THE ENTERIC NERVOUS SYSTEM IN THE GOAT: REGIONAL MORPHOLOGICAL DIFFERENCES AND THE ORGANIZATION OF SUBMUCOSAL AND INTRAMUCOSAL PLEXUSES

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SUMMARY

Regional differences, submucosal and intramucosal organization of ganglia in the enteric nervous system (ENS) of large mammals are not yet clear. The ENS of eight adult goats was studied by S-100 protein, neurofilament proteins, and substance P immunohistochemistry. Numerical density was used to establish intramucosal ganglia distribution. The outer submucosal plexus (OSP) and inner submucosal plexus (ISP) were differentiated using submucosal vascular arcade landmark. Primary nerve strands emerged beneath OSP ganglia and the latter were coarse textured and their primary nerve strands displayed a definitive orientation relative to circular muscle. The OSP and ISP were subdivided into the *plexus submucous extremus* and intermediate plexus, respectively based on topography, size, shape, outline and density of ganglia and nerve meshworks, and size of nerve cell bodies. Mucosal plexus contained many ganglia and isolated nerve cell bodies with higher numerical densities in caecum, colon and ileum. Myenteric, OSP, ISP and mucosal plexuses showed a similar pattern of regional differences in the size of ganglia and nerve strands. Larger ganglia and large nerve strands were found in the colon and caecum. They were medium sized in the duodenum, rectum, ileum and distal jejunum and smaller in the proximal and middle jejunum. The present study elaborates on the morphological features to differentiate OSP from ISP and suggests that *plexus submucous extremus* and intermediate plexus exist in intestine of goat. Regional variations of the sizes of ganglia and nerves, stratification of submucosal and intramucosal ganglia suggest a functional significance in regulating muscle, vascular, immune system and mucosal functions.

INTRODUCTION

The gastrointestinal tract (GIT) contains a large number of neurons in the ganglia of its intrinsic neural plexuses that constitute the enteric nervous system (ENS). The ENS, which is comprised of sensory, motor and interneurons is capable of regulating gastrointestinal functions independent of the brain and is regarded as the brain of the gut or "little" brain (Gershon, 1999; Wood, 2000). In all mammals, the ENS is composed of the myenteric plexus located between the circular and longitudinal muscle layers and the submucosal plexus situated in the submucosal layer (Furness and Costa, 1987, Gershon, 1999).

In large mammals however, anatomical and functional studies have revealed two or three plexuses in the submucosal layer (Balemba *et al*., 1998; Timmermans *et al*., 1997, 2001; Hens *et al*., 2001) that have clear differences from small laboratory animal models (Brown and Timmermans, 2005). The existence of two interconnected but, morphologically and functionally distinct submucosal plexuses, namely the outer submucosal plexus (OSP) located near the circular muscle and the inner submucosal plexus (ISP) located near the mucosa, has been confirmed in the intestine of large mammals. Identification of these plexuses is based on their differences in the topography, ultrastructure, size and shape of ganglia and neurons, size of nerve strands, electrophysiological properties, neurochemical coding and projection of neurons (Timmermans *et al*., 1997, 2001; Balemba *et al*., 1998; Hens *et al*., 2001). A third plexus, an intermediate plexus located between the OSP and ISP, which was first reported in the inner submucosal layer of the small intestine of the pig (Gunn, 1968) and the colon of opossum (Christensen and Rick, 1987) has been confirmed to exist throughout the intestine of humans (Dhatt and Butchan, 1994; Timmermans *et al*., 1997, 2001; Hens *et al*., 2001).

In the small intestine of the pig (Scheuermann *et al*., 1987a; Balemba *et al*., 1998) and cattle (Balemba *et al*., 1999) the ISP ganglia and nerve fibre strands are situated at different topographic levels with the smallest ganglia being situated close to *lamina muscularis mucosae* and larger ganglia being located close to the vascular arcades. Due to these observations, the ISP was subdivided into the outer and inner sub-plexuses (Balemba *et al*., 1998, 1999). These findings are in agreement with the existence of an intermediate plexus in larger animals other than man (Gunn, 1968; Christensen and Rick, 1987).

A delicate ganglionated network closely associated with the inner circular muscle at its inner side,

the *plexus submucous (entericus) extremus* observed first by Stach (1972) in the colon of rat and guinea pigs has been recently confirmed in the human colon (Hoyle and Burnstock, 1989a; Wedel *et al*., 1999). It is unclear whether this is a distinct plexus or part of the OSP. Existence of OSP ganglia at different topographical levels has been recorded in the small intestine of the pig and cattle (Balemba *et al*., 1998, 1999) suggesting that the organization of OSP in these animals may be similar to that observed in the colon of rat, guinea pig and human (Stach, 1972; Hoyle and Burnstock, 1989a; Wedel *et al*., 1999). Clearly, based on marked species differences in the ENS of mammals, particularly the intricate organization of ganglionated meshworks in the submucosal layer (Timmermans *et al*., 1997; Brehmer *et al*., 1999), there is a need for more data from large mammals. Furthermore, morphological features for the differentiation of OSP, ISP and intermediate plexuses are not well described. Apparently there is only one report of the ENS in the goat in which silver impregnation and histochemical staining for cholinesterase were used (Gunn, 1968). Various other immunohistochemical methods have been deployed to study the ENS. Use of S-100 protein to delineate superimposed submucosal plexuses in the small intestine of the pig has been reported (Scheuermann *et al*., 1989). Neurofilament protein (NF) has also proved to be useful to visualise the structure of the ENS in the omasum of sheep (Yamamoto *et al*., 1994) and small intestine of cattle (Balemba *et al*., 1999). To study the submucosal plexuses, substance P (SP) has been successfully used (Timmermans *et al*., 1990). The SP has also been used in the study of ganglia and isolated neurons in the mucosal plexus in the intestine of pig (Balemba *et al*., 2002a).

In the present study, S-100 protein, NF and SP immunohistochemistry were used to study the differences between OSP and ISP, the extent of distribution of intramucosal ganglia and to clarify regional and inter-plexus variations of the ENS plexuses in the intestine of goat.

MATERIALS AND METHODS

Eight male, Tanzanian local goats aged 1½- 2 ½ years and weighing 8-12 kg were anaesthetised using Ketamine ® at a dose rate of 20 mg/Kg followed by exsanguination. The stomach and intestine were then exposed and two ligatures, one at the pylorus and another at the distal part of rectum were applied. The entire intestinal tract was removed from the carcass and in order to prevent drying, it was put into a 5-litre container with cold (about 4^{o}C) 0.01M PBS, pH 7.3 while the sampling was going on. Within 20 minutes after the removal of the

intestinal tract from the carcass, tissue samples (~ 3 - 4 cm long) were collected from the duodenum, proximal, middle and distal parts of the jejunum, ileum, caecum, ascending colon and rectum. Samples were cut open along the mesenteric attachment and washed thoroughly using cold (4°C) 0.01M PBS, pH 7.3. Each sample was cut into two pieces and each piece was pinned mucosal surface upwards while being maximally stretched on polystyrene plastic. One piece was immersion-fixed in 4.5% buffered formaldehyde while the other piece was fixed in 4.5% buffered formaldehyde containing15% saturated picric acid. The tissues were removed from the polystyrene after 1 hr and thereafter fixation continued by immersing the tissues in fresh fixatives for 47 hr at 4°C. They were then washed with PBS and dissected as described by Balemba *et al*. (1998).

Freshly dissected whole mounts were stored in 2 ml of 0.01M PBS, pH 7.3 containing 0.5% triton X-100 and 0.1% sodium azide in plastic tubes at 4°C. They were washed and thereafter stained for S-100 protein, NF, SP and Vasoactive Intestinal Polypeptide (VIP) immunoreactivities (IR) by the two steps indirect streptavidin-ABComplex/HRP method as described by (Guesdon*, et al*. 1969). Rabbit anti-cow S-100 protein (Z 0311; 1:400) was kindly donated by Dako A/S, Glostrup, Denmark. Mouse anti-human neurofilament protein (Cat. no. 168: 1:40) was obtained from Immunotech, France. Rabbit anti-swine SP (SP 250- 2; 1:2000) and rabbit anti swine-VIP (VIP, 8084-4; 1:1400) were kindly donated by Dr. P.J. Larsen and Dr. J. Fahrenkrug of University of Copenhagen and Bispebjerg Hospital, Copenhagen, Denmark, respectively. The specificity of immunostaining, dehydration and tissue mounting was done as described earlier (Balemba *et al*., 1998).

Mucosal whole mounts were mounted serosal surface upwards and evaluated by light microscopy using an Olympus BH-2 microscope, and some of them were photographed. Mucosal wholemounts stained for S-100 protein IR were then embedded in paraffin, serosal surface downwards. Each paraffin block was cut into 10 serial sections of either 10 or 4 μm thick sections that were counterstained with Haematoxylin for 30 seconds, dehydrated in ethanol, cleared in xylene and mounted in DPX. During microscopic evaluation that was done using an Olympus BH-2 microscope, the sections were compared with pictures taken from wholemounts before embedding to confirm the topographical location, architectural and structural features of intramucosal ganglia seen in wholemounts. *Lamina muscularis mucosae* was used as a landmark to demarcate mucosal plexus from ISP. Tissues stained

for S-100 protein IR (n = 4) were used to estimate sizes of ganglia, primary and secondary nerve strands and IR nerve cell bodies. The average size of ganglia was estimated using a calibrated eyepiece and the total number of neurons determined using the criteria set by Young *et al*. (1993). Nerve strands were classified based on the interconnections between ganglia and other nerve strands, size, shape and tortuousness as described by Scheuermann *et al*. (1986, 1987 a, b). Ganglia sizes were estimated using 10x and 20x objectives while nerve strands and nerve cell bodies were estimated at 40x objectives. In each plexus ganglia were classified into large, medium and small ganglia during estimation of their lengths and widths. In a whole mount from a given segment, five replications were considered enough for each category of the structures measured. The means of each measured structure per animal and for the 8 animals were then statistically evaluated using Statistical Analysis Systems (edition 6, 1987) and statistical significance was assumed at $p<0.05$.

Numerical densities of intramucosal ganglia were determined by using mucosal wholemounts (n = 5 goats) that were stained for S-100 protein IR. A set of field of vision were examined under projection onto a table using a Leica DMLB microscope fitted with a projection arm at 20x objective (333x total magnification). The counting was done using the unbiased counting frame (Gundersen, 1978). With a random start, each field of vision was systematically randomly sampled using a predetermined fraction of an 18 cm^2 sampling frame. The selected fraction of the frame was used as an unbiased sampling frame. Ganglia completely inside and those only intersecting the "inclusion" edges were sampled, provided they in no way intersected the "exclusion" edges. Numerical densities were calculated based on the sampling fractions. Results were analysed for variance using Graphpad Prizm 4 (Hollogram, Aurora, CO, USA) and significance was assumed at $P<0.05$.

RESULTS

Immunoreactivity in ganglia and nerve strands

All the ganglia and nerve strands in the myenteric, OSP, ISP and mucosal plexuses were revealed by S-100 protein- and NF-immunohistochemistry. SP- and VIP-IR displayed the differences between these plexuses (Figures 1 a-f; 2 a-g; 3 a-f; 4 a-l). In the preparations labelled with S-100 and NF, the OSP ganglia and primary nerve strands were larger, coarse textured and visible by naked eyes. Primary nerve strands of the OSP emerged beneath the body of the ganglia and were oriented perpendicular to the inner circular muscle layer

(ICML) while ganglia and tertiary nerve strands were oriented parallel to the ICML. The study also revealed that in the OSP and ISP, meshworks (or networks) of larger ganglia were located close to the submucosal vascular arcade (SVA). Many intramucosal ganglia were observed in the intestine, and were more prominent in the large intestine. The size and structure of the ganglia and nerve strands, and size and amount of nerve cell bodies varied greatly between the regions of the intestine (Figure 5 a-d). Generally, larger ganglia and nerve strands as well as dense nerve meshworks were observed in the colon and caecum followed by rectum, duodenum and ileum, while small ganglia with wider meshwork were displayed by the jejunum.

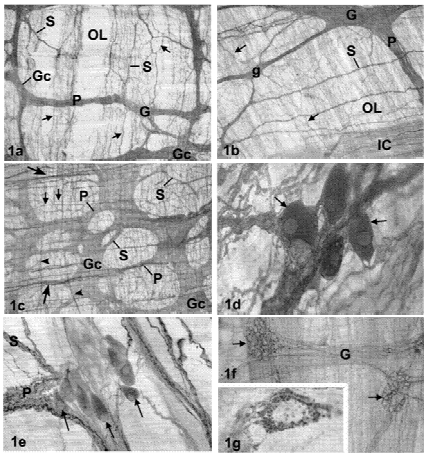


Figure 1 ***a-g.*** S-100 protein-, NF- and SP- IR in the myenteric plexus. **(a)** S-100 protein IR, distal jejunum showing large (Gc) and medium (G) ganglia, primary strand (P), secondary strands (S), and tertiary nerve strands (arrows). The outer longitudinal muscle layer (OL) is seen in the background. 42x. **(b)** S-100 protein- IR, caecum showing large (G) and small (g) ganglia, primary (P), secondary (S) and tertiary nerve strands (arrows) and outer longitudinal muscle layer (OL). Secondary strands are oriented parallel to ICM (IC). 42x. **(c)** S-100 protein- IR, colon showing large (Gc) ganglia, primary (P), secondary (S) and nerve strands coursing parallel (small arrows) and perpendicular (arrow heads) to ICM. 42x. (Compare the blending and sizes of ganglia and nerve strands in Figure 1 a-c. **(d)** Ileum, S-100 protein- IR in a small ganglion. The IR nerve cell bodies (arrows) have smooth outline, are principally adendritic, pseudo-uniaxonal to multiaxonal. 413x. **(e)**. Caecum, NF- IR. Note adendritic, pseudo-uniaxonal to multiaxonal IR nerve cell bodies (arrows) in a ganglion, primary (P) and secondary (S) nerve strands. 250x. Figure 1 f-g. SP- IR in the rectum. **(f)** Shows clusters of intense, pericellular SP- IR varicosities around non reactive nerve cell bodies (arrows) in a ganglion (G). 103x. **(g)** A close up of pericellular SP-IR varicosities around a non reactive nerve cell body. 413x.

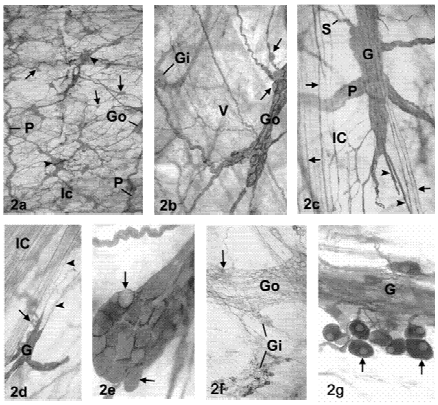


Figure 2*a-g*. The S-100 protein-, NF- and SP- IR in the OSP. **(a)** Duodenum, S-100 protein- IR viewed from the serosal side. The OSP shown by two primary strands (P) that are oriented perpendicular to the ICM revealed by remnant nerves (IC) and a part of the ganglion (Go) is positioned above ISP (arrow heads and arrows) meshwork. Larger outermost ISP ganglia (arrow heads) are located in meshwork situated closer to the OSP. Smaller ISP ganglia (arrows) are located closer to *lamina muscularis mucosae* (not shown). 42x. **(b)** S100 protein- IR; middle jejunum. Demonstration of OSP (Go) and ISP (Gi) (not in focus) ganglia topographical differences and demarcation by the SVA (V). S-100 protein- IR nerve cell bodies are shown by arrows. 103x. **(c)** S100 protein- IR in the proximal jejunum showing a large OSP ganglion viewed from serosal side. The primary nerve strands (P) emerging beneath the body of the ganglion (G) are oriented perpendicular to ICM, which is shown by remnant nerves (arrows). The ganglion and tertiary nerve strands (arrow heads) are oriented almost parallel to ICM. 103x. **(d)** S-100 protein- IR in the duodenum showing a small ganglion (G) in the OSP meshwork equivalent to the *plexus submucous extremus* located very close to the ICM (IC). Tertiary nerve strands (arrow heads) course into the ICM. A secondary nerve strand in the OSP meshwork below the ganglion is shown by arrow. 103x. **(e)** S-100 protein- IR, middle jejunum, a close up of a large ganglion in the OSP showing ovoid, smooth outlined, adendritic, type II nerve cell bodies (arrows). 400x. **(f)** SP- IR in the OSP and ISP ganglia in caecum. The OSP ganglion (Go) has abundant IR varicosities but, few IR nerve cell bodies (arrow). The ISP ganglion (Gi) has many SP-IR neurons. 103x. **(g)**. NF- IR, colon, in the OSP ganglion (G) showing many, intense, elongated, smooth outlined adendritic, pseudo-uniaxonal to multiaxonal type II IR nerve cell bodies (arrows). 400x. Compare sizes of the OSP ganglia in Figure 2 b-d; f.

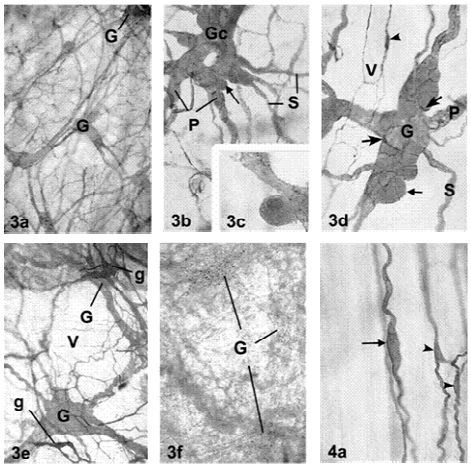
Immunoreactivity in nerve cell bodies

In all plexuses, S-100 protein-, SP- and NF- IR nerve cell bodies differed in size and shape. The majority of S-100 protein-, and NF- IR nerve cell bodies were ovoid or elongated, had smooth outlines and peripherally located nuclei. They were adendritic, pseudo- or uniaxonal or multiaxonal hence considered to be Dogiel type II neurons (Figures 1 d-e; 2 e, g; 3 b; 4 a, l). S-100 protein- IR was seen in Schwann cells and enteric glial cells that were compressed between nerve cell bodies. Schwann cells were small spindle shaped with elongated, cork screw-like or indented nuclei with very little cytoplasm (Figures 3d; 4a, i). Nerves and Schwann cells in the perivascular plexus were distinctly stained with S-100 protein- IR enabling the tracing of the outline of the vascular arcades in all layers (Figures 2 b; 3 d), whereas NF-IR showed mainly innervation of the SVA. Nerve cells that were positive for SP- IR were ovoid or rounded and their neuronal processes were hardly revealed. Dense pericellular SP- and VIP- IR varicosities were commonly seen around non-reactive nerve cell bodies (Figures 1 f-g; 3 f). S-100 protein- IR was observed in the cytoplasm and nuclei of nerve cell bodies.

There were many S-100 protein-IR nerve cell bodies in the OSP and ISP, few in MP and the mucosal plexus. There were many NF-IR nerve cell bodies in the mucosal plexus, OSP and ISP and few in the mucosal plexus. SP- IR nerve cell bodies were abundant in the ISP, few in the OSP and the mucosal plexus, and very few in the myenteric plexus. VIP- IR nerve cell bodies were only occasionally seen in the mucosa and ISP.

The myenteric plexus

Results from the present study showed that the myenteric ganglia in the cecum and colon were longer compared to those in the rectum and small intestine (Figures 1 a-c, 5 a-c). Ganglia, primary and secondary nerve strands in the colon, caecum, duodenum and rectum were wider than those in the jejunum and ileum. The occurrence of smaller ganglia between the main meshwork of larger ganglia was a typical observation in the colon followed by duodenum and ileum. The meshwork formed by ganglia and primary nerve strands was wider in the rectum, less so in the caecum, ileum and distal jejunum, and narrow in the duodenum, proximal and middle jejunum and narrower in the colon. Larger S-100 protein- IR neurons were commonly seen in the large intestine and duodenum. In the small intestine, the majority of S-100 protein- and NF- IR were seen Dogiel type II neurons and were oriented parallel to the ICM cells.



Figures 3 a-f; 4 a. S-100 protein-, NF-, SP- and VIP- IR in the ISP. **(a)** S-100 protein- IR, proximal jejunum. Note the density of the meshwork of ganglia (G) and nerve strands, variation in the size and shape of ganglia, and lack of specified orientation. 103x. **(b)** S-100 protein IR, duodenum, showing a large ganglion (Gc), primary (P) and secondary (S) strands, and IR nerve cell body (arrow). 206x. **(c)** Close up of S-100 protein- IR neuron in Figure3b showing intranuclear IR. 413x. **(d)** S-100 protein- IR, middle jejunum viewed from the serosal side, SVA (V) overly the ISP ganglia. Note the IR nerve cell bodies (small arrow) and enteric glia (large arrows), secondary strans (P) and a Schwann cell (arrow head). 413x. **(e)** S-100 protein- IR, rectum with the ISP viewed from the luminal side. Two small ISP ganglia (g) in the inner ISP meshwork are overlying two large ganglia (G) in outer ISP meshwork, which is equivalent to the intermediate plexus described in other mammals. V, submucosal vascular arcade. 206x. **(f)** VIP- IR , caecum. Note absence of the IR nerve cell bodies in the ISP ganglia (G). 100x. **(4a)** S-100 protein- IR in the caecum showing an isolated nerve cell body (arrow) in the *lamina muscularis mucosae* and Schwann cells (arrow heads). 403x.

The outer submucosal plexus (OSP)

The OSP was situated between the inner side of the ICM and the SVA. Ganglia and nerve strands in the OSP varied in size and were situated at different topographical levels (Figure 2 a-e). Small, broad-meshed, compact ganglia containing small nerve cell bodies and isolated nerve cell bodies were seen immediately beneath the ICML. This meshwork, which was considered to be the *plexus submucous (entericus) extremus* was conspicuous in the caecum, colon and duodenum (Figure 2 d). Larger, coarse ganglia containing loosely arranged nerve cell bodies and few small ganglia in the meshwork were situated closer to the SVA. Large primary nerve strands formed the major axis, which was oriented perpendicular to the ICML. These strands emerged beneath the body of larger ganglia (see Figure 2 a, c). A majority of the large ganglia, S-

100 protein- and NF- IR nerve cell bodies were elongated and oriented parallel to the ICML. The orientation of the major axis was most pronounced in the duodenum and jejunum. In tissues stained for S-100 protein- and NF-IR, the meshwork of larger ganglia and larger primary strands were visible by unaided eyes. The majority of tertiary nerve strands were long, coursed immediately under and parallel to ICML and gave fine branches that linked the OSP to the muscular plexus in the ICML (see Figure 2 c-d).

The ganglia, primary and secondary nerve strands in the caecum, colon and rectum were larger than in the rest of the intestine (Figure 5 b). Larger ganglia and S-100 protein IR nerve cell bodies were most common in caecum and colon while the smaller ganglia and nerve cell bodies were more common in the duodenum.

The inner submucosal plexus (ISP)

The ISP was situated between the SVA and *lamina muscularis mucosae* (Figures 2 b; 3 d). Like in the OSP, the ISP, ganglia and nerve strands varied in size, shape and outlines, and were situated at different topographical levels (Figure 3 a, e). Large ganglia, slightly coarser in outline and less compact, formed a broader mesh, which was located close to the SVA. These ganglia containing larger nerve cell bodies, gave rise to tortuous nerve fibre tracts, and secondary nerve strands interconnecting ISP to the OSP. This meshwork was more prominent in the large intestine (Figure 3 a, e). Smaller, rounded, ovoid or polygonal, narrow meshed, smoothly outlined, compact ganglia with relatively smaller nerve strands and nerve cell bodies were situated closer to the *lamina muscularis mucosae* than to the SVA (Figure 3 a, e). The two ISP neural meshworks were interconnected by nerve strands.

The ganglia in the caecum and colon were longer and broader than those in other parts of the intestine (Figure 5 c). The width of ganglia and nerve strands of other regions did not differ. Larger ganglia were most common in the caecum, colon and duodenum, and few in the jejunum, ileum and rectum. The meshwork of ganglia and primary strands was dense in the colon and small intestine and broad in the caecum and rectum. The largest S-100 protein- IR nerve cell bodies were commonly seen in the caecum while the smallest nerve cell bodies were mostly seen in the duodenum.

In the Peyer's patches, the OSP and ISP ganglia underlying the base of the follicles were still separated by the SVA. The ISP formed three meshworks (subplexuses) around the follicles namely: 1, subfollicular ISP that was situated beneath the base of the follicles; 2, interfollicular ISP that was located in the

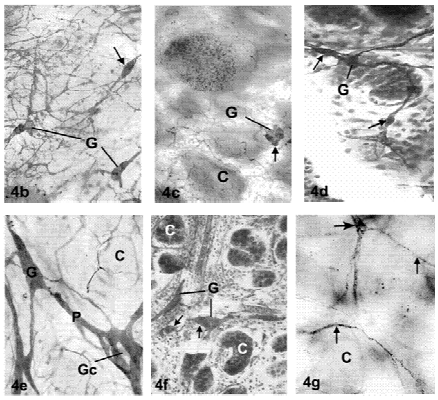


Figure 4 b-*g*. S-100 protein-, NF-, SP- and VIP- IR in the mucosal plexus in wholemounts and sections. **(b)** Middle jejunum, S-100 protein- IR in the ganglia (G) and an isolated nerve cell body (arrow) in the outer proprial subplexus in the subglandular region. 206x. **(c)** S-100 protein- IR in a ganglion (G) and IR nerve cell body (arrow) in the outer proprial subplexus of ileum. An intestinal crypt is shown at C. x 206. **(d)** Paraffin section of a wholemount of the ileum in 4c counter stained by H & E showing a ganglion (G) and isolated nerve cell bodies (arrows) in the interglandular proprial subplexus. x 413. **(e)** S-100 protein IR in caecum. Note the density of intramucosal ganglia (G) meshwork, and primary stands (P) in the outer proprial subplexus. Intestinal crypts (C) are lightly stained. 206x. **(f)** Paraffin section from a wholemount in 4 e and counter stained by H & E showing two small ganglia (G) and IR nerve cell bodies (arrows) in interglandular proprial subplexus. Intestinal crypts are shown at C. 403x. **(g)** VIP-IR, caecum showing nerve strands (small arrows) and one isolated IR nerve cell body (large arrow) in a node in the outer proprial subplexus. (C) show intestinal crypts. 200x.

interfollicular areas and 3, the coronary ISP that was located in the corona. Parts of Peyer's patches follicles that extended into the mucosa were surrounded by nerves that originated from the mucosal plexus. The follicle, corona and dome were all innervated by respective sub-plexuses with dense innervation in the corona, and the tip of the dome and associated epithelium. The S-100 protein revealed dense innervation of the vasculature supplying or draining the follicles.

The mucosal plexus

The mucosal plexus was composed of many intramucosal ganglia and isolated neurons situated at different topographical levels (Figure 4 a-l). There were sparse ganglia and isolated neurons in the *lamina muscularis mucosae* subplexus (Figure 4 a), many in the outer proprial subplexus (OPP, Figure 4 b-c, e, g-i) and few in the interglandular

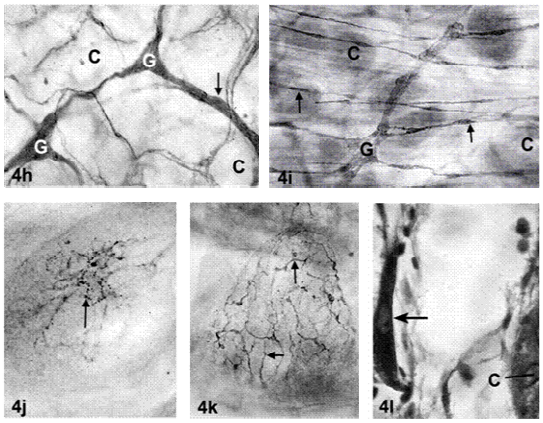


Figure 4 h-k. S-100 protein-, NF-, SP- and VIP- IR in the mucosal plexus in wholemounts and sections. **(h)** S100 protein- IR in the colon showing IR in the ganglia (G) and an isolated nerve cell body (arrow) in the outer proprial subplexus. Intestinal crypts (C) are lightly stained. x 206. **(i)** S-100 protein- IR in the rectum showing a ganglion (G) in the outer proprial subplexus underlying *lamina muscularis mucosae* which is revealed by nerve fibres (arrows). (C) shows crypts. x 206. **(j)** SP- IR, distal jejunum showing dense IR varicosities (arrow) in the dome of a lymphoid follicle. x 206. **(k)** Distal jejunum, showing S-100 protein IR in the villous plexus (arrows). x 206. **(l)** S-100 protein-IR, duodenum. A paraffin section cut from a wholemount showing an isolated neuron (arrow) in the interglandular proprial subplexus. C, shows part of an intestinal crypt. x 1030.

proprial subplexus (IGPP) (Figure 4 d, f, l) close to the base of the crypts and very few high up close to luminal side. Nerve cell bodies that were positive to with S-100 protein- and SP- IR were seen in ganglia and as isolated nerve cell bodies in these subplexuses. A few NF-IR nerve cell bodies were seen in the ganglia and large nerve strands in the OPP and IGPP. Intense SP- IR varicosities were seen in the dense nerve fibre network of the Peyer's patches dome and in the very sparse nerve fibres of the lymphoid follicles (Figure 4 j).

The highest numerical density of intramucosal ganglia and highest number of nerve cell bodies were encountered in the caecum, followed by ileum, colon, distal jejunum, rectum, duodenum, middle jejunum and the proximal jejunum in that order (Figure 5 d). The largest ganglia and primary strands were observed in the caecum followed by colon, rectum, ileum and duodenum in that order. The meshwork of mucosal plexus was narrower in the small intestine and colon, broad in the caecum and rectum where it followed the "honey-comb" organisational pattern of mucosal

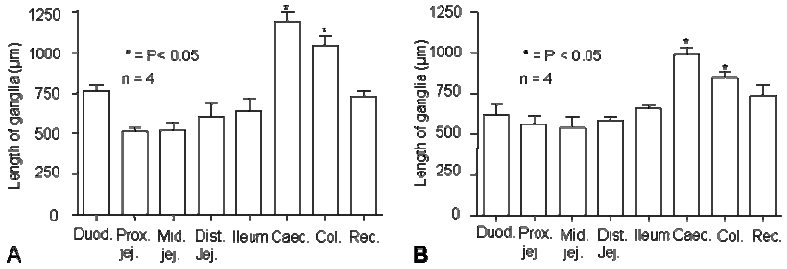


Figure 5 A-D. Summary data of regional differences in the lengths of ganglia and numerical density of intramucosal ganglia. A-C. Lengths of ganglia in the myenteric plexus **(A)**, OSP **(B)** and ISP **(C)** of different regions of the goat intestine demonstrating that cecum and colon have longer ganglia followed by rectum, duodenum and ileum. A similar trend was observed for ganglia widths and sizes of neurons (not shown). **(D)** Numerical density of intramucosal ganglia in the intestine of goat. Cecum, ileum and colon contained higher density of intramucosal ganglia compared to other regions of the intestine (* = P<0.05). The differences between cecum, ileum and colon were also significant.

vascular arcades around the crypts. In the large intestine, the ganglia were mainly elongated with the major axis being clearly oriented parallel to mucosal vascular arcades. The majority of ganglia in the small intestine were small, round or ovoid and sometimes elongated. Large nerve cell bodies were mostly seen in the large intestine whereas the smallest nerve cell bodies were frequently seen in the duodenum.

DISCUSSION

The overall organisation and morphological features of the myenteric plexus, OSP, ISP and the mucosal plexus in the intestine of the goat was in many ways similar to previous observations in the pig, sheep, horse, goat and man (Gunn, 1968; Stach, 1977a,b; Mannl *et al*., 1984; Scheuermann *et al*., 1986, 1987*a, b;* Gabella, 1987; Hoyle and Burnstock, 1989a, b; Timmermans *et al*., 1990,1997, 2001; Krammer and Kühnel, 1992; Brehmer *et al*., 1994, 1999; Pearson, 1994; Balemba *et al*., 1998, 1999, 2002a). The present study has however, revealed that the OSP and ISP are morphologically different and that within the OSP ganglia, large primary nerve strands emerge beneath the main body of the larger ganglia. Furthermore, the ISP presented two types of ganglia and nerve meshwork. This observation is an agreement to other reports and supports a proposition of subdividing the ISP of large mammals (Scheuermann *et al*., 1987*a*; Balemba *et al*., 1998, 1999). The ISP component located closer to SVA, and which is more

prominent in the large intestine is equivalent to the intermediate plexus in humans (Dhatt and Buchan, 1994; Crowe *et al*., 1992).

The sizes of enteric ganglia and nerve strands as well as density of meshworks differ between regions. The variation pattern appear to be conserved across all plexuses with larger ganglia, nerve strands as well as S-100 protein- IR nerve cell bodies being found in the caecum and colon, medium sized in the duodenum, rectum, ileum and distal jejunum and smaller in the proximal and middle jejunum. The variations in the distributions of the NF-, S-100 protein- and SP- IR nerve cell bodies in the myenteric plexus, OSP, ISP and the mucosal plexus with SP- IR being seen more frequently in the mucosal plexus than in the myenteric plexus is new information for the ENS of goat. S-100 protein IR has also been reported in the enteric neurons of the myenteric and submucosal plexuses in the intestine of cow and goat (Albuerne *et al*., 1998).

Regional variations in the size of ganglia has been correlated with the regional variations of nerve cell bodies per unit of ganglionic area in the myenteric plexus of the colon and small intestine of the guinea pigs (Karaosmanoglu *et al*., 1996). Ganglia are not regarded as distinct anatomical entities (Gabella, 1987), but estimation of their relative sizes could still be useful in comparative morphology of different plexuses and relative distribution of neurons (Karaosmanoglu *et al*., 1996) as well as to functional studies of the enteric nervous system. The observed differences may be used to explain differences in the total number and numerical densities of neurons as well as functional differences between intestinal compartments and their ability to react and adapt differently during pathophysiological conditions. The reasons for the observed pattern of variation, and the close similarity between rectum, duodenum and ileum are not apparent. The larger meshwork of all plexuses in the rectum compared to the colon and caecum support the findings in human rectal biopsies and the concept that knowledge of regional differences is crucial in histopathologic diagnosis of ganglia and neuronal pathology (Tafazzoli *et al*., 2005).

It was clearly demonstrated in the present study that a large proportion of S-100 protein- and NF- IR nerve cell bodies in the myenteric plexus and OSP spread in the direction of the ICML. This observation supports the finding of Gabella (1987) and is suggestive of a tendency for polarisation of nerve cell bodies that differs among subsets of neuronal phenotypes. Thus the lesser degree of polarisation of ganglia and nerve strands recorded in the caecum and colon

show segmental differences.

The size, coarseness, orientation of ganglia and nerve strands, and arrangement of neurons in the ganglia of the OSP support the concept that the OSP is more similar to myenteric plexus than to the ISP (Gunn, 1968; Timmermans *et al*., 2001). Small ganglia were observed in the meshwork located close to ICML with nerve network coursing parallel to the ICML. This meshwork of smaller OSP ganglia located adjacent to the circular muscle layer constitutes the plexus submucous (entericus) extremus described in other animals (Stach, 1972; Hoyle and Burnstock, 1989a; Wedel *et al*., 1999). Such a plexus has also been reported in the colons of the rat and guinea pigs (Stach, 1972) as well as in the human (Hoyle and Burnstock 1989a; Wedel *et al*., 1999) and it is, therefore, evident that the goat also has this plexus, and probably other large mammals as well. This also tallies with earlier reports of the existence of OSP ganglia at different topographical levels in small intestine of the pig and cattle (Balemba *et al*., 1998, 1999).

Tertiary nerve strands were seen linking the OSP to the intramuscular plexus. This is a morphological evidence for the involvement of the OSP (and probably the ISP as well) in regulating the gut muscle function (Timmermans *et al*., 2001; Hens *et al*., 2002).

The organization of the ISP in the intestine of the goat is similar to that in the small intestine of the pig (Scheuermann *et al*., 1987a; Balemba *et al*., 1998) and cattle (Balemba *et al*., 1999) and suggests presence of an intermediate plexus in larger mammals as it is in man (Gun, 1968; Christensen and Rick, 1987; Dhatt and Buchan, 1994; Timmermans *et al*., 2001). However, the anatomical stratifications of the submucosal and intramucosal ganglia, and the regional differences shown by this study should not be considered of minor physiological significance. Studies on neurochemical coding and projections of neurons (Porter *et al*., 1999; Hens *et al*, 2000, 2001) as well as on functions are required to elucidate on the functional significance of the subdivisions in OSP and ISP of large mammals.

Within the Peyer's patches, the organisations of the OSP, ISP and mucosal plexuses are similar to those of the pig (Balemba *et al*., 1998; Kulkarni-Narla *et al*., 1999). Substance P immunoreactivity in lymphoid follicles has also been reported by other workers (Kulkarni-Narla *et al*., 1999) and this provides anatomical evidence for modulation of immune responses by neuronal SP as suggested by Stanisz *et al*. (1987).

The present study demonstrated

presence of intramucosal ganglia and isolated nerve cell bodies within the mucosa of the intestine. This is consistent with earlier reports in colon of the rats (Mestres *et al*., 1992a, b), the small intestine and colon of humans (Fang *et al*., 1993; Wedel *et al*., 1999) as well as in the intestine of the pig (Balemba *et al*., 2001, 2002a, b). There was a clear regional difference in the number and size of the intramucosal ganglia and nerves that could explain some mucosal functional differences between the various intestinal segments. These observations support the occasional finding of nerve cell bodies in the lamina propria in the appendix of normal humans and the possibility of their involvement in the pathogenesis of neurogenic appendicopathy (Papadaki *et al*., 1983; Naik *et al*., 1999). However, contrary to an earlier report in the pig (Balemba *et al*., 2002a), there were many intramucosal ganglia in the ileum compared to the colon.

Similar to reports in the rat (Gabella, 1971), the largest neurons in the myenteric plexus were encountered in the caecum of the goats of this study. The smallest S-100 protein- IR nerve cell bodies in all plexuses were however, observed in the duodenum while such small cells were seen in the duodenum and ileum of humans. The reason for this variation is not known. Variation in the size of ganglia and S-100 protein- and NF-IR nerve cell bodies has also been reported in mouse, guinea pig and sheep (Gabella, 1987).

The failure of VIP- IR to reveal IR nerve cell bodies is probably because the VIP antibody did not cross-react fully with VIP antigens or required colchicine treatment (Costa, *et al*., 1985).

In conclusion, the present study in the intestine of goat revealed that the size and orientation of ganglia and major nerve stands, and the position of nerve strands relative to the body of the ganglia are useful morphological features to differentiate the OSP from ISP. In both cases, the OSP and ISP larger ganglia are located near SVA whereas smaller ganglia are located on outer side of each plexus. The outermost component of the OSP meshwork constitutes the *plexus submucous extremus* whereas the ISP meshwork situated close to SVA is the intermediate plexus. The stratification of the submucosal plexuses and regional differences suggest functional grouping of ENS neurons with respect to the regulation of circular muscle, vascular, immune system and epithelial functions by these plexuses that need to be evaluated. The mucosal plexus contains many ganglia and isolated nerve cell bodies suggesting that it should also be considered as a ganglionated plexus. Regional differences revealed in the ENS of goat indicate that larger ganglia and

nerve strands occur in the caecum and colon, they are medium sized in the duodenum, rectum, ileum and distal jejunum and smaller in the middle and proximal jejunum. These variations suggest a similar trend in the population of neurons per unit length and are factors that need to be considered when evaluating the pathology of the ENS and age related degenerative changes.

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