Research in Veterinary Science 95 (2013) 8–14

Contents lists available at SciVerse ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Orexin system expression in the gastrointestinal tract of pigs

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ARTICLE INFO

Article history:
Received 5 October 2012
Accepted 3 February 2013

Keywords:
OXA
OXB
OX1R
OX2R
Ghrelin
Gastrin

ABSTRACT

The aim of the present study was to characterize the expression of both proteins and gene transcripts for orexins (OXA and OXB) and their cognate receptors (OX1R and OX2R) in the different gastrointestinal sections of pigs. Using immunohistochemistry, OXA and OXB were found to be co-expressed in the same endocrine cells localized in the basal third of the glands of the body portion of the stomach. Using double immunostaining technique, these orexin-immunoreactive (IR) cells co-stored ghrelin and gastrin. Apparently, OX1R was also expressed within the same cells, forming the tubular gastric gland which displayed positive immunostaining for orexins and the other peptides. Neurons of the enteric nervous system of the stomach were not immunolabeled. We did not find any definite OXA- or OXB-IR cells as well as any immunosignal for orexin receptors in sections of the duodenum, ileum, cecum and rectum. PPOX, OX1R, OX2R mRNA were similarly expressed in all the gastrointestinal tracts. Gastrin and ghrelin showed the highest levels of expression in the gastric mucosa, but their abundance decreased along the subsequent tracts. Thus, in pigs, orexins do not play any role in the local control of intestinal motility and secretion but may rather be involved as endocrine modulators for the regulation of feeding and metabolic homeostasis. However, the co-localization of ghrelin and gastrin with both orexins in the same endocrine cells of the gastric glands suggests that these gut peptides may collaborate in the regulation of gastric secretion, energy homeostasis, body weight and food intake.

1. Introduction

The orexin family is a complex system consisting of two peptides, orexin-A (OXA) and orexin-B (OXB), derived from the proteolytic processing of a common 130 amino acid precursor, prepro-orexin (PPOX) which is, in turn, derived from the HCRT (hypocretin) gene, and two specific, but distinct, G protein-coupled receptors, OX1R and OX2R (Sakurai et al., 1998). The two orexin peptides share 40% homology. The sequence of OXA is fully conserved in rats, humans, mice, pigs and cows. The orexin receptors are 64% homologous and highly conserved across species. Whereas OX1R selectively binds orexin A, OX2R binds both orexins with a similar, but 100- to 1000-fold lower affinity (Smart and Jerman, 2002).

The orexins were initially discovered in the neurons of the lateral hypothalamus (de Lecea et al., 1998), a region which regulates feeding behavior (Bernardis and Bellinger, 1996; Sakurai, 1999). In addition, orexin producing neurons were also identified in different hypothalamic areas which sub-serve the neuroendocrine axes and are the likely target of peripheral metabolic and endocrine systemic signals (Girault et al., 2012). Orexinergic neurons project widely to multiple brain sites of the central nervous system (CNS) and to the dorsal vagal complex, where efferent and afferent electrical signals via vagal innervation to and from the gut are generated and integrated (Grabauskas and Moises, 2003). Orexin receptors were found in several hypothalamic areas, including those involved in the regulation of food intake and different brain regions (Hervieu et al., 2001; Cluderay et al., 2002). Transgenic mice, in which orexin neurons were genetically ablated, showed narcolepsy, hypophagia and obesity (Hara et al., 2001) whereas intracerebroventricular injection of OXA increased food intake in rats fed *ad libitum* (Semjonous et al., 2009) and induced an irregular postprandial-like motility pattern in the stomach and duodenum (Bülbül et al., 2010). Taken together, these findings confirmed that the orexins and cognate receptors in the CNS are involved in the central regulation of food intake, gastrointestinal functions and energy homeostasis.

Subsequently, the orexins and their receptors were identified in the neurons of the submucosal and myenteric ganglia, and in the

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0034-5288/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.rvsc.2013.02.001

nerve fibers of the gastrointestinal tracts of laboratory animals and humans (Nakabayashi et al., 2003; Ehrström et al., 2005a,b). Their wide distribution across the enteric nervous system (ENS) indicates that the orexins may also locally regulate several gastrointestinal functions by exerting region-specific contractile or relaxant actions as well as by modulating gastrointestinal secretion and hormone release from pancreatic endocrine cells (Kirchgessner, 2002; Baccari, 2010). Orexins and cognate receptors were also localized in numerous endocrine and enterochromaffin cells of the gastrointestinal tract (Solcia et al., 2000; Näslund et al., 2002) and were found to be co-expressed with gastrin and OX1R (Kirchgessner, 2002), thus leading to the hypothesis of an endocrine control on different gastrointestinal functions mediated by the orexin system. Thus, the presence of efferent and afferent orexinergic neurons in the CNS, directed to and from the gut together with the orexin producing gastrointestinal endocrine cells and neurons of the ENS laid the theoretical basis for the existence of an orexin brain-gut axis capable of modulating multiple (metabolic, endocrine, mechanical, and emotional) signals and regulating feeding behavior and energy homeostasis (Kirchgessner and Liu, 1999; Korczynski et al., 2006).

Recently, however, we observed that, in the fallow-deer, the ENS did not display any definite immunoreactivity for the orexins whereas OX1R-immunopositivity was observed in the enteric neuron ganglia localized in the submucosal and muscular intestinal layers (Dall'Aglio et al., 2012). However, in neonatal dogs and horses, OXA and OX2R were found to be expressed in the neurons and fibers of the ENS (Dall'Aglio et al., 2008, 2009). In these species, OXA and OXB were also found in different cell types of the mucosal glands of the stomach and other intestinal tracts, but with species specific differences. Collectively, our previous findings indicated the possibility of some inter-species variability not yet fully understood. Thus, to further expand our knowledge of the physiological functions of the orexin system in domestic animals, we studied the presence and cell type distribution of orexins and their cognate receptors as well as the abundance of their encoding mRNA in the digestive apparatus of pigs. In addition, we examined the co-expression of the orexins with ghrelin and gastrin for their relevance in gastrointestinal functions.

2. Materials and methods

2.1. Reagents

The anti-OXA and anti-OXB mouse monoclonal antibodies (MAB763 and MAB734, respectively) were purchased from R&D Systems (R&D Systems, Inc., Minneapolis, MN 55413, USA). The anti-OX1R (O4514) and anti-OX2R (AB3094) rabbit polyclonal antibodies were purchased from Sigma (Sigma–Aldrich, MO, USA) and from Millipore (Corporate Headquarters, Billerica, MA 01821, USA), respectively. The anti-ghrelin goat (sc-10368) and anti-gastrin rabbit (L1811) polyclonal antibodies were purchased from Santa Cruz Biotechnology and DAKO Corporation (Carpinteria, CA, USA), respectively. The biotinylated secondary antibodies, goat anti-mouse IgG and goat anti-rabbit IgG used for immunohistochemistry were purchased from Vector Laboratories (Burlingame, CA, USA). Normal goat serum was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Avidin/Biotin blocking kit (SP-2001) and Vector VIP substrate kit for peroxidase were purchased from Vector Laboratories. The Canada Balsam Natural was purchased from BDH (Poole, Dorset, England).

Nucleo Spin RNA II (total RNA isolation) was purchased from Macherey–Nagel, (Duren, Germany); iScript cDNA Synthesis Kit and IQ SYBR Green BioRad Supermix were purchased from Bio-RAD Laboratories Inc. (CA, 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 the University of Bologna 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 were fed twice a day with a standard growth diet. The day before sacrifice, food was withheld from the animals overnight but they had access to water *ad libitum*. The pigs were sacrificed using 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 with 0.2 ml/kg ketamine cloridrate (Ketavet 100, Intervet). All the tissue samples were collected from six 13 week-old female pigs, each 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, different portions of the gastrointestinal (GI) tract of each pig were promptly removed and divided into two parts: one was fixed by immersion in 4% (w/v) formaldehyde solution in phosphate buffered solution (PBS) (0.1 M, pH 7.4) for 24 h at room temperature and was subsequently processed for embedding in paraffin, following routine tissue preparation procedures; the other was rinsed with RNAse-free PBS and frozen at −80 °C, for later evaluation of the gene expression. Specimens were collected from the fundic region of the stomach, including scrapings of the mucosa (SM) and muscular wall (SMUS), duodenum (DU), jejunum (JE), cecum (CE), colon (CO) and rectum (RE).

2.3. Immunohistochemistry of OXA, OXB, ORX-1, and ORX-2 on GI tracts

The single immunohistochemical reaction was visualized on 5 μm serial sections, mounted on poly-L-lysine coated glass slides, utilizing the avidin–biotin-complex (ABC) and the 3,3′diaminobenzidine-4-HCl (DAB) as the chromogen. To reduce variation in staining, the tissue sections from each of the above-mentioned gastrointestinal portions were incubated together during each immunohistochemical procedure. In brief, the dewaxed sections were microwaved for 15 min in 10 mM citric acid (pH 6.0) for antigen retrieval. All subsequent steps were carried out in a moist chamber at room temperature. To prevent the non-specific binding of primary antibodies, after a proper cooling, the sections were pre-incubated for 30 min with normal serum. Subsequently, each section was incubated overnight with one of the following primary antibodies (1:100): anti-OXA, anti-OXB, anti-OX1R, or anti-OX2R. The following day, after washing in PBS, the sections were incubated for 30 min at room temperature with the corresponding secondary biotin-conjugated antibodies goat anti-mouse IgG (for OXA and OXB) or goat anti-rabbit IgG (for OX1R and OX2R), and were then processed for 30 min using the Vectastain ABC kit. Subsequently, the tissue samples were repeatedly rinsed with PBS and developed with chromogen solution. After several rinses in PBS, the sections were dehydrated and mounted in Canada Balsam Natural.

2.4. Double-label immunohistochemistry for OXA, OXB, ghrelin, and gastrin on GI tracts

For the double-label localization of OXA with either OXB, ghrelin or gastrin, we followed a previously described method (Dall'Aglio et al., 2010). Briefly, OXA-containing cells were first stained