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Differential gene expression and immune localization of the orexin system in the major salivary glands of pigs

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ABSTRACT

The aim of the present work was to characterize the expression and cell type distribution of the orexinic system in the major swine salivary glands by RT-PCR and immunohistochemistry techniques. Tissue samples of the mandibular (MSG), sub-lingual, and parotid glands were obtained from six pigs. Marked differences in the expression of the orexinergic system were observed among the salivary glands. Prepro-orexin (PPOX) mRNA abundance was approximately 15-fold higher ($P<0.05$) in MSG than in the others salivary glands. The expression level of OX1R mRNA did not differ among the salivary glands, while that for OX2R transcript was undetectable. The hypothalamus, used as positive control tissues, showed the highest expression level for each component of the orexinic system. Immunoreactivity (IR) for both orexins A and B (OXA and OXB), and cognate receptors (OX1R and OX2R) were identified only in the excretory striated ducts of the MSG while acinar cells were not immunoreactive. Both sub-lingual and parotid glands completely lacked IR for any component of the orexinergic system. In the MSG, parasympathetic neurons and axons of local salivary gland ganglia were IR to both OXA and OXB and also to their receptors.

1. Introduction

Salivary glands are of utmost importance for several physiological functions; they produce an abundant volume of their mixed secretion that facilitates mastication and deglutition, lubricates the oral cavity and protects teeth and the surrounding soft tissues. Among them, the three major salivary glands, mandibular (MSG), sub-lingual, and parotid, are characterized by excretory units consisting of secretory acini and a peculiar intralobular duct system made up of intercalated and striated ducts [1]. Although structurally similar, these glands provide secretions which are qualitatively variable, depending on type (serous–mucous or mixed in MSG and sub-lingual *vs.* only serous in the parotid gland), neural stimulation, and endocrine signals. In fact, the control of salivary secretion is regulated by the autonomic nervous system through parasympathetic, sympathetic, and peptidergic (neuropeptide Y, vasoactive intestinal peptide, and enkephalin) secretomotor fibers directed to the salivary glands [2]. While fluid and electrolyte secretion is promoted by parasympathetic nerve impulses, protein secretion is favored by sympathetic stimulation. Several hormones, including growth hormone, prolactin, androgens, thyroid hormones, and corticosteroids, also regulate protein synthesis.

There is increasing evidence that the mandibular gland displays distinct immune-neuro-endocrine functions mediated by different peptides such as nerve growth factor, epidermal growth factor, and transforming growth factor-beta and by the independent release of hormones and a large array of cytokines [3]. The MSG-derived peptide and hormones, secreted into the bloodstream and/or the saliva, can affect immune response of mucosal tissues, cell proliferation and wound repair as well as several endocrine glands [4,5]. Growth factors can also influence the nervous system trough their retrograde axonal transport from the MSG to the superior cervical ganglion and to other nervous centers [4]. Thus, the MSG integrates both exocrine and independent endocrine functions similarly to what is found in the stomach, duodenum, and pancreas. In addition, the presence of low molecular mass proteins and androgen-derived pheromones secreted by the MSG into the saliva of pigs suggests that this gland may have a role in olfactory communication to facilitate mating behavior in this species [3,6].

The orexin family consists of two separate peptides, orexin-A (OXA) and orexin-B (OXB), derived from proteolysis of a common

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130-amino acid precursor, prepro-orexin (PPOX). Both OXA and OXB share 40% homology, and the sequence of OXA is fully conserved among rats, humans, mice, pigs, and cows [7]. The biological actions of these peptides are mediated by two specific G protein-coupled receptors, OX1R and OX2R. Whereas OX1R binds selectively orexin A, OX2R binds both orexins with similar, but 100- to 1000-fold lower affinity [8]. The receptors are 64% homologous and highly conserved across species [9]. The presence of orexins has been described in several regions of the central nervous system [7] where they participate in the control of feeding behavior, energy metabolism, and the sleep–wake cycle [9–11]. Several studies in humans [12,13], in rodents [14,15] and, more recently, in various domestic species [16–18] have demonstrated the presence of the "orexinergic system" in the endocrine cells of the gastro-entero-pancreatic system and in neurones and nervous fibers localized in the gastrointestinal submucosa and muscular layer where they likely exert a regulatory function.

Therefore, the main objective of this study was i) to characterize whether orexins and their receptors are differentially expressed among the major salivary glands of pigs intended as an organ system, and ii) to localize the immunoreactivity of the orexin system within each cell type to support previous comparative and functional findings in the gastrointestinal tract of several animal species.

2. Materials and methods

2.1. Reagents

Anti-OXA and anti-OXB mouse monoclonal antibodies (MAB763 and MAB734 respectively), were from R&D Systems (R&D Systems, Inc, Minneapolis, MN 55413, USA); anti-OX1R rabbit polyclonal antibody (O4514) was purchased from Sigma, (Sigma-Aldrich, MO, USA); anti-OX2R rabbit polyclonal antibody (AB3094) was obtained from Millipore (Corporate Headquarters, Billerica, MA 01821, USA). The biotinylated secondary antibodies, a goat anti-mouse IgG and a goat anti-rabbit IgG, used for immunohistochemistry (IHC), were purchased from Vector Laboratories (Burlingame, CA, USA). Normal goat serum was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The avidin–biotin complex (ABC, Vector Elite Kit) and the chromogen 3,3′-diaminobenzidine tetrachloride (DAB) were from Vector Laboratories; Canada Balsam Natural came from BDH (Poole, Dorset, England).

Tri-Pure reagent was purchased from Roche Diagnostic GmBH (Mannheim, Germany); RNeasy Lipid Tissue Mini Kit was obtained from Qiagen GmBH (Hilden, Germany); DNA-free RNA kit was from Zymoresearch (Orange, CA, USA); iScript cDNA Synthesis Kit and IQ SYBR Green BioRad Supermix were purchased from Bio-RAD Laboratories Inc. (California, USA), whereas all other pure grade chemical and reagents were obtained locally.

2.2. Animals and tissue collection

Purebred Large White pigs were raised in the facility of the Department of Veterinary Medical Science of Bologna University, in accordance with national and international guidelines for the use of animals in research. The pigs were maintained under controlled conditions of light (10 h L/14 h D) and temperature (22 °C) and fed twice a day with a standard growing diet. The day before sacrifice the animals were starved overnight leaving water *ad libitum*. The pigs were sacrificed by an overdose (0.2 ml/kg of body weight) of barbiturate (Tanax, Intervet, Milan, Italy) after surgical anesthesia induced by 0.1 ml/kg azaperone (Stesnil, Jansenn Cilag, Cologno Monzese, Italy) and then by 0.2 ml/kg ketamine cloridrate (Ketavet 100, Intervet). All tissue samples were collected from six 13 week-old female pigs weighing 45.0 ± 1.47 kg. The protocol involving the care and use of the animals for these experiments was approved by the Bioethics Committee of the University of Bologna.

Upon sacrifice, specimens of the parotid, mandibular, and sublingual salivary glands of each pig were promptly removed and divided into two parts. For each tissue sample, one part was immediately fixed by immersion in 4% (w/v) formaldehyde solution in PBS (0.1 M, pH 7.4) for 24 h at room temperature and subsequently processed for embedding in paraffin, following routine tissue preparation procedures for later immunohistochemical detection of orexins and cognate receptors; the other part, together with specimens of the pituitary gland and hypothalamus, were immediately frozen in liquid nitrogen and stored at −80 °C for later evaluation of gene and protein expression.

2.3. Real-time PCR quantification of PPOX, OX1R and OX2R on salivary glands and control tissues

Total RNA was extracted from the parotid, mandibular, sub-lingual, and pituitary glands and the hypothalamus using Tri-Pure reagent, according to the manufacturer's instructions. Total RNA was extracted from the hypothalamus using RNeasy Lipid Tissue Mini Kit according to manufacturer's instructions.

Purified RNA was spectrophotometrically quantified (A260 nm) and its quality was checked by gel electrophoresis on 1% agarose gel. Five micrograms of RNA was treated with DNA-free RNA kit according to the manufacturer's instructions, and then 1 μg of RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit, to a final volume of 20 μl. Real Time quantitative PCR was performed using iCycler Thermal Cycler (Bio-RAD). Primers for swine orexin (PPOX), OX1R and OX2R and the housekeeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) were designed using the Beacon Designer 2.07 Software (Premier Biosoft International, Palo Alto CA, USA).

Primer sequences, expected PCR product lengths and accession numbers in the EMBL database, are shown in Table 1. A master-mix of the following reaction components was prepared to obtain the indicated end-concentrations: 0.5 μl forward primer (0.2 μM), 0.5 μl reverse primer (0.2 μM), 9.5 μl water and 12.5 μl IQ SYBR Green BioRad Supermix (Bio-RAD). Two microliters of cDNA was added to 23 μl of the master mix. All samples were analyzed in duplicate. The real-time PCR protocol employed was: initial denaturation for 3 min at 95 °C, 40 cycles at 95 °C for 15 s and 60 °C for 30 s, followed by a melting step with slow heating from 55 °C to 95 °C at a rate of 0.5 °C/s.

The relative mRNA level was determined as the PCR cycle number that crosses an arbitrarily placed signal threshold (Ct). The Ct value inversely correlates with the amount of target mRNA in the sample. The housekeeping gene GAPDH was used to normalize the amount of RNA. The expression levels of PPOX, OX1R and OX2R expressions were examined using the ΔCt method with $\Delta Ct = Ct_{GAPDH} - Ct_{target}$ that directly correlate with the expression level. To quantify the increase in expression among the different samples, the $2^{\Delta\Delta Ct}$ method was used (ABI Prism, 7700: Sequence detection system User B Bulletin #2 Relative quantification of gene expression. Applied Biosystems 1997). Using the hypothalamus as positive control tissue, real-time efficiencies were acquired by amplification of a standardized dilution

Table 1
Primers for prepro-orexin (PPOX) and orexin receptors type 1 (OX1R) and 2 (OX2R) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as internal standard.

Gene	Primer sequence (5′-3′)	Product length (bp)	Accession no.
PPOX	For.: TTCCTGGAGACCCCACTCTG Rev.: GGCAGCAACAGTAGCAGAAG	101	NM_214156
OX1R	For.: CTGCCTGAACTAGCCAACCG Rev.: CGATGAAGAAGCAACTGTGGTAG	100	NM_001043346
OX2R	For.: TGATTCCTTACTTACAGACTG Rev.: AGATGATGACGATGCTATTC	144	NM_001129951
GAPDH	For.: TGGTGAAGGTCGGAGTGAAC Rev.: TGTAGTGGAGGTCAATGAAGGG	120	AF017079