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Interspecies study of the enteric nervous system and related pathologies

Presentata da: Fiorella Giancola

Coordinatore Dottorato:

Chiar.mo Prof. Carlo Tamanini

Relatore:

Chiar.mo Prof. Roberto Chiocchetti

Correlatore:

Chiar.mo Prof. Roberto De Giorgio

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INDEX

LIST OF ABBREVIATIONS

5-HT, 5-hydroxytryptamine or serotonin

Ach, acetylcholine

ADP, Adenosine diphosphate

AGEs, Advanced glycation end products

AHPs, after hyperpolarizing potentials

AJs, adherentes junctions

AM anorectal manomety

ANS, autonomic nervous system

ATP, Adenosine triphosphate

BEI, intestinal epithelial barrier

BN, bombesin

CALB, calbindin

CC, chronic constipation

CCK , cholecystokinin

CGRP, calcitonin gene-related peptide

ChAT, choline acetyltransferase

CIPO, chronic intestinal pseudo-obstruction

CML, circular muscle layer

CNS, central nervous system

CTRL, control

DBH, dopamine beta-hydroxylase

DCC, deleted in colorectal cancer

DM, diabetes mellitus

ECs, enterocromaffin cells

EDNRB, endothelin receptor B

EGCs, enteric glial cells

ENCCs, enteric neuralcrest-derived cells

ENS, enteric nervous system

ESMP, external submucous plexus

ETB, endothelin-3 or endothelin B

GABA, gamma-Aminobutyric acid

GDNF, glial cell derived neurotrophic factor

GFAP, fibrillary acidic protein

GI, gastrointestinal

GLP2, glucagon like peptide 2

GRP, gastrin releasing peptide

HuC/HuD, human neuronal protein

HO-2, heme oxygenase-2

HY, Hoehn & Yahr

ICCDMP; deep muscular plexus interstitial cells of Cajal

ICCIM, intramuscular interstitial cells of Cajal

ICCMY, myenteric plexus interstitial cells of Cajal

ICCs, interstitial cells of Cajal

ICCSM, submucosal layer interstitial cells of Cajal

*IFAN*s, intestinofugal neurons

*IPAN*s, intrinsic primary afferent neurons

IR immunoreactive

IR, immunoreactivity

ISMP, internal submucous plexus

LB, Lewy bodies

LES, lower esophageal sphincter

LML, longitudinal muscle layer

LN, Lewy neurites

MAO, monoamine oxidases

MASH1, mammalian achaete-scute homologue 1

MDS-UPDRS, Movement Disorder Society - Unified Parkinson's Disease Rating Scale

MEN2B, multiple endocrine neoplasia type 2B

mm, muscularis mucosae

MMC, migrating myoelectric complex

MNGIE, mitochondrial neurogastrointestinal encephalopathy

MP, myenteric plexus

NADPH

NeuN, NEUronal Nuclei

NF200, neurofilament 200 KDa

NFP, neurofilament triplet protein

nNOS, neuronal nitric oxide synthase

NO, nitric oxide

NPY, neuropeptide Y

OSMP, outer submucosal plexus

PACAP, pituitary adenylate cyclase-activating polypeptide

PBS, phosphate-buffered saline

pChAT, peripheral choline acetyltransferase

PD, Parkinson disease

PGP9.5, protein gene product 9.5

PHOX2B, Paired-like homeobox 2b gene

PNS, peripheral nervous system

PVG, prevertebral ganglion

RAGE, Advanced glycation end products receptor

RT, room temperature

S100b, S100 calcium-binding protein B

SDS-PAGE, Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

SERT, serotonin reuptake transporter

SMP, submucous plexus

SO, sphincter of Oddi

SOM, somatostatin

SOX, SRY-box

SP, substance P

TBS, tris-buffered saline

TC, transiently catecholaminergic

TH, tyrosine hydroxylase

TJs, tight junctions

TPH, tryptophan hydroxylase

TRPV1, transient receptor potential cation channel subfamily V member 1

TT, total colonic transit time

TUJ1, neuron-specific class III beta-tubulin

VIP, vasoactive intestinal polypeptide

VIPR1, vasoactive intestinal polypeptide receptor 1

VIPR2, vasoactive intestinal polypeptide receptor 2

WB, western blotting

ABSTRACT

The enteric nervous system (ENS) modulates a number of digestive functions including well known ones, i.e. motility, secretion, absorption and blood flow, along with other critically relevant processes, i.e. immune responses of the gastrointestinal (GI) tract, gut microbiota and epithelial barrier . ENS is critical to preserve body homeostasis as reflect by its derangement occurring in pathological conditions that can be lethal or seriously disabling to humans and animals.

The knowledge of the ENS organization can contribute to better understanding its evolution and morphological and functional features. Furthermore, the characterization of the anatomical aspects of the ENS in large mammals and the identification of differences and similarities existing between species may represent a fundamental basis to decipher several digestive GI diseases in humans and animals**.** In this perspective, severe GI disorders due to ENS malfunction are referred to as "enteric neuropathies" and can be classified into primary or secondary forms. The term 'primary' is used for enteric neuropathies when the ENS is considered to be the main target of the disease, while 'secondary' enteric neuropathy is used for several systemic conditions that can damage several organs / systems including the ENS.

The aim of the present thesis is to highlight the ENS anatomical basis and pathological aspects in different mammalian species, such as horses, dogs and humans.

Firstly, I designed two anatomical studies in horses:

– "Excitatory and inhibitory enteric innervation of horse lower esophageal sphincter".

– "Localization of 5-hydroxytryptamine 4 receptor (5-HT_4R) in the equine enteric nervous system".

Then I focused on the enteric dysfunctions, including:

– A primary enteric aganglionosis in horses (the equine version of the human Hirschsprung's disease): "Extrinsic innervation of the ileum and pelvic flexure of foals with ileocolonic aganglionosis".

– A diabetic enteric neuropathy in dogs: "Quantification of nitrergic neurons in the myenteric plexus of gastric antrum and ileum of healthy and diabetic dogs".

– An enteric neuropathy in human neurological patients: "Functional and neurochemical abnormalities in patients with Parkinson's disease and chronic constipation".

Excitatory and inhibitory enteric innervation of horse lower esophageal sphincter

The lower esophageal sphincter (LES) is a specialized, thickened muscle region with a high resting tone mediated by myogenic and neurogenic mechanisms. During swallowing or belching the LES undergoes strong inhibitory innervation. In the horse, the LES seems to be organized as a "one way" structure, enabling only the oral-anal progression of food. We characterized the esophageal and gastric pericardial inhibitory and excitatory intramural neurons immunoreactive (IR) for the enzymes neuronal nitric oxide synthase (nNOS) and choline acetyltransferase (ChAT). The high percentage of nitrergic inhibitory motoneurons observed in the caudal esophagus reinforces the role of the enteric nervous system in the horse LES relaxation. Those findings might allow an evaluation of whether selective groups of enteric neurons are involved in horse neurological disorders such as megaesophagus, equine dysautonomia, and white lethal foal syndrome.

Localization of 5-hydroxytryptamine receptor 4 (5-HT_4R) in the equine enteric nervous system

The 5-hydroxytryptamine (5-HT) controls the visceral sensitivity, gastrointestinal motility, and fluid secretion acting on specific 5-HT receptor. The interest in 5-HT_4R agonists in the field of equine motility disorders is considerably growing. Despite several functional studies indicate a prokinetic effect of 5-HT_4R agonists, the presence of the 5-HT_4R in the equine gastrointestinal innervation remain to be determined. Furthermore, no data are available in the horse on the presence of 5-HT_4R in extrinsic visceral innervation.The aim of the present study was to identify the 5-HT_4R in the enteric neurons and spinal ganglia of healthy horses and in visceral extrinsic sensory fibers of lethal white foal syndrome (LWFS) foals. Immunohistochemistry was used to characterize the expression of 5-HT_4R in enteric neurons and spinal ganglia of healthy horses, and in extrinsic nervous fibers of LWFS foals. 5-HT_4R -IR myenteric and submucosal plexus neurons were quantified as a relative percentage (mean ± St. Dev), in consideration of the total number of HuC/HuD neurons. Furthermore, 5-HT_4R-IR nervous fibers were quantified in the mucosa and *tunica muscularis* as percentage of 5-HT_4R-IR area (mean ± SEM). 5-HT_4R was localized to large percentages of enteric neurons ranging from 28±9 %

(descending colon) to 63±19% (ileum) in the myenteric plexus, and from 54±6 % (ileum) to 68±14 % (duodenum) in the submucosal plexus. 5-HT_4R–IR was co-expressed by Substance P (SP) -IR spinal ganglion neurons and by SP-IR extrinsic sensory fibers of LWFS foals. 5-HT_4R is localized to large percentages of enteric neurons and extrinsic sensory nervous fibers. These findings represent a morphological support as a reinforcement for the functional investigations carried out on the horse intestine. Furthermore, the expression of 5-HT_4-R-IR in extrinsic sensory fibers and extrinsic sensory neurons opens a new window on the pharmacological treatment of equine visceral nociception.

Extrinsic innervation of the ileum and pelvic flexure of foals with ileocolonic aganglionosis

Equine ileocolonic aganglionosis - or lethal white foal syndrome - is a congenital severe condition characterized by neural crest progenitors' unsuccessful colonization of the caudal part of the small intestine and of the entire large intestine. LWFS, due to a mutation in the endothelin receptor B gene, is the horse equivalent of Hirschsprung's disease in humans. Affected foals suffer from aganglionosis or hypoganglionosis of the enteric ganglia resulting in intestinal akinesia and colic. In other species with aganglionosis, fibers of extrinsic origin show an abnormal distribution pattern within the gut wall, while we have no information to date regarding this happening in horses. The aim of the present research was to investigate the distribution of extrinsic sympathetic and sensory neural fibers in the LWFS, focusing on ileum and the pelvic flexure of the colon of two LWFS foals compared to a control subject. The sympathetic fibers were immunohistochemically identified with the markers tyrosine hydroxylase and dopamine beta-hydroxylase. The extrinsic sensory fibers were identified with the markers SP and calcitonin gene-related peptide (CGRP). Since SP and CGRP are also synthetized by subclasses of horse intramural neurons, LWFS represents a good model for the selective study of extrinsic fibers distribution. Affected foals showed large bundles of extrinsic fibers, compared to the control, as observed in Hirschsprung's disease. Furthermore, altered adrenergic pathways were observed, prominently in the pelvic flexure. Since the ENS contains peptidergic neurons, in LWFS tissues the SP- and CGRP-immunoreactivities were dramatically reduced in both ileum and pelvic flexure; the remaining sensory extrinsic fibers resulted largely distributed around submucosal blood vessels and were in part dedicated to the innervation of the mucosa and serosa. These findings

highlight that the extrinsic innervation, contributing to modulate the enteric functions, might also be affected during LWFS.

Quantification of nitrergic neurons in the myenteric plexus of gastric antrum and ileum of healthy and diabetic dogs.

Diabetes mellitus (DM) in humans and mammals determines a wide array of severe clinical complications including gastrointestinal motility disorders. While the effects of experimentally induced DM on the enteric nervous system of rodents are widely investigated, the information is limited in domestic animals. The present study investigates the effects of spontaneous DM on the nitrergic neurons of the myenteric plexus of the canine gastric antrum and ileum, which, in other species, seem to be susceptible to the development of diabetic neuropathy. Specimens of gastric antrum and ileum from eight control dogs and five insulin-dependent DM dogs were collected. Myenteric plexus neurons were immunohistochemically identified with the anti-HuC/HuD antibody as a pan-neuronal marker, while nitrergic neurons were identified with the antibody anti- nNOS. Nitrergic neurons were quantified as a relative percentage, in consideration of the total number of HuC/HuD neurons. In the stomachs of the control dogs, the percentage of nitrergic neurons was 30±6%, while in the DM dogs, it was 25±2% (P=0.112). In the ileum of the control dogs, the percentage of nitrergic neurons was 29±5%, while in the DM dogs, it was significantly reduced 19±5% (*P*=0.006). Notably, the ileal ganglia of DM dogs showed a thickening of the periganglionic connective tissue and an altered HuC/HuD labeling. These findings indicate that DM in dogs alters intestinal nitrergic innervation more rather than the gastric one.

Functional and neurochemical abnormalities in patients with Parkinson's disease and chronic constipation

Chronic constipation (CC) represents one of the most common gastrointestinal complaints in Parkinson’s disease (PD). The pathogenetic mechanisms underlying CC in PD remain poorly understood. The present study has been designed to shed light on clinical, manometric and neurochemical/molecular findings in constipated PD patients.

Twenty-nine PD/CC and 10 CC Rome III defined patients were enrolled and assessed via colonic transit time and conventional anorectal manometry. Twenty asymptomatic (screening colonoscopy) age-sex matched subjects served as controls. Whole-mounts of colonic submucosa from PD/CC, CC and control subjects were processed for immunohistochemistry and vasoactive intestinal polypeptide (VIP) and related receptor mRNA expression. Four groups of PD/CC patients were characterized: 1) delayed transit and altered manometry (65%); 2) delayed transit (13%); 3) altered manometric pattern (13%); 4) no functional impairment (9%). There were no differences in the number of HuC/HuD neurons/ganglion between PD/CC *vs*. CC *vs*. controls. However, a reduced number of HuC/HuD/VIP submucosal neurons was found in PD/CC (72.3±14.6%) *vs.* controls (87.2±9.2%) ($P = 0.007$). *VIP* mRNA expression was also reduced in PD/CC *vs.* CC ($P = 0.036$) and controls ($P < 0.0001$). Both *VIPR1* and *VIPR2* were lower in PD/CC *vs.* CC ($P = 0.001$) and controls ($P<0 .0001$). Most PD/CC patients showed an impairment of colonic motor and rectal sensory functions. The decrease of VIP expressing secretomotor neurons suggests that both neurally-mediated secretory mechanisms along with sensory-motor abnormalities represent a prominent peculiar mechanisms underlying PD/CC patients.

The present collection of studies was focused on three different species (horses, dogs and humans). The physiology of the GI tract is characterized by a high complexity and it is mainly dependent on the control of the intrinsic nervous system, in all the species considered. There are great differences between the ENS features across similar species, therefore it is very difficult to extrapolate and speculate among animals of different size and alimentary tract morphology and physiology. As consequence, the vast majority of the information that we have about the ENS are not adequate to understand completely the physiology and the pathophysiology in a given species. Any damage able to alter the morpho-functional integrity of the ENS may have a severe impact on the GI balance, resulting in many different pathological conditions both in humans and domestic animals. Therefore, the knowledge of the anatomy and the pathology of the ENS represents a new important and fascinating topic, which deserves more attention in the veterinary medicine field.

BACKGROUND

INTRODUCTION

The enteric nervous system (ENS) is large, complex, and exquisitely able to control gastrointestinal (GI) behavior independently of the central nervous system (CNS). An intact ENS is essential for life and ENS dysfunction is often associated to digestive disorders. Nevertheless, the mechanisms underlying ENS changes and the resultant GI dysfunction are starting to be only recently understood. The gut is a complicated organ since a number of processes / events are necessary for digestion and absorption to occurr. Muscular sphincters compartmentalize the bowel, dividing it into functionally distinct regions with radically different luminal environments. A constant detection of luminal contents allows for ingested material to be transported anally at a physiological rate, i.e. allowing each segment of the gut to accomplish their respective function.

Smooth and skeletal (in the esophagus and anus) muscle contractions are thus coordinated into activity patterns, such as segmentation (small intestine) or haustration (colon) that grind, mix, and propel aborally the ingested food. Secretory mechanisms maintain a regionally appropriate pH, as well as tightly regulated concentrations of electrolytes, enzymes, and mucous. A thin semipermeable epithelial barrier, which is continuously regenerated from stem cells, separates the lumen from the body's internal *milieu*. This barrier facilitates absorption, but also prevents the leakage of essential molecules into the intestinal lumen as well as the translocation of digestive enzymes, toxins, and microbiota into the body from the lumen. A scaffold of loose connective tissue, which contains the body's largest array of immune effector cells, provides mechanical and defensive support for the barrier. All of these functions, i.e. secretion, motility, mucosal maintenance, and immunological defense, require an exquisite degree of regulation and coordination, provided by the ENS.

The ENS dysplays integrative neuronal activity and the ability to control GI behavior independently of input from brain or spinal cord. More than any other part of the peripheral nervous system (PNS), the ENS has at least as many neurons as the spinal cord organized in microcircuits, with interneurons and intrinsic primary afferent neurons (*IPAN*s), which are able to initiate reflexes. Enteric neuronal phenotypic diversity is extensive and virtually every class of neurotransmitter found in the CNS has also been detected in the ENS. Although the ENS can function without input

from the CNS, the latter influences enteric behavior and the gut responde by sending information to the brain. Some of the signals that the brain receives initiate vago-vagal reflexes in which motor neurons within the CNS respond to intra-enteric stimuli to regulate motility patterns in the esophagus or stomach. From this picture it emerges clearly that the ENS contributes significantly for body homeostasis and therefore there is no surprise that when any noxae interfere with ENS maintenance and integrity, disease state can occur and their severity may be such to be imcompatible with life.

The physiology of the GI tract is quite complex. A wide spectrum of cellular types such as muscle and epithelial cells, vasculature, blood cells and nerves are involved. All these elements co-exist and should be in balance likewise musicians in a big orchestra, which need the accurate and devoted supervision of the music director for the execution of wonderful symphonies. In the GI tract, we can identify, as master conductor, the enteric nervous system.

The history of the ENS started in the 19th century, when Meissner (Meissner 1857) and Auerbach (Auerbach 1864) identified an intrinsic nervous system in the human GI wall (Timmermans et al. 1997). Subsequently, Ramón y Cajal described the existence of non - neuronal cell type between the muscle cells of the intestine (Cajal 1893). Some years later, the Russian scientist Dogiel classified the enteric neurons according to their morphologic features (Dogiel 1899). A the same time Bayliss and Starling observed that an isolated segment of small bowel / colon from the dog was able to respond to mechanical stimulation in the absence of the extrinsic innervation (Bayliss and Starling 1899; Bayliss and Starling 1900). In particular, they identified a polarized movement consisting in a contraction (cranially) coupled with a relaxation (caudally), hence providing the description of what they called 'the law of the intestine', later on referred to as 'peristalsis' (from the ancient greek "περιστέλλω" meaning "to squeeze" or "push all around") by Trendelenburg (Trendelenburg 1917). Finally, in the 1905, Langley and Magnus demonstrated that the peristalsis was persistent after the degeneration of extrinsic neuronal input, thus implying the autonomy of the ENS from the CNS (Langley and Magnus 1905). It is thought that this discovery marked the beginning of enteric neuroscience as an independent discipline subsequently redefined as neurogastroenterology, a term coined by Wingate in 1988 (Wingate 2008).

The innervation of the digestive tract is fundamental to many different functions: determining the patterns of GI motility, controlling gastric acid secretion, regulating movement of fluid between the gut lumen and body fluid

compartments, changing local blood flow, releasingof gut hormones, modifying nutrient handling and interacting with the gut immune system.

The ENS represents one of the main players in the neural control of GI physiology, especially of the small and large intestines. The ENS activity is integrated by the two other sections of the autonomic nervous system (ANS), i.e. the sympathetic and the parasympathetic divisions through which is connected to the CNS. In fact, intrinsic (enteric) reflexes, as well as those that pass through sympathetic or vagal / sacral ganglia, or intestinofugal reflexes (going back to the CNS) interact each other and provide an integrated physiology of gut function referred to as "*brain-gut axis*" or, more appropriately the "*gut-brain axis*".

The present thesis is a collection of five experimental studies investigating the ENS in species of veterinary interest (such as dog and horse) and also in humans. In particular, the aim of the present research is to highlight the ENS anatomical basis and related pathological aspects.

Firstly, we developed two anatomical studies in horses:

1) "Excitatory and inhibitory enteric innervation of horse lower esophageal sphincter".

2) "Localization of 5-hydroxytryptamine 4 receptor (5-HT_4R) in the equine enteric nervous system".

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4) A secondary enteric neuropathy in diabetic dogs: "Quantification of nitrergic neurons in the myenteric plexus of gastric antrum and ileum of healthy and diabetic dogs".

5) A secondary enteric neuropathy in human neurological patients: "Functional and neurochemical abnormalities in patients with Parkinson's disease and chronic constipation".

The knowledge of the ENS organization can contribute to better understanding its evolution and morphological and functional features. Furthermore, the knowledge of morphological and functional ENS organization is pivotal to comprehend numerous human and animal digestive diseases. The ENS of different species, such as rodents, guinea-pig and other easy-handling small mammals, have been widely studied as experimental animal to decipher the complexity of

the ENS. However, numerous papers dealing with small and large mammals have also been published over the last ten years. There are great differences between the ENS features across similar species, therefore it is very difficult to extrapolate and speculate among animals of different size and alimentary tract morphology and physiology. As consequence, the vast majority of the information that we have about the ENS are not adequate to understand completely the physiology and the pathophysiology in a given species.

Undoubtly, the characterization of the anatomical aspects of the ENS in large mammals and the identification of differences and similarities existing between species may represent a fundamental basis to decipher several digestive GI diseases in humans and animals**.**

CHAPTER 1

The Enteric Nervous System

The ENS is a complex neuronal network embedded in the gut wall, which extends from the esophagus to the internal anal sphincter. It is composed of ganglia, i.e. aggregates of neuronal cell bodies and glial cells, along with neural bundles connecting enteric ganglia, and individual nerve fibers projecting to the effectors, including smooth muscle cells, epithelial cells, blood vessels, endocrine cells (Furness 2012).

1.1 Stucture of the Enteric Nervous System

The ENS is composed of about 400-600 millions neurons (in humans) and even more supporting cells (enteric glia) outnumbering from 3 to 5 times enteric neurons (Furness 2006). In the ENS, neuronal cell bodies and glial cells are grouped in ganglia, interconnected by nerve fiber bundles, forming two major plexuses, the myenteric (MP or Auerbach's) and submucosal (SMP or Meissner's) plexus (Costa and Brookes 2008). The MP is located between the outer (longitudinal, LML) and inner (circular, CML) muscle layers of the whole GI tract (Furness 2006). The SMP is located between the *muscularis mucosae* and the CML. Although MP and SMP are single entities, they are connected by *vertical fibers* running perpendicular to the CML (Furness et al. 1990; Brehmer et al. 1998) (**Fig. 1.1**).

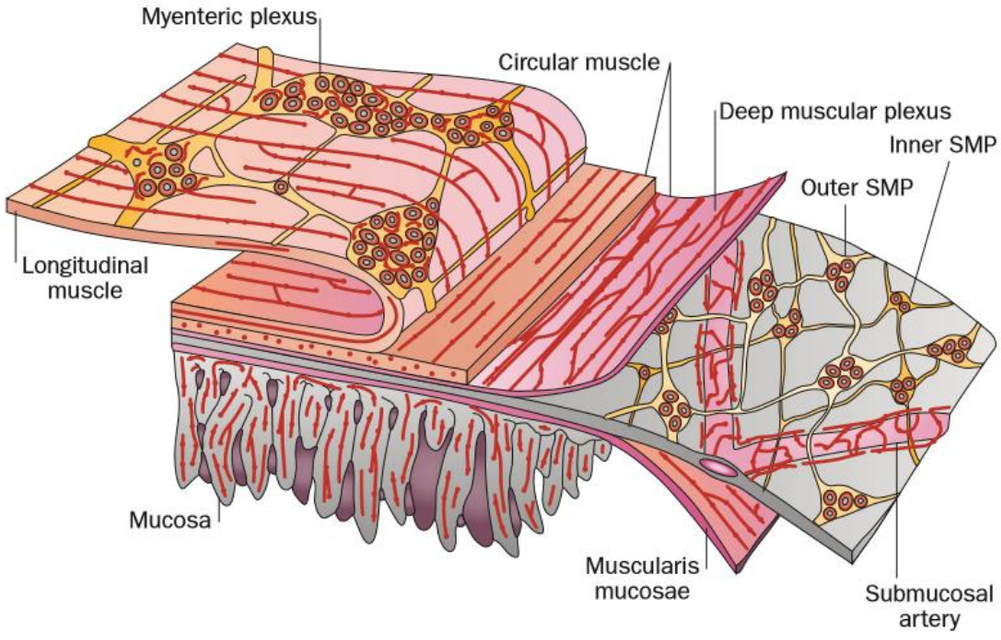

Fig. 1.1 The organization of the ENS of human and medium–large mammals.
The ENS has ganglionated plexuses, the myenteric plexus between the longitudinal and circular layers of the external musculature and the SMP that has outer and inner components. Nerve fiber bundles connect the ganglia and also form plexuses that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries and the mucosa. Modified from Furness 2012.

In addition to ganglionated plexuses, the ENS also contains aganglionated plexuses formed by nerve fibers. These plexuses are constituted by axons of neurons located in the MP and SMP, as well as by extrinsic fibers (Furness 2006). The intrinsic innervation of the extrahepatic biliary tract and pancreas are considered part of the ENS because of their embryological origin emanating from diverticula of the small intestine (Furness 2006).

Submucosal plexus (SMP)

The SMP is well developed in both small and large intestine, while only a few submucous ganglia can be found in esophagus and stomach (Lefebvre et al. 1995; van Ginneken et al. 1996; Teixeira et al. 2001; Izumi et al. 2002; Furness 2006). In general, the interconnecting strands of the SMP are quite fine and SMP ganglia are small. The SMP is organized in a single layer in small mammals (Timmermans et al. 2001; Furness 2006), while it is a multilayered, double or triple layered in the large mammals (Brehmer et al. 2010). In large mammals two different ganglionated plexuses are usually identified: the internal submucous plexus (ISMP) and the external submucous plexus (ESMP) (Schabadasch

1930) (Gunn 1968) (Messenger and Furness 1990; Timmermans et al. 1992a; Timmermans et al. 1992b; Pearson 1994; Timmermans et al. 1997). The ganglia of the ESMP and ISMP, are separated by a thin connective tissue layer (Gunn 1968; Christensen and Rick 1987; Hoyle and Burnstock 1989; Balemba et al. 1998) and by submucosal vascular arcades (Balemba et al. 1998). ESMP and ISMP appears different in relation to the species and the GI tract investigated. Generally, they can be distinguished on the basis of their location, architecture, meshwork density, size and form of the ganglia, vascularization, and light microscopic appearance (Stach 1977b; Stach 1977c; Stach 1977a; Stach 1978; Scheuermann and Stach 1984). The ESMP shows the most irregularly organized nerve meshwork, while the ISMP meshwork is smaller and more regular compared to the ESMP (Gunn 1968; Scheuermann et al. 1987; Hoyle and Burnstock 1989; Timmermans et al. 1990; Balemba et al. 1999).

Additionally, the two compartments of SMP neurons also show differences in their neurochemical code, being the phenotypes of ESMP neurons more similar to that of myenteric neurons (Scheuermann W.D. 1998; Hens et al. 2000). In fact, some neurons of the ESMP, beyond the mucosa, also supply innervations to the CML and LML (Sanders and Smith 1986; Furness et al. 1990; Timmermans et al. 1994b; Timmermans et al. 1997; Porter et al. 1999; Timmermans et al. 2001). The ISMP neurons, in large part, supply the mucosa, and only a small number have projections to the muscle layers (Porter et al. 1999; Timmermans et al. 2001).

ESMP and ISMP neurons overlap in terms of functional control of fluid movements, local blood flow and sensory functions; in addition, however, the ESMP also exerts a role on motility (Timmermans et al. 1990).

Myenteric plexus

The myenteric plexus forms a continuous network, around the circumference of the gut and extending from the upper esophagus to the internal anal sphincter. It lies between the LML and CML and is composed / constituted by a network of cells grouped in ganglia and nerve strands (Furness 2006).

The MP shows numerous differences in its morphological organization, among different gastrointestinal tracts and different species. In the horse ileum, in addition to the classical MP, two other ganglionated MP divisions have been described, located within the inner portion of the LML and in subserosal location (Vittoria et al. 2000; Domeneghini et al. 2004; Chiocchetti et al. 2009b). A subserosal ganglionated plexus was also detected in the cattle abomasum (Vittoria et al. 2000) and in the human sigmoid colon (Crowe and Burnstock 1990). However, in all other human colonic levels

examined (ascending, transverse and descending colon) the subserosal plexus showed no ganglia but only nerve fibers (Ibba-Manneschi et al. 1995).

Generally, in the MP, ganglia are bigger than the SMP in terms of number of neurons, and are linked by interconnecting strands (or primary strands) which constitute the MP primary plexus (Furness 2006). The myenteric ganglia are aligned parallel to the CML, although it can vary between species (Irwin 1931); primary interconnecting strands show longitudinal course, an organization that seems to be a distinctive feature of the MP in the major part of small and large mammals (Scheuermann et al. 1986; Gabella 1987; Santer and Baker 1988; Pearson 1994; Furness 2006; Freytag et al. 2008; Bodi et al. 2009). However, the orientation can vary from one part of the intestine to another (Furness 2006) and between species, including large and small mammals (Balemba et al. 1999; Chiocchetti et al. 2004; Furness 2006; Bodi et al. 2009). The other two components of MP are the secondary and tertiary plexuses (Furness 2006). The secondary plexus is constituted by fine bundles of nerve fibers, mainly oriented parallel to the CML and primary plexus (Balemba et al. 1999; Furness 2006). The tertiary plexus is constituted by the smallest sized interconnecting strands supplying the LML (Scheuermann et al. 1986; Balemba et al. 1999; Furness 2006).

Extrahepatic biliary tree and pancreatic divisions of ENS

The structural and architectural organization of the innervations of the biliary tract have been most extensively studied in the guinea-pig and Australian brush-tailed possum and appear very similar even in large mammals, such as human, rhesus monkey, pig, dog and cat (Alexander 1940; Burnett et al. 1964; Sutherland 1967; Kyosola 1978; Talmage et al. 1996; Mawe 2000; Balemba et al. 2004).

The extension of the biliary ENS is limited to the extrahepatic biliary tree, which includes the gallbladder, the sphincter of Oddi (SO), and the bile ducts. All these portions show a three layered wall (serosa, muscularis, and mucosa); ganglionated nerve plexuses are located in subserosal, muscular and subepithelial (lamina propria) layers.

Gallbladder. The subserosal plexus is constituted by ganglia which have an appearance similar to submucosal ganglia of the intestine. Due to the topographical distribution of nerves and ganglia on different levels, the subserosal plexus has been subdivided into the secondary and tertiary plexuses (Burnett et al. 1964; Sutherland 1966; Mawe and Gershon 1989; Mawe 2000). The muscular plexus is situated deeply, near or within the muscular layer, whereas, the subepithelial plexus is located just underneath the epithelium (De Giorgio et al. 1995; Balemba et al. 2004). The prominent presence

of ganglionated plexuses in the lamina propria is a data consistent in all large species studied such as opossum, Australian brushtailed possum, monkey, and human (Sutherland 1967; Talmage et al. 1996; Balemba et al. 2004). The subepithelial plexus is exclusively composed of neurons projecting to the mucosa; it is similar to the intestinal ISMP, which is thought to innervate primarily the epithelium (Burnett et al. 1964). The ganglionated subserosal and muscular plexuses are functionally similar to the intestinal MP, which innervates the smooth muscle layers (Padbury et al. 1993; Meedeniya et al. 2001; Meedeniya et al. 2003).

Bile ducts. The walls of the cystic duct, hepatic ducts and common bile duct have an intrinsic innervation similar to that of the gallbladder, but since they also have a thinner and more irregular muscle coat than gallbladder, a comparable reduction of intramural plexuses can be observed (Burnett et al. 1964). Furthermore, ganglia are less numerous than in the gallbladder and are usually located in the outer aspect of the wall (Padbury et al. 1993). It has been shown that intrahepatic bile ducts and peribiliary glands are innervated mainly by extrinsic nerves (Balemba et al. 2004).

Sphincter of Oddi. The intrinsic nerve components of the SO show a noticeable increase in their complexity. The nerve plexuses are more developed and the ganglia are larger and numerous. In the SO of the Australian brush-tailed possum Padbury et al. (1993) have identified various neural plexuses, namely the external sphincteric plexus, the intersphinteric plexus, the internal sphincteric plexus, ganglia situated in the fibromuscular septum between common bile duct and pancreatic duct, and ganglia located in the lamina propria. The external sphincteric plexus was located between the longitudinal and circular muscle layers and was continuous with the duodenal MP. The inter- and internal sphincteric plexuses could be continuous with the duodenal SMP.

Pancreatic division. The pancreatic division of the ENS shows a different morphological organization from the other divisions previously described. In fact, the pancreas is not a hollow organ but a solid organ, with a typical anatomical organization that include both a parenchymal and a stromal divisions. It is in the latter division that the nervous component is located, adapting to the available spaces. The ganglia and interganglionic nerve strands constitute an extensive interconnecting network in the interlobular and intralobular connective tissue (Coupland 1958). Nerve fibers and multipolar neurons were observed in association with blood vessels, acinar tissue, and with the islets of Langerhans (Gentes 1902; Pensa 1905; DeCastro 1923). The size of ganglia and the number of their neurons decrease from lobus dexter to lobus sinister of the pancreas (Richins 1945; Honjin 1956; Coupland 1958; Sha et al. 1996; Love and Szebeni

1999). Furthermore, in a typical pancreatic ganglion there are fewer neurons compared to the number present in small intestine MP ganglia (Sha et al. 1996).

Until recently, pancreatic ganglia were considered postganglionic parasympathetic elements which function as relay centers for incoming signals from the preganglionic parasympathetic neurons and postganglionic sympathetic ones (Liu and Kirchgessner 1997). However, it is now known that pancreatic ganglia share some neurochemical and functional characteristics of ENS ganglia (De Giorgio et al. 1992). These similarities include the organization of pancreatic ganglia into a network anatomically and functionally interconnected (King et al. 1989; Kirchgessner and Pintar 1991; Liu and Kirchgessner 1997) and the content of many substances known to be present in enteric neurons (De Giorgio et al. 1992; Kirchgessner 1999). Furthermore, pancreatic ganglia have been shown to be directly connected with gastric and duodenal MP (Kirchgessner and Gershon 1990). The reciprocal connection between pancreatic and myenteric neurons may be at the basis of local reflexes coordinating pancreatic secretion and gastrointestinal function and originate in either the gut or the pancreas.

1.2 Enteric neurons

Neurochemical code

Enteric neurons are able to synthesize and release several (about 30) substances that may act as messengers, i.e. neurotransmitters, neuromodulators and neuropeptides. Each functional class of enteric neurons can be characterized based on the combination of messengers that it contains. This property is known as neurochemical code (Costa et al. 1996).

Enteric neurotrasmitters are classified as primary and secondary neurotrasmitters. Primary neurotrasmitters are conserved across mammals and they exert the same role in different species and along the GI tracts; these substances include acetylcholine (ACh) and tachykinins in enteric excitatory motor neurons, and nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) in inhibitory motor neurons. Inhibitory motor neurons may be immunohistochemically identified by the presence of the neuronal nitric oxide synthase (nNOS), the neuronal form of the enzyme synthesizing the nitric oxide, the primary neurotransmitter utilized by ENS neurons , while excitatory motor neurons may be

immunohistochemically identified by the presence of the synthesizing enzyme choline acetyltransferase (ChAT).Secondary neurotransmitters or modulators include substances which may vary among different groups of neurons depending on the GI tract and the species considered (Furness 2006) (**Table 1.1**).

Type of neuron	Primary transmitter	Secondary transmitters, modulators	Other neurochemical markers
Enteric excitatory muscle motor neuron	ACh	Tachykinin, enkephalin (presynaptic inhibition)	Calretinin, γ-aminobutyric acid
Enteric inhibitory muscle motor neuron	Nitric oxide	VIP, ATP or ATP-like compound, carbon monoxide	PACAP, opioids
Ascending interneuron	ACh	Tachykinin, ATP	Calretinin, enkephalin
ChAT, NOS descending interneuron	ATP, ACh	ND	Nitric oxide, VIP
ChAT, 5-HT descending interneuron	ACh	5-HT, ATP	ND
ChAT, somatostatin descending interneuron	ACh	ND	Somatostatin
Intrinsic sensory neuron	ACh, CGRP, tachykinin	ND	Calbindin, calretinin, IB4 binding
Interneurons supplying secretomotor neurons	ACh	ATP, 5-HT	ND
Noncholinergic secretomotor neuron	VIP	PACAP	NPY (in most species)
Cholinergic secretomotor neuron	ACh	ND	Calretinin
Motor neuron to gastrin cells	GRP, ACh	ND	NPY
Motor neurons to parietal cells	ACh	Potentially VIP	ND
Sympathetic neurons, motility inhibiting	Noradrenaline	ND	NPY in some species
Sympathetic neurons, secretion inhibiting	Noradrenaline	Somatostatin (in guinea pig)	ND
Sympathetic neurons, vasoconstrictor	Noradrenaline, ATP	Potentially NPY	NPY
Intestinofugal neurons to sympathetic ganglia	ACh	VIP	Opioid peptides, CCK, GRP

Table 1.1 Multiple transmitters of neurons that control digestive function.

Abbreviations: 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; CCK, cholecystokinin; ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; GRP, gastrin releasing peptide; ND, not determined; NPY, neuropeptide Y; NOS, nitric oxide synthase; PACAP, pituitary adenylyl-cyclase activating peptide; VIP vasoactive intestinal peptide. (Furness 2012)

During years of studies on the ENS, enteric neurons have been classified on the basis of various criteria: shape, histochemical and immunohistochemical staining, projections, electrophysiological and functional properties.

Morphological aspects.

Enteric neurons were subdivided based on their shape by Dogiel in (Dogiel 1899), using a methylene blue staining technique. MP and SMP neurons were morphologically classified in three types, referred as Dogiel types I, II and III. After Dogiel, many other authors proposed additional classifications based on individual neuronal morphology revealed by silver impregnation techniques, neuronal immunoreactivity for neurofilaments and / or other markers (Stach 1989; Brehmer et al. 1999; Stach 2000; Brehmer et al. 2002; Brehmer et al. 2004b). Taken together, these studies confirmed and extended the Dogiel classification into type I, II and III up to IV, V, VI and VII as well as 'mini-neurons'. Often the importance of the morphological classifications of enteric neurons is underestimated (Brehmer 2006). However, the ability to correlate the morphological appearance of a neuron to its neurochemical code, as well as to its potential functional features has also reassessed the importance of studying the morphology of the enteric nerve cells.

Functional aspect

Functionally, the enteric neurons can be divided in motor neurons, interneurons, *IPANs* (Furness 2000), and intestinofugal neurons *IFAN*s (Furness 2003) (**Fig. 1.2**).

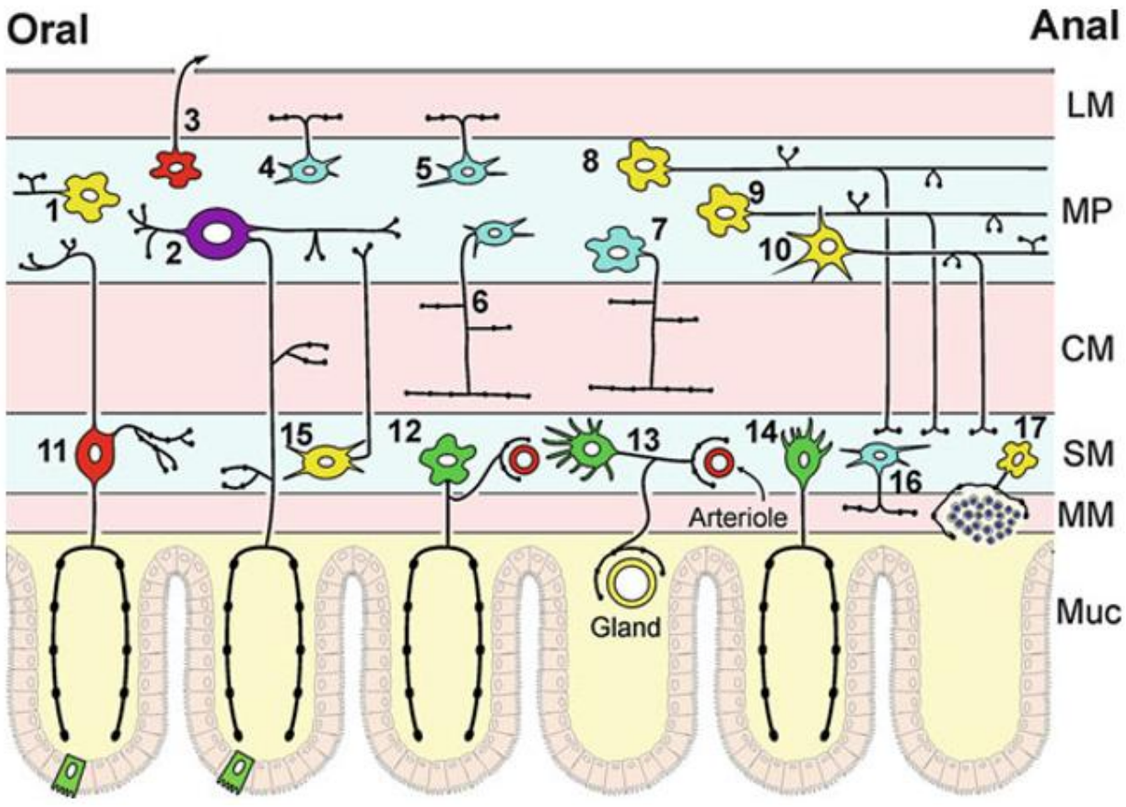

Fig. 1.2 Neuron types in the ENS. The types of neurons in the small intestine.

Neuron Types: Ascending interneurons (1); Myenteric intrinsic primary afferent neurons (IPANs) (2); Intestinofugal neurons (3); Excitatory longitudinal muscle motor neurons (4); Inhibitory longitudinal muscle motor neurons (5); Excitatory circular muscle motor neurons (6); Inhibitory circular muscle motor neurons (7); Descending interneurons (local reflex) (8); Descending interneurons (secretomotor and motility reflex) (9); Descending interneurons (migrating myoelectric complex) (10); Submucosal IPANs (11); Non-cholinergic secretomotor/ vasodilator neurons (12); Cholinergic secretomotor/vasodilator neuron (13); Cholinergic secretomotor (non-vasodilator) neurons (14); Uni-axonal neurons projecting to the myenteric plexus (15); motor neuron to the muscularis mucosa (16); innervation of Peyer's patches (17). Abbreviations: LM longitudinal muscle, MP myenteric plexus, CM circular muscle, SM submucosal plexus, Muc mucosa. Modified from Furness 2012.

Motor neurons. The category of motor neurons includes excitatory and inhibitory neurons directed to gut musculature; secretomotor/vasodilator neurons are able to regulate mucosal secreting cells and vasodilation / vasoconstriction of intestinal vasculature. Furthermore another subset is represented by secretomotor neurons and neurons innervating entero-endocrine cells (Furness et al. 2000).

The motor neurons innervating the smooth muscle of digestive tract are located within the MP of rodents (Wilson et al. 1987; Song et al. 1998) and within myenteric and external and internal submucous plexuses of large mammals [MP>ESMP>ISMP] and humans [MP>ESMP > ISMP] (Sanders and Smith 1986; Timmermans et al. 1990; Timmermans et al. 1994b; Porter et al. 1999; Hens et al. 2001). These neurons could be distinguished in circular muscle motor neurons, longitudinal muscle motor neurons, and motor neurons innervating the *muscularis mucosae (mm)*. According to the description of the peristaltic reflex, the excitatory motor neurons are especially localized aborally to the innervated circular and longitudinal muscle (Brookes et al. 1991) (Michel et al. 2000), and the inhibitory motor neurons are generally localized orally to the innervated circular and longitudinal muscle (Brookes et al. 1992; Brookes et al. 1996; Brookes et al. 1997; Pfannkuche et al. 1998; Yuan and Brookes 1999; Michel et al. 2000). However, the polarized projection patterns of enteric neurons apparently are species- and region-dependent.

Motor neurons can be also distinguished, on the basis of the distance from their target, in long and short (excitatory and inhibitory) motor neurons (Brookes et al. 1991; Brookes 2001). Other well preserved features of enteric motor neurons can be found in their neurochemical code. In fact, it is well established that the primary transmitter of excitatory muscle motor neurons is Ach, although tachykinins (especially substance P, SP) are co-localized with ACh in these neurons (Vassileva et al. 1978; Veenendaal et al. 1982; Wong and McLeay 1988; Bornstein et al. 2004). The NO is considered the main transmitter of inhibitory muscle motor neurons; however it is clear that different transmitters are implicated in the inhibitory neurotransmission (Furness 2000) including ATP (Yamanaka et al. 1985; Bian et al. 2000), VIP (Fahrenkrug and Emson 1982; Costa et al. 1992) (Furness et al. 1992; Aimi et al. 1993; Grider 1993; Barbiers et al. 1994; Timmermans et al. 1994a; Timmermans et al. 1994b; Keranen et al. 1995; Lefebvre et al. 1995; Porter et al. 1999; Lomax and Furness 2000; Munnich et al. 2008), pituitary adenylate cyclase-activating polypeptide (PACAP) (Grider et al. 1994; Ny et al. 1995) and carbon monoxide (De Backer and Lefebvre 2007) being all these substances often co-localized in the same cells.

Many similarities exist in the organization of small and large mammals ENS. Muscle motor neurons with analogous functions can employ the same neurotransmitters in different species, however important differences in the distribution of these cells can be observed by comparing the same gastrointestinal region in different mammals. The polarized

organization of enteric motor neurons is important to allow the peristaltic reflex, as originally implied by Bayliss and Starling (1899).

The organizational principles just described could constitute a general rule applicable to the ENS of all studied species. Concerning large mammals, also in the bowel of pig, horse and human, can be found similar adaptations. In fact, in the swine and horse ileum as well as in human descendant and sigmoid colon, segments with high tone and propulsive functions showed an higher proportion of MP cholinergic than proportion of nitrergic ones (Hasler et al. 1990; Takahashi and Owyang 1998; Brehmer et al. 2004b; Freytag et al. 2008; Wattchow et al. 2008; Chiocchetti et al. 2009a). On the contrary, in the human ascendant colon, a tract with a low tone and functions of accommodation and mixing (Hasler et al. 1990; Takahashi and Owyang 1998), the MP contains a number of nitrergic neurons greater than cholinergic ones (Wattchow et al. 2008).

Among motor neurons, two important subgroups are secretomotor and vasomotor neurons. Hens et al. (2000) showed that in all the ganglionated plexuses of the pig small intestine there are mucosal projecting neurons [ISMP (78%) > ESMP (10%) ≈ MP (12%)]. Dogiel type II neurons exhibiting calcitonin gene-related peptide (CGRP) immunoreactivity (-IR) (MP>ESMP>ISMP) are probably afferent in function; however other mucosal projecting neurons, showing different morphology and phenotype, are present in MP and in both divisions of SMP. The majority of ISMP neurons projecting to mucosa are ChAT/SP- or VIP/Galanin-IR minineurons (Hens et al. 2000); (Timmermans et al. 2001; Brown and Timmermans 2004), whereas in both ESMP and MP most of neurons projecting to mucosa are multidendritic in morphology and ChAT/Somatostatin (SOM)-IR (Hens et al. 2000). Hens et al. (2001) showed that in human jejunum the greater proportion of mucosal projecting neurons are located in the ESMP [ESMP (54%) > MP (23%) > ISMP (13%) > interSMP (10%)]. However, in both species most of these neurons are located in the SMP, while in rodents, mucosal projecting neurons are more equally distributed between the SMP and MP [61% SMP and 39% MP in mouse 50% SMP and 50% MP in guinea-pig (Song et al. 1991).

Considering the dense network of SP-IR nerve fibers found in the *mm*, as well as pharmacologic data showing an involvement of SP as mediator in excitatory response of the *mm* (Angel et al. 1984; Steele and Costa 1990; Messenger 1993; Holzer and Holzer-Petsche 1997), it has been suggested that SP-IR small neurons could mainly be involved in the *mm* innervation, rather than of the epithelium (Hens et al. 2000). However SP is also widely distributed in the nerve

fibers in relationship to the cores of the villi, intestinal glands, and muscular sheath of blood vessels (Costa et al. 1981; Brodin et al. 1983; Domeneghini et al. 2004; Chiocchetti et al. 2009a; Mitsui 2010).

The anatomical distribution of a dense network of VIP-IR nerve fibers in the intestinal villi, together with the evidence of its secretory actions in porcine intestine (Brown 1991; Wood 2006), suggests that VIP minineurons are secretomotor neurons. SOM-IR nerve fibers in the lamina propria have been described in both human (Keast et al. 1984) and pig (Timmermans et al. 1990) small intestine. Pharmacological and physiological evidence suggests an antisecretory role for SOM-IR neurons (Dharmsathaphorn et al. 1980; Sandle et al. 1999). Furthermore, the presence of specific SOM binding sites on the basolateral membranes of enterocytes indicates a direct action of SOM onto the epithelium (Dharmsathaphorn et al. 1985; Cooke 1986; Weber et al. 1986).

Although mucosal projecting neurons show different topographical organization between small and large mammals, these cells seem to show a certain degree of consistency in their neurochemical code. In fact, in all studied species, small neurons expressing VIP-IR are mucosal projecting neurons that are likely to be involved in secretion processes (Hens et al. 2001; Beyer et al. 2013).

The distribution of SP- and VIP-IR has been widely studied in some other large mammals, while SOM-IR has been less studied than the other two substances. In the horse and cattle intestine, both SP- and VIP-IR neurons have been especially detected in the SMP; furthermore a rich network of SP- and VIP-IR nerve fibers has been also detected in the mucosa in relationship to the *mm*, the lamina propria of the villi and the intestinal glands (Cummings et al. 1985; Burns and Cummings 1993; Pearson 1994; Balemba et al. 1999; Vittoria et al. 2000; Domeneghini et al. 2004; Chiocchetti et al. 2009a). These data reflect a possible secretagogin role for these neurons.

Interneurons. Interneurons seem to be the ENS neurons with the longest projections. These cells, which have been identified with certainly only in the guinea-pig (Costa and Brookes 2008), mouse, rat, and human are mainly localized in the MP. Interneurons form long functional chains of ascending and descending elements through which information may travel for short or long distance (Pompolo and Furness 1993). Interneuron projections extend up to 14 mm anally and up to 136 mm orally in guinea pig small intestine (Song et al. 1997). It is worth noting that interneurons may also function also as mechanoceptors (Stebbing and Bornstein 1996; Spencer et al. 2006; Dickson et al. 2007; Smith et al. 2007). At least four types of interneurons have been identified in the guinea-pig small bowel ENS, one type of ascending

and three types of descending interneurons. The ascending interneurons are MP Dogiel type I cholinergic neurons (Furness 2000; Brookes 2001) and may contain also calretinin, SP, neurofilament triplet protein (NFP), and enkephaline (Brookes et al. 1997). Descending MP interneurons (5% of all ENS cells in guinea-pig) are phenotypically cholinergic neurons differentiable in three types, on the basis of their immunoreactivity also for NOS/VIP, SOM and 5-hydroxytryptamine (5HT) (Portbury et al. 1995; Song et al. 1997; Brookes 2001). nNOS/VIP/ChAT/immunoreactive Dogiel type I interneurons can contain also neuropeptide Y (NPY) (Uemura et al. 1995), gastrin releasing peptide (GRP), bombesin (BN) (Brookes, 2001) and the enzyme alkaline phosphatase (Song et al. 1994). SOM/ChAT immunoreactive Dogiel type III MP neurons (Portbury et al. 1995; Song et al. 1997) project to other MP ganglia and also to SMP ganglia (Brookes 2001). Also 5HT/ChAT immunoreactive Dogiel type I MP neurons send their projections to MP and SMP and seem to have significant roles in excitatory pathways regulating both motility and secretion (Neal and Bornstein 2007).

Data on human interneurons is scanty but it seems that MP interneurons project up to 36 mm anally and up to 70 mm orally. In the human colon, 90% of orally projecting interneurons contain ChAT alone, whereas the other neurons are ChAT- and nNOS-negative; three main types of anally projecting interneurons have been identified: neurons co-expressing ChAT- and NOS-IR, neurons immunoreactive for NOS-IR alone, and neurons immunoreactive for ChAT-IR alone (Porter et al. 2002). Descending nNOS-IR interneurons of human ENS may co-express VIP-IR (Brehmer et al. 2002). Descending neurons with an impressive long projection (up to 80 cm) have been identified in the horse ileum (Russo et al. 2010); these cells co-expressed ChAT- and nNOS-IR, or contained ChAT- or nNOS-IR alone (Chiocchetti et al. 2009b). Also in the sheep ileum, descending (up to 18 cm) and ascending (up to 12–14 cm) long projecting neurons have been observed (Mazzuoli et al. 2007). In the same species, the presence of long projecting descending abomasal (up to 14 cm) and ascending duodenal (up to 8 cm) neurons has been reported (Mazzuoli et al. 2008). The phenotype of sheep putative long projecting interneurons (or motoneurons) is peculiar, since markers such as ChAT, nNOS, and SP are widely co-expressed in both categories of orally and anally projecting neurons.

Intrinsic primary afferent neurons (*IPAN*s). The *IPAN*s are the first neurons of the reflex pathways in the intestine (Furness et al. 2004a). They are involved in the control of physiological functions as motility, blood flow and secretion,

being responding to several stimuli, such as distention, luminal chemistry and mechanical stimulation of the mucosa (Furness 2006).

*IPAN*s have typical electrophysiological properties. In fact, they have broad action potentials that are carried by both sodium and calcium currents and are followed by early and late (slow) after hyperpolarizing potentials (AHPs) (Furness et al. 2004a). Their targets are represented by mucosa and other MP and SMP neurons (Bornstein et al. 1989; Kirchgessner et al. 1992; Song et al. 1992; Evans et al. 1994).

Cell bodies of multi-axonal *IPAN*s are 10-30% of neurons in SMP and MP ganglia of the small and large intestine; no *IPAN*s are detected in the esophagus and they are rare in the stomach, where motility is primary controlled by vagal efferents (Furness et al. 2014).

All the *IPAN*s of guinea-pig small intestine show Dogiel type II morphology (non-dendritic multi-axonal type II neurons) (Kirchgessner et al. 1992; Kunze et al. 1995; Bertrand et al. 1997; Kunze et al. 1998; Kunze et al. 1999), and analogous have been found in other species and regions. A large percentage (about 82-84%) of myenteric *IPAN*s of the guinea-pig ileum expresses immunoreactivity for the calcium-binding protein calbindin (CALB) (Furness et al. 1988; Iyer et al. 1988; Costa et al. 1996), and almost all (MP and SMP *IPAN*s) express cytoplasmic NeuN-IR (Chiocchetti et al. 2003; Van Nassauw et al. 2005). Furthermore, all of them seem to be immunoreactive for ChAT (Steele et al. 1991; Li and Furness 1998). To note that only 30% of submucosal *IPAN*s of the guinea-pig ileum appear to be immunoreactive for CALB (Iyer et al. 1988; Song et al. 1991; Quinson et al. 2001), and that CALB is not confined to the *IPAN*s since it is also localized in 50% of submucosal calretinin-IR secretomotor / vasodilator neurons (Quinson et al. 2001). Many researchers studied CALB-IR also in other species, with the aim to establish whether CALB could be considered an *IPAN*s marker. In the pig small intestine, CALB cannot be considered markers of *IPAN*s, being mainly localized in interneurones and intestinofugal neurons (Brehmer et al. 2004a; Brown and Timmermans 2004). Dénes and Gábriel (Denes and Gabriel 2004) described CALB-IR myenteric neurons in rabbit small intestine. These cells showed essentially Dogiel type I morphology, ChAT-IR, and were identified as interneurons. Also in the mouse colon, CALB cannot be considered a good marker of *IPAN*s, while the anti-CGRP antibody is considered the most specific marker of these cells (Furness et al. 2004b). Non-dendritic multiaxonal type II neurons involved in mucosal innervations have been demonstrated also in porcine (Hens et al. 2000) and human (Hens et al. 2001) small intestine. Unlikely from guinea-pig,

porcine and human (and mouse) *IPAN*s express CGRP, which has been considered a specific marker of type II neurons in these species(Scheuermann et al. 1986) (Scheuermann et al. 1991; Timmermans et al. 1992a; Balemba et al. 1998; Hens et al. 2000; Wolf et al. 2007). However, Brehmer et al. (Brehmer et al. 2004a)showed that in human small intestine only a minority of type II neurons displayed distinct reactivity for CGRP, while most of them were immunoreactive for SOM, calretinin, and SP. Notably, in the human small intestine this neurochemical code is common also to type V neurons; thus, the morphological distinction has an important role to recognize *IPAN*s. The importance to couple morphology and chemical code to identify ENS neurons has been confirmed also in the pig intestine; in fact, Jungbauer et al. (Jungbauer et al. 2006) showed that at least three typology of neurons were CGRP-IR: type I like morphology neurons, type V neurons, and type II ones. Thus, CGRP cannot be considered a sufficient marker to specifically identify type II neurons.

Also in sheep (Chiocchetti et al. 2004; Chiocchetti et al. 2005) and horse (Chiocchetti et al. 2009a) ileum, CGRP is considered a marker of Dogiel type II neurons; however, in these species there are not tracer studies confirming the involvement of these cells in mucosal innervations. In fact, the CGRP-IR neurons of sheep and horse ileum have been hypothesized to be *IPAN*s on the basis of their morphology and on the basis of distribution of CGRP-IR fibers. In pig small intestine, Krammer et al. (Krammer and Kuhnel 1992) and Balemba et al. (1998) showed that also anti-Neurofilament 200 KDa (NF200) antibody identify only type II neurons. In the cattle small intestine, Balemba et al. (1999) showed that the pooled antibodies anti-all the three forms of neurofilament proteins revealed type II neurons in the myenteric, external and internal SMP, differently from the pig in which the same antibodies has been reported to show also types IV and VI neurons (Balemba et al. 1998).

A difference between small and large mammals is in the localization of neurons with mucosal projections; in guinea-pig and mouse intestine these neurons are localized approximately in equal part in SMP and MP. In the pig intestine are especially localized in ISMP (about 80%) with a lesser quantity of neurons also in the ESMP and MP, about 10% each (Hens et al. 2000), whereas in the human intestine greater part of these neurons are localized in ESMP (Hens et al. 2001). It is difficult to speculate on the situation of other species for scantiness of studies. In the horse ileum the neuropeptide CGRP, a putative marker of large mammal *IPAN*s, has been largely found in the submucosal ganglia

(Chiocchetti et al. 2009a) while in the MP these neurons are only scantly represented. This result is consistent with the observation made in pig and human intestine.

Concluding, another important difference between small and large mammals is in the proportion of *IPAN*s and secretomotor neurons. In fact, almost all neurons with mucosal projections in the MP of the guinea-pig small intestine exhibit type II morphology, and are assumed to be *IPAN*s (Song et al. 1992). In both pig and human small intestine, the number of primary afferent neurons in the ESMP and MP seem to represent only a minority (less than 30%) of these neurons (Timmermans et al. 2001).

Intestinofugal primary afferent neurons – *IFAN*s- Intestinofugal neurons represent a unique subset of enteric neurons, presenting their cell body in the myenteric ganglia and projections out of the intestinal wall (Szurszewski et al. 2002). Most of them show Dogiel type I and some Dogiel type II morphology (Lomax et al. 2000; Ermilov et al. 2003). *IFAN*s act as mechanoceptors, being able to detect changes in volume and to respond to the stretching (but not to the tension) of the muscle cells (Crowcroft et al. 1971; Szurszewski and Weems 1976; Kreulen and Szurszewski 1979). Once activated, *IFAN*s usually release Ach at the sympathetic neurons in the prevertebral ganglion (PVG), evoking nicotinic fast post synaptic potentials (Szurszewski JH 1994). A subset of *IFAN*s also determines gamma-Aminobutyric acid (GABA) release in PVG in response to colonic distention, facilitating the Ach release from cholinergic *IFAN*s. As consequence, sympathetic neurons in the prevertebral ganglion, reflexy release noradrenaline in GI wall, modulating muscle contractions or MP neurons control (Parkman et al. 1993; Ma and Szurszewski 1996; Walter et al. 2016). The functional significance of this reflex arc is that it acts against large increases in tone and intraluminal pression during filling (Szurszewski et al. 2002).

1.3 Other cell types interacting with enteric neurons

Glial cells

In addition to neurons, enteric glial cells (EGCs) are identified as key players in the ENS in which these cells represent the largest cell population. They are small cells showing a starlike appearance (Ruhl 2005). A population of

EGCs wraps around neuronal cell bodies in MP and SMP, while another population surrounds mucosal nerves (Gabella 1981; Gershon and Rothman 1991; Ruhl 2005). The EGCs outnumber neurons with a ratio ranging from 1.3 to 1.9 and from 5.9 to 7.0 in the human submucosal and myenteric plexuses, respectively (Komuro et al. 1982; Ruhl 2005; Hoff et al. 2008). The glial cells partly surround nerve cell bodies and axons in the ganglia, leaving bare large areas of neuronal membrane at the surfaces of ganglia. There is a marked contrast in relationships of glial cells to axons in small mammals (for example, guinea-pig or rat) and large mammals (such as cat or human). In small mammals, glial cell processes fail to penetrate all the interstices between nerve cell bodies and between axons in the neuropil (Baumgarten et al. 1970; Gabella 1972; Komuro et al. 1982). In contrast, in enteric ganglia of human and monkey, axons are separated from one another by intervening glial cell processes (Baumgarten et al. 1970). It is noteworthy that Auerbach (1864) recognized the different relations of neurites and supporting cells between humans and some other mammals (Furness 2006). Glial cells in nerve strands of the MP of small mammals give rise to radiating lamellae which divide the axons into large bundles, and up to 600 neurites may be associated with one glial cell (Gabella 1981).

EGCs originate from neural crest progenitors which migrate and colonize the GI tract. Upon colonization of the embryonic gut, neural-crest derived progenitors mature into neurons and glia via Hedgehog/Notch pathway (Gershon and Rothman 1991; Ruhl 2005). Once developed, EGCs share morphological features with the astrocytes of the CNS (Gabella 1981). Compared with other peripheral glial cells (e.g., Schwann cells), EGCs do not form basal laminae and they ensheath nerve bundles and not individual axons (Bannerman et al. 1986). Available immunohistochemical markers for EGC labeling in the adult gut include glial fibrillary acidic protein (GFAP), S100b, and Sox (SRY-box) 8/9/10, the first two being the most frequently used (Ruhl 2005; Hoff et al. 2008). Recently, marker expression analysis showed that the majority of glia in the myenteric plexus co- express GFAP, S100b, and Sox10. However, a considerable fraction (up to 80%) of glia outside the myenteric ganglia, did not label for these markers. These alternative combinations of markers reflect dynamic gene regulation rather than lineage restrictions, revealing an extensive heterogeneity and phenotypic plasticity of enteric glial cells (Boesmans et al. 2015).

EGCs have long been thought to exert a mere mechanical property by supporting neurons (hence the ancient Greek name glia, which means “glue,” a term coined by the German pathologist Rudolf Virchow). Many evidences, however, indicates that these cells exhibit a number of functions, ranging from neurotransmission to enteric neuronal maintenance

and survival (De Giorgio et al. 2012; Gulbransen and Sharkey 2012; Neunlist et al. 2013; Neunlist et al. 2014; Boesmans et al. 2015). In fact, EGCs are involved in many crucial tasks, such as synthesis of neurotransmitter precursors, uptake and degradation of neuroligands (i.e., detoxification of glutamate and g-aminobutyric acid), and expression of neurotransmitter receptors, thereby contributing to neuron-glia cross talk and neurotransmission (De Giorgio et al. 2012; Neunlist et al. 2013). Furthermore, EGCs exhibit immunological properties (Ruhl 2005; Neunlist et al. 2008; Da Silveira et al. 2011), participate in epithelial barrier functions (Steinkamp et al. 2003; Neunlist et al. 2007; Savidge et al. 2007a; Savidge et al. 2007b; Bouchard et al. 2008; Van Landeghem et al. 2011) and evoke neuroprotection (De Giorgio et al. 2012; Neunlist et al. 2014) (**Fig. 1.3**). Therefore, enteric glial cells have neurogenic potential and are capable of generating enteric neurons in response to injury (Joseph et al. 2011; Laranjeira et al. 2011).

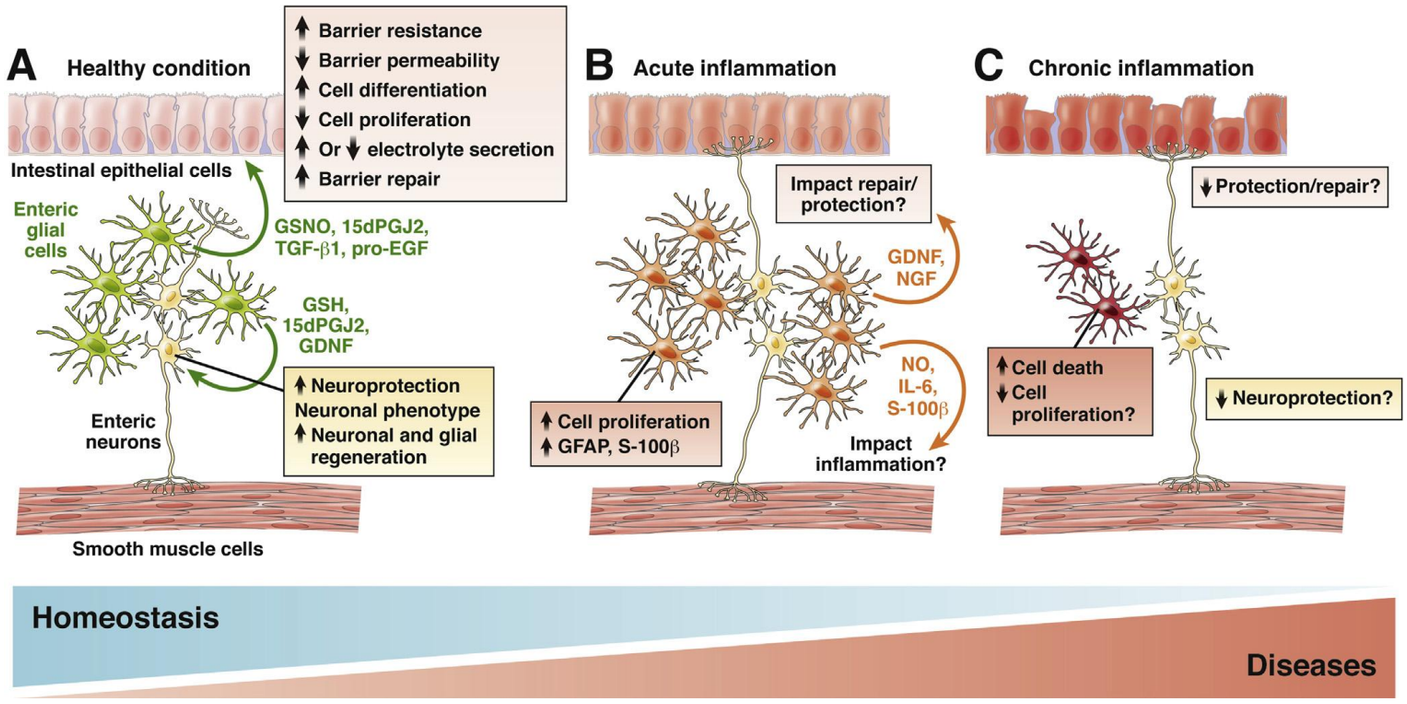

Fig. 1.3. EGCs are central regulators of gut homeostatic processes and might be actors of gut diseases. (A) Under physiological conditions, EGCs regulate various neuronal functions such as neuroprotection, neuromediator expression, or neuronal renewal via liberation of different mediators. In addition, EGCs are central regulators of intestinal epithelial barrier homeostasis via the liberation of functions specific gliomediators. Altogether, EGCs exert protective and reparative functions on the gut. (B) Under environmental stressors such as inflammatory mediators or bacterial stimulation, reactive enteric gliosis (similar to astrogliosis in the brain) can occur, which could participate in the development of intestinal inflammation but also concomitantly participate in protection/repair of IEB/neuronal lesions

induced by these processes. (C) EGC death (induced by specific virus or pathogens) or altered enteric gliosis could contribute to neuronal degeneration or barrier dysfunctions observed in some chronic intestinal or extraintestinal diseases. Modified from Neunlist et al. 2014

Recent works also demonstrated that primary enteric glial cells respond to fast excitatory neurotransmitters such as Ach, 5-HT (5-hydroxytryptamine or serotonin) by changes in intracellular Ca^{2+}, show that enteric glia are not only directly responsive to purinergic (ATP) (Gomes et al. 2009; Gulbransen and Sharkey 2009; Gulbransen et al. 2010) but also to serotonergic and cholinergic signaling mechanisms (Boesmans et al. 2013).

Interstitial cells of Cajal

Interstitial cells of Cajal (ICCs) are characterized by their spontaneous pacemaker-like activity. The first who identified ICCs histologically was Cajal in the 1890s (Cajal 1893). At the discovery he described these cells likely of neuronal origin. Only later, Sir Arthur Keit suggested that ICCs constituted a pacemaker system within the walls of the intestine (Thuneberg 1999). At morphological level, ICCs have small cell bodies and several elongated processes, showing a fibroblast-like shape. During years, the study of ICCs was improved by the discovery of a cellular marker, c-Kit. Kit is a tyrosine kinase receptor for the stem cell factor and was demonstrated that ICCs express c-Kit (Furness 2006).

ICCs have been identified at the level of the submucosal layer (ICCSM), near the inner surface of the circular muscle (ICCDMP; deep muscular plexus), in the myenteric plexus (ICCMY), and within the circular muscle layer (intramuscular ICC, ICCIM) (Komuro 1999; Komuro et al. 1999).

One of the main features of ICCs is the electrical coupling between these cells and smooth muscle cells. It has been demonstrated that ICCs form gap junctions to each other and with smooth muscle cells, thus providing structural evidence of these electrical connectivity (Dickens et al. 1999; Komuro et al. 1999; Hirst and Edwards 2001; Hirst et al. 2002; Cousins et al. 2003). Patch clamping studies suggested that when smooth muscle cells were isolated, they were not able to spontaneous depolaryzations; on the contrary, when ICCs were isolated smooth muscle showed spontaneous depolarisation (Langton et al. 1989; Farrugia 1999). In fact, in many, but not all, regions of the GI tract, spontaneous

pacemaker activity generated by ICC conducts to smooth muscle cells and drives electrical and phasic contractions (**Fig. 1.4**). Spontaneous pacemaker activity is termed slow waves; they occur in continuous rhythmic depolarization/repolarization cycles, and different regions of the bowel display distinct intrinsic slow wave frequencies (Sanders et al. 2014). Each small region of the intestine is electrically coupled to another, thus, regions of higher frequency drive those of lower frequency (Diamant and Bortoff 1969). As consequence, there is a conduction of slow waves from oral to anal direction.When slow waves are raised above threshold by nerve activity in the small intestine, they tend to push the contents in an anal direction, according to the frequency gradient along the small intestine. Slow waves only cause physiologically significant contractions in the small intestine when they are of sufficient amplitude to generate action potentials (Daniel et al. 1959; Bass et al. 1961; Hara et al. 1986). In general, slow waves without action potentials cause very small changes in tension, and the contractions of the muscle are graded according the number of action potentials. The small intestine requires the activity of enteric excitatory motor neurons for the slow waves to be brought to threshold for significant contraction (Quigley JP 1934; Reinke et al. 1967). On the contrary, gastric slow waves reach threshold to generate propulsive contractions; although they do not require neural activity, they are influenced by neural activity (Furness 2006).

ICCs also respond to neural input, in fact they are also approached by nerve fiber varicosities and they exhibit receptors for neurotransmitters (Komuro et al. 1999), such as receptors for NO (Young et al. 1993)and tachykinins (Sternini et al. 1995; Portbury et al. 1996). Excitatory and inhibitory neural inputs have been shown to affect the amplitude of slow waves resulting in effects on the smooth muscle (Hennig et al. 2010).

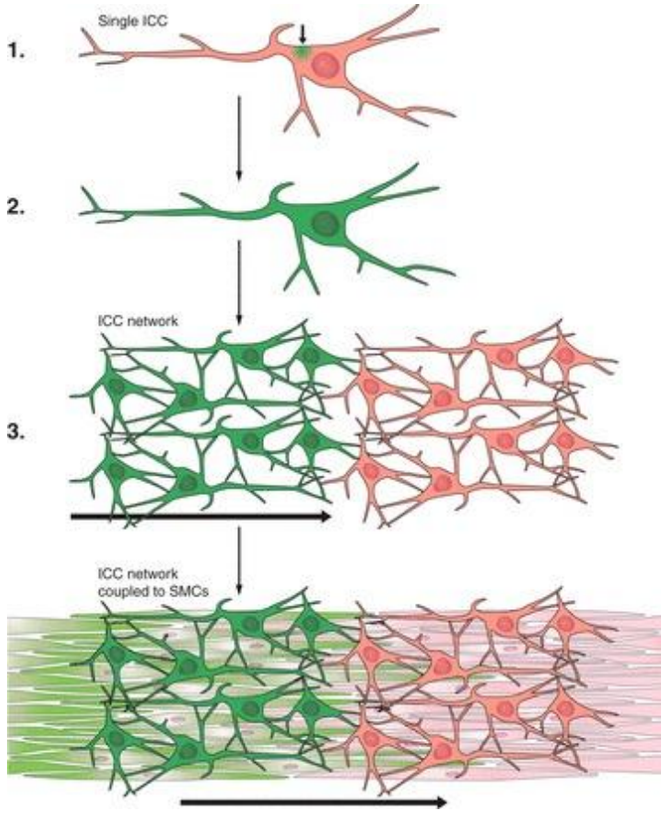

Fig. 1.4 Active propagation of slow waves.

1: The initiating step occurs by localized Ca^{2+} transients (elevated Ca^{2+} depicted by green color in all cells; arrow), initiating STICs in an ICC. *2*: Depolarization caused by STICs activates Ca^{2+} entry and Ca^{2+}-induced Ca^{2+} release raising Ca^{2+} throughout the ICC and activating whole cell slow wave current. *3*: Depolarization causes active propagation of slow waves through the ICC network (horizontal black arrow shows direction of propagation), and slow wave currents are activated cell to cell, as the wavefront spreads. *4*: As slow waves propagate through the ICC network, they conduct passively into electrically coupled smooth muscle cells (SMCs). Slow waves depolarize SMCs and activate L-type Ca^{2+} channels. Ca^{2+} entry (green) triggers SMC contraction. Spread of slow waves in the ICC network leads spread of contractions necessary for segmental and peristaltic contractions. Modified from Sanders et al 2014.

CHAPTER 2

The development of the ENS

The ENS of vertebrates develops from neural crest cells which migrate and colonize the embryonic gut. These complex processes require transcription factors, cell surface adhesion molecules, receptors, extracellular ligands, cytoskeletal rearrangements, and diverse intracellular signaling molecules (Avetisyan et al. 2015).

2.1 Migration of the vagal progenitors and colonization of the embryonic gut

Enteric precursors originate mainly at the vagal and, to a lesser extent, the sacral levels of the neural tube (Kuntz 1910; Yntema and Hammond 1954; Le Douarin and Teillet 1973). Enteric neurons and glial cells originate from vagal neuronal crest progenitors emerging from the caudal hindbrain at level of somites 1-7; a small proportion of the foregut ENS also derives from migratory anterior trunk neural crest cells of the posterior vagal region (Burns and Le Douarin 2001; Newgreen and Young 2002). After delamination from the neural tube, vagal ENS progenitors migrate ventrolaterally and then ventromedially until they reach the dorsal aorta (Burns 2005). As the vagal precursors enter the foregut (mouse embryonic day 9.5, 4th weeks gestation in humans), they are termed enteric neuralcrest-derived cells (ENCCs) (Heanue and Pachnis 2007). ENCCs migrate along the gut and begin the colonization of the foregut (esophagus, stomach and duodenum), the midgut (small intestine, caecum, ascending and rostral trasverse colon), and the hindgut (caudal trasverse colon, descending colon, sigmoid and rectum), following a rostro-caudal direction (**Fig. 2.1**). The colonization of the gut by vagal progenitors is complete by E15 in mice or after the 7 weeks in humans (Fu et al. 2003). Sacral neural crest cells begin their migration along the caudal portion of embryonic gut at late stages, contributing to only a small proportion of enteric neurons and glial cells (Burns and Douarin 1998; Kapur 2000).

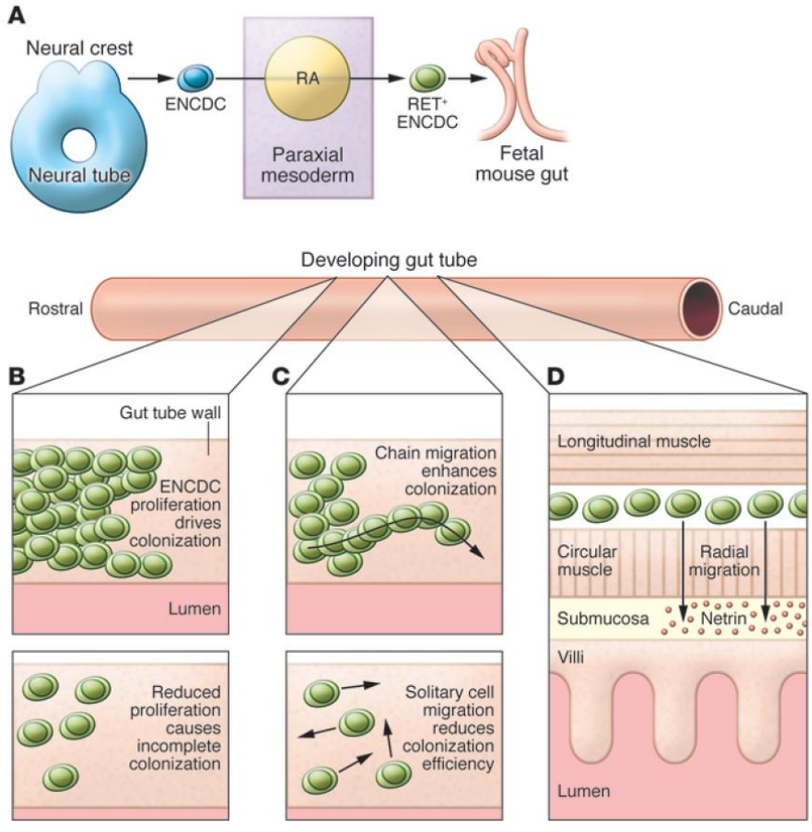

Fig. 2.1 ENS development.

(A) Murine vagal neural crest cells destined for the ENS delaminate from the neural tube at E8.5. These ENCDCs are exposed to retinoic acid (RA) as they migrate by paraxial mesoderm on their way to the foregut at E9. (B) Once ENCDCs are in developing bowel, efficient caudal migration relies on vigorous ENCDC proliferation (top panel), as disorders that reduce ENCDC proliferation (bottom panel) commonly cause incomplete bowel colonization. (C) Efficient ENCDC migration is facilitated by contact between migrating cells. Chain migration of ENCDCs is quicker and more directed than migration of isolated ENCDCs. Disorders that alter ENCDC cell adhesion also delay bowel colonization and may cause HSCR. (D) After ENCDCs have populated the whole developing bowel (E13.5 in mice) in the region of the future myenteric plexus, a subset of ENCDCs migrates inward radially to form the submucosal plexus. Radial migration is regulated by the RET-GDNF signaling axis and by netrin/ DCC chemoattraction. Modified from Avetisyan et al. 2015.

During the colonization and migration along the gut, vagal ENCCs at leading edge of the migratory stream (the 'wavefront') appears as chains or strands of interconnected cells; some cells appears isolated in advance of the wavefront (Druckenbrod and Epstein 2005), while ENCC following the wavefront are randomly distributed (Fu et al.

2004; Wallace and Burns 2005). The proliferation of ENCCs is important during migration and colonization of the entire gut (Avetisyan et al. 2015). In fact, the proliferation occurring at the wavefront, provides progenitor cells to progressively colonize distal regions, while, behind the wavefront, ENCCs proliferation increase the number of cells to fully populate the intestine (Burns and Le Douarin 2001; Simkin et al. 2013). It seems that ENCCs proliferation drives the rostro-caudal bowel colonization thought a mechanism of competition for space and trophic factor (Landman et al. 2007).

Bowel colonization by ENCCs is enhanced by chain migration, a process whereby ENCCs preferentially contact each other while migrating (Young et al 2004; Druckenbrod et al 2005).

The reason why contact among ENCCs enhances bowel colonization is not understood. One hypothesis is that ENCCs at the wavefront pull trailing ENCCs via cellular adhesion mechanisms; Another possibility to enhance migration is to create spaces through the alteration or the degradation of the extra-cellular matrix (Akbareian et al. 2013).

2.2 Genetic control of the colonisation and migration of ENCCs

ENS development is driven by regulatory molecules. During the early migration, ENCCs start to express the tyrosine kinase receptor RET, in response to the local production of Retinoic Acid by the paraxial mesoderm. RET is the receptor for the glial cell derived neurotrophic factor (GDNF) (Avetisyan et al. 2015). Through GDNF and its co-receptor GRFα1, RET signaling supports ENCCs survival, proliferation, migration and differentiation. The actions of GDNF vary depending on the timing of its presentation to susceptible precursor cells and the location of its source (Gershon 2010). GDNF attracts migrating vagal ENCCs, which do follow a GDNF gradient to and within the bowel (Young et al 2001; Nataranjan et al 2002). In fact, when ENCCs first begin to colonize the gut, GDNF expression is most intense in the stomach, then moves caudally, reaching higher concentration in the caecum. Recent evidences showed that a population of ENCCs crosses from the midgut to the hindgut via the mesentery during a developmental time period in which these gut regions are transiently juxtaposed; also this migratory process requires GDNF signaling (Nishiyama et al. 2012). An additional factor would be necessary to enable vagal ENCCs migrating in the thrall of GDNF to escape what would otherwise be a cecal trap. Endothelin-3 (ETB) has been postulated to be such a factor (Barlow et al. 2003).

ETB signaling enhances Ret-driven ENCCs proliferation (Barlow et al. 2003; Nagy and Goldstein 2006)and inhibits ENCCs migration (Barlow et al. 2003). These mechanism might allow migrating ENCCs to escape the cecal trap and complete the colonization of the bowel (Barlow et al. 2003). Nevertheless, ETB/EDNRB (Endothelin receptor B) pathway inhibits the generation of enteric neurons (Wu et al. 1999) (Davenport and Maguire 2006), being responsible of the postponement of enteric neuronal differentiation, which involves withdrawal from the cell cycle and probably also cessation of migration (Wu et al. 1999). ETB/EDNRB functions with SOX10 to maintain ENCCs in an uncommitted state (Bondurand et al. 2006). ETB/EDNRB signaling might therefore be needed to prevent ENCCs from differentiating before they have completed their colonization of the gut (**Fig. 2.2**).

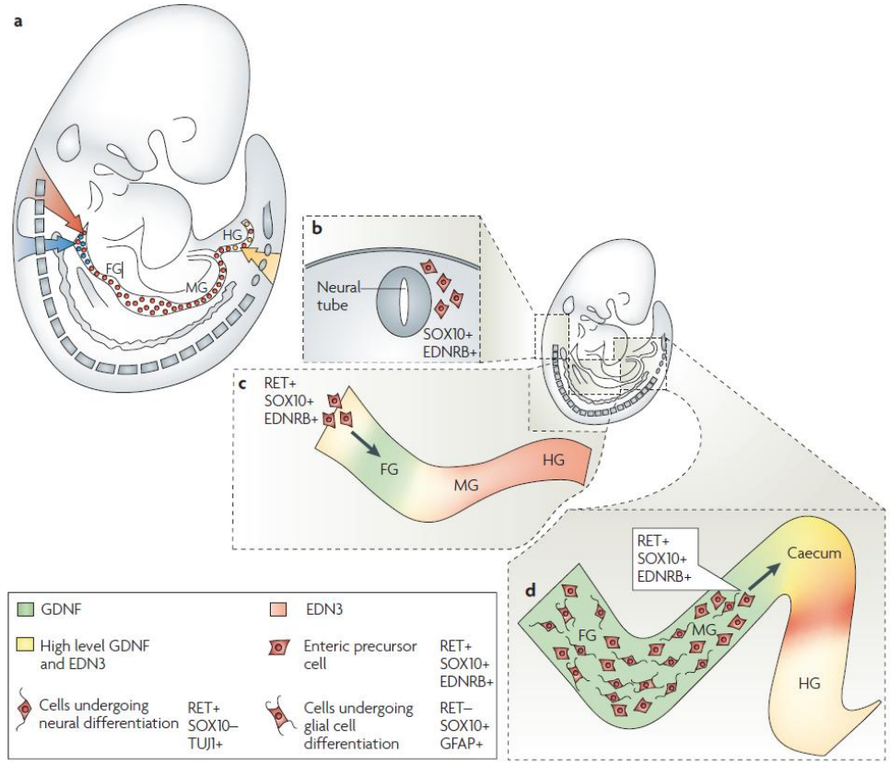

Fig. 2.2 Sources, migratory routes and gene expression in neural crest cells contributing to the ENS.

a) At approximately embryonic day (E) 8.5–9 in the mouse, vagal neural crest cells (red arrow) invade the anterior foregut and migrate in a rostral to caudal direction to colonize the entire foregut (FG), midgut (MG), caecum, and hindgut (HG) and give rise to the majority of the enteric nervous system (ENS, red dots). Colonization is complete by E15.5. The most caudal vagal neural crest cells, emanating from a region overlapping with the most anterior trunk neural crest cells (blue arrow), make a small contribution to the ENS of the oesophagus and the anterior stomach (blue dots).

Finally, sacral neural crest cells (yellow arrow) also make a small contribution, beginning their migration at approximately E13.5 and migrating in a caudal to rostral direction to colonize the colon (yellow dots). b) As vagal neural crest cells (red) emigrate from the neural tube, they express SRY-box 10 (SOX10) and endothelin receptor B (EDNRB). c) Upon entering the foregut at E9–9.5, enteric neural crest-derived cells (ENCCs) begin to express RET. Within the gut mesenchyme, the RET ligand glial cell-line-derived neurotrophic factor (GDNF) is expressed at high levels in the stomach (green) and the EDNRB ligand endothelin 3 (EDN3) is expressed in the midgut and hindgut (pink). d) As ENCCs migrate caudally at approximately E11, they encounter high levels of GDNF and EDN3 expression in the caecum (yellow). Cells behind the wavefront begin progressive differentiation towards neural and glial cell fates. Beginning at E11.5, GDNF and EDN3 are expressed in the distal hindgut (not shown). Modified from Heanue and Pachnis 2007.

2.3 Differentiation of ENCCs

A subset of ENCCs undergoes radial migration to form the submucosal plexus or into the pancreas to form ganglia near the Islet of Langerhans (Jiang et al. 2003; Lake and Heuckeroth 2013; Uesaka et al. 2013). This process is driven by the chemoattractant netrin and its receptor DCC (Deleted in colorectal cancer) (Jiang et al. 2003), secreted by fetal gut epithelium and pancreas. Cells that move to the SMP also have less RET signaling than do adjacent ENCCs (Uesaka et al. 2013). During radial migration, perimuscular gut mesenchyme downregulates GDNF and mesenchyme closer to the lumen upregulates GDNF (Uesaka et al. 2013). This change in GDNF localization provides additional stimulus for ENCCs with low RET activity to migrate inward. How cells modulate levels of RET and how neighboring cells communicate to ensure that only a subset of cells leave the myenteric plexus is unknown (Avetisyan et al. 2015).

ENCCs differentiate into neuronal subtypes or enteric glia (Grundy et al. 2006), form ganglia, extend neurites, and establish and refine functional neural circuits to control the bowel (Young et al. 2003) (**Fig. 2.3**). Although Ret and ETB signaling are necessary for the migration/survival of adequate numbers of ENCCs in the gut, they are not sufficient to form an ENS. ENCCs must also differentiate into glia and neurons of appropriate phenotypes and form correct synaptic connections (Gershon 2010). Different transcription factors have a critical role in driving the ENCCs differentiation process. Among these factors, SOX10 and mammalian achaete-scute homologue 1 (MASH1) are relevant during the differentiation of ENCCs in enteric neurons (and subtypes) and glia. SOX-10 is a transcription factor expressed by neural crest progenitors before the migration, in ENCCs and in mature glial cells. It has a critical role in maintaining

ENCCs in an undifferentiated state (Bondurand et al. 2006). MASH-1 is a transcription factor involved in the development of transiently catecholaminergic cells. In particular, MASH-1 expression is required for the differentiation of serotonergic neurons (Blaugrund et al. 1996).

Self-renewing progenitors express SOX10 and the stem marker p75. Upon commitment to neurons and glial cells, precursors upregulate RET and MASH1 expression. Neurogenic precursors cells downregulate SOX10, MASH1 and p75, maintain RET expression, and upregulate the neural marker TUJ1 and other neuronal subtype-specific markers. Gliogenic precursor cells maintain SOX10 and p75 expression, downregulate RET and MASH1, and upregulate glial markers such as S100 and GFAP (Lo and Anderson 1995; Young et al. 1999; Young et al. 2003; Heanue and Pachnis 2007) (**Fig. 2.3**).

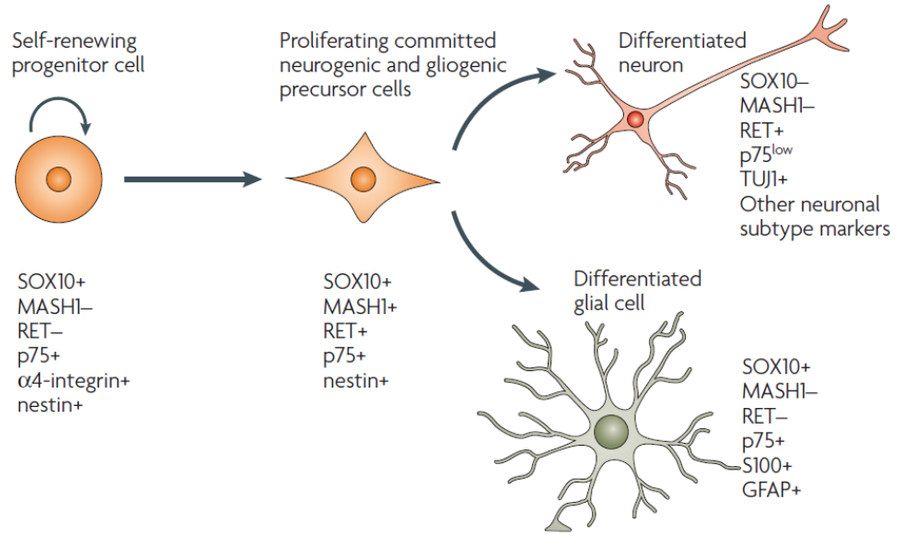

Fig. 2.3 Differentiation of enteric neural stem cells follows a normal ENS developmental profile.
Self-renewing progenitor cells express SOX10, p75, α4-integrin and nestin, but do not express RET or MASH1. Upon commitment to neurogenic and gliogenic precursor cells, RET and MASH1 expression is upregulated. Cells that differentiate into neurons downregulate SOX10, MASH1 and p75, maintain RET expression, and upregulate the neural marker TUJ1 and other neuronal subtype-specific markers. Alternatively, cells differentiating into glial cells maintain Sox10 and p75 expression, downregulate RET and MASH1, and upregulate glial markers such as S100 and GFAP Modified from Heanue and Pachnis 2007.

Enteric ganglia contain at least 20 distinct neuronal subtypes that differ in function, transmitters, neurite patterning, and electrophysiology (Furness 2000). One model for enteric neuron subtype lineage relationships include the transiently catecholaminergic (TC) cells. TC ENS precursors arise early in development (Pham et al. 1991)and absolutely require the transcription factor MASH1 (Blaugrund et al. 1996; Obermayr et al. 2013) (**Fig. 2.4**). Serotonergic neurons, but not other subtypes, also require MASH1 and were thought to arise exclusively from TC precursors. Fate-mapping studies of tyrosine hydroxylase–expressing cells now suggest that TC precursors give rise to only 30% of serotonergic neurons, but also can become calbindin-, calretinin-, and neurofilament- M–expressing neurons (Obermayr et al. 2013). Importantly, TC precursors give rise to less than 3% of myenteric neurons and to 13% of submucosal neurons in the mouse distal small intestine. It is unclear whether TC precursor–derived serotonergic neurons differ from non–TC derived serotonergic neurons (**Fig. 2.4**). The complexity of these data highlights how little we understand enteric neuron subtype specification (Avetisyan et al. 2015). Several transcription factors are essential for the differentiation of ENCCs in the different subtypes. In general, neuronal subtype specification appears to depend on a combination of intrinsic (genetic) and extrinsic factors (Avetisyan et al. 2015).

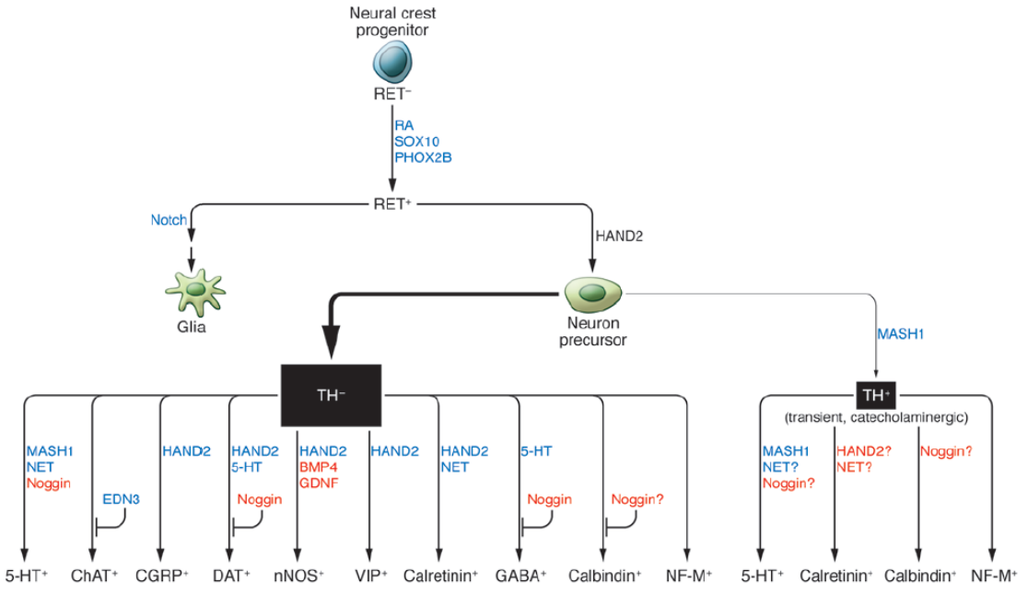

Fig. 2.4 ENS precursor lineage relationships and neuronal subtype specification.

Lineage relationships among enteric neuron subtypes remain poorly understood. This figure summarizes in vivo observations. Gain-of-function data are indicated in red. Loss-of-function data are indicated in blue. Most myenteric neurons arise from TH-negative precursors, as indicated by the relative thickness of arrows. Modified from Avetisyan et al.2015.

CHAPTER 3

The extrinsic innervation of the gastrointestinal tract.

The neural controls of the GI functions also involves an extrinsic innervation, beyond the ENS. The ENS works in concert with the CNS through a bidirectional information flow.

Connections between the gut and the central nervous system can be classified in three major divisions: vagal, spinal thoracolumbar and spinal lumbosacral, each including an afferent (sensory) and an efferent (motor) compartment (Furness 2012) (**Fig 3.1**).

Sensory afferents are specialized: their nerve endings originate within the gut wall, but project centrally, following spinal and vagal nerve connections (Furness et al. 2014). There are, at present, 5 different functional classes of spinal afferent that respond to different sensory modalities (Brierley et al. 2004). The location of the nerve endings of spinal afferents includes intraganglionic laminar endings in the myenteric ganglia, mucosal endings, intramuscular endings, muscular-mucosal endings close to the *muscularis mucosae,* and vascular endings on the blood vessels feeding into the gut (Brookes et al. 2013).

The efferent pathways contain pre-enteric neurons that end within enteric ganglia and control or modify the activities of enteric neurons. Pathways from the CNS also contain neurons that directly innervate a restricted number of gastrointestinal effectors, such as striated muscle of the esophagus (vagal innervation), sphincters (sympathetic innervation) and intrinsic blood vessels (also sympathetic innervation). The parasympathetic connections are predominantly cranio – sacral, while the thoracolumbar pathways contribute sympathetic input into the gut (Furness et al 2014).

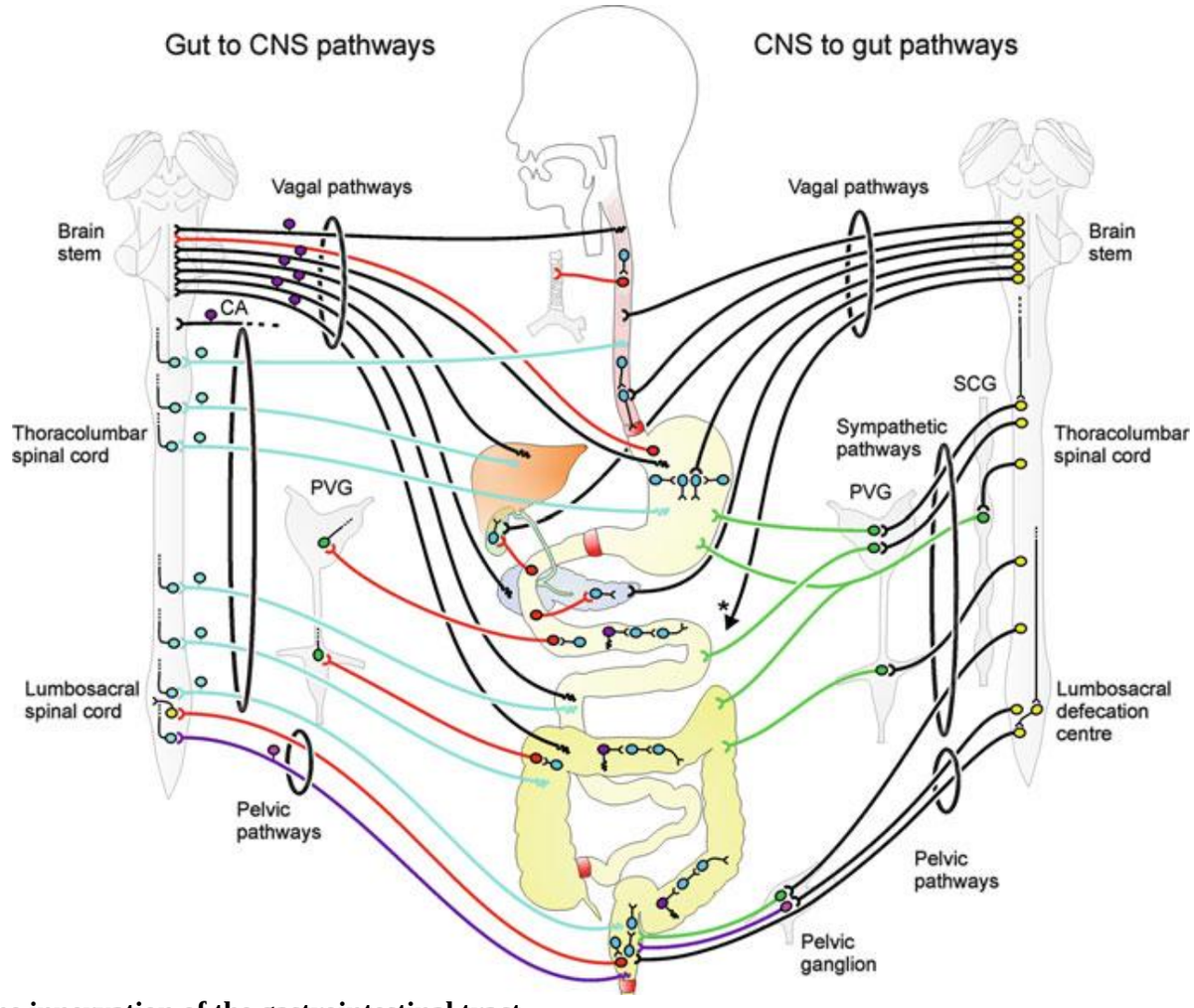

Fig. 3.1 The innervation of the gastrointestinal tract.

The neural connections between the enteric nervous system (ENS), the central nervous system (CNS) and sympathetic ganglia, and neural connections between gastrointestinal organs are illustrated.Connections from the ENS to other organs and the CNS are at the left, and connections from the CNS are at the right. The small and large intestines (middle of figure) contain full ENS reflex circuits (motor neurons and interneurons in blue, sensory neurons in purple). Pathways from the gastrointestinal tract (left) project outwards, via intestinofugal neurons (red), to the CNS, sympathetic ganglia, gallbladder, pancreas and trachea. Some neurons in sympathetic prevertebral ganglia (PVG, green neurons) receive both CNS and ENS inputs. Sensory information goes both to the ENS, via intrinsic primary afferent (sensory) neurons (purple) and to the CNS via extrinsic primary afferent neurons (left of figure) that follow spinal and vagal nerve connections. Cervical afferents (CA) connect the esophagus to the cervical spinal cord. Pathways from the CNS reach the ENS and gastrointestinal effector tissues through vagal, sympathetic and pelvic pathways (right of figure). Vagal medullary and pelvic spinal outflows include pre-enteric neurons (ending in enteric ganglia) and most gut-projecting sympathetic neurons with cell bodies in PVG are also pre-enteric neurons. SCG sympathetic chain ganglia. Modifies from Furness et al 2014.

3.1 Vagal pathways

Vagal afferent fibers exerted the role of mucosal mechanoceptors, chemoceptors and tension receptors in the esophagous, stomach and proximal small intestine; in liver and pancreas they act as sensory endings. The functions that are regulated by the vagal sensory innervation include appetite and satiety, esophageal propulsion, gastric volume, contractile activity and acid secretion, contraction of the gallbladder and secretion of pancreatic enzymes (Furness et al. 2014). In the caudal small intestine and in the rostral colon, the vagal afferent innervation is less prominent. In fact, sensory information are detected by enteroendocrine cells, which release gut hormones. Once released, these molecules, such as Cholecystokinin (CCK) and 5-HT, act on vagal afferent endings and determine a mechanism of indirect chemoreception important to respond to luminal stimuli, including nutrients, noxious agents and to regulate satiety, appetite, esophageal and gastric activities (Raybould 2010; Furness et al. 2013). The vagal efferent pathways arise from the dorsal motor nucleus of the vagus nerve and the nucleus ambiguous. Most of these neurons are pre-enteric and they form synapses with myenteric neurons, while other neurons project directly to the striated muscle of the esophagus to control the esophageal propulsion, the lower esophageal sphincter (LES) relaxation and gastric accommodation, contractile activity and acid secretion (Furness et al. 2014). In the small intestine a sparse vagal innervation is present, but efferent fibers are predominantly connected to enteric neurons (Furness 2006). Vagal pre-enteric neurons innervate all intrinsic gallbladder neurons and they also have e strong influence on pancreatic neurons for the secretion of pancreatic enzymes (Mawe 1998; Furness 2006).

3.2 Thoracolumbar innervation

The sympathetic innervation of the GI tract, derived from the thoracolumbar region of the spinal cord (T5-L2), forms neuronal centers in larger mammals such as the human or pig (Taubin et al. 1972). The thoracolumbar division includes afferent fibers arising from neuronal bodies located in the dorsal root ganglia. Axons from these neurons are almost all unmyelinated c-fibers (Furness et al. 2014). They are mostly CGRP and SP immunoreactive; also TRPV1channel (Transient receptor potential cation channel subfamily V member 1) is highly expressed, triggering visceral pain signals (Green and Dockray 1988; Tan et al. 2008). Sympathetic fibers innervate arterioles in gut wall, mucosa and submucosal

layer; many varicosities are found around myenteric ganglia and rare fibers are detected in muscle layers. Axons of spinal afferent neurons also provide collaterals forming synapses with cell bodies of postganglionic neurons. The sympathetic efferents from thoracolumbar division mainly supply MP and SMP ganglia, blood vessels and sphincter muscle (Taubin et al. 1972). The preganglionic sympathetic neurons have their cell bodies in the intermediolateral columns of the spinal cord (Furness et al. 2014). Postganglionic neurons of vasoconstrictor pathways are in sympathetic chain and pre-vertebral ganglia. There are three major pre-vertebral ganglia which are known as the coeliac ganglia, superior mesenteric and inferior mesenteric ganglia (Szurszewski 1994). The superior mesenteric ganglia supply the stomach, small intestines and the proximal colon, and the inferior mesenteric ganglia supplying the distal colon. Postganglionic projections from sympathetic chain and pre-vertebral ganglia both exert inhibitory effects: in the MP they inhibit excitatory neurons of the stomach and intestine and contract sphincters; in the SMP they inhibit secretomotor neurons. The resulting effect is of delayed transit of the luminal content along the intestine (Taubin et al. 1972; Szurszewski 1994; Furness 2006).

3.3 Lumbosacral innervation

The more distal regions of the colon and rectum receive afferent and efferent innervation from the sacral nerve roots (S2-4) arising from the lumbosacral spinal cord (Furness et al. 2014). Afferent projections include pain fibers. Pelvic afferents carry information from low threshold mechanoceptors, in response to mild stroking of the mucosa in the colon and small intestine (Ness and Gebhart 1990; Larsson et al. 2003). On the contrary, in the rectum, mucosal mechanoceptors of the rectum are activated by stretch over a wide range, including into the level for pain; they also respond to wall distension (Zagorodnyuk et al. 2011). The efferent pelvic pathways supply innervation to enteric ganglia in distal colon and rectum (Brookes et al. 2009). Nerve cells of the spinal cord and pelvic ganglia project directly to the colon (Olsson et al. 2006). The motility control involves neurons of the defecation centers located in the lumbosacral spinal cord (L5-S3, depending on the species). Reflexes through this center can be initiated by rectal distention or irritation. In healthy condition, the propulsive reflexes of distal colon and rectum are under the control of central centers. Central centers are also located in the spinal defecation center, and they are responsible of the fecal continence (voluntary control)(Shimizu et al. 2006).When the defecation is appropriate, central commands cause co-ordinated

emptying of the colon, via ENS. Pelvic pathways also carry pathways that cause vasodilatation in colorectum (Hulten 1969; Furness et al. 2014).

CHAPTER 4

Neuronal control of gastrointestinal functions

The ENS is responsible for the control of intestinal functions, such as motility, secretion, maintenance of epithelial barrier, regulation of the blood flux, interaction with the immune system.

4.1 Motility

The intestinal motility is an essential function in which different mechanisms act together, including the activity of the muscle layers, the neural control of both the intrinsic and extrinsic nervous systems, the pace-maker activity of the ICCs. The muscle layer of GI tract direct propulsion, mixing of contents, reservoir capacity and expulsion of pathogens. The role of the ENS is essential to coordinate the GI motility and it varies depending on the region (Furness 2012).

Esophagus – Although the esophagus contain a well developed ENS, enteric neurons have little influence on the esophageal peristalsis. The propulsive activity of the striated muscles in the upper esophagus is controlled by motor neurons of the nucleus ambiguous, through nerve circuits starting from the medulla oblongata in the CNS (Jean 2001). About a third of the endplates in the esophagus receive a dual innervation, i.e. an excitatory vagal transmission and a presynaptic inhibition exerted by MP nitrergic neurons (Neuhuber et al. 1994; Kuramoto et al. 1996; Wu et al. 2003a). In the lower esophagus, the smooth muscle cells are innervated by enteric ganglia, which are directly controlled by pre-enteric neuron of the dorsal motor nucleus of the vagus(Jean 2001). The lower esophageal sphincter (LES) is essential to allow passage of food from the last portion of the esophagus into the stomach. The relaxation of the LES is mainly controlled by vagus nerve through a descending inhibitory fibers; however a local reflex can be elicited in response to distension. The LES has also an important role in limiting reflux of the corrosive gastric contents and its contraction is mediated by vago-vagal reflex pathway that passes through the brain stem (Furness 2012; Furness et al 2014).

Stomach – The fundic portion of the stomach is primarly associated with the gastric reservoir function, while the corpus-antrum is associated with the gastric mixing and antral propulsion (Kelly 1981).

The reservoir function is explained by a relaxation of the fundus (due to a volume increase), coupled with a contraction (due to a volume reduction). This activity is mainly dependent by vago- vagal reflexes. A vagally mediated gastro-gastric reflex relaxation is also elicited when distention is confined to the antrum(Abrahamsson and Jansson 1969); in addition a small residual accommodation is due to an intrinsic reflex (Andrews et al. 1990).

Gastric peristalsis is generated by slow waves. Neuronal circuits are not required to co-ordinate peristaltic movements. In fact, the direct excitation of the muscle by the pacemaker activity of the ICCs is sufficient to evoke gastric muscle contraction(Sanders et al. 2006). The neuronal control of the corpus and antrum is exerted through vago-vagal reflexes and there is only a little evidence for a gastric intrinsic reflex (Andrews et al. 1980).

Small intestine – The ability of small intestine to function when isolated from CNS was demonstrated over a century ago. The intestinal motility is under the control of the ENS and the nerve circuits for the control of intestinal motility are similar in many species, including humans. There are various pattern of movement: rapid orthograde propulsion of contents (peristalsis), mixing movements (segmentation), slow orthograde propulsion (the migrating myoelectric complex, MMC) and retropulsion (expulsion of noxious substances associated with vomiting)(Furness 2012). Although the structural organization of the circuits that detect the state of the intestine, integrate the information and direct the activities of motor neurons is known, the integrative circuitry through which one pattern is converted to another remains to be defined. Some of the signals triggering changes of the patterns of movements in the small intestine have been identified (Furness 2006) (**Fig. 4.1**).

Mechanical and chemical stimuli are detected by enterocromaffin cells (ECs) in the mucosal layer and are able to induce the release of 5-HT. *IPAN*s displaying specific serotonin receptors, are activated by 5-HT and synapse with ascending and descending inteneurons releasing ACh and SP. Ascending interneurons activate excitatory motor neurons, evoking a muscle contraction in the oral side of the tube, through the release of excitatory neurotransmitters (Ach and SP); on the contrary, descending interneurons activate inhibitory motor neurons responsible for a relaxation in the anal side, through the release of inhibitory neurotransmitters (nNOS, VIP and ATP). The coordination of the oral muscle contraction and anal relaxation make possible the propulsion of the content along the intestine (Furness 2006; Furness et al. 2014).

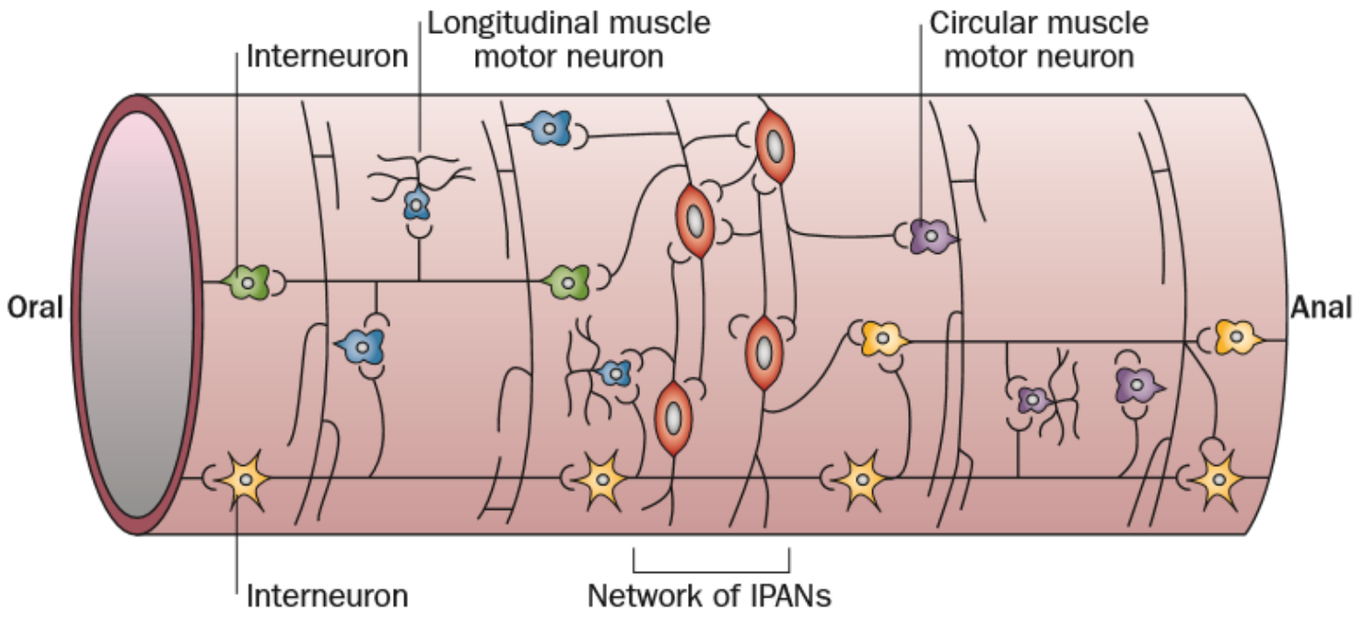

Fig. 4.1 Nerve circuits for control of motility in the small intestine.

Networks of interconnected intrinsic sensory neurons (*IPAN*s; red) detect mechanical distortion and lumenal chemistry. These synapse with descending (yellow) and ascending (green) interneurons, excitatory muscle motor neurons (blue) and inhibitory muscle motor neurons (purple) Modifies from Furness 2012.

Large intestine - In the large intestine the pattern of movements are similar to the small intestine: orthograde propulsion, including both peristalsis and MMC, and segmentation. The ENS is responsible for the neuronal control of the motility, except for the distal colon and the rectum. In fact, in these tracts, the propulsive reflexes are regulated by central commands; when it is appropriate, neurons of the defecation center located in the lumbosacral spinal cord, trigger defecation, evoking coordinated emptying of the colon via ENS (Shimizu et al. 2006; Furness et al. 2014).

4.2 Secretion

The control of fluid movement between the intestinal lumen and the body fluid compartment is essential. The regulation of the fluid balance is exerted by ENS and sympathetic reflexes (Furness 2006) (**Fig.4.2**). One of the reasons for the large flux is the absorption from the lumen with nutrients, such as sugars and amino acids. Large volumes of fluids are absorbed during the absorption of glucose. In fact, glucose is absorbed through a cation-coupled transporter,

which is responsible for the internalization of a molecule of glucose, coupled with a molecule of sodium ion and counter ions, mostly chloride ions (Wright and Loo 2000). Lumenal glucose act via the glucose receptor expressed by the enteroendocrine cells, which release a gut hormone, namely glucagon like peptide 2 (GLP2). GLP2 diffuses through the lamina propria and activates enteric secretomotor neurons. Once activated, enteric reflexes are modulated to control whole body fluid balance and to return water and electrolyte to the lumen (Furness 2012). This control is exerted through blood volume and blood pressure detectors that change the activity of two sympathetic pathways, vasoconstrictor pathways and secretomotor inhibitory pathways (Sjovall et al. 1986).

Secretomotor enteric reflexes are mainly mediated by non cholinergic neurons through the release of VIP or Substance P and neuromodulator such as ATP and PACAP. Another important molecule is the 5-HT which act on *IPAN*s determining the activation of cholinergic and VIPergic secretomotorneurons. Ach and VIP released, are able to activate specific receptors located on the epithelial cells, determining the secretion of chloride ions and fluids. Substances such as glutamate and PACAP are able to modulate the activity of secretomotor neurons, to increase or inhibit the secretion (Furness 2006).

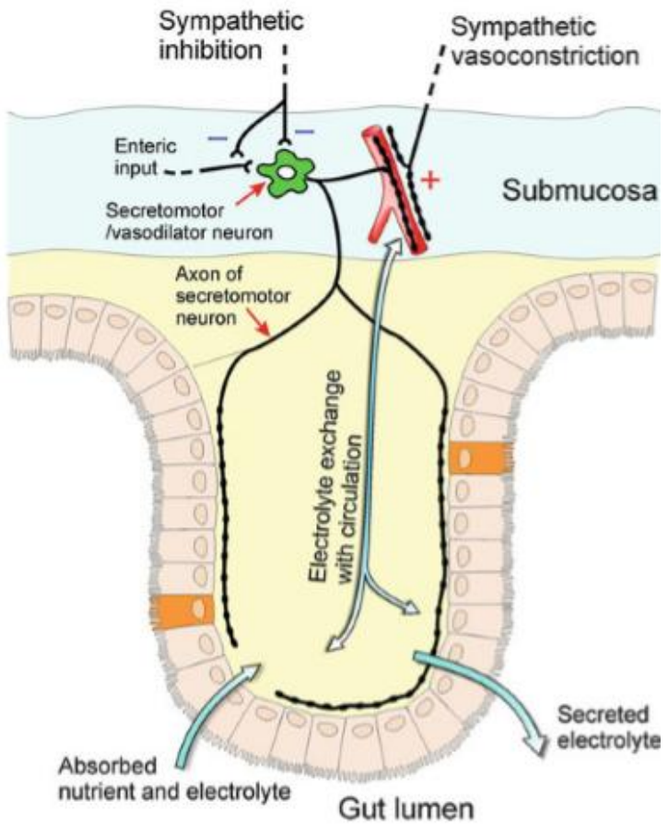

Fig. 4.2 Neural control of transmucosal water and electrolyte movement in the small intestine. The final secretomotor neuron of reflexes that play an essential role in balancing local fluid fluxes and in whole body water and electrolyte balance is illustrated. Large volumes of fluid are absorbed from the lumen with nutrients, such as glucose. These fluids are returned through secretomotor reflexes. The absorption of nutrients with fluid activates enteric secretomotor reflex pathways that impinge on the secretomotor neurons. It is important that the balance of this fluid exchange is modulated by sympathetic vasoconstrictor and secretomotor inhibitory pathways. Activity in these sympathetic pathways, which inhibit secretion and reduce local blood flow, is determined by whole body fluid status, which includes sensory detection through blood volume detectors, baroreceptors and osmoreceptors. Modifies from Furness et al. 2014.

4.3 Barrier function

The ENS plays a role in controlling the intestinal epithelial barrier (BEI) functions. In particular, ENS is involved in mechanisms involved in the maintenance of BEI integrity, such as the proliferation of epithelial cells and permeability.

The gut barrier is a functional unit, organized as a multi-layer system, made up of two main components: a physical barrier surface, which prevents bacterial adhesion and regulates paracellular diffusion to the host tissues, and a deep functional barrier, that is able to discriminate between pathogens and commensal microorganisms, organizing the immune tolerance and the immune response to pathogens (Muller et al. 2005). The intestinal epithelium is organized in a single layer of 20 μm, and consists of 5 different cell types: enterocytes, endocrine cells, M cells, goblet cells and Paneth cells. The enterocytes are the most represented type (Moens and Veldhoen 2012). They act as a physical barrier, inhibiting the translocation of luminal contents in the inner tissues. They are connected by intercellular junctions, characterized by transmembrane proteins that interact with near cells and with intracellular proteins associated with the cytoskeleton. Together, these components form a complex and homogeneous network. In the intestinal epithelium there are two main types of junctions: the adherentes junctions (AJs) and the tight junctions (TJs). AJs are mainly formed of cadherins connected to the actin cytoskeleton via a family of catenins, while TJs are the results of interactions of occludin, claudins and JAM-A connected to the actin cytoskeleton via zonula occludens proteins and α-catenin (Yuan and Rigor 2010). The phosphorylation of myosin and contraction of the actinmyosin complex regulates the strength of such connections, and therefore the permeability of the epithelial barrier (Naydenov and Ivanov 2010).

The permeability of the BEI is based on paracellular and transcellular components. It is known that the epithelial permeability is composed of two parts, the transcellular permeability and paracellular permeability (Kapus and Szaszi 2006). The transcellular permeability is the movement of ions or molecules through apical and basolateral transporters or channels across the membrane, while the paracellular permeability is the diffusion of ions or molecules via the intercellular space, driven by the transepithelial electrochemical gradient. Paracellular permeability is featured as size and charge selectivity and the physiological function is various, to a great extent, dependent on the cell type (Hu et al. 2013).

Neurotransmitters such as Ach, VIP and SP may have a role in controlling BEI permeability (Hallgren et al. 1998; Hardin et al. 1999; Hayden and Carey 2000). In fact, it was demonstrated that Ach release evoke the increase of transcellular permeability acting on muscarinic receptor 3, expressed by intestinal epithelial cells (Cameron and Perdue 2007).

In vitro studies showed that VIP containing neurons are able to induce an increase of the paracellular BEI permeability, modulating the expression of tight junctions proteins (Neunlist et al 2003). Therefore, the ENS is involved in mechanisms controlling the epithelial cells proliferation, both increasing (via GLP-2) or inhibiting (via VIP) cells proliferation (Bjerknes and Cheng 2001; Toumi et al. 2003). There are other neuromediators, such as somatostatine and its analogues, which act inhibiting the epithelial cells proliferation, and ATP or ADP, which are able to promote the migration of intestinal epithelial cells (Jessen and Mirsky 1985; Thompson et al. 1993; Dignass et al. 1998).

The permeability of the IEB has a central role in the regulation of fluid and nutrient intake as well as in the control of the passage of pathogens. The functions of the IEB are highly regulated by luminal as well as internal components, such as bacteria or immune cells, respectively. Recent evidence indicates that enteric neurons and enteric glial cells are potent modulators of IEB functions, giving rise to the novel concept of a digestive 'neuronal–glial–epithelial unit' akin to the neuronal–glial–endothelial unit in the brain (Neunlist et al. 2013).

CHAPTER 5

The Serotonergic system in the gut

Serotonin, was isolated by Vittorio Erspamer in 1935 from the intestinal mucosal in frog, it was initially named enteramine. 5-HT represents one of the most important molecules in controlling different neural system, in the CNS and PNS (Gershon and Tack 2007). In fact, the serotonergic system is involved in motor activity, pain control and regulation of autonomic processes (descending circuitries) and also in the regulation of higher integrative function, such as mood, anxiety, stress, aggression, cognition, sleep, sexual behavior, and appetite behavior and appetite (ascending projections) (Olivier 2015).

5.1 Serotonin synthesis and secretion

5-HT belongs to the monoamine neurotransmitter family.Despite 5-HT is the most widely utilized neurotransmitter in the brain, only 5% of the whole bodies serotonin content is localized in CNS (Olivier 2015). The 95% of the 5-HT synthesis occurs in the gut, mostly in the ECs of the mucosal layer and in less amount in the neurons of the myenteric plexus of some species (Spiller 2008). 5-HT is synthetized from tryptophan. L-tryptophan is hydroxylated to 5-hydroxy-L-tryptophan which is then catalyzed by the rate limiting enzyme tryptophan hydroxylase (TPH) to 5-HT (Walther et al. 2003). TPH consists in two forms, TPH1 expressed by ECs and THP2 expressed by enteric neurons. Serotonergic enteric neurons represent only 2% of all enteric neurons (in guinea pig small intestine, (Furness 2006) and account for the 10% of the serotonin production, thus, the most of 5-HT synthesis occurs in ECs. Calcium influx into the ECs stimulates release of serotonin via activation of L-type Ca2+ channels. ECs produce and secrete far more serotonin than either CNS or ENS neurons to reach GI lumen and blood (Tamir et al. 1985). Overflowing serotonin from ECs, which is taken up and concentrated in platelet, is virtually the sole source of blood serotonin; platelets lack TPH. Similar to other neurotransmitters, 5-HT is constantly catabolised by monoamine oxidases (MAO) into its corresponding aldehyde. Aldehyde is then metabolised by aldehyde dehydrogenase to 5 hydroxyindolacetic acid in the liver and excreted via the

kidneys(Sikander et al. 2009). In the gut, a specialized serotonin reuptake transporters (SERT) mediate the reuptake into epithelial cells, platelets and other cells. SERT is able to binds 5-HT and internalize the molecule through the cellular membrane; it is present both in the mucosa and enteric nerves. As consequence, SERT represent a critical inactivating mechanism for the serotonin signal and in maintaining the 5-HT homeostasis (Wade et al. 1996) (**Fig. 5.1**).

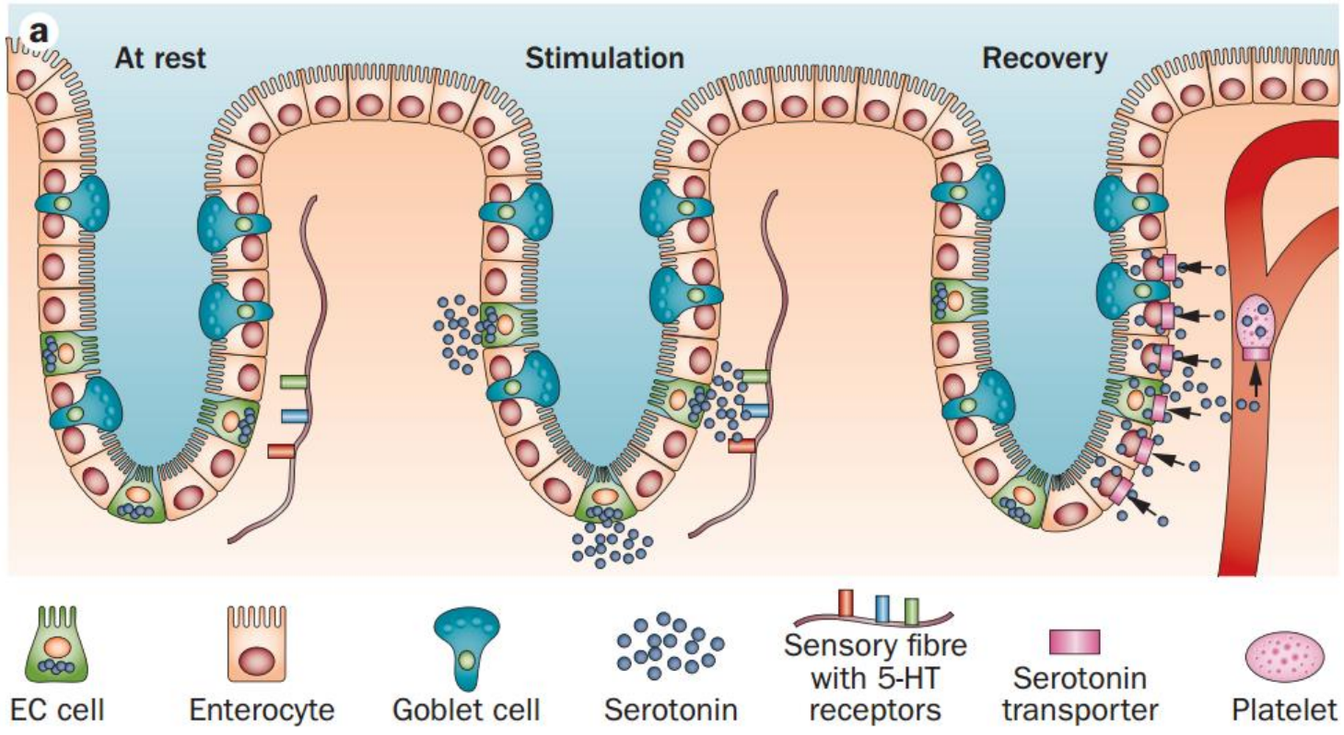

Fig. 5.1 5-HT signaling in the gut.

a) The sequence of events involved in 5-HT signaling in the gut. At rest, 5-HT is synthesized by enterochromaffin cells. Upon mechanical or chemical stimulation, 5-HT is released into the interstitial space of the lamina propria and binds to receptors on nearby nerve fibers. 5-HT signaling is terminated during the recovery phase: 5-HT is transported by serotonin transporter into epithelial cells where it is enzymatically degraded, or it enters the blood stream where it is transported into platelets and stored for future use. Modified from Mawe and Hoffman 2013.

5.2 Serotonin actions in the gut

Mechanical, chemical and nervous stimuli leads to the release of 5-HT in the lamina propria and in the gut lumen from ECs (Spiller 2008). The importance of the 5-HT in the GI physiology and pathophysiology is due to its double action as mucosal molecule (targeting the enterocytes) and as neurotransmitter (acting on nerves) (De Ponti 2004; Gershon 2012b). In fact, 5-HT has been defined as "polyfunctional signaling molecule" in the gut, exerting multiple actions both in developing and mature animals as a neurotransmitter, paracrine factor, endocrine hormone and growth factor (Gershon 2013). 5-HT initiates responses as diverse as nausea, vomiting, intestinal secretion and peristalsis. The 5-HT secreted in the lamina propria acts on the mucosal projection of the extrinsic primary afferent neurons (extrinsic nerves, spinal and vagal sensory neurons) and on the intramural neurons of the ENS presenting specific receptors (Mawe and Hoffman 2013)(**Fig 5.2**).

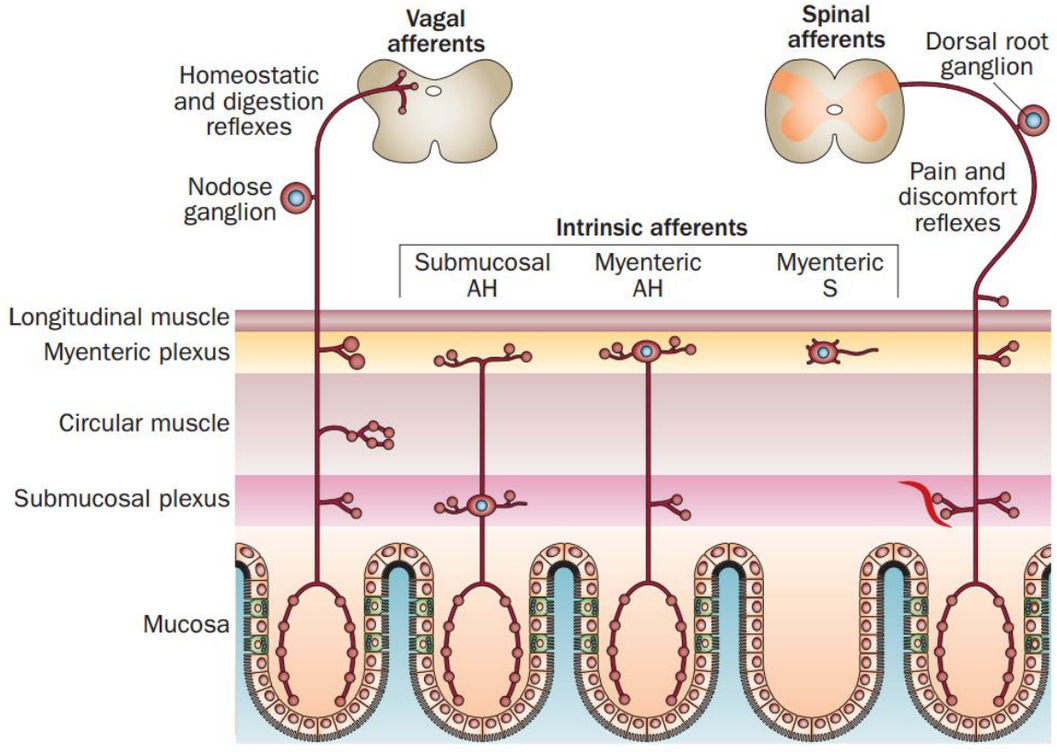

Fig. 5.2 Intrinsic and extrinsic afferent nerve fibers.
Most of the intrinsic and extrinsic primary afferent neurons that innervate the gut extend processes into the lamina propria of the mucosal layer where they can become exposed to 5-HT released by enterochromaffin cells. These include vagal afferent fibers arising from the nodose ganglion, spinal afferent fibers arising from dorsal root ganglia, and

intrinsic AH neurons located in submucosal and myenteric ganglia. There is also a class of mechanosensitive S neurons in the myenteric ganglia that do not project to the mucosal layer. Modified from Mawe and Hoffman 2013.

In particular, 5-HT can activate submucosal and myenteric *IPAN*s. Once activated by the 5-HT, the extrinsic component transmits nausea and discomfort (and pain) to the CNS, while the *IPAN*s initiate peristaltic and secretory reflexes. The 5-HT secreted by MP neurons acts directly on the GI motility pattern, regulating the fast and slow neurotransmission (Gershon and Tack 2007; Mawe and Hoffman 2013).

5.3 Serotonin receptors

The variety of serotonin functions in the body is determined by the activation of different pathways, depending on the receptors involved. In fact, there are 14 classes of serotonin receptors (5-HT1A, 1B, 1D, 1E, 1F, 2C, 2B, 2C, 3, 4, 5A, 5B, 6, and 7), located in presynaptic and postsynaptic neurons (Millan et al. 2008) and specifically distributed in different organs. All the classes of 5-HT receptors are G protein-couple receptors, except for 5-HT_3, which is a ligand-gated cation-permeable ion channel (Millan et al. 2008).

Actually, in the scenario of GI disorders, some the most interesting targets for pharmacological intervention are 5-HT receptors that are known to affect gut motor function, in particular they are those belonging to the 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 subtypes (**Fig. 5.3**) (De Ponti 2004).

Neuronal 5-HT receptors may inhibit or enhance transmitter release and include the 5-HT1A (inhibitory), 5-HT3, and 5-HT4 (both excitatory) subtypes. Smooth muscle 5-HT receptors may contract or relax effector cells and belong to the 5-HT2A (mediating contraction), 5-HT4, and 5-HT7 subtypes (both mediating relaxation). In the human small bowel, 5-HT2A receptors mediating contraction and 5-HT4 receptors mediating relaxation coexist on smooth muscle cells (De Ponti 2004).

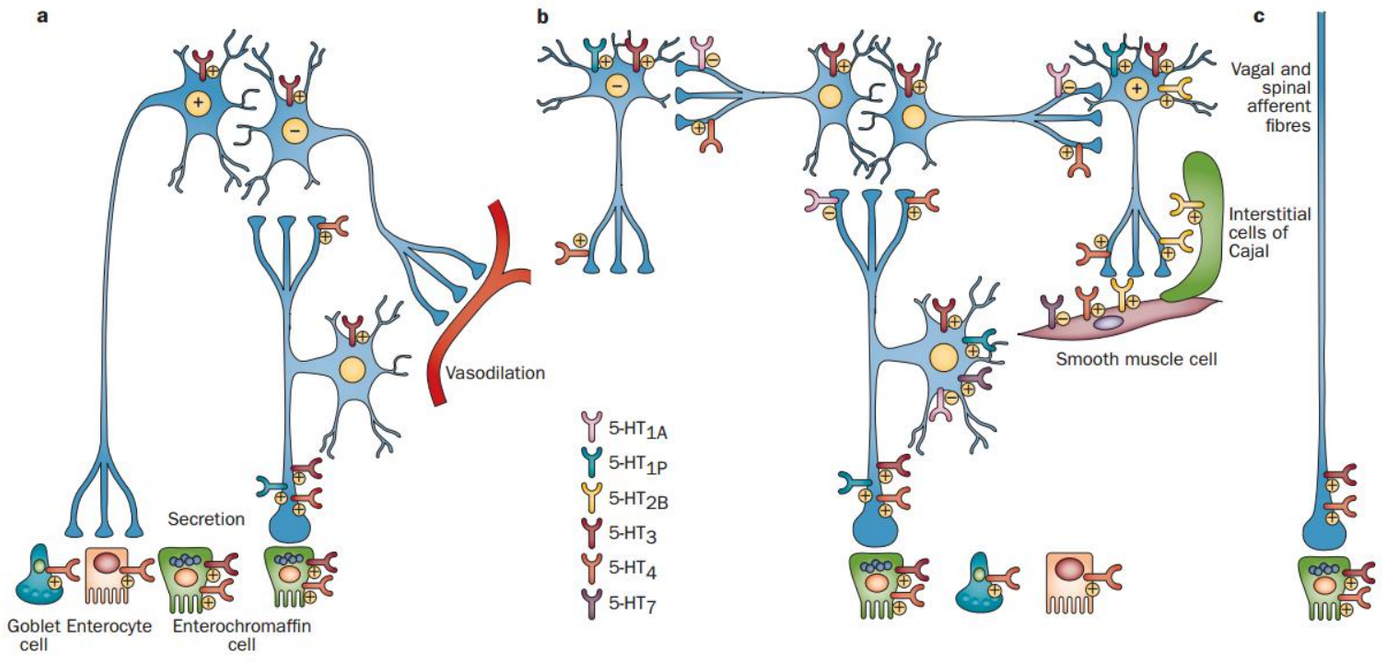

Fig.5.3 Distribution of 5-HT receptors on enteric neurons, extrinsic nerve fibers and other excitable cells in the gut.

The importance and complexity of 5-HT signaling in gut function, and its appeal as a target for pharmacotherapies, is reflected by the distribution of 5-HT receptors in the wall of the gut. Serotonin receptors are located on epithelial cells, including enterochromaffin cells, goblet cells and enterocytes. They are also distributed throughout the neuronal reflex circuitry, where they are located presynaptically and/or postsynaptically on all types of enteric neurons, and on extrinsic afferent nerve fibers. Furthermore, 5-HT receptors are expressed by interstitial cells of Cajal and smooth muscle cells. At least six subtypes of 5-HT receptors are expressed in the wall of the gut, and they can exert excitatory and/or inhibitory influences depending on their location and on the target cell type. A) Intrinsic circuits for epithelial secretion and vasodilation. A given motor neuron might serve both secretory and vasodilatory functions, but it promotes secretion via actions on epithelial cells and vasodilation via relaxation of vascular smooth muscle. B) Intrinsic circuit for propulsive motility. C) Extrinsic vagal and spinal afferent fibers. The + and – symbols indicate excitatory and inhibitory actions, respectively. Modified from Mawe and Hoffman 2013.

Peristaltic and secretory reflexes are initiated by submucosal *IPAN*s, which are stimulated by 5-HT acting at 5-HT(1P) receptors. Sensory signaling to the CNS is predominantly 5-HT_3R mediated, although serotonergic transmission within the enteric nervous system and the activation of myenteric *IPAN*s are also 5-HT_3 mediated. 5-HT acting at 5-HT_4R determine a neurotransmission in prokinetic pathways, through the enhancement of Ach and CGRP release by

submucosal *IPAN*s. The increase of Ach and CGRP enhances neurotransmission, in turn increasing the spread of excitation in the bowel following mucosal stimulation, and thus the tendency of the gut to manifest propulsive peristaltic and secretory reflexes (Gershon 2004).

In the past decades, the viability of 5-HT_3 and 5-HT_4 receptor antagonists and agonists, has determined an explosion of studies aimed to investigate the possibility of a pharmacological approach for different GI disorders (De Ponti 2004; Gershon and Tack 2007; Spiller 2008; Mawe and Hoffman 2013).

The current knowledge on the serotonin system in the GI lead to conclude that the modulation of the bioavailability of gut-derived serotonin and of the serotonin pathway, represent a very significant and promising way of treating patients with GI dysmotility conditions.

CHAPTER 6

Enteric neuropathies

Any damage, noxae or dysfunction involving the main effector or the control system (mainly the ENS) of the gut physiology may be responsible for the onset of a wide array of pathological conditions involving some lethal or seriously disabling manifestations (Schemann and Grundy 1999; Knowles et al. 2013). Human pathologies that involve the ENS include dysmotilities (e.g., achalasia and chronic intestinal pseudo-obstruction), some of which are potentially fatal (e.g., infantile hypertrophic pyloric stenosis, Hirschsprung's disease) (De Giorgio and Camilleri 2004).Serious gastrointestinal GI pathologies due to ENS malfunction are reported in humans as well as in domestic mammals, e.g., abomasal displacement in cattle (Geishauser et al. 1998) and lethal white foal syndrome in horses (Metallinos et al. 1998), an equine version of Hirschsprung disease; damage of autonomic ganglia has been reported in dysautonomia of dogs, cats (Key-Gaskell syndrome) (Nash 1987), and horses (grass sickness) (Cottrell et al. 1999) which is associated with severe gastrointestinal malfunctions.

Pathological conditions involving the ENS are defined as "enteric neuropathies". Several GI disorders can result from enteric neuropathies, including both primary and secondary forms. These neuropathies have been grouped as congenital or developmental neuropathies; sporadic and acquired neuropathies; neuropathies associated with other disease states and iatrogenic or drug-induced neuropathies (Furness 2012) (**Table 6.1**).

Congenital or developmental neuropathies
Hirschsprung disease (colorectal aganglionosis)
Hypertrophic pyloric stenosis
Multiple endocrine neoplasia 2B
Neuronal intestinal dysplasia
Mitochondriopathies affecting enteric neurons
Sporadic and acquired neuropathies
Chagas disease
Neurogenic forms of intestinal pseudo-obstruction
Slow transit constipation
Chronic constipation, including constipation of aging
Pathogen-induced diarrhea
IBS
Autoimmune enteric neuritis, paraneoplastic syndrome
Enteric neuritis of unknown etiology
Internal anal sphincter achalasia
Neuropathies secondary to, or associated with, other disease states
Diabetic gastroparesis and other diabetes-related motility disorders
Enteric neuropathy of Parkinson disease
Enteric neuropathy of prion disease
Enteric neuropathies associated with mental retardation or other central nervous system disorders
Ischemic enteric neuropathy, such as in ischemic colitis
Iatrogenic or drug-induced neuropathies
Disorders initiated by antineoplastic drugs (including vinca alkaloids and cisplatin)
Postoperative ileus
Neuropathy of ischemia, reperfusion injury, such as that associated with intestinal transplantation
Opioid-induced constipation (usually caused when opioids are used to treat chronic pain)

Table 6.1 A classification of enteric neuropathies. Modified from Furness 2012.

Obviously, the most of the current knowledge on enteric neuropathies is related to human medicine and experimental animal models.

6.1 Histopathological aspects

The current literature shows the existence of many morphological and molecular features in enteric neuropathies resulting in gut dysmotility, in the absence of systemic or easily identified neuromuscular disorders. A combination of molecular derangements may contribute to the degenerative process that leads to enteric neuronal loss. Among these, there are disorders of intracellular Ca^{2+} signaling, mitochondrial dysfunction, oxidative stress and alterations in signal

transduction pathways. Enteric pathological features of GI neuromuscular diseases include aganglionosis, neuronal apoptosis and inclusions, neural degeneration, intestinal neuronal dysplasia, hyperplasia and ganglioneuromas, mitochondrial dysfunction, inflammatory neuropathies, neurotransmitter disorders, interstitial cell pathology (De Giorgio and Camilleri 2004).

Aganglionosis is characterized by the complete absence of ganglion cells in the submucosal and myenteric plexuses; it occurs most frequently in the congenital form, such as Hirschsprung's disease (Kapur 1999).

Apoptosis of myenteric neurones has been described in diseases associated with myenteric ganglionitis and are often associated to pseudo obstruction (De Giorgio and Camilleri 2004); a distinct process is characterized by apoptotic bodies, which are features of programmed cell death (Nijhawan et al. 2000).

Neuronal inclusions are composed of proteinaceous material and electron microscopy documented membrane-bounded filaments. Similar intranuclear inclusions (i.e. Lewy bodies and Lewy neuritis) occur in neurones of patients with central nervous system diseases (Patel et al. 1985) such as Parkinson disease (PD)(Wakabayashi et al. 1988) . Lewy inclusions have also been observed in myenteric and submucosal neurones in parkinsonian patients and seems to be associated to GI dysfunctions (Fasano et al. 2015).

Neural degeneration is associated with a reduction in the total number of neurones in chronic intestinal pseudo-obstruction; remaining neurons may result enlarged, with thick, clubbed processes; some ganglia show an increase in the number of glial cells and hypertrophy of the muscularis propria.

Intestinal neuronal dysplasia is classified in type A and B. Type A intestinal neuronal dysplasia is extremely rare, while type B is more common. Type A is characterized by hypoplasia or immaturity of the extrinsic sympathetic nerves supplying the gut. Type B is characterized with a wide spectrum of changes in the ENS, including submucosal hyperganglionosis, increased numbers of cells per ganglion (giant ganglia), associated with ectopic neurones localized throughout the lamina propria.

Neuronal hyperplasia and ganglioneuromas consists in an increased number and nodular proliferations of ganglion cells (neurons and glia), associated with abundant nerve fibres, occurring in the myenteric plexus (Shekitka and Sobin

1994). This histopathological appearance is almost pathognomonic of multiple endocrine neoplasia type 2B (MEN2B) (Carney et al. 1976), a heritable disorder associated with tumours of the neuroendocrine system (Moline and Eng 2011).

Mitochondrial dysfunctions resulting from structural, biochemical or genetic derangements are known to be associated to neuronal death both in CNS and ENS. Mitochondrial impairment associated with neural cell death may involve complex mechanisms, including disorders of intracellular Ca2+ signaling, oxidative stress, and alterations in mitochondrial pathway(Moudy et al. 1995; De Giorgio and Camilleri 2004). These mechanisms may result in necrotic and apoptotic enteric neuronal loss, as documented in severe constipation in elderly and neurogenic type of chronic idiopathic intestinal pseudo-obstruction. Mitochondrial disorders may also determine syndromic diseases with severe GI dysfunction, such as the Mitochondrial neurogastrointestinal encephalopathy (MNGIE)(Mueller et al. 1999).

Inflammatory neuropathies are determined by inflammatory or immunological insult to the ENS GI , resulting in enteric ganglionitis and axonitis (Krishnamurthy et al. 1986). Cellular mechanisms in myenteric ganglionitis involve inflammatory infiltrate surrounding ganglion cell bodies, including cytotossic T lymphocytes (in achalasia and chronic intestinal pseudo-obstruction), B-lymphocytes producing antibodies against antigens expressed by myenteric neurons, and also osinophils and neutrophils (De Giorgio and Camilleri 2004).

Neurotransmitter disorders are described in dysfunction of sphincteric regions i.e. achalasia and congenital hypertrophic pyloric stenosis, in which loss of intrinsic inhibitory neurones has been documented. Many studies also described different neurotransmitter alterations in patients affected by idiopathic slow transit constipation. A variety of genetic defects as well as inflammatory or degenerative processes may be responsible for neurotransmitters imbalance, and as consequence, for GI dysmotilities (De Giorgio and Camilleri 2004).

Interstitial cell pathology is well documented in various dysmotilities, including achalasia and hypertrophic pyloric stenosis, idiopathic or diabetes-related gastroparesis, Hirschsprung's disease, chronic intestinal pseudo-obstruction, slow transit constipation and Chagasic and idiopathic megacolon. Different pathological features related to ICCs are described: reduced number, loss of processes and damage to the intracellular cytoskeleton and organelles, as well as alteration during the development or maturation of ICCs (De Giorgio and Camilleri 2004; Knowles et al. 2013).

6.2 Primary neuropathies

Enteric neuropathies are classified as "primary" in the case that the ENS is the major target of the disease process. Most are termed as 'idiopathic' to denote the poor aetiological understanding of these conditions. Among primary neuropathies are listed Hirschsprung's disease, idiopathic achalasia, idiopathic gastroparesis, chronic intestinal pseudo-obstruction (Knowles et al. 2013) .

Hirschsprung's disease is characterized by an absence of enteric neurons (aganglionosis) in terminal regions of the gut, leading to tonic contraction of the affected segment, intestinal obstruction and massive distension of the proximal bowel (megacolon). In humans, Hirschsprung's disease occurs in 1:5,000 live births and can be either familial or sporadic. Mutations occurring in genes involved in the development, colonization and differentiation of enteric neurons are responsible for genetic forms. On the contrary, sporadic forms seems to have multifactorial genesis (Di Nardo et al. 2008; Panza et al. 2012).

Idiopathic achalasia is characterized by hypoganglionosis or aganglionosis in the LES region, with or without inflammatory and degenerative features (Goldblum et al. 1994). Evidences demonstrated specific inhibitory neuronal loss, including nitrergic motorneurons, in LES and gastric fundus (Gockel et al. 2008; Knowles et al. 2013).

Idiopathic gastroparesis, seems to be due to generic neuronal loss, decreased nNOS expression and in the most of the cases to ICCs reduction or abnormalities. These mechanisms are responsible for gastric dismotility (Knowles et al. 2013).

Chronic intestinal pseudo-obstruction (CIPO) indicate a condition in which affected subjects show failure of the propulsive forces of intestinal peristalsis to overcome the natural resistances to flow. Myogenic and neurogenic altered mechanisms contribute to CIPO (De Giorgio et al. 2011). Concerning CIPO-related neuropaty, there are evidences of neuronal degeneration and loss, with or without ganglionitis due to lymphocytic and eosinophilic infiltrate (De Giorgio and Camilleri 2004).

6.3 Secondary neuropathies

Secondary neuropathies are defined as pathological conditions in which the ENS is not the prymary target of the disease. They can be classified as degenerative neuropathies and inflammatory neuropathies, based on the mechanisms underlying the enteric neuronal pathology. Degenerative neuropathies include diabetes mellitus (DM) and PD, while inflammatory neuropathies include paraneoplastic enteric neuropathy and Chagas disease (in humans) (Knowles et al. 2013).

Diabetes mellitus (DM) can affect all GI tracts. In fact, symptoms such as abdominal pain, heartburn, dysphagia, post prandial fullness, nausea, diarrhoea and constipation are common in diabetic patients(Bytzer et al. 2001). GI dysfunctions during DM seems to be due to ICCs abnormalities, neuronal degeneration and loss and specific enteric subclasses impairment, including mostly inhibitory and less excitatory motor neurons(Chandrasekharan and Srinivasan 2007). Concerning diabetic gastroparesis, several key cell types are affected by diabetes. These changes include abnormalities in the extrinsic innervation to the stomach, loss of key neurotransmitters at the level of the enteric nervous system, smooth muscle abnormalities, loss of interstitial cells of Cajal, and changes in the macrophage population resident in the muscle wall. Although oxidative stress may play an important role in the genesis of GI alteration in DM, mechanisms leading to neuropathy and loss of ICC remains to be determined (Farrugia 2015).

Parkinson disease has been associated with intra neuronal accumulation of hyperphosphorylated α-synuclein (a typical component of classic Lewy bodies and neurites), in the ENS, with or without neuronal degeneration and loss in different GI tracts (Fasano et al. 2015). Parkinsonian patients complains a wide arrays of GI symptoms, including dysphagia, gastroparesis and severe constipation (Pfeiffer 2003). To date, the association between the presence of intra neuronal inclusions and GI dysfunction remains to be demonstrated .

Paraneoplastic enteric neuropathy is characterized by gastroparesis, intestinal pseudo-obstruction, intractable constipation, colonic inertia or megacolon (Lee et al. 2001). Anti-neuronal antibodies directed against the RNA-binding protein family Hu (anti-Hu) are the most common autoantibody expressed in affected patients and seems to elicit

myenteric neuronal apoptosis(Lucchinetti et al. 1998). In addition, ICCs alterations and ganglionitis were also reported in affected patients (Pardi et al. 2002).

Chagas disease is caused by infection with the protozoan *Trypanosoma cruzi*. Affected patients showed enteric neuropathy (myenteric neuronal loss) and in 10% of cases associated with gastrointestinal dilation, preferably in oesophagus and colon. In particular, enteric neuropathy in is responsible for the failure of colorectal propulsion and megacolon development in adults (Knowles et al. 2013). This neuropathy has classically been reported as being one of generalized neuronal loss with inflammation although selective loss and survival of neuronal subpopulations were reported (Da Silveira et al. 2009). Functional autoantibodies that modify muscarinic receptor functions have been detected in a notable proportion of patients with chagasic heart and gut disease and might include direct damage from autoimmunity(Goin et al. 1999).

Enteric neuropathy	Estimated lifetime clinical prevalence	Histological prevalence
Primary		
Hirschsprung disease	1 in 5,000	100% have aganglionosis[12]
Congenital CIPO	1 in 10,000–20,000	25% have neuropathy,[3,146] of which the majority are myopathies[3,150]
Idiopathic achalasia	1 in 10,000	100% have neuropathy[15]
Idiopathic gastroparesis	1 in 4,000	70% have neuropathy[21]
Acquired CIPO*	<1 in 2,000	60% have neuropathy[27,28]
Chronic constipation	Estimated 1 in 50–100	Some evidence[10,36]
Idiopathic megacolon/rectum	1 in 4,000	Some evidence[63,65]
Secondary		
NF1	1 in 4,000	<1 in 100,000‡[147]
MEN-2B	1 in 35,000	96% have gastroinestinal symptoms[148]
Paraneoplastic gastrointestinal syndromes	<1 in 50,000	100% where biopsied
Parkinson disease	1 in 500	70% based on submucosal Lewy body pathology[108]
Diabetes mellitus	1 in 20	1 in 4,000§
Systemic inflammatory disease	1 in 1,000	80% in in primary Sjögren syndrome and unknown in systemic lupus erythematosus
Crohn's disease	1 in 400	54–58%[143,144]
Chagas disease with gastrointestinal involvement	10% disease carriers	60% where biopsied[149]

Table 6.2 Enteric neuropathies with estimated prevalence data in humans. Modified from Knowles et al. 2013. Abbreviations: CIPO, chronic intestinal pseudo‐obstruction; MEN‐2B, multiple endocrine neoplasia type 2B; NF1, neurofibromatosis type 1.

EXPERIMENTAL STUDIES

CHAPTER 7

Excitatory and inhibitory enteric innervation of horse lower esophageal sphincter

Modified from

"**Excitatory and inhibitory enteric innervation of horse lower esophageal sphincter"**

R. Chiocchetti, F. Giancola, M. Mazzoni, C. Sorteni, N. Romagnoli, M. Pietra

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Introduction

The LES is a specialised, thickened part of the circular smooth muscle located at the junction between the esophagus and the stomach (Boeckxstaens 2005). It shows a high resting tone (Christensen 1973) mediated by myogenic and neurogenic mechanisms, and undergoes strong inhibitory innervation during swallowing or belching. The origin of basal LES tone is primarily myogenic, although the cellular mechanisms that impart and control it are multilayered, and the underlying pathways not fully understood. Myogenic LES tone depends on smooth muscle properties (Farre and Sifrim 2008); in fact, myocytes have a less negative resting membrane potential, which favors the occurrence of spontaneous spike-like action potentials that cause Ca^{2+} influx in the muscle during the resting state (Kwiatek and Kahrilas 2012).

The esophageal *tunica muscularis* shows different conformations, based on the domestic mammalian species (Worl and Neuhuber 2005); in the ruminant and dog, the musculature is totally striated whereas in the pig, cat, and horse (Barone 1981; Delmann 2000), the caudal portion of the esophagus and LES is composed of smooth muscle cells.

Peristalsis in the esophageal striated muscle is controlled by the nucleus ambiguous; peristalsis in the smooth muscle is controlled by the vagal dorsal motor nucleus and the MP (Mittal and Bhalla 2004). Nevertheless, in several mammalian species, including humans, striated muscle fibers are co-innervated by vagal and ENS neurons (Worl and Neuhuber 2005; Hempfling et al. 2009).

The LES is controlled by the parasympathetic, sympathetic and ENS (Niel et al. 1980; Clerc 1983; Collman et al. 1992; Collman et al. 1993; Brookes et al. 1996; Yuan et al. 1998; Kwiatek and Kahrilas 2012). The vagal preganglionic fibers innervate LES smooth muscle via MP neurons (Goyal and Chaudhury 2008), providing both inhibitory and excitatory innervation to the LES (Goyal and Rattan 1975; Furness 2012).

The equine LES is organized as a “one way” structure, enabling only the oral-anal progression of food; its resting tone increases by about 6-8 fold after deglutition (Stick et al. 1983; Clark et al. 1987) and may prevent vomiting. It interesting to note that, in this species, vomiting is an undesirable event because of the anatomical conformation of

equine soft palate. In fact, it is so elongated as to possibly hamper the passage of the rejected bolus to the mouth, deflecting it into the nasal cavities, increasing the probability of aspiration pneumonia.

Although horses have a very delicate digestive system, still few exhaustive investigations have been carried out on equine ENS (Pearson 1994; Freytag et al. 2008; Chiocchetti et al. 2009a; Chiocchetti et al. 2009b; Russo et al. 2010) and its disorders. Among horse neurological disorders affecting the ENS (for instance equine dysautonomia, megaesophagus, and overo lethal white syndrome), satisfactory investigations have been published only on the grass sickness (equine dysautonomia) (Cottrell et al. 1999; Pirie et al. 2014), although no data are related, to the best of our knowledge, to the modification of the esophageal ENS. To date, the literature on the alteration of the esophageal ENS during achalasia (and the related megaesophagus) is scarce (Komine et al. 2013). Likewise, concerning the overo lethal white syndrome, a congenital disorder of neural crest cell migration considered the equine variant of human Hirschsprung disease, few papers are available on the absence of intestinal neurons (Hultgren 1982; Muniz et al. 2013); however, no indications are present about the esophageal intramural neuronal subclass composition.

With the aim to study the intramural innervation involved in the control of the horse LES, we characterized immunohistochemically the inhibitory and excitatory enteric neurons of the caudal tract of the esophagus and the proximal portion of the gastric *fundus*. Inhibitory motor neurons may be immunohistochemically identified by the presence of the nNOS, the neuronal form of the enzyme synthesizing the nitric oxide, the primary neurotransmitter utilized by ENS neurons (also in the esophagus; (Conklin 1998; Farre and Sifrim 2008) during smooth muscle relaxation. Excitatory motor neurons utilize acetylcholine as main neurotransmitter and these neurons may be immunohistochemically identified by the presence of the synthesizing ChAT. Enteric neurons immunoreactive for nNOS and ChAT represent the two largest neuronal subpopulations occurring in the ENS (Furness 2006), also in the horse (Freytag et al. 2008; Chiocchetti et al. 2009b).

Materials and methods

Animal and tissue collections – Tissues were collected from six young Spanish Warmblood horses (1.5 years) slaughtered at the public slaughterhouse, and from three Italian standardbred trotter horses (two 1-week old foals and

one 19-years old horse) euthanized because of cardiac (foals) and musculoskeletal problem. None had gastrointestinal disorders.

En block specimens consisting of the caudal third of the esophagus (about 40 cm) and the stomach were immediately removed and then longitudinally cut open, flushed out with phosphate-buffered saline (PBS: 0.15M NaCl in 0.01M sodium phosphate buffer, pH 7.2), gently pinned on wood board (thickness 5 mm) and fixed in 2% paraformaldehyde containing 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.0) at 4°C for 48 hours. After rinsing in PBS, the tissues were stored in PBS containing 30% sucrose and 0.1% sodium azide (pH 7.4) at +4°C. The tissue was then separated taking a caudal portion of the esophagus (20 cm) and a portion (10 cm) of non-glandular stomach (*fundus*); the site of maximum muscular thickness (about 2 cm) within the gastro-esophageal junction served as a landmark for the determination of the measurement site. Pieces of tissues (2 x 1 cm) were subsequently cut, transferred to a mixture of PBS-sucrose-azide and OCT compound (Tissue Tek®, Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) at a ratio of 1:1 (overnight) and then embedded in 100% OCT. The tissues were frozen, mounted in Tissue Tek® mounting medium and sectioned at 14-16 μm on a cryostat. The sections were collected on gelatin-coated slides.

Immunohistochemistry - Cryosections were washed in PBS and processed for immunostaining. To block non-specific bindings, the sections were incubated in a solution containing 10% normal goat serum (Colorado Serum Co., Denver, Colorado, USA) and 0.5% Triton X-100 (Merck, Darmstadt, Germany) in PBS for 1h at RT. Cryosections were then incubated overnight in a humid chamber at RT in a cocktail of primary antibodies (**Table 7.1**) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. Enteric neurons were identified with the anti-human neuronal protein (HuC/HuD) antiserum or the green fluorescent Nissl stain solution (NeuroTrace®, Molecular Probes, Eugene, OR, USA).

The specificity of the employed antibodies has already been tested in the horse by Western blot (Russo et al. 2010).

After washing in PBS (3x10 min), the tissues were incubated for 1 h at RT in a humid chamber with the secondary antibodies (**Table 7.2**) diluted in PBS. The cryosections were then washed in PBS (3x10 min) and mounted in buffered glycerol at pH 8.6. The specificity of the secondary antibodies was tested as described in a previous work (Russo et al. 2010).

Analysis of the sections - The sections were observed with a Zeiss Axioplan (Carl Zeiss, Oberkochen, Germany) microscope equipped with the appropriate filter cubes for immunofluorescence. The images were recorded with a Zeiss Axiocam MRm (Carl Zeiss) and Axiovision software (Carl Zeiss). Contrast and brightness were slightly adjusted with Adobe Photoshop CS (Adobe Systems Software Ireland, Dublin, Ireland), whereas the figure panels were prepared using Corel Draw (Mountain View, Ottawa, Canada).

For co-localization analysis, the cells were first located by the presence of a fluorophore which labelled one antigen; thereafter the filter was switched to a different color fluorophore to determine whether the neurons were also labelled for a second antigen. In this way, the proportions of cholinergic and nitrergic neurons were determined, as were the percentages of co-localizations between these two markers. Data obtained are reported as mean±standard deviation.

Results

Histological esophageal features

At 18 cm from the LES, the esophageal *tunica muscularis* was composed of muscular striated fibers intermingled with few smooth muscle cells. Approaching the caudal portion of the esophagus, the striated elements disappeared progressively, although they were also observed at 8 cm from the cardias (**Fig. 7.1 a-b**). The LES was entirely composed of smooth muscle cells. The single layered *mm* was continuous and thick. Large glands were observed in the submucosa (**Fig. 7.1 c**).

Esophageal ENS

In large ganglia, the myenteric neurons were often eccentrically distributed, with cell bodies showing a crown-like distribution (**Fig. 7.2 a-c**).

The SMP appeared double-layered; the inner layer (ISMP) was composed of clusters of small neurons occupying an intermediate position between the *mm* and the CML. The outer layer (OSMP) was represented by small groups or, more often, single rows of larger neurons located very close or attached to the inner portion of the CML (**Fig. 7.2 d-f**). Occasionally small groups of neurons appeared “embedded” in the inner portion of the CML.

Nitrergic neurons – In the MP, nNOS-IR neurons represented 72±9% of the total neuronal myenteric population (530/727 cells; n=7). These neurons were large and showed irregular outline and short lamellar processes, resembling Dogiel type I neurons (**Fig. 7.2 a-c**). nNOS-IR fibers were mainly seen in the CML but were well represented also in the LML. Large bundles of NOS-IR fibers ran longitudinally in the space between the LML and CML.

In the SMP, a large percentage of Dogiel type I neurons expressed nNOS-IR (69±8%, 118/173 cells, n=4); these neurons were observed in both layers of the SMP (**Fig. 7.2 g-i**). In the submucosa as well, large nNOS-IR bundles of fibers were seen in continuity with the CML. Also noteworthy is the abundance of nNOS-IR thin fibers (with their long axis parallel to the LML) running within the *mm* (**Fig. 7.2 g-i**).

Cholinergic neurons - In the MP there were 29±14% of faintly labelled cholinergic neurons (149/434 cells, n=4), whereas in the SMP the percentage of these neurons was greater (65±24%, 64/97 cells, n=4) and their labeling stronger (**Fig. 7.3 a-c**).

Co-localizations of *nNOS- and ChAT-IR* - In the MP and SMP, the nNOS-IR neurons co-expressing ChAT-IR were 15±11% (66/381 cells, n=3) and 30±17% (34/106 cells, n=3), respectively, whereas cholinergic neurons co-expressing nNOS-IR were 34±24% (65/150 cells, n=3) and 44±23% (42/90 cells, n=3). Myenteric neurons showing strong ChAT-IR were often nNOS-negative, whereas those showing moderate ChAT-IR were also nitrergic (**Fig. 7.3 d-f**). In the SMP instead, ChAT- and nNOS-IR neurons showed the same brilliant fluorescence (**Fig. 7.3 g-i**).

Fundic ENS

The myenteric ganglia were, in general, smaller than the esophageal ones and also in the stomach most of the large neurons observed showed Dogiel type I morphology. The MP neurons were also scattered between fascicles of smooth muscle and connective tissue. In the submucosa, few small ganglia, composed of elongated small neurons, were visible. The SMP was monolayered.

Nitrergic neurons– As many as 57±17% (395/813 cells, n=6) of MP neurons showed nNOS-IR (**Fig. 7.4 a-c**), and the few SMP ganglia contained 45±3% of nNOS-IR neurons (78/150 cells, n=3).

Cholinergic neurons - Cholinergic MP and SMP neurons accounted for 36±8% (266/739 cells, n=3) (**Fig. 7.4 d-f**) and 38±20% (41/98 cells, n=3), respectively.

Co-localizations of *nNOS- and ChAT-IR* - In the MP, the nNOS-IR neurons co-expressing ChAT-IR showed wide variability, ranging from 16% to 83% (32±31%; 86/280 cells, n=3), whereas in the SMP these cells were 54±30% (30/95 cells, n=3). Cholinergic MP and SMP neurons co-expressing nNOS-IR were 41±16% (89/306 cells, n=3) (**Fig. 7.4 g-i**) and 54±26% (28/62 cells, n=3). Data related to the caudal esophagus and gastric fundus are illustrated in **Fig. 7.5.**

Discussion

Esophageal structure

Horse striated esophageal musculature extended far caudally and striated and smooth musculature co-existed in the caudal esophagus up to few centimeters from the LES.

In the horse, the transition from striated to smooth musculature is described as very abrupt, with the striated muscle giving way to smooth muscle cells at the level of the tracheal division (Barone 1981) or more gradual in the caudal third of the esophagus (Delmann 2000). The submucosa contains glands which, in the horse, have been described only in the rostral portion of the esophagus (Nickel 1973; Barone 1981; König 2006); in the mucosa, the *muscularis mucosae* is well developed and the epithelium is keratinized (Delmann 2000).

Submucosal glands, never described so far in the equine caudal esophagus (Nickel 1973; Barone 1981; Delmann 2000; König 2006), produce mucous and bicarbonate (Long and Orlando 1999), which is able to provide local protection from the injurious effects of refluxed gastric juice proximal to the cardias, thus leaving the squamous epithelium in this area intact. This hypothesis does not seem to apply to the horse, given the absence of vomit in this species. Although these glands seem to be under the control of the vagus nerve (Rossowski et al. 1996; Long and Orlando 1999) it is not excluded that the ENS might play a role in their innervation.

Esophageal ENS

It is known that the mammalian MP well developed from the esophagus to the internal anal sphincter whereas the submucosa of the esophagus and stomach lacks a continuous ganglionated SMP (Lefebvre et al. 1995; van Ginneken et al. 1996; Teixeira et al. 2001; Furness 2006). Although the equine esophagus and gastric fundus lack the fluid fluxes across the mucosal epithelium that occur in the small and large intestines, we observed a continuous and double layered

SMP. This finding reinforces both the role of the ENS in this tract of the horse esophagus and the notion that the ENS is no less important than the vagal circuitries.

Almost all MP and SMP neurons showed Dogiel type I morphology; this observation is consistent with the rare evidence of Dogiel type II myenteric neurons in the esophagus (Furness 2006). Dogiel type I neurons are cells showing irregular (angular) outline, short lamellar dendrites and only one axon; these features usually characterize inhibitory and excitatory motor neurons or interneurons (Furness 2006). Dogiel type II neurons are large or oval cells with smooth outline showing up to 11 long processes which have been demonstrated to be, on morphological and functional grounds, axons (Stach 1981). These cells are considered intrinsic primary sensory neurons (Furness 2006).

nNOS-IR neurons – We observed a largely greater percentage (about 72%) of nNOS-IR MP neurons than in the other tracts of the equine digestive system (Freytag et al. 2008; Chiocchetti et al. 2009a). This evidence reinforces the role of inhibitory esophageal neurons which are necessary for the LES relaxation.

It is interesting to note that the percentages of nNOS-IR neurons observed in the foals were comparable to the percentage seen in the adult subjects, despite the LES of foals is not a strong barrier to reflux than in adults. It is known that the development of the ENS (and its functions) begins in fetal life and continues for some time post-partum, depending also on the maturity of the species at birth (Bueno and Ruckebusch 1979; Branchek and Gershon 1989; Milla 1993), thus it is plausible that at birth the two main ENS neuronal subclasses (nitrergic and cholinergic neurons) are well represented in the horse.

It is interesting to note that the percentage of MP nitrergic neurons observed in the horse, a species considered unable to vomit, was larger than the percentage of nitrergic neurons counted in species able to vomit, such as the cat (30-45%) (Rodrigo et al. 1998), monkey (45%) (Rodrigo et al. 1998), human (about 55%) (Singaram et al. 1994), opossum (35–51%) (Fang and Christensen 1994), but also of those unable to vomit, such as the guinea-pig (from about 53% to 69%) (Furness et al. 1994; Brookes et al. 1996; Morikawa and Komuro 1998) and rat (about 65%) (Wu et al. 2003b; Dong et al. 2013).

Contradictory data are also related to the mouse (unable to vomit) esophagus; Grozdanovic et al. (Grozdanovic et al. 1992) observed that 100% of the MP neurons were nitrergic whereas a later study showed that not all myenteric neurons expressed nNOS (Sang and Young 1998; Sang et al. 1999). Taken together, these data suggest that animals unable to

vomit show a stronger tone of the LES and, consequently, the need of a greater percentage of esophageal nitrergic inhibitory neurons able to relax this sphincter during swallowing.

Another interesting piece of evidence is the presence of large amounts (about 69%) of nNOS-IR neurons in the SMP of the horse esophagus; this finding is quite different compared to the percentage of nitrergic neurons observed in the ileal SMP of the same species (about 5%) (Chiocchetti et al., 2009a). The SMP is well developed in both the small and large intestine, whereas only scattered submucosal ganglia can be found in the esophagus and stomach (Lefebvre et al., 1995; Teixeira et al., 2001; Furness, 2006). While the SMP in small mammals is organized in a single layer (Furness, 2006), it is multilayered in large mammals (Timmermans et al. 1992b; Pearson 1994) and human (Brehmer et al., 2010). In particular, the horse SMP is double layered (Pearson et al., 1994; Chiocchetti et al., 2009a). Studies on other large mammals have shown that the neurons of the outer SMP project to the circular muscle and, possibly, also to the longitudinal muscle (Sanders and Smith, 1986).

ChAT-IR neurons – We observed a small percentage of MP cholinergic neurons (about 29%) but a large percentage (about 65%) in the SMP. In the horse ileum these neurons account for approximately 66% of MP and 74% of SMP neuronal populations (Chiocchetti et al. 2009a).The paucity of MP cholinergic neurons in the caudal esophagus of the horse is interesting, although we should take into account that, as discussed above, the SMP of large mammals contains also neurons acting on muscle layers.

We noted that smooth muscle cells are intermingled with striated fibers also very close to the LES; the paucity of MP cholinergic neurons could also be correlated to the presence of vagal cholinergic innervation regulating the esophageal motility of striated muscle, but not of smooth muscle. Furthermore, the vagus nerve is able to act on contraction and relaxation of the LES directly via cholinergic and nitrergic release (Hornby and Abrahams 2000; Smid and Blackshaw 2000; Kuramoto and Kadowaki 2006; Goyal and Chaudhury 2008; Furness 2012) but also indirectly, via stimulation of MP nitrergic neurons (Goyal and Rattan 1975; Yuan et al. 1998); this could mean that, in the horse, in which the tonic closure of the LES is consistent, nitrergic neurons are more necessary than cholinergic ones, to regulate the inhibition of the LES.

Few studies are available on other species, regarding the quantification and characterization of LES cholinergic neurons. In the opossum and guinea-pig cholinergic MP neurons represent, respectively, 38% and 47% of local LES

neurons (Seelig et al. 1984; Brookes et al. 1996). In the rat, Nakajima et al (Nakajima et al. 2000) observed that cholinergic neurons were largely represented in the esophageal MP (76%) whereas no neurons were observed in the SMP of esophagus and stomach.

The studies reported above took into account the nitrergic and cholinergic local ENS neuronal subpopulations, but did not identify the descending neurons specifically innervating the LES, except for the investigation by Brookes et al. (1996) who identified nitrergic and cholinergic neurons innervating the LES by the injection of the lipophilic fluorescent tracer DiI in organotypic culture.

Gastric ENS

The stomach has two main functions: the first is to relax prior to food intake and act as a "reservoir" by increasing its volume as it fills; the second is to mix the food with gastric juice and push it down into the duodenum. The *fundus* is primarily associated with gastric reservoir functions while the *corpus* and *antrum* are associated with gastric mixing and propulsion (Kelly 1981; Lefebvre et al. 1995).

The adjustment of gastric volume is mediated through the vagus nerve, although the ENS may play a role in accommodation reflexes (Furness 2006). The intensity of rhythmic gastric movements is modulated by enteric neurons which, nevertheless, do not have the same prominent role shown in the intestinal reflexes. In the present research the MP ganglia were scantly represented and SMP ganglia have rarely been observed. As reported for the caudal esophagus, also in the fundic portion of the stomach, no Dogiel type II neurons (putative intrinsic primary afferent neurons) were seen; this evidence strengthens the role of the vagus nerve in oeasophago-gastric sensitive functions.

nNOS-IR neurons - Gastric relaxation seems to be mediated also through inhibitory motor neurons (nNOS-IR), and this function justifies the presence of a high percentage of nNOS-IR neurons in the *fundus* (about 57%).

The *mm* is innervated by excitatory and inhibitory motor neurons (Onori et al. 1971); in large mammals such as cat and dog, the *mm* receives innervation from SMP neurons (Onori et al. 1971; Furness et al. 1990), and it is very plausible that also in the horse – although gastric SMP ganglia were not frequently observed – the gastric double layered *mm* is innervated by SMP neurons. We observed that the equine gastric *mm* was thinner than the esophageal one which could justify the smaller percentage of nNOS-IR neurons observed in the gastric SMP (69±8% *vs*. 45±3%).

ChAT-IR neurons – The reduced percentage of ChAT-IR MP neurons (about 36%) could reflect the prevalence of cholinergic vagal innervation and the inability to contract of this gastric portion. The absence of gastric glands in the equine *fundus* could justify the reduced percentage of SMP cholinergic neurons (about 38%), which should be gastric secretomotor neurons (Furness 2006).

In the human gastric *fundus* MP, cholinergic neurons are 34±6% whereas nNOS-IR neurons are 24±4%(Pimont et al. 2003). In the rhesus monkey MP, cholinergic neurons are 70±5% (twice as many as in humans) whereas nitrergic neurons are 22±3% (similar to humans) (Noorian et al. 2011).

In the guinea-pig gastric antrum, cholinergic neurons are 56% and nitrergic one are 41% (Vanden Berghe et al. 1999).

Co-localizations of *nNOS- and ChAT-IR* - The mixed phenotype seems to be, in general, a distinctive feature of MP interneurons; in the guinea-pig small intestine, neurons co-expressing ChAT and nNOS-IR account for 5% of all the neuronal population (Furness 2006). Also in the mouse esophagus, 5% of ChAT-IR neurons co-express nNOS-IR and *viceversa* (Sang and Young 1998; Sang et al. 1999).

In large animals, the percentage of enteric neurons co-expressing ChAT- and nNOS-IR is greater; in the pig ileum, the percentage of MP cholinergic neurons co-expressing nNOS-IR does not exceed 9.6% (Brehmer et al., 2004). In the horse and sheep ileum, the cholinergic neurons co-expressing nNOS-IR are about 30% and 48% (Chiocchetti et al., 2009a; Mazzuoli et al., 2007).

In the human intestine neurons co-expressing ChAT- and nNOS-IR account for about 3-4% of the total neuronal MP population, in both the small and large intestine (Beck et al., 2009; Murphy et al., 2007) and about 14% in the gastric fundus (Pimont et al., 2003).

Table 7.1. Details of the primary antibodies and NeuroTrace® (NT) marker used.

Antisera	Host species	Serum Code	Dilution	Source
ChAT	Rabbit	P3YEB	1:250	kindly provided by Prof. Michael Schemann, Technische Universität München, Germany
HuC/HuD	Mouse	A21271 #833294	1:400	Molecular Probes
nNOS	Mouse	(A-11) sc-5302	1:50	Santa Cruz
nNOS	Rabbit	SA-227 #T4254	1:200	Biomol
NT		N21480	1:200	Molecular Probes

Abbreviations: ChAT, choline acetyltransferase; HuC/HuD, human neuronal protein; nNOS, neuronal nitric oxide synthase; NT, the green fluorescent Nissl stain solution.

Suppliers: Biomol Research Laboratories, Butler Pike Plymouth Meeting, PA, USA; Molecular Probes, Eugene, OR, USA; Santa Cruz Biotechnology, California, USA.

Table 7.2. Details of the secondary antisera used.

Antibody	Dilution	Source
Goat anti-mouse IgG Alexa 594	1:200	Molecular Probes
Goat anti-rabbit IgG FITC	1:200	Calbiochem-Novabiochem

Abbreviation: FITC, Fluorescein Isothiocyanate.

Suppliers: Calbiochem-Novabiochem, San Diego, CA, USA; Molecular Probes, Eugene, OR, USA.

Figures

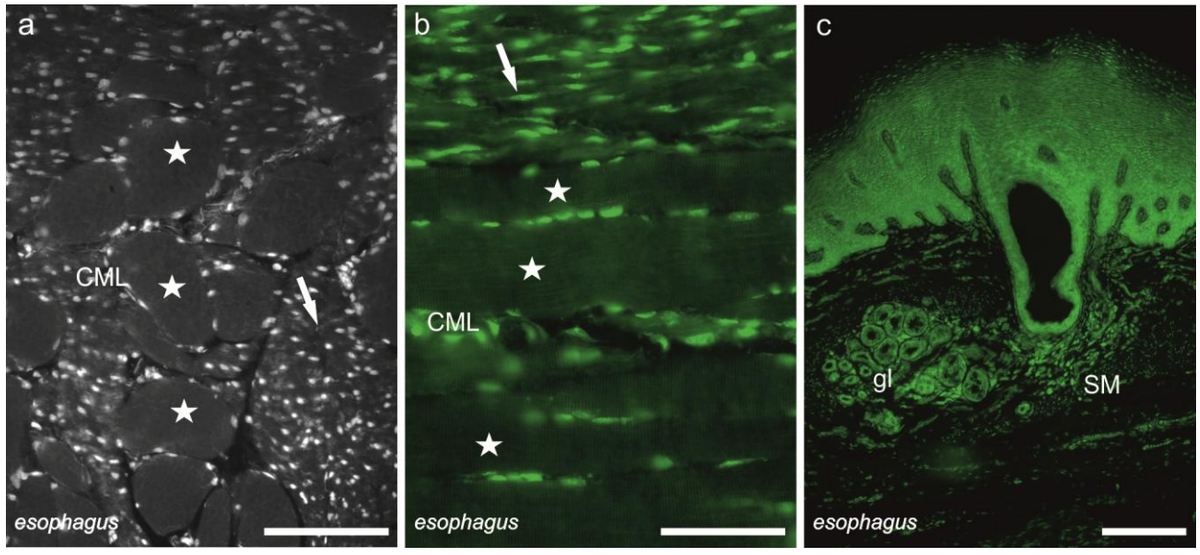

Fig. 7.1 Photomicrograph showing horse horse esophagus 8 cm from the cardias. (a) Transverse and longitudinal (b, c) cryosections labelled with NeuroTrace®. a-b) The stars indicate circular muscle striated fibers in the circular muscle layer whereas the arrows indicate smooth muscle cells. c) A large gland (gl) is visible in the submucosa (SM). Scale bar: a-b 50 µm; c 100 µm.

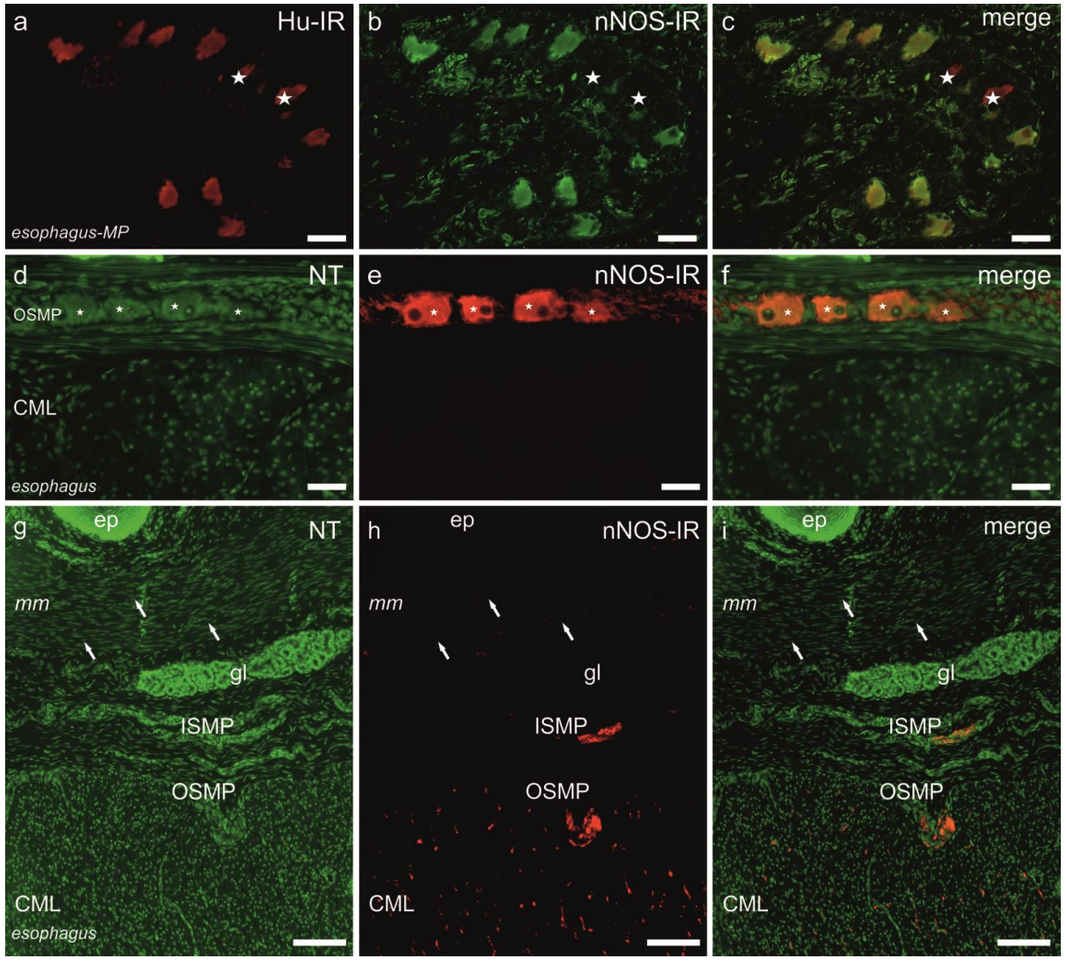

Fig. 7.2 Myenteric (MP) and submucosal plexus (SMP) neurons of the caudal portion of the equine esophagus immunoreactive (IR) for the pan-neuronal marker HuC/D (Hu) and neuronal nitric oxide synthase (nNOS). Enteric neurons were identified also by NeuroTrace® (NT) green fluorescent Nissl stain solution. Merged images are shown on the right (c, f, i). (a-c) The photomicrographs show a large myenteric ganglion in which the neurons occupy a peripheral position; the stars indicate two nNOS-negative Hu-IR myenteric neurons. (d-f) The stars indicate NT-labelled neurons (d) belonging to the outer submucosal plexus (OSMP) showing strong nNOS-IR (e). The neurons were aligned in a single row close to the inner portion of the circular muscle layer (CML). (g-i) Photomicrograph showing neurons of the outer (OSMP) and inner (ISMP) submucosal plexus labelled with the fluorescent tracer NT (g) and nNOS-IR (H). Small ISMP nNOS-IR neurons close to the esophageal gland (gl). nNOS-IR fibers were abundant in the circular muscle layer (CML) and along the muscularis mucosae (mm) (arrows). Scale bar: a-f 50µm; g-i 100µm.

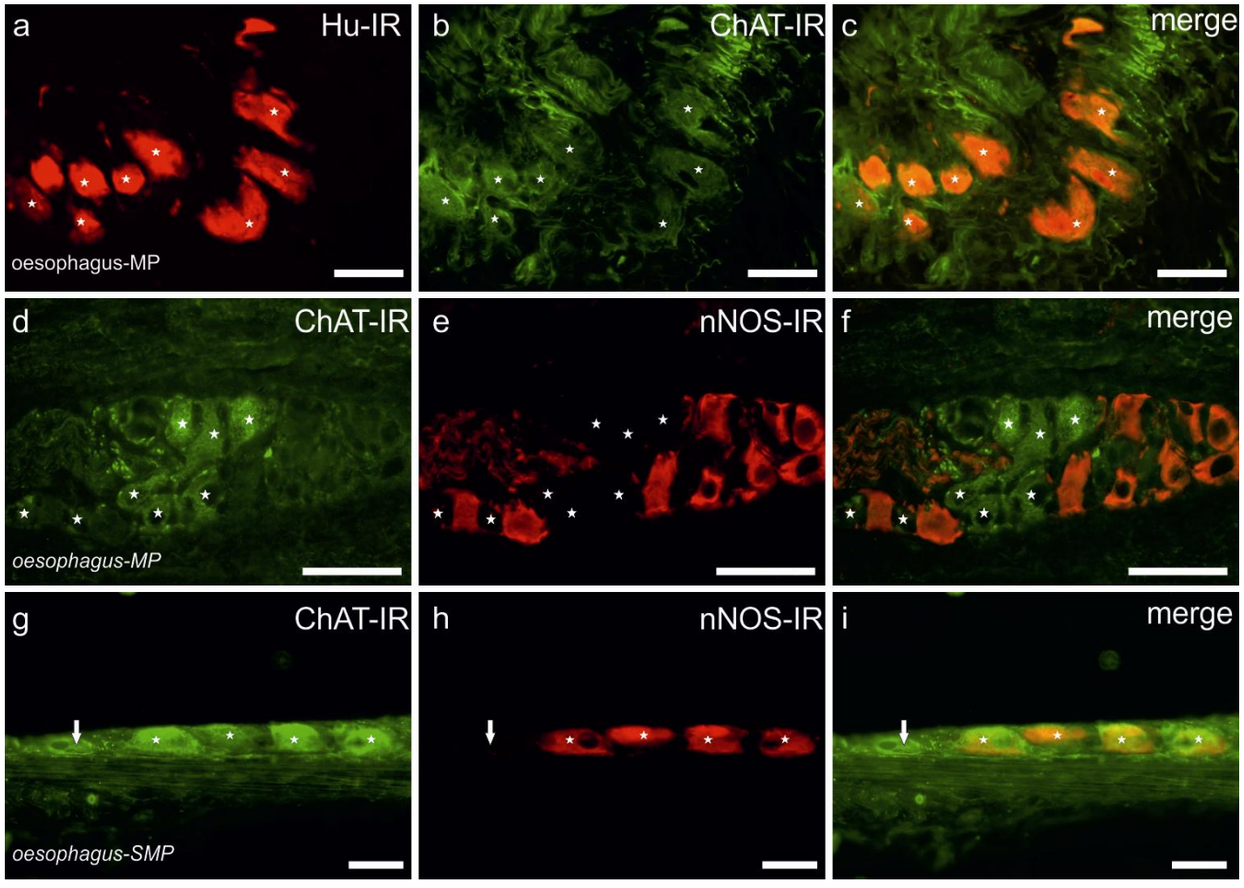

Fig. 7.3 Myenteric (MP) and submucosal plexus (SMP) neurons of the caudal portion of the equine esophagus immunoreactive (IR) for the pan-neuronal marker HuC/D (Hu), neuronal nitric oxide synthase (nNOS) and choline acetyltransferase (ChAT). Merged images are shown on the right (c, f, i). a-b) Stars indicate MP Hu-IR neurons co-expressing weak ChAT-IR. d-f) Stars indicate myenteric strong ChAT-IR neurons which were nNOS-negative. g-i) Stars indicate SMP neurons co-expressing ChAT-(M) and nNOS-IR (N). The arrow indicates a cholinergic neurons negative for nNOS. Scale bar: a-i 50µm.

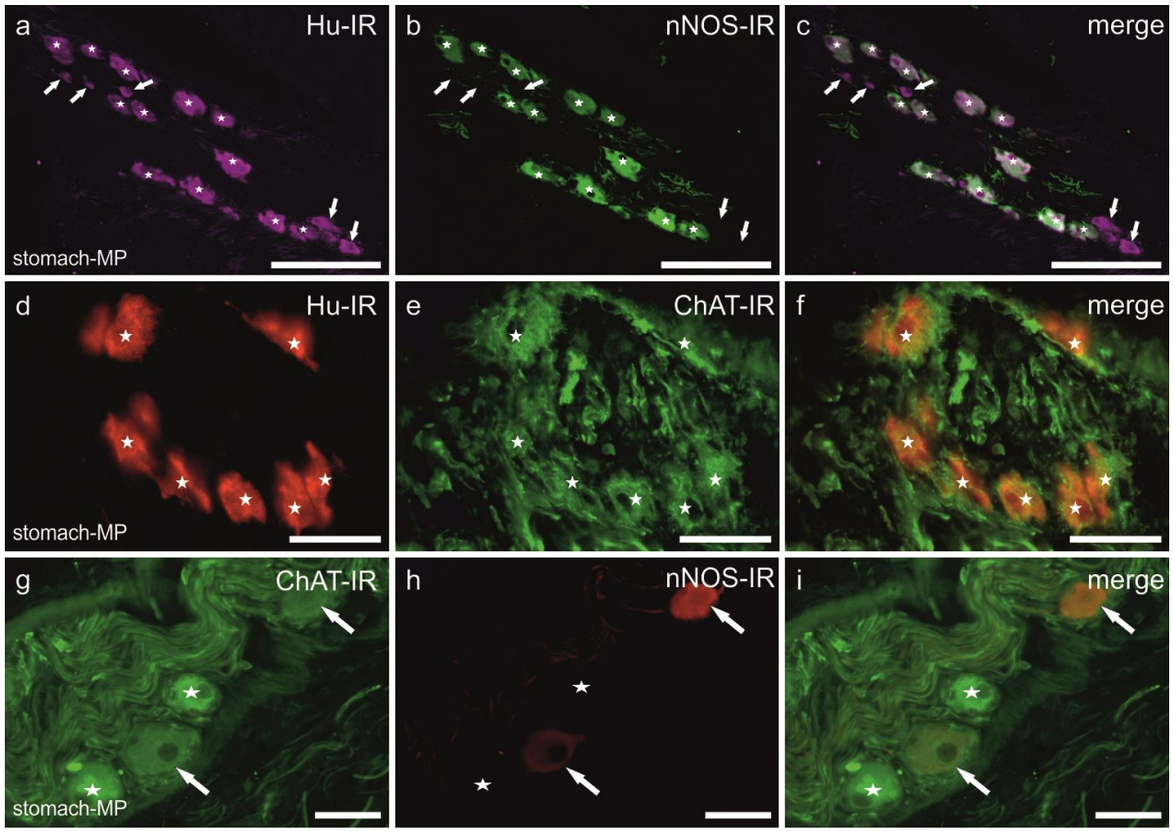

Fig. 7.4 Myenteric plexus (MP) neurons of the fundic portion of the equine stomach immunoreactive (IR) for the pan-neuronal marker HuC/D (Hu), the neuronal nitric oxide synthase (nNOS) and choline acetyltransferase (ChAT). Merged images are shown on the right (c, f, i). (a-c) Stars indicate some Hu-IR MP neurons which were also nNOS-IR; the arrows indicate nNOS-negative Hu-IR neurons. (d-f) Stars indicate Hu-IR MP neurons showing strong ChAT-IR. (g-i) Arrows indicate two cholinergic MP neurons co-expressing nNOS-IR while the stars indicate two neurons which were strongly ChAT-IR but nNOS-negative. Scale bar: a-i 50µm.

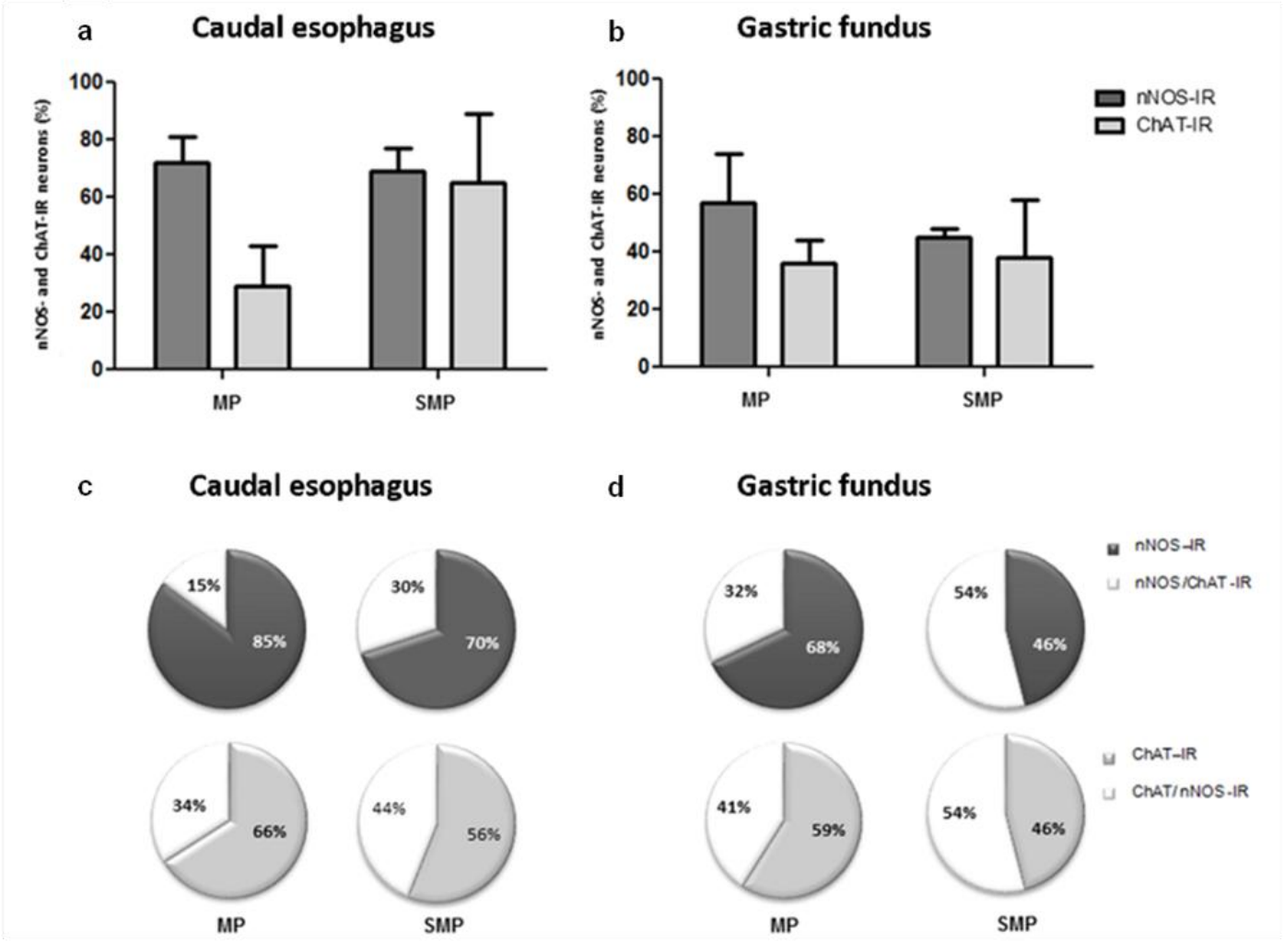

Fig. 7.5 Graphical representation of the percentages of intramural inhibitory neurons immunoreactive for the neuronal nitric oxide synthase (nNOS-IR) and excitatory neurons immunoreactive for the choline acetyltransferase (ChAT-IR) in the horse caudal esophagus and gastric fundus. a-b) Proportion of nNOS- and ChAT-IR neurons in the myenteric (MP) and submucosal plexus (SMP) of the caudal esophagus (a) and gastric fundus (b). Mean value are presented in percentage ± standard deviation. c-d) Co-localization studies: on the top are represented the percentages of nNOS-IR neurons co-expressing ChAT-IR in the MP and SMP of the caudal esophagus (c) and gastric fundus (d); on the bottom are represented the percentages of cholinergic neurons co-expressing nNOS-IR.

CHAPTER 8

Localization of 5-hydroxytryptamine receptor 4 (5-HT_4R) in the equine enteric nervous system

Introduction

The 95% of the serotonin synthesis occurs in the gut ECs (Gershon and Tack 2007; Spiller 2008). Mechanical, chemical and nervous stimulation of ECs leads to the release of serotonin in the *lamina propria* (Spiller 2008), which acts on the serotonin receptors (Gershon and Tack 2007; Millan et al. 2008) of mucosal projection of the intrinsic and extrinsic sensory neurons. In particular, serotonin activates submucosal and myenteric intrinsic primary afferent neurons, which initiate, respectively, peristaltic and secretory reflexes. Once activated by serotonin, the extrinsic component transmit nausea, discomfort, and pain to the CNS (Gershon and Tack 2007).

Actually, in the scenario of GI disorders, the most interesting target for pharmacological intervention is the 5-HT_4R subtype, which is involved in the control of motility, fluid secretion and visceral sensitivity(Schikowski et al. 2002; De Ponti 2004; Hoffman et al. 2012).

There are several studies showing the presence of the 5-HT_4R in different intestinal cell types of humans and rodents (McLean et al. 1995; Prins et al. 2000; Leclere et al. 2005; Liu et al. 2005; Cellek et al. 2006; van Lelyveld et al. 2007; Yaakob et al. 2015).

Since there are evidences that 5-HT_4R agonists such as cisapride, mosapride, and tegaserod can induce important adverse effects, a new selective 5-HT_4R full agonist was developed, namely prucalopride (De Maeyer et al. 2008), which is currently used in human medicine as prokynetic agent (Camilleri et al. 2010; Diederen et al. 2015; Yiannakou et al. 2015). Prucalopride stimulates colonic propulsion, mucosal secretion and seems also to exert an anti-nociceptive action (Hoffman et al. 2012) and enteric neuroprotection (Gershon and Liu 2007; Liu et al. 2009; Bianco et al. 2016).

Also in the horse 5-HT_4R seems to play a fundamental role in the gastrointestinal physiology, representing a promising pharmacological target (Gerring and King 1989; van der Velden and Klein 1993; Steiner and Roussel 1995; Nieto et al. 2000; Weiss et al. 2002; Lippold et al. 2004; Sasaki et al. 2005; Delesalle et al. 2006; Delco et al. 2007; Prause et al. 2009; Prause et al. 2010), as showed *in vitro*(Nieto et al. 2000; Sasaki et al. 2005; Prause et al. 2010) and *in*

vivo experiments(Delco et al. 2007; Okamura et al. 2009). The unique immunohistochemical study (Prause et al. 2010) carried out on horse failed to detect 5-HT_4R in enteric neurons.

The existence of a contrasting literature on the presence of the 5-HT_4R in equine enteric neurons, lead us to design the present research.

The first aim of our study was to localize, immunohistochemically, the 5-HT_4R on enteric neurons of the small and large equine intestine.The second aim was to evaluated the role of 5-HT_4R receptor in the extrinsic sensory innervation; to obtain this information, we analyzed tissues of foals with ileocolonic aganglionosis (lethal white foal syndrome, LWFS), the horse equivalent of Hirschsprung's disease in humans, in which only extrinsic fibers are present. Furthermore, we localized the 5-HT_4R on spinal ganglion sensory neurons.

Materials and methods

Animals and tissues collection- Intestinal tissue samples were collected from three horses of different breed aging 1 year and six months slaughtered at the public slaughterhouse. None had a story of gastrointestinal disorders. Lumbar spinal ganglia were immediately collected from the half-carcasses. Furthermore, tissues from the ileum and pelvic flexure of two new-born American paint male foals affected with LWFS were utilized. According to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, the Italian legislation (D. Lgs. n. 26/2014) does not require any approval by the competent Authorities or by ethics committees.

The blood samples of selected horses were analyzed; haemato-biochemical parameters confirmed the general healthy state of the subjects.

Cryosections were obtained (as described previously) (Russo et al. 2010; Chiocchetti et al. 2015) from small and large intestine (descending duodenum, jejunum, ileum, pelvic flexure, and descending colon) and spinal ganglia of three adult horse, and from ileum and pelvic flexure of two LWFS foals.

Whole-thickness piece of pelvic flexure was immediately frozen in liquid nitrogen and stored at -80°C for testing the anti-5-HT4 antiserum specificity by western blot (WB) analysis.

Immunofluorescence- Cryosections were hydrated in PBS and processed for double immunostaining. To block non-specific bindings, the sections were incubated in a solution containing 20% normal goat or donkey serum (Colorado Serum Co., Denver, CO, USA) and 0.5% Triton X-100 (Sigma Aldrich, Milan, Italy, Europe) in PBS for 1h at RT. The cryosections were incubated overnight in a humid chamber at RT with a cocktail of primary antibodies (**Table 8.1**) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. Enteric neurons were identified with the anti-HuC/HuD antiserum.

After washing in PBS (3 x 10 min), the sections were incubated for 1 h at RT in a humid chamber with the secondary antibodies (**Table 8.1**) diluted in PBS. The cryosections were then washed in PBS (3 x 10 min) and mounted in buffered glycerol at pH 8.6.

Specificity of the primary antibodies - The polyclonal rabbit anti-5-HT4R antibody (AB 60359, Abcam, UK) utilized in the present research is predicted to work on horse tissues. To confirm its specificity, we tested this antibody by WB. The specificity of the secondary antibodies has been tested in a previous work (Russo et al. 2010). The specificity of the secondary antibodies has been tested in a previous work (Russo et al. 2010).

Analysis of the sections- Preparations were examined on a Nikon Eclipse Ni microscope (Nikon Instruments Europe BV, Amsterdam, Netherlands, Europe) equipped with the appropriate filter cubes. The images were recorded with a DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01(Nikon Instruments Europe BV, Amsterdam, Netherlands, Europe). Slight contrast and brightness adjustments were made using Corel Photo Paint, whereas the figure panels were prepared using Corel Draw (Mountain View, Ottawa, Canada).

Quantitative analysis- At least 200 HuC/HuD-IR neurons were counted for in the Myenteric and Submucosal plexus (MP and SMP, respectively) in each gastrointestinal tract (n=3). Double-labeling immunofluorescence using HuC/HuD and the polyclonal rabbit anti-5-HT_4 antibody on tangential crysections, allowed us to quantify the proportions 5-HT_4 R-

IR neurons. The ratio between the 5-HT_4 neurons and HuC/HuD-IR neurons was determined and data were expressed as relative percentage (mean ± St. Dev).

The quantitative analysis for the densities of 5-HT_4 positive fiber was performed in all intestinal tracts considered. For each layer (*tunica mucosa*, circular muscle layer and longitudinal muscle layer), three high power fields (40x, longitudinal sections) randomly selected were acquired at the same exposure time. Images were converted into 8-bit files and they were analyzed using ImageJ software (http://imagej.nih.gov/ij/). Threshold values were determined empirically by selecting a setting which gave the most accurate binary image. The same threshold was used for all images. The resulting numbers of pixels corresponding to the percentage of immunoreactive area on the total area were measured. Data were expressed as mean ± St. Dev.

Western blotting - Colonic tissue sample was collected, frozen in liquid nitrogen, and stored at -80 C°. Tissue was thawed and homogenized. Total protein content from human (SHSY5Y) and murine (Neuro 2A) neuroblastoma cell lines, were included as positive and negative controls, respectively (Bianco et al. 2016).

Tissue samples (horse colon) was collected, frozen in liquid nitrogen, and stored at -80°C. Tissues were later thawed and homogenized. Total protein contents from horse colon and cell lines lysate (i.e. SHSY5Y and Neuro 2A) were extracted using T-PER tissue protein extraction reagent in the presence of a protease inhibitor cocktail (Thermo Scientific, Italy, Europe) according to the manufacturer's instructions, and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Italy, Europe). Aliquots containing 80 ug of proteins were denatured by heating at 95°C for 5 min in Laemmli buffer, separated by 12.5% Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and transferred overnight (70 mV) onto a nitrocellulose membrane (GE Healthcare, UK, Europe). After blocking treatment, the membranes were incubated at 4°C overnight with the primary antibodies (**Table 8.1**) diluted in Tris-buffered saline-T20 (TBS-T20 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% T-20). After washes, the blots were incubated 30 min at room temperature with respective peroxidase-conjugated secondary antibodies (**Table 8.1**). Immunoreactive bands were visualized using chemiluminescent substrate (Pierce ECL Western Blotting Substrate, Thermo Scientific, Italy, Europe). The intensity of luminescent signal was acquired on a C-DiGit

Chemiluminescent Western Blot Scanner using Image Studio Digits Software Ver 3.1 (LI-COR Biotechnology, UK, Europe).

For $5HT_4R$ antibody, a unique band of ~45 kDa (theoretical molecular weight ~44 kDa) (http://www.uniprot.org/) was present in extracts from the descending colon as well as in the positive control; no band was detected in the negative control (**Fig. 8.1**). WB analysis confirmed the specificity of the primary antibody utilized in the present study.

Results

5-HT_4R immunoreactivity in enteric neurons and nervous fibers

Neurons – The HuC/HuD-immunolabeling was localized in the cytoplasm and nucleus of MP and SMP neurons. The intensity of HuC/HuD-IR varied, from weak to bright; notably, in the submucosa, some neurons showed very faint or undetectable HuC/HuD-IR, whereas the 5-HT_4R-IR was well defined. Neurons not recognizable for their HuC/HuD-IR were not considered in the counts.

Large percentages of HuC/HuD-IR neurons showed 5-HT_4R-immunoreactive (IR) in the MP and SMP of all the intestinal tracts considered (**Fig. 8.2**). The 5-HT_4R-immunoreactivity (IR) showed different degrees of brightness, varying from weak to strong. The pattern of immunolabeling was preferentially located into the cytoplasm rather than along the plasma membrane. In the myenteric neuropil, nervous fibers and varicosities embracing neurons showed bright immunolabeling (**Fig. 8.2 a-f)**.

In the small intestine MP, the greatest percentage of immunolabelled neurons was observed in the ileum (63±19 %), followed by duodenum (44±25 %) and jejunum (35±20 %). In the large intestine MP, pelvic flexure 5-HT_4R-IR neurons largely outnumbered (47±13 %) the density of those observed in the descending colon (28±9 %) (**Fig. 8.2 a-f**).

Submucosal neurons, which showed in general brighter 5-HT_4R-IR than myenteric ones, were observed in the inner and outer SMP layers (**Fig. 8.2 g-l**). In the duodenal submucosa, 5-HT_4R-IR neurons were closely related to the Brunner's glands (Fig. 8.2 j-l); nevertheless, no 5-HT_4R-IR nervous fibers were observed within the glands. The percentages of 5-HT_4R-IR SMP neurons were similar in the pelvic flexure, duodenum and jejunum (69±13 %, 68±14 %, and 67±3 %, respectively), and slightly decreased in the descending colon (60±23 %) and ileum (54±6 %).

Nervous fibers - Bright 5-HT_4R-IR nervous fibers were widely distributed in all the layers of the small and large intestine.

The duodenal mucosa showed the greatest density of 5-HT_4R-IR fibers, which were however, well represented also in the mucosa of the other intestinal tracts considered. 5-HT_4R-IR nervous fibers were distributed in the *muscularis mucosae* (*mm*), around intestinal glands and in the lamina propria (**Fig. 8.3 a-b**). In the mucosa of small intestine, 5-HT_4R-IR fibers were more visible on the upper half of the villi, whereas in the large intestine these fibers were more observable in the basal portion of the glands.

In the submucosa, a thin network of 5-HT_4R-IR fibers and varicosities encircled SMP neurons (**Fig. 8.3 b**); a few immunolabelled fibers were visible around blood vessels.

In the *tunica muscularis*, the greatest density of 5-HT_4R-IR fibers were observed in the CML (**Fig. 8.3 c**) of the duodenum and descending colon. In the LML, 5-HT_4R-IR fibers were well represented in the descending colon and ileum, while were scantly represented in the other tracts.

Data related to the distribution of 5-HT_4R-IR neurons and fibers are graphically represented in the **Fig. 8.4a** and **Fig. 8.4b**, respectively.

5-HT_4R immunoreactivity in extrinsic innervation

Intestinal extrinsic sensory fibers – In the horse, the majority of SP-IR innervation derives from enteric neurons, which largely innervate the CML (**Fig. 8.3 d**). Double immunohistochemistry carried out on adult horse tissues showed that 5-HT_4R- and SP-IR fibers widely co-localized. Interestingly, also in LWFS tissues, in which only extrinsic innervation is present, the extrinsic sensory SP-IR nervous fibers co-expressed 5-HT_4R-IR (**Fig. 8.3 e-f**).

Spinal ganglion neurons – Weak to moderate 5-HT_4R-IR was expressed by small- and medium-sized spinal ganglion neurons. Double immunohistochemistry showed that large percentages of 5-HT_4R-IR neurons co-expressed SP-IR (**Fig. 8.2 g-i**).

Extra-neuronal 5-HT_4-IR distribution – 5-HT_4R-IR was expressed by the *tunica muscularis*, ECs cells, and endothelial cells of small vessels (data not shown). 5-HT_4R-IR was not detectable in enterocytes and interstitial cells of Cajal.

Discussion

This is the first evidence of 5-HT_4R-IR in the myenteric and submucosal neurons of the horse intestine. Our results represent a strong morphological support as a reinforcement for the functional investigations carried out on horse small and large intestine (Gerring and King 1989; van der Velden and Klein 1993; Nieto et al. 2000; Weiss et al. 2002; Lippold et al. 2004; Sasaki et al. 2005; Delesalle et al. 2006; Delco et al. 2007; Prause et al. 2009; Prause et al. 2010). The presence of large percentages of MP and SMP 5-HT_4R-IR neurons suggests that 5-HT_4R agonists may influence intestinal motility and secretion, respectively.

In relation to the putative secretory role of 5-HT_4R-IR submucosal neurons, it is known that the active mucosal secretion can be triggered by serotonin activation of 5-HT_4R (and 5-HT_3 and 5-HT_{1p}) of intramural sensory submucosal neurons, which in turn excite secretomotor neurons releasing acetylcholine and VIP. This neurogenic mechanism induces Cl^- and bicarbonate secretion (Mawe and Hoffman 2013).

The expression of 5-HT_4R-IR by spinal ganglion neurons does not necessarily indicate that these neurons innervate the intestine; nevertheless, the presence of 5-HT_4R-IR on extrinsic sensory SP-IR fibers observed in LWFS tissues suggest that drugs acting on 5-HT_4R might influence the visceral sensitivity, as shown in human(Bharucha et al. 2000; Coffin et al. 2003). This is, to the best of Author's knowledge, the first morphological evidence of 5-HT_4R-IR at spinal ganglia levels. Our findings are consistent with those showing that 5-HT_4R is involved in nociception, being the RNAm of this receptor expressed by nociceptive neurons of rat spinal ganglia(Cardenas et al. 1997; Nicholson et al. 2003).

5-HT_4R-IR was also observed in SP-negative spinal ganglion neurons; this indicates that 5-HT_4R might be involved in other kinds of sensitivity. In fact, it has been shown that 5-HT_4R agonist (tegaserod) may have an inhibitory effect on intramural mechanoceptors of the cat rectum (Schikowski et al. 2002). Nevertheless, to identify and confirm a role of 5-HT_4R in the visceral sensitivity of the horse, functional and pharmacological investigations are needed.

Gut vasodilation is regulated by intrinsic reflex circuitry involving SMP neurons via the activation of 5-HT_3 and 5-HT_4R (Nicholson et al. 2003). In the present study, endothelial cells of small vessels expressed 5-HT_4R -IR(Nishikawa

et al. 2010; Machida et al. 2013); this finding is consistent with studies indicating that 5-HT_4R mRNA (together with 5-HT_1, 5-HT_2, and 5-HT_7 mRNA) is expressed by endothelial and vascular smooth muscle cells. Furthermore, it is known that 5-HT_4R of endothelial cells regulates angiogenesis and that mosapride might inhibit proliferation and migration (Nishikawa et al. 2010) of endothelial cells in human umbilical vein (Ullmer et al. 1995).

There are growing evidences that 5-HT_4R stimulation enhances the development, survival, and neurogenesis of enteric neurons neuroprotection (Gershon and Liu 2007; Liu et al. 2009; Takaki et al. 2015; Bianco et al. 2016) and that 5-HT_4R agonists facilitates neurogenesis from transplanted stem cells in intestinal anastomosis (Goto et al. 2016). This last finding may have relevance to regenerative potential of intestinal intramural innervation and survival in horses subjected to colic surgery.

A large variety of infectious and noninfectious inflammatory diseases may affect the gastrointestinal system of horses; it has been shown that, in mice, colitis promotes enteric neurogenesis through a 5-HT_4R -dependent mechanism driving glial cells to transdifferentiate into neurons (Belkind-Gerson et al. 2015; Uzal and Diab 2015).

Table 8.1 Primary and secondary antibodies used in the study.

Primary antibody	**Host**	**Code**	**Dilution**	**Source**
HuC/HuD	Mouse	A21271	IHC 1:200; WB 1:200	Life Technologies
5-HT_4	Rabbit	AB60359	IHC 1:200; WB 1:200	Abcam
Substance P	Rat	10-S15A	IHC 1:400	Fitzgerald
Secondary antibody	**Host**	**Code**	**Dilution**	**Source**
Anti-mouse IgG Alexa 594	Goat	A11005	IHC 1:200	Life Technologies
Anti-mouse IgG Alexa 488	Donkey	20014	IHC 1:100	Biotium
Anti-rabbit IgG FITC	Goat	401314	IHC 1:200	Merck Millipore
Anti-rabbit IgG HRP coniugated	Goat	A2304	WB 1:35000	Sigma Aldrich
Anti-rat IgG Alexa 594	Donkey	A21209	IHC 1:50	Life Technologies

Abbreviations: FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IHC, Immunohistochemistry; HuC/HuD, human neuronal protein;WB, Western Blot.

Suppliers: Abcam, Cambridge, United Kingdom, Europe; Biotium Inc., Hayward, California, USA; Fitzgerald Industries Int., Inc. Concord, MA, USA Merck Millipore, Darmstadt, Germany, Europe; Life Technologies, California, USA; Sigma Aldrich, Milan, Italy, Europe.

Figures

Fig. 8.1 Validation of 5-HT_4R antibody in horse tissue by Western Blot. Total protein lysate form horse colon (lane 1), marker of molecular weight (M) (lane 2), SH-SY5Y human cell line as positive control (lane 3), murine Neuro2A (N2A) cell line as negative control (lane 4). A specific band of ~ 45 kDa was detected in horse tissue (lane 1) as well as in the positive control (lane 3).No bands were detected in the negative control (lane 4).

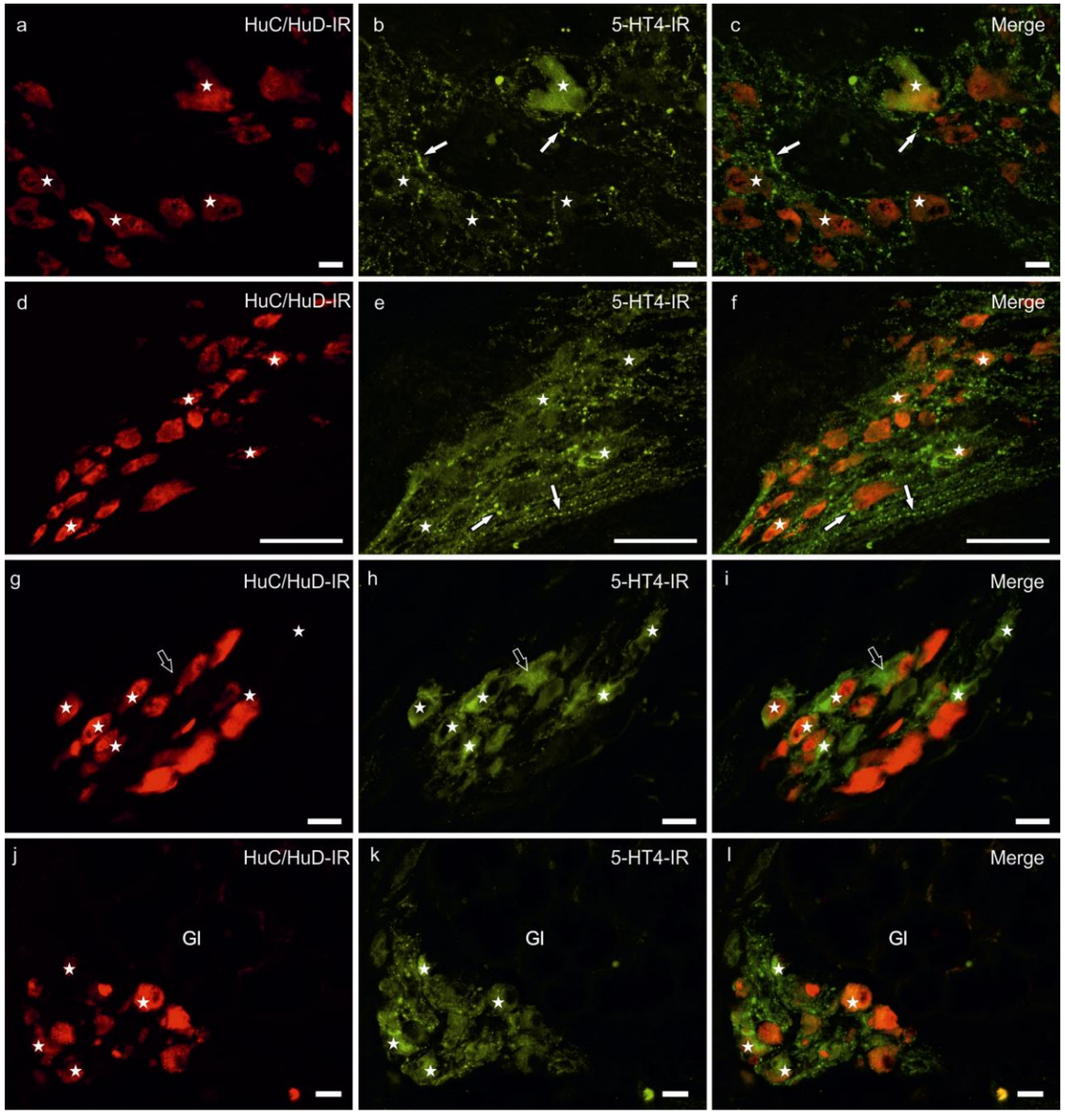

Fig. 8.2 Micrographs showing HuC/HuD- and 5-HT_4R -immunoreactivity (IR) in tangential cryosections of myenteric and submucosal plexus of horse ileum, pelvic flexure, and duodenum. a-f) Stars indicate some HuC/HuD-IR myenteric plexus neurons of the ileum (a-c) and pelvic flexure (d-f) which showed 5-HT_4R -IR. Arrows indicate nervous fibers varicosities showing strong 5-HT_4R -IR. g-l) Stars indicate some ileal and duodenal submucosal plexus HuC/HuD-IR neurons co-expressing strong 5-HT_4R -IR. g-i) The open arrow indicates one 5-HT_4R -IR ileal submucosal neuron showing very faint HuC/HuD-IR. HuC/HuD-IR showed several degrees of immunolabeling, from strong nuclear and cytoplasmic immunoreactivity to very weak (or almost undetectable). Bars = a-c; g-l: 20 µm; d-f: 100 µm.

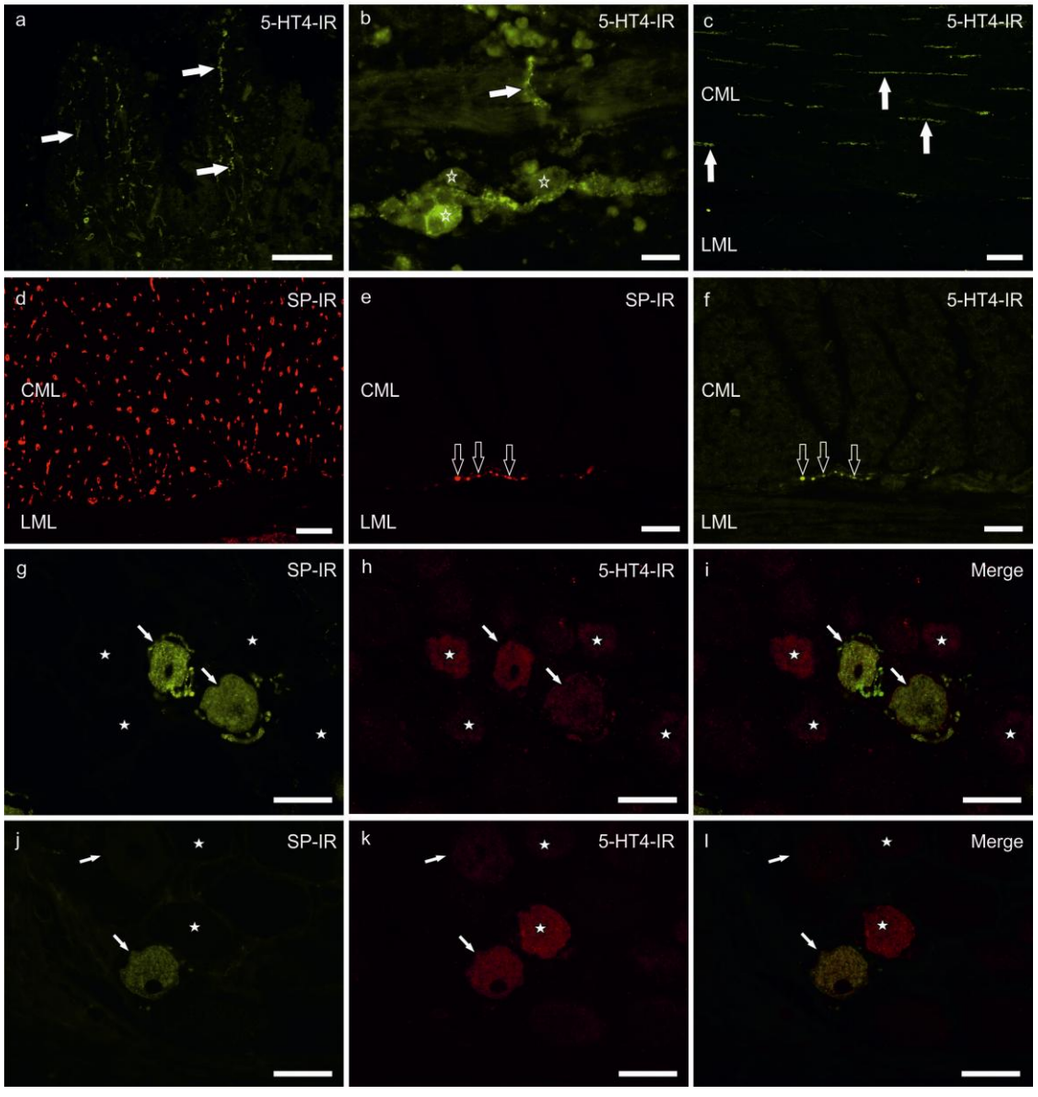

Fig. 8.3 5-HT_4R-immunoreactivity in healthy and LWFS foals and in spinal ganglia. a-c) Micrographs showing 5-HT_4R-immunoreactive nervous fibers (arrows) in the *lamina propria* (a), *muscularis mucosae* (b) and circular (CML) and longitudinal muscle layer (LML) (transverse cryosections; c) of the horse ileum; stars (b) indicate some 5-HT_4R-IR submucosal neurons very close to the *muscularis mucosae*. d) Longitudinal ileal cryosections showing the dense CML innervation by substance P (SP) nervous fibers. e-f) Longitudinal cryosections of the ileum of a lethal white foal syndrome foal, characterized by the absence of enteric neurons. The arrows indicate SP-IR sensory fibers of extrinsic origin running between CML and LML and co-expressing strong 5-HT_4R -IR. g-l) Cryosections of horse spinal ganglion neurons; the arrows indicate SP-IR sensory neurons which co-expressed 5-HT_4R -IR; stars indicate other 5-HT_4R -IR neurons which were SP-negative. Bars = a, c, d, g-i: 100 µm; b, e, f: 20 µm.

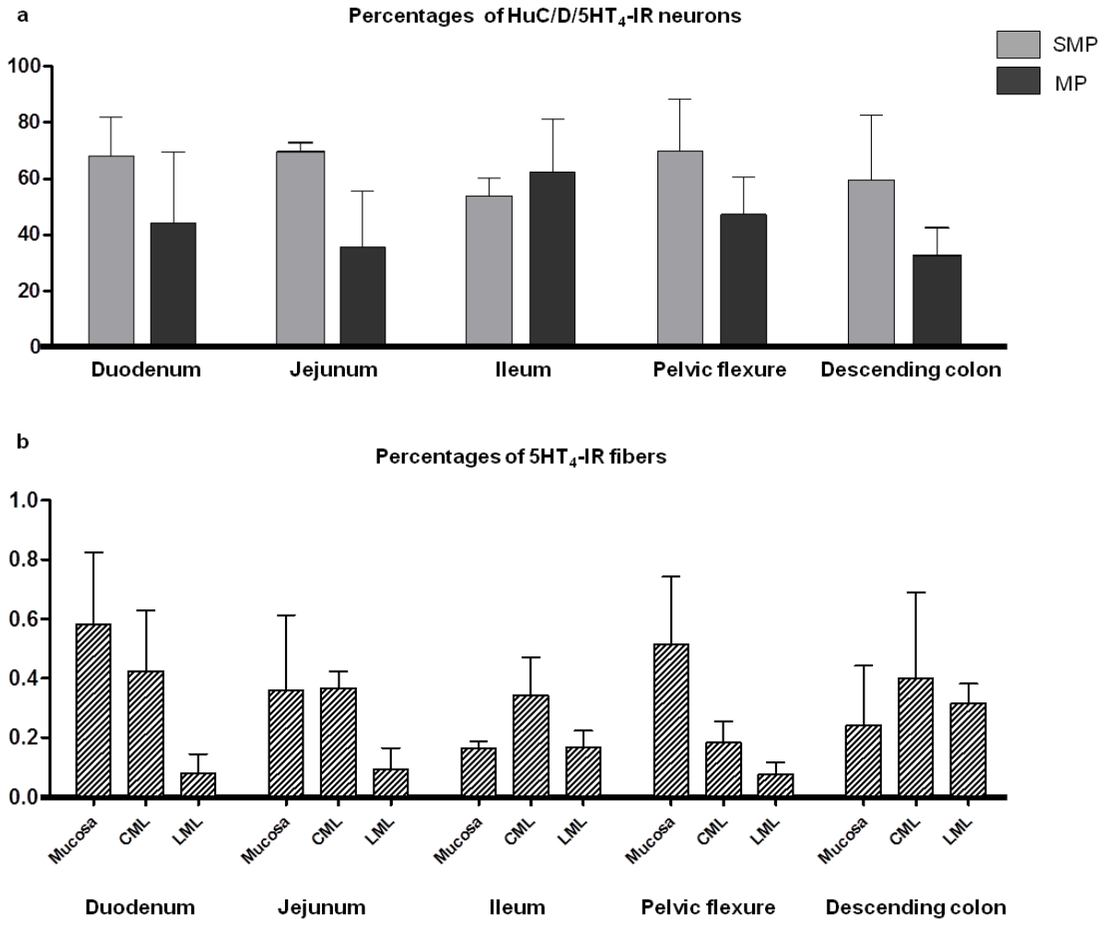

Fig 8.4 Graphical representation of the percentages of 5-HT_4R -immunoreactive neurons and fibers in the horse small and large intestine. a) Black bars indicate the percentages of 5-HT_4R-IR neurons in the myenteric plexus (MP) of duodenum (161/452 cells), jejunum (147/497 cells), ileum (348/571 cells), pelvic flexure (297/606 cells), and descending colon (165/469 cells). Gray bars indicate the percentages of 5-HT_4R-IR neurons in the submucosal plexus (SMP) of duodenum (394/586 cells), jejunum (262/404 cells), ileum (324/599 cells), pelvic flexure (377/540 cells), and descending colon (248/402 cells). Data are represented as mean ± St. Dev. b) Density of 5-HT_4R-IR nervous fibers in the mucosa and muscular layers of the horse small and large intestine. a) Percentages of 5-HT_4R-IR area (fibers) in the *tunica mucosa* (mucosa), circular muscle layer (CML) and longitudinal muscle layer and (LML) of duodenum, jejunum, ileum, pelvic flexure and descending colon. Data are represented as mean ± St. Dev.

CHAPTER 9

Extrinsic innervation of the ileum and pelvic flexure of foals with ileocolonic aganglionosis

Modified from

"**Extrinsic innervation of the ileum and pelvic flexure of foals with ileocolonic aganglionosis**"

Giancola F, Gentilini F, Romagnoli N, Spadari A, Turba ME, Giunta M, Sadeghinezhad J, Sorteni C, Chiocchetti R

Under revision

Introduction

Intestinal aganglionosis is a congenital severe condition caused by aberrant development of the ENS. The main feature is the absence of enteric neurons, determined by neural crest progenitors' unsuccessful migration and colonization of the gut. Natural variants of such pathological condition are described in humans - Hirschsprung's disease - (Heanue and Pachnis 2006; Moore 2015) as well as in horses - lethal white foal syndrome, LWFS - (McCabe et al. 1990; Metallinos et al. 1998). LWFS is an inherited syndrome occurring in foals born prevalently from American Paint Horse parents of the overo coat-pattern lineage (Finno et al. 2009). Affected foals are totally or almost totally white and die within few days (between 23 and 132 hours) (Julian 1994) from complications due to intestinal (ileocolonic) aganglionosis or hypoganglionosis (Hultgren 1982; Vonderfecht et al. 1983; Metallinos et al. 1998; Lightbody 2002; Finno et al. 2009; Muniz et al. 2013) and consequent intestinal akinesia.

The mutated EDNRB gene is responsible for the LWFS (Metallinos et al. 1998; Santschi et al. 1998; Yang et al. 1998). Mutations in EDNRB gene are also reported in the 5% of familiar cases of human Hirschsprung's disease (Heanue and Pachnis 2006) and in rodents (Robertson et al. 1997; Moore 2015). In horses, as in other mammalian species, mutations occurring in the EDNRB gene reveal the association between intestinal aganglionosis and coat-colours phenotypes (Reissmann and Ludwig 2013), since this receptor is involved in the development of neural crest cells committed to differentiate in both ENS elements and melanocytes (Robertson et al. 1997; Santschi et al. 1998; Santschi et al. 2001). In mice (Baynash et al. 1994; Hosoda et al. 1994), rats (Ceccherini et al. 1995; Gariepy et al. 1996) and humans (McCallion and Chakravarti 2001) the defects of peripheral pigmentation are associated with aganglionosis, while in sheep (Luhken et al. 2012) the EDNRB gene deletion is associated with hypopigmentation, megacolon, not with aganglionosis.

The mutation responsible for LWFS is a TC to AG transversion causing an isoleucine to lysine substitution at codon 118 of the ENDRB gene. The disease has a recessive inheritance pattern (Yang et al. 1998). Horses with white Overo patterning are more likely carriers of the mutation than solid-colored horses (Vrotsos PD 1999). Affected foals suffer

from aganglionosis of the myenteric and submucosal ganglia of the caudal part of the small intestine and of large intestine, resulting in intestinal akinesia and colic (Muniz et al. 2013).

At present no treatment for LWFS has been suggested; therefore the genetic test is essential to prevent its occurrence (McCabe et al. 1990). PCR tests are now available, making possible the identification of carriers horses, heterozygous for the specific mutation in the EDNRB gene (Lightbody 2002).

While it is evident that LWFS and Hirschsprung's disease share genetic characteristics and, in some cases, the same aganglionosis pattern (Moore 2015), it is not yet known whether humans and horses share also the same pattern of extrinsic innervation in the aganglionic tracts. In fact, in humans, as well as in rodents with aganglionosis, extrinsic fibers show an abnormal distribution pattern (Facer et al. 2001; Rabah 2010; Nagashimada et al. 2012). Furthermore, since extrinsic fibers also arise from neurons derived from neural crest elements, these might also be irregularly distributed in the intestinal aganglionic segments. To address this question we immunohistochemically characterized, for the first time, the distribution and phenotype of extrinsic neural fibers in the ileum and pelvic flexure (ascending colon) of LWFS foals, compared to a control foal. In this research, we benefitted also from our previous findings on the origin and neurochemistry of the extrinsic sensory and sympathetic innervation supplying the equine ileum (Chiocchetti et al. 2009b; Russo et al. 2010; Russo et al. 2011; Russo et al. 2012).

Materials and methods

Animals –Two American paint male foals affected with LWFS were included in the present research (**Fig. 9.1 a-e**). The two foals had the same dam and different sires. While the dam and the sire # 1 showed a clear white frame- overo pattern, the sire # 2 had a likely combination pattern. The coat of the first foal was entirely white (**Fig. 9.1 a-b**) and the iris was totally light blue; the second foal's coat showed pigmentation of upper eyelid, nasolabial area, lower lip, foreskin, and perianal area while the iris was pigmented (**Fig. 9.1 c-e**). Within 24 hours from the birth, on physical examination, the foals were recumbent, depressed, and frequently rolling on their back. The first alarming observation was the absence of defecation. In particular, foals stopped feeding and started showing signs of abdominal pain, without feces production. In order to genetically confirm the clinical diagnosis of LWFS, blood samples were collected from the mare and foals and stored in the EDTA tubes. The foals survived up to day 3. Necessarily, foals were euthanized for

ethical reasons and their tissues have been collected following the owners' written consents. According to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, the Italian legislation (D. Lgs. n. 26/2014) does not require any approval by the competent Authorities or by ethics committees. As a control, we also collected tissues from a one-week old foal which had died for reasons not related to gastrointestinal problems.

Genetic analysis - The TC to AG transversion responsible for the p.118Ile>Lys substitution in EDNRB (Metallinos et al. 1998; Santschi et al. 1998; Yang et al. 1998) was genotyped *intra-vitam* using a High Resolution Melting (HRM) Assay and later confirmed by direct sequencing. The disease locus was amplified using the primers pair forward 5' CTCCCCCGTGCGAAAGA 3' and reverse 5' AAATGATTCTCAGCAGTGTGGAGTT 3' which amplifies a 120 bp target of the EDNRB. The reaction was carried out using 1 µL of genomic DNA purified from K3EDTA anticoagulated blood, 1x MeltDoctor HRM master mix (Applied Biosystems, Thermo Fisher Scientific, Italy, Europe), 200 nM each of forward and reverse primer and molecular biology grade water in a final volume of 15 µL. The assay was accomplished on a StepOne thermal cycler with a program composed by an initial denaturation stage at 95°C x 10 min followed by a 40 cycles, two-steps protocol including denaturation at 95°C for 15 s and annealing/extension at 62° C for 45 s with signal acquisition. The amplification stages were followed by a High Resolution melt stage. Generated melting data were then analyzed using the High Resolution Melt software v 3.0.1 (Applied Biosystems, Thermo Fisher Scientific, Italy, Europe) which automatically calls the variant.

Tissue collection – The ileum and the pelvic flexure were collected within 2 hours from euthanasia (details in Russo et al. 2010). Tissues were immediately removed and then longitudinally cut open, flushed out with PBS, pinned flat on balsa wood and fixed in 2% paraformaldehyde containing 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.0) at 4°C for 48 hours.

Tissue preparation -Cryosections - This technique was described in detail elsewhere (Chiocchetti et al. 2015). After rinsing in PBS, the tissues were stored in PBS containing 30% sucrose and 0.1% sodium azide (pH 7.4) at +4°C. Pieces of tissues (2 x 1 cm) were subsequently cut, transferred to a mixture of PBS-sucrose-azide and OCT compound (Tissue

Tek®, Sakura Finetek Europe, Alphen aan den Rijn, the Netherlands, Europe) at a ratio of 1:1 (overnight) and then embedded in 100% OCT. The tissues were frozen, mounted in Tissue Tek® mounting medium and sectioned at 14-16 µm on a cryostat. The sections were collected on gelatin-coated slides. *Wholemounts* – This technique was described in detail elsewhere (Chiocchetti et al. 2009b). Briefly, small specimens of the ileum and pelvic flexure were pinned flat, mucosa side up, in a Sylgard-covered Petri dish containing PBS. The mucosa was carefully scraped off using a scalpel. To obtain thin SMP wholemounts, we removed two distinct SMP layers, the inner submucosal plexus and the outer submucosal plexus. To obtain MP preparations, we gently removed the strips of circular muscle layer.

Immunohistochemistry - All the neuronal markers utilized in this study have been shown to be expressed by extrinsic and intrinsic nervous fibers and ENS neurons of healthy horses (Freytag et al. 2008; Chiocchetti et al. 2009a; Chiocchetti et al. 2009b; Hartig et al. 2009; Russo et al. 2010). Enteric neurons were identified with the anti- HuC/HuD antiserum (Freytag et al. 2008; Hartig et al. 2009). Sympathetic postganglionic pathways were identified with antisera against the tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) (Russo et al. 2010).

For the identification of the spinal ganglia sensory pathways, we utilized antisera directed against the neuropeptides SP and CGRP (Russo et al. 2010; Russo et al. 2011).

Furthermore, enteric glial cells were identified with antisera anti-S100b (Hudson et al. 2000; Chiocchetti et al. 2009b).

Cryosections and wholemount preparations were incubated with the primary and secondary antisera listed in Table 9.1. Double labeling studies using the indirect immunofluorescence method were performed, as described elsewhere (Chiocchetti et al. 2006; Russo et al. 2010).

Antibody specificity - The specificity of the employed antibodies anti- CGRP, -DBH and TH has already been tested in the horse by WB (Russo et al. 2010; Russo et al. 2012). Furthermore, two antibodies anti-TH were used (from mouse and sheep), which identified the same neuronal structures. The antibody sheep anti-TH was utilized in combination with the antibody anti-DBH to verify the co-localization between the two different neuronal markers of the adrenergic pathways.

The rat anti-SP antibody has already been well-characterized in other species and it results raised against highly conserved peptides; it should recognize the appropriate antigens also in the horse, as showed recently (Chiocchetti et al. 2009a; Chiocchetti et al. 2009b; Russo et al. 2011; Russo et al. 2012). The specificity of the secondary antibodies has been well described elsewhere (Russo et al. 2010).

Analysis of the sections and wholemount preparations – Preparations were examined on a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes. The images were recorded with a DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). To obtain large images, single fields were scanned automatically using a motorized XY stage with auto-focus capability and then stitched by the software. Slight contrast and brightness adjustments were made using Corel Photo Paint, whereas the figure panels were prepared using Corel Draw (Mountain View, Ottawa, Canada).

The quantitative analysis of the fiber densities was performed for SP, CGRP, and TH in the ileum and pelvic flexure of control and LWFS foals. For each layer (*tunica mucosa*, CML and LML), three high power fields (40x, longitudinal sections) randomly selected were acquired at the same exposure time. Images were converted into 8-bit files and analyzed using ImageJ software (http://imagej.nih.gov/ij/). Threshold values were determined empirically by selecting a setting, which gave the most accurate binary image for a subset. The same threshold was used for all images of the same marker and the resulting number of pixels corresponding to the percentage of immunoreactive area on the total area was measured. All graphical representations were obtained using a commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean ± standard error (SEM).

Results

Genetic diagnosis - Both affected foals were demonstrated to carry the mutated allele in a homozygous state by HRM analysis carried out intra-vitam. Also the dam and one of the two sires (namely sire #2) were analyzed and found to be carriers of the disease. All the HRM screening diagnosis were ultimately confirmed with direct sequencing.

Innervation of the ileum and pelvic flexure

Sympathetic fibers (TH- and DBH-immunoreactivity)

Control – TH- and DBH-immunoreactivity (IR) co-localized in nervous fibers (data not shown). As already observed in the *tunica mucosa* of adult horse (Russo et al. 2010), a dense network of adrenergic fibers was observed within the *muscularis mucosae* (*mm*) and some fibers were also seen in the *lamina propria* of the foal as well. A delicate network of TH- or DBH-IR fibers and varicosities was scattered in the submucosa, along blood vessels around which it formed a dense perivascular plexus (**Fig. 9.2 a, c, e**) encircling, although with larger meshes, also large veins (**Fig. 9.2 e**). Adrenergic fibers encircled also SMP and MP neurons, which, however, did not express adrenergic phenotype (**Fig. 9.3 a, b**). In the *tunica muscularis*, adrenergic innervation was homogenously distributed in the whole thickness of the CML of the ileum (**Fig. 9.3 c**) and pelvic flexure (**Fig. 9.3 e**). In the CML and LML, the adrenergic innervation was largely more represented in the pelvic flexure than in the ileum. Some adrenergic fibers were observed in the *tunica serosa*.

LWFS – In the mucosa and submucosa of pathological subjects, the adrenergic innervation did not seem to vary, compared to the control. In fact, some thin TH-IR fibers were seen in the *tunica mucosa* (pelvic flexure>ileum) along the major axis of the villi (ileum) and in the *mm* (**Fig. 9.2 b**). In the submucosa, as observed in control tissues, adrenergic fibers were abundantly seen, mainly along large blood vessels around which they formed a delicate network of innervation (**Fig. 9.2 b, d, f**). In cryosections (**Fig. 9.3 d, f**) and wholemount preparations (**Fig. 9.4 b, d, f**), large bundles of adrenergic fibers were visible in either the plexuses; from these fibers arose smaller bundles of fibers which never formed varicosities, as was observed in the SMP (**Fig. 9.3 a; Fig. 9.4 a**) and MP (**Fig. 9.1 b; Fig. 9.4 c**) of control tissues. Affected and control foals showed a comparable distribution of adrenergic fibers in the CML of the ileum (**Fig. 9.3 d**). It is to remark that, in the pelvic flexure CML, the distribution of adrenergic fibers was different if compared to the pattern observed in the control tissues, being these fibers more concentrated in the outer portion of the layer and scantly represented in the inner portion of the CML (**Fig. 9.3 f**). Moreover, in the space between CML and LML, as well in the LML of the pelvic flexure, a dense network of large and thin bundles of adrenergic fibers was seen (**Fig. 9.3 f**), and this evidence was more appreciable in wholemount preparations (**Fig. 9.4 f**).

Semi-quantitative data related to the distribution of sympathetic fibers are summarized in **Table 9.2**. Quantitative data concerning the density of TH-IR fibers in the ileum and pelvic flexure of control and LWFS foals are graphically represented in **Fig. 9.5**.

Peptidergic innervation (SP- and CGRP-immunoreactivity)

Control - A large number of SP-IR fibers was seen in all the layers of the ileum and pelvic flexure. A dense network of mucosal SP-IR fibers co-expressing CGRP-IR was visible in the *lamina propria* and in the *mm* (**Fig. 9.6 a-d**). In the submucosa, SP-IR fibers encircled vessels and SMP neurons, many of which largely expressed both markers. The most abundant concentration of SP-IR fibers was seen in the CML, while in the LML these fibers were thinner and showed less density (**Fig. 9.6 i, k**). Bundles of SP-IR fascicles run between the two muscle layers, and thin varicosities were seen encircling myenteric neurons which partly showed SP-IR in both intestinal tracts considered. SP-IR fibers were also observed in the serosa. In the ileum and pelvic flexure, some CGRP-IR fibers were seen in either muscular layers (CML>LML); this finding was consistent with the presence of some CGRP-IR myenteric neurons (**Fig. 9.7 a**).

LWFS – The SP- and CGRP-IR was dramatically reduced in the ileum and pelvic flexure (**Fig. 9.6 e-h**). In the mucosa, only a few thin SP- and CGRP-IR fibers were seen (**Fig. 9.6 e-h**). Notably, in the submucosa of the ileum very few CGRP-IR neurons (co-expressing SP-IR) were seen; it is to remark that in the mucosa above these submucosal neurons, the peptidergic fibers were more represented (**Fig. 9.7 c**). In the submucosa, peptidergic fibers were most abundantly represented around blood vessels (CGRP>SP) (**Fig. 9.6 e-h; Fig. 9.7 d**). Very few SP-IR fibers were observed in the CML of the ileum and pelvic flexure (**Fig. 9.6 j, l**); a few CGRP-IR fibers were seen in the CML of the pelvic flexure, while a few fibers were seen in the LML of both intestinal tracts considered (**Fig. 9.7 b**). Peptidergic fibers of different dimensions were seen between the CML and LML.

Semi-quantitative data related to the distribution of peptidergic sensory fibers are summarized in **Table 9.2.**

Quantitative data concerning the density of sensory fibers in the ileum and pelvic flexure of control and LWFS foals are graphically represented in **Fig 9.5.**

HuC/HuD- and S100b-immunoreactivity

Control – HuC/HuD-IR was strongly expressed by cytoplasm and nucleus of SMP and MP neurons (**Fig. 9.3 a, b: 9.8 a**). Bright S100-IR was visible in all the layers of the ileal and pelvic flexure wall and around MP and SMP neurons (**Fig. 9.8 a**). The MP ganglia of the ileum appeared smaller than those observed in the pelvic flexure. MP ganglia often occupied often a position within the LML; furthermore, some small ganglia were observed in ileal subserosal space (data not shown). The equine SMP were organized in two layers in the ileum (Chiocchetti et al. 2009a) and in a single layer of small ganglia in the pelvic flexure, distributed near the mucosa (data not shown).

LWFS – In the two subjects, a few HuC/HuD-IR small neurons (co-expressing nitrergic phenotype; data not shown), usually grouped in small clusters, were seen in the MP of the ileum (**Fig. 9.8 b-c**). No neuronal somata were identified in the pelvic flexure. Owing to the absence of ENS neurons and fibers, S100-IR was reduced in density and limited to cells ensheathing bundles of extrinsic nervous fibers (**Fig. 9.8 b**); nevertheless, S100-IR was visible around the few HuC/HuD-IR neurons migrated in the MP (**Fig. 9.8 b-c**).

Discussion

LWFS vs. Hirschsprung's disease

Aganglionosis – In the horse, the LWFS displays total or almost total lack of enteric neurons from the caudal half of the jejunum to the whole length of the large intestine (Vonderfecht et al. 1983). In human Hirschsprung's disease, the aganglionosis affects frequently short segments of the colon but may also involve larger portions of the bowel; in fact, in few cases (2-13%), i.e. in the so called Zuelzer-Wilson Syndrome, ENS neurons are missing in the whole colon and also in the caudal portion of small intestine (Zuelzer and Wilson 1948). Thus, it seems reasonable to compare, in terms of aganglionosis width, the LWFS to the human Zuelzer-Wilson Syndrome or to another form of aganglionosis which involves the whole colon (total colonic aganglionosis) (McCabe et al. 1990; Moore 2015).

Genetic – The endothelin system is considered, at present, as the unique responsible for the LWFS pathogenesis (Yang et al. 1998; Finno et al. 2009). In the most investigated Hirschsprung's disease, it seems that at least twelve genetic variations are involved in the aberrant colonization of the ENS during development (Heanue and Pachnis 2006; Ou-Yang et al. 2007; Liu et al. 2015; Moore 2015); however, the endothelin pathway has been shown to be one of the

most important genetic factors in its pathogenesis (Moore 2015). It has been shown that, in 30–50 % of patients with familiar Hirschsprung's disease, in addition to EDNRB alteration, other genes are involved, such as mutations in the receptor tyrosine kinase (RET) or its ligand GDNF and the co-receptor GFRA1 (Moore 2015; Tomuschat and Puri 2015). Another pair of genes, PHOX2B and SOX10, is strictly correlated in the aberrant colonization of the ENS and the faults in formation of enteric neurons and sympathetic ganglia (Gershon 2012a; Nagashimada et al. 2012). It is to remark that mutations both in RET and in SOX10 seem to be also important for alteration of the sympathetic nervous system (Robertson et al. 1997; Moore 2015). This represents another finding of the present research that will be discussed later on.

LWFS features

ENS - In the ileum of the two subjects analyzed, only very few HuC/HuD-IR neurons were identified in the MP and SMP. While the presence of SMP neurons is a new evidence, the presence of some MP neurons is consistent with the recent findings of Muniz et al. (Muniz et al. 2013); in fact, in their accurate paper, authors showed that in the ileal MP of LWFS foals a small percentage (about 1%) of atrophied neurons was still identifiable. The two foals considered in the present research were affected by hypoganglionosis in the ileum and complete aganglionosis in the pelvic flexure.

Extrinsic nerve fibers - The presence of hypertrophied nerve bundles running in the two intestinal plexuses seems to characterize LWFS (Julian 1994), Hirschsprung's disease (Moore 2015), and murine models of aganglionosis (Watanabe et al. 1995; Nagashimada et al. 2012). Nevertheless, thickened nerve trunks may be completely absent in the human total colonic aganglionosis bowel (Kapur 2009; Knowles et al. 2009).

It must be considered that extrinsic fibers derive from extrinsic neurons, which in turn are derived from neural crest cells; thus, it is conceivable that also these neurons and fibers are not perfectly programmed to reach their natural target. In fact, there is evidence that other components of the autonomic nervous system (in particular the sympathetic elements, see below) might be altered during neurocristopathies (Carnahan et al. 1991; Gershon 2012a; Nagashimada et al. 2012) and Hirschsprung's disease (Robertson et al. 1997; Ding et al. 2013).

Sympathetic fibers – These fibers, which innervate all the layers of the horse intestine, derive (at least for the horse ileum) from paravertebral (thoracic) and prevertebral ganglionic neurons (Russo et al. 2010; Bombardi et al. 2013). One interesting finding of the present research is the conspicuous contribution of the sympathetic innervation to the *tunica muscularis* of the pelvic flexure in control foal. This observation remarks the role of the sympathetic innervation on the pelvic flexure motility in the horse, in which postoperative ileus frequently occurs (Lefebvre D 2015). In the LWFS ileum and pelvic flexure we noticed an increased density of large bundles of adrenergic fibers between the two layers of the *tunica muscularis*, coupled with a "bizarre" distribution of adrenergic fibers in the CML and LML of the pelvic flexure. The presence of large nervous fascicles might be justified by the absence of enteric neurons, which represent a specific target of the extrinsic fibers; although just a fanciful hypothesis, the extrinsic bundles might be considered as bundles wandering in search for a final destination. It has been shown that a great percentage of rat postganglionic sympathetic fibers innervate simultaneously the muscular smooth muscle cells and myenteric neurons (Walter et al. 2016), suggesting a crucial role of sympathetic system in the direct control of the gut motility. In line with our findings, we can state that in the LWFS foals also the development of the sympathetic system may be altered, exactly as observed in human (Watanabe et al. 1998) and rodents (Watanabe et al. 1995). This finding, never reported in the LWFS horse before, might be useful for further genetic investigations.

Sensory fibers – We demonstrated by using retrograde fluorescent tracers, that CGRP- and SP-IR extrinsic fibers reaching the horse ileum arise almost exclusively from spinal ganglia (Russo et al. 2010; Russo et al. 2011). However, since no data are reported about the phenotype of equine vagal sensory neurons, we cannot exclude that a vagal contingent of sensory CGRP- and SP-IR fibers might be present in the equine ileum.

Neuropeptides SP and CGRP seem to act synergistically in modulating the nociceptive neurons in the dorsal horn of the spinal cord (Hanesch and Schaible 1995).

In the dog ileum it has been shown that the mucosal SP-IR fibers largely arise from SMP neurons, while those innervating the CML arise from MP neurons (Daniel et al. 1987); data obtained in the present research are consistent with the observations of Daniel et al. (1987); in fact, in LWFS tissues lacking enteric neurons, only some SP-IR fibers were seen in the tunica mucosa, CML and tunica serosa, while a considerable number of SP-IR fibers encircled

submucosal arteries. SP-IR extrinsic fibers were seen reaching the aganglionic MP; these SP-IR, partially distributed also within the CML, might indicate a close relationship with the interstitial cells of Cajal (Pavone and Mandara 2010; Matsumoto et al. 2011).

Our findings indicate that the extrinsic SP-IR fibers supply sensory innervation to the submucosal vessels, mucosa, and serosa. It is well established that primary sensory neurons can be activated antidromically and have efferent effects; in response to noxious stimuli, CGRP- and SP-IR can be released peripherally to induce vasoactive effects, and to promote inflammatory responses, thus having a healing effect on the tissue (Brain and Williams 1985; Brain and Williams 1988). As a result of this evidence, we can suppose that also in the horse CGRP- and SP-IR extrinsic sensory fibers might be also vasomotor.

While it is quite easy to compare and verify whether sympathetic fibers are altered in LWFS tissues owing to the exclusively extrinsic origin of these fibers, it is not so easy to draw conclusions regarding sensory fibers immunoreactive for SP and CGRP. In fact, although in general our results do not seem to present a picture of abnormality regarding the distribution of extrinsic sensory fibers, it has been reported that also extrinsic SP- and CGRP-IR sensory fibers might be altered in aganglionosis (Robertson et al. 1997). To address this topic, the neurochemical and morphological characterization of the spinal ganglia neurons in LWFS foals would be useful, also considering the current knowledge about the horse sensory innervation (Russo et al 2010; 2011; 2012).

Fibers with different phenotype - In the horse intestinal wall, extrinsic fibers immunoreactive for other neuronal markers have been observed: neuronal nitric oxide (nNOS), 200-kDa neurofilament protein (NF200), and neuropeptide Y (NPY) (Russo et al. 2010; Russo et al. 2011; Russo et al. 2012). nNOS-IR fibers belong to spinal ganglia sensory neurons and largely (about 82%) co-express both SP- and CGRP-IR (Russo et al. 2011). NPY-IR is expressed by sympathetic prevertebral neurons co-expressing also TH- and DBH-IR (Russo et al. 2010). NF200-IR is expressed by spinal ganglia (Russo et al. 2012) but also by sympathetic ganglia (personal observation, Dr. R. Chiocchetti); being expressed by two different populations of neurons (sensory and sympathetic) with different patterns of peripheral distribution, we excluded the investigation of the marker NF200 (Russo et al. 2012) in the present study. For the same reason, although the literature reports the presence of an increased number of cholinergic fibers in Hirschsprung's

disease tissues, in the present research we did not utilize the antibody anti-choline acetyltransferase (ChAT), a neurochemical marker which should be expressed by the majority of extrinsic primary sensory fibers and vagal efferent and afferent fibers (Russo et al. 2010).

Glial marker S100 - Enteroglial cells derive, as enteric neurons, from neural crest cells; these elements do not contribute only to the structural activity and to the nourishment of the enteric neurons but are also essential for a variety of functions, such as the control of homeostasis, mucosal integrity, neuroprotection, neurogenesis, neuro-immune interactions, and synaptic transmission (Ruhl 2005; Boesmans et al. 2015). S100-IR is expressed both by ENS glial cells and by Schawann cells accompanying the extrinsic fibers; these cells have already been studied in Hirschsprung's disease tissues (Kato et al. 1990). Theoretically, the absence of enteric neurons should be combined by that of enteric glia. Thus, the great majority of the S100-IR observed in LWFS is related to the presence of glial cells surrounding extrinsic fibers. In this line, it is tentative to speculate about a possible role of the Schwann cells in the postnatal neurogenesis. In fact, it has been demonstrated in mice that a subset of Schwann cell precursors are able to invade the gut alongside extrinsic nerves and give rise to new born neurons in the small and large intestine, in absence of vagal neuronal crest derived ENS precursors (Uesaka et al. 2015). Although this mechanism might explain the presence of few neurons surrounded by S-100-IR processes in the ileum of LWFS, it remains a fascinating hypothesis and further studies are needed.

Table 9.1. Details of the primary and secondary antibodies.

Primary antibody	**Host**	**Code**	**Dilution**	**Source**
CGRP	Rabbit	T-4032	1:1000	Peninsula
DBH	Mouse	MAB 308	1:250	Merk Millipore
HuC/HuD	Mouse	A21271	1:200	Life Technologies
S100B	Rabbit	Z0311	1:200	Dako Cytomation
SP	Rat	10-S15A	1:400	Fitzgerald
TH	Mouse	NCL-TH	1:80	Novocastra
TH	Sheep	AB 1542	1:80	Merk Millipore
Secondary antibody	**Host**	**Code**	**Dilution**	**Source**
Anti-mouse IgG Alexa 594	Goat	A11005	1:200	Life Technologies
Anti-rabbit IgG FITC	Goat	401314	1:200	Merck Millipore
Anti-rabbit IgG Alexa 594	Donkey	AB150132	1:600	Abcam
Anti-mouse IgG Alexa 488	Donkey	20014	1:100	Biotium
Anti-rat IgG Alexa 594	Donkey	A21209	1:50	Life Technologies
Anti- rat IgG Alexa 488	Goat	20023	1:100	Biotium
Anti-sheep IgG Alexa 594	Donkey	A11016	1:200	Life Technologies

Abbreviations: CGRP, calcitonin gene-related peptide; DBH, dopamine beta-hydroxylase; FITC, Fluorescein isothiocyanate; HuC/HuD, human neuronal protein; S100b, S100 protein; SP, Substance P; TH, tyrosine hydroxylase. Suppliers: Abcam, Cambridge, United Kingdom, Europe;Biotium, Inc. Hayward, CA, USA; Dako Cytomation, Denmark, Europe; Fitzgerald Industries Int., Inc. Concord, MA, USA.; Life Technologies, Carlsbad, California, USA; Merck Millipore, Merck KGaA, Darmstadt, Germany, Europe; Novocastra, Leica Microsystems - Biosystems Division, Newcastle, UK; Peninsula Laboratories, San Carlo, CA, USA; Santa Cruz Biotechnology, CA, USA.

Table 9.2. Semiquantitative evaluation of the density of the adrenergic-(TH and DBH) and peptidergic-(CGRP and SP) immunoreactive nerve cell bodies [(c)] and fibers [(f)] innervating the ileum and pelvic flexure of control and lethal white foal syndrome foals. Immunoreactive cells and fibers are graded as: –, not found; +, a very small number; ++, a moderate number; +++, a large number of nerve cell bodies and/or fibers.

	Control foal			**Lethal white foal syndrome foals**		
	Adrenergic fibers	Peptidergic fibers		Adrenergic fibers	Peptidergic fibers	
	TH/DBH	**CGRP**	**SP**	**TH/DBH**	**CGRP**	**SP**
Mucosa	$+^{f}$ lamina propria $++^{f}$ *mm*	$+++^{f}$	$+++^{f}$	$+^{f}$	$+^{f}$	$+^{f}$
Submucosa	$+++^{f}$	$+++^{c,f}$	$+++^{c,f}$	$+++^{f}$	$+++^{f}$	$++^{f}$
SMP	$++^{f}$	$+++^{c,f}$	$+++^{c,f}$	$+^{f}$	$^{+f}$	$^{+f}$
CML	$++^{f}$	$+^{f}$	$+++^{f}$	$+^{f}$/ileum $++^{f}$/pf*	-/ileum $+++^{f}$/pf	$+^{f}$
MP	$+++^{f}$	$++^{f}$ $+^{c}$	$+^{c}$ $+++^{f}$	$+++^{f}$	$++^{f}$	$++^{f}$
LML	$+^{f}$/ileum $++^{f}$/pf	$+^{f}$	$+^{f}$	-/ileum $+++^{f}$/pf	-	-
Serosa	$+^{f}$	$+^{f}$	$+^{f}$	$+^{f}$	$+^{f}$	$+^{f}$

* In the circular muscle layer (CML) of the pelvic flexure (pf) of LWFS foals, the adrenergic fibers were concentrated in the outer portion of the layer.

Abbreviations: circular muscle layer (CML), longitudinal muscle layer (LML), *muscularis mucosae* (*mm*), myenteric plexus (MP), pelvic flexure (pf), submucosal plexus (SMP).

Figures

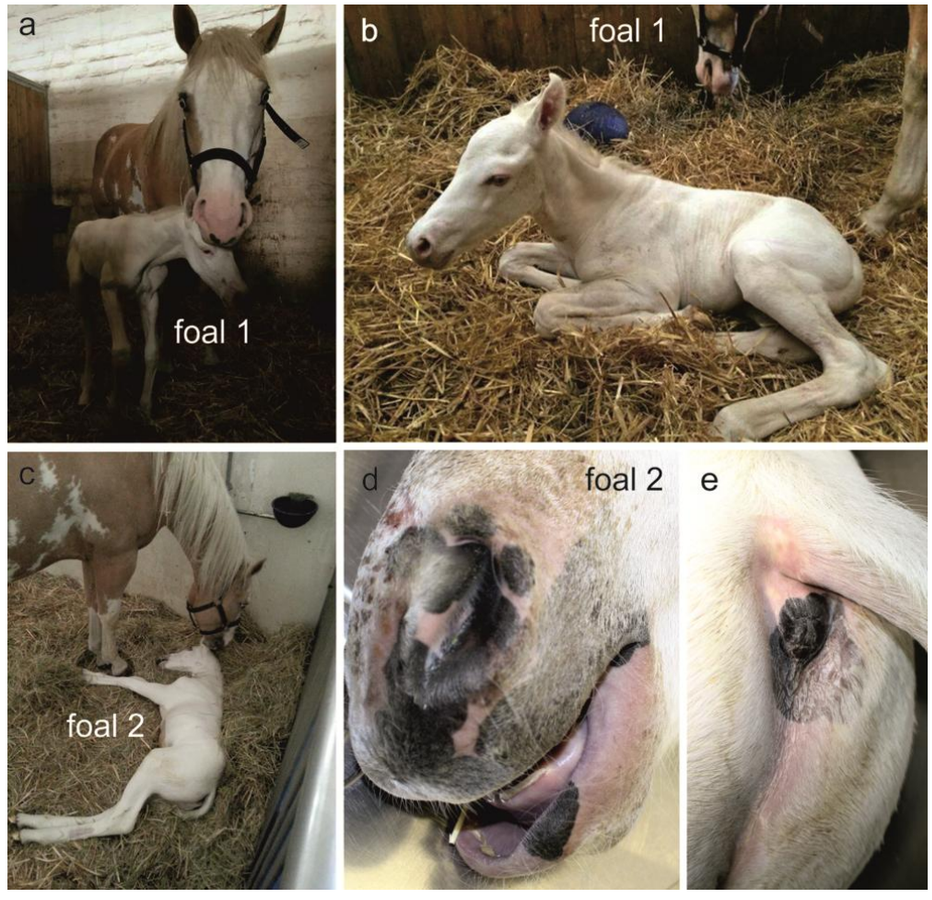

Fig. 9.1 The figures show the two American paint male foals investigated, in which the lethal white foal syndrome (LWFS) was clinically suspected. The two foals had the same dam (a, c) and a different sire. The two foals were quickly accepted by the mare and were active in the box (a), but one day after birth they were depressed and recumbent (c). The coat of the first subject was entirely pure white (a-b) and the iris was totally light blue; in the second foal (c-e) the coat showed pigmentation of upper eyelid, nasolabial area (d), lower lip, foreskin, and perianal area (e), while the iris was pigmented.

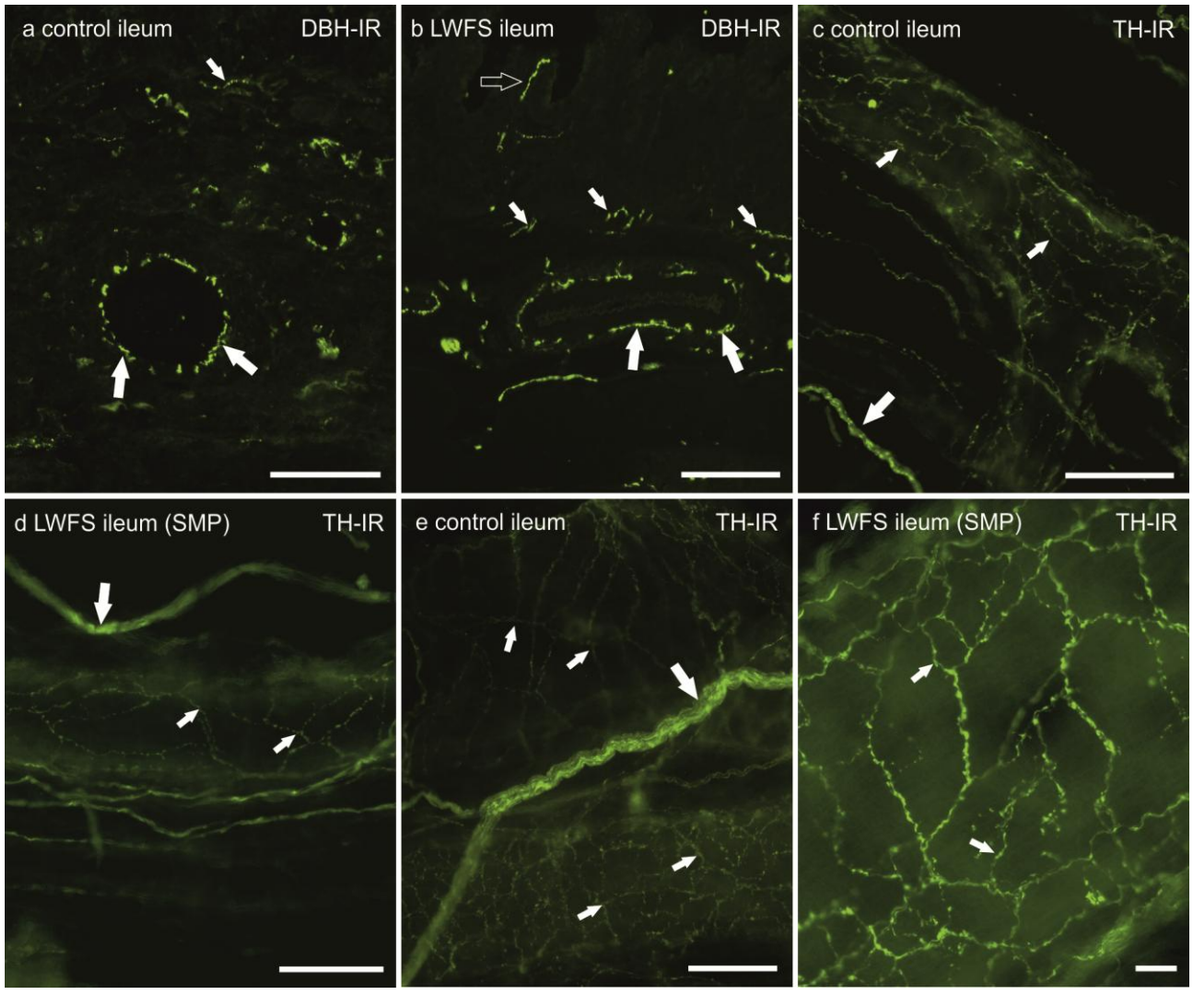

Fig. 9.2 Photomicrographs showing TH- and DBH-immunoreactivity (IR) of adrenergic innervation observed in in the submucosa of one week old foal (control) (a, c, e) and lethal white foal syndrome (LWFS) affected subjects (b, d, f). In either subjects no differences in the mucosal and submucosal adrenergic innervation were noted. a, b) Longitudinal cryosections of control (a) and LWFS ileum (b) showing adrenergic DBH-IR nervous fibers distributed around submucosal vessels (large arrows), within the *muscularis mucosae* (small arrows) and in the *lamina propria* (empty arrow). c-f) Submucosal wholemount preparations of control (c, e) and LWFS (d, f) ileum: the large arrows indicate large bundles of adrenergic TH-IR fibers running along submucosal blood vessel (c, d, e); the small arrows indicate the delicate network of adrenergic TH-IR fibers encircling large submucosal arteries. Note the thin perivascular adrenergic network surrounding an artery (e) and also a large vein (above the large bundle of fibers).Scale bar: a-e 20 µm; f 100 µm.

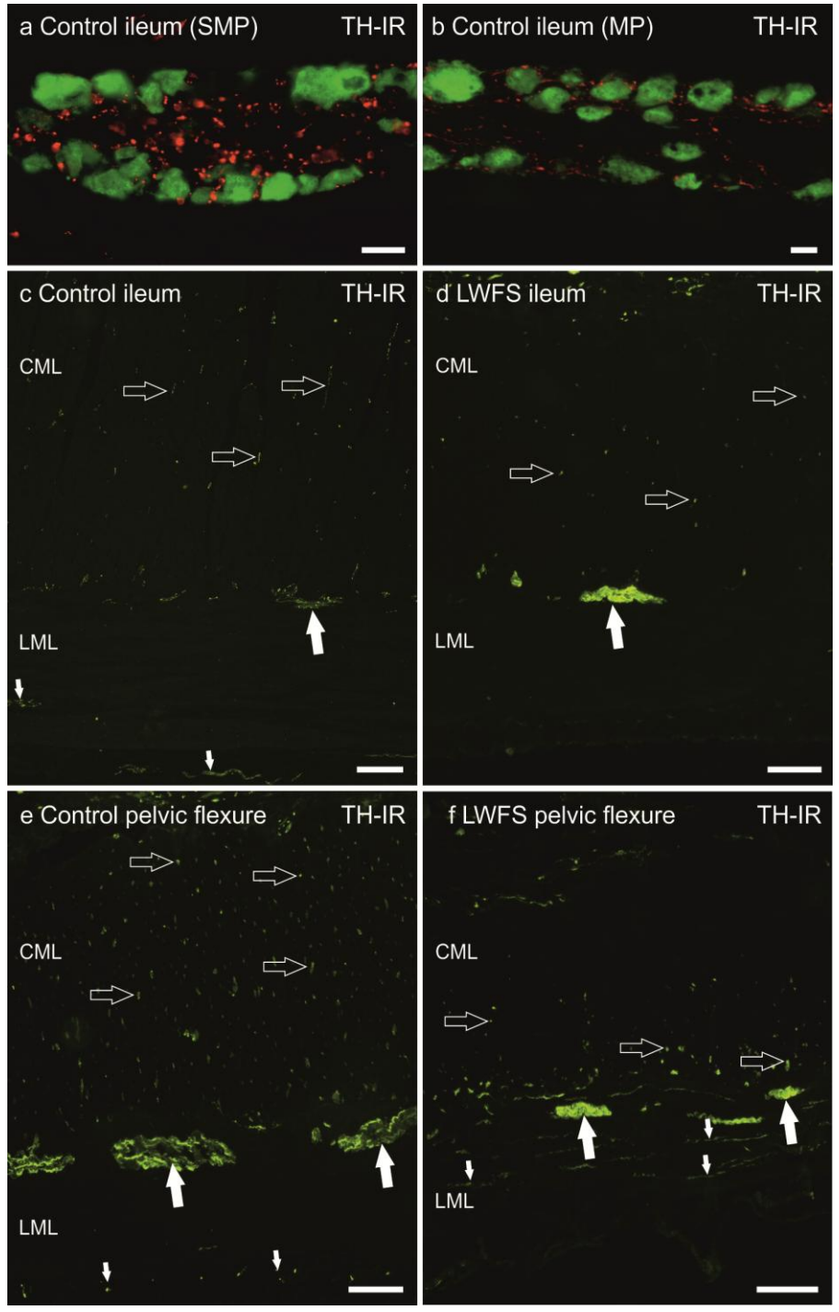

Fig. 9.3 Photomicrographs showing TH-immunoreactivity (TH-IR) of adrenergic innervation observed in cryosections of the ileum (a-d) and pelvic flexure (e-f) of one week old foal (control) (a-c, e) and lethal white foal syndrome (LWFS) affected subjects (d, f). a-b) A delicate network of TH-IR fibers and varicosities (red color) was scattered around submucosal plexus (SMP) (a) and myenteric plexus (MP) (b) HuC/HuD-IR neurons (green color). c-f) Longitudinal cryosections of control (c, e) and LWFS (d, f) ileum and pelvic flexure; empty arrows indicate TH-IR nervous fibers scattered in the circular muscle layer (CML). Small arrows indicate TH-IR fibers within the longitudinal muscle layer and serosa; large arrows indicate TH-IR fibers between the CML and LML. In the control tissues (c, e), the adrenergic

fibers were homogenously scattered in the whole thickness of the CML layer (empty arrows). In the LWFS ileum (d) adrenergic fibers were slightly reduced in the CML and LML. Note the abundancy of adrenergic fibers in the CML of the pelvic flexure of control foal (e). In the LWFS pelvic flexure (f), adrenergic fibers were more concentrated in the outer portion of the CML (empty arrows) and formed packed bundles of nervous fibers between CML and LML (large arrows) and in the LML (small arrows).Scale bar: a-b 20 µm; c-f 100 µm.

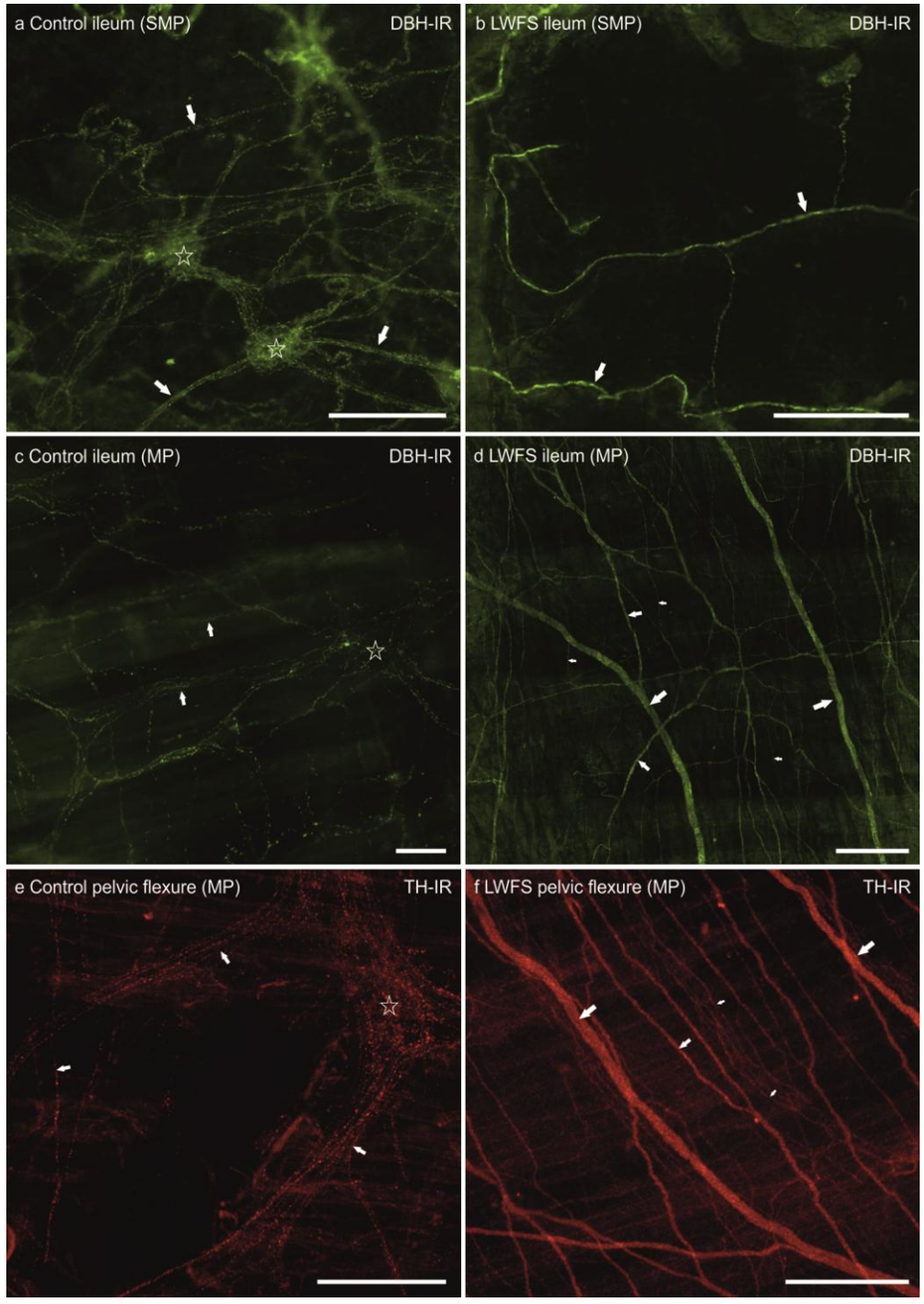

Fig. 9.4 Photomicrographs showing DBH-and TH-immunoreactivity (IR) of adrenergic innervation observed in the wholemount preparations of ileum (a-d) and pelvic flexure (e-f) of one week old foal (control) and lethal white foal syndrome (LWFS) affected foal. a-b) In the submucosa of control ileum (a), a large mesh network of adrenergic fibers (arrows) were seen, forming baskets of varicosities at the level of SMP ganglia (stars). In the submucosa of LWFS ileum (b) lacking enteric neurons, only large bundles of adrenergic DBH-IR fibers (arrows) were seen, which never formed varicosities. c-f) In the myenteric plexus (MP) of control ileum (c) and pelvic flexure (e), DBH- and TH-IR nervous fibers were thin and showed varicosities within the myenteric ganglia (star). In the LWFS ileum (d) and pelvic flexure

(f), DBH- and TH-IR was observed in large bundles of adrenergic fibers (large arrows) running longitudinally; from these large bundles of nervous fibers arose medium (medium sized arrows) and small diameter bundles of fibers (small arrows), which never form varicosities. Scale bar: a, c 100 µm; b, d-f 500 µm.

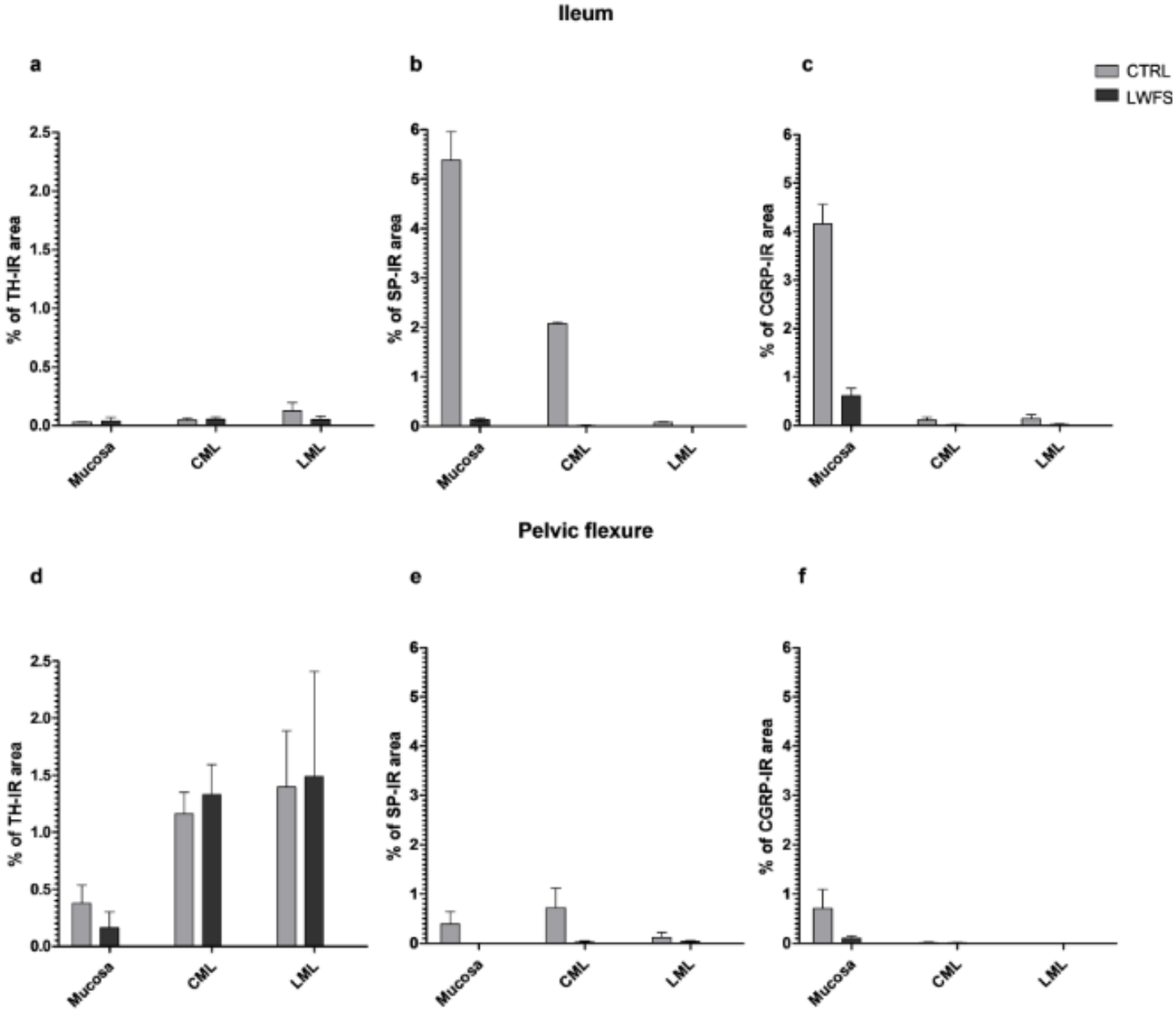

Fig. 9.5 Quantification of the densities for TH- (a, d), SP- (b, e), and CGRP-immunoreactive fibers (c, f) of one week old foal (control, CTRL) and LWFS foals. a, d) Percentages of TH-IR area in the mucosa, circular muscle layer (CML), and longitudinal muscle layer (LML) of the ileum (a) and pelvic flexure (d). b, e) Percentages of SP -IR area in the mucosa, CML and LML of the ileum (b) and pelvic flexure (e). c, f) Percentages of CGRP-IR area in the mucosa, CML and LML of the ileum (c) and pelvic flexure (f). Data are represented as mean ± SEM.

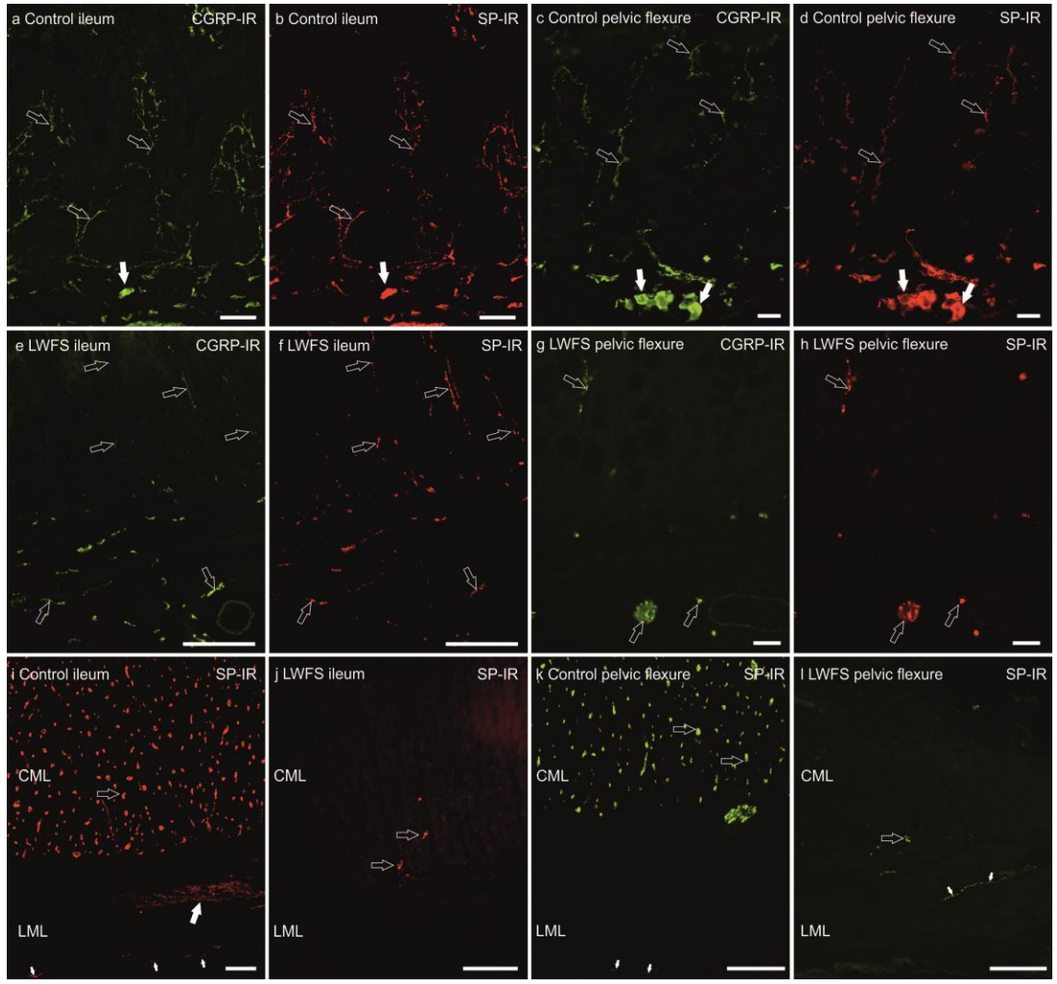

Fig. 9.6 a-h) Photomicrographs showing the co-localization of CGRP- and SP-immunoreactivity (IR) in the cryosections of ileum and pelvic flexure of the one week old foal (control) and lethal white foal syndrome (LWFS) affected subjects. In the control ileum (a-b) and pelvic flexure (c-d), CGRP- and SP-IR were co-expressed in the nervous fibers (empty arrows) and submucosal neurons (white arrows); in the LWFS ileum (e-f) and pelvic flexure (g-h), there was a decreased density of peptidergic immunoreactive nervous fibers in the mucosa (empty arrows) compared to the control tissues. Furthermore, in LWFS sections (e-h), no submucosal neurons were visible. i-l) Photomicrographs showing SP-IR in longitudinal cryosections of the ileum (i-j) and pelvic flexure (k-l) of control and LWFS foals. Note that SP-IR was brightly expressed in the nervous fibers (empty arrows) in the circular muscle layer (CML) of control ileum (i) and pelvic flexure (k) and was dramatically reduced in the CML of the LWFS ileum (j) and pelvic flexure (l). In the longitudinal muscle layer (LML) the large arrow (i) indicates SP-IR nervous fibers in a myenteric plexus ganglion embedded in the LML, while small arrows indicate think SP-IR fibers running along the LML (i; k-l).
Scale bar: a-b; e-h; j: 20 µm; c-d; i; k-l:100 µm.

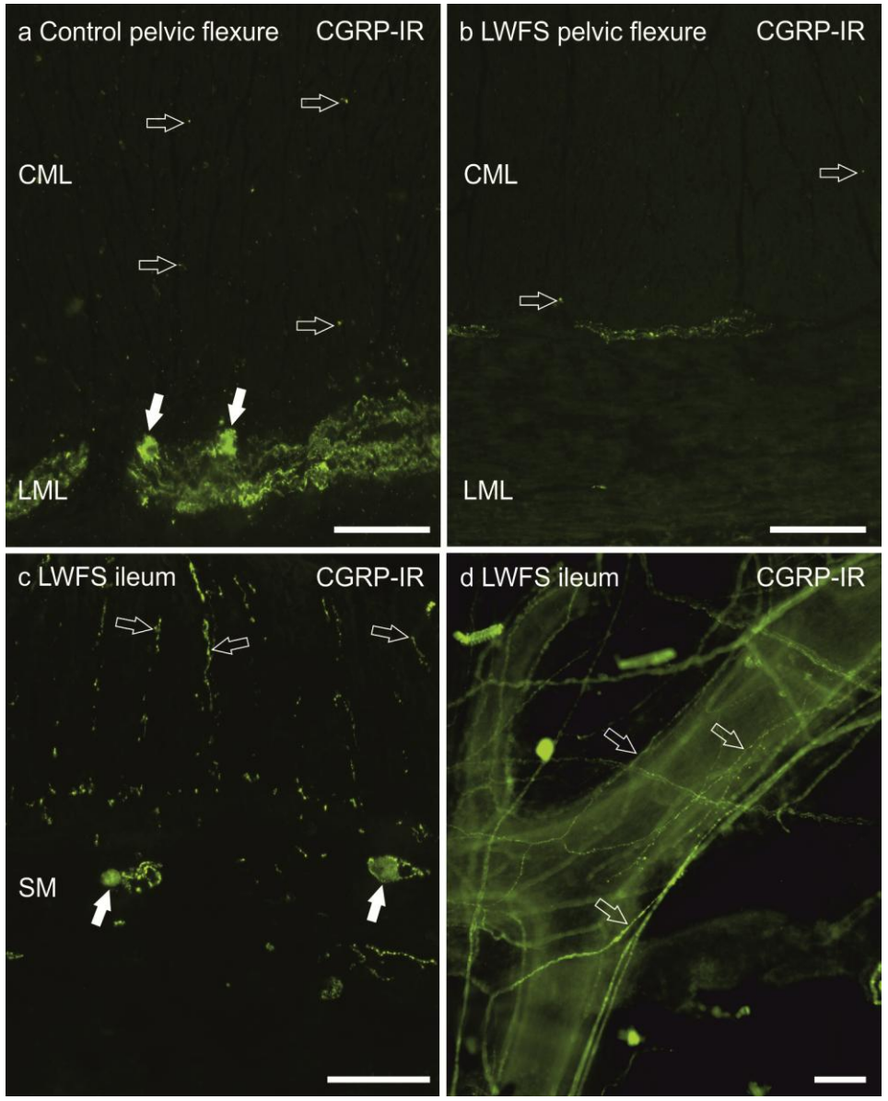

Fig. 9.7 a-c) CGRP-IR in longitudinal cryosections of the pelvic flexure (a-b) and ileum (c) of one week old foal (control) and lethal white foal syndrome (LWFS) affected subject. a-b) In the control pelvic flexure (a) CGRP-IR was expressed by a few CML nervous fibers (empty arrows) and myenteric plexus neurons (white arrows). In the LWFS pelvic flexure (b), no enteric neurons were visible and there was a great reduction of CGRP-IR nervous fibers in the CML and longitudinal muscle layer (LML). c) In the submucosa (SM) of LWFS ileum, only very few submucosal plexus neurons were observed (white arrows) which showed CGRP-IR. Notably, in the mucosa above these CGRP-IR neurons, the CGRP-IR fibers were more concentrated (empty arrows) compared to the mucosa above submucosa lacking enteric neurons (see Fig. 5 e). d) Wholemount preparation of the ileum of LWFS foal in which a large submucosal blood vessel showed a rich network of CGRP-IR fibers (empty arrows). Scale bar: a-d 100 µm.

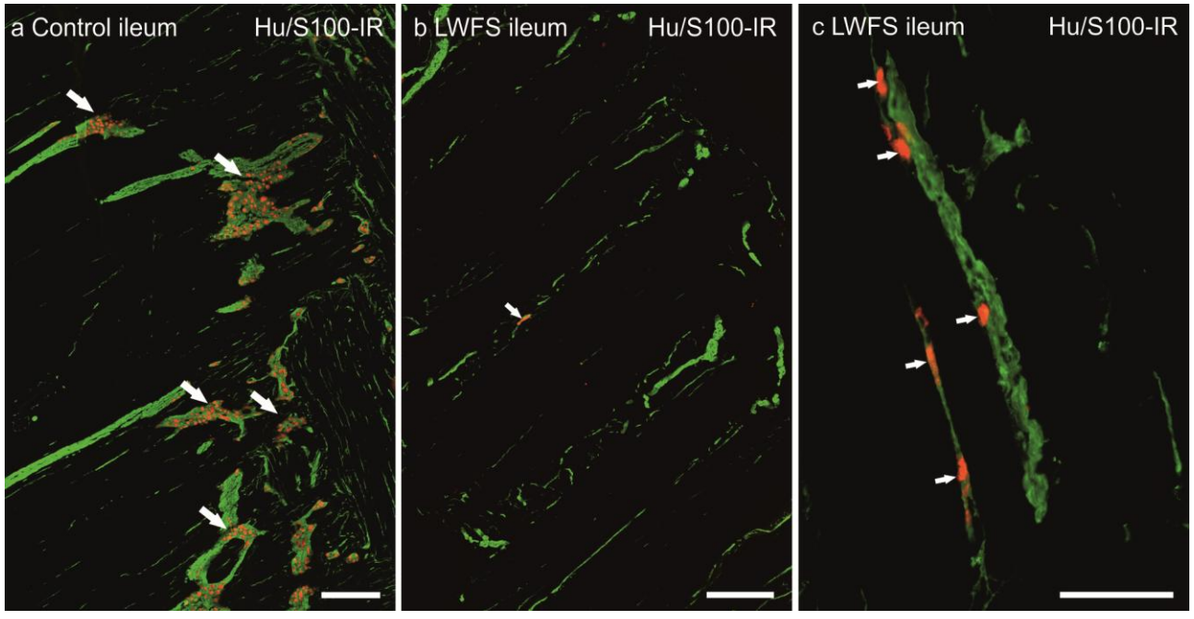

Fig. 9.8. Photomicrographs showing S100- and HuC/HuD-immunoreactivity (Hu-IR) in tangential cryosections of the ileum of one week old foal (control) (a) and lethal white foal syndrome (LWFS) affected subjects (b-c). a) Hu-IR was strongly expressed by myenteric plexus neurons which formed ganglia of different dimensions (arrows). In the picture is also visible the S100-IR (green color) around HuC/HuD-IR neurons, along the strands of nervous fibers running between ganglia and within the muscle. b-c) In the LWFS tissues, very few HuC/HuD-IR neurons were visible only in the ileum (arrows, b, c) and S100-IR was reduced in density and limited mainly to cells ensheathing bundles of extrinsic nervous fibers and the very few HuC/HuD-IR neurons. Scale bar: a-c 100 µm.

CHAPTER 10

Quantification of nitrergic neurons in the myenteric plexus of gastric antrum and ileum of healthy and diabetic dogs

Modified from

"Quantification of nitrergic neurons in the myenteric plexus of gastric antrum and ileum of healthy and diabetic dogs"

F. Giancola, F. Fracassi, A. Gallucci, J. Sadeghinezhad, G. Polidoro, E. Zini, M. Asti, R. Chiocchetti

Under revision

Introduction

The gastrointestinal physiology is mainly under the control of the ENS, which consists of millions of neurons harbored in the wall of the digestive system from the esophagus to the inner anal sphincter. Enteric neurons are organized in two ganglionated plexuses: the MP and SMP, which interact in coordinating gut functions almost independently from the central nervous system (Furness 2006). The gastrointestinal peristalsis is triggered by sensory fibers responsive to the radial distension of the lumen or by chemical stimuli. Once exited, the intramural sensory neurons activate ENS excitatory and inhibitory muscle motor neurons. The excitatory neurons release acetylcholine, whereas the inhibitory neurons release nitric oxide (Furness 2006). Nitrergic neurons and fibers, which are usually immunohistochemically identified by the use of an antibody against the enzyme nNOS, have been already characterized in the canine gastrointestinal tract (Berezin et al. 1994; Ward et al. 1994). Nevertheless, none of these studies quantified the percentage of nitrergic neurons.

A wide spectrum of damages affecting the structural and functional integrity of the ENS can be responsible for many gastrointestinal symptoms and dysfunction. Among the secondary enteric neuropathies, i.e. heterogeneous disease in which the primary target of the disease is not the ENS (that results however damaged), diabetes mellitus (DM) is classified as a "predominantly degenerative neuropathy"(Knowles et al. 2013).

DM is a worldwide endocrine disease affecting humans but also domestic mammals, such as dogs and cats (Nelson and Reusch 2014). The common feature of DM is hyperglycemia, which must be controlled to avoid severe DM complications such as retinopathy, vascular damage, generalized neuropathy, and gastrointestinal motility disorders (i.e. vomiting, constipation, diarrhea, and fecal incontinence), in both human and animal models (Rothstein 1990; Zandecki et al. 2008; Adewoye et al. 2011; Ciobanu and Dumitrascu 2011). Seemingly, generalized neuropathy and gastrointestinal motility disorders are two strictly correlated complications. As a matter of fact, a growing body of evidence suggests that gastric and intestinal symptoms in human and animal diabetic patients derive from intestinal motility abnormalities related to enteric neuropathy.

A few studies have focused on the gastrointestinal dysfunction in DM dogs (Takeda et al. 2001; Onoma et al. 2008), and to date, no information is available on the effects of DM on canine ENS.

The present research was focused on the nitrergic enteric subpopulation of dogs, since in other species (mainly rodents) these neurons are susceptible to diabetic neuropathy. The aims of the present study were to histochemically quantify the percentage of MP nitrergic neurons of the gastric antrum and ileum in healthy dogs and to characterize the effects of spontaneous DM on these neurons.

Material and methods

Animals-Tissues were collected from eight control (CTRL) dogs (none had evident gastrointestinal disorders) (**Table 10.1**) and five non-obese DM-affected dogs (**Table 10.2**).

DM type I was diagnosed through a documented clinical history and blood biochemical analysis. All animals died spontaneously or were euthanized and their tissues were collected following owner permissions. According to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, the Italian legislation (D.Lgs. n. 26/2014) does not require any approval by competent Authorities or ethics committees.

Tissue collection-The gastrointestinal tracts were removed within 2 h after each animal's death. The stomach and ileum were longitudinally cut open respectively along the greater curvature and the mesenteric border. The stomach and intestine of CTRL dogs did not present apparent mucosal hyperemia or inflammatory lesions, whereas the ileum of DM-affected dogs showed severe (two dogs) or mild (three dogs) mucosal hyperemia. The pyloric portions of the stomach and the ileum were treated to obtain tangential (1.0 cm x 1.0 cm) and longitudinal (2.0 cm x 0.5 cm) cryosections. Specimens from all the subjects were processed for immunohistochemistry as described previously (Sadeghinezhad et al. 2013).

Immunohistochemistry -The antibody anti- HuC/HuD was utilized as a pan-neuronal marker to identify all the enteric neurons.

Nitrergic neurons and nervous fibers were immunohistochemically identified by the use of two antibodies against the nNOS enzyme. Table 10.3 lists the primary and secondary antibodies employed.

Specificity of the primary antibodies-The antibodies utilized in the present research (mouse anti-HuC/HuD, mouse anti-nNOS, and rabbit anti-nNOS were tested for their specificity by WB analysis, which indicated that they were specific for the targeted molecules in dogs (**Fig. 10.1 a**). Furthermore, the two anti-nNOS antibodies were tested in a double-staining protocol and were totally co-localized (**Fig. 10.1 b**).

The specificity of the secondary antibodies was tested as described in a previous work (Sadeghinezhad et al. 2013).

Western blot- Tissue samples (dog ileum) were collected, frozen in liquid nitrogen, and stored at -80°C. Tissues were later thawed and homogenized. Total protein content was extracted using T-PER tissue protein extraction reagent in the presence of a protease inhibitor cocktail (Thermo Scientific, Italy, Europe) according to the manufacturer's instructions, and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Italy, Europe). Aliquots containing 50 ug of proteins were denatured by heating at 95°C for 5 min in Laemmli buffer, separated by SDS-PAGE (12.5% to test HuC/HuD and 7.5% to test nNOS specificity) and transferred onto a nitrocellulose membrane (GE Healthcare, UK, Europe). After blocking treatment, the membranes were incubated at 4°C overnight with the primary antibodies (**Table 10.3**) diluted in Tris-buffered saline-T20 (TBS-T20 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% T-20). After washes, the blots were incubated with respective peroxidase-conjugated secondary antibodies (Table 10.3). Immunoreactive bands were visualized using chemiluminescent substrate (Pierce ECL Western Blotting Substrate, Thermo Scientific, Italy, Europe). The intensity of luminescent signal was acquired on a C-DiGit Chemiluminescent Western Blot Scanner using Image Studio Digits Software Ver 3.1(LI-COR Biotechnology, UK, Europe).

For HuC/HuD antibody, a two band of ~42 kDa and ~40 kDa (theoretical molecular weight 41,770 kDa for HuD and 39,547 kDa for HuC, respectively) (http://www.uniprot.org/) were present in extracts from the ileum (Fig. 10.1). The band revealed by the two nNOS antibodies showed a molecular weight of ~155 kDa (theoretical molecular weight 160,970 kDa) in the ileum (**Fig. 10.1 a**). The blotting of the monoclonal antibody (mouse anti nNOS) was more clear and defined compared to that obtained with the polyclonal antibody (rabbit anti-nNOS); since the perfect co-localization between the two anti-nNOS sera utilized (**Fig. 10.1 b**), we also considered the polyclonal antibody suitable for our research.

WB analysis confirmed the specificity of the primary antibodies utilized in the present study.

Fluorescence microscopy- Preparations were examined on a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes to distinguish the fluorochromes employed. The images were recorded with a Nikon DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). Slight adjustments to contrast and brightness were made using Corel Photo Paint, whereas the figure panels were prepared using Corel Draw (Corel Photo Paint and Corel Draw, Ottawa, ON, Canada).

Quantitative analysis- At least 300 HuC/HuD-IR neurons were counted for each gastrointestinal tract from each animal. Double-immunofluorescence using HuC/HuD and the rabbit anti-nNOS antibodies on tangential sections, allowed us to quantify the proportions of nitrergic neurons. The ratio between the nNOS-IR neurons and HuC/HuD-IR neurons was determined and data were expressed as relative percentage.

Statistical analysis- The percentages of nitrergic neurons quantified in DM-affected dogs were compared with those observed in the control dogs. According to the Gaussian distribution of the data (Kolmogorov-Smirnoff test), the *Student t* test was used to analyze the difference between the mean values of the percentages of nitrergic neurons in the antrum and ileum of the two groups of dogs (CTRL *vs.*DM). Data are presented as mean ± St. Dev.

The level of significance was set at $P<0.05$. All analyses were performed using a commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software Inc., La Jolla, CA, USA).

Results

Control dogs- Gastric and ileal HuC/HuD-IR neurons showed bright and homogenous labeling that was more concentrated in the cytoplasm than in the nucleus; nNOS immunoreactivity (-IR) was strongly expressed by neurons and fibers (**Fig. 10.2**). Tangential sectioning allowed us to observe that, in the stomach, some ganglia contained several nitrergic neurons, sometimes grouped in clusters (**Fig. 10.2 a-c**), but also some ganglia were completely free of nNOS-IR cell bodies (**Fig. 10.2 d-f**). In the stomach, 30±6% of the total neuronal population showed nNOS immunoreactivity (902/3129 cells). Ileal ganglia were, in general, larger than gastric ones and almost all contained homogenously distributed nitrergic neurons (**Fig. 10.2 g-i**), which represented 29±5% (795/2800 cells) of the total neuronal population.

Diabetic dogs- In all DM dogs, regardless of the age of the subjects, duration of the hyperglycemic status, and diabetes severity, the common denominator was a thickening of the connective tissue surrounding the ganglia, which was limited in the antrum but more pronounced in the ileum (**Fig. 10.3 a-l**). In the most affected ganglia, this connective thickening showed marked autofluorescence (**Fig. 10.3 d-l**). While HuC/HuD and nNOS immunoreactivity was quite preserved in the stomach (Fig. 10.3 a-c), in some ileal ganglia, the HuC/HuD-IR was compromised, showing morphological changes (**Fig. 10.3 d-f**). In the worst condition, neuronal somata were barely identifiable (**Fig. 10.3 d-e**) and HuC/HuD-labeling seemed dispersed in small granules (**Fig. 10.3 f**); in some neurons, HuC/HuD-labeling was confined only in the nucleus. In a few ganglia in which nNOS-IR was still brightly expressed by cell somata and fibers, HuC/HuD-neurons were no longer identifiable (**Fig. 10.3 g-i**). In the ileum, nNOS immunoreactivity was altered also in neuronal fibers, which appeared often deranged or discontinued (**Fig. 10.3 j-l**). In the stomach the percentage of nNOS-IR neurons was 25±2% (727/2926 cells). In the ileum the percentage of nitrergic neurons was 19±5% (308/1508 cells).

Control and diabetic comparison

The statistical analysis did not show differences between the percentages of nitrergic neurons observed in the gastric antrum of the two groups CTRL= 30±6% *vs.* DM= 25±2% (P= 0.112). On the contrary, there was a statistically relevant reduction in the percentage of nNOS-IR neurons in the ileum of pathological animals: CTRL= 29±5% *vs.* DM= 19±5% (P= 0.006). **Fig. 10.4** graphically represents the data.

Discussion

In the present study, we firstly evaluated the percentage of nitrergic neurons harbored in the myenteric plexus of the canine antrum and ileum.

In the control dogs, similar percentages of nitrergic neurons were observed in the antrum (about 30%) and ileum (about 29%). In laboratory rodents, the percentage of nNOS-IR neurons was about 20% in the stomach (Furness 2006) and may vary from 24–29% in the ileum (Lawson et al. 2010; Sadeghinezhad et al. 2013), respectively. No data are available on the percentage of nNOS-IR myenteric neurons in the stomach of large animals, whereas this information is available in relation to the ileum of pigs (about 19%) (Brehmer et al. 2004b), horses (about 28%) (Chiocchetti et al. 2009a), and sheep (about 33%) (Chiocchetti et al. 2006).

Knowing the proportion of the nitrergic subclass of ENS neurons in healthy subjects is crucial to compare any alteration (by percentage) of the same category of neurons during pathological conditions, such as DM.

Concerning the effects of DM in the canine nitrergic myenteric neurons, our findings indicate that, in the stomach of diabetic dogs, the percentage of nitrergic neurons was not significantly decreased (about 25%). On the contrary, in the ileum, this subclass of enteric inhibitory neurons was meaningfully reduced (about 19%). To the best of our knowledge, the present study represents the first contribution related to diabetic enteric neuropathy in dogs.

Path et al. (Phat et al. 1978) documented the first evidence of human ENS alteration during DM. In the last few years, many researchers investigated the alterations caused by spontaneous type I or streptozotocin-induced DM on the ENS using laboratory rodents (Fregonesi et al. 2001; Yoneda et al. 2001; Cellek et al. 2003; Alves et al. 2006; Izbeki et al. 2008) and showed a reduction in the number of ENS neurons in different tracts of the digestive system (Fregonesi et al. 2001; Furlan et al. 2002; Alves et al. 2006). Enteric neuronal subpopulations are known to respond differently to diabetes: some exhibit degeneration, some undergo changes in neurotransmitter content without degeneration, and some others are unaffected (Chandrasekharan and Srinivasan 2007).

In the early stages of animal (Takahashi et al. 1997; Cellek et al. 1999; Cellek et al. 2003; Takahashi 2003; Cellek et al. 2004; Demedts et al. 2013) and human DM (Miller et al. 2008; Chandrasekharan et al. 2011), only inhibitory nitrergic neurons seem to be involved. Furthermore, at least in human, DM type I seems to determine clinical problems mainly in the upper gastrointestinal tract, whereas the large intestine seems to be spared (Schvarcz et al. 1996).

The data presented here lead to the question of why the nitrergic subpopulations in the stomach and ileum were differently affected by the diabetic condition. Our neuroanatomical findings in the DM dog stomach are consistent with functional evidence from Takeda et al. (Takeda et al. 2001) and Onoma et al.(Onoma et al. 2008); these researchers observed that delayed gastric emptying in streptozotocin-induced diabetic dogs may require long DM duration (from 18–60 months). The average time of hyperglycemic state of the insulin-treated DM dogs considered in the present study (20 months; ranging from 1–48 months) was probably not long enough to develop gastric alteration. Another explanation might be related to the insulin treatment of the dogs considered in our study; in fact, as shown in rats, insulin replacement may restore nNOS expression in the stomach (Watkins et al. 2000).

Regarding the significant reduction of nitrergic neurons in the ileum of DM dogs, functional studies do not exist. Furthermore, in some ileal ganglia of the DM dogs, the pattern of HuC/HuD immunolabeling was abnormal, i.e. not homogenously distributed in the cytoplasm and nucleus, showing several degrees of alteration (weakness, fragmentation, nuclear internalization). It is important to remark that HuC/HuD cannot be considered only as a pure pan-neuronal marker; in fact, recent investigations reported nuclear internalization of the HuC/HuD protein in mouse MP neurons during experimental infectious, metabolic, and ischemic/reperfusion conditions (Lawson et al. 2010; Thacker et al. 2011; Rivera et al. 2014). Furthermore, a recent investigation confirmed that the subcellular localization of HuC/HuD-IR reflects the health status of the neurons, with oxygen deprivation being the most probable trigger of intracellular HuC/HuD modifications (Desmet et al. 2014). It is surely admissible that, in the presence of diabetes, many ganglia might undergo hypoxia.

The etiology of nerve damage in diabetes is complex and many possible mechanisms have been proposed that may be interrelated (Voukali et al. 2011). During diabetes, oxidative stress and advanced glycation end products (AGEs) also play an important role in the pathophysiology of vessel alteration and ENS changes (Chandrasekharan and Srinivasan 2007; Bagyanszki and Bodi 2012).

The involvement of the oxidative stress in the development of neuropathy in nitrergic enteric neurons during DM, is associated to absence of the antioxidant enzyme heme oxygenase-2 (HO-2); in fact, nNOS-IR neurons lacking HO-2 are more susceptible to the changes caused by diabetes, while nitrergic neurons containing HO-2, seem to be protected from neuropathy (Shotton and Lincoln 2006). Concerning the presence of HO-2 in the canine gastrointestinal tract, Ny and colleagues (Ny et al. 1997) showed that the percentage of nitrergic neurons co-expressing HO-2 in the pylorus and ileum was the same; this result does not contribute to our understanding of the regional differences observed in the DM dogs of the present research.

The loss of nNOS expression in the ENS seems to be mediated by AGEs, which interact with the receptor for AGE (RAGE) expressed by MP nitrergic neurons (Korenaga et al. 2006; Voukali et al. 2011). AGEs and RAGE seem to be also responsible for the vascular damages occurring in diabetes (Yamamoto et al. 2000). Furthermore, AGE accumulation in tissues seems to begin at the time point when nNOS depletion becomes irreversible. This action mechanism suggests that nitrergic apoptosis is triggered by the irreversible AGE increase in the serum and its

accumulation in tissues. It should be mentioned that at present, no data are available on the effects of AGEs in the canine digestive system or on the distribution of RAGE in canine enteric microvessels and neurons.

Another aspect that deserves attention is the observation (from a macroscopic point of view) that the diabetic dogs in our study showed mild to severe intestinal inflammation, which can derive from the diabetic vascular damages (Bodi et al. 2012) and consequently ENS alteration (or vice versa) (Brierley and Linden 2014; Uranga-Ocio et al. 2015).

In the present preliminary study, we considered only one (large) subpopulation of enteric neurons. It is plausible that other subclasses of neurons might be involved in DM (for instance cholinergic neurons) and other studies are needed to better understand the impact of hyperglycemia on the ENS.

Considering the findings of the present research and a lack of functional studies on the digestive system of spontaneous diabetic dogs, further functional investigations in DM dogs are necessary.The present research turns the spotlight on the effect of spontaneous diabetes on the gastrointestinal intramural innervations, indicating that DM can potentially affect the ileal motility in dogs.

Table 10.1 Clinico-pathological data of the control dogs included in the present research.

Control dogs	Breed	Gender	Age	Cause of death
CTRL1	German shepherd	M [a]	10 yr	Euthanasia due to progressive physical deterioration
CTRL2	German shepherd	M	9 yr, 6 mo	Heart cancer
CTRL3	Boxer	M [a]	8 yr	Cardiovascular disease
CTRL4	German shepherd	M	10 yr	Cardiovascular disease
CTRL5	Siberian Husky	M [a]	16 yr	Neurological (CNS) disorders
CTRL6	English Setter	F	2 yr	Road accident
CTRL7	Chihuahua	F	8 mo	Head trauma
CTRL8	West Highland White Terrier	M	17 yr	Intracranial neoplasia

Abbreviations: M, male; F, female; M [a] male neutered

Table 10.2 Clinico-pathological data of the diabetic dogs included in the present research.

Diabetic dogs	Breed	Gender	Age	Time of insulin treatment
DM 1	Cairn Terrier	F [a]	15 yr	2 yr, 5 mo
DM 2	Labrador retriever	F	12 yr, 7 mo	4 yr
DM 3	Mongrel	M	11 yr, 1 mo	6 mo
DM 4	German Shepherd	F [a]	13 yr, 5 mo	3 yr
DM 5	Bull Terrier miniature	F	4 mo	1 mo

Abbreviations: M, male; F, female; F [a] female spayed

Table 10.3 Primary and secondary antibodies used in the study.

Primary antibody	**Host**	**Code**	**Dilution**	**Source**
HuC/HuD	Mouse	A21271	IHC 1:400; WB 1:200	Life Technologies[a]
nNOS	Mouse	sc-5302	IHC 1:50; WB 1:200	Santa Cruz[b]
nNOS	Rabbit	AB5380	IHC 1:500; WB 1:1000	Merck Millipore[c]
Secondary antibody	**Host**	**Code**	**Dilution**	**Source**
Anti-mouse IgG	Goat	A11005	IHC 1:200	Life Technologies[a]
Anti-rabbit IgG	Goat	401314	IHC 1:200	Merck Millipore[c]
Anti-mouse IgG	Goat	A2304	WB 1:1000	Sigma Aldrich[d]
Anti-mouse IgG	Goat	A0545	WB 1:3000	Sigma Aldrich[d]

Abbreviations: IHC, Immunohistochemistry; HuC/HuD, human neuronal protein; nNOS, neuronal nitric oxide synthase; WB, Western Blot.

Suppliers: [a] Life Technologies, California, USA; [b] Santa Cruz Biotechnology, California, USA; [c]Merck Millipore, Merck KGaA, Germany, Europe; [d] Sigma Aldrich, Italy, Europe.

Figures

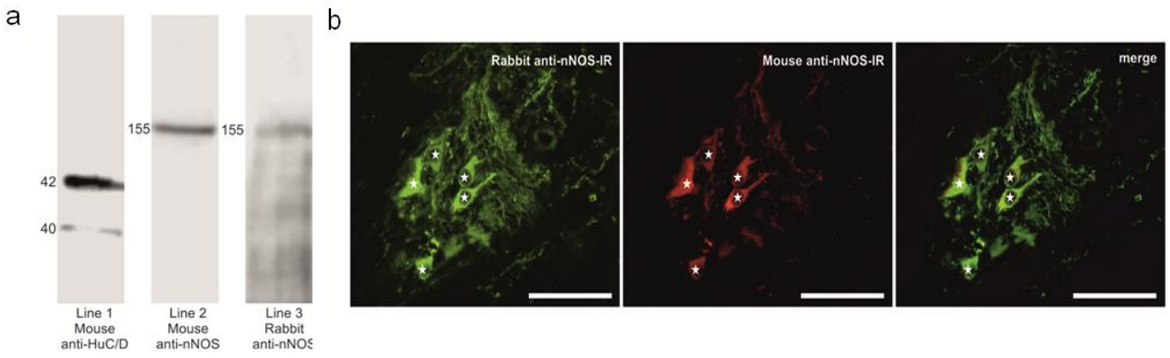

Fig. 10.1 a) Western blot (WB) analysis showing the specificity of the primary antibodies utilized (mouse anti-HuC/HuD, mouse anti nNOS, and rabbit anti-nNOS). Each antibody showed a major band close to the theoretical molecular weight. The number on the left of each line indicates the molecular weight. The images of the different immunoblots were slightly adjusted in brightness and contrast to match their backgrounds. b) Photomicrograph showing a tangential cryosection of the myenteric plexus of the ileum in a control dog with some myenteric neurons (*stars*) immunoreactive for both the anti-nNOS antibodies utilized in the study (rabbit anti-nNOS and mouse anti-nNOS). Scale bar: 50 µm.

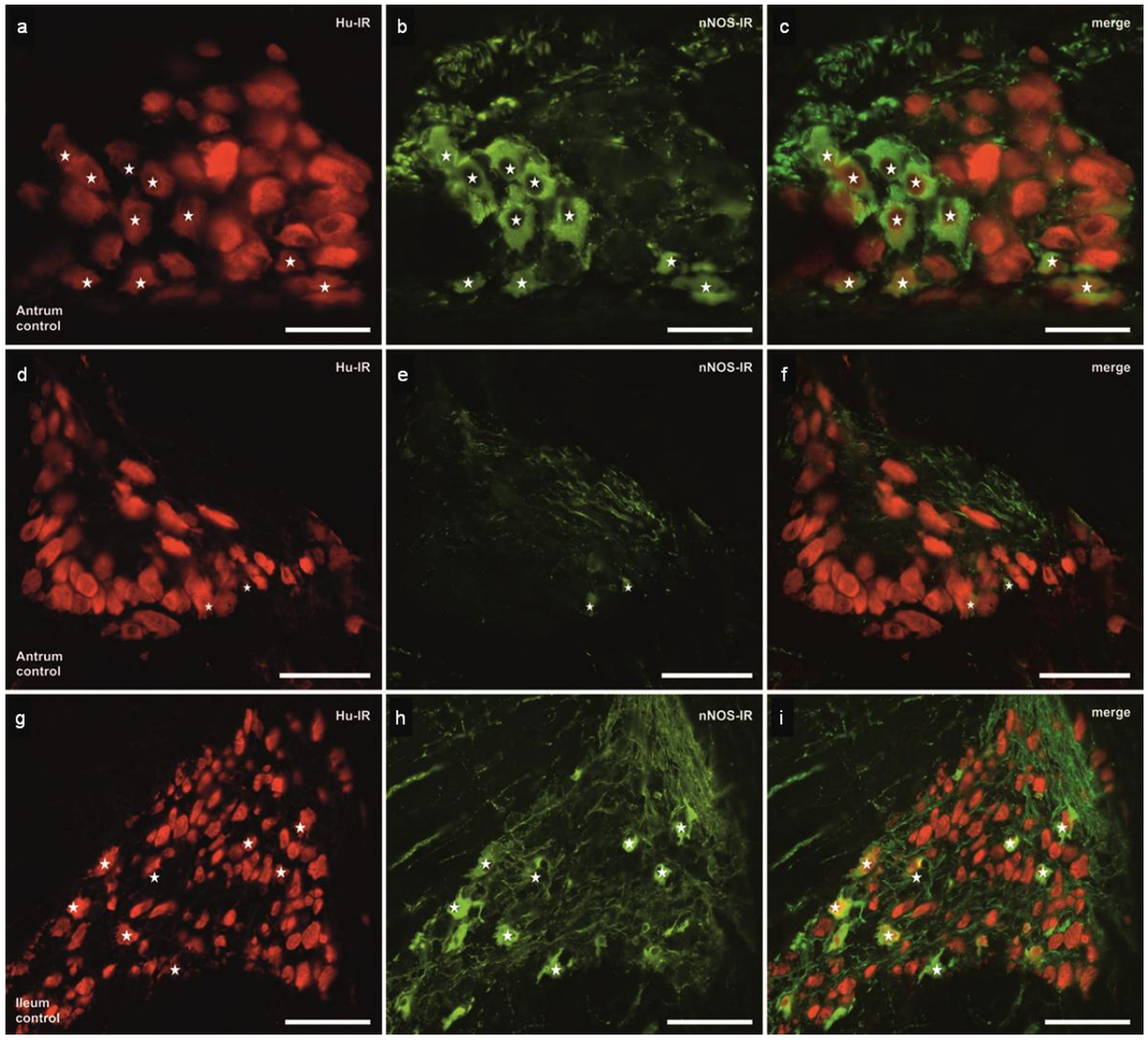

Fig. 10.2 Photomicrograph showing tangential cryosections of the myenteric plexus of the pyloric antrum (a-f) and ileum (g-i) of control dogs in which all the myenteric plexus neurons were immunolabeled with the antibody anti-HuC/HuD and the nitrergic neurons with the antibody anti-neuronal nitric oxide (nNOS). a-f) Of note, nitrergic neurons were not homogenously distributed in the gastric ganglia; in some ganglia nNOS-immunoreactive neurons (nNOS-IR) (*stars*) were well represented and grouped in clusters (a-c) while in others nNOS-IR neurons were poorly represented (d-f). g-i) In the ileum nitrergic neurons were diffusely distributed (g-i). Scale bar: a-c, 50 µm; d-i, 100 µm.

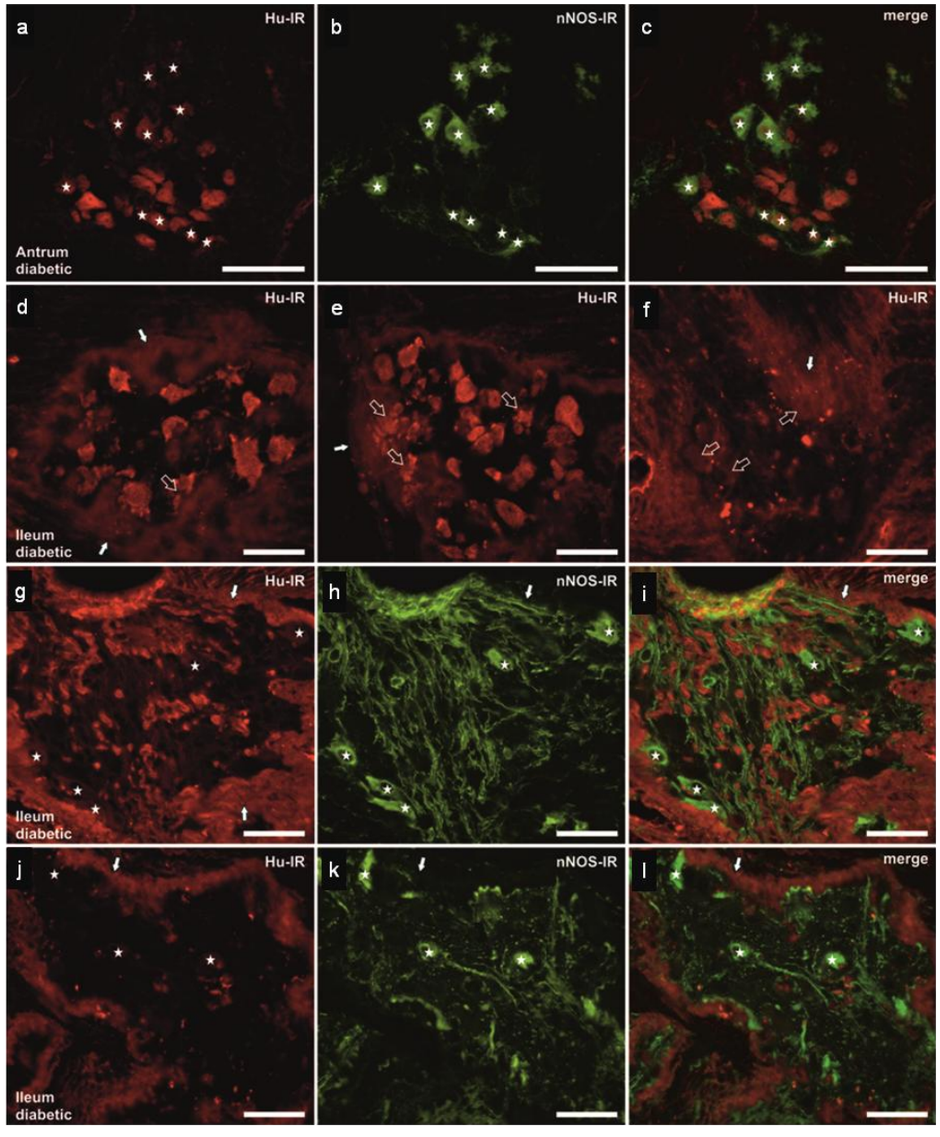

Fig. 10.3 Photomicrograph showing tangential cryosections of the myenteric plexus of the antrum (a-c) and ileum (d-l) of diabetic dogs. a-c) in all the antral plexuses, Hu- and nNOS-IR neurons were easily recognizable. d-l) in the ileum, an evident thickening of the connective tissue (*white arrows*) surrounding the ganglia is visible. Hu-IR was severely compromised in many ganglia in which neurons showed structural changes with different degrees of morphological disorganization and disintegration (*open arrows*; d, e), or were barely identifiable due to the very faint immunolabeling (*open arrow*, f). in some more deranged ganglia, HuC/HuD-labeling was confined in small granules (f). g-i) in some ganglia nNOS-IR preserved bright immunolabeling (h, stars), whereas HuC/HuD-neurons were no longer identifiable (g). j-l). in other more deranged ganglia, also nNOS-IR was altered in both cell somata (*stars*) and in fibers, which appeared often discontinued/interrupted.

Scale bar: a-c, 100 µm; d-l, 50 µm.

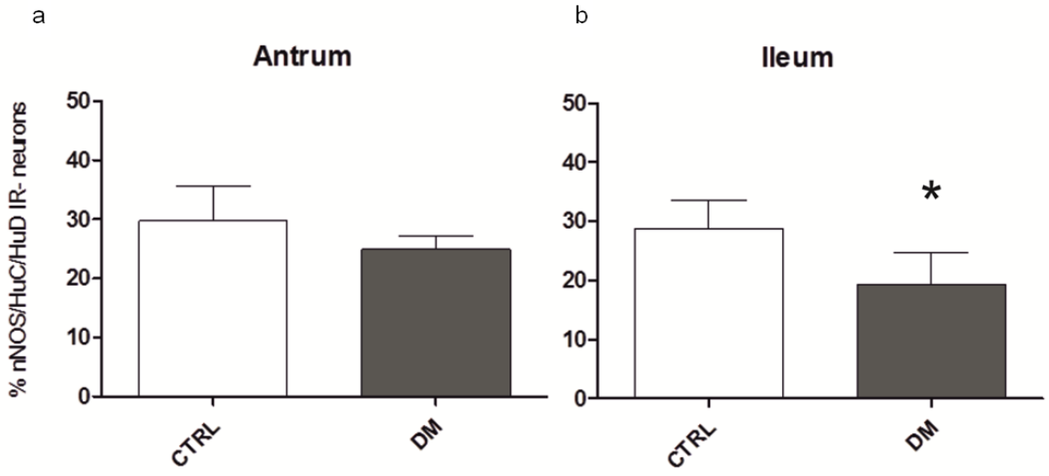

Fig. 10.4 Graphical representation of the percentages (mean ± St. Dev) of nNOS-IR neurons in the antrum (a) and ileum (b) of control (CTRL) and diabetic (DM) dogs. The statistical analysis carried out by the Student t test evaluated the differences between mean values of nNOS/HuC/HuD–IR neurons in DM *vs.* CTRL dogs. The level of significance was set at P<0.05. A) In the antrum, the averages were similar: CTRL= 30±6% *vs.* DM= 25±2% (*P*=0.112). B) In the ileum, the averages resulted significantly different: CTRL= 29±5% *vs.* DM= 19±5% (* *P*= 0.006).

CHAPTER 11

Functional and neurochemical abnormalities in patients with Parkinson's disease and chronic constipation

Introduction

Parkinson disease is a chronic neurodegenerative multi-system condition affecting about 1% of the elderly population, although 10% of all patients develop symptoms before 50 years of age (De Rijk et al. 1997). The two pathological hallmarks of PD are a progressive degeneration of the dopamine-containing neurons in the *substantia nigra* along with aggregates of eosinophilic inclusions (mainly phosphorylated alpha-synuclein), i.e. Lewy bodies (LBs) and neurites (LNs), in the remaining surviving neurons (Schulz-Schaeffer 2010). Although PD is regarded as a prototypical movement disorder, non-motor manifestations, such as autonomic dysfunctions, in particular those involving the GI tract, are increasingly recognized as being part of a wider clinical picture (Poewe 2008).

Virtually all parkinsonian patients experience GI dysfunctions showing dysphagia (up to 98% of cases), nausea and other severe dyspeptic symptoms attributable to gastroparesis, as well as chronic constipation (CC) (Pfeiffer 2003; Kaye et al. 2006). Specifically, CC is a dominant manifestation in up to 80% of PD patients (Fasano et al. 2015) and it occurs 2-4 times more commonly in PD compared to age-sex matched controls (Lin et al. 2014). Furthermore, the administration of L-Dopa, which is a fundamental and unavoidable therapy for PD patients, exerts a well-known inhibitory action on GI motility, worsening the severity of CC (Pagano et al. 2015), even though a delayed colonic transit has been reported in PD patients independently of drugs (Jost 2010). CC in PD is usually severe since it poorly responds to first-line treatment (e.g. osmotic laxatives) and at times evolves to severe complications such as megacolon and intestinal pseudo-obstruction (Wedel et al. 2002; Tateno et al. 2011; Knowles et al. 2013).

Previous data showed that the ENS, the third component of the autonomic nervous system, can be targeted by the pathological process of PD as confirmed by the presence of LBs in myenteric and submucosal neurons throughout the GI tract of patients with PD (Wakabayashi et al. 1988; Braak and Del Tredici 2009). In addition, it is well acquired that ENS changes can occur in patients with idiopathic CC as emerged by studies based on full thickness biopsies of patients undergoing colectomy for treatment-resistant, severe slow transit CC (Bassotti et al. 2006). Taken together these features provide a strong support to the regulatory role of the ENS since any abnormality affecting its integrity may

result in GI dysfunction and symptoms such as those detectable in patients with PD and CC. The recent evidence that mucosal biopsies from the colon can be used to retrieve submucosal tissue with its ganglionated plexus provided an exciting tool which can be easily exploitable to investigate enteric neurochemical and molecular changes occurring in patients with PD and GI dysfunction, including CC (Lebouvier et al. 2010).

Thus, the present study was conceived to investigate functional and neuronal features in a cohort of PD/CC patients and compared neurochemical findings of PD/CC to non-parkinsonian CC. Our neuronal analysis focused on submucosal specimens, i.e. submucosal plexuses, which play a critical role in controlling secretomotor mechanisms in the GI tract. Deciphering the mechanisms underlying bowel dysfunction in PD would ultimately allow for a better knowledge of the management and treatment of CC in these patients.

Material and methods

Patient recruitment - The study design included three groups of patients: n= 29 PD/CC; n= 10 patients with chronic constipation CC; n= 20 control subjects. PD/CC patients were enrolled at the Movement Disorder Center of the Neurology Unit of St. Orsola-Malpighi Hospital in Bologna, Italy. The diagnosis of PD was defined according to well established guidelines of the United Kingdom Parkinson's Disease Survey Brain society (Hughes et al. 1992). Patients with a significant cognitive impairment or a Mini Mental State Examination score < 19 were excluded. Data concerning duration of PD, Hoehn & Yahr (HY) stage, parkinsonian features evaluated by the Movement Disorders Society revised unified PD rating scale (MDS-UPDRS), along with daily medication dosage of L-Dopa, were collected for each PD patient. CC patients with or without PD were diagnosed according to Rome III criteria (Longstreth et al. 2006) at the Gastroenterology outpatient clinic of St. Orsola-Malpighi Hospital, Bologna, Italy. Each PD/CC and CC patient reported an average Bristol stool scale of 1-2 (Saad et al. 2010). Asymptomatic, otherwise healthy subjects undergoing screening colonoscopy for polyps served as control group.

Each patient / subject signed an informed consent form before entering the study. The study protocol was approved by Ethical Committee of St. Orsola-Malpighi Hospital, Bologna, Italy (N° 66/2011/U/Tess).

GI functional assessment - Both colonic transit time and ano-rectal manometry were performed in any CC patients with or without PD based on standardized methods. Colonic transit time was performed using radiopaque markers. Briefly, on morning of day 1, patients ingested 24 polyethylene markers (Sitzmark-Konsyl Pharmaceutical, Inc., Edison,

NJ) orally with water. On day 5, abdominal radiographs were obtained in the erect position. The patients were instructed to maintain their regular diet and avoid laxatives and suppositories for the duration of the test (Evans et al. 1992). The manometric study was performed with a four-channel water perfused catheter using a stationed pull-through technique (Mui Scientific, Mississauga, Ontario, Canada) connected to an electronic manometer and to a computer which generated the graphic register (Sandhill Scientific Inc., Littleton, Colorado, USA) with the patient in the left lateral decubitus position. The catheter is placed through the anal canal and advanced into the rectum. A complete manometric evaluation includes determination of the resting pressure, squeeze pressure, the ability of the IAS to relax with straining, the RAIR and the rectal sensation were performed according with S.S.C. Rao et al (Rao et al. 2002).

Tissue collection - During colonoscopy, n= 4 standard mucosal biopsies were taken from the descending colon in each PD/CC, CC and control patient / subject. One biopsy was immediately snap-frozen in liquid nitrogen and kept at -80°C until use. The remaining biopsies were place into a Sylgard-coated Petri dish with ice-cold Hank's balanced salt solution (H4641, Sigma-Aldrich, Italy, Europe) and were microdissected under a steromicroscope (Leica S6E, Leica mycrosistems, Italy, Europe). Whole mounts of the submucosal layer were isolated, pinned flat and fixed in 4% paraformaldehyde buffered solution for 3 hours at room temperature. After three washes in phosphate-buffered saline (PBS, pH 7.2) solution, submucosal whole mounts were processed for immunohistochemistry.

Immunohistochemistry and neuronal counts- Each submucosal specimen was incubate for 3h RT in solution containing 2% Triton X-100 and 20% Goat serum (Colorado Serum Co., Denver, CO, USA) in PBS. Tissues were incubate overnight +4°C in primary antibodies (Table 11.1). Whole mount tissues were then washed in PBS (3 × 10 min) and were then incubated for 2 h RT with a cocktail of secondary antibodies (**Table 11.1**). Finally, after three more washes in PBS, preparations were mounted with buffered glycerol (pH 8.6) and examined on a on a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes to distinguish the fluorophores employed. The images were recorded with a Nikon DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands).

The following primary antibodies were used: two general paneuronal markers, i.e. PGP9.5, recognizing perikarya and nerve fibers, and HuC/HuD, detecting only neuronal cell bodies (the latter also used for quantitative analysis). Neuronal counts were expressed as number of cell bodies / ganglion (mean ± SD). A total number of at least 20-30 neurons (about

5-6 ganglia / whole mount) was quantitated in each whole mount / patient or controls subjects. Cholinergic neurons were identified by using the anti-peripheral choline acetyl-transferase (pChAT) antibody (rabbit polyclonal; diluted 1:150, Justus-Liebig-University, Giessen, Germany) and VIP (rabbit polyclonal; diluted 1:2,500; CURE/DDRC, DDD, University of California Los Angeles, Los Angeles, California, USA;) as markers for secretomotor neurons. Double labeling immunohistochemistry included experiments with HuC/HuD and either pChAT or VIP. The percentage of HuC/HuD positive neurons colocalizing with either pChAT or VIP were counted and data expressed as mean ± St.Dev. For each colocalization in each patient, a total number of at least 20 neurons (about 5-6 ganglia / whole mount) was quantitated in each whole mount / patient or control subjects. Experiments aimed at testing antibody specificity were performed by omitting the primary antibody and co-incubating each primary antibody (namely for pChAT and VIP) with an excess of the homologous molecule. Specificity for pChAT and VIP was demonstrated by the lack of immunoreaction in sections in which the primary antiserum was omitted or in sections incubated with primary antibodies preabsorbed with the appropriate molecule (i.e. acetylcholine and VIP peptide).

Gene expression assay by RT-qPCR- For each patient, the frozen biopsy (10 mg of tissue) was thawed, mechanically disrupted with sterile forceps and homogenized using QIAshredder (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA Extraction was performed using RNeasy mini kit as indicated and eluted in a final volume of 30 µl. Extracted RNA was firstly purified from genomic DNA incubating for 30 min at 37°C in a thermocycler (Termal Cycler 2027, Applied Biosystem, USA) with a mixture containing the specific DNasi enzyme (Fermentas, Thermo Scientific, USA) according to the instructions. The product was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Italy, Europe) and the quality was checked by electrophoresis in 1% agarose. Each sample (200 ng of total DNA-free RNA in a 20 µl total reaction volume) was reverse transcribed to obtained cDNA using TaqMan® Reverse Transcription Reagents (Life Technologies, USA). RT conditions used were: 15 min 45°C, 3 min 95°C and 5 min 4°C. Resulted cDNA was store at -20°C. Relative gene expression analysis was performed on an Applied Biosystem 7500 Fast real time PCR system (Life Technologies, Milan, Italy) by Duplex TaqMan® Gene Expression Assays (Life Technologies, Milan, Italy). Amplification was performed in a 20 µl final volume including 2 µl of cDNA as template and TaqMan® Fast Advanced master mix (Applied Biosystem, USA). Each sample was tested in duplicate and each assay was performed in triplicate. Amplification conditions were: 2 min at 50°C, 20 sec 95°C

followed by 45 cycles (30 sec at 95°C, 30 sec at 60°C). The relative gene expression analysis evaluated the following genes: *VIP*, *VIP receptor 1* (*VIPR1) and VIP receptor 2* (*VIPR2).* Primer probes were from Applied Biosystem: VIC labelled for *18S* (Hs99999901_S1), FAM labelled for *VIP* (Hs00175021_m1), *VIP receptor 1 – VIPR1*- (Hs00910453_m1), *VIP receptor 2 – VIPR2*- (Hs00173643_m1). Data were calculated with $\Delta\Delta C_T$ method using *18S* as a reference gene.The mean value of CTRL group for each gene was the calibrator at unit value.

Statistical analysis - Statistical analysis was performed with the commercial software SPSS (for Windows, version 13.0; SPSS Inc, Chicago, Illinois) according to the appropriate tests for each considered variable. A Kolmogorov-Smirnov non-parametric test was applied to verify the normality of the distributions. Continuous data were reported as mean ± SD, and categorical data were described as frequencies. One-way analysis of variance, Fisher exact test, and χ^2 test were applied. Correlation analyses were performed by Pearson χ^2 and Spearman's rank test. Two- tailed P values less than 0.05 were considered significant. Graphical representations of data were obtained using a commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software Inc., La Jolla, CA, USA).

Results

Functional constipation assessment in PC/CC and CC

Among PD/CC patients, n= 17 (58.6%) completed the study; n= 7 (24.1%) refused colonoscopy, n= 1 (3.5%) refused both TT and AM assessment, n= 3 (10.3 %) refused TT and n= 1 (3.5%) refused AM. Regarding CC patients, n= 9 (90%) underwent TT and AM, while n= 1 performed only AM.

Evaluation of colonic TT and AM was performed n= 24 and n= 28 of PD/CC patients and the main features of those functional tests were summarized in **Table 11.2**. In the investigated PD/CC cohort, about 75% (n= 18 / 24) showed a delay colonic TT, while 69% (n= 22 / 28) had AM abnormalities, i.e. a single or, more often, combined motor abnormalities. We found 1 / 28 patient with an increased basal pressure of the anal sphincter, 4 / 28 with a decreased basal pressure and a normal pressure in 23 / 28. Ultra-slow waves were present in 1 / 28 patient and the squeezing attempts, showing inability to contract the anus, in 7 / 28 patients (2 cases lacking and 5 with short duration anal contractions). In 8 / 28 the straining attempts revealed anismus, i.e. lack of anal inhibition and paradoxical sphincter contraction. The rectal sensory dysfunction was also observed in PD/CC patients characterized by a reduced ampullary

threshold (7 cases), although not associated with hyposensibility. The recto-anal inhibitory reflex was detected in all patients. Both colonic TT and AM were performed in the 23 / 29 PD/CC and the resultant features allowed for the identification of four subgroups of patients: 1) delayed TT and altered AM (65%); 2) delayed TT (13%); 3) altered AM pattern (13%); 4) no functional impairment (9%) (**Fig. 11.1a**). Regarding CC patients, colonic TT and AM identified two groups of patients: 1) delayed TT and altered AM (33%) and 2) delayed TT (67%) (**Fig. 11.1b**).

Concerning TT assessment, there were no differences in the mean number of pellets found in PD/CC *vs*. CC patients (12.4±7.78 vs. 11.9± 4.65; *P*=.28) (**Fig. 11.1 c**).

Submucosal neuronal count

In order to define possible differences existing in the number of neurons among PD/CC, CC and controls, submucosal whole mount preparations were analyzed by labeling neuronal cell bodies with HuC/HuD. Virtually all perikarya were labeled by HuC/HuD and quantitative assessment revealed no significant differences in terms of the mean number of HuC/HuD immunoreactive ganglion cell bodies in the three groups (4.4±0.86 *vs*. 4.0±1.23 *vs.* 4.0±1.35 in PD/CC, CC and controls, respectively, *P*= 0.357) (**Fig. 11.2 a-d**).

Submucosal cholinergic and VIP containing neurons- Both cholinergic and VIP containing neurons are widely represented in the human colonic submucosal plexus (Anlauf et al. 2003). pChAT and VIP-IRs were readily detectable in the cell bodies as well as nerve processes running off the identified ganglia. The proportion of pChAT-IR neurons was calculated for each patient and control on the total number of HuC/HuD-IR ganglion cell bodies. There was no changes in the number of HuC/HuD /pChAT-IR neurons in the three groups (87.3±8.67% *vs.* 85.9±10.97% *vs.* 89.3±8.71% in PD/CC, CC and controls, respectively, *P*= 0.770) (**Fig. 11.2 e-h**). In contrast, the percentage of HuC/HuD /VIP-IR neurons was significantly reduced in PD/CC (72.3±14.59%) *vs.* controls (87.2±9.24%) (*P*= 0.007), while no differences were observed between PD/CC *vs.* CC (78.3±16.44%; *P*= 0.292) and between CC *vs.* controls (*P*= 0.321) (**Fig. 11.2 i-l**).

VIP pathway gene expression

Based on the quantitative data, showing a reduced number of VIP containing neurons unrelated to changes of the total number of HuC/HuD and cholinergic neurons, we tested whether *VIP* and its receptors, *VIPR1* and *VIPR2*, gene expression was altered in PD/CC, CC and controls (**Fig. 11.3 a-c**). Figure 11.3 a demonstrated a significant reduction of

VIP mRNA expression in PD/CC *vs.* controls (0.012 ± 0.045 *vs.* 1.059 ± 0.177; *P*< 0.0001). A significant reduction of VIP was also detected in PD/CC *vs.* CC (0.012 ± 0.045 ± *vs.* 0.189±0.39; *P*= 0.036) and in CC *vs.* controls (1.059 ± 0.177 *vs* 0.189±0.39; *P*= 0.001). Compared to controls, *VIPR1* expression (**Fig. 11.3b**) was significantly reduced in both groups of constipated patients, PD/CC *vs.* controls (0.00008±0,000085 *vs.* 1.04268±0.226402; *P*< 0.0001) and CC *vs*. controls (0.00315±0.005571 *vs.* 1.04268 ±0.226402; *P*< 0.0001). Notably, PD/CC *VIPR1* mRNA expression was significantly lower than that detected in CC (0.00008±0.000085 *vs.* 0.00315±0.005571; *P*< 0.0001). Similar results were obtained for *VIPR2* expression (**Fig. 11.3c**). Our data showed reduced *VIPR2 mRNA* expression levels in PD/CC *vs.* controls (0.00218±0.006309 *vs.* 0.93140±0.379026; *P*< 0.0001) and CC *vs*. Controls (0.05102±0.126542 *vs.* 0.93140±0.379026; *P*< 0.0001). Finally, a significant reduction was also evident in PD/CC *vs.* CC (0.00218±0.006309 *vs.* 0.05102±0.126542; *P*= 0.001).

Considering functional GI assessment, PD/CC and CC patients with slow transit showed no significant changes in *VIP* mRNA levels compared to patients with normal transit (*P*=0. 07); however they showed a significant decrease of *VIPR1*and *VIPR2* mRNA expression (*P*=0. 014 and *P*= 0.002 respectively) (**Fig. 11.4 a-c**).

Clinico-pathological correlations

The association between age, sex, clinical and experimental data was evaluated. No correlations were detected among age, sex, neuronal counts and gene expression, neither with functional gastrointestinal parameters (Data not showed).

In the PD/CC group, age resulted positively associated with the neurological and motor scale score UPDRSIII (*P*=0.023; **Fig. 11.5 a**). The number of HuC/HuD/VIP-IR neurons resulted positively correlated with UPDRSIII (*P*=0.018) , i.e. the lower the number of VIP containing neurons, the worst the score of PD was (Fig. 11.5 b). The severity of CC in PD (Rome III criteria) was significantly correlated to UPDRSIII (*P*= 0.023), i.e. more severe was the CC in PD/CC patients, the worst the score of PD was (**Fig. 11.5 c**). The number of intracolonic residual pellets (TT) was positivelycorrelated with the duration of PD (years) (*P*= 0.028), i.e. longer was the duration of the disease, highest was number of retained pellet in the colon (**Fig. 11.5 d**).

Discussion

The present study was developed to investigate the pathophysiological, neurochemical and molecular features of the severe constipation in parkinsonian patients. Thus, we performed a thorough characterization of PD/CC patients and

compared this group with non-parkinsonian CC patients and control subjects. Most (91%) of PD/CC patients enrolled in our study showed abnormalities to at least one of the two considered parameters, i.e. TT or AM, while 65% had a delayed TT combined with ano-rectal sensory-motor impairment (i.e., dyssynergic defecation). Taken together our data indicate a severe functional motor impairment in the colon and rectum of PD/CC patients. In contrast, in the CC group, only 33% of patients showed slow TT and altered AM pattern, while most cases displayed slow transit, suggesting the referral origin of CC, including those cases usually not easily managed in primary or secondary care levels. Thus, it appears that the two groups, PD/CC and CC, were comparable in terms of severity of constipation assessed by objective measurements. Our functional data on PD/CC confirmed and expanded previous studies evaluating constipated parkinsonian patients (Edwards et al. 1994; Sakakibara et al. 2003). In fact, Sakakibara et al. demonstrated that slow transit constipation was prevalent in PD/CC patients, while ano-rectal abnormalities were identifiable only in a small proportion of patients (Sakakibara et al. 2003). In contrast, Edwards et al. found ano-rectal alterations / dyssynergic defecation in 77% of the PD/CC cases and slow transit only in 31%. In that study, the acute administration of apomorphine has been shown to improve dyssynergic defecation symptoms in a subset of patients with PD/CC, thus implying that a dopaminergic dysfunction is also responsible for dyssynergia (Edwards et al. 1994). The differences between our findings and those reported by Sakakibara et al. and Edwards et al. may be explained with a number of reasons, including the heterogeneity of pathogenetic factors contributing to CC in PD (e.g., delayed transit vs. sensory-motor abnormalities), the severity of PD per se (known to impact of CC) and sample size, i.e. the number of subject enrolled in the two mentioned studies (i.e. approximately 50% less patients than current study). In light of the data emerged by our study and integrating previous experience one can conclude that both a delayed TT and a dyssynergic pattern contribute to CC in PD patients. The origin of these abnormalities is quite complex and to date two mechanisms seems to be plausible: *i*) ENS changes mainly in the gut myenteric plexus and associated to Lewy bodies and neurites with or without neuronal loss (Wedel et al. 2002; Annerino et al. 2012); and *ii*) an altered extrinsic nerve input to the lower gut thereby affecting the colonic and ano-rectal sensory-motor function (Furness 2012). An accurate histopathological evaluation of the myenteric plexus was clearly impracticable in our study since we cannot obtain full-thickness biopsies from the enrolled patients. Therefore we cannot conclude whether myenteric neuron abnormalities and, most likely, associated Lewy pathology, occurred in the investigated PD/CC patients. In the human colonic

submucosa, using specific antibodies to phosphorylated synuclein, Lebouvier et al. showed Lewy neurites (not bodies) in about 72% of PD/CC cases. The number of Lewy neurites positively correlated with the severity of Rome III defined PD/CC patients, although such correlation was lost when data were adjusted by age (Lebouvier et al. 2010). The neuropathological impact of Lewy neurites on altered gut physiology (transt time and sensory-motor function) remain plausible, but not yet proven. Thus, Lewy pathology may be a marker of PD involvement in the ENS, although its actual role in terms of neutrally-driven gut dysfunction remains to be assessed. Finally, it should be stressed that the identification of Lewy pathology does not represent a reliable bio-marker of PD as many evidence points to its demonstration in the nervous system (either central or peripheral) in patients with a variety of neurological disorders (Pouclet et al. 2012), ageing people and even in healthy (Visanji et al. 2015).

Concerning the assessment of the ENS, our study applied the newly established technique aimed at separating the submucosa (and related plexuses) from the mucosa in routine biopsies obtained during colonoscopy (Lebouvier et al. 2010). Our focus was directed to the submucosal plexus which is composed primarily of two major neuronal subsets, i.e. secretomotor / vasomotor neurons (Furness 2003; Banks et al. 2005). In order to provide a neurochemical analysis of the secretory neuron component, we performed immunohistochemistry on two major transmitters, namely acetylcholine (identified via pChAT immunoreactivity) and VIP. Both are well known to participate significantly to the control of enteric secretory mechanisms in the submucosal plexus of several mammalian species, including humans (Banks et al. 2005; Furness JB. 2014). Upon release from submucosal neurons, VIP activates the specific constitutive receptor VIPR1 expressed by the enteric epithelial lining. This results in a cAMP-related $HCO3^-$ excretion and entrance of Na^+ and H_2O in the gut lumen, thereby enhancing fluid secretion (Chandrasekharan et al. 2013). As widely reported, both acetylcholine and VIP are largely costored in submucosal plexus, with VIP containing neurons being a subset of the broader family of cholinergic neurons (Anlauf et al. 2003). In this respect, we showed for the first time that VIP containing neurons in PD/CC patients were significantly less than those of CC and controls, although the total number of neurons and cholinergic neurons did not change in the three groups. In support of a selective decrease of VIP in submucosal neurons of PD/CC patients, we showed a more pronounced downregulation of the VIP mRNA and related receptors, i.e. VIPR1 and VIPR2, than in CC and controls. The mechanisms underlying the reduced mRNA expression of VIP, VIPR1 and VIPR2, the inducible receptor, remain unknown. Probably, inflammatory changes, which are known

to occur in colonic mucosal biopsies of PD patients (Devos et al. 2013), can be thought to affect VIPR2 expression. However, since our data showed that VIPR1 was also downregulated it is conceivable that other mechanisms may alter the whole pathway through which VIP exerts its biological effects in PD/CC patients. Also, the altered VIP and related receptors did not appear to be a unique feature underlying PD/CC only; in fact, although to a lower extent, also CC patients had a lower expression of VIP, VIPR1 and VIPR2 compared to controls. Taken together, our data suggest that an altered VIP and related pathway can contribute to the pathophysiology of CC in parkinsonian patients as well as in CC by impairing secretion in addition to colonic and ano-rectal sensory-motor dysfunction. Beyond motility abnormalities, altered secretion conditioning the composition of the fecal content is correlated to a delayed colonic TT (Saad et al. 2010).

Finally, given the key role exerted by VIP and related VIPRs, it is tentative to speculate that an abnormal expression of this neurotransmitters (and/or of their receptors), can affect other mechanisms, i.e. the intestinal epithelial barrier and immunomodulation, thereby contributing to the constipation in PD and even in non-parkinsonian patients by perturbing the neuroenteric environment. Clearly further research is eagerly awaited to prove this pathogenetic link in PD/CC patients.

Table 11.1. Details of the primary and secondary antibodies.

Primary antibody	**Host**	**Code**	**Dilution**	**Source**
PGP9.5	Rabbit	AB1761	1:500	Merck Millipore
HuC/HuD	Mouse	A21271	1:50	Life Technologies
VIP	Rabbit	#7913	1:2500	Generous gift of Prof. C. Sternini[a]
pChAT	Rabbit		1:150	Generous gift of Dr. K. Lips[b]
Secondary antibody	**Host**	**Code**	**Dilution**	**Source**
Anti-mouse IgG Alexa 594	Goat	A11005	1:200	Life Technologies
Anti-rabbit IgG FITC	Goat	401314	1:200	Merck Millipore

Abbreviations: HuC/HuD, Human neuronal protein; PGP9.5, Protein Gene Product 9.5; VIP, Vasoactive Intestinal Polypeptide; pChAT, peripheral Choline Acetyl-Transferase

Suppliers: Life Technologies, Carlsbad, California, USA; Merck Millipore, Merck KGaA, Darmstadt, Germany, Europe; a CURE/DDRC, DDD, University of California Los Angeles, Los Angeles, California, USA; b Justus-Liebig-University, Giessen, Germany.

Table 11.2 Clinical and functional gastrointestinal features of PD/CC patients.

PD/CC	TT	AM	RP	SQ	SP	S
P1	0	Normal	Altered	Normal	Normal	Normal
P2	-	Normal	Decreased	Normal	Normal	Ipersensitive
P3	15	-	-	-	-	-
P4	0	Altered	Altered	Normal	Normal	Normal
P5	-	Altered	Altered	Normal	Paradoxical increase	Normal
P6	0	Normal	Altered	Normal	Normal	Normal
P7	14	Altered	Altered	Normal	Paradoxical increase	Ipersensitive
P8	20	Altered	Altered	Normal	Paradoxical increase	Normal
P9	19	Altered	Altered	Normal	Normal	Normal
P10	15	Altered	Altered	Normal	Normal	Normal
P11	0	Altered	Decreased	Normal	Normal	Normal
P12	17	Normal	Altered	Normal	Normal	Normal
P13	19	Altered	Altered	Normal	Paradoxical increase	Normal
P14	20	Altered	Altered	Normal	Normal	Normal
P15	0	Altered	Altered	Normal	Normal	Ipersensitive
P16	17	Altered	Altered	Normal	Paradoxicalincrease	Normal
P17	17	Altered	Altered	Normal	Normal	Ipersensitive
P18	18	Altered	Altered	Normal	Normal	Normal
P19	-	Altered	Altered	Normal	Paradoxical increase	Ipersensitive
P20	8	Altered	Increased	Normal	Normal	Normal
P21	-	-	-	-	-	-
P22	20	Altered	Altered	Normal	Normal	Ipersensitive
P23	14	Normal	Decreased	Incapable	Normal	Normal
P24	15	Altered	Altered	Normal	Normal	Normal
P25	0	Altered	Increased	Normal	Normal	Normal
P26	15	Altered	Altered	Normal	Normal	Ipersensitive
P27	20	Altered	Altered	Normal	Paradoxical increase	Normal
P28	15	Altered	Altered	Normal	Normal	Normal
P29	-	Altered	Decreased	Normal	Paradoxical increase	Normal

Abbreviations: AM, Anorectal manomety diagnosis; RP, resting pressure; S, sensitivity; SQ, squeezing pressure; SP, strain pattern; TT, total colonic transit time (number of pellets).

Figures

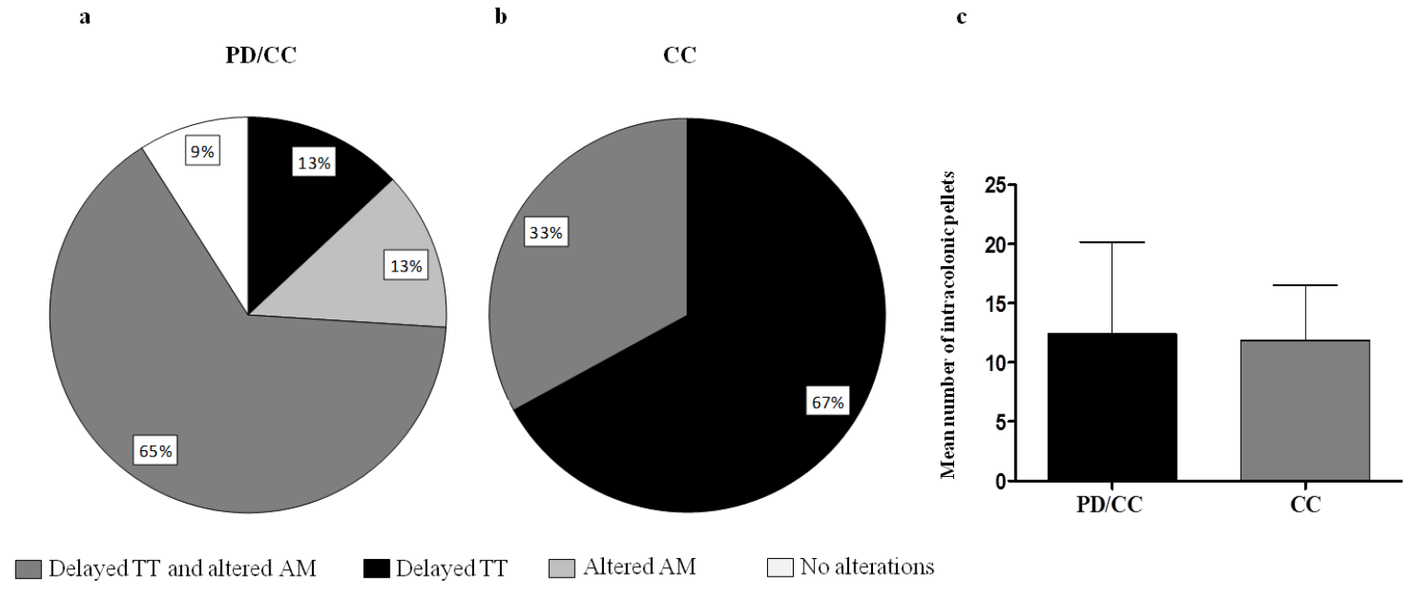

Fig. 11.1 Functional assessment of total colonic transit time (TT) and anorectal manometry (AM) in PD/CC and CC patients. a) Percentages of PD/CC (n= 23) showing delayed TT and altered AM (65%) (dark gray), delayed TT (13%) (black) ; altered AM (13%) (light gray); no alterations (9%) (white). b) Percentages of CC (n= 9) patients showing delayed TT and altered AM (33%) (dark gray), delayed TT (67%) (black); no patients showed AM alteration and no functional impairment (%). c) No differences were present in the mean number of residual intracolonic pellets in PD/CC (black) *vs.* CC (dark gray) patients.

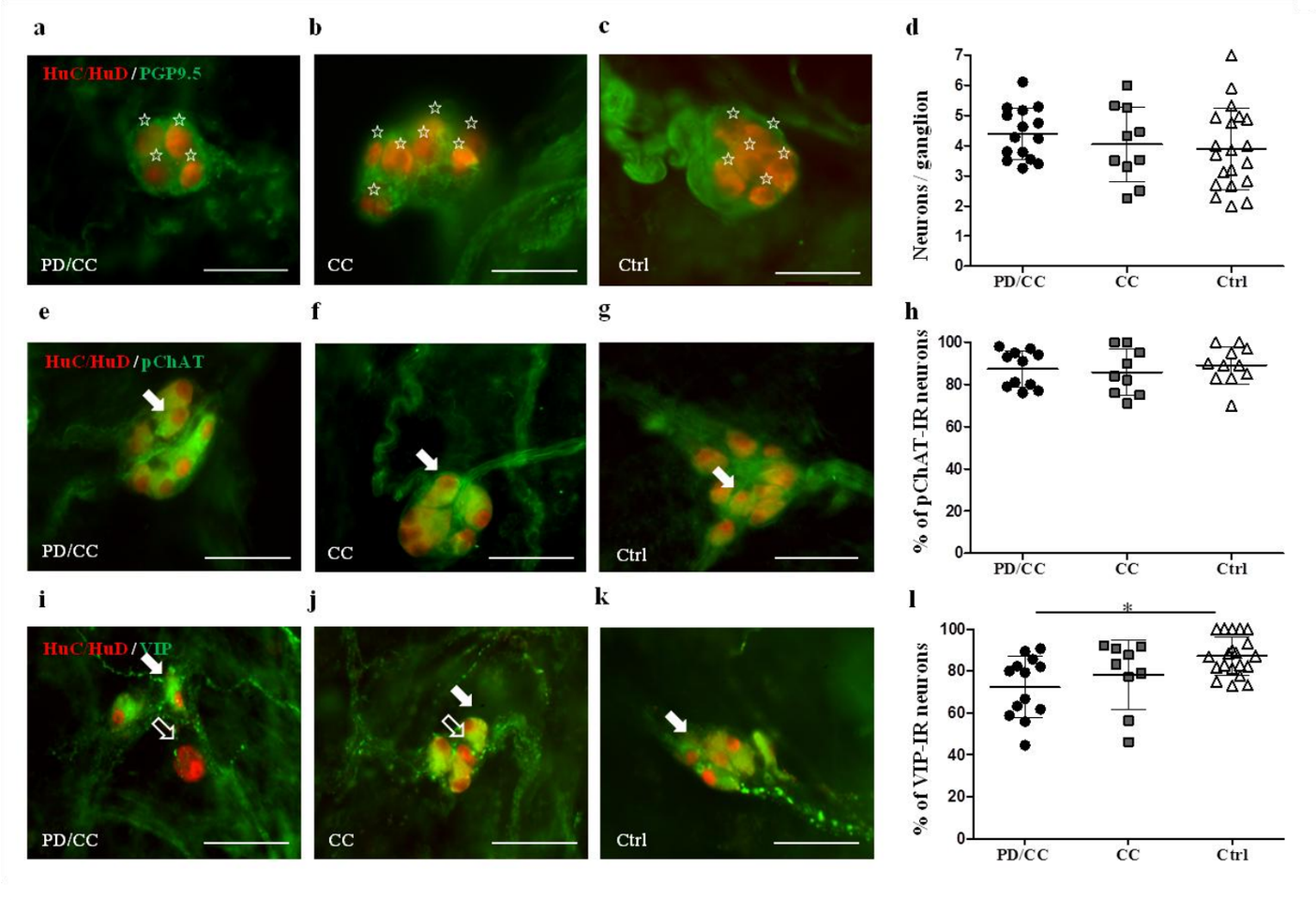

Fig. 11.2. Submucosal neuronal count performed by immunohistochmistry in PD/CC, CC and Ctrl groups. a-c) Photomicrographs showing the double-labeling of HuC/HuD (red) and PGP9.5 (green) to identify neuronal cell bodies and ganglionic structures. Stars are used to indicate a single neuron. Scale bar: 50 µm. d) Box plot representing the number of neurons / ganglion. Data are represented as mean ± SD. e-g) photomicrographs showing the double-labeling of HuC/D (red) and pChAT (green) to identify neuronal cell bodies and colinergic neurons. White filled arrows are used to indicate examples of pChAT immunoreactive (-IR) neurons. Scale bar: 50 µm. h) Box plot representing the percentages of HuC/HuD /pChAT-IR neurons. Data are represented as mean ± SD. i-k) Photomicrographs showing the double-labeling of HuC/HuD (red) and VIP (green) to identify neuronal cell bodies and VIP-containing neurons. White filled arrows are used to indicate examples of VIP immunoreactive (-IR) neurons. White empty arrows are used to indicate an example of VIP not containing neurons. Scale bar: 50 µm. l) Box plot representing the percentages of HuC/HuD /VIP immunoreactive (-IR) neurons. Data are represented as mean ± SD; * $P < 0.05$.

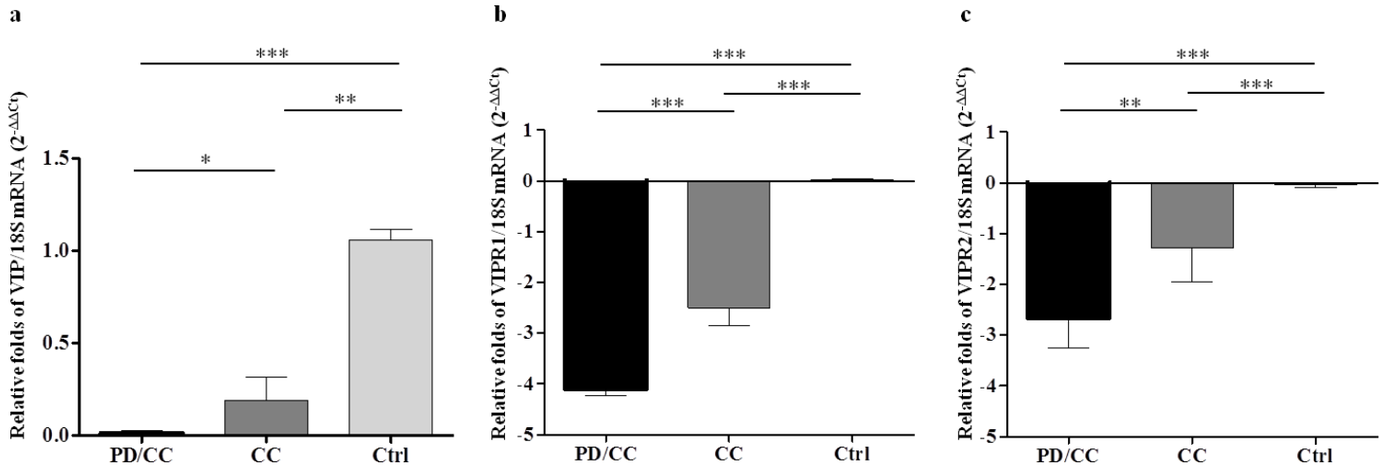

Fig. 11.3 *VIP pathway* gene expression in PD/CC, CC and Ctrl groups. a) VIP mRNA levels. Data are expressed as relative folds compared to the mean value of the Ctrl group (mean ± SEM; ***P< 0.0001; **P<0 .001; * P< 0.05). b) VIPR1 mRNA levels. Data are expressed in logarithmic scale as relative folds compared to the mean value of the Ctrl group (mean ± SEM; ***P< 0.0001). c) VIPR2 mRNA levels. Data are expressed in logarithmic scale as relative folds compared to the mean value of the Ctrl group (mean ± SEM; **P< 0.001; ***P< 0.0001).

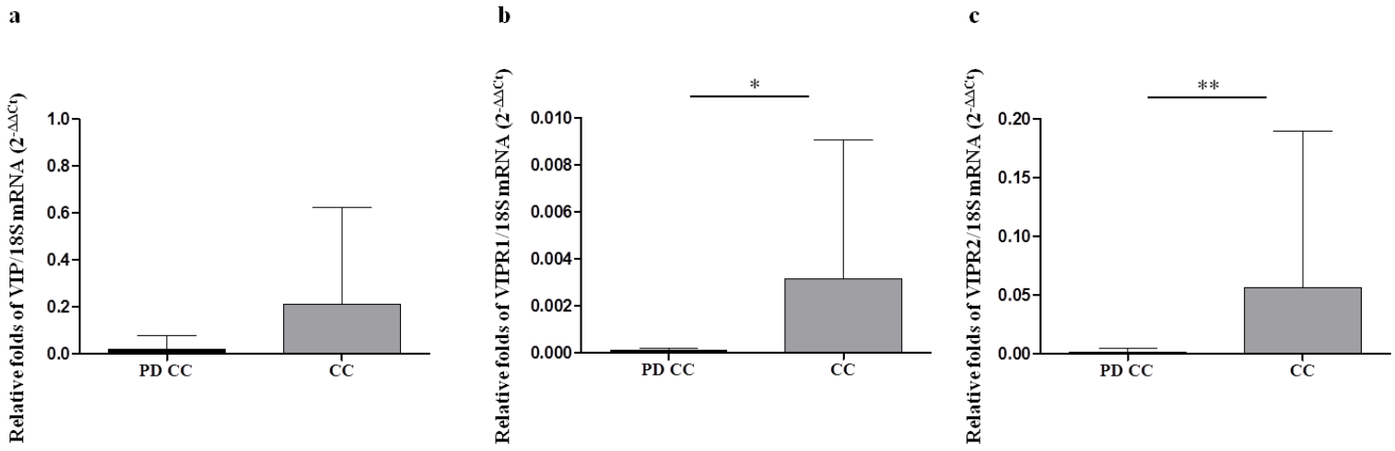

Fig. 11.4 *VIP pathway* gene expression in patients showing delayed transit time of PD/CC *vs.* CC groups. a) VIP mRNA levels showed no differences in PD/CC *vs.* CC patients (mean ± SEM; ns). b) VIPR1 mRNA levels resulted significant decreased in PD/CC *vs.* CC patients (* P<0 .05). c) VIPR2 mRNA levels resulted significant decreased in PDCC *vs.* CC patients (**P< 0.001;). Data are expressed as relative folds compared to the mean value of the Ctrl group.

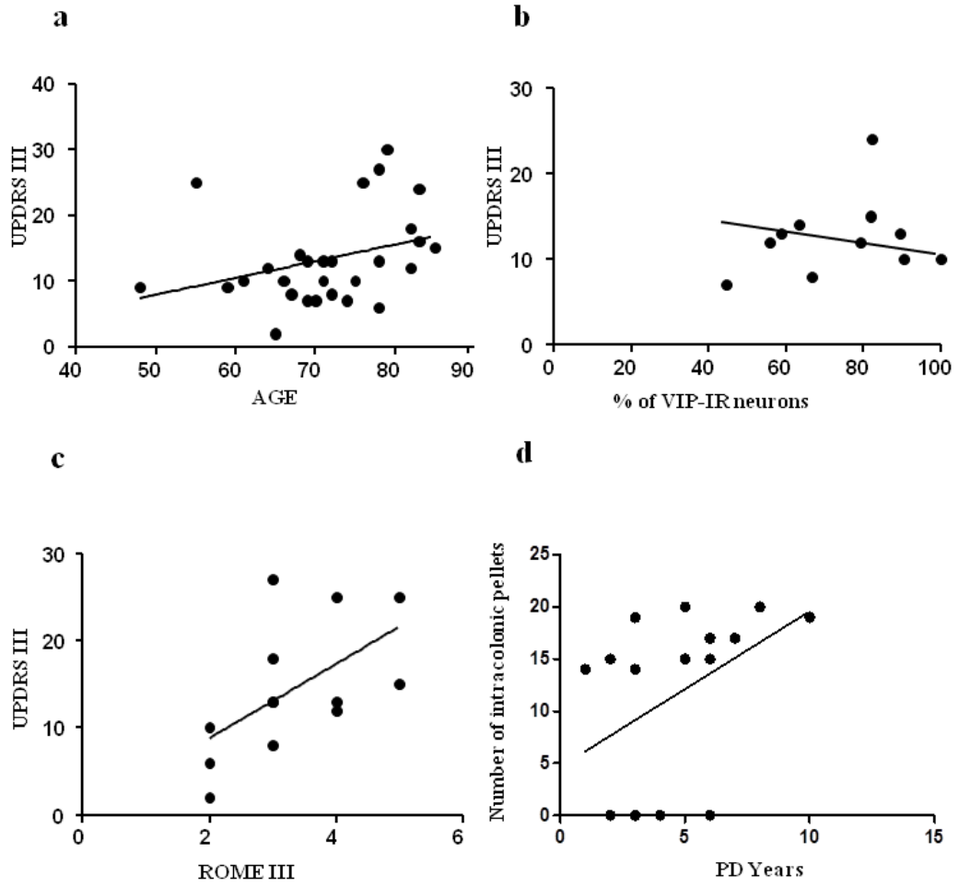

Fig. 11.5 Clinical correlations in PD/CC group. a) Age resulted positively correlated with the severity of motor impairment (UPDRSIII scale) *(P*=.023). b) Percentages of VIP containing neurons resulted negatively correlated with the UPDRSIII scale (*P*=0.018). c) Rome III severity scale resulted positively correlated with the UPDRSIII scale (*P*= 0.023). d) The number of intracolonic residual pellets (TT) was positively with the duration of PD (years) (*P*= 0.028).

CONCLUSIONS

The ENS of different species, such as rodents, guinea-pig and other easy-handling small mammals, have been widely studied as experimental animal to decipher the complexity of the ENS. However, numerous papers dealing with small and large mammals have also been published over the last ten years. The characterization of the anatomical aspects of the ENS in large mammals and the identification of differences and similarities existing between species may represent a fundamental basis to decipher several digestive GI diseases in humans and animals. In this perspective, the present thesis is a collection of studies in which three different species (horses, dogs and humans) were examined.

Two studies were focused to investigate anatomical aspects in the horse ENS.

In the first study, the inhibitory and excitatory enteric neurons of the caudal tract of the esophagus and the proximal portion of the gastric *fundus* were immunohistochemically characterized. Although the equine esophagus and gastric *fundus* lack the fluid fluxes across the mucosal epithelium that occur in the small and large intestines, we observed a continuous and double layered SMP. We also observed a largely greater percentage (about 72%) of nNOS-IR MP neurons than in the other tracts of the equine digestive system. Those findings reinforces both the role of the ENS in the lower esophageal sphincter relaxation and the notion that the ENS is no less important than the vagal circuitries. Taken together, those findings represent an anatomical basis to allow an evaluation of whether selective groups of enteric neurons are involved in horse neurological disorders such as megaesophagus, white lethal foal syndrome, and equine dysautonomia.

The second study was designed to investigate the loacalization of the 5-hydroxytryptamine receptor 4 (5-HT4R) in the equine enteric nervous system and spinal ganglia of healthy horses, and in extrinsic nervous fibers of LWFS foals (lacking intrinsic innervation). 5-HT_4R was localized to large percentages of enteric neurons and extrinsic sensory nervous fibers. These findings represent the first morphological support for the functional investigations carried out on 5-HT_4R in the horse intestine. In fact, despite several functional studies indicate a prokinetic effect of 5-HT_4R agonists,

the presence of the receptor in the equine gastrointestinal innervation was not yet demonstrated. Furthermore, no data were available in the horse on the presence of 5-HT_4R in extrinsic visceral innervation. Taken together, these findings reinforce the role of the 5-HT_4R in controlling the equine intestinal motility through a direct effect on enteric neurons and opens a new window on the pharmacological treatment of visceral nociception in this species.

Concerning enteric dysfunctions, three studies were designed to investigate one primary and two secondary neuropathies in horses, dogs and humans.

Equine ileocolonic aganglionosis - or lethal white foal syndrome - (the equine version of the human Hirschsprung's disease) is a primary neuropathy characterized by neural crest progenitors' unsuccessful colonization of the caudal part of the small intestine and of the entire large intestine. As consequence, LWFS foals represent a unique model to analyzed the distribution of extrinsic sympathetic and sensory neural fibers in absence of ENS development. Affected foals showed large bundles of extrinsic fibers, compared to the control, as observed in Hirschsprung's disease. Furthermore, altered adrenergic pathways were observed, prominently in the pelvic flexure. Since the ENS contains peptidergic neurons, in LWFS tissues the SP- and CGRP-immunoreactivities were dramatically reduced in either ileum and pelvic flexure; the remaining sensory extrinsic fibers resulted largely distributed around submucosal blood vessels and were in part dedicated to the innervation of the mucosa and serosa. These findings highlight that the extrinsic innervation, contributing to modulate the enteric functions, might also be affected during LWFS.

Diabetes mellitus represents one of the most common secondary enteric neuropathy. In fact, as demonstrated in humans and in rodents, DM can determine severe GI symptoms, associated to enteric neuronal degeneration, in particular involving the inhibitory nitrergic subclass of motor neurons. No date were available on enteric neuropathy in spontaneous diabetic dogs, also showing gastrointestinal complication. While in the antrum of DM dogs, nitrergic neurons resulted not damaged, in the ileum, the percentage of myenteric nitrergic resulted significantly reduced compared to a control group and structural alterations were also evident. These findings turn the spotlight on the effect of spontaneous diabetes on the gastrointestinal intramural innervation, indicating that DM can potentially affect the ileal motility in dogs. Considering the lack of functional studies on the digestive system of spontaneous diabetic dogs, further functional investigations in DM dogs are necessary.

Parkinson's disease is a neurological human pathology. Although PD is regarded as a prototypical movement disorder, virtually all parkinsonian patients experience autonomic dysfunctions, in particular GI manifestations associated to ENS neuropathy. The pathogenetic mechanisms underlying the severe chronic constipation affecting the majority of PD patients, remain poorly understood. Most (91%) of PD patients enrolled in our study showed a severe functional motor impairment in the colon and rectum. Concerning ENS, PD constipated patients showed a significant reduction of submucosal secretomotor VIP-containing neurons, compared to constipated (CC)and controls patients, although the total number of neurons and cholinergic neurons did not change in the three groups. Furthermore, PD constipated patients showed a more pronounced downregulation of the VIP mRNA and related receptors in PD constipated patients than in CC and controls. These findings support a selective decrease of VIP expressing secretomotor neurons, suggesting that both neurally-mediated secretory mechanisms along with sensory-motor abnormalities represent a prominent peculiar mechanisms underlying PD constipated patients.

Concluding, the physiology of the GI tract is characterized by a high complexity and it is mainly dependent on the control of the intrinsic nervous system, in all the species considered. There are great differences between the ENS features across similar species, therefore it is very difficult to extrapolate and speculate among animals of different size and alimentary tract morphology and physiology. As consequence, the vast majority of the information that we have about the ENS are not adequate to understand completely the physiology and the pathophysiology in a given species. Any damage able to alter the morpho-functional integrity of the ENS may have a severe impact on the GI balance, resulting in many different pathological conditions, in humans and domestic animals. Therefore, the knowledge of the anatomy and the pathology of the ENS represents a new important and fascinating topic, which deserves more attention in the veterinary medicine field.

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