Acta Histochemica 120 (2018) 768–779

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis

Expression profile of some neuronal and glial cell markers in the ovine ileal enteric nervous system during prenatal development

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ARTICLE INFO

Keywords:
Sheep
Enteric nervous system
S100A6

ABSTRACT

The enteric nervous system (ENS) is a network of neurons and glia found in the gut wall and governs this gastrointestinal function independently from the central nervous system (CNS). ENS comprises the myenteric plexus (MP) and the submucous plexus (SP). In this study, we examined the expression profile of neurofilament heavy chain (NF-H), neuron-specific enolase (NSE), calcyclin (S100A6), vimentin and glial fibril acidic protein (GFAP) in ovine ileal enteric neurons and enteric glia cells (EGCs) during prenatal development using an immunohistochemical method. The material of the study consisted of 15 different fetal ileum tissues obtained between days 60 and 150 of pregnancy. NF-H was observed in the majority of ganglion cells in SP and MP throughout the fetal period. It was determined that there was no NF-H reaction in some ganglion cells in Peyer's patches of internal submucosal plexus (ISPF). In the early stage of pregnancy (60–90 days), there was no expression of NSE and S1006 in ileum. After this period, NSE and S1006 were expressed in the ganglion cells of the plexus, indicating an increase in the amount of expression towards the end of pregnancy. In the early period, vimentin expression was only detected in intramuscular interstitial cells (ICs) (60–90 days), but later (90–150 days) it was also seen in the cells around the ganglion cells in the plexus. On days 60–90 of gestation, GFAP expression only occurred in MP, but in later stages, staining was also detected in SP. In the plexus, an immunoreactivity was present in EGCs forming a network around the ganglion cell. During the last period of gestation (120–150 days), the number of GFAP-positive plexus increased, with the majority of these stained cells being observed in MP. Interestingly, weak staining or reaction did not occur in ISPF, unlike other plexuses. In conclusion, this is the first study that demonstrated the expression of NF-H, vimentin, S100A6, NSE and glial fibril acidic protein (GFAP) in ovine ileal ENS in the prenatal period. In the last period of gestation (120–150 days), the expression profile of ENS was similar to that of adult animals. The expression of the used markers increased toward the end of pregnancy. Our results suggest that neurons and EGCs show heterogeneity, and GFAP and NF-H cannot be used as panenteric glial or panneuronal markers, respectively. We also demonstrated, for the first time, the prenatal expression of S100A6 in enteric neurons and the possibility of using this protein for the identification of enteric neurons.

1. Introduction

Considered as the largest and complex component of the peripheral nervous system, the enteric nervous system (ENS) is an integrative network within the gastrointestinal tract (Benarroch, 2007). Despite a normal interaction between ENS and the central nervous system (CNS), ENS may autonomously function in many regions of the gastrointestinal tract; therefore, it has been defined as the 'second brain' (Benarroch, 2007).

ENS comprises the myenteric plexus (MP) and the submucous plexus (SP) (Goyal and Hirano, 1996). Within large animal species, SP is further divided into an internal submucosal plexus (ISP) near the lamina muscularis mucosae and an external submucosal plexus (ESP) closest to the tunica muscularis (Hansen, 2003). ISP is localized in the base of lymphoid follicles (ISPF) and various sites in the sections of the gastrointestinal tract that contain intense lymphoid tissues, such as the ileum (Peyer's patches) (Balemba et al., 1998). Plexuses are composed of ganglion cells, primary interganglionic fibers, and secondary orifice

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https://doi.org/10.1016/j.acthis.2018.09.002
Received 17 May 2018; Received in revised form 7 August 2018; Accepted 3 September 2018
0065-1281/ © 2018 Published by Elsevier GmbH.

Table 1
The antibodies used for immunohistochemical analysis.

Primary antibodies	Clonity	Host	Dilution	Source	Code
Neurofilament 200 (NF-H)	Polyclonal	Rabbit	1:400	Sigma-Aldrich	N4142
NSE	Monoclonal, Clone BBS/NC/VI-H14	Mouse	Ready-to-Use	Dako	IR612
S100A6	Polyclonal	Rabbit	1:200	Thermo Fisher Scientific	RB-1805-A
Vimentin	Monoclonal, Vim 3B4	Mouse	1:200	Abcam	ab28028
GFAP	Polyclonal	Rabbit	1:50	Thermo Fisher Scientific	RB-087-A

nerve fibers leading to effector organs (Gershon, 1999). MP is primarily responsible for motility, while SPs are responsible for secretion and absorption (Goyal and Hirano, 1996).

Enteric neurons have a number of structural proteins, some of which form the neuronal intermediate filament (NF), a filamentous structure with a thickness of 8–10 nm (Eaker et al., 1990). NF are found abundantly in axons, but they are also expressed in the perikaryon and dendrites. These intermediate filaments are necessary for the radial growth of axons, preservation of the constant diameter of the axon, and the transmission of electrical stimuli through axons (Yum et al., 2009). In CNS, NF are composed of light (NF-L), medium (NF-M) and heavy (NF-H) polypeptide subunits and alpha internexin proteins (Yan et al., 2007). During embryonic development, intermediate filaments, such as vimentin, can be seen in nerve cell precursors (Perrin et al., 2005). Vimentin is responsible for balancing the cell shape, cytoplasm integrity, and cytoskeletal interactions (Lowery et al., 2016). During axonal growth, vimentin expression may occur with the NF-L and NF-M subunits. When axonal growth reaches the desired level, vimentin begins to disappear (Cochard and Paulin, 1984). Another protein used to detect neuron maturation and differentiation is neuron-specific enolase (NSE), which is the major enolase form found in mature neurons and neuroendocrine cells. All known eukaryotic enolases have a dimeric structure (Haque et al., 2016).

Unlike other autonomous ganglions, under normal conditions, fibroblasts, macrophages, mast cells, collagen, blood vessels, and chromaffin cells are not present around the enteric ganglia. In addition, enteric ganglia lack perineural connective tissue capsule and satellite cell sheath (Gulbransen, 2014). Another group of cells that surround the enteric neurons in ENS are EGCs that are usually large in number. EGCs were originally considered to be the intestinal Schwann cells since they originated from crista neuralis. This classification began to change when electron microscopy studies revealed differences between the Schwann cells in the peripheral nerves and glia in the intestine (Rao et al., 2015). Gabella described glia cells in the MP as small cells with a central perikaryon and a large number of nerve processes. Considering the stellate morphology of these cells, Gabella suggested that these cells resemble astrocytes more than the Schwann cells (Gabella, 1971). Jessen and Mirsky (1980) showed that the glial fibrillary acidic protein (GFAP), an astrocyte marker, was expressed in the enteric ganglion, indicating the similarities between the glia in ENS and astrocytes in CNS astrocytes (Jessen and Mirsky, 2005). Another characteristic of EGCs in resembling astrocytes is that glial filaments are predominantly composed of vimentin during development (Jessen and Mirsky, 1985). EGCs cover both cell bodies and processes of neurons throughout the intestinal tract. These cells exhibit extraganglionic, intramuscular and mucosal localization, as well as MP and SP (Rühl et al., 2004; Gulbransen and Sharkey, 2009).

The neurodegenerative diseases such as animal prion diseases including scrapie, bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD) is charecterized by the presence of prion protein depositions. Prions spread via autonomic nervous system from the gastrointestinal tract to the CNS (Hoffmann et al., 2007; Seelig et al., 2011). One of candidate facilitator of prion infectivity is ENS (Savidge et al., 2007; Rühl, 2005). Furthermore, prions is found to be constitutively expressed in both human and animal ENS (Shmakov and Ghosh, 2001). In addition, some prenatal intestinal tract anomalies, such as atresia, affect the development of ENS (Fourcade et al., 2009). Therefore, the examination of ENS in healthy animals may further an understanding of the pathogenesis of the neurodegenerative diseases mentioned above. In our review of the literature, we did not find a study that investigated the prenatal development of ENS in the ileum of sheep. In this study, we aimed to investigate the expression profile of NF-H, NSE, S1006, vimentin, and GFAP in enteric neurons and EGCs during prenatal development in sheep.

2. Materials and methods

2.1. Animals and tissue preparation

This study was approved by the Department of Experimental Animals Ethic Committee of Ankara University, Turkey (approval number: 22724). The fetal tissues used consisted of specimens collected from 20 Akkaraman sheep fetuses from various slaughterhouses. The gestational age of the fetuses ranged from 63 to 90 days in seven, 90 to 120 days in six, and 120 to 147 days in six. The age of the fetuses was determined using Richardson's 'x = 2.1 * (y + 17)' correlation formula, where 'y' refers to the length from forehead to the chest in cm and 'x' indicates age of fetus in days (Richardson et al., 1976).

The terminal ileal samples from the fetuses were fixed in Bouin's solution, followed by washing in 70% alcohol. After routine procedures, the samples were embedded in paraffin.

2.2. Immunohistochemistry (IHC)

For the immunohistochemical (IHC) analysis, 5 μm-thick sections were placed on Poly-L-Lysine-coated slides and the streptavidin-biotin-peroxidase technique was performed, as detailed in our previous work (Beyaz et al., 2017). Briefly, the sections treated with xylol and alcohol series were incubated for 15 min in PBS; then, antigen retrieval was performed by boiling the specimens in a solution of citrate buffer 10 mM (pH: 6) for 20 min. To prevent endogenous peroxide activity, the sections were incubated in a 3% hydrogen peroxide solution for 20 min in the dark. The sections were then washed again with PBS for 15 min and encircled with Dako Pen. In order to prevent non-specific antigenic binding, the sections were left in an Ultra V Block solution (Ultra V Block®, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 10 min. Subsequently, they were incubated using primary antibodies (Table 1) for overnight at + 4 °C. Then, the sections were

Table 2
Scoring for specific immunostaining of NF-H, NSE, S100A6, Vimentin and GFAP in the ovine ileum during the prenatal period.

Antibody	Cell	Gestational ages											
		60-90				91-120				121-150			
		MP	ESP	ISP	IM	MP	ESP	ISP	IM	MP	ESP	ISP	IM
NF-H	EN	+	+	−	+	+ +	+ +	+ +	+	+ + +	+ + +	+ + +	+
	EGCs	−	−	−	−	−	−	−	−	−	−	−	−
NSE	EN	−	+	+	−	+ +	+ +	+ +	−	+ + +	+ + +	+ + +	+
	EGCs	−	−	−	−	−	−	−	−	−	−	−	−
S100A6	EN	+	+	−	−	+ +	+ +	+ +	−	+ + +	+ + +	+ + +	−
	EGCs	−	−	−	−	−	−	−	−	−	−	−	−
Vimentin	ICs	+	−	−	+	+ +	+	+	+ +	+ +	+	+	+ +
GFAP	EN	−	−	−	−	−	−	−	−	−	−	−	−
	EGCs	+	−	−	−	+ +	+ +	+	−	+ + +	+ + +	+ +	−

washed with PBS for 15 min and incubated for 30 min using biotinylated goat secondary antibody (Biotinylated Goat Anti-Polyvalent, anti-mouse and anti-rabbit, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA). After washing with PBS again, the sections were placed in a solution of streptavidin peroxidase (Streptavidin Peroxidase, TS-060-HR, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 30 min. 3-Amino-9-Ethylcarbazole (AEC) was used as a chromogen (TA-125-SA, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) to visualize the immune reaction. Nuclei were stained using Gill's Hematoxylin and sealed with a water-compatible adhesive.

For negative control, the primary antibody was replaced with non-specific rabbit immunization serum (sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA), and for positive control, rat colon tissue was used. The same protocol was applied to all sections including the positive and negative controls.

2.3. Quantification of immunoexpression

The sections were examined under light microscope. Olympus BX51

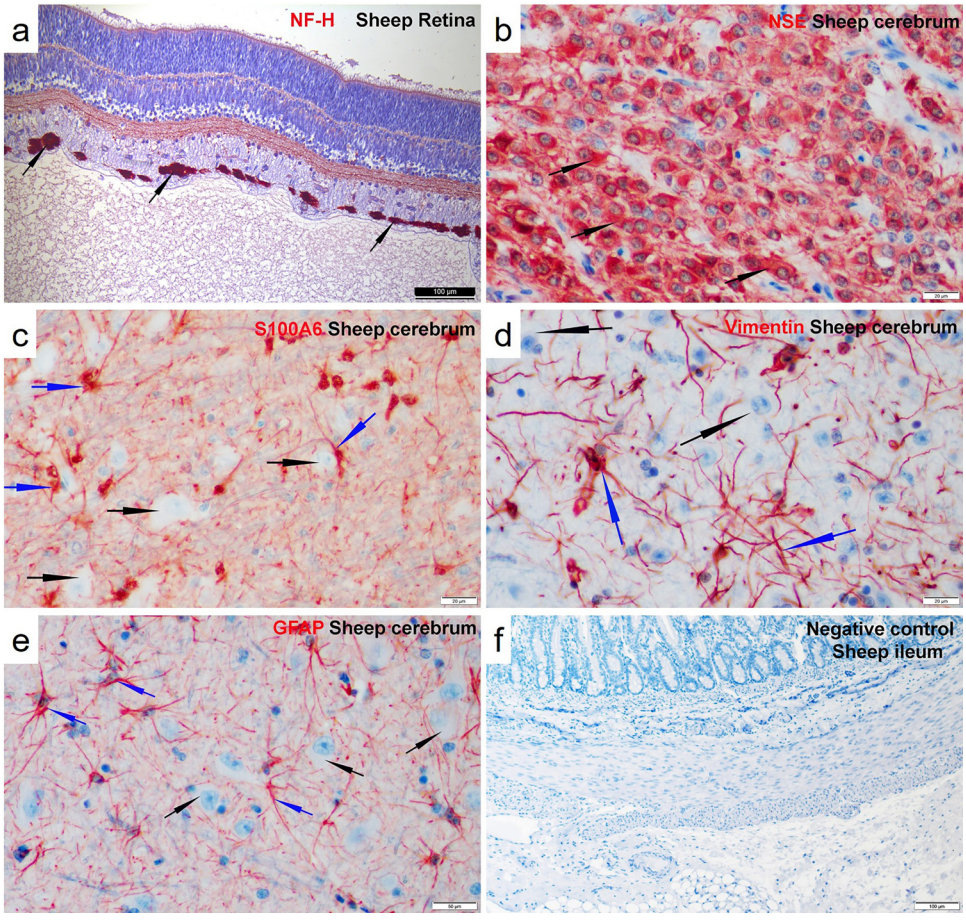


Fig. 1. Sheep retina tissue section as positive control for NF-H and sheep cerebrum tissue section as positive control for NSE, S100A6, Vimentin and GFAP. Strept-ABC, AEC, Paraffin. (a) NF-H positive ganglion cells (black arrows) in ganglion cell layer. (b) NSE positive neurons (black arrows) in substantia grisea. Glial cells exhibited any immunostaining for NSE. (c) S100A6 positive astrocyte (blue arrows) and S100A6 negative some neurons (black arrows) in substantia grisea. (d) Vimentin positive astrocyte (blue arrows) in substantia grisea. Neurons (black arrows) exhibited any immunostaining for vimentin. (e) GFAP positive astrocyte (blue arrows) in substantia grisea. Neurons exhibited any immunostaining for GFAP (black arrows). (f) No immunostaining was observed in the sheep ileal tissue sections that were incubated without the primary antibody. Scale bars: 100 µm (a, f), 20 µm (b, c, d, e). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

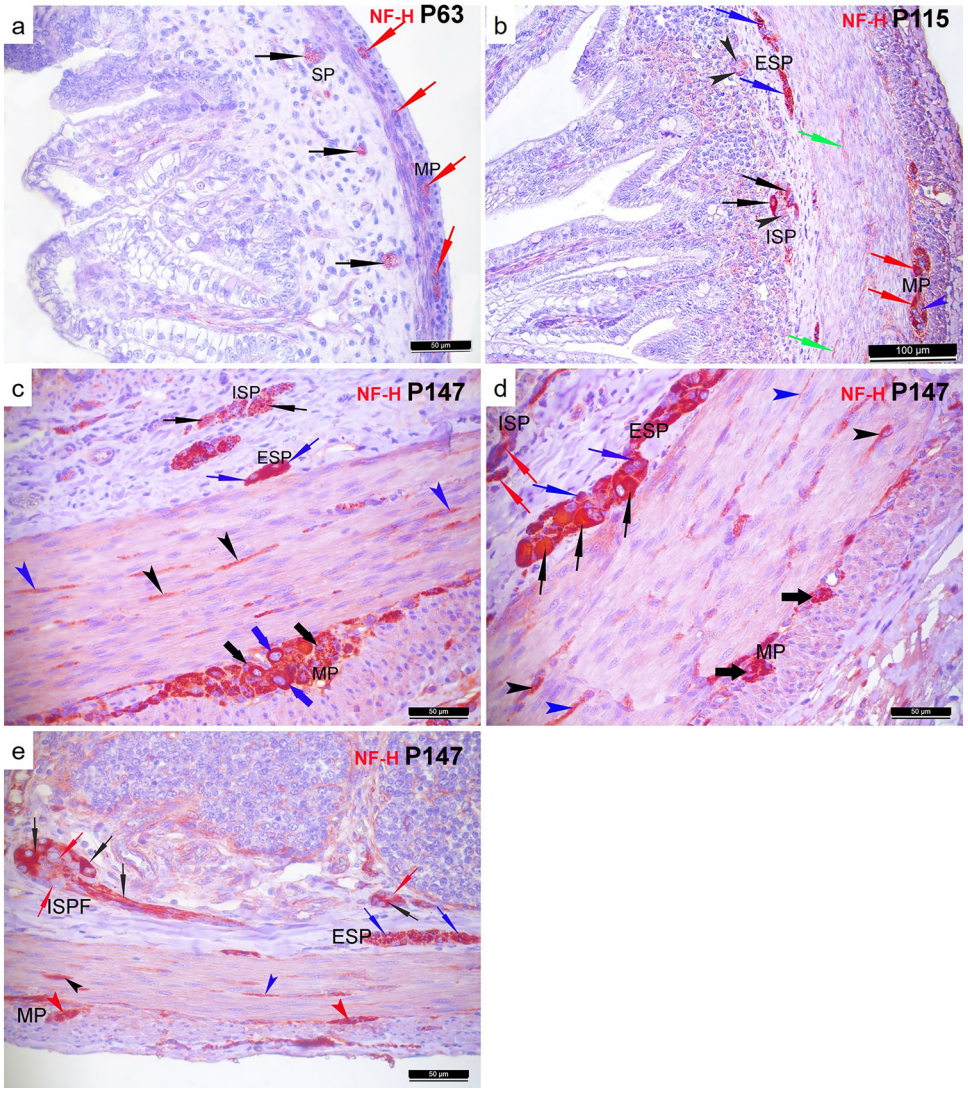


Fig. 2. Representative samples showing expression of NF-H in ovine ileum of day 63 prenatal (P63; a), day 115 prenatal (P115; b), day 147 prenatal (P147; c, d, e). Strept-ABC, AEC, Paraffin. (a) A representative figure of NF-H positive ganglion cells in ileal SP (black arrows) and MP (red arrows) from 63 day old ovine fetus. (b) A representative figure of NF-H positive ganglion cells in ileal MP (red arrows), ESP (blue arrow) and ISP (black arrows) from 115 day old ovine fetus. Some ganglion cells in MP (red arrowheads) and ISP (black arrowheads) exhibited any immunostaining for NF-H, while the intramuscular nerve processes (green arrows) exhibited positive immunostaining. (c, d, e) Representative figures of NF-H positive ganglion cells in ileal MP (red arrows), ESP (blue arrows) and ISP (black arrows) from 147 day old ovine fetus. Small type (thick black arrows) and large type (Thick blue arrows) ganglion cells and intramuscular nerve processes were also positive for NF-H. Scale bars: 50 µm (a, c, d, e), 100 µm (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

light microscope digital camera (Olympus DP74; Olympus, Tokyo, Japan) was used for imaging the immunohistochemical staining. Transverse sections from the ileal tissue of each animal were first examined with low magnification (x10) to individually assess the quality of the immunoreactivity, cell labeling patterns and histomorphological changes during the prenatal period. Since the IHC scoring algorithms of the markers used were not optimized or standardized, in this study, IHC staining was semiquantitatively evaluated based on a score (density score, IS) of staining intensity (-, negative reactivity; +, weak staining; ++, moderate staining; +++, strong staining). In three different prenatal periods (early; 60–90, medium; 91–120, late; 121–150), IHC staining was assessed separately, and the scores are summarized in Table 2.

3. Results

Tissue sections from the ileum were analyzed for the expression and localization of NF-H, NSE, S100A6, Vimentin, and GFAP. In the positive control tissue section, the immunostaining for these glial and neuronal markers are presented in Fig. 1. Furthermore, as shown in Fig. 1f no immunostaining was observed in the ileal tissue sections that were incubated without the primary antibody, indicating the specificity of the immunohistochemical procedure.

3.1. NF-H immunoreactivity

In the early stage of gestation, SP comprising ganglia was present in small groups in the submucosal area near the tunica muscularis. The tunica muscularis consisted of circular and longitudinal smooth muscle cells in several rows, and MPs were observed in groups among these muscular fibers. Positive staining was observed in the majority of ganglion cells in both plexuses (Fig. 2a). In the middle stage of gestation, SP was composed of two parts; ISP and ESP, and the number of ganglion cells was increased. There was positive staining in the majority of these ganglion cells. In addition, immunoreactivity for NF-H was found in the intramuscular nerve processes (Fig. 2b). In the late stage of gestation, small and large types of ganglion cells were observed to form plexuses. These cells also had positive staining for NF-H. There was a positive reaction in the processes of the neurons. In this period, round-shaped ISPF was also seen at the bottom of follicles with the

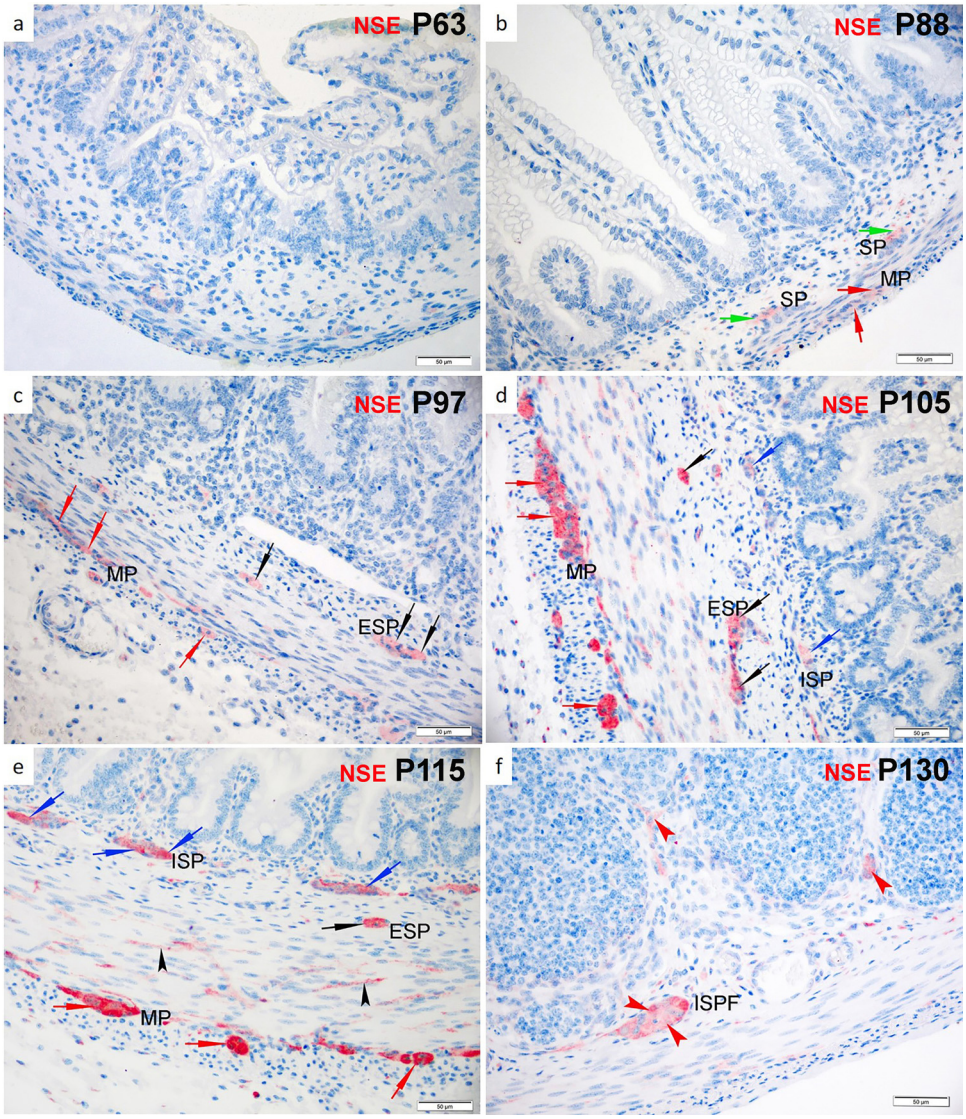


Fig. 3. Representative samples showing expression of NSE in ovine ileum of day 63 prenatal (P63; a), day 88 prenatal (P88; b), day 97 prenatal (P97; c), day 105 prenatal (P105; d), day 115 prenatal (P115; e), day 130 prenatal (P130; f). Strept-ABC, AEC, Paraffin. (a) A representative figure of ganglion cells exhibited any immunostaining for NSE. (b, c, d, e, f) Representative figures of NSE positive ganglion cells in ileal SP (green arrows), ESP (black arrows), ISP (blue arrows), ISPF (red arrowheads), MP (red arrows) and intramuscular nerve processes (black arrowheads). Scale bars: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

formation of Peyer's patches (Fig. 2c, d). These plexuses were connected to each other by interneuronal nerve fibers adjacent to the submucosa of the follicles. Some of the neurons in the ISPF had positive staining, while others did not. Positive staining was also noted in the nerve fibers that connected the plexuses to each other (Fig. 2e).

3.2. NSE immunoreactivity

NSE expression was not present on days 60–85 of gestation (Fig. 3a). From the 85th day of the prenatal period, a weak reaction appeared in the ganglion cells of SP and MP (Fig. 3b), and the reaction intensity gradually increased during the 90–120 day period of the prenatal development. Although reaction was also present in ESP and ISP, the most intense immunoreaction was detected in MP (Fig. 3c, d). In the last period of gestation (120–150 days), a more intense reaction was observed in the all plexus and there was also staining in some intramuscular nerve processes (Fig. 3e). In the ISPF, staining was strong in some plexuses and weak in others (Fig. 3f).

3.3. S100A6 immunoreactivity

In the tissues examined, S100 immunoreaction was not observed between days 60 and 88 of gestation (Fig. 4a). After 88 days of the prenatal period, weak staining began to occur in MP and SP (Fig. 4b). Moderate reaction for S100A6 was observed in ganglion cells of SP (ISP and ESP) and MP on days 90–120 (Fig. 4c, d). In this period, round-shaped ISPF was detected with the formation of Peyer's patches and moderate immunostaining was present in the ganglion cells of these plexuses (Fig. 4e). On days 120–150, immunostaining intensity for S100A6 increased in the ganglion cells of ISPF, and there was strong staining in the interneuronal nerve fibers where the ISPF were connected with each other (Fig. 4f and 5 a, b). In addition, intense

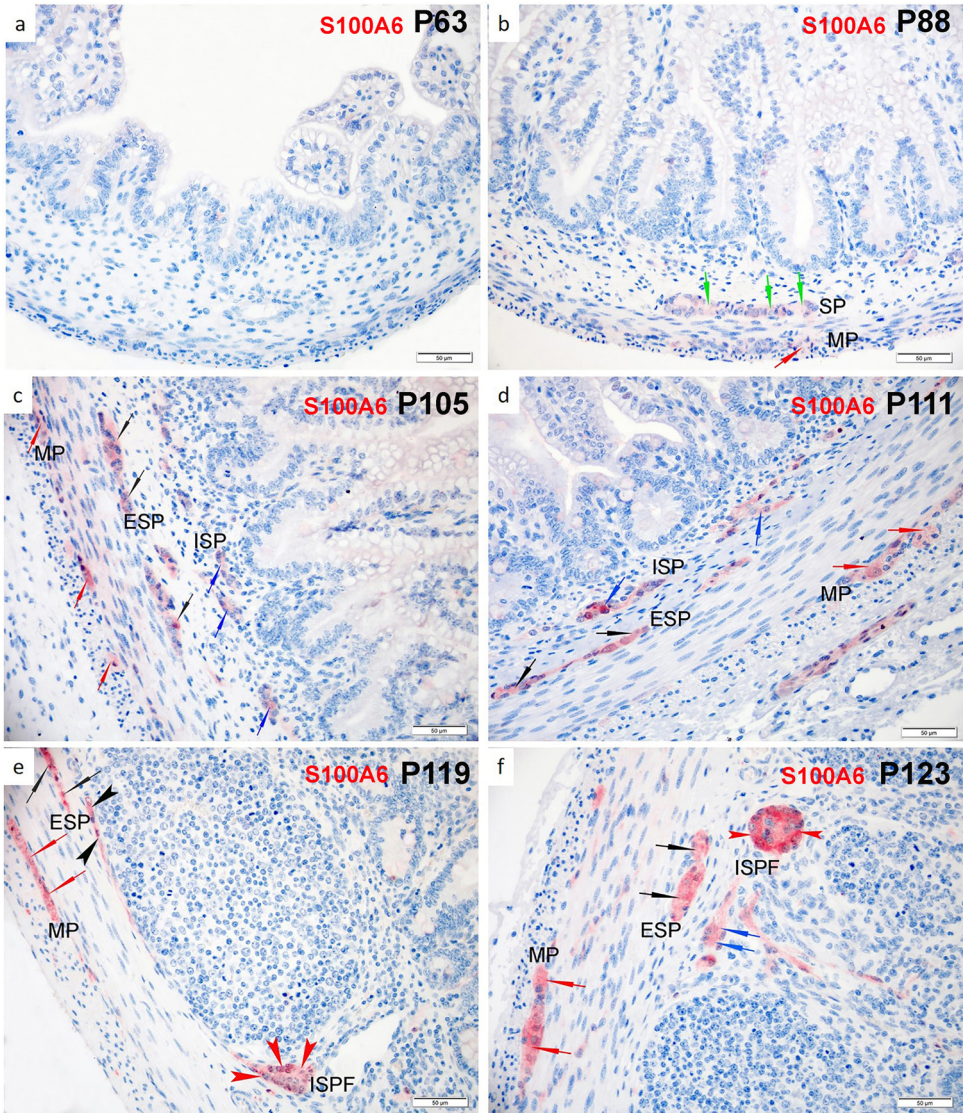


Fig. 4. Representative samples showing expression of S100A6 in ovine ileum of day 63 prenatal (P63; a), day 88 prenatal (P88; b), day 105 prenatal (P105; c), day 111 prenatal (P111; d), day 119 prenatal (P119; e), day 123 prenatal (P123; f). Strept-ABC, AEC, Paraffin. (a) A representative figure of ganglion cells exhibited any immunostaining for S100A6. (b, c, d, e, f) Representative figures of S100A6 positive ganglion cells in ileal SP (green arrows), ESP (black arrows), MP (red arrows), ISP (blue arrows), ISPF (red arrowheads) and neural networks linking ISPF (black arrowheads). Bar: 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

immunoreactivity was observed in MP and ESP ganglion cells (MP, ISP, ESP) during this period (Fig. 5c, d).

3.4. Vimentin immunoreactivity

In the early period of gestation (60–90 days), staining was present in some ICs showing a bipolar structure between circular and longitudinal muscles (Fig. 6a). The processes of these cells were found to be in close contact with MPs in some areas (Fig. 6b). No reaction was detected in SP during this period (Fig. 6c, d). In the following stages (90–120 days), reaction also began to appear in the cells around the ganglion cells, as well as ICs. There were strong or weak vimentin immunoreactivity in the cells of MP and SP, respectively (Fig. 6e, f), but no reaction in ISPF (Fig. 7a). During the last period of gestation (120–150 days), strong staining was observed in the cells around the ganglion cells of MP and ICs (Fig. 7b, c), and staining was also present in ISPF (Fig. 7d).

3.5. GFAP immunoreactivity

In the early stage of gestation (day 63), GFAP reaction was only found in MP (Fig. 8a). In the following stages (90–120 days), GFAP immunoreactivity was also detected in SP, with more positive staining in ESP than in ISP (Fig. 8b, c). In the plexuses, GFAP immunoreactivity was present in EGCs in the form of a network surrounding the ganglion cells (Fig. 8d). In the last period of gestation (120–150 days), the number of GFAP-positive plexuses (MP, ISP, ESP) increased (Fig. 8e), however the majority of GFAP-positive cells were localized in MPs. Interestingly, no or weak staining was observed in ISPF, unlike other plexuses (Fig. 8f1, f2)

Staining intensity: -, negative (no staining even at high magnification), +, weak (only visible at high magnification), ++, moderate (readily visible at low magnification), +++, strong (strikingly positive at low power magnification) staining. EN: Enteric neuron, EGC: Enteric glial cell, MP: Plexus myentericus, ESP: External submucosal plexus, ISP: Internal submucosal plexus, IM: Intramuscular cell or cell process.

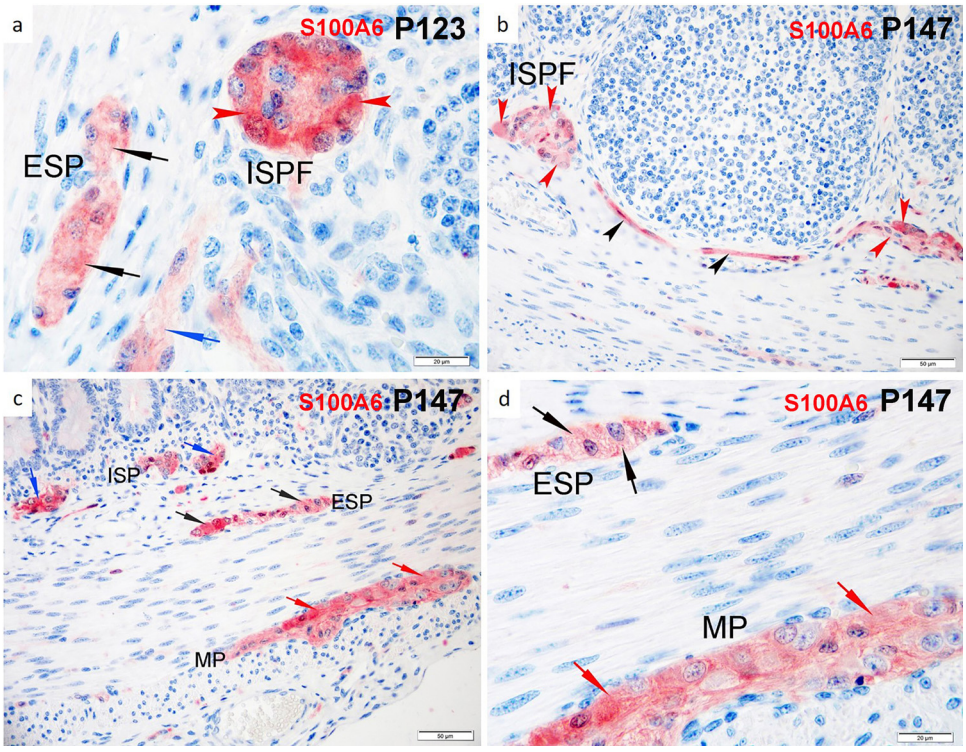


Fig. 5. Representative samples showing expression of S100A6 in ovine ileum of day 123 prenatal (P123; a), day 147 prenatal (P147; b, c, d), Strept-ABC, AEC, Paraffin. (a, b, c, d) Representative figures of NSE positive ganglion cells in ileal ESP (black arrows), MP (red arrows), ISP (blue arrows), ISPF (red arrowheads) and neural networks linking ISPF (black arrowheads). Scale bars: 20 µm (a, d), 50 µm (b, c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

We here examined the expression of NF-H, NSE, S100A6, vimentin and GFAP in the ileal ENS of sheep between days 60 and 150 of gestation. During the developmental period, we observed differences in the expression of the indicated markers in neurons and EGCs.

During embryonic development, NF-L is the first marker to be detected in nerve progenitors, followed by NF-M subunit expression during neurite formation (Cochard and Paulin, 1984). NF-H expression occurs later when the axon reaches its target and starts to grow radially, corresponding to the putative role of NF-H in determining axonal diameter. The distribution of these neuronal IFs differs with the neurofilament triplet proteins (NF-L, NF-M, NF-H) being observed throughout the mature nervous system and being especially plentiful in cells with large axons. However, the neurofilament triplet proteins are not produced by all neurons (Brehmer et al., 2002, 2004; Ganns et al., 2006). NF-H has been reported to be an important marker for distinguishing type II neurons from other neurons in many species (human, pig, guinea pig) in the small intestine (Ganns et al., 2006; Brehmer, 2007; Wolf et al., 2007). It has also been determined that NF-L (Mazzuoli et al., 2007) and NF-M (Qu et al., 2008) is molecules that can detect type II neurons in the ENS of sheep and mice, respectively. These type II cells have a round-shaped cell body and a number of nerve processes originating from this body (Rivera et al., 2009). During all the prenatal periods examined in this study, we observed NF-H expression in some nerve cells in the plexus and intramuscular nerve processes. Considering previous studies, we conclude that round-shaped ganglion cells that are positively stained in the plexus may be type II neurons. Unlike other plexuses in ISPF, the number of positively stained ganglion cells was lower, indicating that the neural cell profile of these plexuses may be different from that of MP and ESP.

NSE consists of gamma homodimers (γγ) weighing 78 kDa and has a half-life of approximately 24 h in body fluids. The predominant isoforms at the beginning of the enzyme are αα dimers. When cells begin to divide and migrate during embryonic development, αα dimers are converted to γγ dimers (Haque et al., 2016). NSE is a good indicator of neuronal maturation and differentiation since γγ dimer levels increase during neurogenesis and early neuronal differentiation. NSE is a marker for all neuronal species and neuroendocrine cells (Sarnat, 2013). In the human enteric system, it has been reported that there is no specific NSE expression in neurons until 11–12th week of gestation. Poor expression has been seen in MPs from the 12th week of gestation, with no reactions in the submucosa. From the 14th week of gestation, its expression has been observed in the ganglion cells in both MP and SP, with the intensity gradually increasing in the cell body and processes of nerve cells in later stages (Tam and Lister, 1986). Studies conducted in various species (human, pig, cat) in the postnatal period have shown that NSE expression is present in the body and processes of nerve cells in the plexus of ENS (Bishop et al., 1985; Scheuermann et al., 1989; Kleinschmidt et al., 2011). The findings from the present study were similar to those related to NSE expression in ENS in the fetal period in humans. In addition, the presence of strong NSE expression in all three plexuses at the end of the prenatal period was consistent with previous studies undertaken in various species. This research revealed the absence of NSE expression in the ileal ENS up to 85 days of gestation, indicating that neuronal maturation is not complete in the early stages of the fetal period. This suggests that in this period, the enolase enzymes in the nerve cells are comprised of α subunits, and these enzymes are gradually transformed to γγ subunits only after days 85 to 90 of gestation.

Vimentin is a class III intermediate filament produced in almost all neuronal precursor (Cochard and Paulin, 1984) and mesenchymal cells. The expression of vimentin is reduced when the neuronal precursors cease dividing and neurites start to be produced. Before the NF

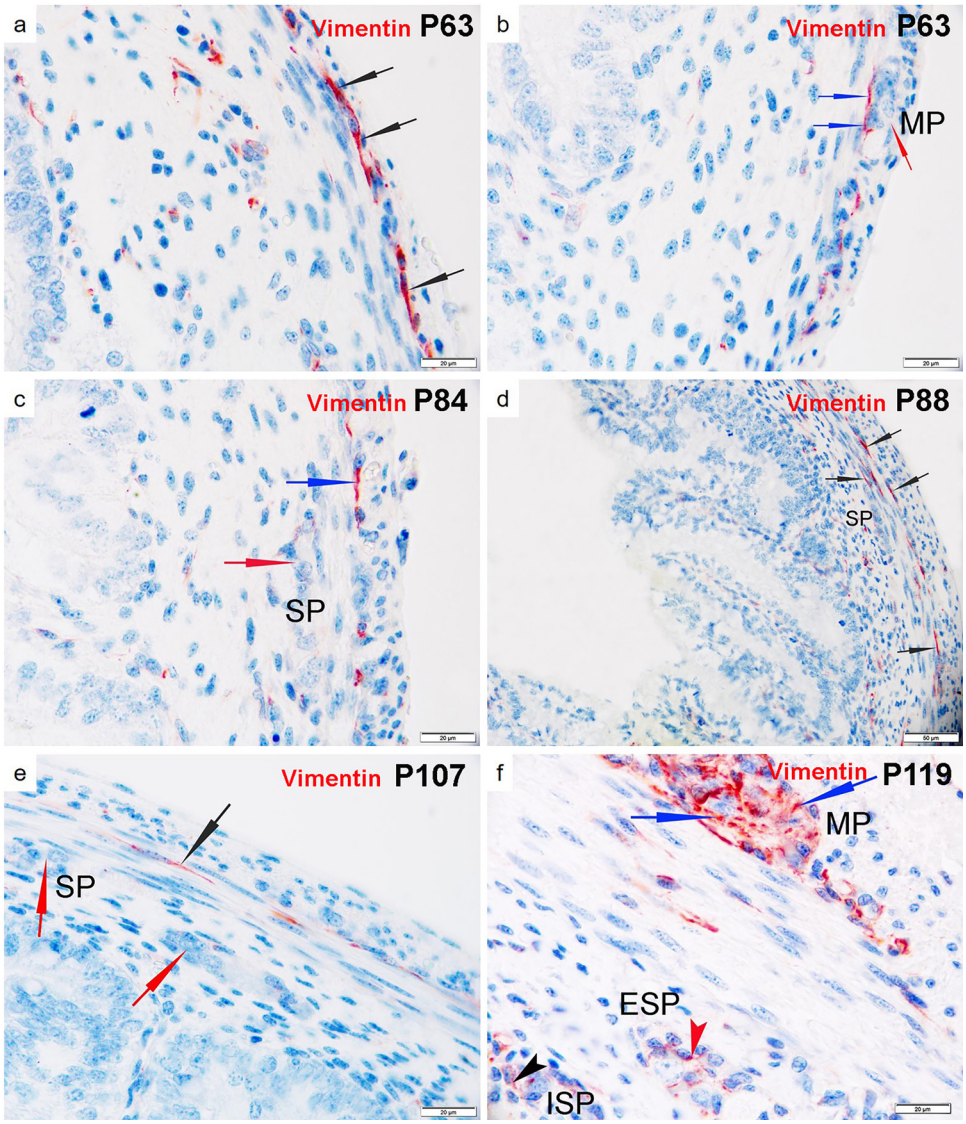


Fig. 6. Representative samples showing expression of Vimentin in ovine ileum of day 63 prenatal (P63; a, b), day 84 prenatal (P84; c), day 88 prenatal (P88; d), day 107 prenatal (P107; e), day 119 prenatal (P119; f) Strept-ABC, AEC, Paraffin. (a, b, c, d, e, f) Representative figures of vimentin positive ICs showing a bipolar structure (black arrows), vimentin positive cells around the ganglion cells in MP (blue arrows), ESP (red arrowhead), ISP (black arrowhead). Ganglion cells exhibited any immunostaining for vimentin (red arrows). Scale bars: 20 µm (a, b, c, e, f), 50 µm (d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

accumulation in the axon, a short-lived increase in vimentin is seen during axogenesis (Shea, 1990). The transition from the cells producing vimentin to those expressing NF is an overlapping gradual process(Yabe et al., 2003). During this process, co-polymerization of vimentin with NF occurs, in which these filaments are replaced by filaments possessing only the NF proteins of mature neurons. Within this neuronal regeneration, the up-regulation of vimentin is observed (Perlson et al., 2005; Shim et al., 2008; Toth et al., 2008). In addition to the nerve precursors in ENS, vimentin expression is also seen in EGCs in different developmental stages of the prenatal period. Moreover, unlike nerve cells, vimentin expression in EGCs has been reported to continue in adulthood (Masot et al., 2007; Gulbransen, 2014). Jessen and Mirsky (1985) reported that typical S100-positive EGC cells exhibiting vimentin expression were localized in MP in the mouse colon. Another cell group that expresses vimentin in the gastrointestinal tract is ICs, which reside in muscles, SP, and MP (Nahar et al., 1998; Sanders et al., 2014). Since ICs have a heterogeneous structure, markers such as c-kit, PDGFR α and Ano1 are used to distinguish their subgroups (Sanders et al., 2014). It has been determined that ICs show a bipolar structure, parallel to smooth muscles (Komuro, 2012). It has also been reported that ICs located in the plexus show a multipolar structure and surround ganglion cells (Sanders et al.). These cells have a pacemaker and mechanosensitive function (Komuro, 2012). Consistent with the literature, we did not observe vimentin expression in differentiated nerve cells in plexuses. As also stated in postnatal studies (Sanders et al., 2014), we detected vimentin expression in ICs, parallel to intramuscular smooth muscles in the prenatal period. Vimentin expression in the plexus was heterogeneous in both periodical and regional terms. Vimentin was expressed only in MP in the early phase (60–90 days), while it was also present in SP, albeit weak in the later stages (90–150 days). In all the periods examined, staining was prominently strong in MP. Given the current literature, cells stained in the plexus may be either EGCs or ICs. Therefore, double immunofluorescence staining with specific markers should be performed to precisely identify these stained cells.

The S100 family consists of more than 20 $Ca2^{+}$ binding proteins with a low molecular weight and have many important functions in

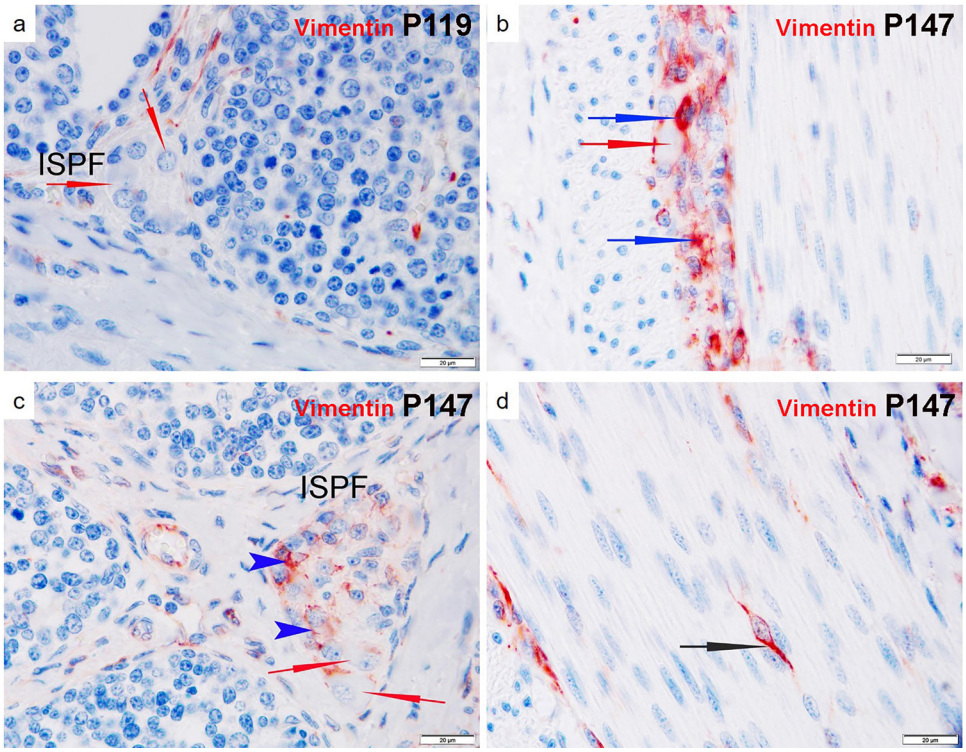


Fig. 7. Representative samples showing expression of Vimentin in ovine ileum of day 119 prenatal (P119; a), day 147 prenatal (P147; b, c, d), Strept-ABC, AEC, Paraffin. (a, b, c, d) Representative figures of vimentin positive ICs showing a bipolar structure (black arrows), vimentin positive cells around the ganglion cells in MP (blue arrows), ISPF (blue arrowheads). Ganglion cells exhibited any immunostaining for vimentin (red arrows). Scale bars: 20 µm (a, b, c, d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

both normal and pathological tissues. S100A6, a member of this family, comprises 90 amino acids in humans and 89 amino acids in mice and rats (Donato et al., 2017). Being a predominantly cytoplasmic protein, S100A6 may become associated with the plasma membrane and nuclear membrane in the presence of $Ca2^{+}$ (Leśniak and Filipek, 1996; Stradal and Gimona, 1999). S100A6 interacts with many target proteins and plays a role in the regulation of Ca^{2+} homeostasis, cell growth and differentiation, apoptosis, and cytoskeleton dynamics (Donato et al., 2017). Its expression level can be altered by the effects of tumor necrosis factor, estrogen, retinoic acid, palmitate, vasopressin, gastrin, and various stress factors (Leśniak et al., 2009). S100A6 is expressed in many different tissues and cells (Kuźnicki et al., 1992). In the fetal period, S100A6-positive reaction has been observed in some nerve cells and glia cells in the human cerebral cortex (Tiu et al., 2000). In a study on the rat nervous system, Yamashita et al. (1999) found S100A6-positive cells in the body and cellular processes of some nerve cells, some astrocytes, ependymal cells covering canalis centralis, and Schwann cells. In mice, S100A6 expression was detected in nerve cells, but S100A6 expression was not present in S100ß-positive glia cells (Filipek et al., 1993). There exists no study in the literature on the expression of S100A6 in ENS. In the present study, S100A6 expression was not present between 60 and 88 days of gestation, but after 90 days, there was a positive reaction in nerve cells in the plexuses, similar to those previously reported on CNS. We consider that the absence of S100A6 expression in nerve cells in the early period may be associated with cell growth and differentiation. Thus, double immunofluorescence staining with panneuronal markers should be undertaken to prove the presence of S100A6 expression in all nerve cells.

GFAP is an intermediate filament involved in the cytoskeletal structure of glia cells, and it supports adjacent neurons and blood-brain barrier, as well as helping to tolerate mechanical stress (Eng et al., 2000). In addition to astrocytes and radial glia cells in CNS, GFAP is expressed in non-myelinated Schwann cells in the peripheral nervous system and in EGCs in ENS (Gulbransen, 2014). Following nerve cell differentiation in the prenatal period, this intermediate filament begins to be expressed in EGCs of ENS, (Rothman et al., 1986). Studies on the stomach compartments of deer in the prenatal period have shown that GFAP expression is seen from day 67 of gestation (45% gestation) in ENS, gradually increasing approaching the completion of pregnancy (Franco et al., 2004; Redondo et al., 2005; Masot et al., 2007). Research conducted in recent years has reported that EGCs display a heterogeneous structure and GFAP expression is restricted to a specific subset of enteric glycans. In more than half the mucosal and submucosal glia in the ileum and colon, there is no GFAP expression. It has been particularly noted that the majority of extraganglionic and intramuscular glia do not have GFAP expression. In contrast, most enteric glia exhibit S100ß, PLP1 and Sox10 expression (Boesmans et al., 2015; Rao et al., 2015). Studies on astrocytes have reported high GFAP levels indicative of "reactivity" and cellular activation (Silver and Miller, 2004), and it has been suggested that GFAP expression may have a similar dynamic in enteric glia (Rao et al., 2015). Boesmans et al. (2015) observed cells with GFAP expression in myenteric ganglia, and 23% of intraganglionic myenteric glia showing GFAP but this was not found after seven days. Similar to other studies conducted in the prenatal period (Franco et al., 2004; Redondo et al., 2005; Masot et al., 2007), we found positive reactions in enteric glia only in MPs in the middle period of gestation, while this reaction was also present in SPs in later periods. Consistent with those reported in the literature (Boesmans et al., 2015; Rao et al., 2015), our finding related to the absence or weak nature of reaction in the plexuses positively stained with NF-H and S100A6 in ISPF supports the idea that GFAP expression only occurs in some but not all EGCs in the prenatal period.

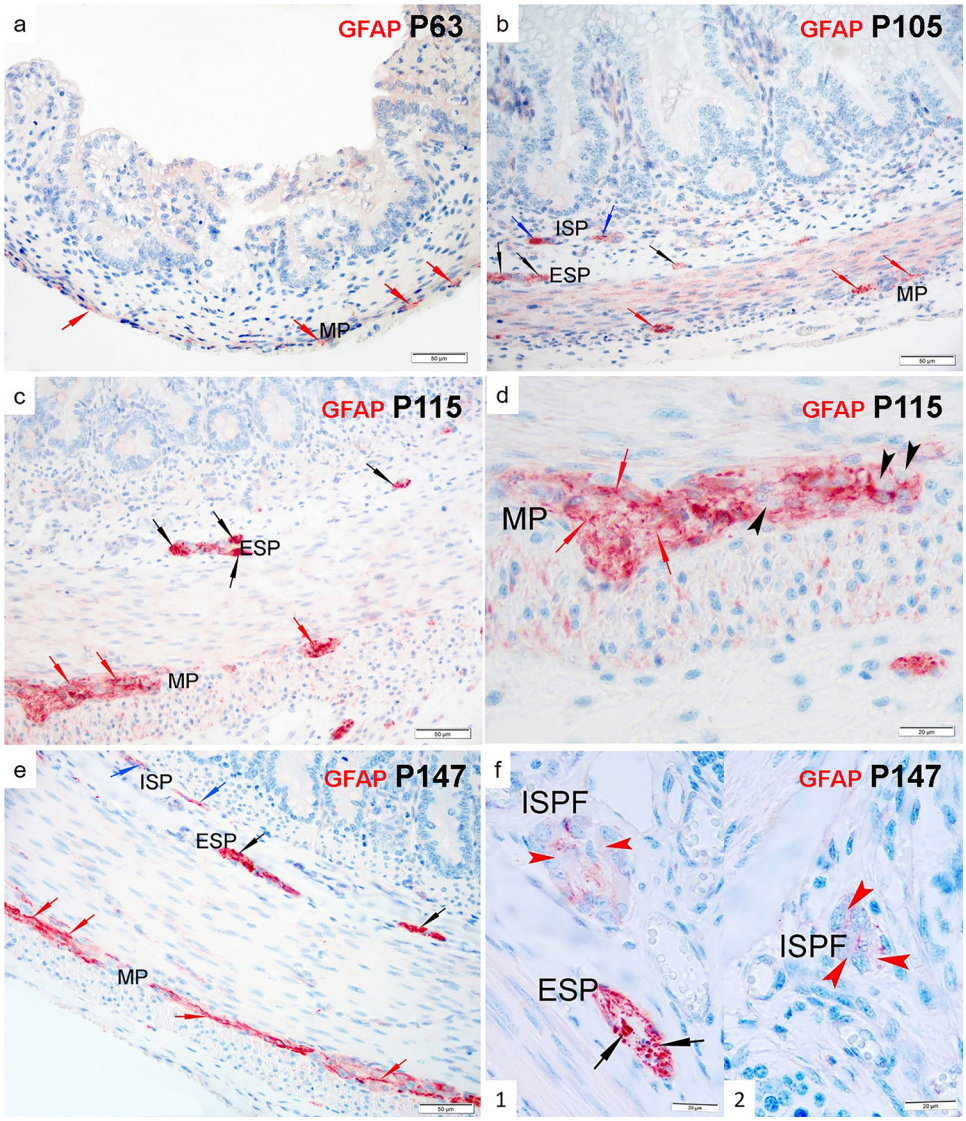


Fig. 8. Representative samples showing expression of GFAP in ovine ileum of day 63 prenatal (P63; a, b), day 84 prenatal (P84; c), day 88 prenatal (P88; d), day 107 prenatal (P107; e), day 119 prenatal (P119; f) Strept-ABC, AEC, Paraffin. Representative figures of GFAP positive EGC surrounding the ganglion cells in MP (red arrows), ESP (black arrows), ISP (blue arrows). Ganglion cells in all plexus (black arrowheads) and EGC in ISPF exhibited any immunostaining for GFAP (red arrowheads). Scale bars: 50 µm (a, b, c, d, e), 20 µm (f). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

In conclusion, this is the first study to demonstrate the expression of NF-H, vimentin, S100A6, NSE, and GFAP in the ovine ileal ENS during the prenatal period. ENS had a primitive structure in the early period of gestation (60–90 days), gradually developed, and acquired an expression profile similar to that in adulthood in the last period of gestation (120–150 days). It was observed that the expression of the examined markers increased toward the end of pregnancy. Our results also indicate that neurons and EGCs show heterogeneity. It is unlikely that GFAP is used as a panenteric marker or NF-H as a panneuronal marker. The expression of S100A6 in enteric neurons in the prenatal period is shown for the first time in the literature.

5. Authorship contributions

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Conception and design of study: M. Özbek, M.F. Bozkurt, F. Beyaz.

Acquisition of data: M. Özbek, M.F. Bozkurt, L. Ergün, E. Ergün.

Analysis and/or interpretation of data: M. Özbek, F. Beyaz, M.F. Bozkurt.

Category 2

Drafting the manuscript: M. Özbek, M.F. Bozkurt, L. Ergün, F. Beyaz, E. Ergün.

Revising the manuscript critically for important intellectual content: M. Özbek, M.F. Bozkurt, L. Ergün, F. Beyaz, E. Ergün.

Category 3

Approval of the version of the manuscript to be published (the names of all authors must be listed): Mehmet ÖZBEK, Mehmet Fatih BOZKURT, Feyzullah BEYAZ, Emel ERGÜN, Levent ERGÜN.

Acknowledgements

All persons who have made substantial contributions to the work reported in the manuscript (e.g., technical help, writing and editing assistance, general support), but who do not meet the criteria for authorship, are named in the Acknowledgements and have given us their written permission to be named. If we have not included an Acknowledgements, then that indicates that we have not received substantial contributions from non-authors.

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