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Hypoxia stress and Hsp 70 expression by Pseudobranchial Neurosecretory System in Indian catfish, *Clarias batrachus*

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ABSTRACT

The pseudobranchial neurosecretory system- third system of neurosecretion known to exist in air-breathing fishes and in some other fresh water fish species. The pseudobranchial neurosecretory cells showed significant changes in their secretory activity, which was found consistent with the varying lengths of stress of hypoxia. It may be noted that all the group of teleosts, in which this system has been found to be present, so far share one unusual thing in common not possessed by other teleosts, i.e., capability to tolerate low oxygen tension in surrounding waters. The present work is an attempt to construct the protein profile of this novel system in, Clarias batrachuus. The work is attempted both on normoxic and hypoxic condition. Stress protein hsp 70 is also reported in the hypoxic condition indicating the role of this system to help the fish overcoming hypoxic condition in water.

Keywords: hypoxia stress, SDS-PAGE, 2-D PAGE, hsp70, *Clarias batrachus*

INTRODUCTION

Various types of paraneuronal cells are the sources of neurologically active substances, typical of the endocrine cells belonging to the diffuse neuroendocrine cell systems scattered throughout the animal body. Similar types of cells have been observed in the gills and air breathing organs of air-breathing fishes (Dunel-Erb, *et al.,* 1982; Zaccone, *et al.,* 1989, 1992; Goniakowaska, *et al.,* 1995). These groups of neuroendocrine cells with putative chemoreceptors and paracrine function have been reviewed by Zaccone *et al.* (1994, 1995) and have been found to be belonging to the category of receptosecretory cells-"Paraneuron". It is a term given to the group of cells which are endocrine and sensory but not counted as neuron, though share structural, functional and metabolic features with neurons on the basis of the production of neuromodulators, neurotransmitters or neurohormones in response to any stimulus (Fujita, 1976, 1989).

Investigations have revealed similar type of cells in several Indian teleosts, slightly away from the gills, but situated in the gill region, close to pseudobranch or carotid labyrinth (Fig.1). These cells occur in groups and their cell process run together as bundles ending up in close proximity of blood capillaries of first two blood vessels. Several lobes, comprising pseudobranchial neurosecretory cells lie in rather diffused condition without forming any compact neurohaemal organ. The cytological and morphological attributes of these cells were investigated using neurosecretion specific histological techniques and on the basis of this account, a third system of neurosecretion, **the pseudobranchial neurosecretory system**, was revealed, besides, the two known

ones, viz., hypothalamo-hypophyseal and the caudal neurosecretory system. This has now been included among the category of neuroendocrine systems known for fishes (C.E.Bond 1996).These neurosecretory cells identified seems to display all the principal morphological attributes for a neurosecretory role- a site for production- the perikaryon, axon process for transport and a neurohaemal contact site for release of bioactive substances.

To maintain oxidative metabolism active animals must be able to sense and respond to changes in environmental oxygen availability and metabolic oxygen demand. This needs special attention in regard to aquatic animals such as fish because they are subject to extreme spatial and temporal changes in environmental oxygen tension. Aquatic hypoxia is a common occurrence, especially in fresh water, and may be due to a variety of biological and physical factors. The most noticeable effect of O_2 receptor stimulation are decreased heart rate, increased blood pressure and increased ventilatory frequency and amplitude. These responses are generally regarded as the typical 'chemoreflex' responses of a water-breathing fish to hypoxia. In practice, however, depending upon the species, if it is an air-breather, and physical condition of the fish (i.e. anaesthetic, stress, temperature, etc.) the response may vary considerably. It has been observered earlier that the PNS plays an important role in overcoming the hypoxia stress of fish. In order to have a better understanding of this novel system the SDS PAGE and 2-D PAGE profile of this system is done in both, the normal and hypoxic condition. The immunoblot was done and HSP 70 protein was also reported in this system.

MATERIALS AND METHODS

TEST ANIMALS

Fig. 1. *Clarias batrachus (*Indian magur)

1) HYPOXIA INDUCTION

The air-breathing fishes, viz., *C. batrachus* was set for hypoxia. Live specimens of the above mentioned fish were kept in a jar of 5 litres and set for hypoxia. Water was filled in the jar up to the brim and covered with lid and finally sealed with wax to avoid any entry of air in it. The fish was kept in it for few hours (according to

size) and was taken out from it just before it was to hang. Then it was cut opened from the ventral side to collect the pseudobranchial neurosecretory tissue.

PREPARATION OF PROTEIN EXTRACTS

The tissue was cleaned thoroughly in chilled normal saline solution (0.89% NaCl in distilled water) and was homogenized thoroughly with the help of tissue homogenizer (REMI). The homogenates were centrifuged 5000rpm for 5min at 4^0C in a biofuge (Heraeus Fresco) and supernatants were collected, made in to small aliquots and were stored frozen at -40^o C till further use.

2) PROTEIN ESTIMATION

Protein concentration of the samples was determined by Folin-phenol method (Lowry *et al.,* 1951), using BSA as the standard.

3) SDS-PAGE

SDS-PAGE was carried out in a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories) using 5% (w/v) stacking gel and 10% (w/v) separating gel (Laemmli 1970). After electrophoresis, the gels were stained with Coomassie blue R-250 (CBB) for visualization of the proteins. Molecular weight of the protein bands was determined with reference to standards (SIGMA MARKER, M-4038). Densitometric scanning of the Coomassie-stained polyacrylamide gels were carried out using Gel Base/Gel Blot™ Pro, Gel Analysis Software, UVP.

2-D PAGE

For 2D-PAGE, the isoelectric focussing (first dimension) was performed in a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. The gel solution was prepared as follows (O' Farrell 1975): 9.2 M urea, 4% acrylamide, 2.0% (w/v) CHAPS, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte. Polymerization was initiated by 0.01% ammonium persulfate and 0.1% (v/v) TEMED. The upper chamber was filled with cathode electrode buffer (100mM NaOH). The anode electrode buffer was 10mM ortho-phosphoric acid. First-dimension gels were prefocussed at 200V for 10 min, 300V for 15 min, and 400V for 15 min. The electrode buffers were exchanged against fresh and degassed buffers. Samples were prepared by adding an equal volume of sample buffer (final conc.: 9.5M urea, 2.0% CHAPS, 5% □-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte) and incubated at room temperature for 10-15 min after which sample proteins (50-60□g) were loaded into the sample reservoirs. The samples were overlaid with 20-40 □l of sample overlay buffer (final conc.: 9M urea, 0.8% Bio-Lyte 5/7 ampholyte, 0.2% Bio-Lyte 3/10 ampholyte, and 0.05% Bromophenol blue). The 2D SDS-PAGE Standards were also run parallel to samples. The proteins were electrophoresed at 500V for 10 min followed by 750V for 3.5hr. After the first dimension run, the gels were equilibrated for 10-15 min at room temperature and were loaded on

to the slab gel for second-dimension run. The IEF gels not used immediately were stored at -70^{0}C in sample equilibration buffer. The second dimension run (SDS-PAGE) was carried out as described above. The gels were stained with either Coomassie for visualization of the proteins (Anonymous 1992)

IMMUNOBLOT

The highly conserved stress proteins/heat shock proteins (HSPs) are ubiquitous and occur in all organisms from bacteria and yeast to humans (Kregel, 2002). These proteins play important role in both normal cellular homeostasis and the stress response. Out of all HSPs the 70 kDa HSPs have been of particular interest because of their cellular abundance and their protective effects. HSP 70 has two main forms: the constitutive form, referred to as HSC 70 (HSC 70) and the predominantly inducible form, referred to as HSP 72. Diverse cells and tissues have been shown to not only have an increase in HSP 72 with stress, but also early accumulation of HSP 72 in the nucleus. Thermo tolerance with increased resistance to heat killing has been shown to correlate with the level of HSP 70 in the cells. In the present study we have analysed the PNS proteins for the presence of HSP's if any. Constitutive HSP 70 could be identified in the PNS cells of *C. batrachus* by immunoblot analysis. It would be of interest to see if the inducible form of HSP 70 is expressed during stress, as it would be of help to confirm the hypothesis that PNS play significant role in hypoxia stress. Further immunological analysis would help to identify and characterize the pseudobranchial neurosecretory proteins.

PNS proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose membranes. Transfer of the proteins was checked by reversible staining with Ponceau S. Mouse monoclonal HSP 70 antibody (Sigma H 5147) was used as the primary antibody (1/5000 dilution) and anti-mouse IgG-HRP conjugate (Sigma A 2304) was used as the secondary conjugate. The side lane shows location of the HSP 70 band in the SDS-PAGE resolved PNS proteins, along with mol. wt. markers.

RESULTS

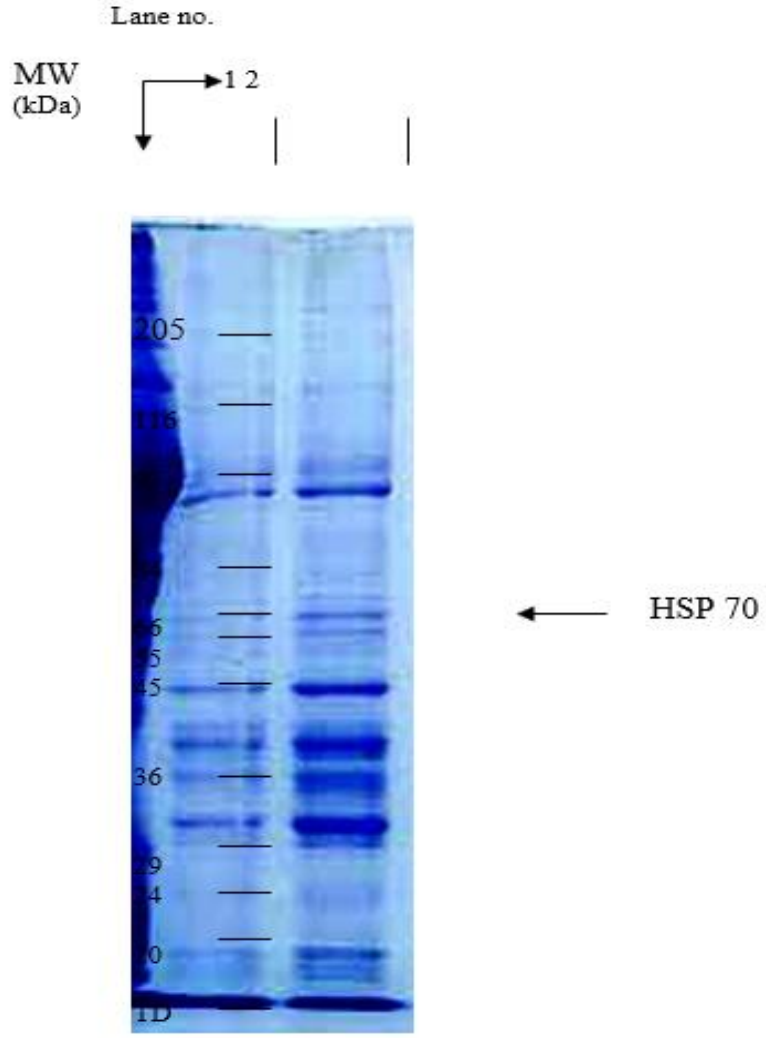

Fig.2. SDS-PAGE (Laemmli, 1970, 10% separating gel) profile of protein extract from pseudobranchial neurosecretory cells in *C. batrachus*, Lane 1: *C. batrachus* normal, Lane 2: *C. batrachus* hypoxic; ~20 g protein was loaded in each lane. The gels were Coomassie stained for visualization of the proteins.

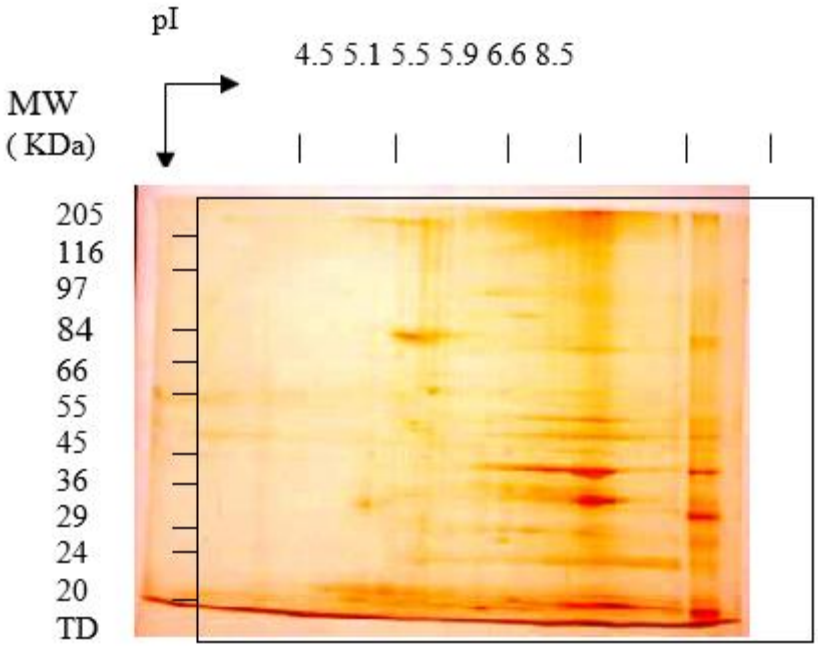

Fig.3. 2-D protein profile of pseudobranchial neurosecretory system in *Clarias batrachus*, under normal condition.

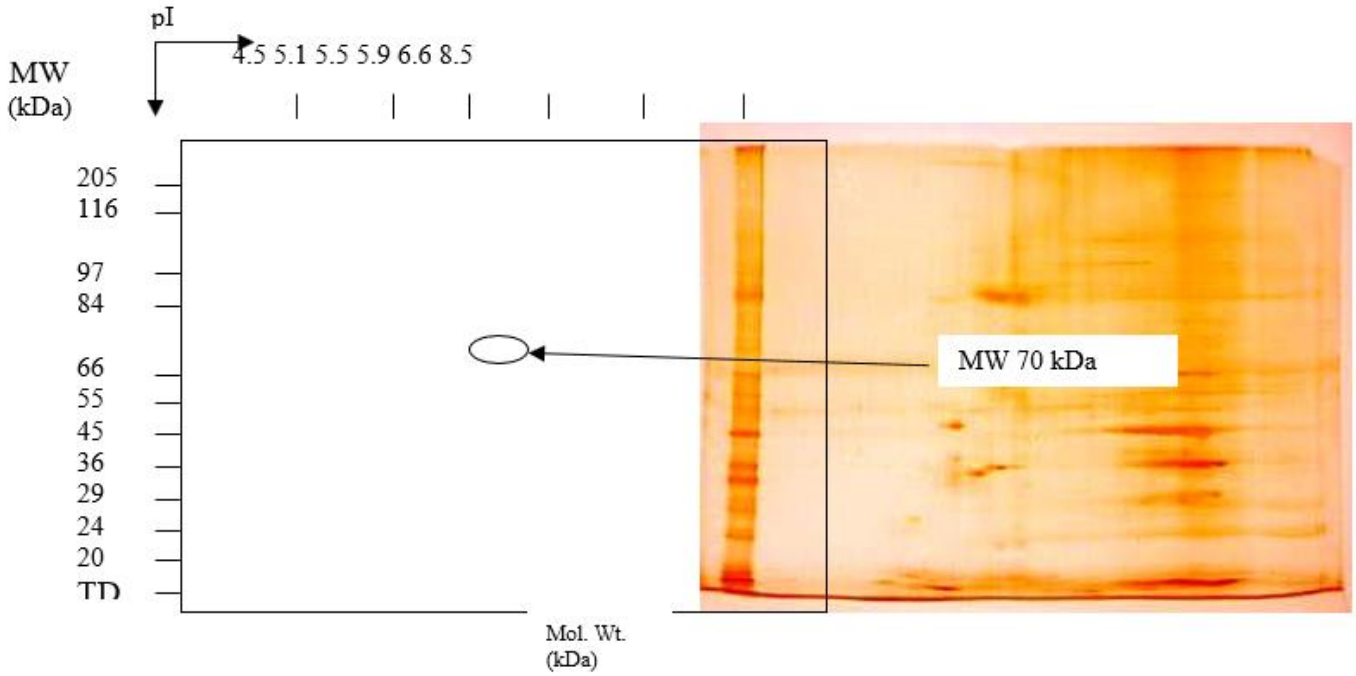

Fig.4. 2-D protein profile of pseudobranchial neurosecretory system in *Clarias batrachus*, under hypoxia.

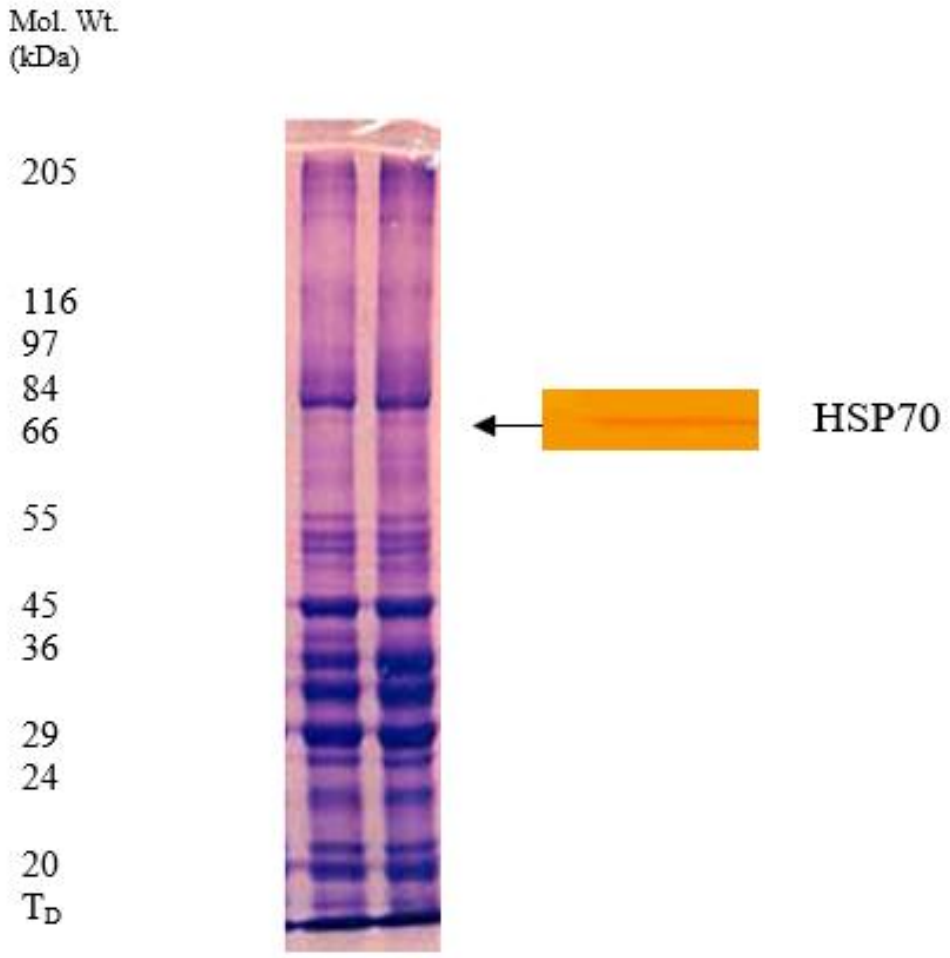

Fig.5. **Detection of constitutive HSP 70 in the Pseudobranchial neurosecretory cells of *Clarias batrachus* by immunoblot.**

DISCUSSION

The electrophoretic technique can be regarded as a reliable tool for the identification of related species (Thangaraj and Lipton, 2004). Since there is no information on pseudobranchial neurosecretory protein, identification of these specific proteins is a maiden attempt. These proteins may be useful for fish species identification and can be further categorized and used as a tool for upgrading the existing information on teleost taxonomy. Morphological and histochemical studies, already done, could be supplemented by biochemical studies. Earlier reports indicated that electrophoresis could be adopted effectively for species identification (Yoshihiro *et al.*, 1989). Image analysis of the SDS-PAGE gels helped to precisely compare the protein profiles in different species (Alan and Mackie, 1988). The two profiles, i.e. normal and hypoxic condition profile of *Clarias batrachus* shows very mild difference in the number of bands and peak absorbances.

In the molecular weight range of 116 kDa to 205 kDa there are only two bands in case of *C. batrachus*. The protein density is high in molecular weight range below 45 kDa. Four thick bands can be located in molecular weight range 45 to 29 kDa and two thick bands around 20 kDa. After mol. Wt. 84 kDa few more bands are visible in hypoxic condition. These bands may be probably the neurosecretions in response to cope up with the hypoxic stress. We can locate a band of molecular weight 70 kDa in hypoxic condition.

In case of 2-D PAGE, most of the proteins lie in the mol. wt. range 20 kDa to 205 kDa and pI 5.0 to 8.5 (Fig. 3-4), though most of them are concentrated towards the basic end. The proteins at pI zone 4.9, 5.1 and 6.6 are very prominent and more in quantity, probably may be some structural proteins. There are four major proteins A, B, C and D forming a 'tetrad' which can be used as landmark to identify the polarity of pseudobranchial neurosecretory proteins. The 2-D maps inform that most of the proteins in pseudobranchial neurosecretory tissue are basic. From this we could find out the quantity of a particular protein present. This is the first approach of such type of study in these proteins.

The study has provided fish pseudobranchial neurosecretory cell protein database, as has been constructed for several mammalian organs (Hochstrasser, *et al*., 1992). In recent years construction of 2-D maps for various proteins in different organisms ranging from bacteria to humans has been worked out to create extensive database. These maps are useful references for further studies involving pathogenicity, vaccine development, design novel drugs, etc.(Liao, *et al*., 2003).

One of the major area of current research involves a family of highly conserved proteins known as 'heat shock proteins'. The principle HSPs range in molecular mass from ~15 to 110kDa and are divided into groups based on both size and function (42, 87, 106). They are present in cytosol, mitochondria, endoplasmic reticulum and nucleus, although these locations vary depending on the particular protein. HSP70 family of proteins are the most temperature sensitive and highly conserved of the HSPs (Kregel, 2002).

Inducible heat shock protein Hsp70 is a stress protein whose expression is up regulated when the cell or organism is placed under conditions of stress. Hsp70 is essential for cellular recovery, survival and maintenance of normal cellular function. It is also a molecular chaperone (these are proteins, which stabilize unfolded or partially folded structures and prevent the formation of inappropriate intra- or interchain interactions) which prevents protein aggregation and refolds damaged proteins in response to cellular stress caused by environmental insults, pathogens and disease.

The highly conserved stress proteins/heat shock proteins (HSPs) are ubiquitous and occur in all organisms from bacteria and yeast to humans (Kregel, 2002). These proteins play important role in both normal cellular homeostasis and the stress response. Out of all HSPs the 70 kDa HSP have been of particular interest because of their cellular abundance and their protective effects. HSP 70 has two main forms: the constitutive form, referred to as HSC 70 (HSC 70) and the predominantly inducible form, referred to as HSP 72. Diverse cells and

tissues have been shown to not only have an increase in HSP 72 with stress, but also early accumulation of HSP 72 in the nucleus. Thermo tolerance with increased resistance to heat killing has been shown to correlate with the level of HSP 70 in the cells. Members of the HSP70 family are involved in:

- The import of proteins in cellular components.
- The folding of proteins in the cytosol, endoplasmic reticulum (ER) and mitochondria.
- The degradation of unstable proteins and heat shock transcription factor rho.
- Dissolution of protein complexes.
- Control the activity of regulatory proteins.
- HSP70 chaperones assist in the refolding of misfolded proteins.

Thus presence of HSP70 in the pseudobranchial neurosecretory cells of *C. batrachus* is an indicative of the fact that this system plays an important role in overcoming hypoxia stress in these fishes. Apart from this most of these fishes are hardy fishes and are able to cope up with low oxygen tension, justifying the presence of these cells in them.

It is now an established fact that fish gill are thus a multifunctional organ while sensing as gas exchangers, they are also involved in the acid base balance, osmoregulation and calcium uptake. Recent investigation has shown (Zaccone, *et al*., 1992) for the first time that there are neuroendocrine cells in the gill epithelium of certain fish species. In the evolution of vertebrates, the neuroendocrine cell systems of respiratory epithelia have undergone extensive changes between the shift from gill to pulmonary respiration, probably to the fluctuations in the atmospheric oxygen during the geological eras leading to the evolution of lungs in Dipnoi and Holosteans and accessory air breathing organs of advanced group of teleosts (Zaccone *et al*., 1997). It is postulated that the pseudobranchial neurosecretory cells might have originated in the gill epithelia and have shifted to gill region, close to pseudobranch/carotid labyrinth secondarily at some stage of evolution.

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