT positive cells found in this species with 1.6 cells/mm^2 section in the primary and 4 cells/mm^2 section in the secondary epithelia. The last species which had TH positive cells, did not have any in the lamellae as mentioned earlier, but had 12.7 cells/mm^2 section in the filament which is five times the number of cells found in zebrafish.

Catecholamine Stores in Fish Gill Tissue

The presence of TH$^+$ cells in the gills of some of the species examined, raises the question whether there was a corresponding selective accumulate of catecholamines (CA), since TH is the rate limiting enzyme in CA biosynthesis. Therefore, tissues from gills rich in TH$^+$ cells were compared to those that were devoid of such cells, with respect to CA storage. HPLC (see methods) with electrochemical detection was used to separate the CA, norepinephrine (NOR), epinephrine (EPI) and dopamine (DOP).

Figure 13. Histogram represents mean number ± SEM of TH positive cells per mm^2 /section (na= number of animals) in primary and secondary epithelia in perch (na= 3), zebrafish (na= 3) and killifish (na= 3). Killifish lacked TH positive cells in the secondary epithelium and is represented by cell numbers in the primary epithelium only. The numbers above the histogram represent the number of sections sampled (ns).


TH positive cells/mm2 section
14
12
10
8
6
4
2
0
primary epithelium
secondary epithelium
8
46
12
64
70
Perch
Zebrafish
Killi

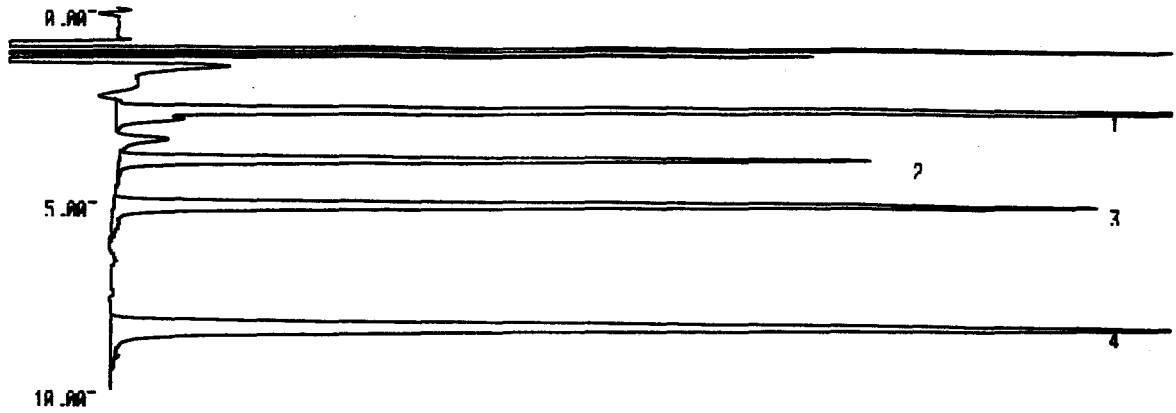
Examples of HPLC peaks representing injected CA controls as well as CA stores in tissue samples are illustrated in Figure 14. Peaks from the control correspond to 25 nM samples of NOR, EPI, di-3,4-hyroxybenzylamine hydrobromide (DHBA) and DOP. Figure 14b is an example of a trace from trout gill homogenate and 14c is a sample from perch. Comparative quantitative analyses of these data are represented in Figure 15. Zebrafish had the highest concentrations of NOR and EPI with 3.3 and 6 nmols/gram wet weight tissue respectively. Though trout did not show any TH positive cells the gill contained higher EPI and DOP concentrations, (NOR 0.8, EPI 1.8 and DOP 1.6 nmols/gram wet weight tissue) than perch (NOR 0.8, EPI 0.85 and DOP 0.2 nmols/gram wet weight tissue). Perch overall had the lowest concentrations of CA stores in the first arch except for NOR, killifish having less NOR with 0.35 nmols/gram wet weight tissue. killifish also had a fairly low amount of DOP (0.34 nmols/gram) and except for zebrafish had a relatively high amount of EPI (2.7 nmols/gram). With the exception of perch a general observation noted was the high concentrations of EPI in the gill tissue relative to the other catecholamines.

Dispersed Cell Cultures

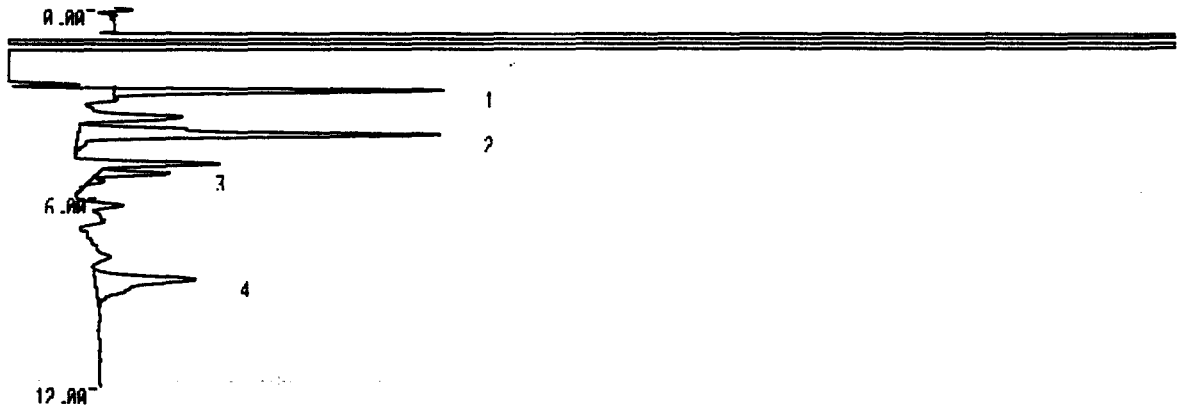
The process of dispersing and culturing cells is a beneficial technique, making it possible to study membrane properties and electrophysiological characteristics of the isolated cells. After culturing techniques are established, identification of the specific cell which is to be studied is crucial. Culturing methods for fish gill cells are

Figure 14. HPLC chromatograms showing readings from 25 nm external controls (a), trout gill homogenate (b) and perch gill homogenate (c). The peaks in all three chromatograms represent norepinephrine (1), epinephrine (2), di-3,4-hyroxybenzylamine hydrobromide (3), and dopamine (4).

a



b



c

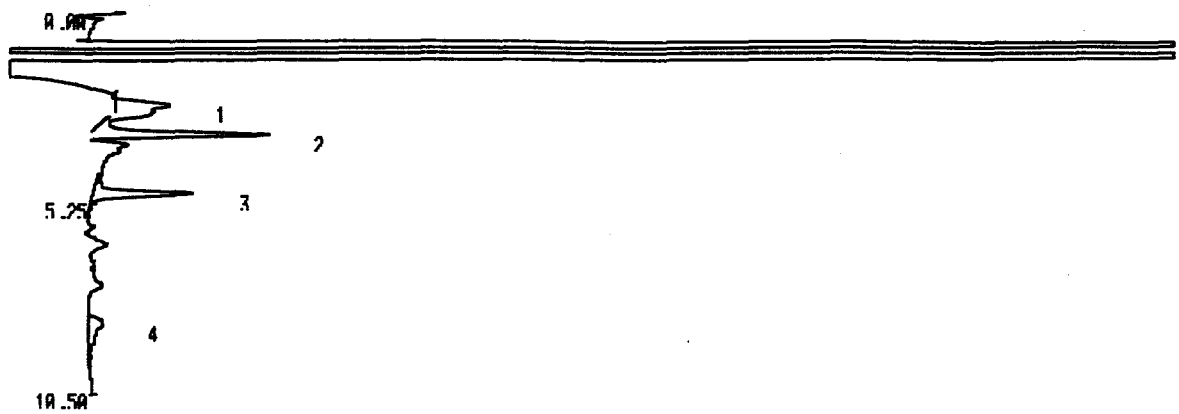
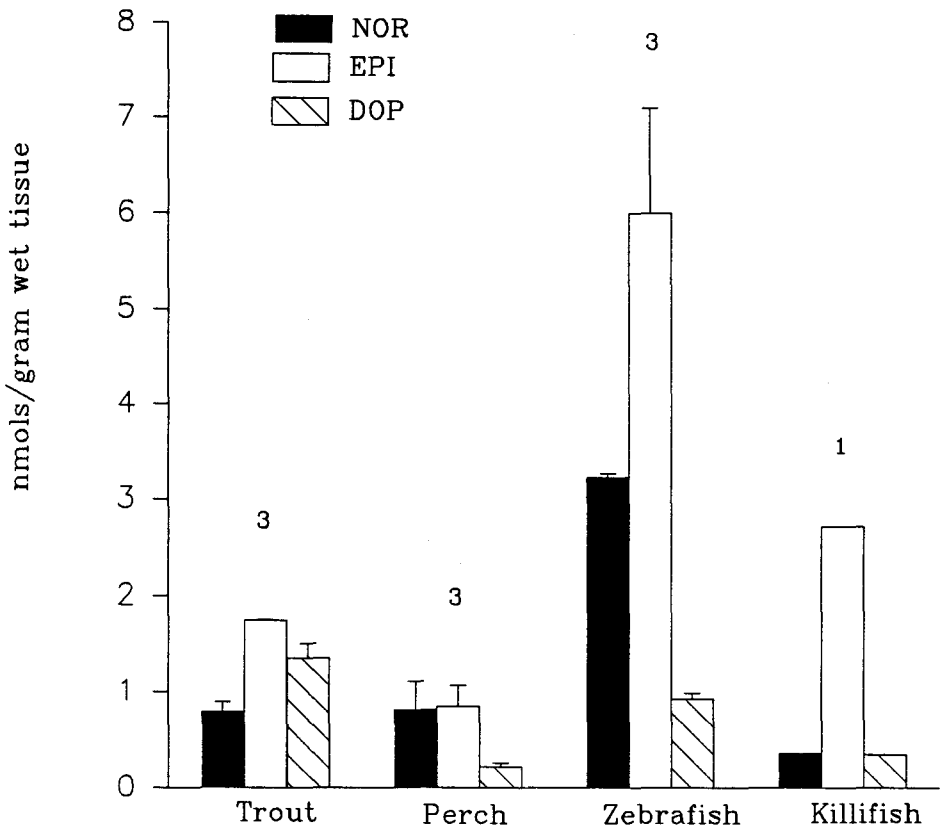


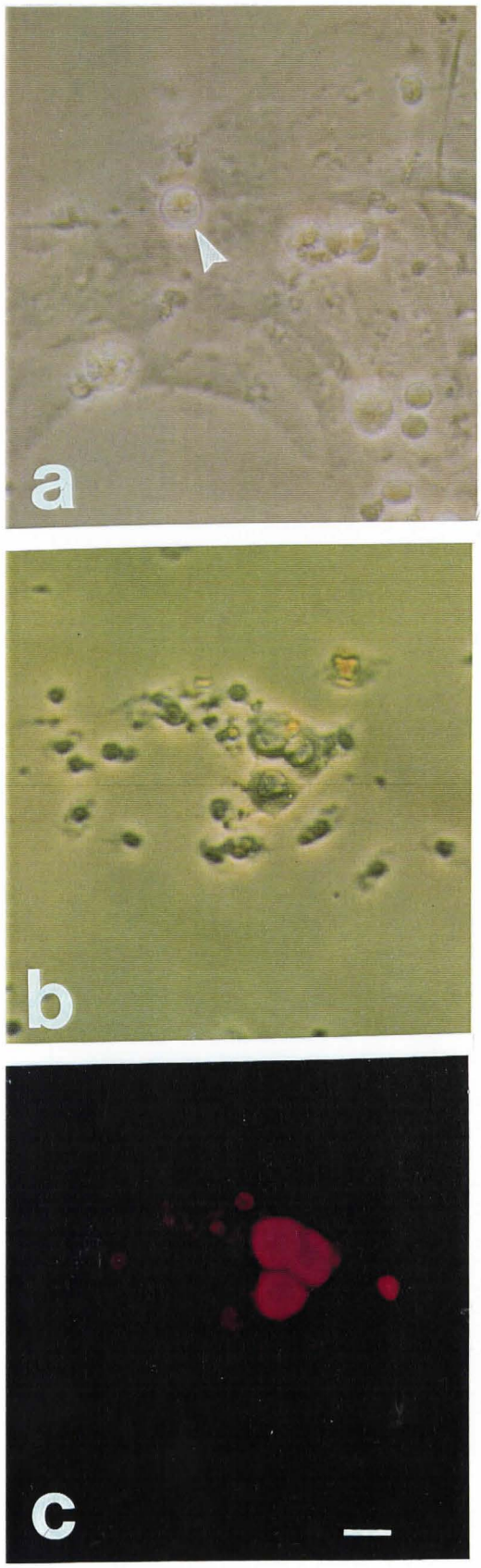
Figure 15. Histogram represents the mean ± SEM of the total catecholamine (CA) stores separated by high performance liquid chromatography (HPLC) and represent norepinephrine (NOR), epinephrine (EPI) and dopamine (DOP). Values representing the means are expressed in nmols/ gram wet tissue weight of the filaments in the first arches from trout, perch, zebrafish, and killifish. Numbers above the histograms represent the total number of fish from which tissue homogenates were extracted for each species.


nmols/gram wet tissue
8
7
6
5
4
3
2
1
0
NOR
EPI
DOP
3
3
3
1
Trout
Perch
Zebrafish
Killifish

established, and though NECs from mammals have been identified in culture, there have not been any studies which have identified NECs in dispersed gill cell cultures. After 48 hr *in vitro*, a monolayer (sometimes forming a bilayer in areas) of various kinds of cells originating from the gill was present. An example of cells in an unfixed culture is given in Fig. 16a. Many cells in culture were erythrocytes, which were identified by their typically flat appearance, oval shape and a visible nucleus (not shown). Other cells present in the cultures consisted of the major cell types in the gill epithelium including mucous cells, chloride cells, pavement cells and NECs. The pavement cells typically become very flat in culture and extend numerous processes appearing much like fibroblast type cells (Fig. 16a).

In order to identify the NECs present in cultures immunocytochemical staining against 5-HT was performed. Due to the fixation and immunostaining procedures, some cells which had not adhered to the substrate sufficiently were lost. When the procedure was complete however, a majority of the cells were still intact. Examination of the cultures with the aid of a microscope equipped with epifluorescence revealed 5-HT positive cells (Fig.16b,c). These cells were dispersed throughout the culture occasionally in groups or clusters of two to three, more frequently they were observed as isolated-single cells. The NECs were approximately 8 to 10μm in diameter and infrequently, short processes extending from the cells were observed. The 5-HT positive NECs appeared to make up a small portion of the total cell population *in vitro*.

Figure 16. Dispersed and cultured perch gill epithial cells after a 48 hr incubation are shown in (a). The arrow points to an unidentified cell along with other cells positioned on a background fibroblast like cell (pavement cell). A cluster of cells in a different culture incubated for 48 hr and fixed with paraformaldeyde and immunostained for 5-HT (see methods) are seen with phase contrast (b) the corresponding image © depicts three 5-HT positive cells in a cluster. Scale bar = 10 μm.


a
b
c

Discussion

Since the first description of NECs in the filamental gill epithelium of fish by Dunel-Erb et al. (1982), and in the lamellar epithelium by Zaccone et al. (1992), there have only been qualitative comparisons made in the number of NECs among fish species. The first study identified NECs in the primary epithelium of various species and described their location along the entire length of the filament on the efferent side, with the larger population of cells at the distal tip. The cells were adjacent to the basal lamina near the filament parenchyma and contained the efferent filament artery as well as the central venous sinus. Distal and proximal NECs were innervated by efferent and afferent catecholaminergic nerves. These NECs had processes which extended through the primary epithelium to the external environment. Zaccone et al. (1992) observed NECs in the secondary epithelium of catfish and these were occasionally found traversing the epithelial cell bilayer, in contact with the mucosal surface and the basal lamina. The results of this thesis are consistent with previous findings (Dunel-Erb et al. 1982; Zaccone et al. 1992). The closed type cells which never reach the external environment are located along the efferent artery of the gill filament and the NECs in the lamellae were often of the open type which were exposed to the mucosal surface. Bailly et al. (1992) found NECs in the distal part of the filament epithelium while the present study only found them in the lamellae. An obvious explanation for this could be that the previous study used the tilapia, *Oreochromis mossambica*, and this study examined the NECs in *Oreochromis niloticus.*

An interesting finding in the present study is the fact that some species such as

trout, killifish, and bass had NECs exclusively in the primary epithelium, whereas in tilapia NECs were located strictly in the secondary or lamellae epithelium. A third group including perch and zebrafish had them in both epithelia. The quantitative aspect of the study revealed differences in the cell densities both between the two epithelial locations in the same species, as well as among the different species examined. The reasons for the variability in cell location and number are not clear. One possibility is that it may be due to behavioural differences and/or environmental conditions to which the bottom dwellers reside in water with relatively low environmental O_2 content, compared to other species which inhabit streams. Similar situations arose in comparative studies of other chordate subclasses including Reptilia. Some species examined such as *Pseudemis scipta elegans*(Scheuermann et al. 1983) revealed NEBs exposed to the lumen while others did not, *Basiliscus vittatus* (Van den Steen, 1994). The most probable explanation for the differences among classes and species are evolutionary factors.

It has been conjectured that the NECs in the gills of fish are homologous to the NEBs in mammalian lungs and/or type I cells in the carotid body based on morphological similarities (Dunel-Erb et al. 1982), and similar physiological responses of the gills to the carotid body (Burleson and Milsom, 1993). In the latter study, O_2-sensitive electophysiological responses from the first gill arch resemble those from the carotid body, as revealed in studies where hypoxia or cyanide were used as the stimulus. These comparisons inevitably led to the conclusion that the chemoreceptors in the gill are evolutionary precursors to the those in mammalian carotid body and lung. While

considering the phylogeny of the NEC, Goniakowska-Witalinska, (1997) theorised that there were three main steps in the evolutionary path taken by this cell. The first step is characterized by the single closed type NECs found in some salamanders deep within the epithelial tissue and amphibians where the cells are closer to the lumen surface. Another characteristic of the first step is seen in bowfin with open single NECs. None of these cells were found to be innervated. The second step involves the innervation of these single cells both the closed and open types. The final stage is the grouping of the single cells to form a closed NEB found in some amphibians and then the open NEB in higher vertebrates. During this transition there is an increased innervation and complexity of the organ.

One possible explanation for NECs in the two locations of gill epithelia is that they represent the early ancestors or evolutionary precursors of different types of O_2 chemoreceptors. Cells in the lamellae which are exposed to the external water, maybe analogous to NEC clusters or NEBs in the mammalian lung which are exposed to the airways. On the other hand, the closed type NECs of the filament epithelium, which are hypothesized to sense arterial O_2 levels, may well be the precursors to the O_2 chemoreceptor or type I cells in the mammalian carotid body. At the moment this is highly speculative, though there is strong evidence that there are three types of O_2 sensors in the gills (Burleson and Mislom, 1993). NECs in the primary epithelium are likely to be at least one of these sensors based on location and functional responses to hypoxia. NECs in fish gills have not yet been directly shown to have O_2 sensing capabilities. Further

investigations are required to determine the functioning of both NEC populations, especially those located in the lamellae (open type).

Seasonal Comparison of NEC numbers in perch Gill

A previous study by Dunel-Erb et al. (1994) examined seasonal differences in NECs found in the primary epithelial layer of the trout (*Oncorhynchus mykiss*). They found that the cells doubled in number in the spring (April) compared to winter (January-February). In this thesis perch were examined from this viewpoint, and there was approximately a five fold increase in primary epithelial NECs in July compared to November. The seasonal difference in cell numbers may be due to the different species (trout vs. perch) examined, or the variation in the months that the data were collected. Regardless, the data confirm that NECs in the primary epithelium do decrease during winter months. Dunel-Erb et al. (1994) speculated that a change in the photoperiod between the months examined might have contributed to the seasonal variation since there was only a 2°C change in environmental temperature between spring and winter experiments. In the present study, however, the perch were maintained indoors and the photoperiod remained constant throughout the year; the temperature on the other hand fluctuated by 10°C. Another interesting finding in this study was that the NECs in the secondary epithelium did not change in number significantly between July and November. Dunel-Erb et al. (1994) suggested that because salmonids tend to be more active in the spring due to feeding, and less active in the winter, a time of reproduction, monitoring

blood O_2 levels may not be as important. This may explain why the NEC density decreased in the primary filament during the winter since the need for internal O_2 receptors may not be crucial. However, the fish may still require an external lamellar O_2 sensor during this dormant time to give early warnings of hypoxic conditions in the environment, possibly resulting in behavioural avoidance. On the other hand, if the lamellar or open type NECs are in fact calcium modulators, rather than O_2 sensors, as suggested by Zaccone et al., (1992), then they may be required to maintain calcium homeostasis during all seasons. This could also explain the lack of change in lamellar NEC numbers observed in the perch gill in the present study between November and July.

Catecholaminergic cells in the fish gill

In the present study tyrosine hyroxylase (TH) positive cells were observed in gill epithelia of perch, killifish, and zebrafish. To our knowledge, this is the first report of TH positive cells in fish gills. The data from perch and killifish indicate that the TH positive cells do not correlate with the 5-HT positive NECs because of the differences in numbers. Perch had many more 5-HT positive than TH positive cells, whereas the converse was true for killifish. Secondly, the locations of the TH positive cells do not correlate with the 5-HT positive cell in this study. Zebrafish on the other hand do demonstrate similar numbers of TH positive and 5-HT positive cells as well as similar locations, determined by serial sectioning. Hence, some species appear to express two populations of monoamine containing cells, one containing primarily 5-HT and the other, catecholamines (CA), as

revealed by the presence of TH, the rate limiting enzyme in CA biosynthesis. It is possible that in zebrafish, both monoamines are made by the same cells. Further studies are required to determine whether TH positive cells in the gill represent a new subpopulation of NECs, or a different type of neuroendocrine cell. A recent study examined the effects of exogenously applied 5-HT on the gill and questioned the hypothesized role of NECs as O_2 chemoreceptors which release 5-HT. Sundin et al. (1995) found that exogenously applied 5-HT caused a constriction of the efferent filamental vasculature, resulting in an increased blood flow to more proximal parts of the filament. This study demonstrated that 5-HT did not result in the recruitment of lamellae and was not effective in compensating for hypoxic conditions though it may help during anoxia by preventing the loss of O_2 to the environment. This may validate the reason for the existence of a TH positive cell population in the gill. For example, a study by Ostlund and Fange (1962) found that perfusion of isolated gills with 5-HT and acetylcholine resulted in a constriction of the vessels, whereas the catecholamines, epinephrine (EPI) and norepinephrine (NOR) had a strong dilatory effect. In this case, cells which release catecholamines (as opposed to 5-HT) could cause vasodilation of the efferent artery, thereby recruiting more lamellae and improve O_2 uptake, as well as increasing Hb-O_2 affinity (for review see Thomas and Perry, 1992).

It has been shown that blood CA levels increase during hypoxia (Randall and Perry, 1992) and improves O_2 transport across the gill epithelium. In addition there is a release of red blood cells from the spleen and an increase in ventilation followed by

bradycardia. In the present study quantification of CA stores in the gill could not be correlated to the number of TH positive cells because measurements of the CA stores included contributions from other sources such as nerves and plasma. It is noteworthy, that a catecolaminergic innervation of NECs has been described in the gills of trout (Bailly et al., 1992).The examination in this study of CA stores in the gill does not reflect the contents of the TH positive cells alone, since NECs have been shown to have both indolaminergic and catecholaminergic innervation (Bailly et al. 1992). Epinephrine is the most prominent CA released into the bloodstream from the chromaffin tissue due to hypoxia (Perry and Reid, 1994) and may show a correlation with the gills. Gill tissue had higher stores of epinephrine than norepinephrine or dopamine in all the species examined with the exception of perch. If gill TH positive cells also act as O_2 chemoreceptors they may release epinephrine locally during hypoxia and regulate the blood flow or contribute in an endocrine-like manner to plasma CA. The proposed roles for the TH positive cells found in the gill during this study are only speculative. However it is evident that CA play an intrinsic part in O_2 chemoreception in fish and further study regarding the role of these cells is required.

5-HT positive cells in perch gill cultures

This study has for the first time identified 5-HT positive cells in isolated fish gill cell cultures. The fact that the cells contain 5-HT suggests that they are NECs and are the same cells as those described *in vivo*. Another fact consistent with the idea that 5-HT positive cells seen in cultures are NECs is their size. NECs were found to have an average

size of approximately 8 to 10 μm in situ, which is similar with the size of the 5-HT positive cells in culture. The sparse density of these cells *in vitro* may reflect the relatively low number of NECs found in the gill epithelia.

Most of the outstanding questions regarding the function of NECs in fish gills might be more directly addressed with the isolation of these cells in culture. For example, patch clamp electrophysiology on identifiable NECs might be used to investigate whether these cells are O_2 sensitive, as was shown in mammalian lung (Youngson et al., 1993), and carotid body (see Gonzalez at al. 1992). Techniques for enriching the cultures or increasing the NEC population have not been attempted. In future studies however, techniques such as density gradients using percol, or selection for these cells using fluorescent 5-HT labelling and flow cytometry should be explored. Another method for enriching NEC populations is to incubate the gill tissue in lysis-medium (9 parts 0.17 M NH_4Cl, 1 part 0.17 M Tris/HCL pH 7.4; see Yust et al., 1976) prior to enzyme exposure. This solution results in the specific lysis of red blood cells, which make up the majority of the cell population in these cultures. The next step is the consistent identification of these cells *in vitro,* especially using vital markers to aid electrophysiological studies. Previous studies have shown that the vital stain neutral red labels neuroendocrine cell such as Merkel cells from salamander skin and Xenopus (Nurse et al., 1983) and rabbit lung NECs (Youngson et al., 1993). It would be interesting to determine if NECs from teleost fish gills have the same physiological characteristics as mammalian NECs such as an O_2-sensitive potassium channel linked to an O_2-sensitive protein (NADPH oxidase)

(Youngson et al., 1993). In addition, controlled CA release experiments from isolated NECs could determine if 5-HT is the principle neurotransmitter released from these cell when exposed to hypoxic conditions, and secondly, if this release is Ca^{++} dependent. These studies may also help to elucidate the difference, if any, in the roles of open and closed type NECs. More selective cell isolation procedures would make it possible to determine if the cells located in the lamellae are O_2-sensors, calcium modulators, or have some other function. The two populations of NECs would have to be distinguishable, possibly by starting with the presence of the peptide calbindin which is present in open NECs only (Zaccone et al., 1992). A second option may be to disperse the cells from a species containing only lamellar NECs such as tilapia, and compare them to other species such as killifish, where NECs occur exclusively in the primary epithelium. Many questions regarding the evolutionary aspects of the NEC have been addressed through immunocytochemical and electron microscopic analyses. In future studies comparing the physiological responses of isolated cells among species could contribute significantly to our understanding of the phylogeny and role of NECs in vertebrate systems.

Literature Cited

Adriaensen D, Scheuermann DW, Gomi T, Kimura A, Timmermans J, DeGroodt-Lasseel MHA (1994) The pulmonary neuroepithelial endocrine system in the quail, *Coturnix coturnix*. Light- and electron-microscopical immunocytochemistry and morphology. Anat. Rec. 239: 65-74.

Adriaensen D, Scheuermann DW, Timmermans JP, De Groodt-Lasseel MHA (1990) Neuroepithelial endocrine cells in the lung of the lung fish *Protopterus aethiopicus* : An electron and fluorescence microscopical investigation. Acta. Anat. (Basel), 139: 70-77.

Bailly Y, Dunel-Erb S, Laurent P (1992) The neuroepithelial cells of the fish gill filaments: indolamine-immunocytochemistry and innervation. Anat. Rec. 233: 143-161.

Bailly Y, Dunel-Erb S, Geffard M, Laurent P (1989) The vascular and epithelial serotonergic innervation of the actinopterygian gill filament with special reference to the trout, *Salmo gairdneri*. Cell Tissue Res. 258: 349-363.

Bailly Y, Dunel-Erb S (1986) The sphincter of the efferent filament artery in teleost gills. I. Structure and parasympathetic innervation. J. Morpohol. 187: 219-237.

Burleson ML and Milsom WK (1993) Sensory receptors in the first gill arch of rainbow trout. Resp. Physiol. 93: 97-110.

Burleson ML and Milsom WK (1995a) Caridio-ventilatory control in rainbow trout: I. Pharmacology of branchial, oxygen-sensitive chemoreceptors. Resp. Physiol. 100: 231-238.

Burleson ML and Milsom WK (1995b) Caridio-ventilatory control in rainbow trout: II. Reflex effects of exogenous neurochemicals. Resp. Physiol. 101: 289-299.

Conte FP (1969) Salt secretion. In "Fish Physiology" (W. S. Hoar and D. J. Randall, eds), vol. I, pp. 241-283. Academic Press, New York.

Cutz E, Chan W, Wong V, Conen PE (1975) Ultrastructure and fluorescence histochemistry of endocrine (APUD-type) cells in tracheal mucosa of human and various animal species. Cell Tissue Res. 158: 425-437.

Cutz E, Gillan JE and Tryan AC (1985) Neurondocrine cells in the developing human lung: Moropohologic and functional consierations. Pediatr. Pulmonaol. 1(suppl): S21-S29.

Cutz E (1997) Intoduction to pulmonary neuroenocrine cell system, structure-function correlations. Microsc. Res. Tech. 37: 1-3.

Cutz E, Speirs V, Yeger H, Newman C, Wang D, Perrin DG (1993) Cell Biology of Pulmonary Neuroepithelial Bodies-Validation of an in vitro model. Anat. Rec. 236: 41-52.

Donald JA (1987) Comparative study of the adrenergic innervation of the teleost gill. J. Morph. 193: 63-73.

Dunel-Erb S, Bailly Y, Laurent P (1982) Neuroepithelial cells in fish gill primary lamellae. J.Appl. Physiol. 53: 1342-1353.

Dunel-Erb S, Bailly Y, Laurent P (1989) Neurons controlling the gill vasculature in five species of teleosts. Cell Tissue Res. 255: 567-573.

Dunel-Erb S, Chevalier C, Laurent P (1994) Distribution of Neuroepithelial cells and neurons in the trout gill filament: comparison in spring and winter. Can. J. Zool. 72: 1794-1799

Feyrter F (1938) Uber diffuse endocrine epitheliale organe. J. A. Barth, Leipzig.

Frohlich F (1949) Die "helle-zelle" der bronchialschleimhaut und ihre beziehungen zum problem der chemoreceptoren. Frankf. Z. Pathol. 60: 517-559.

Fryer JL and Lannan CN (1994) Three decades of fish cell culture: A current listing of cell lines derived from fishes. J. Tis. Cult. Meth. 16: 87-94.

Fujita T (1989) Present status of paraneuron concept. Arch Histol. Cytol. 62 (suppl.), 1-8.

Glorieux R (1963) Les cellules argentaffines du poumon et leurs connexions avec le systeme nerveux. Arch. Biol. 74: 377-390.

Goniakowska-Witalinska L (1980) Endocrine-like cells in the lungs of the newt *Triturus alpestris* Laur. Cell Tissue Res. 210: 521-524.

Goniakowska-Witalinska L (1997) Neuroepithelial bodies and solitary neuroendocrine cells in the lungs of amphibia. Microsc. Res. Tech. 37: 13-30.

Goniakowska-Witalinska L, Zaccone G, Fasulo S, Youson J (1995) The neuroendocrine cells in the gills of the bowfin *Amia calva*. The ultrastructural and immunocytochemical study. Folia Histochem. Cytobiol. 33: 171-177.

Goniakowska-Witalinska L, Lauweryns JM, Zaccone G, Falsulo S, Tagliafierro G (1992) Ultrastructure and immunocytochemistry of the neuroepithelial bodies in the lung of the tiger salamander, *Ambystoma tigrinum* (Urodela, Amphibia). Anat. Rec. 234: 419-431.

Gonzalez C, Almaraz L, Obeso A, and Rigual R (1992) Oxygen and acid chemoreception in the carotid body chemoreceptors. Trends Neurosci. 15: 146-153.

Jackson A and Nurse CA (1997) Dopaminergic properties of cutltured rat carotid body chemoreceptors grown in normoxic and hypoxic environments. J. Neurochem. 69: 645-654.

Laurent P and Dunel S (1980) Morphology of gill epithelia in fish. Regulatory Integrative Comp. Physiol. 7: R147-R159.

Laurent P (1984) Gill internal morphology. In "Fish Physiology" (W. S. Hoar and D. J. Randall, eds), vol. X, pp. 73-172. Academic Press, New York.

Lauweryns JM, Lommel AV (1982) Morphometric analysis of hypoxia-induced synaptic activity in intrapulmonary neuroepithelial bodies. Cell Tissue Res. 226: 201-214.

Lauweryns JM, Cockelaere M, Theunynck P (1972) Neuroepithelial bodies in the respiratory mucosa of various mammals. A light optical, histochemical and ultrastructural investigation. Z. Zellforsch. 135: 569-592.

Manger PR and Hughes RL (1992) Ultrastructure and distribution of epidermal sensory receptors in the beak of the echidna, *Tachyglossus aculeatus.* Brain Behav. Evol. 40: 287-296.

McLelland J and MacFarlane CJ (1986) Solitary granular endocrine cells and neuroepithelial bodies and neuroepithelial bodies in the lungs of the ringes turtle dove (*Strptopelia risoria*). J. Anat. 147: 83-93.

Milsom WK and Brill WB (1986) Oxygen sensitive afferent information arising from the first gill arch of yellowfin tuna. Resp. Pysiol. 66: 193-203.

Moll I, Moll R, Franke WW (1986) Fomation of epidermal and dermal Merkel cells during fetal skin development. J. Invest. Dermatol. 87: 779-789.

Morgan M (1974) Developoment of secondary lamellae of the gills of the trout, *Salmo gairdneri* (Richardson). Cell Tissue Res. 151: 509-523.

Nilsson S (1984) Innervation and pharmacology of the gills. In "Fish Physiology" (W. S. Hoar and D. J. Randall, eds), vol. X, pp. 185-219. Academic Press, New York.

Nurse CA, Mearow KM, Holmes M, Veshern B and Diamond J (1983) Merkel cell distribution in the epidermis as determined by quinacrine fluorescence. Cell Tissue Res. 228(3): 511-524.

Ostlund E and Fange R (1962) Vasodilation by adrenaline and noradrenaline, and the effects of some other substances on perfused fish gills. Comp. Biochem. Physiol. 5: 307-309.

Pastor LM, Ballesta J, Hernandez F, Perez-Tomas R, Zuasti A, and Ferrer C (1987) A microscopic study of the tracheal epithelium of *Testudo graeca* and *Pseudemis scripta elegans.* J. Anat. 153: 171-183.

Patrizi G, and Munger BL (1966) The ultrastructure and innervation of rat vibrissae. J. Comp. Neurol. 126: 423-435.

Pearse AGE (1968) The cytochemistry and ultrastructure of polypeptide hormone producing cells of the APUD series and the embryologic, physilogic and pathologic implications of the concept. J. Histochem. Cytochem. 17: 303-313.

Perry SF and Reid SG (1994) The effects of acclimation temperature on the dynamics of catecholamine release during acute hypoxia in the rainbow trout *Oncorhynchus mykiss.* J. exp. Biol. 186: 289-307.

Perry SF and Reid SG (1992) Relationships betwen blood oxygen content and catecholamine levels during hypoxia in reainbow trout and american eel. Am. J. Physiol. 263: R240-R249.

Polak JM, Becker KL, Cutz E, Gail DB, Goniakowska-Witalinska L, Gosney JR, Lauweryns JM, Linnoila I, McDowell EM, Miller YE, Scheuermann DW, Springall DR, Sunday ME, Zaccone G (1993) Lung endocrine cell markers, peptides and amines. Anat. Rec. 236: 169-171.

Randall DJ, and Perry SF (1992) Catecholamines. In "Fish Physiology" Randal DJ et al (eds), vol 12B. Academic Press, New York, pp 255-300.

Scheuermann Dw, De Groodt-Lasseel MHA, Stilman C, and Meisters ML (1983) Acorrrelative light-, fluorescent- and electron microscopic study of neuroepithelial bodies in the lung of the red-eared turtle, *Pseudemys scripta elegans.* Cell Tissue Res. 234: 249-269.

Scheuermann Dw, Stilman C and De Groodt-Lasseel MHA (1988) Microspectrofluorimetric analysis of the formaldehyde induced fluorophores of 5-hydroxytryptamine and dopamine in intrapulmonary neuroepithelial bodies after administration of L-5-hydroxytryptophan and L-dopa. Histochemistry, 88: 219-225.

Scheuermann DW (1997) Comparative histology of pulmonary neuroendocrine cell system in mammalian lungs. Microsc. Res. Tech. 37: 31-42.

Sonstegard KS, Wong V, and Cutz E (1976) Dissociation of epithelial cells from rabbit trachea and small intestine with demonstration of APUD endocrine cells. Am. J. Anat. 147: 357-374.

Sorokin SP (1988) The respiratory system. In "Cell and Tissue Biology" Sixth edition (L. Weiss, ed.) Chapter 25, pp. 753-813. Urban and Schwarzenberg, Baltimore.

Speirs V, Cutz E (1993) An overview of culture and isolation methods suitable for in vitro studies on pulmonary neuroendocrine cells. Anat. Rec. 236: 35-40.

Sundin L, Nisson GE, Block M, and Hofman CO (1995) Control of gill filament blood flow by serotonin in the rainbow trout, *Oncorhynchus mykiss.* Am. J. Physiol. 268: R1224-R1229.

Thomas S and Perry SF (1992) Contol and consequences of adrenergic activation of red blood cell Na+/H+ exchange on blood oxygen and carbondioxide transport. J. Exp. Zool. 263: 160-175.

Van den Steen P, Van Lommel A, Lauweryns JM (1994) Neuroepithelial bodies in the lung of *Basiliscus vittatus* (Reptilia, Iguanidae). Anat. Rec. 239: 158-169.

Verbost PM, Flik G, Cook H (1994) Isolation of gill cells. In "Biochemistry and Molecular Biology of Fishes" (Hochachka and Mommsen eds.) Vol III, chapter 21, pp. 239-247.

Walsh C, McLelland J (1974) Granular "endocrine" cells in avian respiratory epithelia. Cell Tissue Res. 153: 269-276.

WhitearM (1989) Merkel cells in lower vertebrates. Arch. Histol. Cytol. 52 (suppl.): 415-422.

Young JZ (1981) The life of vertebrates. Third edition, Oxford University Press, New York.

Youngson C, Nurse C, Yeger H, Cutz E (1993) Oxygen sensing in airway chemoreceptors. Nature 365: 153-155.

Yust I, Smith RW, Wunderlilch JR and Mann DL (1976) Temporary inhibition of antibody-dependent cell-mediated cytotocicity by retreatment of human attacking cells with ammonium chloride. J. Immuno. 116: 1170-1172.

Zaccone G, Fasulo S, Ainis L, Licata A (1997) Paraneurons in the gills and airways of fishes. Microsc. Res. Tech. 37: 4-12.

Zaccone G, Fasulo S, Ainis L (1994) Distribution patterns of the paraneuronal endocrine cells in the skin, gills and airways of fishes as determined by immunohistochemical and histological mehtods. Histochem. J. 26: 609-629.

Zaccone G, Wendelaar Bonga SE, Flik G, Fasulo S, Licata A, Lo Cascio PL, Mauceri A, Lauriano ER (1992) Localization of Calbindin D28K-like immunoreactivity in the fish gill: a light microscopic and immunoelectron histochemical study. Regulatory Peptides 41: 195-208.

Zaccone G, goniakowska-Witalinska L, Lauweryns JM, Fasulo S, Tagliafierro G (1989) Fine structure and serotonin immunohistochemistry of the neuroendocrine cells in the lungs of the bichirs *Polypterus delhezi* and *P. Ornatipinnis.* Basic Appl. Histochem. 33: 277-287.