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Original article

Enteric neuronal development in canine small intestine – an immunohistochemical study

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Abstract

The development of the enteric nervous system (ENS) is still a valid and intensely studied issue. However, literature in the field has no data on this topic in the dog. The present investigations were performed in three groups of fetuses from mongrel dogs – from the third, sixth--seventh, and ninth week of pregnancy – and in 3-5-day-old puppies (3 specimens for each age group). The tissues (the medial parts of the duodenum, jejunum, and ileum with the cecum and a small portion of the adjacent ascending colon) were cut using a cryostat and the sections were processed for single- and double-labeling immunohistochemistry using antisera against acetylated tubulin (AcTub), vesicular acetylcholine transporter (VAChT), nitric oxide synthase (NOS), vasoactive intestinal polypeptide (VIP), galanin (GAL), neuropeptide Y (NPY), substance P (SP), and calcitonin gene-related peptide (CGRP). In the 3-week-old fetuses, some oval cells invading the gut wall were found. From the seventh week of pregnancy onwards, two different enteric ganglia were present: submucosal and myenteric. The estimated number of nerve elements in the 9-week-old fetuses was much higher than that observed in the 6-7-week-old individuals. There was no significant difference in the estimated number of nerve structures between the 9-week-old fetuses and the 3-5-day-old puppies. The colonization pattern and the development of the ENS in the canine small intestine are very similar to those observed in other mammals. However, a few exceptions have been confirmed, regarding the time of appearance of the VIP-, GAL-, and CGRP-immunoreactive neurons, and their distribution in different portions of the canine bowel during development.

Key words: dog, fetus, small intestine, enteric nervous system, development, neurons, immunohistochemistry

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Introduction

The enteric nervous system (ENS) consists of ganglionated plexuses located in the walls of the gastrointestinal tract (GIT) that are responsible for contraction of the muscular tunic, glandular secretion, intestinal transport, and mucosal blood flow (Arciszewski and Zacharko-Siembida 2008, Evans and de Lahunta 2013). The structure of the ENS differs significantly among species. Basically, two ganglionated plexuses, referred to as submucosal and myenteric, are distinguished (Gabella 1987, Furness 2006). However, in large mammals, the submucosal plexus is divided into the inner network located at the abluminal side of the muscularis mucosae and the outer plexus laying adjacent to the luminal side of the circular muscle coat; additionally, in the human intestine, a third intermediate plexus was recognized between those two (Hansen 2008). The major source of ENS precursors to the small intestine is the vagal neural crest (Yntema and Hammond 1954, Young et al. 2004, Goldstein et al. 2013, Brierley and Costa 2016). When colonizing the gut, neural crest-derived cells (NCDC) differentiate into glial cells and many different types of neurons, and form the most complex part of the peripheral nervous system (Karaosmanoglu et al. 1996, Gershon 1998, Anderson et al. 2006). In our research, we investigated morphological and immunohistochemical changes during the development of the ENS in the canine small intestine in the third, sixth-seventh, and ninth week of pregnancy, as well as in the 3-5 days after birth. The aim was to establish the complete forming process of ganglia, paying great attention to the changes in the chemical coding of newly formed neurons, and to the rearrangement of their fibers providing innervation to different layers of the gut wall. Although authors are aware that there are many subtypes of enteric glial cells which are very important for the majority of ENS functions (Boesmans et al. 2015, Grubišić and Gulbransen 2017), this study is focused only on the development of enteric nerve cells and fibers since they first appear in the bowel. Even though some studies investigating the ENS in the canine stomach (Horiguchi et al. 2003, Musara and Vaillant 2013) or focusing on the expression of particular substances in the bowel (McDonald et al. 1993) have been conducted, the development of the ENS in the canine small intestine has never been thoroughly described, and thus we believe that our research provides valuable observations in this regard.

Materials and Methods

Investigations were performed in three groups of fetuses from mongrel dogs – from the third (group 1, G1), sixth-seventh (group 2, G2), and ninth week of pregnancy (group 3, G3) – and in 3-5-day-old puppies (group 4, G4) (3 specimens for each age group). The fetuses were obtained during ovariohysterectomy performed for clinical reasons, and the puppies were euthanized because of medical indications. In accordance with Polish legislation and the Act on animal protection (Act of 21 August 1997, Dziennik Ustaw, 1997 No. 111, item 724, with later amendments, uniform text Dziennik Ustaw 2017, item 1840), experiments performed on tissues collected from animals during routine veterinary procedures, or in a slaughterhouse, do not require the consent of the Institutional Animal Care and Use Committee. The fetuses were fixed by immersion in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4 (PBS). After 24 hours of fixation, they were rinsed in PBS, transferred into 18% saccharose solution in PBS, and stored until they sank to the bottom of the container. The tissues (the middle parts of the duodenum, jejunum, and ileum with the cecum and a small portion of the adjacent ascending colon) were subsequently removed and immersed in a matrix for cryostat sectioning (Tissue-Tek O.C.T. Compound). This preparation method was applied to all of the specimens, except for the 3-week-old fetuses, which were entirely immersed in the aforementioned matrix due to their small size. Serial cryostat sections 16 μm thick were placed on chrome alum-coated slides and stored in a freezer (-30˚C) until further processing. After washing with PBS (3 × 10 min), the sections were processed for single- and double-labeling immunohistochemistry using antisera against acetylated tubulin (AcTub), vesicular acetylcholine transporter (VAChT), nitric oxide synthase (NOS), vasoactive intestinal polypeptide (VIP), galanin (GAL), neuropeptide Y (NPY), substance P (SP), and calcitonin gene-related peptide (CGRP) (Table 1). Due to the very limited amount of nerve fibers in the 3-week-old fetuses immunolabeling with substances other than AcTub was not performed. The sections were incubated in a blocking mixture containing 1% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.5% Triton X100 in PBS for 1 hour. They were then rinsed in PBS (3 × 10 min) and incubated with the primary antiserum for 24 hours at room temperature (RT). After rinsing in PBS (3 × 10 min), the sections were incubated with a secondary antiserum for 1 hour at RT (Table 1). They were subsequently rinsed in PBS (3×10 min) and mounted with 70% glycerole solution in PBS. The stained sections were studied using a confocal laser scanning microscope (Zeiss LSM 700 microscope).

In order to visualize the progressive alterations in the ENS during its development, the density of nerve fibers and the amount of nerve perikarya in the different

Table 1 Antisera used in this study.

Primary antibodies					
Antigen	Species	Catalog no.	Dilution	RRID	Supplier
AcTub	mouse monoclonal	32-2700	1:1500	AB_2533073	Invitrogen, UK
VAChT	rabbit polyclonal	V5387	1:4000	AB_261875	Sigma-Aldrich, USA
NOS	mouse monoclonal	N2280	1:2000	AB_260754	Sigma-Aldrich, USA
VIP	mouse monoclonal	ABS-023-02-1	1:2000	AB_1084155	Enzo Life Sciences, USA
GAL	rabbit polyclonal	#AB2233	1:2000	AB_1587050	Millipore, USA
NPY	rabbit polyclonal	ab123951	1:5000	AB_11000800	Abcam, USA
SP	rat monoclonal	8450-0505	1:200	AB_2200292	AbD-Serotec, UK
CGRP	rabbit polyclonal	C8198	1:8000	AB_259091	Sigma-Aldrich, USA
Secondary antibodies					
		Catalog no.	Dilution	RRID	Supplier
Alexa Fluor 555 goat anti-rabbit IgG (H+L)		A-21428	1:2000	AB_2535849	Invitrogen, UK
Alexa Fluor 488 goat anti-mouse IgG (H+L)		A-11001	1:2000	AB_2534069	
Alexa Fluor 488 goat anti-rat IgG (H+L)		A-11006	1:2000	AB_2534074	

Table 2. Estimated amount of nerve fibers/perikarya in studied tissues.

		3-week-old fetuses (G1)	6-7-week-old fetuses (G2)			9-week-old fetuses (G3)			3-5-day-old individuals (G4)		
		Midgut	Duod.	Jej.	Il.	Duod.	Jej.	Il.	Duod.	Jej.	Il.
AcTub	Cells	+	++	++	++	+++	+++	+++	+++	+++	+++
	Fibers	+	++	++	++	+++	+++	+++	+++	+++	+++
VAChT	Cells	Not investigated in this study	+	+	-	++	++	++	++	++	++
	Fibers		+	+	+	++	++	++	++	++	++
NOS	Cells		++	++	-	++	++	+	++	++	++
	Fibers		+	+	-	+	+	+	++	++	++
VIP	Cells		-	-	-	+	+	+	+	+	+
	Fibers		-	-	-	++	++	++	++	++	++
GAL	Cells		-	-	-	+	+	+	+	+	+
	Fibers		++	++	-	++	++	++	+++	+++	+++
NPY	Cells		+	+	-	+	+	+	+	+	+
	Fibers		++	++	-	++	++	++	++	++	++
SP	Cells		+	+	-	+	+	+	+	+	+
	Fibers		++	++	-	+++	+++	++	+++	+++	+++
CGRP	Cells		+	+	-	+	+	+	+	+	+
	Fibers		++	++	-	++	++	++	++	++	++

(-) – no nerve structures, (+) – single nerve structures, (++) – moderate number of nerve structures, (+++) – numerous nerve structures. Duod. – duodenum, Jej. – jejunum, Il. – ileum.

portions of small intestine were estimated according to the method described in our previous paper (Majewski et al. 1995). Arbitrary evaluation of the nerve structures in all specimens was performed by the same two authors who cooperated to obtain an objective estimation and the outcome of their work is shown in Table 2. The secondary antibody controls were run in parallel with each experiment by processing a sample with the omission of each primary antibody and replacing it with the same amount of NGS to ensure that the labeling observed was due only to binding of the secondary antibodies to the primary antibodies (Burry 2011). Additionally, control of specificity of staining was performed by preabsorbtion of a diluted antiserum with 20 μg/ml of an appropriate antigen (except for AcTub and VAChT), which abolished the specific immunoreaction completely.

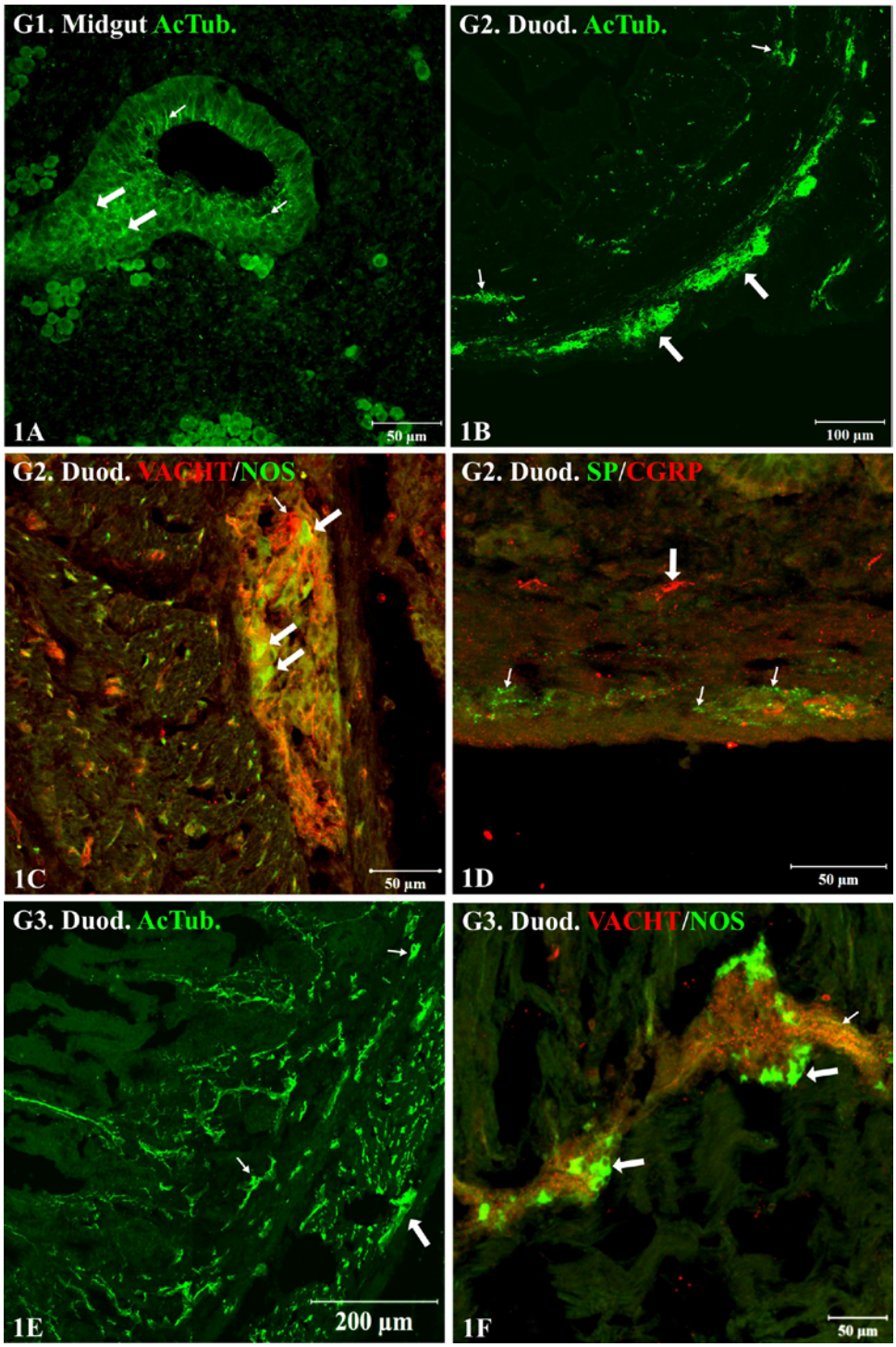

Fig. 1A. Nerve fibers in the 3-week-old fetus's midgut in the labeling with antibodies against AcTub (thin arrows). Note the presence of some putative nerve cells captured while invading the midgut wall (thick arrows).

Fig. 1B. Transverse section through the duodenum (Duod.) of the 6-7-week-old fetus stained with an AcTub antiserum showing numerous nerve cells within the myenteric plexus (thick arrows). Note the less-well developed submucous ganglia (thin arrows).

Fig. 1C. Nerve cells in the myenteric plexus of the 6-7-week-old fetus's duodenum stained with antibodies against VAChT (thin arrow) and NOS (thick arrows).

Fig. 1D. Sixth-seventh week of pregnancy. Section of the duodenum showing a moderate amount of intraganglionic neurites immunoreactive for SP (thin arrows) and single CGRP-positive nerve fibers, both within the myenteric plexus. Single CGRP-IR nerve cells are visualized in the submucus plexus (thick arrow).

Fig. 1E. Transverse section through the 9-week-old fetus's duodenum stained with an AcTub antiserum. Numerous nerve cells and fibers within the myenteric (thick arrows) and submucous (thin arrows) plexuses are observed.

Fig. 1F. Higher magnification of the myenteric ganglion in the 9-week-old fetus's duodenum. Note the presence of nerve cells immunoreactive for NOS (thick arrows) surrounded by a high-density network of VAChT-positive nerve fibers (thin arrow).

Results

Third week of pregnancy

In the specimens stained with an AcTub antibody, rare nerve fibers sparingly distributed throughout the midgut were observed (Fig. 1A). Additionally, some oval cells with conspicuous nuclei were found invading the gut wall (Fig. 1A).

Sixth-seventh week of pregnancy

Staining with AcTub antiserum revealed the presence of numerous nerve elements in the duodenum (Fig. 1B) as well as in the jejunum and ileum, uniformly distributed across the intestinal wall. The nerve cells in the myenteric plexus outnumbered the submucous population of neurons (Fig. 1B). The submucous ganglia were starlike in shape, which distinguished them from the elongated myenteric ganglia. A moderate number of nerve fibers was found, many of which interconnected both plexuses or penetrated into the intestinal villi.

Many of the nerve cells observed in the duodenum and jejunum were immunoreactive to NOS, whereas the population of VAChT-positive neurons was quite small and hardly seen (Fig. 1C). NOS-positive perikarya were, however, underrepresented in the submucosa. Rare VAChT-containing neurons and neurites seemed to be chaotically distributed in the submucosa, the myenteron, and within the myenteric plexus. Importantly, apart from a few putative VAChT-containing neurites, no immunoreactive nerve elements were observed in the ileum.

The sections used for anti-VIP immunostaining were simultaneously labeled with anti-VAChT antibodies to evaluate the colocalization of both substances. However, no VIP-immunoreactive (IR) nerve structures were found.

The myenteric plexus of the duodenum and jejunum was found to contain a comparable density of neurites immunoreactive to GAL or NPY, whereas in the submucous plexus, NPY-positive fibers clearly predominated. Interestingly, GAL- or NPY-positive nerve elements were absent in the ileum at this developmental stage.

SP-IR nerve structures were mostly found in the muscular plexus of the duodenum, whereas CGRP-positive neurites and neurons predominated in the submucosa (Fig. 1D). A moderate density of intraplexial nerve fibers in the myenteric plexus of the proximal small intestine was found to penetrate into the circular muscles of the gut wall, and into the intestinal villi. In contrast, no SP- or CGRP-positive nerve elements were observed in the ileum.

Ninth week of pregnancy

Numerous intramural nerve elements were found in the duodenum (Fig. 1E), jejunum, and ileum. Most of the neurons were contained in the myenteric plexus, whereas they were less well-represented in the submucosa. Numerous nerve fibers were distributed in the circular muscular layer, while they were poorly seen in the longitudinal muscle coat. No variations were observed in the number of nerve structures between different parts of the small intestine.

Immunoreactivities to VAChT or NOS were noted in many neurons distributed between the circular and longitudinal muscle layers. They formed separate populations with no simultaneously positive cells present. The population of NOS-positive neurons in the submucosa was much less numerous compared with that in the myenteron (Fig. 1F); however, the estimated amounts of VAChT- and NOS-positive neurons in both plexuses were very similar. The only exception was the ileum, in which cholinergic neurons predominated to some extent. Furthermore, plenty of VAChT-IR nerve fibers were determined, the vast majority of which were intraplexial (Fig. 1F), whereas multiple NOS-positive fibres were distributed in the myenteron and in the mucosa.

In the specimens stained with antibodies against VIP, a quite dense network of VIP-positive axons within the gut wall was visualized. Most of the nerve fibres were intraplexial, of which many were basket-like formations. Some nerve terminals were also observed in the myenteron and in the mucosa.

Staining with antibodies against GAL revealed a high density of nerve elements widely distributed throughout the intestinal wall, with fibers predominating. They were preferentially located in the circular muscular layer and within the plexuses. There were only a few nerve fibers in the mucosa and single neurons within the ganglia.

NPY-positive neurons were found exceptionally rarely in the submucosa and sparingly distributed within the myenteric plexus. A substantial amount of the fibers interposed among the myenteric neurons was found; however, they were rarely seen in the circular muscular layer.

Nerve fibers containing SP were extensively distributed in the muscularis externa of the duodenum and jejunum, with the highest density of the axons within the myenteric plexus (Figs. 2A). In comparison, their density in the submucosa and inside the villi was low. CGRP- or SP-containing neurons were dispersed solely within the myenteric ganglia (Fig. 2A) and in the submucosal plexus. However, in contrast to the duodenum and jejunum, the ileum contained far fewer nerve elements immunoreactive to SP. CGRP-positive fibers were a lesser component in the myenteron of the proxi-

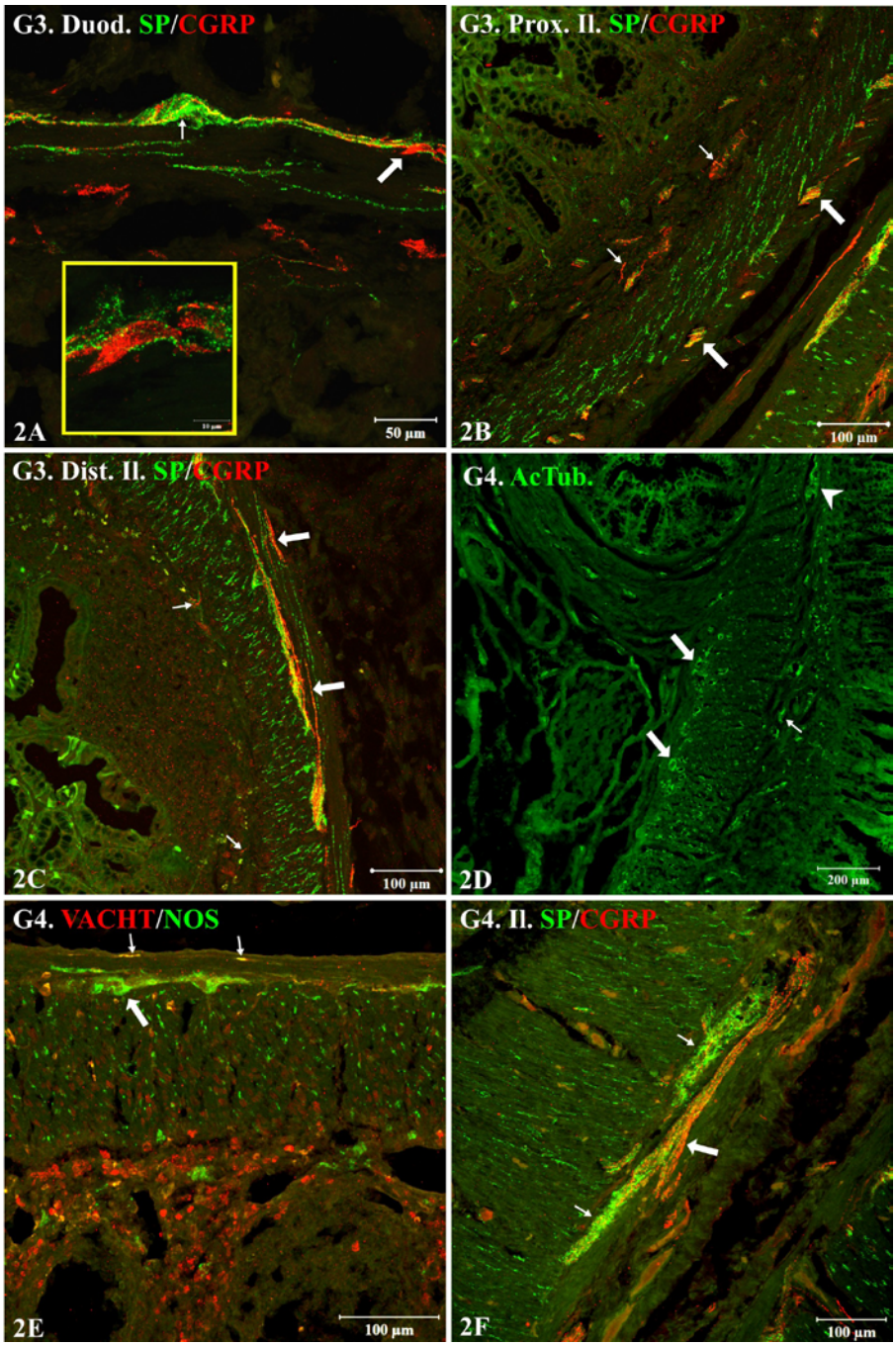

Fig. 2A. The duodenal myenteric plexus of the 9-week-old fetus in the labeling with antibodies against SP and CGRP. SP- (thin arrow) or CGRP-positive (thick arrow) perikarya, surrounded by a dense network of SP-IR nerve fibers, are observed. Higher magnification of a myenteric CGRP-IR nerve cell indicated by the thick arrow is visualized in the lower left corner of the picture.

Fig. 2B. Ninth week of pregnancy. Section of the proximal part of ileum (Prox. Il.) stained with antibodies against SP and CGRP. A moderate density of CGRP-positive neurites in the submucosa (thin arrows), as well as some double-positive nerve fibers in the myenteric plexus (thick arrows), are also seen.

Fig. 2C. Ninth week of pregnancy. Section of the distal part of ileum (Dist. Il.) stained with antibodies against SP and CGRP. Note that the density of CGRP-IR nerve fibers in the myenteric plexus (thick arrows) is higher than in the submucosa (thin arrows).

Fig. 2D. Longitudinal section through the ileal papilla (Il. P.) of 3-5-day-old puppy stained with an AcTub antiserum showing numerous nerve cells within the myenteric (thick arrows) and submucous (thin arrow and arrowhead) plexuses.

Fig. 2E. Longitudinal section of the ileum of 3-5-day-old puppy stained with VAChT and NOS antisera showing a moderate amount of NOS-positive nerve cells within the myenteric plexus (thick arrow) and some double-positive nerve fibers in the subserosal space (thin arrows).

Fig. 2F. Three-five-day-old puppy. Section of the ileum stained with antibodies against SP and CGRP showing dense network of the SP- (thin arrows) or CGRP-IR (thick arrow) nerve fibers within the myenteric plexus and muscular layers. Some fibers and nerve cells are double-positive.

mal portions of the small intestine, but they were particularly prominent in the submucosa and in the mucosa, where they predominated over SP-IR axons (Fig. 2A). Surprisingly, the density of CGRP-IR nerve fibers was higher in the myenteric plexus and lower in the submucous plexus of the distal part of the ileum than in the other portions of the small intestine, including the proximal part of the ileum (Figs. 2B, Fig 2C). No simultaneously positive cells were noted; however, a substantial number of the nerve fibers located within the myenteric ganglia contained both neuropeptides, especially in the ileum.

Three-five days postpartum

The general organization of nerve structures marked with the panneuronal marker AcTub was very similar to that observed in the 9-week-old fetuses (Fig. 2D).

In the sections stained with antibodies against VAChT and NOS, many single-positive neurons were observed. NOS-IR nerve cells accompanied by numerous axons were preferentially located in the myenteric ganglia (Fig. 2E). The estimated amount of VAChT-IR neurons in both plexuses was comparable to that of NOS-positive cells. Several simultaneously positive nerve fibers were distributed beneath the serous membrane (Fig. 2E).

Staining with anti-VIP antibodies revealed a substantial density of the nerve fibres in the longitudinal and circular muscular layers, whereas the density of the fibers in the mucosa was quite small. In addition, a limited amount of VIP-positive neurons were found in ganglia, of which nearly all were simultaneously immunoreactive to VAChT.

The myenteric plexus was abundant in GAL-IR nerve fibers, whereas in the submucosa they were less well represented. Multiple axons containing this neuropeptide were located in the circular muscular layer of the duodenum and jejunum, and some of these also projected into the intestinal villi. In contrast, the density of NPY-positive nerve fibers was distinctly lower along the entire intestinal wall, however, the general distribution pattern was similar to that of the GAL-containing nerve terminals.

Most of the SP-IR nerve structures were fibers, the vast majority of which were located in the myenteric plexus and in the muscularis externa (Fig. 2F). The arrangement of SP- and CGRP-positive nerve elements was similar to that observed in the 9-week-old fetuses. The only exception was the ileum, where the SP-IR neurites were more numerous.

Discussion

Many investigations on the progressive alterations in the ENS during its development have already been conducted in several species, including rats (Heitkemper and Marotta 1983, Gershon et al. 1993, Tanano et al. 2005), mice (Lake and Heuckeroth 2013), and even humans (Okamoto and Ueda 1967, Tam 1986, Wallace and Burns 2005). This study provides for the first time the general characterization of the development of the ENS in the small intestine of the dog.

Analysis of our data indicates that the ganglia formation process in canine fetuses was wery consistent with the colonization pattern observed by Wallace and Burns (2005) in humans. In 3-week-old fetuses, only a few lone oval cells were noted invading the gut wall. These cells may represent part of a wave of NCDC, suggesting that the NCDC enter the canine midgut later than in the first three weeks of fetal life. However, it is known that AcTub is not a marker of neuronal progenitors, thus it is possible that their emergence within the midgut occurs much earlier. From the seventh week of pregnancy onwards, two types of enteric ganglia were observed: submucosal and myenteric. Importantly, there was no significant difference in the density of nerve structures between the specimens from the ninth week of pregnancy and those from 3-5-day-old puppies, implying that the enteric ganglia are already developed to a great extent a few days before parturition.

In our research, we observed that VAChT-IR nerve structures were present for the first time in the 6-7-week-old fetuses, which corresponds with the results obtained by Erickson et al. (2014) in mice. We also notedthat up to the ninth week of pregnancy, the estimated number of VAChT-positive nerve cells rose. As reported in previous investigations (Erickson et al. 2014), the percentage of cholinergic neurons does not change significantly between E13.5 (embryonic day 13.5) and P30 (postnatal day 30) in the distal small intestine of mice. These data correspond with our results, providing evidence that the cholinergic neurons reach adult levels early in development.

NOS-positive neurons first appeared in the 6-7-week-old fetuses. Up to the ninth week of pregnancy, the number of nerve cells showing immunoreactivity for NOS increased. Importantly, it was found that in the 9-week-old fetuses, NOS expression stabilized and remained almost unchanged in 3-5-day-old individuals, except in the ileum, where more NOS-containing neurons were seen after birth. These observations are in agreement with the results obtained in mice (Arnhold et al. 2004).

Both neurons and neuritic processes containing VIP were first seen in 9-week-old fetuses, suggesting that

VIP-immunoreactivity appears late in canine ontogeny. Although this hypothesis corresponds to Tharakan's et al. (1995) conclusions, who did not observe the expression of VIP in neurons of the rat foregut until day E20, it differs from findings obtained by other authors. Our conclusions, for instance, contradict the observations reported by Rothman et al. (1984), who found VIP-immunoreactivity in the murine duodenum at day E14 of development, while it could not be determined in the colon as late as day E16. However, these discrepancies may be species-related.

Thin fibers expressing GAL-immunoreactivity were visualized in the rat gut by day E18 (Tharakan et al. 1995). In contrast to that, in the canine duodenum and jejunum GAL-positive fibers appear much earlier, in 6-7-week-old fetuses. No nerve elements containing this neuropeptide seem to be present in the ileum until the ninth week of pregnancy. There is, however, a significant gap between the 9-week-old fetuses and the 3-5-day-old puppies in the density of GAL-IR nerve fibers. Speculatively, this periparturient increase may be attributable to GAL-mediated neuronal survival being essential for the proper maturation of the ENS.

In our research, we noted that NPY-containing fibers were initially present in the duodenum and jejunum of the 6-7-week-old canine fetuses before appearing in the ileum, which had no NPY-immunoreactivity at this developmental stage. In the 9-week-old fetuses, the density of NPY-containing nerve structures in the duodenum and jejunum did not change, however; the ileal ganglia exhibited immunoreactivity for this neuropeptide. The development of NPY-IR nerve elements progresses rapidly in the first days of its appearance in the bowel, then stabilizes and remains almost unchanged in the periparturient period.

Our research confirms that the proximodistal colonization pattern of SP- and CGRP-IR nerve structures observed in previous studies (Rothman et al. 1984, Branchek and Gershon 1989, Sasselli et al. 2012) can be also applied to the development of the canine ENS. However, our data indicate that the CGRP-positive nerve elements appear in the canine bowel during the sixth or seventh week of pregnancy. This is counter to the observations made by Branchek and Gershon (1989) in mice, according to which CGRP-immunoreactivity was first observed at day E17.

The colonization pattern and the development of the ENS in the canine small intestine described in this contribution are very similar to those observed in other mammals. However, a few exceptions have been confirmed, regarding the time of origin of the VIP, GAL-, and CGRP-IR nerve elements, and the distribution of particular substances in different portions of the canine bowel during its development. Our study, then, provides a helpful overview of the ENS development in the dog.

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