Acta Histochemica 115 (2013) 371–375

Contents lists available at SciVerse ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Identification and location of the cocaine and amphetamine regulated transcript (CART) in the abomasum of cattle

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ARTICLE INFO

Article history:
Received 16 August 2012
Received in revised form
19 September 2012
Accepted 19 September 2012

Keywords:
Cocaine and amphetamine regulated transcript (CART)
Abomasum
Immunohistochemistry
Cattle

ABSTRACT

The cocaine and amphetamine regulated transcript (CART) belongs to the group of peptides with anorexigenic properties and is present in many areas of the central and peripheral nervous systems of numerous mammalian species. Research has suggested an effect on the feeling of appetite and satiety; however, there are no clear clues as to the role of CART in specific organs, including the stomach. Considering the specificity of cattle feeding and digestion, CART may play a highly significant role possibly associated with the option of administering greater amounts of high-volume feeds. Based on the results of immunohistochemical staining of abomasum samples prepared from hybrid bulls, the presence of CART-positive structures and CART distribution were determined in the mucosa, submucosa and muscularis layers of the stomach. Abundant sites of CART were found in the myenteric plexus, nerve fibers innervating the myocytes of the myenteron, neuroendocrine cells of the diffuse neuroendocrine system and the submucous plexus. The preliminary stage of abomasal CART detection suggests that CART is an agent that strongly affects the regulation of motor activity involved in stomach emptying and in secretory functions of the stomach. However, further research is necessary to explain the relationship.

Introduction

The control of food intake is multi-faceted and usually regulated by the central nervous system (CNS), the gastrointestinal system and autonomic nerves (Schwartz et al., 2000). Peptide neuromodulators produced and released by both the CNS and the gastrointestinal mucosa are significantly active at each of the above levels. The role of the neuromodulators relates primarily to the mechanisms that integrate the signals sent by the brain to specific sections of the digestive tract. It is widely known that the integration of the signals is effected by two antagonistic systems: the orexigenic system that stimulates appetite and the anorexigenic system that inhibits hunger (Vettor et al., 2002).

In recent years there has been considerable progress in understanding the anorexigenic cerebrogastrointestinal peptides that function as food intake inhibitors (Kuhar and Dall Vechia, 1999; Vicentic and Jones, 2007). The group of appetite-controlling animal peptides includes the cocaine and amphetamine regulated transcript (CART). This neuropeptide was first isolated from the sheep hypothalamus by Spiess et al. (1981); however, precise analysis and description of this compound was only achieved much later (Douglass et al., 1995).

The discovery of CART significantly influenced the development of many scientific disciplines, including endocrinology and physiology and initiated many experiments to try and explain the role of CART in the organism. It is known that CART acts through specific receptors located virtually throughout the body with the greatest activity observed in the hypothalamus, hypophysis, adrenal glands, thyroid, ovaries, pancreas, lungs and the urinary bladder (Koylu et al., 1997; Davidowa et al., 2005; Zvarova and Vizzard, 2005; Wierup et al., 2007; Sen et al., 2008). In addition, CART has been detected in several organs of the alimentary tract (Ellis and Mawe, 2003; Ekblad et al., 2003; Gonkowski et al., 2009). Experimental studies have identified an effect of CART on gastric acid secretion, stomach emptying and enhanced colon peristalsis (Okumura et al., 2000; Tebbe et al., 2004).

CART has been identified in the stomach, duodenum, ileum, colon and the islets of Langerhans (Jensen et al., 1999; Cowles et al., 2001; Ellis and Mawe, 2003; Wierup et al., 2006, 2007; Arciszewski et al., 2008; Gonkowski et al., 2009).

Several studies have shown that a large proportion of gastrointestinal CART is released by the fibers of the gastrointestinal

Abbreviations: BW, black and white breed cows; CART, cocaine and amphetamine regulated transcript; CNS, central nervous system; DAB, 3,3′-diaminobenzidine; DNES, diffuse neuroendocrine system; IR, immunoreactivity; LI, like immunoreactivity; LIM, Limousin breed; NE, neuroendocrine.

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http://dx.doi.org/10.1016/j.acthis.2012.09.010

myenteric and submucous plexus, as well as by the G cells in the stomach (Wierup et al., 2007; Gonkowski et al., 2009). Apart from its anorexigenic effect that causes reduced food demand (Cummings and Schwartz, 2000) and a consequent decrease of body weight, CART also strongly stimulates pancreatic secretion. Moreover, it is associated with the energetic homeostasis of the organism, participates in gonad development, in the regulation of hormonal balance and body temperature, and in the control of blood circulation and mineral homeostasis (del Giudice et al., 2001; Hunter and Kuhar, 2003; Baranowska et al., 2004; Tebbe et al., 2004; Bai et al., 2005).

Considering the above, the cocaine and amphetamine regulated transcript (CART) should be regarded as an important factor in the pathways that regulate appetite. Owing to its role in food intake, it consequently plays an important role in the nutritional status of the organism, growth and development. It should be noted that numerous studies concerning the presence and distribution of CART in mammals, especially in the stomach, were based on experimental studies in laboratory animals with only limited information on this peptide in tissues of pigs, sheep and humans (Gunnarsdóttir et al., 2007; Arciszewski et al., 2009; Gonkowski et al., 2009). In the case of ruminants, including cattle, the available data are very limited, owing to difficulties in obtaining the experimental material. The present study was undertaken to broaden and supplement our knowledge of CART distribution in the abomasum in cattle.

Material and methods

The study was performed on 9 hybrid bulls, crosses of Polish Lowland black-and-white breed cows (BW) in which the gene share of the Holstein Friesian breed did not exceed 25% with bulls of the Limousin breed (LIM). Bulls were kept under similar conditions, but came from different farms. Fattening started when the calves weighed 150–180 kg. In terms of weight gain and fattening time, growth intensity was calculated ($GI_{g/day}$). In the autumn–winter period, animals from the first group were fed hay *ad libitum* and corn silage (approximately 10 kg/24 h). In the summer, green fodder and straw were provided *ad libitum*. Compound cereal meal was used as a supplement to the main diet for animals in the second group at approximately 1.0 kg/24 h throughout the fattening period. The hybrids had mean values: age 573 ± 32 days, slaughter weight 483.9 ± 45 kg and growth intensity 852 ± 47 g/day.

Sampling and preparation of the experimental material

The material for analyses was sampled in meat plants located in Eastern Poland. Directly after slaughter, the stomachs of the analyzed animals were prepared. Equal sized fragments (1 mm^2) of the abomasum walls were cut out (always from the same spot). The sampled tissue was *ex tempore* fixed in 4% buffered formaldehyde for 72 h at room temperature. Subsequently, paraffin wax blocks were made using standard procedures.

The preparations were analyzed and photographed with an Olympus BX41 light microscope (Olympus Corp., Tokyo, Japan) with a video channel connected to a PC with an installed morphometric program for Cell-B image analysis (Olympus Corp., Tokyo, Japan). When recording the microscopic images, particular attention was paid to the distribution of the structures showing immunoreactivity to the analyzed antigen. Morphometric analysis was applied to brown stained cells of the DNES (diffuse neuroendocrine system) that were easily identifiable.

Immunopositive neuroendocrine (NE) cells were counted in 10 randomly selected fields of view (0.785 mm^2) at a 200× zoom (20× objective and 10× eyepiece). The number of positively stained cells was expressed as a mean per field of view. As for the semi-quantitative evaluation of the density of CART-IR structures (nerve fibers, submucous plexus and myenteric plexus), an arbitrary scale was used, where (0) = absence of stained structures; (1) = single structure; (2) = few structures; (3) = moderate number; and (4) = dense.

Immunohistochemical procedure

In the immunohistochemical study, the Dako EnVision technique (Dako, Glostrup, Denmark) was used according to Herman and Elfont (1991). The paraffin blocks were cut into 4 μm sections and mounted on Superfrost Plus slides (Menzel, Braunschweig, Germany) and dried overnight at 37 °C followed by 1 h at 60 °C. Immunohistochemistry was performed using the EnVision (+) HRP Rabbit Detection System (no. K4011, Dako, Glostrup, Denmark). Immunostaining was performed using the following protocol. Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of pure ethanol. For antigen retrieval, the sections were subjected to pretreatment in a pressure chamber heating for 1 min at 21 psi (one pound force per square inch (1 psi) equates to 6.895 kPa, conversion factor provided by United Kingdom National Physical Laboratory) at 125 °C using Target Retrieval Solution pH, 9.0 (S2367; Dako, Glostrup, Denmark). After being cooled to room temperature, sections were incubated with peroxidase blocking reagent for 10 min to block endogenous peroxidase activity. CART (Phoenix Pharmaceuticals, Burlingame, CA, USA, code H 003-61) was diluted (1: 12,000) in antibody diluent (S 0809, Dako, Glostrup, Denmark).

The sections were incubated overnight at 4 °C in a humidified chamber with the diluted antibody, followed by incubation with labeled polymer for 1 h. Bound antibodies were visualized by 1 min incubation with liquid 3,3′-diaminobenzidine (DAB) substrate chromogen. The sections were finally counterstained in hematoxylin QS (H-3404; Vector Laboratories, Burlingame, CA, USA), mounted, and evaluated under a light microscope. Appropriate washing with wash buffer (S 3006; Dako; Glostrup, Denmark) was performed between each step.

The specificity test performed for the CART antibody included: negative control, where the antibodies were replaced by normal rabbit serum (Vector Laboratories, Burlingame, CA, USA) at the respective dilution. Positive control was done for specific tissue recommended by the manufacturer, which for bovine CART is human paraventricular nucleus of the hypothalamus.

Results

The results for the distribution of the cocaine and amphetamine regulated transcript (CART) in the abomasum of cattle are presented in Table 1. The results show the presence of the analyzed neuropeptide confirmed by a positive immunohistochemical (IHC) reaction in: the myenteric plexus (MP) (Fig. 1A), the nerve fibers innervating the myenteron (Fig. 1B), in the cytoplasm of the neuroendocrine cells of the diffuse neuroendocrine system (DNES) (Fig. 1C) and the submucous/Meissner's plexus (Fig. 1D). The highest CART-like immunoreactive (CART-LI) density was observed in the thick and long nerve fibers present in the entire muscularis (Fig. 1B and F) and in the myenteric plexus (Fig. 1A). CART was also often observed in numerous perikarya of the myenteric plexus (Fig. 1G).

The lowest density, on the other hand, was identified in the submucous plexus in all the analyzed animals (Table 1).

Analysis of the obtained images revealed that the peptide was also present in the DNES cells that usually either appeared as single cells and were dispersed among other mucosal epithelial cells or formed complexes of two or several cells (Fig. 1E). It should be noted