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Glial fibrillary acidic protein-immunoreactive enteroglial cells in the jejunum of cattle

Anna Costagliola*

Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Via F. Delpino, 1, Naples, 80137, Italy

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ABSTRACT

Enteroglial cells (EGCs) play critical roles in human health and disease, however, EGC-dependent neuropathies also affect commercially important animal species. Due to the lack of data on the distribution and phenotypic characterization of the EGCs throughout the bovine gastrointestinal tract, in this study the topographic localization of EGCs in the jejunum of healthy cattle was investigated by immunofluorescence using the glial specific marker glial fibrillary acidic protein (GFAP) and the panneuronal marker PGP 9.5. This analysis was conducted on both cryosections and whole mount preparations including the myenteric and the submucous plexuses of the bovine jejunum. The results obtained showed the presence of a large subpopulation of GFAP-expressing EGCs in the main plexuses and within the muscle layers, whereas only few GFAP-positive glial processes were found within the deeper layer of the mucosa, and they never reached the mucosal epithelium. Three different EGC subtypes, namely I, III and IV types were recognized in the examined tract of the bovine intestine. Overall, our results provide the basis for future investigations aimed at elucidating the functional role of the GFAP-containing EGCs which is crucial for a better understanding of the physio-pathology of the bovine intestine.

1. Introduction

The enteroglial cells (EGCs), a population of small astrocyte-like cells, together with the intrinsic neurons constitute the enteric nervous system (ENS) that extends along the entire mammalian gastrointestinal tract. The ENS coordinates motility, sensory, mucosal permeability and secretion, vascular flow and local immune processes, independently or in cooperation with the central nervous system (Furness, 2006). The EGCs, more numerous than the intestinal neurons, envelop both neuronal cell bodies and cell processes throughout the intestinal wall up to the mucosa. They lie in the ganglia of the myenteric and submucous plexus, and within the smooth muscle layer and the mucosa (Gershon and Rothman, 1991; Ruhl et al., 2004; Gulbransen and Sharkey, 2012). Several cytotypes have been identified, mainly in laboratory animals (Hanani and Reichenbach, 1994; Boesmans et al., 2015). Immunohistochemical markers of EGCs in the adult gut are the glial fibrillary acidic protein (GFAP), S100, and Sox10 (Ferri et al., 1982; Ruhl, 2005; Hoff et al., 2008; Costagliola et al., 2009). In the gut, GFAP is considered a specific mature EGC marker (Jessen and Mirsky, 1980), although it is expressed by extrinsic, non-myelinating Schwann cells too (Jessen et al., 1990). Growing interest in EGCs emerged since the discovery that these cells may contribute to pathological processes (e.g., inflammation) due to their ability to release chemokines/cytokines in response to bacterial or inflammatory processes (Ruhl, 2006; Murakami et al., 2009; Neunlist et al., 2014), or may cause enteric neuro-gliopathy such as constipation (Bassotti and Villanacci, 2011).

Although the majority of the studies on the role and functions of the EGCs have been focused on experimental animals and humans, enteric neuropathies also affect commercially important animal species such as horses and cattle (Furness and Poole, 2012). Prion proteins have been found in EGCs of human and several animal species, cattle included (Davies et al., 2006; Albanese et al., 2008; Hoffmann et al., 2011). In particular, GFAP-expressing EGCs have been involved in the scrapie disease of small ruminants (Marruchella et al., 2007; Natale et al., 2011) or in the experimental infections (Cannas et al., 2011). Nevertheless, little is known on the topographical distribution of GFAP-expressing EGCs in agricultural animals (Marruchella et al., 2007; Di Giancamillo et al., 2010; Furness and Poole, 2012).

The neuronal plexuses in the jejunum–ileum of cattle show similarities but also some differences from the other large animals: The main organisation of the plexuses is similar to that of the pig, horse and man with external and internal submucous plexuses being morphologically distinct, with further subdivisions of the

* Corresponding author.
E-mail address: costagli@unina.it

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internal submucous plexus into the external and internal subplexuses (Balemba et al., 1999). However, in contrast to the other species, the submucous layer is firmly attached to the inner circular muscle layer. Unlike the other large animals, included sheep (Chiocchetti et al., 2004), the submucous plexus comprises an intermediate ganglionic network that is next to the submucous vascular arcades. This plexus is interconnected with the outer submucous plexus that lies close to the depth of the circular muscle layer, and the inner submucous plexus that lies close to the muscularis mucosae (Balemba et al., 1999). However, a precise distribution and phenotypic characterization of the EGCs throughout the bovine intestinal wall remains unexplored.

In order to assess the general topographic EGC distribution in the small intestine of healthy cattle, in this study the jejunum was chosen since previous reports did not highlight morphological differences in the jejunum and ileal segments (Balemba et al., 1999; Vittoria et al., 2000). Single and double immunofluorescence using the glial specific marker GFAP and the pan-neuronal marker protein gene product (PGP) 9.5 (Krammer et al., 1993) was carried out. Both cryosections and whole mount preparations, the latter including the myenteric or the submucous plexus, have been used. In the present study, a large subpopulation of GFAP-expressing EGCs has been found in the main plexuses and within the circular muscle layer, and three different subtypes have been recognized. By contrast, only few GFAP-positive glial processes have been found within the deeper layer of the mucosa without reaching the mucosal epithelium of the jejunum.

2. Materials and methods

The primary and secondary antibodies used in this study are described in Table 1. Both polyclonal antisera specific for GFAP and PGP 9.5 are directed against a molecule extracted from the bovine brain and spinal cord. For the present study, the jejunums of healthy cattle (two male and seven females, aged 16–25 months) were collected in the slaughterhouse of San Marcellino, Aversa (CE), Campania Region, immediately after the death of the animals and their exsanguination. The segments did not show any macroscopic pathological or inflammatory sign. The jejunum of each animal (about 10 cm in length) was removed and flushed with a Krebs solution (117 mM NaCl, 5 mM KCl, 2.5 mM $CaCl{\cdot}2H_2O$, 1.2 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 1.2 mM $NaH_2PO_4{\cdot}2H_2O$ and 10 mM glucose; pH 7.4). For cryosections, small parts of the jejunum were fixed for 6–9 h at room temperature in 4% paraformaldehyde (PF) in 0.1 M phosphate buffer (pH 7.0), as previously described (Vittoria et al., 2000); other parts were fixed for 5–6 h at room temperature in Zamboni's fixative (PF containing 10% picric acid). After fixation, the samples were washed in 0.01 M phosphate buffered saline (PBS; pH 7.4), and those fixed in Zamboni were washed several times in alcohol, and, finally, in PBS. Subsequently, the samples were incubated overnight in PBS containing 20% sucrose at 4 °C, embedded in OCT-embedding medium (Pelko Int., Torrance, CA, USA), cryostat-sectioned at 20 μm, and thaw-mounted on poly-L-lysine-coated slides.

The remaining part of the jejunum was opened along the mesenteric border and pinned out in a Sylgard-lined Petri dish. Both types of fixation for these parts of the jejunum were performed as described for the tissue parts processed for cryo-sectioning, but next the samples were cleared according to the procedure of Llewellyn-Smith et al. (1985). Whole mounts containing the myenteric and submucous plexus were then prepared by dissecting the external musculature and submucosa/mucosa apart and removing the circular muscle layer and the mucosa, respectively. Single and double immunohistochemical incubations were carried out at room temperature as previously described (Costagliola et al., 2009). Unless otherwise indicated, washes with 0.01 M PBS were performed between each incubation step. Primary antibodies were diluted in 0.1 M PBS with 0.05% Thimerosal (PBS*), containing 10% normal horse serum (NHS) and 0.1% Triton X-100. Secondary antibodies were diluted in PBS* containing 1% NHS. In single- and double-labelling experiments, GFAP was detected and visualized using the biotin–streptavidin technique; PGP 9.5 was detected and visualized using a standard fluorophore-labelled secondary antibody. Briefly, to eliminate endogenous avidin/biotin activity, cryosections were treated with a blocking kit of Zymed Laboratories (San Francisco, CA). Next, cryosections and whole-mounts were immersed in PBS* containing 10% NHS and 1% Triton X-100 (1 h, cryosections; 3–5 h, whole-mounts), prior to incubation with a primary antibody (18 h, cryosections; three overnights, whole-mounts). They were subsequently incubated with a biotinylated secondary antibody (1 h, criosections; overnight, whole-mounts). Visualisation was performed using fluorophore-conjugated streptavidin (FITC, Jackson Immunores Labs, West Grove, PA) diluted 1:1000 in PBS (1 h, cryosections; overnight, whole-mounts). Double labelling experiments using two primary antibodies raised in the same species were performed by a sequential procedure according to the method of Negoescu et al. (1994): in the first step, a biotinylated polyclonal monovalent Fab fragment and fluorochrome-conjugated streptavidin were used to detect GFAP. After detection of the first antigen, the preparations were washed prior to incubation for 1 h (cryosections) or overnight (whole-mounts) with unlabelled Fab fragments, diluted in PBS, directed against the first primary antibody to block residual binding sites (Lewis Carl et al., 1993). Next, they were rinsed in PBS and PGP 9.5 was detected and visualized using a standard fluorophore-labelled secondary antibody. Negative controls omitting one of the primary antibody, and interference control staining were performed. Omission of primary antibodies did not yield any signal. Interference control staining showed no cross-reactivity of the secondary antiserum with the primary antiserum used in the previous step. Zamboni fixative gave the best results in terms of reduced background and sharpness of the picture. The whole-mounts and cryosections were evaluated and photographed by a fluorescence microscopy Leica DMRA2. Some whole-mounts were photographed at different depth and their focal optical sections were constructed in order to get a final 3D image by Adobe Photoshop.

Table 1
List of antisera used for immunohistochemistry.

Antigen	Host	Dilution	Source
Primary antisera			
Glial fibrillary acidic protein (GFAP) from bovine spinal cord	Rabbit	1:1000	DAKO (Z0334)
Protein gene product 9.5 (PGP) from bovine brain	Rabbit	1:10000	DAKO (Z5116)
Secondary antisera and streptavidin complex			
Cy3-conjugated donkey anti-rabbit IgG		1:800	Jackson Immunores Labs, West Grove, PA
Biotinylated Fab-fragment of donkey anti-rabbit IgG		1:400	Jackson Immunores Labs, West Grove, PA
FITC-conjugated streptavidin		1:1000	Jackson Immunores Labs, West Grove, PA

3. Results

No pathological sign of inflammatory or degenerative activity was observed in the jejunum of the sampled animals both macroscopically and histologically.