The Orexin System in the Enteric Nervous System of the Bottlenose Dolphin (*Tursiops truncatus*)

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Abstract

This study provides a general approach to the presence and possible role of orexins and their receptors in the gut (three gastric chambers and intestine) of confined environment bottlenose dolphin. The expression of prepro-orexin, orexin A and B and orexin 1 and 2 receptors were investigated by single immunostaining and western blot analysis. The co-localization of vasoactive intestinal peptide and orexin 1 receptor in the enteric nervous system was examined by double immunostaining. Also, orexin A concentration were measured in plasma samples to assess the possible diurnal variation of the plasma level of peptide in this species. Our results showed that the orexin system is widely distributed in bottlenose dolphin enteric nervous system of the all gastrointestinal tract examined. They are very peculiar and partially differs from that of terrestrial mammals. Orexin peptides and prepro-orexin were expressed in the main stomach, pyloric stomach and proximal intestine; while orexin receptors were expressed in the all examined tracts, with the exception of main stomach where found no evidence of orexin 2 receptor. Co-localization of vasoactive intestinal peptide and orexin 1 receptor were more evident in the pyloric stomach and proximal intestine. These data could suggest a possible role of orexin system on the contractility of bottlenose dolphin gastrointestinal districts. Finally, in agreement with several reports, bottlenose dolphin orexin A plasma level was higher in the morning during fasting. Our results emphasize some common features between bottlenose dolphin and terrestrial mammals. Certainly, further functional investigations may help to better explain the role of the orexin system in the energy balance of bottlenose dolphin and the complex interaction between feeding and digestive physiology.

Introduction

Dolphins, like all toothed whales (odontocetes) mainly feed on fish and squid. Since chewing is an impossible act to perform underwater, dolphins grab their prey and ingest it whole, leaving mechanical digestion to the first muscular chamber of their polygastric stomach complex [1]. Frequent feeding is requested by the intense energetic costs of continuous swimming and the whole structure of the gastroenteric apparatus reflects these needs (i.e. cetaceans have no gallbladder). However the whole organization of the gustatory sense is poorly understood: odontocetes have no sense of smell and the tongue of adult dolphins possesses few or no taste buds [2]. Muscular species of fish and cephalopods living in the proximity of the continental shelf are among the favorite prey of bottlenose dolphins [3]; captive bottlenose dolphins are known to prefer capelin (*Mallotus villosus*) and other fish with a relatively high content of water, especially during the warm season [4]. But it is unclear how gustatory discrimination between different varieties of fish occurs. Feeding preferences (when the abundance of food allows them) may therefore rely on mechanisms other than stimulation of receptors in the mouth.

Orexin A (OXA) and B (OXB), initially identified in the "feeding center" of the rat lateral hypothalamus [5,6], are respectively 33- and 28- amino acids peptides that originate from a single precursor produced by the prepro-orexin (PPO) gene [5,6]. Intracerebroventricular (ICV) administration of OXA or OXB is able to increase food intake [5,7–9], hence the name "Orexin" that derives from the Greek word "orexis" [ὄρεξις] which means appetite, desire.

OXA is more powerful than OXB, and the actions of both are mediated through binding to the closely related orexin 1 (OX1R) and orexin 2 (OX2R) receptors that belong to the family of G-protein-coupled receptors [5]. While OX1R is highly selective for OXA, OX2R binds both orexins with similar affinity [5].

The orexinergic system is widely distributed throughout the Central Nervous System (CNS) [10–13] and in peripheral organs and body systems, including heart [14], adipose tissue [15], kidney [16], endocrine glands [14,17–19], urogenital [20,21] and gastrointestinal tracts [22,23]. In addition to a key role in food intake, orexins are also involved in the control of several biological functions such as arterial blood pressure and heart rate [24], sleep/wake cycle [25], water assumption [26], plasma corticosteroid levels [27], testosterone production [28], and endocrine and

exocrine pancreatic secretions [29–31]. A series of recent studies [32–35] proposed a role of the orexin system in the central and peripheral control of gastrointestinal secretion and motility.

In a previous publication on the neuroendocrine regulation of the digestive post-diaphragmatic functions in sea mammals, we reported the distribution of the anorexigenic peptide leptin in the gastrointestinal tract of the bottlenose dolphin [36]. The physiological mechanisms that regulate glucose metabolism and food intake in cetaceans are not fully understood. Here we present a general approach to the presence and possible role of orexins in the gut of the bottlenose dolphin *Tursiops truncatus* (Montagu 1821): the expression and topography of the orexin system (OXA, OXB, OX1R, OX2R) in the gastrointestinal tract were investigated by single immunostaining; the expressions of prepro-orexin and orexin receptors were analyzed by western blotting analysis; and the co-localization of vasoactive intestinal peptide (VIP) and OX1R in the enteric nervous system (ENS) was examined by double immunostaining. Finally, peptide concentrations were measured in plasma samples collected at 10:00 and 17:30 hours to assess the possible diurnal variation of the plasma levels of OXA in this species.

Materials and Methods

Animals and tissue preparations

For the present study we used a series of samples of the gastrointestinal tract of three specimens of bottlenose dolphin *Tursiops truncatus* stored at the *Mediterranean marine mammal tissue bank* (MMMTB) of the Department of Comparative Biomedicine and Food Science of the University of Padova (http://www.marinemammals.eu). The MMMTB stores tissues from stranded animals or from marine mammals who died in captivity. The MMMTB is a recognized CITES institution (IT 020). The samples (see Table 1) were removed within a few hours after death. Each sample was divided into two portions. One portion was frozen and stored at −80°C, while the other was fixed in 10% buffered formalin and later embedded in paraffin. Sections were serially cut in 8 μm thick transversal sections and placed on slide glasses. Frozen plasma samples of two male bottlenose dolphin were obtained from the MMMTB of Padova. The MMMTB stores tissues from stranded animals or from marine mammals who died in captivity. The MMMTB is a recognized CITES institution (IT 020). Archival plasma samples stored in the Bank are excesses of blood sampled from captive dolphin living in different Aquariums or Sea-worlds during routine veterinary medical controls and sent to the MMMTB for laboratory controls and special analyses. No venipuncture was performed specifically for this study. Plasma samples were originally collected at 10:00 am and 17:30 pm in four different days. Each blood sample was drawn from the ventral surface of the flukes of trained dolphins, and collected into vacuum EDTA tubes. The plasma was then separated after centrifugation for 20 min at 2800 g (3500 rpm) and stored at 20°C.

Also, we used as positive control archival samples of male Wistar rat duodenum (Harlan, Italy), stored in the Department of Veterinary Medicine and Animal Production of University of Naples Federico II. The samples were collected during a series of animal experiments for which approval was granted by the Italian Ministry of the University (now called "Ministry of Education") according to the contemporary regulation on animal experimentation [37].

Use of archival samples is encouraged based on the EU Directive 2010/63/ of 22 September 2010 on the protection of animals used for scientific purposes: "Member States should, where appropriate, facilitate the establishment of programmes for sharing the organs and tissue of animals that are killed" (Introduction section #27).

Single immunohistochemistry

The expression and distribution of OXA, OXB, OX1R and OX2R in the gastroenteric tract of the bottlenose dolphin were studied by immunohistochemistry. The sections were de-waxed and incubated with 0.3% hydrogen peroxide for 30 min at room temperature (RT), to block endogenous peroxidase activity. The sections were then rinsed in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 15 min and subsequently incubated for 20 min at RT with normal rabbit serum. Normal serum and the other components of the immunological reaction were contained in the Vectastain Elite ABC kit (PK 6105; Vector Laboratories Inc, CA, USA). In the specific step, polyclonal antibodies raised in goat against OXA (sc-8070), OXB (sc-8071), OX1R (sc-8072) and OX2R (sc-8074) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) were used and diluted 1:300. After incubation with primary antisera the sections were rinsed in PBS for 15 min and incubated for 30 min at RT with biotinylated rabbit anti goat IgG. Subsequently, the sections were rinsed in PBS for 15 min and then incubated for 30 min at RT with an avidin-peroxidase complex. Peroxidase activity was detected using a solution of 3–3′ diaminobenzidine tetrahydrocloride (Sigma, St. Louis, MO, USA) of 10 mg in 15 ml of 0.5 M Tris buffer, pH 7.6, containing 0.03% hydrogen peroxide.

Antigen unmasking procedures always preceded the immunohistochemical reaction and were carried out by dipping the sections in 0.01 M sodium citrate buffer, pH 6.0, and heating them in a microwave oven for 10 min at 750 W.

Double immunostaining

Double immunohistochemical staining was performed as follows: histological sections were de-waxed, rehydrated, rinsed in PBS and incubated for 30 min at RT with normal rabbit serum (1:5; S-5000, Vector Laboratories Inc., Burlingame, CA). After blocking of endogenous biotin was performed (Avidin-Biotin blocking kit, SP-2001, Vector), the sections were incubated with

Table 1. Specimens and samples used for immunohistrochemistry and western blotting analysis.

specimen	First chamber or forestomach	Second chamber or mainstomach	Third chamber or pyloric stomach	Proximal intestine	Distal intestine
ID 107 (♂)		x	x		
ID 110 (♂)	x	x	x	x	x
ID 139 (♂)	x	x	x	x	x

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the first primary antibody (goat anti-OX1R 1:50) over night at 4°C. The sections were then washed with PBS and incubated with a biotinylated secondary rabbit-anti goat antibody (1:20; BA-5000, Vector) for 2 h at RT. After rinsing in PBS, the sections were incubated with Streptavidin Texas Red conjugate (1:50; S-872, Life Technologies Europe, Monza, Italy) for 2 h at RT in dark humid chamber. After rinsing in PBS the sections were incubated with normal donkey serum (1:5; 017-000-121, Jackson Immunoresearch Laboratories Inc., Suffolk, UK) for 30 min at RT in dark humid chamber. The sections were subsequently incubated with the second primary antibody rabbit-anti VIP (1:20; 20077, ImmunoStar, Inc., Hudson, WI, USA) over night at 4°C in dark humid chamber. After rinsing in PBS, the sections were incubated with secondary antibody Alexa Fluor 488-conjugated affinipure donkey anti-rabbit (1:50, 711-545-152, Jackson) for 2 h at RT in dark humid chamber. Finally the sections were washed with PBS and mounted with glycerin diluted with PBS 1:1.

Fluorescent and light images were observed and analyzed by Nikon Eclipse 90i. The digital raw images were optimized for image resolution, contrast, evenness of illumination, and background by using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA).

Controls

Controls of antibody specificity were performed by absorbing each primary antiserum with an excess of the relative peptide (100 mg of peptide/ml of diluted antiserum; OXA/sc-8070p; OXB/sc8071p; OX1R/sc-8072p; OX2R/sc-8074p; Santa Cruz). Internal reaction controls were carried out by substituting primary antisera or secondary antisera with PBS or normal serum in the specific step. Archival samples of rat duodenum, known to be positive to the orexin system, were used as positive reference controls.

Western Blot Analysis

Bottlenose dolphin gastrointestinal tract and rat duodenum samples (1 g) were extracted in 6 ml of RIPA (Radio Immuno-precipitation Assay) buffer with Lysis buffer (50 mM Tris–HCl pH 7.4, 1% Triton X-100, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA), to which protein inhibitors 2 mM PMSF and protein inhibitor cocktail (P8340; Sigma-Aldrich) were added. Samples were homogenized with an Ultra-Turrax T25 (IKA-Labortechnik, Staufer, Germany) at 13,500 rpm. Homogenates were centrifuged 10,000 rpm for 20 min at 4°C, supernatants were collected separately and the protein concentration was determined with Bio-Rad dye protein assay (Bio-Rad Laboratories Inc., Hemel Hempstead, UK). 25 μg of protein for each sample were boiled at 98°C for 10 min in 25 μg of loading buffer 2x (50 mM Tris–HCl pH 6.8, 100 mM β-mercaptoethanol, 2% SDS, 0.1% blue bromophenol, 10% glycerol). Proteins were separated on a 12% (for orexins receptors) and 16% (for prepro-orexin) SDS–polyacrylamide gel electrophoresis with 4% stacking gel in 1% Tris–glycine buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS [pH 8.3]) in a miniprotean cell (Bio-Rad) at 130 V for 2 h. The separated proteins were electrotransferred onto a polyvinylidene fluoride membrane with transfer buffer (25 mM Tris base, 0.2 M glycine, and 20% methanol [pH 8.5]) in a minitransfer cell (Bio-Rad) at 100 V at 4°C for 1 h. Membranes were incubated at 4°C for 1 h in blocking buffer containing 1% PBS and 0.05% Tween 20 with 5% dried non-fat milk and then were probed with polyclonal antibodies in rabbit raised against prepro-orexin AB3096, Millipore, Temecula, CA, USA) and β-actin (A5060, Sigma, Sant Louis, MO, USA), used as internal marker, and polyclonal antibodies in goat raised against OX1R (sc-8072, Santa

Cruz Biotechnologies Inc) and OX2R (sc-8074, Santa Cruz Biotechnologies Inc.). All primary antibodies were diluted 1:1,000 and incubated over night at 4°C. This was followed by incubation with the secondary rabbit anti-goat IgG (Millipore 1:5000), and goat anti-rabbit IgG (Sigma 1:5000) antibodies for 1 h at RT. Signals were detected by chemoluminescence with the Pico Enhanced Chemiluminescence Kit (Pierce Chemical) with Chemidoc (Bio-Rad). A pre-stained molecular-weight ladder (Novex Sharp Pre-Stained Protein Standard, Life Technologies, Monza, Italy) was used to determine protein size.

Specificity was determined by pre-absorption of primary antibodies with their relative control peptides before western blotting.

Orexin A Plasma Levels

Plasma OXA concentration was measured with an enzyme immunoassay kit for orexin A/Hypocretin-1 (EK-003-30; Phoenix Pharmaceuticals Inc, Burlingame, CA, USA), according to the manufacturer's protocols. The assay was validated with a dilution series. The standard curve was 0.01–1,000 ng/mL. According to the manufacturer, OXA antibody cross-reacted with human, rat, mouse and bovine orexin A. The absorbance was read at 450 nm with a microplate reader (Bio-Rad, model 680).

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) and any significant difference was determined at a significance level of 0.05 via the application of a Tukey's test. The analyses were carried out using Statistica version 7.0 (Statsoft inc., Tulsa, OK, USA).

Results

The post-diaphragmatic gastrointestinal tract of the bottlenose dolphin includes a three chambered stomach, a pyloric stomach and a long undivided, unvarying intestine. Since a division between small and large intestine (and respective sub-constituent parts) is impossible to perform, here we considered a proximal and a distal part of the intestine based on their approximate length.
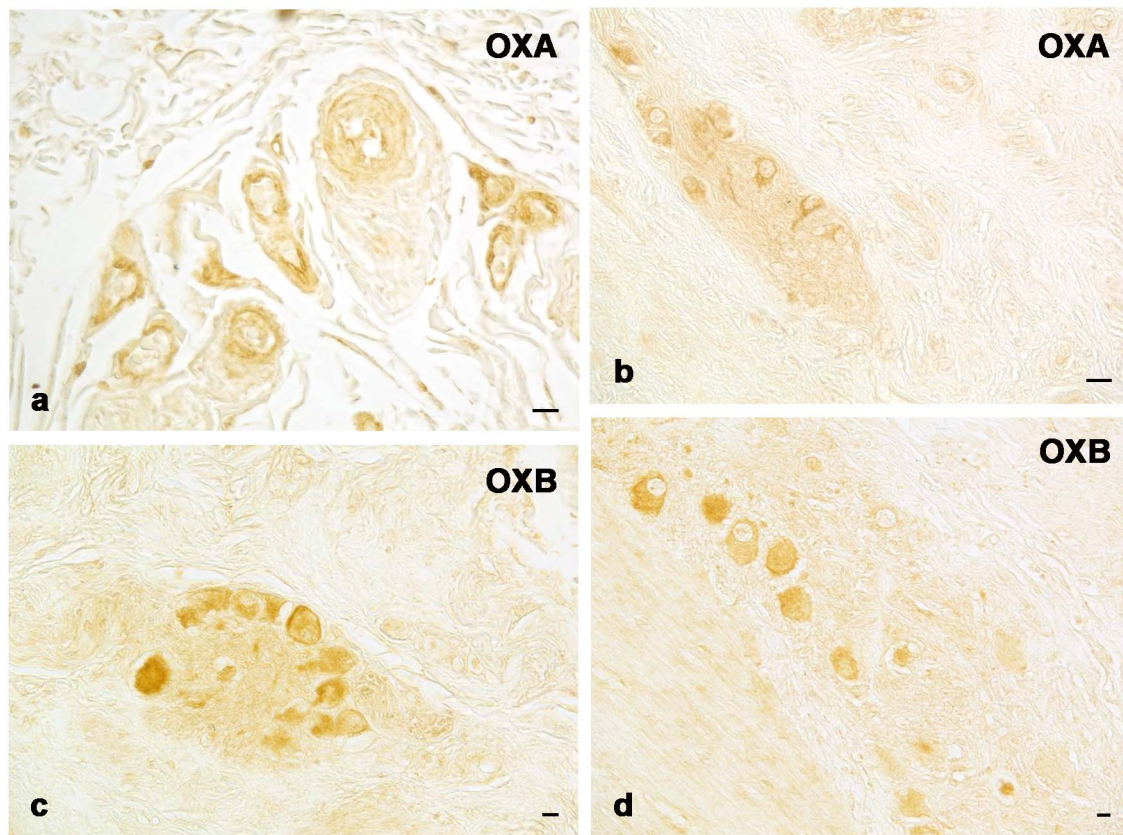
Single Immunohistochemistry

Orexin system immunopositivity was widely distributed throughout the entire bottlenose dolphin gastrointestinal tract. In particular, immunopositivity was evident in the ENS. OXA immunoreactive (ir) perivascular (Fig. 1a), and ganglion nervous fibers, as well as neuronal cells (Fig. 1b) were detected both in the submucosal layer (Fig. 1a) and the myenteric plexuses of the main stomach, pyloric stomach and proximal intestine (Fig. 1b). OXB-ir (Fig. 1c, d) neurons and fibers were found in the ENS of the same gastrointestinal tract.

OX1R-ir neurons and fibers were identified in both plexuses of the ENS (Fig. 2 a–d) throughout the gut. Immunostained neurons were occasionally found scattered (Fig. 2a) or isolated (Fig. 2b, b[1]), but in most cases they were grouped and belonged to the ganglion structures (Fig. 2c, d). OX2R-ir neurons and fibers were observed in the nervous plexuses of the intestinal tracts (Fig. 2e, f). A high intensity of staining was detected in both the submucosal (Fig. 2e) and myenteric ganglion (Fig. 2f) of the distal intestine.

Double Immunohistochemistry

Double immunostaining allowed the identification of a population of neurons and fibers that were positive to both VIP and OX1R. The immunopositive nervous elements were found both in submucosal and in the myenteric plexuses of the pyloric stomach

Figure 1. OXA and OXB single immunohistochemical detection. OXA immunopositive perivascular fibers in the submucosal layer of main stomach (a); OXA ir neurons and nervous fibers in the myenteric plexus of proximal intestine (b); OXB ir neurons of the submucosal plexus of pyloric stomach (c) and myenteric plexus of proximal intestine (d). Scale bars: 5 µm (a); 20 µm (b); 10 µm (c, d).
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(Fig. 3a–c), proximal (Fig. 3d–f) and distal intestine (Fig. 3g–i). Co-localizations of VIP-ir and OX1R-ir elements were more evident in the pyloric stomach and proximal intestine than in the distal intestine.

Protein expression

Western blot analysis was carried out on homogenates of all gastrointestinal tract of bottlenose dolphin (forestomach, main stomach, pyloric stomach, proximal and distal intestine). The results of western blot analysis (Fig. 4) showed the presence of prepro-orexin 16 kDa band in the main stomach, pyloric stomach and proximal intestine. The presence of OX1R immunoreactive band of about 50 kDa and OX2R immunoreactive band of about 38 kDa was detected in all gastrointestinal tracts, with the exception of main stomach where no band for OX2R was present. β-actin, used as internal marker, was detected in all examined samples as a band of about 42 kDa.

The specificity of the response was confirmed by pre-incubation of prepro-orexin, OX1R and OX2R antibodies with their respective blocking peptides. There was no expression of prepro-orexin, Ox1R and OX2R in these preparations, whereas the presence of the proteins was detected in rat duodenum homogenate that was used as positive control.

Orexin A Plasma Levels

OXA plasma concentrations are reported in Figure 5. OXA levels were statistically higher in the morning (10:00) than in the afternoon (17:00).

Discussion

In the last decade, numerous investigations report the presence and the expression of the orexin system in the gastrointestinal tract of several vertebrates [22,23,38,39]. The distribution of orexins and their specific receptors along the gastrointestinal tract varies in terrestrial mammals, depending on the species of animal investigated [40–42] and the respective digestive physiology. Toothed whales, including the bottlenose dolphin, are entirely carnivore species that feed mainly on fish or marine invertebrates. Dolphins do not chew their food, but grab and swallow their prey whole, leaving mechanical digestion to the heavily muscular forestomach [43]. The intestine shows no macroscopic or microscopic subdivisions into a small or large part, but consists of a long unvarying tube of homogenous caliber. Our results showed that in gastrointestinal tract of the bottlenose dolphin the distribution of the orexin system is very peculiar and partially differs from that of terrestrial mammals. OXA, OXB and prepro-orexin are expressed in the main stomach, pyloric stomach and proximal intestine. Orexin receptors are expressed in all the examined tracts, with the exception of main stomach where we found no evidence of OX2R. Therefore our data suggest that the orexin system is widely distributed in bottlenose dolphin ENS. The majority of neurons and fibers with high intensity of staining were identified in the intestinal tract, as also observed in newborn dogs and pigs (carnivorous and omnivorous monogastric animals) [40,42] and in fallow deer (erbivorous poligastric mammal) [44]. The abundant expression of orexins in the post-diaphragmatic
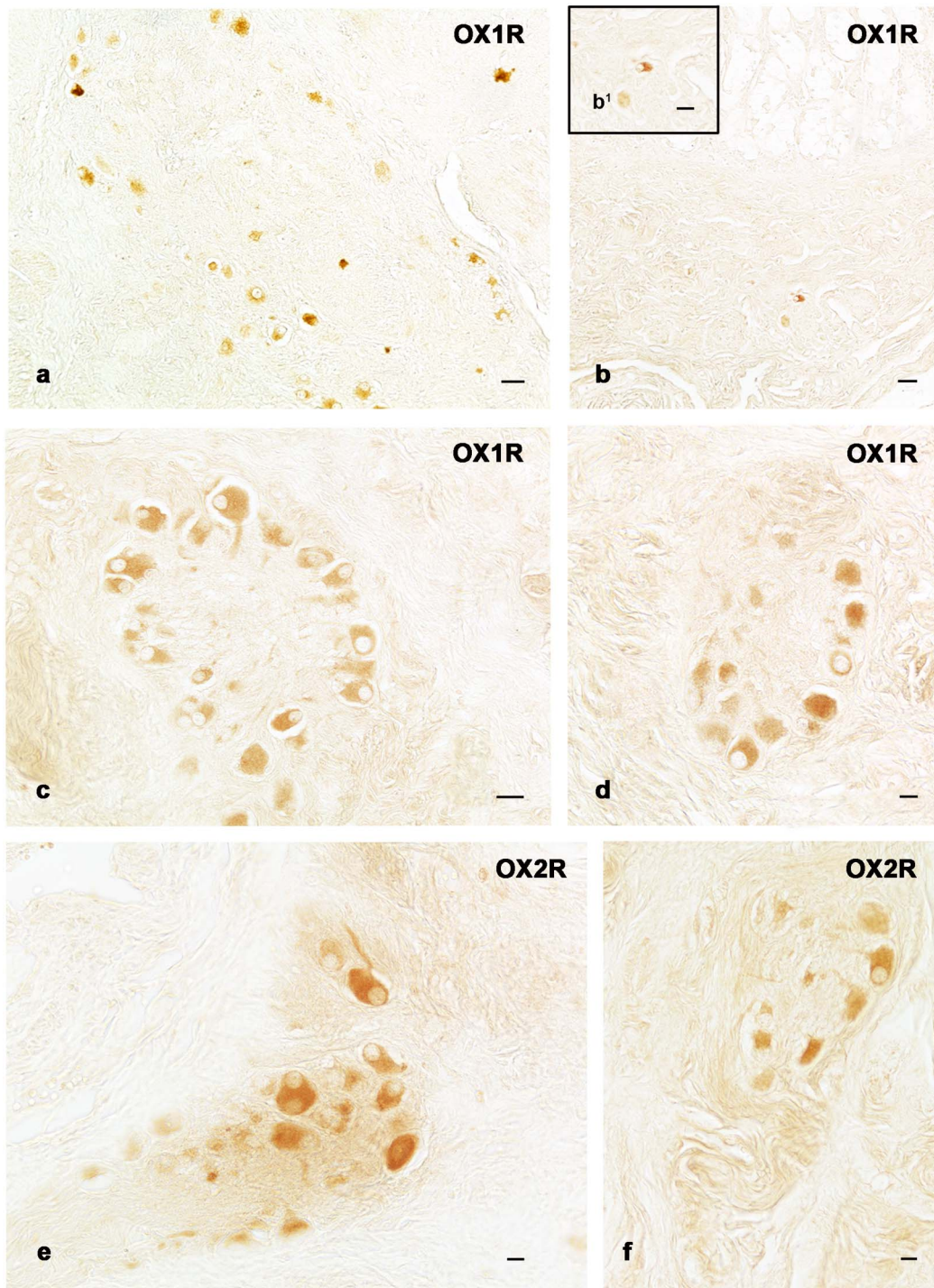
Figure 2. OX1R and OX2R single immunohistochemical detection. Orexin 1 (a–d) and orexin 2 (e, f) receptors ir nervous fibers and neurons in the submucosal plexus of forestomach (a), proximal (b, b^1; b^1 detail of b) and distal (e) intestine and in the myenteric plexus of proximal (c) and distal (d, f) intestine. Scale bars: 30 μm (a, b); 20 μm (c); 10 μm (d–f).
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gastrointestinal tract of the bottlenose dolphin supports the involvement of orexigenic peptides in the regulation of gastrointestinal secretion and motility, as suggested for terrestrial mammals, in which orexin peptides modulate digestive physiology acting centrally or peripherally. The specific anatomy of the intestine of dolphins may explain the apparent uniform distribution of both orexin and their respective receptors.

OXA penetrates the blood-barrier by simple diffusion, contrarily to OXB [45], and this may explain why the different effects of the two peptides. OXA stimulates gastric acid secretion throughout vagal pathways [46], regulates intestinal bicarbonate secretion [32], and gastrointestinal and colon motility [33,35,47]. Studies on the dose-dependent peripheral effects of OXA and OXB on intestinal wall show that they slow down intestinal
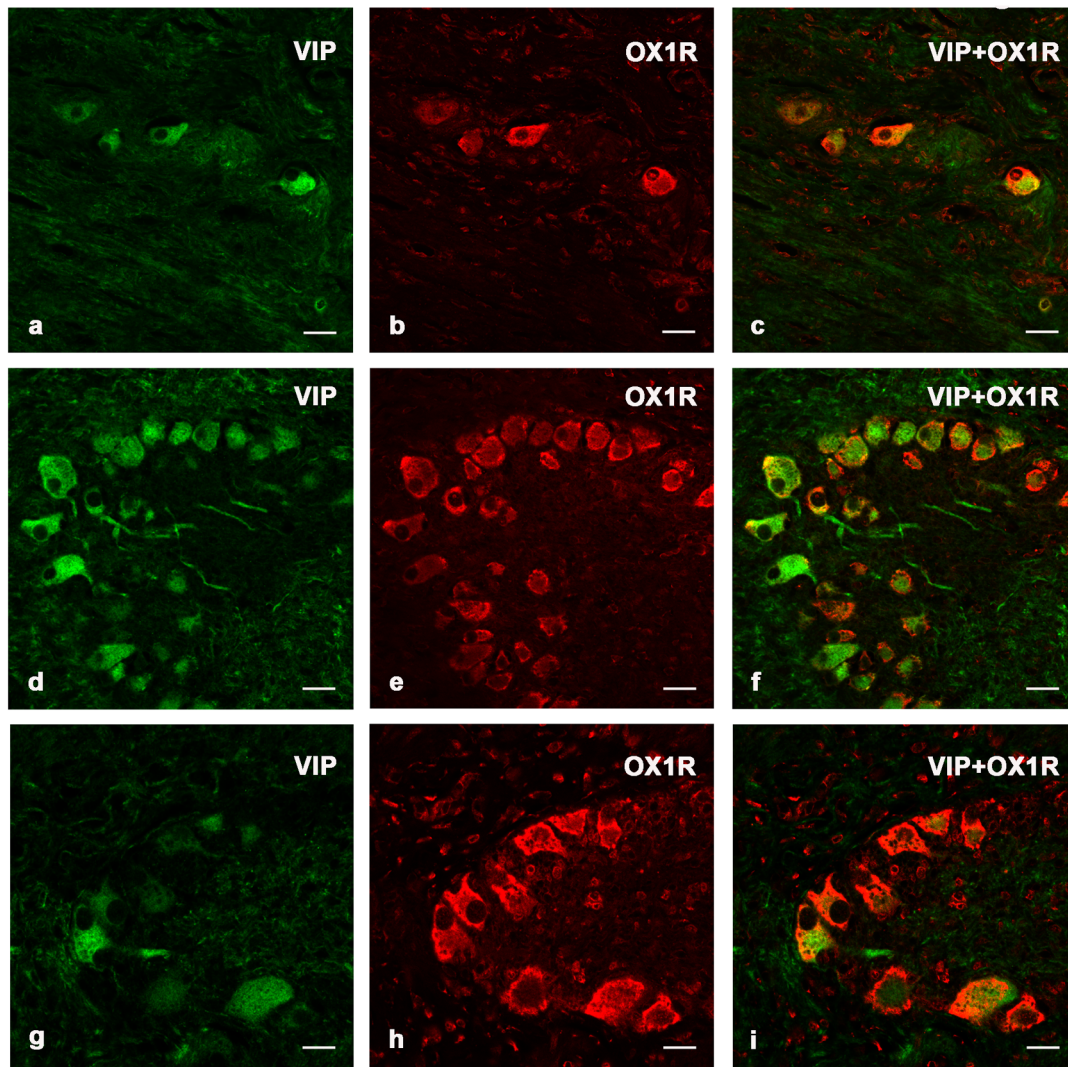
Figure 3. VIP and OX1R double immunostaining. Colocalizations of VIP- (green) and OX1R- (red) immunopositive neurons and nervous fibers located in the myenteric plexus of pyloric stomach (a–c) and submucosal plexus of proximal (d–f) and distal (g–i) intestine. Scale bars: 20 µm (a–i).
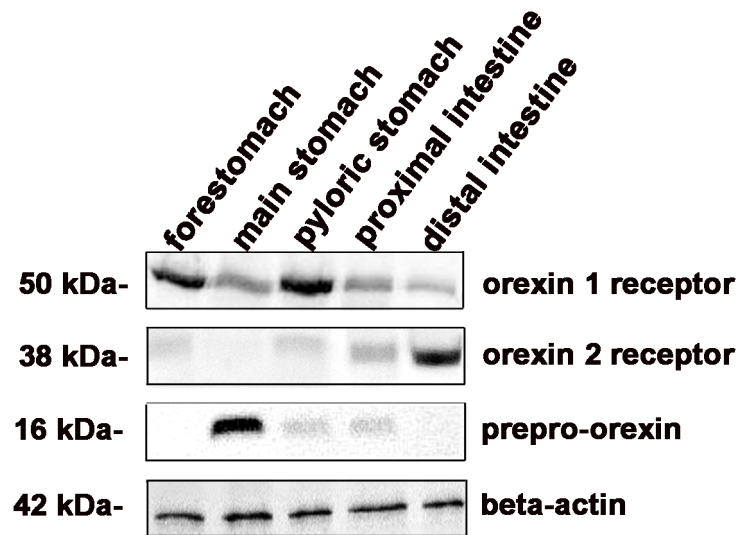doi:10.1371/journal.pone.0105009.g003

motility by extending of duration of migrating motor complex (MMC) [22,23,48 7].

Our double immunostaining results, in agreement with Naslünd et al. (2002), showed OX1R and VIP-ir neurons and fibers in submucosal and myenteric plexuses of the pyloric stomach, proximal and distal intestine. Co-localizations of VIP and OX1R were more evident in the pyloric stomach and proximal intestine than in the distal intestine. VIP exerts various effects on the gastrointestinal tract of terrestrial mammals, including the inhibition of MMC [49]. VIP acts on MMC directly, or indirectly either through L-arginine/nitric oxide, or OXA/OX1R pathways [22,50]. Its co-localization with OX1R could suggest that orexin system act on the intestinal motility also in the bottlenose dolphin, reducing the contractility in the gastrointestinal districts similarly to what described in terrestrial mammals [33,51].

Gastrointestinal and blood samples used in this study were collected during routine veterinary medical procedures from captive animals used to a constant feeding schedule including regular meals and feeding sessions used to positively enforce required behaviors. Since blood cannot be drawn from wild animals, possible differences with free-ranging individuals should

be taken into account. However bottlenose dolphins do not usually undergo long migrations to reach breeding grounds and generally show no fasting period during the mating season or calving. However our data indicate that plasma concentrations of OXA in bottlenose dolphins are higher in the morning before their main meal. This is in agreement with several studies reporting the increase of plasma OXA concentration during fasting in rats and human [22,52,53]. A correlation was found in the bottlenose dolphin between urinary vasopressin values after the meal and urine concentration, a sign that indicates conservation of food-derived water [54]. Orexin concentration may have a direct relationship with the glucose requirements of the brain, and the specific needs of the peculiar central blood flow and metabolism typical of dolphins [55]. However the presence and nature of circadian and circannual rhythms in dolphins remain open, at least based on the origin and concentration of neuroendocrine transducer melatonin [56]. Furthermore, the neurophysiology of sleep and the alternate phases of rest-activity cycles are markedly different in cetaceans [57].

Physiological investigations on marine mammals are obviously mostly limited to observation of wild specimens and occasional

Figure 4. Orexin system protein expression. Western blot for orexin 1 and 2 receptors, prepro-orexin and β-actin (used as internal marker) in forestomach, main stomach, pyloric stomach, proximal and distal intestine.
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studies on captive animals. Feeding strategies, food preferences and even diving behavior can be studied in wild populations of bottlenose dolphins, but actual information on digestive physiology derive largely from trained dolphins living within a confined environment. So i.e. the peculiar glucose metabolism of the bottlenose dolphin that shows postprandial hyperglycemia similar to humans with diabetes mellitus type two [58,59] remains largely unexplained. However orexin concentration may have a direct relationship with - or be linked to - the glucose requirements of the brain, and thus also be part of the mechanism of selective control

of central blood flow and metabolism, important for the integration of auditory and other sensory inputs [55]. We also note that unihemispheric sleep implies uneven distribution of glucose between the active and resting parts of the brain [60].

Our report adds novel information on the presence and distribution of the orexin system in the gastrointestinal tract of dolphins. These results, taken together with data on the plasma concentration of orexins, emphasize some common features between bottlenose dolphin and terrestrial mammals. Further functional investigations may help to better explain the role of the
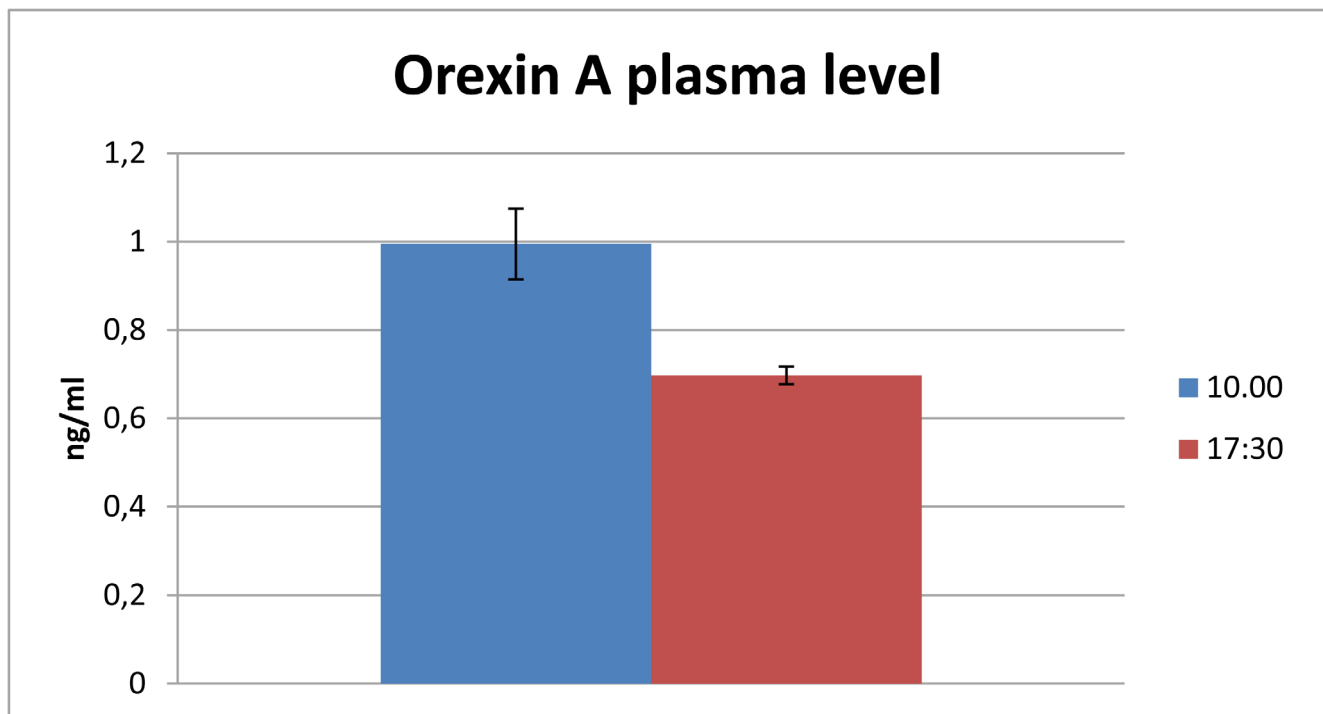
Figure 5. OXA plasma levels. Bottlenose dolphin plasma OXA concentration (mean ± SD) at 10:00 and 17:30 hours.
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orexin system in the energy balance of bottlenose dolphin and the complex interaction between feeding and digestive physiology.

Author Contributions

Conceived and designed the experiments: CG FR MGR EV MP LC CL PdG BC LM. Performed the experiments: CG FR MGR LM. Analyzed the data: FR EV MP PdG. Contributed reagents/materials/analysis tools: BC EV MP PdG. Contributed to the writing of the manuscript: FR BC LC CL.

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