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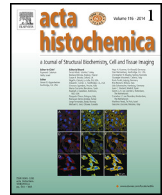

Glial fibrillary acidic protein-immunoreactive enteroglial cells in the jejunum of cattle

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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.

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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

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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 $CaCl_2{\cdot}2H_2O$, 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.

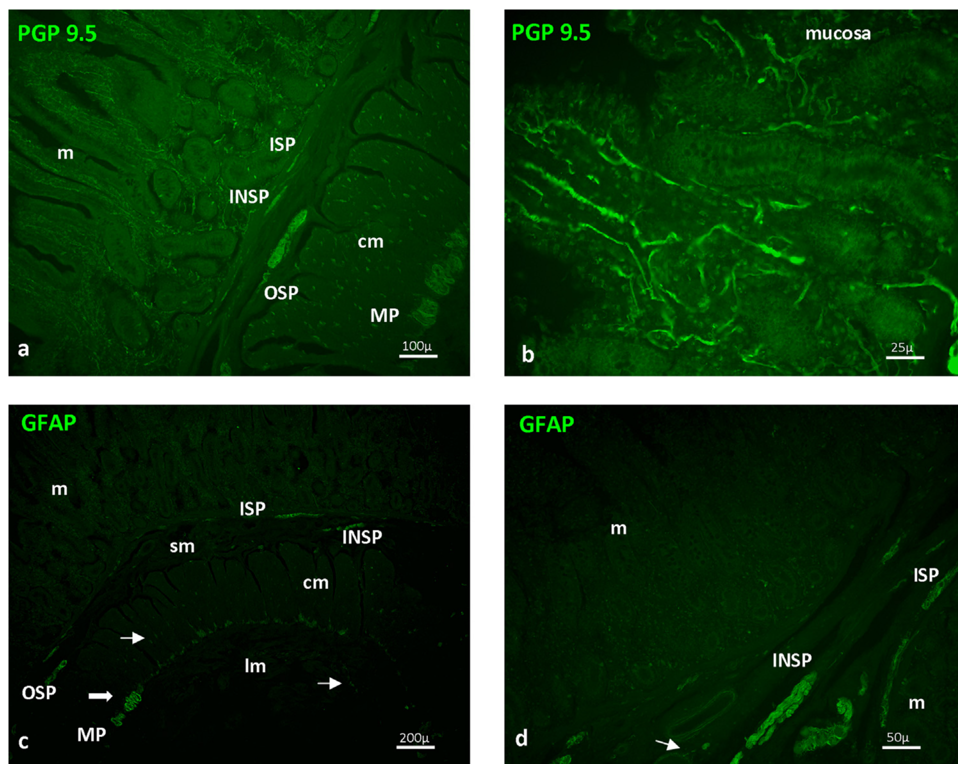

Fig. 1. a–d. Single immunolabelling with PGP 9.5 (a–b) and GFAP (c–d) antibodies on cryosections of the bovine jejunum. (a) PGP 9.5–immunoreactive neurons in the myenteric plexus and the outer, intermediate and inner submucosal ganglia. Positive nerve bundles and fibres in the circular muscle, between the mucosal glands up to the mucosal epithelium. (b) Higher magnification of PGP 9.5-immunoreactive nerve fibres in the mucosal stroma of villi. (c) GFAP-immunoreactive structures in the myenteric plexus (thick arrow) and in the outer, intermediate and inner submucosal ganglia, within the longitudinal and the circular muscle layers (thin arrows). (d) Higher magnification of a mucosal/submucosal fold: GFAP-immunoreactivity is present in the ganglia of the intermediate and inner submucous plexus, but not in the mucosa. A paravascular GFAP-immunoreactive structure is observed in the submucosa (arrow). MP: myenteric plexus; sm: submucosa; cm: circular muscle; m: mucosa; lm: longitudinal muscle; OSP: outer submucous plexus; INSP: intermediate submucous plexus; ISP: inner submucous plexus.

In cryosections, single immunostainings revealed PGP 9.5 immunoreactive nerve structures within the myenteric and the submucous plexus, the latter showing ganglia of different size and extension laying at the outer, intermediate and inner levels. PGP 9.5-immunoreactivity (PGP-IR) was found in nerve fibre bundles in the outer muscle layer, in the muscularis mucosae, between the glandular layer of the mucosa, and extended up to the mucosal epithelium (Fig. 1a–b). As seen for PGP 9.5 immunoreactive nerve structures, GFAP-immunoreactivity (GFAP-IR) occurred in isolated fibre bundles in the longitudinal muscle, numerous within the circular muscle layer, around some sub-mucosal arteries, in the muscularis mucosae, and in few fibre bundles among the deeper mucosal glands, without extending up to the mucosa epithelium. GFAP-IR occurred in the myenteric and submucosal ganglia (Fig. 1c–d), and in single isolated small cell bodies within the circular muscle bundles (data not shown).

Single immunostaining on whole mounts revealed PGP 9.5-immunoreactive nerve cell bodies in the main myenteric ganglia, and immunoreactive nerve fibre bundles in the branches and within the muscular wall (data not shown). GFAP-IR was detected at the periphery of the larger myenteric ganglia or entirely occupying the smallest ganglia (Fig. 2a). Double immunostaining showed GFAP-immunoreactive EGCs and their processes intermingled with the neighbouring, larger PGP 9.5-immunoreactive nerve cell bodies (Fig. 2b and c). GFAP-immunoreactive EGCs were small sized, star-shaped, resembling type I morphology. GFAP-IR also occurred along interganglionic nerve strands (Fig. 2d). In addition, GFAP-IR was detected in intramuscular nerve fibre bundles and in cell bodies provided with two–three cellular processes resembling type III EGC morphology: these cells occurred outside the myenteric ganglia but joined them by their cellular extensions. Finally, GFAP-immunoreactive EGCs occurred within the circular muscle, with a bipolar shape, resembling type IV EGC morphology (Fig. 2e and f).

In the submucous plexus, different sized and shaped PGP 9.5-immunopositive nerve cell bodies localized at the outer, intermediate and inner submucous ganglia as seen in the cryosections. Single PGP-immunoreactive nerve cell bodies were found outside the main ganglia but projecting to them (Fig. 3a). In double labelling with PGP 9.5-IR, GFAP-IR occurred in a large subpopulation of EGCs between the larger PGP 9.5-immunoreactive nerve cell bodies, both in the outer, intermediate and inner submucosal ganglia, or surrounding isolated neurons laying at the base of lymphatic nodules (Fig 3b and c), and in peri- and para-vascular nerve fibres (data not shown). GFAP-immunoreactive EGCs were small sized, star-shaped, resembling type I morphology (Fig. 3c).

4. Discussion

This study describes for the first time the topographical distribution of EGCs in the jejunum of healthy cattle by its main intracellular marker, the intermediate glial fibrillary acidic protein (GFAP). In cryosections, GFAP-immunoreactive enteroglial distribution resembled that shown by the neuronal PGP 9.5 in the main neuronal plexuses and layers of the jejunal wall. GFAP-immunoreactive glial structures were observed at the base of the mucosa, within the glandular layer, without extending up to the mucosal epithelium. These data are consistent with the finding that GFAP-expressing EGCs do not contribute to the integrity of the gut epithelium and are not involved in the regulation of the mucosal permeability, at least in this segment (Yu and Li, 2014).

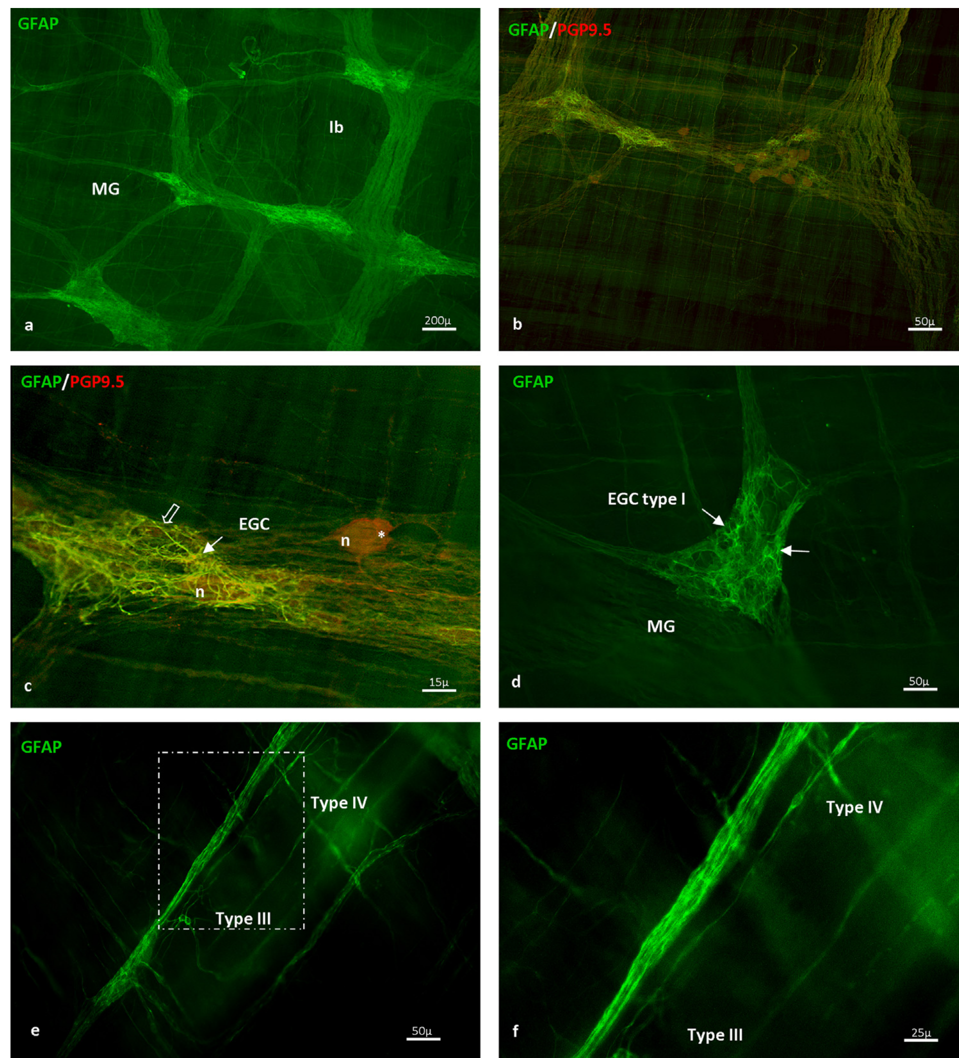

Fig. 2. a–f. Immunolabelling with GFAP and PGP 9.5 antibodies on whole-mounts of the myenteric plexus of the bovine jejunum. (a) Different extension of ganglionic GFAP-immunoreactive EGCs population next the interganglionic strands. (**b**) Merged GFAP/PGP 9.5-immunolabelling in a myenteric ganglion. Several PGP 9.5-immunoreactive nerve cell bodies (Cy3) are surrounded by GFAP-immunoreactive glial processes (FITC). (c) Higher magnification of a merged GFAP/PGP 9.5-immunolabelling in a myenteric ganglion. GFAP-immunoreactive glial cells (arrow) and processes (empty arrow) surround some nerve cell bodies. A neuron (asterisk) not surrounded by GFAP-immunoreactive glial processes is seen. (d) Final image of seven focal optical sections of a myenteric ganglion. Star-shaped EGCs with type I morphology (arrow) are recognizable. (e) Extra-ganglionic, intramuscular, GFAP-immunoreactive EGCs showing type III- and IV-like morphology in single staining. (f) Higher magnification of a final image of four focal optical sections of type III and type IV EGC morphology seen in (e). MG: myenteric ganglion; EGC: enteroglial cell; cm: circular muscle; n: neuron; ib: interganglionic branch.

In whole mounts, GFAP-immunoreactive EGCs surrounded nerve cell bodies in the myenteric and submucosal ganglia, the latter having different size and shape and found at the outer, intermediate and inner depth levels within the submucosa in the jejunm of cattle, a peculiarity that is not found in the same segments of other larger mammals (Timmermans et al., 2001). Consistently with previous findings in other species (Furness, 2006), in cattle, myenteric and submucosal GFAP-expressing EGCs are small cells with a star-like shape and multiple processes. GFAP-immunostaining on fixed intestinal whole mounts is considered a poor technique for a morphological study because contiguous glial cells are stained precluding observations that need single cell resolution (Boesmans et al., 2015). However, in the present study, we were able to observe labelled myenteric and submucosal EGCs showing a cellular shape that resembled type I morphology or protoplasmic EGCs: these cells types have been described in the same ganglia in guinea pig by intracellular filling with horseradish peroxidase and histochemical staining (Hanani and Reichenbach, 1994), or in mouse by genetically encoded fluorescent reporters (Boesmans et al., 2015).

In the myenteric plexus, GFAP-labelled EGCs were intercalated among several, although not all, neurons that were wrapped by the glial processes. They entirely occupied the smaller ganglia, while in the larger ganglia GFAP-immunoreactive EGCs localized at sites where the interconnecting strands arose. This finding suggests that GFAP-labelled EGCs may be involved in the control of neurotransmission of the motor neurons projecting to the muscle wall, i.e., the nitrergic neuromuscular transmission (Aube et al., 2006). It is well known that EGCs store glial L-arginine (Nagahama et al., 2001) as substrate for the synthesis of NO in neurons by NO synthases (nNOS or NOS1), and nitrergic neurons occur in the bovine intestinal wall (Vittoria et al., 2000). Thus, it would be intriguing to further investigate the relationship between the GFAP-expressing EGCs and the NOS-expressing neurons. Type I GFAP-labelled EGCs were also extensively found in the larger outer submucosal ganglia, and less in the intermediate and inner ones. The outer submucosal plexus also contains nitrergic neurons partially colocalized with the vasoactive intestinal peptide (VIP) (Vittoria et al., 2000), probably projecting to the upper circular muscle layer as observed in other

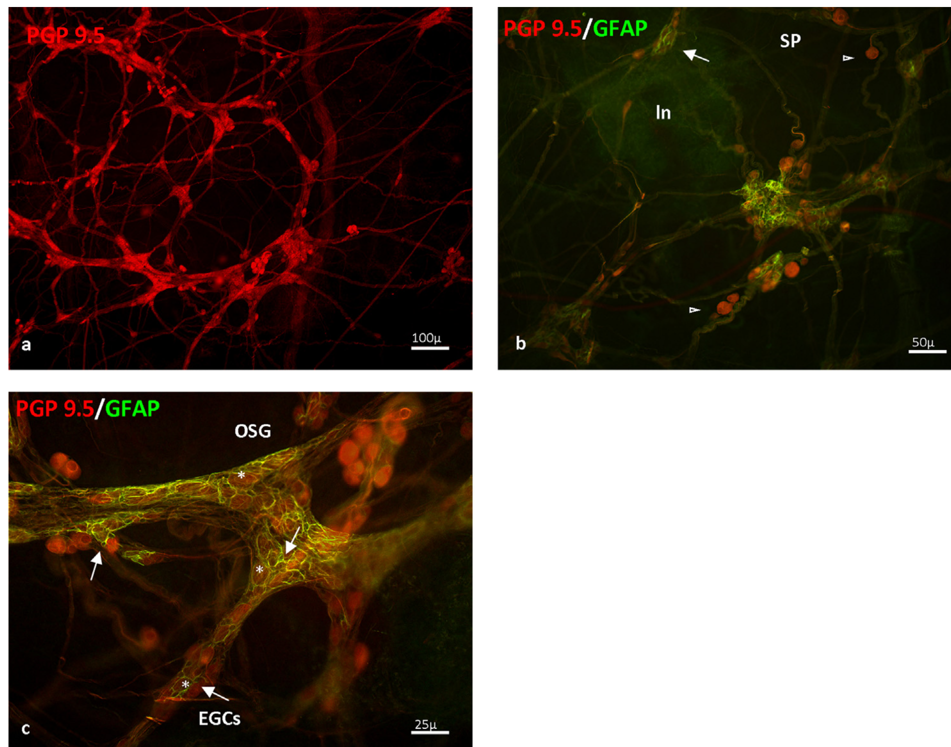

Fig. 3. a–c. Immunolabelling with PGP 9.5 and GFAP antibodies on whole-mounts of the submucous plexus of the bovine jejunum. (a) Single and grouped PGP 9.5 immunoreactive neurons in different sized and shaped submucosal ganglia within the submucosa at different depth. (b) Merged PGP 9.5 and GFAP immunolabelling: GFAP-immunoreactive EGCs mainly lie in the larger submucous ganglia. A small GFAP-labelled ganglion close to a lymphatic nodule (arrow). Single PGP 9.5-immunoreactive nerve cell bodies not surrounded by GFAP-labelled EGCs (arrowhead). (c) Higher magnification of a large submucous ganglion and isolated neurons within the submucosa at different depth. Numerous, small GFAP-labelled EGCs (arrow) surround with their cellular processes the PGP 9.5-immunoreactive nerve cell bodies (asterisk). SP: submucous plexus; In: lymphatic nodule; OSG: outer submucous ganglion; EGCs: enteroglial cells.

large animals such as pig (Timmermans et al., 1994). By contrast, the inner submucosal ganglia receive mainly nitrergic terminals and contains VIP- and substance P-containing neurons (Vittoria et al., 2000).VIP-expressing neurons are moto-secretory in function. Isolated neurons in the intermediate and inner submucous ganglia, as well as in the myenteric plexus, were not surrounded by GFAP-immunoreactive EGCs suggesting the presence of functional EGCs expressing the other markers S100 and/or Sox10 (Neunlist et al., 2014). Further studies in cattle dealing with the (co)-expression of the other glial markers and their relationship with different coded neurons will help in understanding the functional role of EGCs in gut homeostasis and physio-pathology in this species.

Extra-ganglionic GFAP-labelled EGCs, whose cell processes continued with the interconnected branches, were found within the bovine muscle layer too. Due to their shape and localization, these cells resembled type III and IV EGCs described by Boesmans and coworkers (2015) in the mouse. In the mouse, the majority of type-III EGCs did not express GFAP, thus suggesting the occurrence of species differences. Although this study has been carried out on the jejunum, further studies are in progress on the other gut segments. Increased expression of GFAP has been reported in several pathological conditions such as intestinal inflammation, in murine ECGs following ischemia, and in cytokine-treated cultures of rat EGCs (Cornet et al., 2001; von Boyen et al., 2004; Thacker et al., 2011), in the submucous plexus of pigs in presence of probiotics (Di Giancamillo et al., 2010), in sheep following scrapie disease (Marruchella et al., 2007). Although the functional role of the GFAP-containing EGCs in the jejunum of cattle intestine as well as in other gut segments not yet studied is currently unknown, their response to signals from their microenvironment are likely crucial for animal health and disease.

5. Conclusions

The present data show that GFAP-IR EGCs are widely distributed within the wall of the bovine jejunum, except for the mucosal layer. Three different morphological types of EGCs have been identified on the basis of their shape and topographic localization. Further studies are necessary to disclose the role of these specific glial populations in the patho-physiology of the bovine intestine.

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