

Cholinergic, nitrergic and peptidergic (Substance P- and CGRP-utilizing) innervation of the horse intestine. A histochemical and immunohistochemical study

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Summary. The small and large intestine of adult horses were histochemically and immunohistochemically investigated in order to evidence components of the intramural nervous system. The general structural organization of the intramural nervous system was examined by using Nissl-thionin staining as well as the anti-neurofilament 200 (NF200) immunoreaction, which demonstrated the presence of neurons in the submucous as well as myenteric plexuses. The additional presence of subserosal ganglia was shown in the large intestine. Acetylcholinesterase (AChE) activity was observed in both the submucous and myenteric plexuses. Localization of acetylcholine-utilizing neurons was also evidenced by immunohistochemical reactions for choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VACHT). With both histochemistry and immunohistochemistry possible cholinergic nerve fibres were detected in the inner musculature. The two possible cholinergic co-mediators Calcitonin Gene-Related Peptide (CGRP) and Substance P (SP) have been investigated by an immunohistochemical approach. CGRP immunoreactivity was detected in roundish nerve cell bodies as well as in nerve fibres of the submucous plexus, whereas SP immunoreactivity was evidenced in nerve fibres of the tunica mucosa, in nerve cell bodies and fibres of the submucous plexus and in nerve fibres of the myenteric plexus. NADPH-diaphorase reactivity, which is linked to the synthesis and release of nitric oxide, was detected in nerve cell bodies and nerve fibres of both the submucous and myenteric plexuses as well as in a subserosal localization of the large intestine. The nitrergic components were confirmed by the anti-NOS (nitric oxide synthase) immunoreaction. Results are compared with those of other mammals and related to the complex

intestinal horse physiology and pathophysiology.

Key words: Horse, Gut, Acetylcholine, Substance P, CGRP, Nitric oxide

Introduction

Gastrointestinal diseases are frequent in equine species. A dramatic symptom of them is acute abdominal pain (Navarre and Roussel, 1996; Cottrell et al., 1999). The syndromes are often ascribable to motility disturbances, among which diskinctic/ hyperkinetic phenomena are present, with a possible neuro-vegetative origin. Unbalance between the vagal and the sympathetic branches of gut innervation might be at the basis of pathological situations. The extrinsic and intrinsic components of the enteric nervous system appear to be the primary mechanisms involved in motility regulation. It is well known that besides the nor-adrenergic and cholinergic regulations, serotonergic and peptidergic ones are present, too (Furness et al., 1995), some of them having a facilitatory action, others having inhibitory effects upon classical neurotransmitters (Lundberg and Hökfelt, 1983; Surprenant, 1994; Lundberg, 1996; Maggi et al., 1996). Substance P neuropeptide (SP) is a neurokinin known to stimulate smooth muscle contraction and to act as a sensory mediator in pain transmission, frequently present with acetylcholine in components of gut intramural nervous structures (Hens et al., 2000; Brookes, 2001; Timmermans et al., 2001). CGRP is also considered a cholinergic co-mediator (Ren

Abbreviations: AChE: Acetylcholinesterase; C1: right ventral colon; C2: left ventral colon; C3: left dorsal colon; C4: right dorsal colon; CGRP: Calcitonin Gene-Related Peptide; ChAT: choline acetyltransferase; ENS: enteric nervous system; ir: immunoreactive; NADPH-d: nicotinamide adenine dinucleotide phosphate-diaphorase; NANC: non-adrenergic, non-cholinergic; NF200: neurofilament 200; NO/NOS: nitric oxide/nitric oxide synthase; PBS: phosphate-buffered saline; SP: Substance P; VACHT: vesicular acetylcholine transporter

et al., 1992), involved either in sensorial or motor activities (Timmermans et al., 1992a; Jakab et al., 1993). CGRP also shares vasoactive (dilatatory) effects with SP. There are many overlappings of SP and CGRP activities and demonstrations of their presence in many organs of the central and peripheral nervous systems in relationship to cholinergic innervation (Sundler et al., 1991). In addition, other molecules, such as nitric oxide (NO), may be synthesized by neurons of the enteric nervous system (ENS) (Schleiffer and Raul, 1997). NO, a diffusible short-lived mediator not subject to re-uptake, is known in mammalian species as a non-adrenergic, non-cholinergic (NANC) inhibitory molecule (Sanders and Ward, 1992; Rand and Li, 1995).

Although the distribution of the above-mentioned chemical messengers has been studied mostly in laboratory animals, some descriptions for the equine digestive system have appeared (Cummings et al., 1984; Burns and Cummings, 1993; Pearson, 1994; Rakestraw et al., 1996; Hudson et al., 1999), which were mainly directed at studying the small intestine.

The aim of the present work was to add knowledge to this item by examining the entire horse intestine. Particularly, the morphological data on the horse large intestine can be regarded as new.

The presence and localizations of the two neuropeptides SP and CGRP, as well as the nitrergic components of gut intramural innervation were investigated and compared with those referring to the presence of the classical neurotransmitter acetylcholine in the intramural innervation. The latter has been studied by means of the histochemical reaction for acetylcholinesterase (AChE) and by the immunohistochemical demonstration of two markers for the cholinergic innervation, choline acetyltransferase (ChAT) (Fagan et al., 1997) and vesicular acetylcholine transporter (VAChT) (Arvidsson et al., 1997; Takei et al., 1997). The choice of the neuromediators to be studied takes into account the well known involvement of SP in both diskynetic motor effects and pain sensory functions, which are basically present in pathological situations such as the colic syndrome (Cummings et al., 1984).

Studies on the nitrergic components of the horse gut intramural innervation can have important clinical rebounds, as in future years ongoing studies will bring to the therapeutic use for equine motility disorders of NO promoter substances or, on the contrary, of agents that inhibit the synthesis or actions of NO.

Materials and methods

Intestines from healthy horses were collected at the public slaughterhouses of Verdello (Bergamo, Italy), Piove di Sacco (Padova, Italy) and Voghera (Pavia, Italy). N.3 adult (10-12 years old, one female and two males) animals were used. Numerous samples were collected from small and large intestine immediately after death, in anatomically defined sites: 1) duodenum: beginning of the descending duodenum; 2) jejunum:

about midpoint; 3) ileum: midpoint of the ileocecal fold; 4) ileo-cecal valve; 5) cecum: midpoint of the body including lateral taenia; 6) ascending colon: 6.1.) right ventral colon, midpoint, including lateral free taenia (C1), 6.2) left ventral colon, midpoint, including lateral free taenia (C2), 6.3) pelvic flexure, midpoint, 6.4) left dorsal colon, next to the diaphragmatic flexure (where taeniae are discernible), including lateral free taenia (C3), 6.5) right dorsal colon, midpoint, including lateral free taenia (C4); 7) descending colon: midpoint, including free taenia; 8) rectum: midpoint, at the level of the rectal ampulla.

Samples were fixed in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS), pH 7.4 for 4-5 h at 4 °C, rinsed overnight in PBS, then in 20% sucrose in the same buffer for 24 h at 4 °C and finally snap-frozen in liquid nitrogen-cooled isopentane. Other specimens were snap-frozen as above without previous fixation. In parallel, other specimens were dehydrated after fixation and paraffin embedded. Successive microtome sections of the specimens were processed as follows.

Histochemistry

NISSL-thionin

Paraffin-embedded sections (4 μ m thick) were picked up on gelatin-coated glass slides, dewaxed, rehydrated and stained for the general morphology of neurons by Nissl-thionin staining.

Acetylcholinesterase (AChE)

Cryostat sections (40 μ m thick) were picked up on gelatin-coated glass slides and stained for AChE by the method of Beermann and Cassens (1976) which in part modifies the "historical" ones of Koelle and Friedenwald (1949) and Karnovsky and Roots (1964). Sections were incubated for 30 min at room temperature in 2 mg/ml acetylthiocholine iodide (Sigma, Italy) (pH of the incubating medium was 6). Sections were then rinsed in distilled water, incubated for 10 min at room temperature in 0.5M potassium ferricyanide [$K_3Fe(CN)_6$] and then for 1 h at room temperature in a solution containing 20% silver nitrate ($AgNO_3$) and 0.1% copper sulfate ($CuSO_4$). The reaction product was developed for 60 sec at room temperature in a Bodian solution containing 50 mg/ml sodium sulfite and 10 mg/ml hydroquinone. After development, the sections were treated for 5 min at room temperature in 5% sodium thiosulfate, dehydrated and mounted in Eukitt.

The specificity of the AChE method was verified by excluding acetylthiocholine iodide from the incubating medium; as expected this abolished all staining.

NADPH-diaphorase

Cryostat sections (20 μ m thick) from both fixed and unfixed specimens were picked up on gelatin-coated

Innervation of horse gut

glass slides and incubated for 1 h at 37 °C in 0.1M PBS pH 7.4, containing 0.15 mg/ml nitroblue tetrazolium (Sigma, Italy), 0.1% Triton X-100 and 1 mg/ml NADPH (Sigma), according to Scherer-Singler et al. (1983). The sections were then rinsed in PBS, dehydrated and mounted in Eukitt. The specificity of this stain was verified by excluding NADPH from the incubating medium, which abolished all activity.

NADPH-diaphorase is reputed to be a marker of NO synthesis (Hope et al., 1991).

Immunohistochemistry

Fragments of the gut fixed as mentioned above in 4% paraformaldehyde, cryoprotected and frozen, were cryosectioned, and the sections (12 µm thick) were collected on polylysinated glass slides (Poly-D-lysine, Sigma, Italy, 0.1% in PBS pH 7.6). Other sections were obtained from paraffin-embedded samples. A treatment of antigen retrieval was performed before the immunostaining by immersion of the slides in a Coplin jar (covered with a loose fitting screw cap) filled with 10mM citric acid monohydrate (pH 6.0), followed by heating in an autoclave for 2 min at 120 °C under 1 atmosphere pressure. After heating, the Coplin jar was taken out of the autoclave and allowed to cool for 15 min.

After washing in distilled water, the sections were treated with 3% H₂O₂ (20 min) to block the endogenous peroxidase activity and rinsed in phosphate-buffered saline solution (PBS) (pH 7.4) containing 0.2% Triton X-100 and 0.1% bovine serum albumin.

Background was prevented by incubating the sections with 1:5 normal swine (Dako, Italy) or donkey serum (Chemicon, USA) for 30 min prior to the incubation with primary antibodies. The primary antisera indicated in Table 1 were applied overnight at 4 °C in a humid chamber.

Thereafter a Labelled StreptAvidin Biotin (LSAB) system was used, utilizing biotinylated swine anti-rabbit immunoglobulins (Dako) or donkey anti-goat immunoglobulins (Chemicon) as secondary serum, followed by StreptABComplex/HRP (Dako). Tris-Buffered Saline (TBS: 0.05 M Tris/HCl, 0.15 M NaCl) pH 7.6 was used for dilutions and rinses throughout the whole procedure. The immunoreactive sites were

visualized using a freshly prepared solution of 10 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 15 ml of a 0.5 M Tris buffer at pH 7.6 containing 1.5 ml of 0.03% H₂O₂. Sections were counterstained with Mayer's hematoxylin and mounted using a permanent mounting medium (Eukitt).

The specificity of immunostaining was verified: (1) by omission of the 1st layer; (2) by the use of non-immune rabbit or goat serum in place of a primary antiserum at the same dilution; and (3) by incubating sections with antisera preabsorbed with the respective antigens (10-100 mg/ml) (Substance P was purchased from Bachem, UK; human CGRP, rat CGRP and ChAT protein were purchased from Sigma, Italy). The preabsorption procedures were carried out overnight at 4 °C. The results of these controls were negative. Positive controls were performed utilizing sections of the gut of other mammalian species (bovine, swine).

Slides were observed and photographed under an Olympus BX50 photomicroscope. Evaluation of staining intensities was based on subjective estimates of all the authors after examination of many sections per slide of all the animals tested.

Results

Histochemistry

The Nissl-thionin reaction showed that the intramural innervation of the horse gut was composed of neurons in the submucous (Fig. 1A) as well as myenteric (Fig. 1B) plexuses all along the intestine. The Nissl reaction prevalently showed neuronal bodies and very subtle nerve fibres close to their origin; that is within the plexuses only. In the submucous plexus of all the examined tracts nerve cell bodies were mostly grouped in small numbers, forming tiny ganglia (Fig. 1A). These neurons were prevalently located in the inner layer of the tunica submucosa. Limited to the jejunum (which is in this, as in other mammalian species, very long), the submucous plexus showed an inner and an outer layer. The inner layer was near the mucosa, the outer layer was near the muscular sheath. The myenteric plexus was arranged in an almost continuous layer which contained both nerve cell bodies and fibres. This plexus was located between the inner and outer musculature. In

Table 1. Primary antisera employed, their sources and working dilutions.

| ANTISERA | SOURCE | DILUTION |
|--|-------------------------|----------|
| rabbit anti-neurofilament 200 (NF200) | Sigma, Italy, N-4142 | 1: 200 |
| rabbit anti-Substance P | Amersham, UK, RPN 1572 | 1: 600 |
| rabbit anti-rat calcitonin gene-related peptide (CGRP) | Peninsula, UK, IHC 6006 | 1: 500 |
| rabbit anti-human calcitonin gene-related peptide (CGRP) | Peninsula, UK, IHC 6009 | 1: 500 |
| goat anti-choline acetyltransferase (ChAT) | Chemicon, USA, AB144 | 1: 500 |
| goat anti-vesicular acetylcholine transporter (VACHT) | Chemicon, USA, AB1578 | 1: 1000 |
| rabbit anti-neuronal nitric oxide synthase I (n-NOS-I) | Chemicon, USA, AB1552 | 1: 500 |

addition, structures belonging to the myenteric plexus were often embedded in the longitudinal musculature (Fig. 1B). In the tracts of the large intestine, characterized by the presence of taeniae, other ganglia containing Nissl-reactive neurons were present in a subserosal localization.

Cholinergic components of the intramural innervation of the horse gut could be described by the histochemical reaction evidencing the acetylcholinesterase enzyme (Table 2; Fig. 2A,B). AChE activity was detected in nerve fibres of the submucosa as well as the myenteric plexus, in both the small (Fig. 2A) and large intestine (Fig. 2B). In both the plexuses reactive nerve fibres were frequently grouped in bundles. Numerous reactive nerve fibres were discernible in the inner layer of the tunica muscularis (Fig. 2B). The AChE activity was discernible with particular evidence within nerve fibres running in both the musculature and myenteric ganglia, whereas it was very faint within the nerve cell bodies.

NADPH-diaphorase reactivity was detected in nerve cell bodies and fibres of the submucosa of both the small and large intestine (Fig. 2C). In the myenteric plexus of all the examined tracts NADPH-diaphorase reactivity was observed in nerve cell bodies of voluminous ganglia where nerve fibre bundles were also reactive (Fig. 2D). Some NADPH-reactive nerve fibres were also seen contacting the inner musculature (Fig. 2D). In the tracts of the large intestine, characterized by the presence of taeniae, ganglia containing reactive neurons were also present in a subserosal localization (Fig. 2E).

Immunohistochemistry

Anti-neurofilament 200 immunoreactivity was always detected in the submucosa, in nerve fibre bundles and positive nerve cell bodies grouped in

ganglia (Fig. 3A). Limited to the jejunum, immunoreactive neurons were detected in both the inner and outer submucosa. Immunoreactive nerve cell bodies and nerve fibres were also present in different tracts within the myenteric plexus (Fig. 3B,C). Structures belonging to the myenteric plexus were often embedded in the longitudinal musculature (Fig. 3C), as described above by the Nissl-thionin reaction. Immunoreactive nerve terminals were also found in the inner musculature (Fig. 3B). In the tracts of the large intestine ganglia were present in a subserosal localization (Fig. 3D), especially when an intestinal taenia was present.

Data regarding immunolocalization of VACHT and ChAT in the adult horse gut are summarized in Table 2. VACHT-immunoreactivity was observed in nerve cell bodies of the myenteric plexus of both small (Fig. 3E) and large intestinal tracts. ChAT immunoreaction was detected in nerve cell bodies and possibly the initial segments of the processes in the myenteric plexus all along the intestine, as far as the rectum (Fig. 3F). In addition, a few subtle and scarcely VACHT-immunoreactive nerve terminals were observed located in the inner musculature.

Data regarding immunolocalization of CGRP, substance P and NOS in the adult horse gut are summarized in Table 3.

In both the small and large intestinal tracts CGRP immunoreactive nerve cell bodies were present in the submucosa together with immunoreactive nerve fibres, sometimes apposed to negative nerve cell bodies. The immunoreactive nerve cell bodies usually showed a roundish profile. Limited to the jejunum, immunoreactive neurons were detected in both the inner and outer submucosa. Subtle nerve fibres were seen running parallel to muscle cells of the muscularis mucosae (Fig. 4A) especially frequent in the jejunum and ileum. In addition, subtle nerve fibres were seen

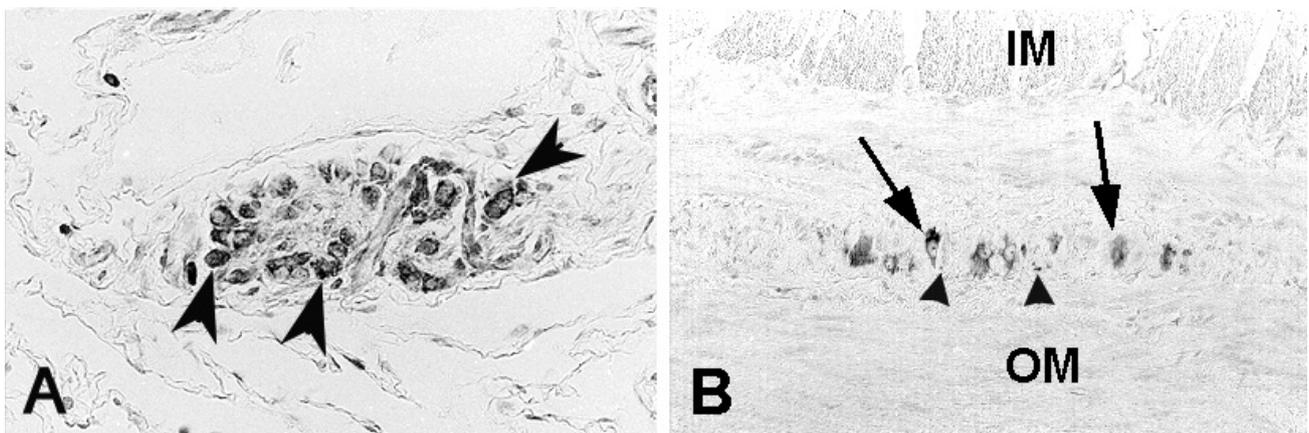


Fig. 1. **A.** Nissl-thionin staining in the cecum submucous plexus. The reaction is detected in the cytoplasm of nerve cell bodies (arrowheads). x 180. **B.** Nissl-thionin staining in the ileum myenteric plexus. The reaction is detected in the cytoplasm of nerve cell bodies (arrows). The ganglion is embedded in the longitudinal musculature. Arrowheads show very thin transversally-sectioned nerve fibres. IM: inner musculature; OM: outer musculature. x 150

Table 2. Localization of AChEase (AChE), VACHT and ChAT in the adult horse gut.

| | TUNICA MUCOSA | | MUSCULARIS MOCOSAE | | INNER SUBMUCOUS PLEXUS | | OUTER SUBMUCOUS PLEXUS ¹ | | INNER MUSCULATURE | | MYENTERIC PLEXUS | | OUTER MUSCULATURE | | TUNICA SEROSA | | | |
|----------------|---------------|-------|--------------------|------|------------------------|------|-------------------------------------|-------|-------------------|------|------------------|------|-------------------|-------|---------------|------|-------|------|
| | AChE | VACHT | ChAT | AChE | VACHT | ChAT | AChE | VACHT | ChAT | AChE | VACHT | ChAT | AChE | VACHT | ChAT | AChE | VACHT | ChAT |
| duodenum | - | - | - | - | - | - | ++f | - | - | ++f | - | ++f | ++f | ++f | - | - | - | - |
| jejunum | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| ileum | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| ileo-cec-valve | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| cecum | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| asc.colon C1* | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| asc.colon C2* | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| pelvic flexure | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| asc.colon C3* | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| asc.colon C4* | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| desc. colon | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |
| rectum | - | - | - | - | - | - | ++f | - | ++f | - | ++f | ++f | ++f | ++f | - | - | - | - |

Number of + identifies quantity of immunoreactive neuronal structures (nerve cell bodies^b and/or fibres^c), e.g.: +/4+++ from few to numerous immunoreactive structures; -, absence of immunoreactivity. ¹: the outer submucous plexus is present only in the jejunum; an empty box represents the absence of the outer submucous plexus. * C1-C2-C3-C4: different ascending colon tracts referred to in materials and methods.

Table 3. Immunolocalization of CGRP, substance P and NOS in the adult horse gut.

| | TUNICA MUCOSA | | | MUSCULARIS MOCOSAE | | | INNER SUBMUCOUS PLEXUS | | | OUTER SUBMUCOUS PLEXUS ¹ | | | INNER MUSCULATURE | | | MYENTERIC PLEXUS | | | OUTER MUSCULATURE | | | TUNICA SEROSA | | | |
|----------------|---------------|------|-----|--------------------|------|-----|------------------------|-----|-----|-------------------------------------|-----|-----|-------------------|-----|-----|------------------|-----|-----|-------------------|-----|-----|---------------|-----|-----|-----|
| | CGRP | SP | NOS | CGRP | SP | NOS | CGRP | SP | NOS | CGRP | SP | NOS | CGRP | SP | NOS | CGRP | SP | NOS | CGRP | SP | NOS | CGRP | SP | NOS | |
| duodenum | ++f | ++f | - | + | ++f | - | + | ++f | + | ++f | ++f | + | ++f | ++f | - | + | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| jejunum | + | +++f | - | ++f | ++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| ileum | ++f | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| ileo-cec-valve | ++f | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| cecum | ++f | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| asc.colon C1* | + | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| asc.colon C2* | + | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| pelvic flexure | ++f | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| asc.colon C3* | ++f | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| asc.colon C4* | ++f | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| desc. colon | + | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |
| rectum | + | +++f | - | ++f | +++f | - | + | ++f | ++f | ++f | ++f | + | ++f | ++f | - | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f | ++f |

Number of + identifies quantity of immunoreactive neuronal structures (nerve cell bodies^b and/or fibres^c), e.g.: +/4+++ from few to numerous immunoreactive structures; -, absence of immunoreactivity. ¹: the outer submucous plexus is present only in the jejunum; an empty box represents the absence of the outer submucous plexus. * C1-C2-C3-C4: different ascending colon tracts referred to in materials and methods

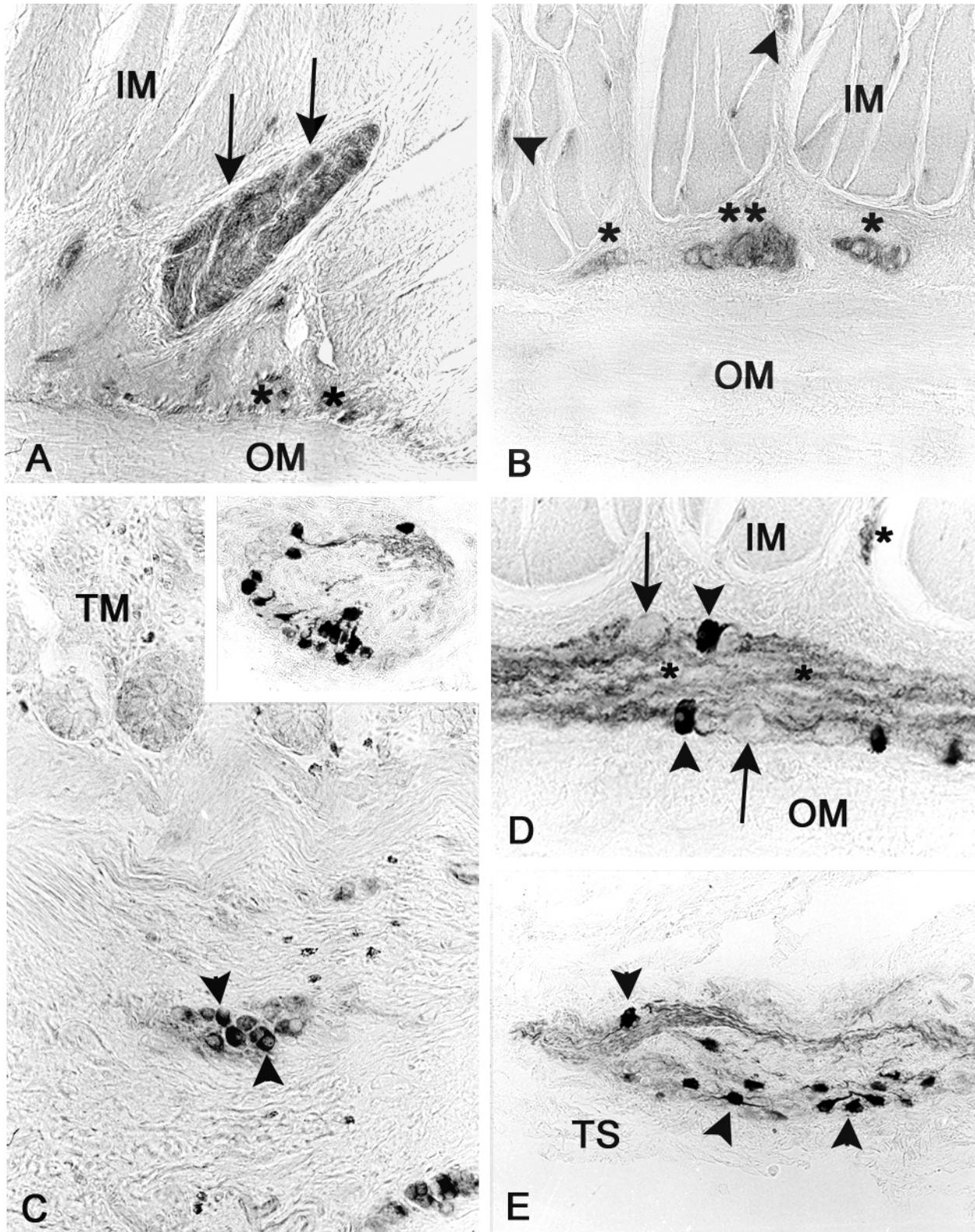


Fig. 2. **A.** AChE reactivity in the ileum myenteric plexus. AChE reactivity is evident in a bundle of nerve fibres (arrows) as well as in single nerve fibres (asterisks). IM: inner musculature; OM: outer musculature. x 150. **B.** AChE histochemistry in the myenteric plexus of the left ventral colon (C2). Asterisks indicate a reactive ganglion in the plexus. Arrowheads indicate nerve fibres located in the inner musculature (IM); OM: outer musculature. x 150. **C.** NADPH-diaphorase histochemistry in the ileum submucous plexus. Reactivity is present in grouped nerve cell bodies (arrowheads). TM: tunica mucosa. x 150. **Inset:** a strong NADPH-diaphorase reactivity can be seen in nerve cell bodies of a ganglion in the submucous plexus of the left dorsal colon (C3). x 120. **D.** NADPH-diaphorase histochemistry in the myenteric plexus of the left dorsal colon (C3). Arrowheads indicate strongly positive nerve cell bodies. Arrows indicate unreactive nerve cell bodies. Strongly reactive nerve fibres (asterisks) can be seen either in the ganglion or contacting the inner musculature. IM: inner musculature; OM: outer musculature. x 180. **E.** NADPH-diaphorase histochemistry in the tunica serosa (TS) of the caecum. Reactivity is evident in nerve cell bodies (arrowheads) of a ganglion in the subserosal connective tissue. Nerve fibres are also immunoreactive. x 150

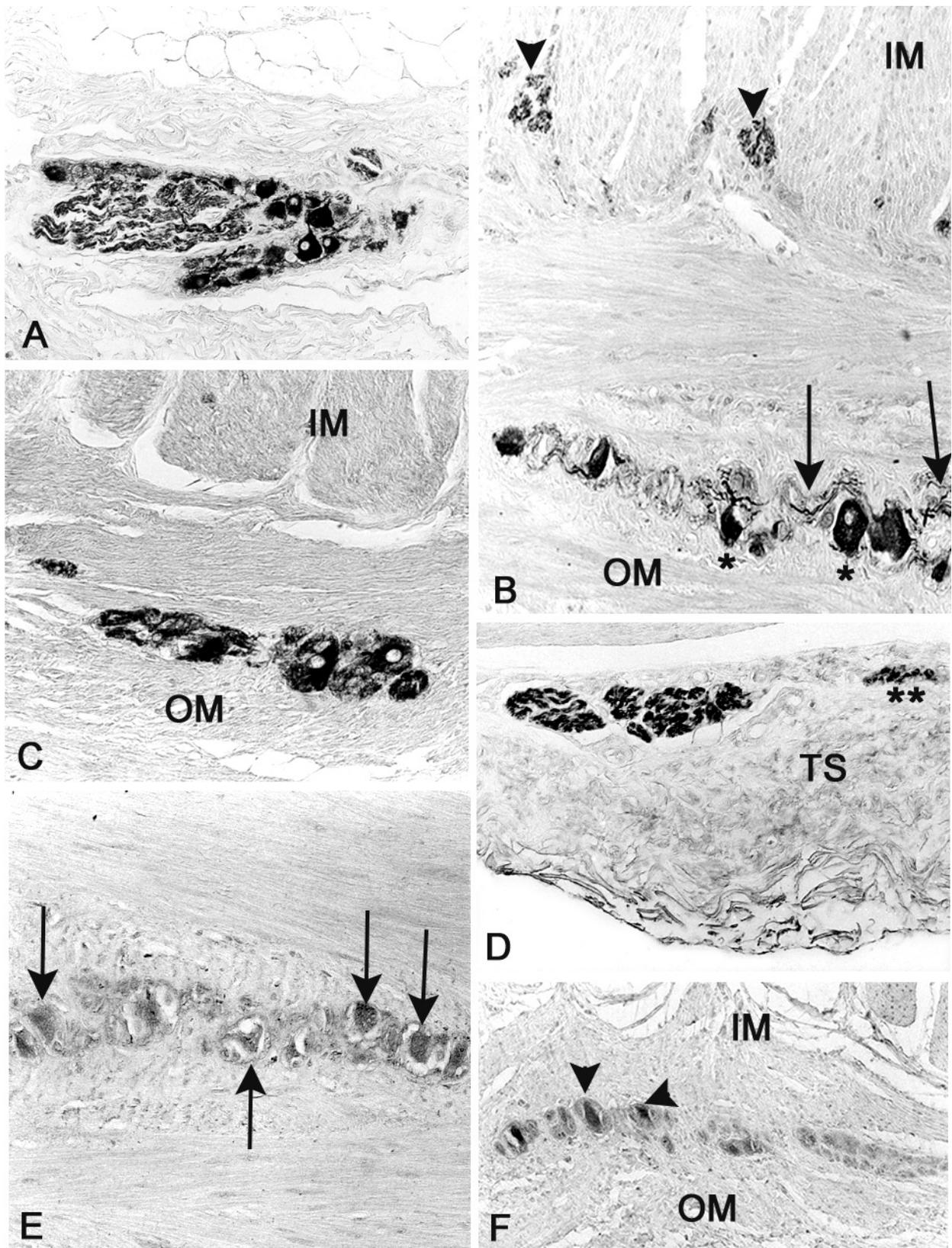


Fig. 3. **A.** NF-200 immunoreactivity in the jejunum inner submucous plexus. Positive nerve cell bodies as well as bundles of nerve fibres can be seen in a ganglion of the inner tunica submucosa. x 150. **B.** NF-200 immunoreactivity in the ileum tunica muscularis. Positive nerve cell bodies (asterisks) as well as nerve fibres (arrows) are present in the myenteric plexus. Note also clusters of immunoreactive nerve fibres in the inner musculature (IM) (arrowheads). OM: outer musculature. x 150. **C.** NF-200 immunoreactivity in the rectum myenteric plexus. Positive nerve cell bodies as well as nerve fibres are present in a ganglion. IM: inner musculature; OM: outer musculature. x 150. **D.** NF-200 immunoreactivity in the tunica serosa (TS) of the cecum. The reaction is detected in a ganglion in the subserosal connective tissue. Asterisks indicate bundles of immunoreactive nerve fibres. x 150. **E.** VACHT immunohistochemistry in the ileum myenteric plexus. Arrows indicate the immunostained nerve cell bodies. x 150. **F.** ChAT immunoreactivity in the rectum myenteric plexus. Reactivity is present in nerve cell bodies (arrowheads). IM: inner musculature; OM: outer musculature. x 150

insinuating into the musculo-vascular axis of the villi and in intestinal glands of small and large intestine (Fig. 4A). Using either of the CGRP antisera, CGRP was not discernible in the myenteric plexus and tunica muscularis of any of the examined intestinal tracts.

Substance P-immunoreactive nerve structures were very numerous in all the examined tracts, with the exception of the ileo-cecal valve and the rectum, which showed a small number of immunoreactive nerve fibres. Reactivity was found in nerve fibres of the tunica mucosa in relationship to the muscularis mucosae, in the connective axis of the villi and of the intestinal glands (Fig. 4B). From the left dorsal colon onward, a network of SP-ir nerve fibres ran parallel to the surface

epithelium (Fig. 4B). The submucous plexus contained both SP-immunoreactive nerve cell bodies and fibres (Fig. 4C). Limited to the jejunum, immunoreactive neurons were detected in both the inner and outer submucous plexus. In the myenteric plexus, only immunoreactive nerve fibres were noticed, sometimes in relationship to negative nerve cell bodies, in such a way as to establish a pericellular basket of varicose fibres. This pattern was present in both the small and large intestinal tracts, with the exception of the pelvic flexure, which did not contain immunoreactive structures. The inner muscular layer in the small and large intestine contained numerous immunoreactive nerve fibres (Fig. 4D). In the distal intestinal tracts, namely in the left

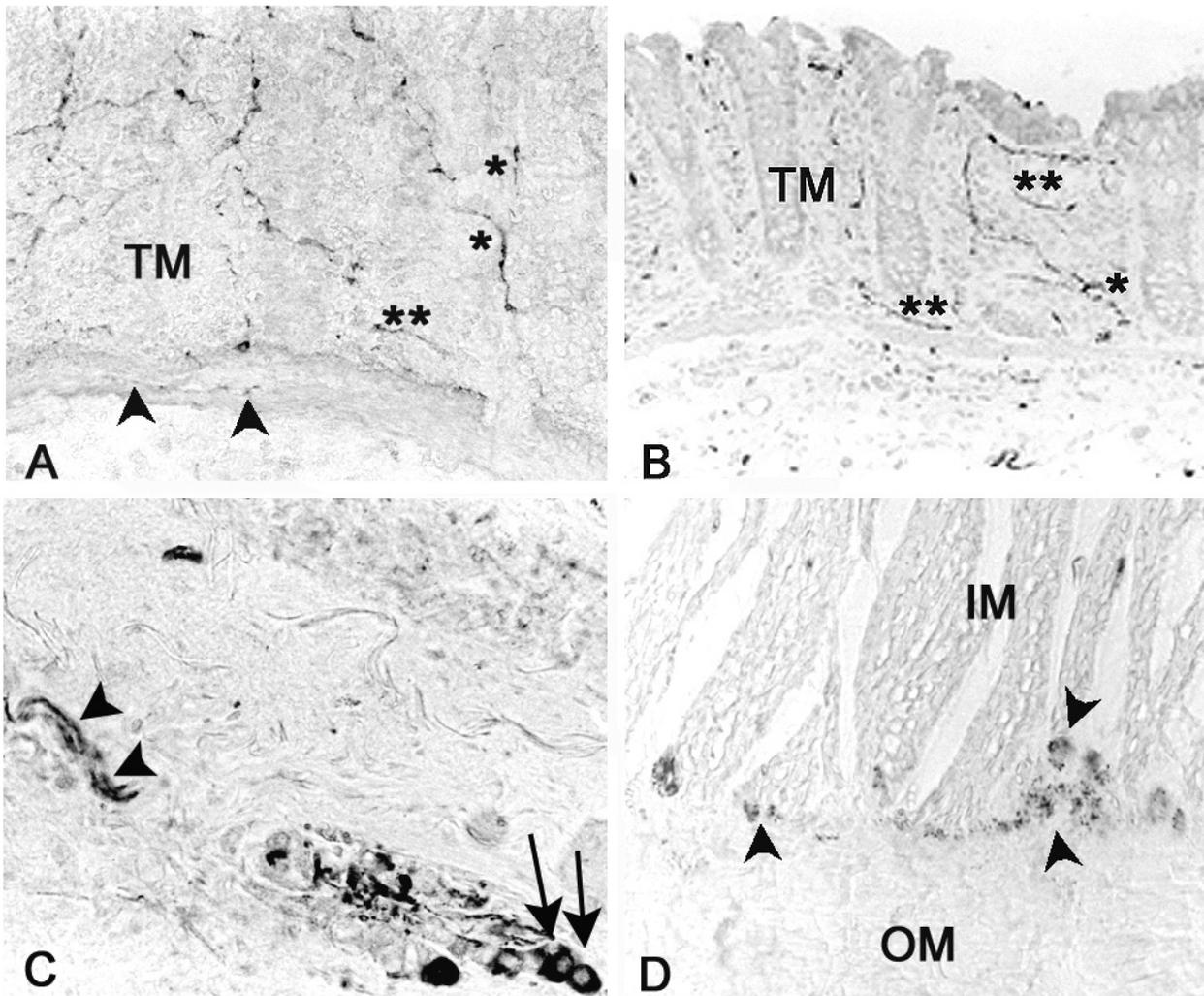


Fig. 4. **A.** CGRP immunoreactivity in the tunica mucosa (TM) of the left dorsal colon (C3). Thin immunoreactive nerve fibres can be seen running parallel to the epithelium lining the intestinal glands (asterisks). Arrowheads indicate tiny nerve fibres in the muscularis mucosae. x 150. **B.** Substance P immunoreactivity in the cecum tunica mucosa (TM). Thin immunoreactive nerve fibres can be seen in the connective tissue of the lamina propria (asterisks). x 150. **C.** Substance P immunoreactivity in the rectum submucous plexus. A group of immunoreactive nerve cell bodies (arrows) as well as nerve fibres (arrowheads) can be seen. x 150. **D.** Substance P immunoreactivity in the ileum myenteric plexus. Immunoreactive nerve fibres (arrowheads) can be seen. IM: inner musculature; OM: outer musculature. x 150

Innervation of horse gut

dorsal colon and successive tracts, the outer muscular sheath also showed SP-immunoreactive nerve fibres.

SP-immunoreactive nerve fibres were constantly present in relationship with the muscular sheath of blood vessels, all along the intestine.

Anti-n-NOS immunoreaction was evidenced at the level of the submucous plexus in both the small and large intestine. Limited to the jejunum, immunoreactive neurons were detected in both the inner and outer submucous plexus. In addition, anti-n-NOS immunoreaction was detected in ganglia of the myenteric plexus in both the small (Fig. 5A) and large (Fig. 5B) intestine. Immunoreactivity was localized in strongly positive nerve cell bodies and nerve fibres, with particular abundance and intensity at the level of the left dorsal colon. Finally, immunoreactive neurons were observed in a subserosal localization in some of the large intestinal tracts.

Discussion

The aim of this study was to give a description of the cholinergic, nitrergic and peptidergic innervation of the entire horse intestine by using either histochemical or immunohistochemical reactions.

An overall morphological examination obtained by both Nissl-thionin staining for neurons and neurofilament (NF200)-immunohistochemistry, showed that the enteric nervous system of the horse intestine, in all the examined tracts, was made up by nerve cell bodies and fibres organized in the submucous and myenteric plexuses. The submucous plexus of the jejunum was organized in an inner and an outer layer. This pattern is in agreement with what has previously been described by Timmermans et al. (1992b) and Pearson (1994) for large mammals. We can similarly hypothesize that the outer layer of the submucous plexus may serve to regulate the circular musculature, in the

horse as in other mammals (Timmermans et al., 1992b). Ganglia were also detected in a subserosal localization in tracts of the large intestine. Subserosal ganglia have been detected in humans by Crowe and Burnstock (1990).

Immunohistochemical reactions for choline acetyltransferase and vesicular acetylcholine transporter gave very similar results, since reactivities for both antibodies were mainly detected in nerve cell bodies of the myenteric plexus all along the intestine. Antibodies against ChAT gave a weak positivity, as previously demonstrated by comparison of the ChAT immunoreactivities in the central nervous system with that of the enteric nervous system (Schemann et al., 1993; Sang and Young, 1998). During the preparation of this manuscript, evidence about the presence of a peripheral form of ChAT (pChAT) has been reported in the enteric neurons of guinea pigs (Chiocchetti et al., 2003). Yet our results are in agreement with those of Mann et al. (1999), who described ChAT immunoreactivity in nerve cell bodies of the myenteric and submucous plexuses of the rat ileum by utilizing the same antiserum which has been used in our work. Scarce VAcHT-immunoreactive nerve terminals were observed contacting the muscle cells of the inner musculature, but the small number of nerve terminals immunodetected is not surprising, because vesicular acetylcholine transporter and choline acetyltransferase share a prevalent localization in the perikaryal cytoplasm (Mann et al., 1999) and consequently the possible immunodetection is also prevalently in the nerve cell body, with neuronal processes poorly stained. We were not able to detect any cholinergic fibres in mucosal localizations. The scarce reactivity or the absence of mucosal immunoreactive nerve fibres could possibly make our results different from those obtained in the guinea pig by Li and Furness (1998) who, however, employed a pre-treatment to enhance ChAT and/or VAcHT immunoreactivities. On the other hand, possible

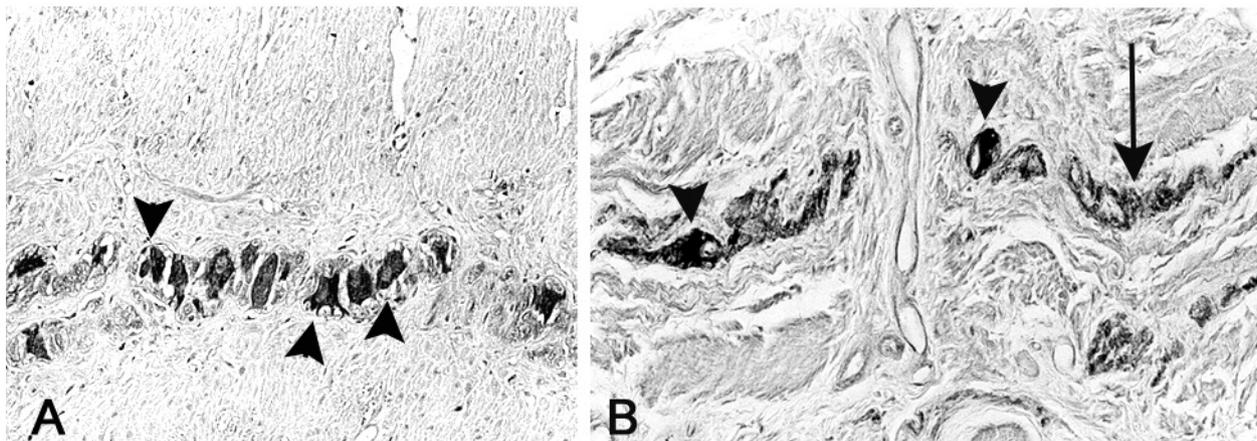


Fig. 5. A. NOS immunoreactivity in the ileum myenteric plexus. Arrowheads indicate strongly positive nerve cell bodies. x 150. **B.** NOS immunoreactivity in the rectum myenteric plexus. Either nerve cell bodies (arrowheads) or nerve fibres (arrow) are intensely reactive. x 150

cholinergic nerve terminals were constantly detected by the histochemical reaction for the acetylcholinesterase activity. Thus, we have shown that either immunohistochemistry or histochemistry are useful to cast light on the structural pattern of intestinal cholinergic intramural innervation in this mammalian species. All these data may anatomically support the hypothesis that the cholinergic innervation could sustain either sensory functions, through neurons located in the myenteric plexus projecting to the submucous plexus in both the small and large intestine (Song et al., 1991; Neunlist and Schemann, 1997, 1998; Li and Furness, 1998; Mann et al., 1999), or excitatory functions mediated by nerve fibres distributing to the inner musculature, presumably originating from neurons of the myenteric plexus itself (Porter et al., 1997; Clerc et al., 1998). Mechanical or chemical stimuli that activate the intrinsic primary neurons might provoke intestinal secretion. Otherwise, excitatory motor functions could suggest a possible role of acetylcholine in regulation of segmental motility. We cannot exclude that a part of the neurons containing choline acetyltransferase and vesicular acetylcholine transporter are ascending and/or descending interneurons (Brookes, 2001; Porter et al., 2002), having an excitatory function over the circular muscle, so giving way to some motor components of the peristaltic reflex of the gut (Neunlist et al., 2001). A more complete understanding of the possible cholinergic components in the intestine has been achieved in laboratory mammals, but it is not fully clear in large domestic mammals. The few data available on horse jejunum (Malone et al., 1999) just consider the acetylcholine/norepinephrine balance in the regulation of smooth muscle contractility, and emphasize the different functional roles of circular versus longitudinal musculature. We found that possible cholinergic nerve fibres are detected in circular musculature only.

Nitric oxide (NO) is a gaseous mediator reputed to be a nonadrenergic-noncholinergic (NANC) neurotransmitter in the gut of mammalian species (Sanders and Ward, 1992), having an inhibitory role over the contraction of smooth muscle cells. The enzyme responsible for its production, the neuronal NO synthase (n-NOS), has been localized in enteric neurons belonging to the two plexuses and in nerve fibres running within the tunica muscularis in the alimentary canal of men and laboratory mammals (Costa et al., 1992; Ekblad et al., 1994; Furness et al., 1994). The NADPH-diaphorase reaction is convincingly reputed to be the selective marking tool for identifying neuronal NOS activity in paraformaldehyde-fixed tissues (Hope et al., 1991; Cracco and Filogamo, 1994). In our samples, staining for NADPH-diaphorase was localized in the horse enteric nervous system, showing the presence of possibly nitrenergic neurons. Reactivity was detected in nerve cell bodies and fibres of the submucous and myenteric plexuses either in the small or large intestine. NADPH-diaphorase reactivity was in addition observed in ganglia localized in the tunica serosa of the large

intestinal tracts. The n-NOS immunoreactivity was prevalently detected in nerve cell bodies and nerve fibres of the myenteric plexus. The larger number of possibly nitrenergic neurons shown by NADPH-diaphorase histochemistry in comparison with n-NOS immunohistochemistry may be explained considering the different intervention levels of the two enzymes during the synthesis of nitric oxide (Grozdanovic et al., 1995). As demonstrated in other mammalian species (Porter et al., 1999; Timmermanns et al., 2001), the presence of nitrenergic neurons in the submucous plexus suggests that these neurons could project to the circular musculature. Other NOS-immunoreactive neurons located in the myenteric plexus could share an inhibitory role and display an ascending and/or descending pattern typical of interneurons (Clerc et al., 1998; Neunlist et al., 2001; Hens et al., 2002). Other possibly nitrenergic neurons located in subserosal ganglia are conceivably aimed at regulating the large number of longitudinal muscle cells grouped in taeniae. The subserosal ganglia limited to large intestinal tracts are described here for the first time in a large mammal. Particularly in the horse, the putative inhibitory action of NOS-utilizing neurons may be conspicuous at the level of the left dorsal colon, where large NOS-immunoreactive nerve fibre bundles and voluminous nerve cell bodies were observed in the myenteric plexus. Actually, this colon tract is often considered to be the origin of diseases characterized by dysmotility in equidae, pathological conditions which can have as a common factor the disruption of progressive motility. Measures of the contractile activity of circular, longitudinal and taenia muscle strips in response to electrical field stimulation have been performed after administration of NO inhibitors in equine ventral colon (van Hoogmoed et al., 2000). These electrophysiological results, in agreement with previous ones on jejunum muscle strips (Rakestraw et al., 1996) helped to elucidate the role of NO in regulating inhibitory contractile activity of the equine ventral colon. Van Hoogmoed et al. (2000) hypothesized that inflammatory intestinal ileus, a common disturbance in horses, could be associated to the release of endogenous NO from inflammatory cells, thus causing the motility disorder. The role of NO in the pathogenesis of slow-transit syndromes via intrinsic NANC inhibitory neuronal input has also been recently elucidated in the colon of human patients (Tomita et al., 2002).

The two accessory neuromediators (SP, CGRP) whose distribution has been immunohistochemically investigated in this work are both reputed to be cholinergic co-mediators. Their involvement in motor and peripheral sensory functions has been hypothesized and demonstrated in many species and localizations (Hens et al., 2000).

The presence of SP-immunoreactive neurons in the submucous plexus could be related to a secretomotor role in mammalian species (Timmermanns et al., 1997; Hens et al., 2000). The conspicuous presence of immunoreactive nerve fibres in the horse intestinal

Innervation of horse gut

lamina propria, probably originating from immunoreactive nerve cell bodies located in the submucous ganglia, suggests a possible peripheral sensory function (Kitamura et al., 1993; Kunze et al., 1995). Other possibly afferent fibres could project through the vagal nerves, which could utilize SP as a cholinergic co-mediator (Cummings et al., 1984). The peculiar disposition of the immunoreactive fibres parallel to the luminal surface of most tracts of the horse large intestine might be indicative of a receptive function. They could be involved in the reception of distension stimuli which are important in the regulation of motility, for instance in evocating defecation reflexes. In the horse, in particular, a correct balance of filling of successive intestinal tracts is important. Cecum or colon constipation accompanied by visceral pain happens when gastric over-distension takes place. The horse is said to be more sensitive than other species to abdominal pain but there is little direct evidence to support this.

The SP neuromodulator can also be implicated in the motor innervation of the inner musculature through neurons of the submucous plexus (Hens et al., 2002). The observation of a network of SP-immunoreactive nerve fibres in the muscularis mucosae of the horse intestine (specially in the ileo-cecal valve) is in accordance with morphological, immunohistochemical and pharmacological studies of other authors (Steele and Costa, 1990; Holzer and Holzer-Petsche, 1997; Hens et al., 2000), indicating that SP can be associated with an excitatory response of the muscularis mucosae.

The data obtained in the horse intestinal musculature suggest a different pattern of regulation for the inner and the outer muscular layers. Part of these SP-immunoreactive terminals of all the intestinal tracts could belong to SP co-storing vagal afferent fibres, as immunoreactive nerve cell bodies are totally absent in the myenteric plexus. A further part of the immunoreactive nerve fibres accompanying the inner musculature possibly modulate segmental motility. In contrast, the presence of SP-immunoreactive nerve fibres in the outer muscular sheath of the distal tracts of the gut, namely from the left dorsal colon, could indicate that this neuromodulator possibly also influences the regulation of the gut longitudinal motility.

It has been demonstrated that although foals have a higher neuron density in their myenteric plexus (Schusser and White, 1994, confirmed by our unpublished results), the gut of adult horses contains a larger number of SP-immunoreactive nerve structures than prepuberal animals. Therefore, in young animals a minor modulatory action of SP in comparison with the adult may exist, which could be related to diskinetic/hyperkinetic gastrointestinal phenomena, which are more frequent in young ages (Dolera et al., 1997). Actually the possible spasmogenic action of SP over gut musculature is well known.

The action of substance P on smooth muscle activity is also consistent with regulation of local blood flow as SP-utilizing innervation of small blood vessels was

observed all along the intestine.

A possible modulatory action upon other neuronal families synthesizing different neuropeptides is otherwise conceivable for the immunoreactive nerve fibres contacting negative neurons in the submucosa (Hens et al., 2001).

CGRP-immunoreactive nerve cell bodies of the submucous plexus could be primary afferent neurons, as has been demonstrated in other mammalian species (Hens et al., 2000; Timmermans et al., 2001) and hypothesized for the horse (Burns and Cummings, 1993). Actually we have here demonstrated the prominent presence of roundish CGRP-immunoreactive neurons. There is, thus, a morphological similarity between them and the sensory neurons within spinal ganglia, which is in agreement with what Kunze et al. (1995) showed in the enteric neurons of the guinea pig. Therefore, on a merely morphological basis we can hypothesize that CGRP-immunoreactive projections through the mucosa could be involved in the reception of chemical stimuli coming from the lumen (Kinoshita et al., 1993; Grider, 1994).

CGRP immunoreactivity is totally absent in the myenteric plexus and tunica muscularis. This pattern appears peculiar to equine species, the presence of CGRP-immunoreactive nerve structures in these latter localizations being demonstrated in many other mammalian species (Sternini et al., 1992).

In conclusion, this paper illustrates, for the first time in a contemporary way, the cholinergic, nitrgergic and peptidergic innervation of the entire intestine in adult horses. Particularly, the morphological data on the horse large intestine can be regarded as new, as previous works focused prevalently on the small intestine. The knowledge of normal mechanisms that are at the basis of gastrointestinal motility is imperative for understanding the relationships between normal gut physiology and pathology (Navarre and Roussel, 1996). In addition, new therapeutic agents could be formulated as a consequence of a detailed knowledge of structural aspects and chemical coding of intramural enteric innervation.

Our morpho-functional observations appear to correlate well with other physiological and clinical ones. Malone et al. (1999) demonstrated by use of electrical field stimulation that either acetylcholine or norepinefrine (NE) are released from the myenteric plexus of the horse jejunum, contraction being strongly influenced by ACh release from the enteric neurons, and NE typically inducing relaxation. Combined adrenergic and cholinergic blockade revealed the likely involvement of NANC neurotransmitters and it was also hypothesized that ACh may stimulate release of nitric oxide. Nitric oxide produced in enteric neurons is probably the main inhibitor of equine oesophageal, gastric and intestinal muscular activity (Rakestraw et al., 1996; van Hoogmoed et al., 2000). Implications of NO production have been invoked to explain certain equine pathologies, e.g. equine grass sickness (Cottrell et al., 1999), in which the increased nitrgergic neuronal activity

can account for many of the clinical signs, namely dysphagia, generalized ileus, gastric dilatation, peripheral vasodilatation and others. As concerns the different colic tracts, our data confirm the peculiarity of the pelvic flexure in the horse intestine. Actually, the distribution of neurotransmitters and neuromodulators differs at the pelvic flexure compared to other sampling sites. In particular, the lack of neuronal SP-ir structures in the myenteric plexus in this localization and the paucity of SP-ir terminals in the inner musculature is noteworthy (see table 2). It is conceivable that in this tract neuromediators different from substance P could coordinate local intestinal motility.

Intervening of interstitial cells of Cajal in the control of intestinal motility should be considered as well. These have been recently described in the equine gastrointestinal tract (Hudson et al., 1999) and the investigation of their relationship with the different neuronal families implicated in regulation of intestinal motility will be the aim of our future studies.

~~**Acknowledgements.** This work was supported by the Italian Ministero Università e Ricerca Scientifica e Tecnologica (MURST 60%). The authors wish to thank the public slaughterhouses of Verdello (Bergamo, Italy), Piove di Sacco (Padova, Italy) and Voghera (Pavia, Italy) for kindly providing the horse guts. In particular, Dr. Giuseppe Lucotti is gratefully acknowledged for his kind collaboration.~~

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Accepted October 27, 2003