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ORIGINAL STUDY

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Immunohistochemical expression and distribution of orexin, orphanin and leptin in the major salivary glands of some mammals

Angelo Leone, Giovanni Francesco Spatola, Daniela Cucco, Vincenzo Tessitore, Giuseppe Bonaventura, Maria Laura Uzzo

Department of Biomedicine and Clinical Neuroscience- BioNec, School of Medicine, Univeristy of Palermo Italy

Abstract: The aim of the study was to determine by immunochemistry the expression of leptin, orexin A and orphanin FQ in the major salivary glands (parotid, submandibular and sublingual) of rat, sheep and cow. These peptides, originally synthesized in central nervous system, adipose tissue and peripheral tissues including gastrointestinal tract, play an orexigenic (orphanin and orexin) or anorexigenic (leptin) roles in the intricate neuronal network appointed to the control of nutritional homeostasis. Peptide-specific immunoreactivity was present in the studied salivary glands with various intensities in different species, in the ductal epithelium, sometimes in the acinar epithelium, and in nervous trunks spread in connective tissue stroma. The obtained data show that salivary glands present an unexpected source of orexigenic and anorexigenic peptides which with their autocrine, paracrine, and endocrine mechanisms of action may participate in the control of salivary gland function. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 4, 504–512)*

Key words: major salivary glands, orphanin FQ, nociception, orexin, leptin, IHC, rat, sheep, cow

Introduction

It is well known that nutrients' digestion is initiated in the mouth, being under the control of both the major salivary glands, including the parotid, sublingual and submandibular glands, and the minor salivary gland that are scattered into the wall of the oral cavity and oropharyngeal mucosa, which justifies the traditional axiom '*prima digestio fit in ore*'.

It has been also classically established that saliva secretion is under the control of the adrenergic and cholinergic branches of the autonomic nervous system [1], which both stimulate salivary secretion.

Experimental data from the last fifteen years have, however, suggested, that the adrenocholinergic control of these organs is also mediated by other neurotransmitters and molecules. E.g. at the end of the 90's nitric oxide (NO) was identified acting as a neurotransmitter in many organs [2]. In the salivary glands, NO was shown to be involved not only in the physiological control of secretion, but also in the pathogenesis of many inflammatory and neoplastic processes [3–7].

In addition to NO, we have found recently by immunohistochemistry that rat's major salivary glands express the CB1 receptor for endocannabinoids, which are lipid derivatives involved, among other activities, in nutritional balance [12, 13]. Continuing our research on these organs, we have investigated the immunohistochemical (IHC) expression and distribution of orphanin FQ, orexin, and leptin, relatively newly-discovered peptides in the major salivary glands of three mammals: cow, sheep and rat. The goal of this study was to provide a morphological basis for the understanding of possible role of these three peptides in the function of major salivary glands.

Orexin A and B (OxA and OxB) and nociceptin/orphanin FQ were originally identified in the neurons of the posterolateral hypothalamic and perifornical areas. Therefore, these neuropeptides were con-

Correspondence address: A. Leone, BioNec. Sezione di Istologia ed Embriologia, Facoltŕ di Medicina e Chirurgia, Via Del Vespro, 129 90127 Palermo; tel. +39 091 655 35 81, fax: +39 091 655 35 86; e-mail: angelo.leone@unipa.it

sidered to be exclusively expressed in the Central Nervous System (CNS) [14–16] and responsible for the central regulation of nutritional balance, relaying orexigenic effects after activation of their receptors Ox1R and Ox2R for the orexin, and ORL-1 for nociceptin/orphanin FQ [17, 18]. Further immunohistochemical studies identified the presence of these neuropeptides in peripheral organs and tissues associated with the energy balance such as the gastrointestinal tract and pancreas [19–22].

Leptin is being considered as the typical hormone with anorectic action, hence its name of 'satiety hormone'. It was identified for the first time in white and brown adipose tissue, and then in many other cells, including chief cells in human oxyntic glands [23–25] and gastroenteropancreatic system (GEP) of Zucker rats. Further unexpected localization of these three mediators of energy balance in other areas which are not associated with the regulation of energy metabolism, such as the urogenital [26, 27] and respiratory systems, adrenal glands [28] and testis [29], suggest new functional roles performed by these peptides.

Material and methods

Animals and tissue preparation. 10 Wistar rats treated in accordance with the Helsinki Convention on the use of animals in biomedical research were used. The animals were sacrificed after anesthesia (50 mg/kg Nembutal administered peritoneally). Also 5 cows and 5 sheeps collected from beef abattoir were used. From all animals parotid, submandibular and sublingual salivary glands were taken. The tissues were fixed in 10% buffered formalin and after 12–24 hours depending on the size of the sample were washed in water, then dehydrated in alcohol at increasing concentrations, cleared in xylene and embedded in paraffin. The samples were sectioned and processed for immunohistochemistry using antibodies against leptin, orexin A, orphanin FQ; the reactions were revealed using detection kit En Vision + + System-HRP with AEC as substrate (Dako, Italy).

Immunohistochemistry. Serial sections 8μm thick were cut on Leica microtome RM2145, dried overnight at 37°C and then stored at room temperature. The day after, the slides were dewaxed and re-hydrated by sequential immersion in a graded series of alcohols and transferred into water for 5 min; to inhibit any endogenous peroxidase activity the slides were treated for five minutes with Peroxidase Block (Dako). Subsequently, the slides were transferred into PBS (Phosphate-Buffered Saline, pH 7.4) at room temperature. The following protocol was realized using the kit En-Vision + + System HRP with AEC as substrate (Dako). After rinsing with PBS for 4 min, the sections were incubated overnight at 4°C with polyclonal anti- leptin anhtibody(Ob A-20, Santa Cruz Biotechnology, Inc.) diluted 1:100; polyclonal anti-orphanin FQ antibody (Gene Tex, Inc.) diluted 1:500; polyclonal anti-orexin A antibody (Chemicon) diluted 1:100. After the incubation, any excess antibody was removed by washing with PBS for 5 minutes. Next, the sections were incubated with Peroxidase labeled polymer conjugated to goat anti-rabbit immunoglobulin in Tris-HCl buffer containing stabilizing protein and an antimicrobial agent. Unbound polymer was removed by washing (2 times with PBS, 5 minutes each) and subsequently AEC chromogen in substrate buffer was added, and washed after 5 minutes with distilled water. Slides were coverslipped with one drop of an aqueous mounting medium (Dako).

Negative controls were performed by omission of primary antibody, and by incubating sections with antiserum saturated with homologous antigen.

The IHC technique was standardized according to the suggestions of the US Biological Stain Commission [35].

Image analysis. The IHC specimens were examined using a Leica Laborlux S Microscope (Leica Microsystem GmbH Wetzlar, Germany) with a Nikon DSL2 photo digital system (Nikon Corp. Tokyo, Japan). Each sample was analyzed with a double-blind system and two different operators. Moreover, the results were compared to an image analysis obtained from digital TIF files acquired with the multispectral system [31, 32]. To apply this method, we made sequential shots using CoKin (Cokin SAS, Rungis Cedex, France) filters to obtain all the different color spectra. Adobe Photoshop CS4 extended (Adobe Systems Inc, San Jose, CA) was used to elaborate images [32, 33]. Choosing the spectrum related to AEC, we converted the image color profile from RGB to CMYK. Then we chose the yellow channel because it was found to have the best linear response to color intensity and thus to protein presence [34]. The quantification of IHC staining was performed by using a score system with values from 1+ to 5+ [34–36].

Results

The major salivary glands (parotid, submandibular and sublingual) in the examined mammals were tubulo-acinar structures. The ductal system was composed of intralobular ducts (intercalated and striated ducts) and excretory interlobular ducts. The secretory parenchyma (acini) contained only serous cells in the parotid gland or mucous and serous cells in the submandibular and sublingual glands. All the results obtained have been resumed in Table 1.

Parotid glands

In cow, peptidergic IHC reactivity was widely distributed in the cytoplasm of intralobular ductal epithelial cells with variable intensity for each peptide (Table 1). Within the same cell type, the immunoreactivity was

Table 1. Comparison of the results of all the structures of the 3 mammalian species studied by the immunohistochemical score (0= negative +5 = max immunoreactivity)

Parotid	**RAT**				**SHEEP**				**CATTLE**			
	DUCTAL epithelium	**ACINAR epithelium**	**Ganglion cells and Nerve fibers**	**Isolated Cytotypes**	**DUCTAL epithelium**	**ACINAR epithelium**	**Ganglion cells and Nerve fibers**	**Isolated Cytotypes**	**DUCTAL epithelium**	**ACINAR epithelium**	**Ganglion cells and Nerve fibers**	**Isolated Cytotypes**
LEPTIN immunoreactivity	++	0	0	–	++	+	0	+++	++	0	0	+++++
ORPHANIN immunoreactivity	+++++	0	++++	–	+	+	++	–	+++	0	–	–
OREXIN immunoreactivity	++	0	+++	–	+	+	++	–	+++	0	–	–
Submand. sublingual	**RAT**				**SHEEP**				**CATTLE**			
	DUCTAL epithelium	**ACINAR epithelium**	**Ganglion cells and Nerve fibers**	**Isolated Cytotypes**	**DUCTAL epithelium**	**ACINAR epithelium**	**Ganglion cells and Nerve fibers**	**Isolated Cytotypes**	**DUCTAL epithelium**	**ACINAR epithelium**	**Ganglion cells and Nerve fibers**	**Isolated Cytotypes**
LEPTIN immunoreactivity	++++	0	–	–	0	0	0	++++	++	+	0	–
ORPHANIN immunoreactivity	++++	0	+++++	–	0	0	+++++	++++	++	+	+++	–
OREXIN immunoreactivity	++++	0	–	–	0	0	+++++	++++	++	+	+++	–

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not always distributed with the same intensity in all cells, and it appeared not uniformly homogeneous (Figure 1). Sometimes leptin immunoreactivity was, mostly in the microgranular form, detectable in isolated prismatic cell types (Figure 2) that showed distinct fluorescence by multispectral analysis. The serous acinar compartment appeared to be not labelled.

In rat, the orphaninergic immunoreactivity was present almost uniformly in the ductal epithelium which was intensely immunoreactive (Figure 3); the ductal leptin immunoreactivity was less uniform and the orexinergic immunoreactivity was weak and often limited to the basal edge of the ductal sialocytes (Figure 4). In the connective tissue stroma orphaninergic and orexinergic immunoreactivity was found in nervous fibers (Figures 5–6). The serous acinar compartment was immunohistochemically not labelled.

In sheep, the ductal epithelium exhibited leptin immunoreactivity only in a few ductal sialocytes (Figures 7–8) which was clearly evident at multispectral analysis. In the ductal epithelium, orphaninergic and orexinergic immunoreactivity was very weak, however, it increased at the apical edge of sialocytes (Figure 9). Orexinergic nervous fibers were found in the connective tissue stroma. The acinar compartment exhibited discrete immunoreactivity.

Submandibular and sublingual glands

The results obtained for the submandibular and sublingual glands have been described together as the immunostained structures were similar in both glands.

In cow, ductal peptidergic immunoreactivity was found in sialocytes, it was sometimes unevenly distributed (Table 1). Serous acini exhibited a modest peptidergic immunoreactivity that increased in small acini located close to the ductal branching (Figure 10). In the periductal stromal connective tissue small orexinergic gangliar cells and fibers were frequently found; orphaninergic gangliar cells and fibers were also found in some areas of the stroma (Figure 11).

In rat, the peptidergic immunoreactivity of all neuropeptides studied of the ductal epithelium was fairly uniform (Table 1, Figure 12).

In sheep, the peptidergic immunoreactivity of the ductal epithelium was not homogeneous. The serous acinar compartment did not exibit peptidergic immunoreactivity.

In rat and sheep, the periductal and perivascular connective tissue contained orphaninergic ganglion neurons (Figure 13). In addition, in intercalated ducts, some cells, irregularly scattered among unreactive epithelial mucous cells, showed peptidergic immunoreactivity (Figures 14–15). Finally peptidergic immunoreactivity, often in the microgranular form, was displayed by small cells, probably basket cells, that were located between the glandular cells and basal lamina (Figure 16).

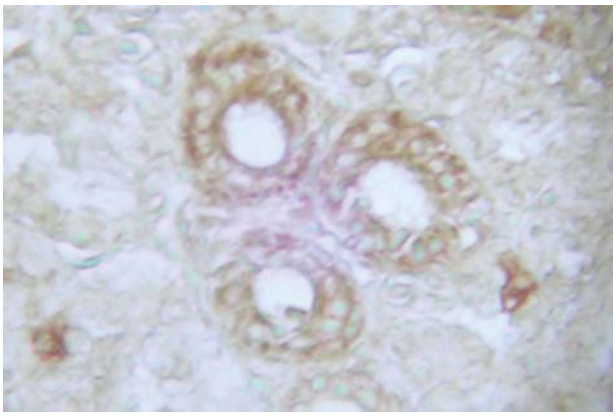

Figure 1. Cow parotid gland: Leptin immunoreactivity (Ir) of ductal epithelium. Magnification of objective 20 ×

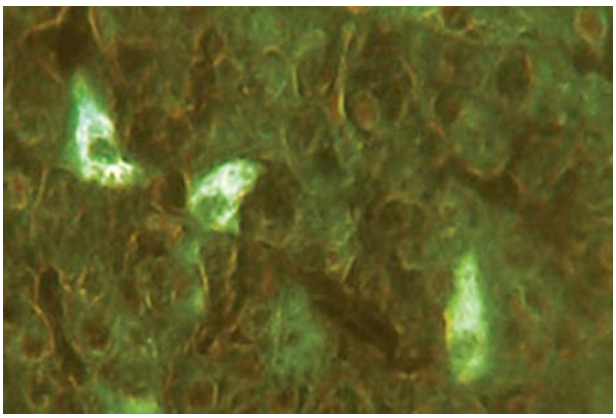

Figure 2. Cow parotid gland: Leptin Ir in some cells. Lack of immunoreactivity in serous acini. Multispectral analysis. 40 ×

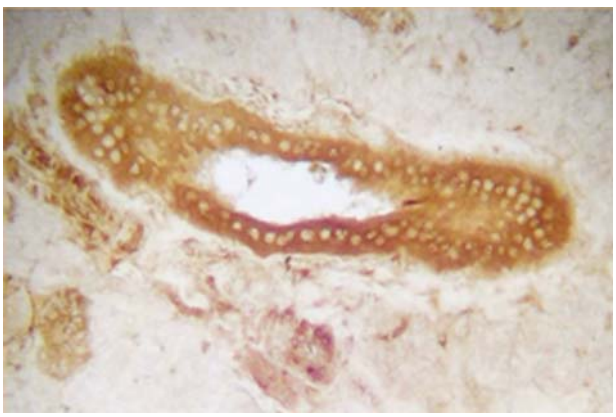

Figure 3. Rat parotid gland: evenly distributed orphanin Ir of ductal ephitelium. 20 ×

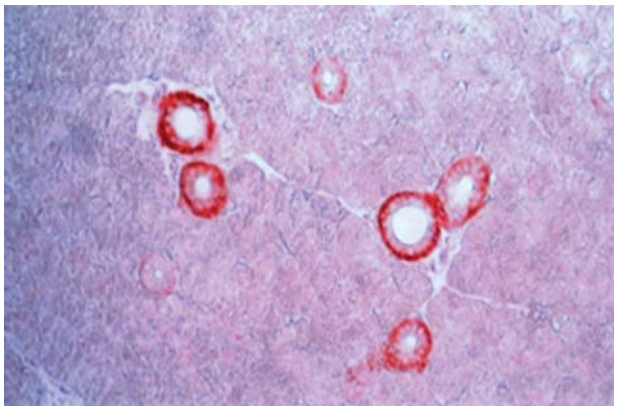

Figure 4. Rat parotid gland: orexin Ir in ductal sialocytes' basal cytoplasm. No Ir in acinar parenchyma. 10 ×

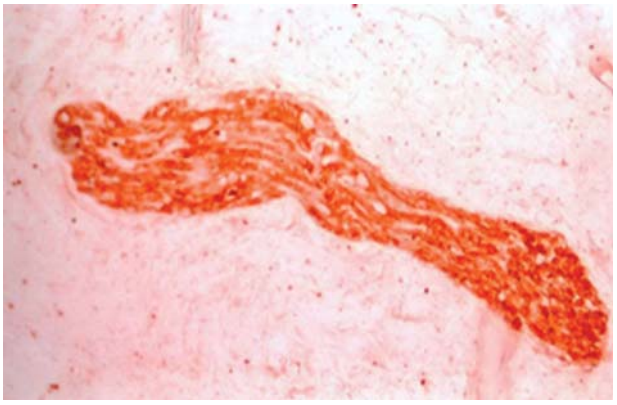

Figure 5. Rat parotid gland: orphanin Ir in nerve fibers. 40 ×

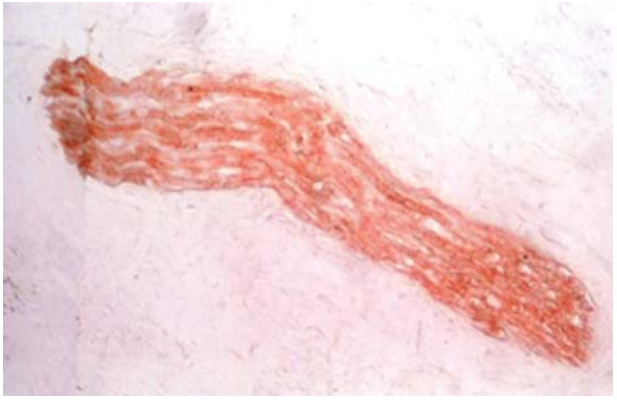

Figure 6. Rat parotid gland: orexin Ir in nerve fibers. 40 ×

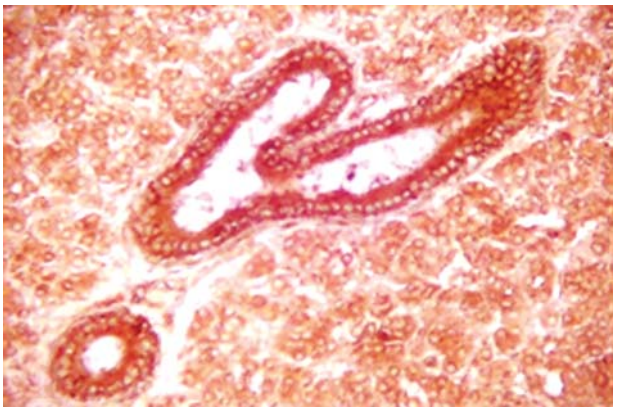

Figure 7. Sheep parotid gland: discrete leptin Ir of ductal epithelium. Weak Ir in acinar compartment. 20 ×

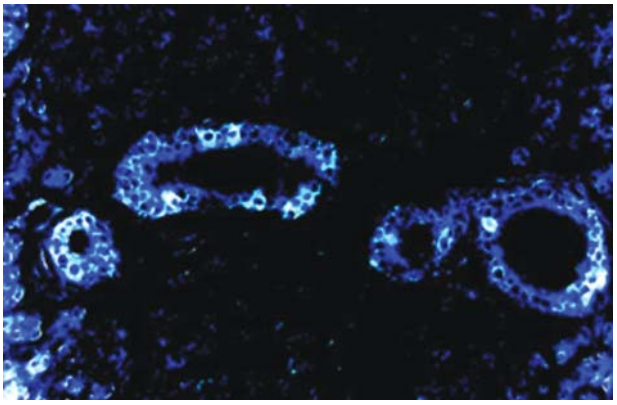

Figure 8. Sheep parotid gland: leptin Ir of some ductal sialocytes at multispectral analysis. 10 ×

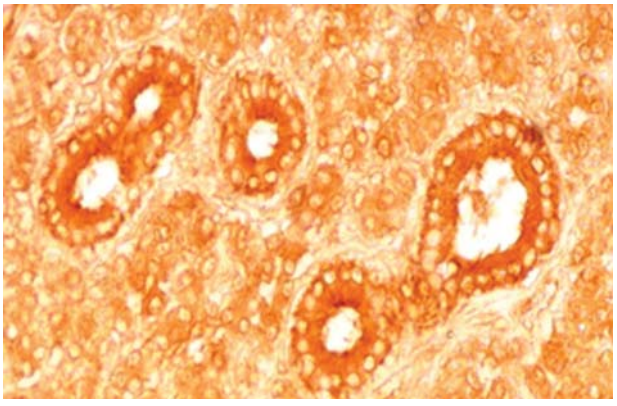

Figure 9. Sheep parotid gland: Intense orexin Ir at the apical pole of ductal sialocytes. Weakly reactive acinar parenchyma. 20 ×

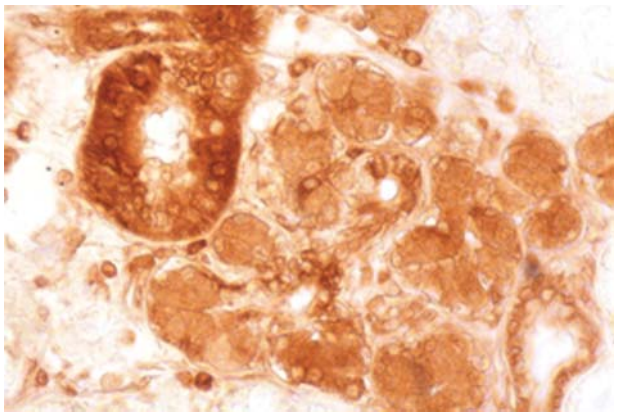

Figure 10. Cow sublingual gland: orphanin Ir in periductal serous acini. 40 ×

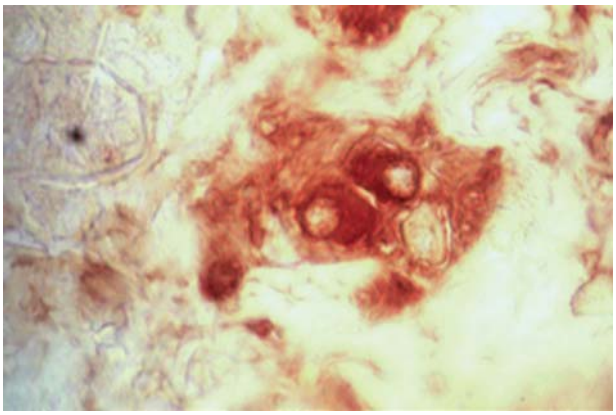

Figure 13. Rat sublingual gland: orphanin Ir of stromal gangliar body nerve cells. 63 ×

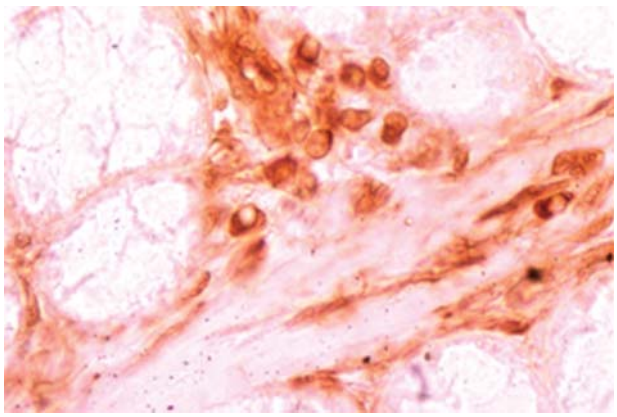

Figure 11. Cow sublingual gland: orexin Ir in stromal cells. 63 ×

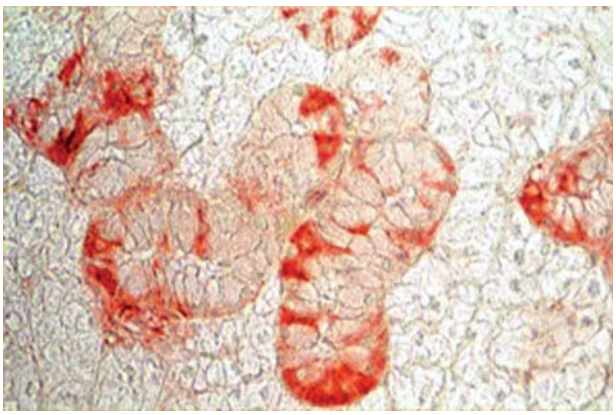

Figure 14. Rat submandibular gland: orexin Ir of some cells irregulary scattered among unreactive epithelial mucous cells in intercalated ducts. 40 ×

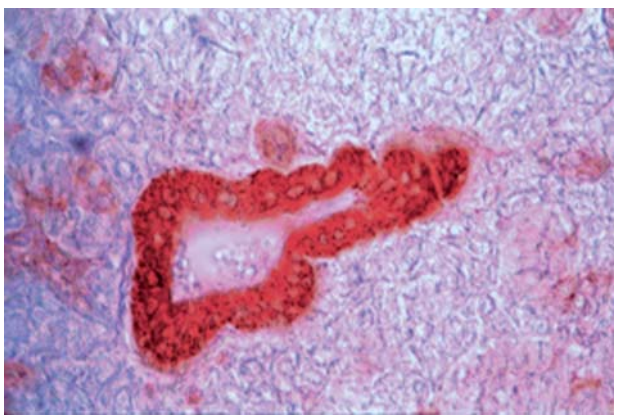

Figure 12. Rat sublingual gland: orexin Ir of ductal epithelium. 20 ×

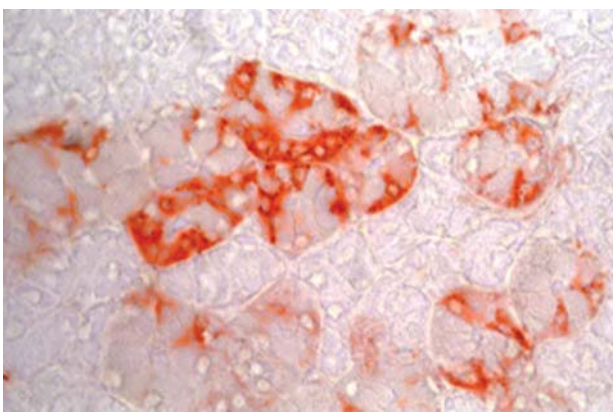

Figure 15. Rat submandibular gland: orexin Ir of some cells irregulary scattered among unreactive epithelial mucous cells in intercalated ducts 40 ×

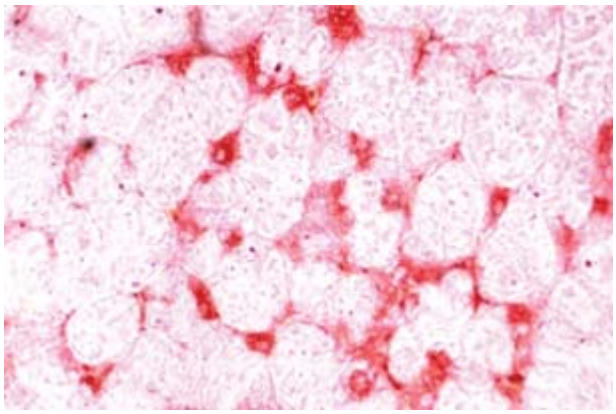

Figure 16. Rat sublingual gland: orexin Ir in some interacinar. cells 40 ×

Discussion

Previous research by other authors[8–11] demonstrated that the major salivary glands produce in humans such hormones as insulin, glucagon, leptin and ghrelin. We have extended immunohistochemical peptidergic mapping to the major salivary glands of three mammal species, cow, sheep and rat, documenting the presence of leptin, nociception/orphanin FQ and orexin. Our results show that these organs represent yet an another organ exhibiting these peptides, alongside the gastrointestinal tract, adipose tissue and skeletal muscles.

It is not easy to assign a functional role to the presence of the appetite-regulating peptides detected in the salivary glands, because neuropeptides derive from the proteolysis of giant precursors (prepro-orexin, prepro-orphanin). In attributing a possible role to the detected peptides, one should consider the orexinergic or anorexinergic activities traditionally attributed to these peptides in the regulation of appetite and energy regulation, acting as hunger (orphanin and orexin) or satiety (leptin) signals.

In making a final assessment on the peptidergic immunoreactivity in the tested animal species it should be pointed out that the immunohistochemical distribution of examined peptides in the three major salivary glands was essentially limited to the ductal system in its various segments (interlobular, intralobular, striated and intercalated ducts). Serous acini were weakly or non-immunoreactive. Regardless of the varying intensity of the IHC signal detected in the different animal species studied, we found that in the same species a variety of structural components were immunopositive (ductal epithelia, isolated sialocytes, nervous cells and nerve fibers). Altogether, these data suggest that the examined peptides may play various roles in functions of salivary glands.

The immunoreactivity detected mainly in the ductal system, strongly suggests that the examined peptides do not control the first step of the classic secretory process by acinar glandular cells but rather control the special functions of ductal sialocytes, involved not only in the transport of the saliva but also in the modification of the primary saliva secreted by the acini through electrolyte reabsorption and protein production [37–41]. Previous studies identified presence of leptin receptor in sialocytes of the human ductal system [10, 43], suggesting that leptin may exert control of salivary function. Our findings together with the identification of leptin in human saliva [11, 42], suggest that salivary leptin derives not only from blood but also from ductul cells of salivary glands.

In cow and rat, the expression of orphanin FQ and orexin in nerves and gangliar cells suggests a role of those neuropeptides in controlling salivary gland function similarly to the classical adrenergic, cholinergic and nitroxidergic innervation.

The leptin and orphanin FQ immunoreactivity found in isolated cells in cow parotid gland (stroma and acini) and in small cells in rat intercalated ducts among mucous negative sialocytes, might be related to the endocrine activity of these peptides. Therefore co-existence of endocrine and exocrine cells in an organ traditionally considered exclusively exocrine, should now be considered. The exocrine glands of the gastrointestinal tract comprise many cells of the Diffuse Neuroendocrine System (DNES), and our findings provide evidence that also major salivary glands contain DNES cells.

Our findings may be also related to the recent studies on a possible role of 'neither-anorexigenic nor-orexigenic'peptides' which may function as growth factors regulating the proliferation of specific cell types in various organs [45–51]. In this context the observation that proliferation of sialocytes was reduced in the presence of recombinant leptin [44] seems noteworthy. However, additional studies are necessary to check if the presence of orexin, orphanin FQ and leptin in the major mammalian salivary glands, documented in our present study, may affect physiology and pathophysiology of these organs.

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