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Research article

GDNF and GFRα co-receptor family in the developing feline gut

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ABSTRACT

Glial cell-line derived neurotrophic factor (GDNF) and the GFRα co-receptors play a role in the developing enteric nervous system. The co-receptors elicit their action by binding receptor tyrosine kinase RET.

This immunohistochemical study reports the presence of GDNF and its specific co-receptor GFRα1 in the cat gastrointestinal apparatus during development, from stage 9 to 22. At stage 9 and 11, immunoreactivity (IR) to GDNF was observed in the cells of mesenchyme of the anterior gut. From stage 14 to 22, GDNF IR was detected in nervous plexuses; moreover, GDNF and GFRα1 IR appeared localized in gastrointestinal endocrine cells. The presence of GDNF in the enteric nervous system and in the endocrine cells suggests an involvement of this neurotrophic factor in the gastrointestinal development. Moreover, the presence of the co-receptor GFRα1 in endocrine cells and its absence in the enteric nervous system seems to indicate a different mode of transduction of GDNF signal. GFRα2 and GFRα3 co-receptors were not detected.

1. Introduction

The GDNF family of ligands (GFLs) consists of four members: glial cell-line derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN) (Baloh et al., 2000). They activate a receptor complex formed by RET and co-receptor (GFRα) which conveys specificity of the transduction signal. GDNF binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4 co-receptors. Some cross-talks were described between GDNF/GFRα2 and ARTN/GFRα1 (for a review see Sariola and Saarma, 2003).

GFLs family factors and their specific co-receptors play an important role in regulating a wide range of processes in adult and developing animals. In adulthood, previous studies have documented differences in neurotrophic factor presence in endocrine cells and the nervous system of the gut in adult higher vertebrates (Lucini et al., 2002), and lower as, e.g., the frog (Maruccio et al., 2004), and the fish (Lucini et al., 1999, 2003, 2005; de Girolamo et al., 1999; Hannestad et al., 2000). During development, GDNF is a trophic factor for many neuronal populations, specifically involved in enteric neurogenesis (Chalazonitis et al., 1998; Hearn et al., 1998; Heucheroth et al., 1998; Taraviras et al., 1999; Worley et al., 2000). Also, GFRα1, GFRα2 and GFRα3 play a role in the developing enteric nervous system (Marcos-Gutierrez et al., 1997; Widenfalk et al., 1998; Golden et al., 1999; Shepherd et al., 2004).

GDNF knockout mice, GFRα1 knockout mice and/or RET knockout mice display an absence of enteric neurons (Schuchardt et al., 1994; Pichel et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998; Tomac et al., 2000) leading to aganglionosis, reminiscent of Hirschprung's disease (Uesaka et al., 2007). In contrast, studies addressing the involvement of GFLs in the development of endocrine cells have not been reported.

The occurrence of GDNF and its specific receptor in gut development has been studied mainly in animal models such as the rat and mouse (Golden et al., 1999), chick embryo (Homma et al., 2000) and zebrafish (Shepherd et al., 2004). Studies investigating the presence of other co-receptors are few (Dolatshad and Saffrey, 2007; Yu et al., 1998).

In order to extend studies to other animal models, we chose to study the cat due to its fairly short period of gestation. Furthermore, we have previously used this species to study the presence of RET in the gut (Lucini et al., 2013). Therefore, the aim of this study has been the investigation of the presence and distribution of GDNF and GFRα co-receptors family in the developing gut of the domestic cat, using samples at different stages.

2. Materials and methods

2.1. Animals

The cat embryos were removed from ovariohysterectomized pregnant queens. The queens, at 54 min before surgery were premedicated with atropine sulphate (ATI) 0.025 mg/kg SC and

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Table 1
Antisera used for IHC experiments.

Antisera	Source	Host	Characteristics	Dilution for light microscopy	Dilution for fluorescence microscopy
GDNF (D-20)	sc-328 Santa Cruz Biotechnology	Rabbit	C-terminus of GDNF human origin	1/400	1/40
GFR alpha 1 (H-70)	sc-10716 Santa Cruz Biotechnology	Rabbit	C-terminus of GFR alpha1 human origin	1/300	1/30
GFR alpha 2 (H-89)	sc-28953 Santa Cruz Biotechnology	Rabbit	C-terminus of GFR alpha2 human origin	1/200	1/20
GFR alpha 3 (M-210)	sc-28954 Santa Cruz Biotechnology	Rabbit	N-terminus of GFR alpha 3 mouse origin	1/200	1/20
Chromogranin	642 DiaSorin Minn, USA	Rabbit	against Bovine antigen	1/1000	1/100

Rimadyl (caprofen, Pfizer Inc., New York, NY, USA) 2 mg/kg. Then, they were sedated with Domitor (medetomide Hydrochloride, Pfizer Inc.) 0.05 ml/kg IM and Altadol (tramadol chlorohydrate Formevet Animal Health) 2 mg/kg IM. Anaesthesia was induced with Rapinovet 0.4 ml/kg (propofol 4 mg/kg, Schering-Ploug Spa) and maintained after tracheal intubation with 1.5% isoflurane in 1 l/min of oxygen.

The embryos identified according to Knospe (2002) as stage 9 (15–17 days), 11 (17–18 days), 14 (21–23 days) and 16 (25–28 days) were fixed in toto, whereas the foetuses at stage 19 (38–44 days) were fixed after decapitation. From foetuses at stage 22 (55 days approximately) the gut was removed and fixed. Fixation was made by immersion in Bouin's fluid for 12–24 h at room temperature (RT). The specimens were dehydrated in an ethanol series and embedded in paraffin wax, then cut in 7 μ-thick sections.

2.2. Single immunohistochemical staining

Peroxidase-antiperoxidase (PAP) method (Sternberger, 1986) was used for immunohistochemical staining. Dewaxed sections were hydrated and subjected to microwave oven treatment to unmask the antigens (0.01 M sodium citrate buffer, pH 6.0, for 10 min at 750 W) (Reynolds et al., 1994), then rinsed in distilled water and treated with 3% H_2O_2 for 20 min to block endogenous peroxidase activity. After rinsing in phosphate-buffered saline (PBS), pH 7.4, containing 0.2% triton X-100 and 0.1% bovine serum albumin (BSA), the sections were incubated for 30 min at RT with 1:5 normal serum of the species in which the primary antiserum was raised (rabbit serum: 011-000-120, goat serum: 005-000-121 Jackson Immuno Research, Baltimore Pike, PA, USA) to block background. The sections were then incubated for 24 h at 4 °C in a humid chamber with each of the respective primary antibodies used (see Table 1).

After incubation, the sections washed in PBS, were further incubated for 30 min at RT with antiserum raised in goat against rabbit IgG (GAR, 1:50, 111-035-003 Jackson Immuno Research). Then the sections were washed in PBS and incubated for 30 min at RT with rabbit PAP complex (1:100; 123-005-025 Jackson Immuno Research). After rinsing, the immunoreactive sites were visualized using a fresh solution of 10 μg of 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.) in 15 ml of Tris buffer 0.5 M, pH 7.6, containing 1.5 ml of 0.03% H_2O_2.

2.3. Double immunohistochemical staining

Double immunohistochemical staining using two primary antisera raised in the same species; i.e. rabbit, was performed according to Wessel and McClay (1986), who used fluorochrome-conjugated Fab fragments to avoid cross-talk of subsequent antisera. Primary antisera used for double immunohistochemical stainings were GDNF/GFRα1, GDNF/Chromogranin, GFRα1/Chromogranin. Dewaxed, rehydrated, blocked sections were rinsed in 0.01 M PBS (pH 7.4) containing 0.2% Triton X-100 and 0.1% BSA. Sections were then incubated with primary antiserum (see Table 1) in 1:5 normal donkey serum for 48 h at RT. The sections were washed in PBS and incubated with GAR-Fab fragment conjugated to FITC

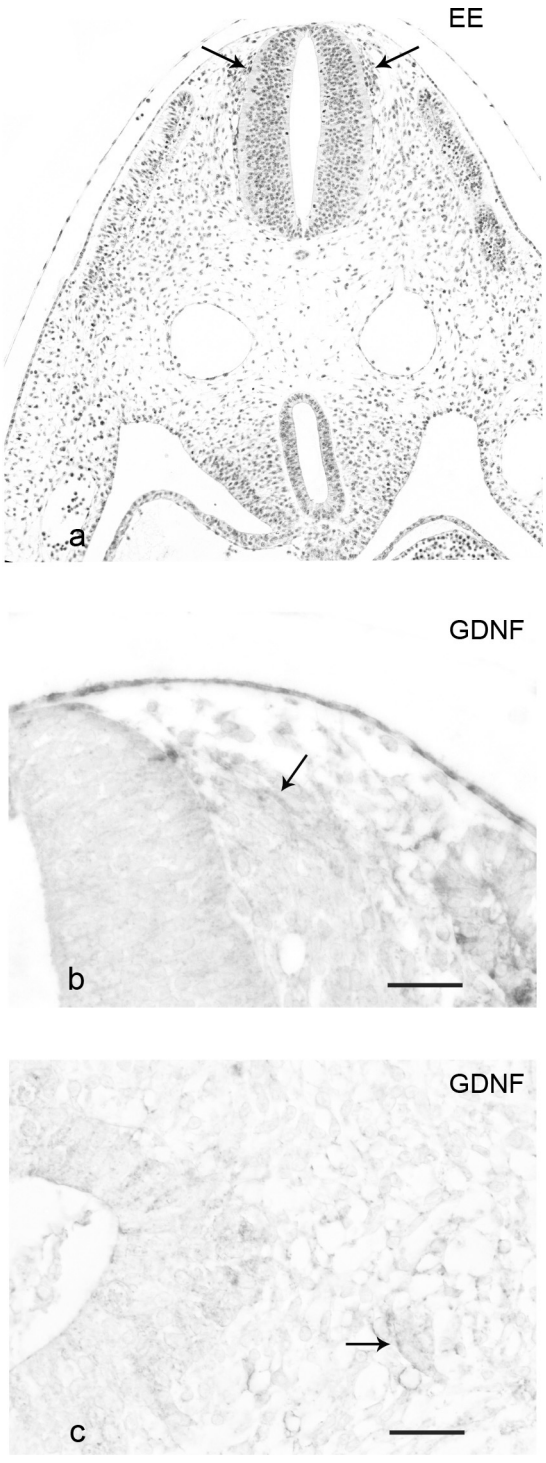

Fig. 1. Cat embryo stage 9. (a) EE staining. Overview of section showing neural tube (nt), neural crest cells (arrows), somites (s), dorsal aort (da), and mesenchyme (arrowhead) around foregut (g). (b) GDNF IR in cells of neural crest (arrow); (c) GDNF IR in some mesenchymal cells around foregut (arrow). Scale bars = 200 μ: (a); 50 μ: (b) and (c).

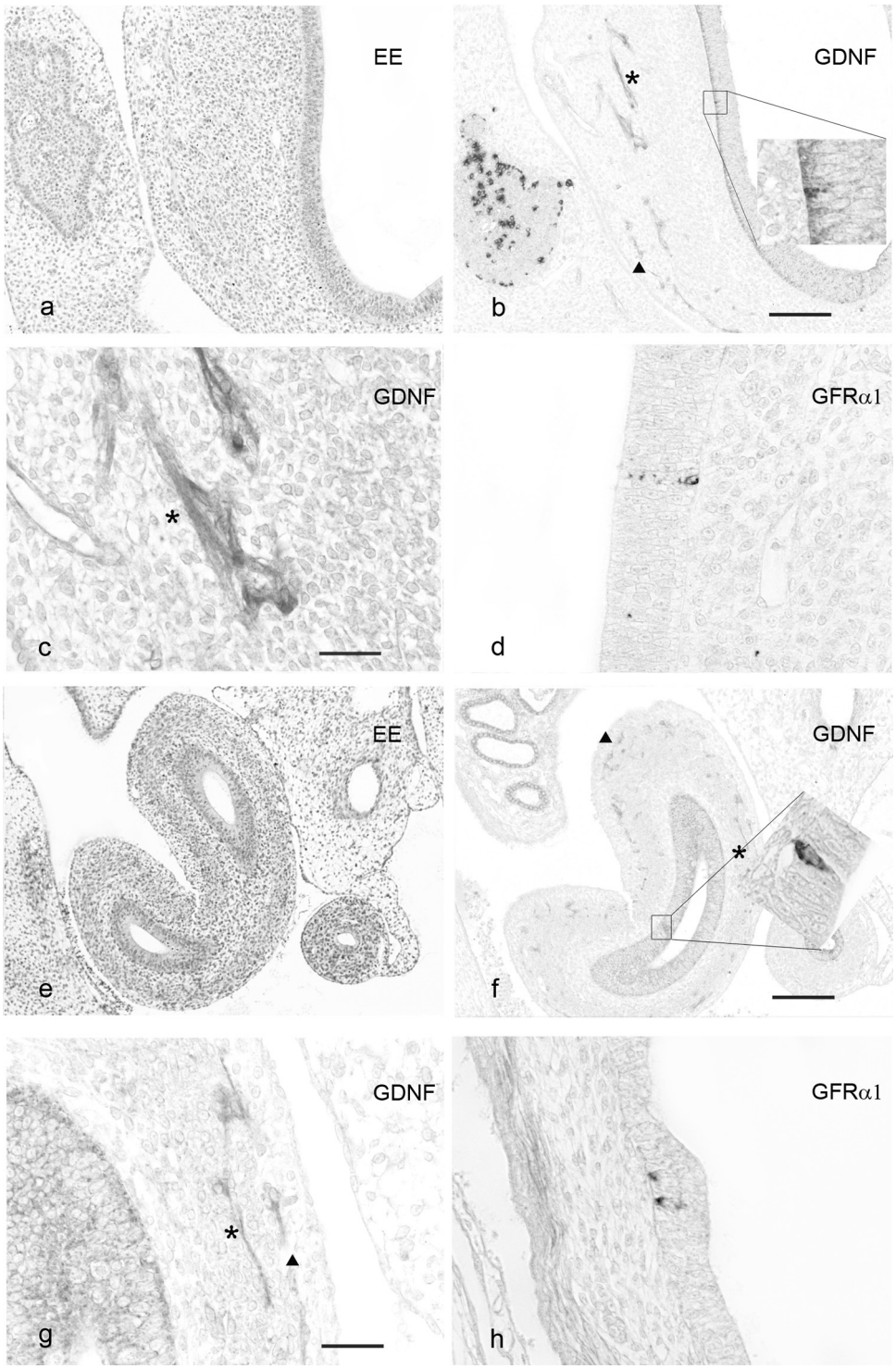

Fig. 2. Cat embryo stage 14. (a) EE staining of section showing pancreas (p) and stomach (s). (b) GDNF IR in neurons and fibers of the myenteric (triangle) and submucous (asterisk) plexuses and endocrine cell of stomach. (b′) and (c) Higher magnification of (b). (d) GFRα1 IR in endocrine cell of stomach epithelium; (e) EE staining of section showing small intestine (i). (f) GDNF IR in neurons and fibers of the myenteric (triangle) and submucous (asterisk) plexuses and endocrine cell of anterior gut. (f′) and (g): Higher magnification of (f). (h) GFRα1 IR in endocrine cells of the anterior gut epithelium. Scale bars= 200 μ: (a), (b), (e) and (f); 50 μ: (b′), (c), (f′) and (g); 100 μ: (d) and (h).

fluorochrome, diluted 1:30, (111-097-007, Jackson), for 2 h at RT. Thereafter the sections were rinsed in PBS and incubated with the other primary antiserum (see Table 1), with normal donkey serum 1:5 for 48 h at RT. After rinsing in PBS, the sections were treated with affinity-pure donkey anti-rabbit IgG conjugated to TRITC fluorochrome (111-025-152, Jackson), diluted 1:50 for 2 h at RT. Finally the sections were washed with PBS, mounted with glycerin diluted with PBS 1:1.

2.4. Microscopical photographs

Immunohistochemical stainings were photographed using a Leica microscope DM RA2 (Leica Camera AG, Solms, Germany) connected to a Leica DC 300F camera for light microscopy and to a Leica DC camera for fluorescence and stored in a Leica IM 1000 archive. Photomicrograph processing and lettering was carried out by using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA). Color balance,

contrast, and brightness of the images were adjusted to a variable extend and most of the color images were converted to gray-scale.

2.5. Controls

Single immunohistochemical staining specificity was tested by successive substitution of the primary or secondary antisera or the PAP complex with normal serum or PBS in repeated trials. Adsorption controls were also made by using homologous and heterologous antigens (Lucini et al., 2008).

The specificity of the immunoreactivity in double labeling with Fab fragments was tested by omitting the primary antibody in the second staining, and by the two assays described below and according to Negoescu et al. (1994).

2.5.1. Test 1

To demonstrate the monovalence of the Fab fragment, after the first staining, sections were incubated for 48 h at RT with a PAP rabbit complex (A200/V, diluted:100, UCB, Braine-l'Alleud, Belgium) which serves as the second-staining primary antibody.

2.5.2. Test 2

To demonstrate the complete Fab saturation of rabbit IgG epitopes, after the first staining, sections were incubated for 24 h at 4 °C with a peroxidase-coupled donkey anti-rabbit Fab fragment (711-036-132, diluted 1:30, Jackson) which serves as the second-staining secondary antibody.

3. Results

This study reports the presence of GDNF and its high affinity receptor GFRα1 in the cat gastrointestinal apparatus during development from 15–17 to 55 days of gestation, using samples at different stages. No immunopositivity to GFRα2 and GFRα3 was detected in any developmental stages analyzed.

No reaction was observed in controls performed by substituting the primary antibodies with PBS, normal serum or antibodies adsorbed by their homologous antigens. On the contrary, the reaction was not modified by the substitution of primary antibodies with antibodies adsorbed by heterologous antigens. Moreover, test 1 and test 2 controls were negative.

In the embryos at stage 9 (Fig. 1a), immunoreactivity (IR) to GDNF was observed in few cells of neural crest extending in the mesenchyme latero-ventrally to the neural tube (Fig. 1b) and in scattered or grouped cells in the mesenchyme around the developing foregut (Fig. 1c). No IR to GFRα1 was observed.

In the embryos at stage 11, GDNF IR was observed in small cell cluster in the mesenchyme around the anterior gut. No IR to GFRα1 was detected.

In the embryos at stage 14, in the gastric (Fig. 2a) and in anterior intestinal region (Fig. 2e) GDNF IR was seen in neurons and nervous fibers of myenteric and submucous plexuses (Fig. 2b, c, f and g). In the posterior intestinal wall, GDNF IR was mainly detected in nervous fibers and few neurons of the myenteric plexus. GDNF IR was also detected in the perinuclear cytoplasm of scattered cells of the gastric (Fig. 2b′) and anterior gut epithelium (Fig. 2f). These cells were round in shape and located at the base of the epithelium. GFRα1 IR was observed in scattered cells in the basal epithelium of the gastric wall (Fig. 2d). These cells appear mainly round or ovoid in shape, with positive staining in the perinuclear cytoplasm. GFRα1 IR was also observed in some cells of the anterior intestine (Fig. 2h). They appeared mainly oval or elongated in shape with positivity in the basal and apical cytoplasm.

In the embryos at stage 16, GDNF IR was observed in neurons and fibers of the both nervous plexuses in stomach and all intestinal tracts (Fig. 3a). In addition, IR to GDNF was frequently observed in the perinuclear cytoplasm of scattered cells localized in the basal epithelium of the gastric wall (Fig. 3b) and more rarely in the intestinal epithelium. The positive cells were mainly round in the stomach, and elongated in the gut. Similarly, GFRα1 IR was detected in scattered epithelial cells of the gastric (Fig. 3c) and intestinal wall. Also, these cells were mainly round in gastric and elongated in the gut epithelium.

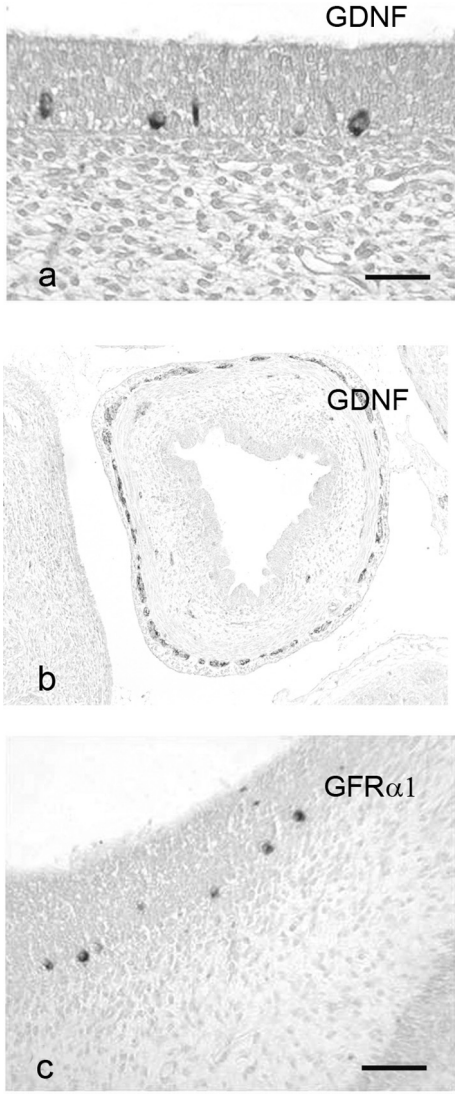

Fig. 3. Cat embryo stage 16. (a) GDNF IR in round endocrine cells of the gastric epithelium (b) GDNF IR in neurons and fibers of myenteric and submucous plexuses of posterior intestine; (c) GFR alpha 1 IR in round endocrine cells of the gastric epithelium. Scale bars = 200 μ.: (a); 50 μ.: (b) and (c).

In the foetuses at stages 19 (Fig. 4a) and 22, intense GDNF IR was observed in neurons and nervous fibers of the myenteric and submucous gastric and intestinal (Fig. 4b) plexuses. GDNF immunopositive fibers were also observed in circular muscle layer, connecting the myenteric and submucous plexuses. In addition, immunopositivity to GDNF was detected in some epithelial cells throughout the whole gastroenteric tract (Figs. 4c and 5d and f). In the gastric mucosa, positive cells, with IR localized in the perinuclear cytoplasm, were distributed in the basal epithelial region, and were round in shape. In the intestinal epithelium, GDNF IR was detected in scattered cells, elongated or ovoid in shape. The positivity was detected in the perinuclear cytoplasm, and sometimes in the apical cytoplasmic portion.

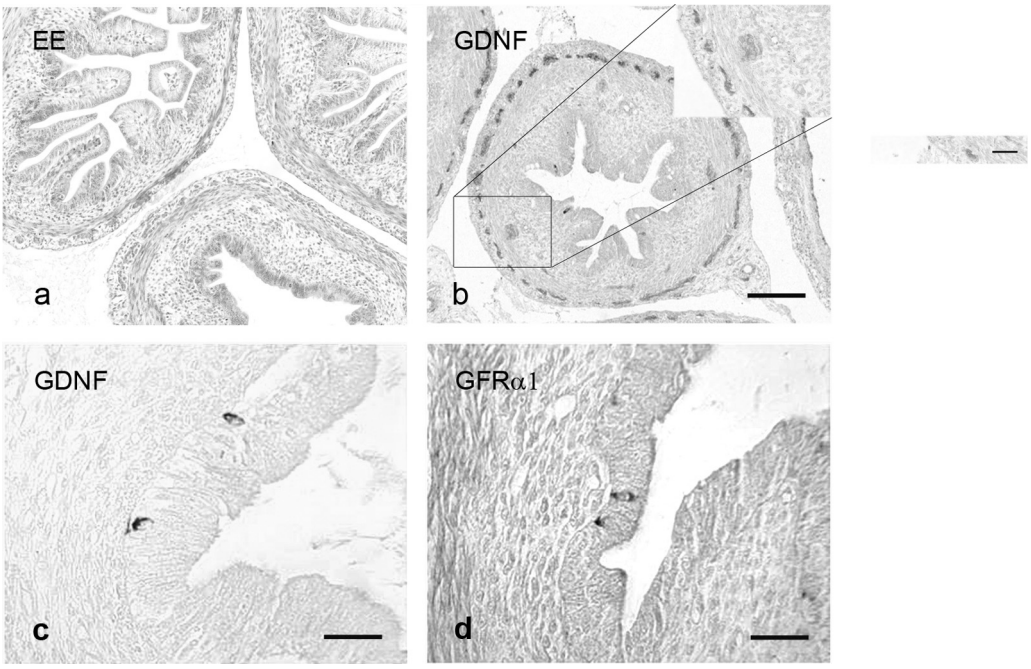

Fig. 4. Cat foetus stage 19. (a) EE staining of section showing anterior and posterior intestine; (b) GDNF IR in neurons and fibers of the myenteric and submucous plexuses of anterior intestine; (b′) Higher magnification of (b). (c) GDNF IR in elongated endocrine cells of the anterior intestinal epithelium; (d) GFRα1 IR in elongated endocrine cells of the anterior intestinal epithelium. Scale bars = 200 μ.: (a) and (b); 50 μ.: (b′), (c) and (d).

GFRα1 IR was detected in few and round cells of the stomach (Fig. 5c) and in elongated cells of the intestinal epithelium (Figs. 4d and 5e). The positivity was localized in the perinuclear cytoplasm and sometimes in the apical cytoplasmic portion.

To characterize the cell epithelial population showing IR to GDNF and the co-receptor, antiserum against chromogranin, an endocrine marker, was employed.

At all analyzed stages, some chromogranin positive cells (Fig. 5a) detected in the epithelium of gastrointestinal apparatus were positive to GDNF (Fig. 5b) and GFRα1. Moreover, the endocrine cells displayed co-presence of GFRα1 and GDNF both in gastric (Fig. 5c and d) and intestinal tract (Fig. 5e and f).

4. Discussion

This study reports the presence of GDNF and its specific co-receptor GFRα1 in the cat gastrointestinal apparatus during development. GFRα2 and GFRα3 co-receptors are not detected. The results obtained with positive and negative controls confirm the specificity of the reactions.

GDNF positive cells observed at stages 9 and 11 may be considered as neuroblast migrating toward the gut. Indeed, it is known that neurons of the vertebrate ENS arise from vagal crest cells, migrate through branchial arches in the foregut mesenchyme and then in the gut wall (for review see Burns, 2005; Burns and Thapar, 2006). GDNF distribution in the cat, at stage 11, corresponds to the start of colonization of the foregut by enteric neural crest cells in rodents (Golden et al., 1999).

From stage 14 onward, GDNF is detected first in the nervous plexuses of gastric region, and then in those of intestinal tracts. In addition, in each region, GDNF appears first in the myenteric plexus, and then in the submucous plexus, Thus, the distribution of GDNF IR progresses cranio-caudally, from the stomach to different tracts of the gut, and centripetally, in an outer–inner progression through the wall of the gut. These findings reflect the vagal crest cell progression along the developing gut and their positioning on both sides of the circular muscle layer in regions corresponding to the presumptive myenteric and submucousal ganglia (for review see Burns, 2005; Burns and Thapar, 2006). Thus, the localization of GDNF parallels the development of ENS in cat as reported in other mammals (Gershon, 1997; Golden et al., 1999; Schafer and Mestres, 1999; Mallappa et al., 2008), and birds (Homma et al., 2000). Interestingly, other trophic factors (Maruccio et al., 2008) and neurotransmitters (Vittoria et al., 1992; Lucini et al., 1993) show similar distribution in the developing gut of duck. In addition, in the cat, GDNF displays a similar distribution to RET (Lucini et al., 2013) confirming that they are both involved in the migration of vagal crest cells in the gut (Young et al., 2001; Natarajan et al., 2002), and in the proliferation and neuronal differentiation of ENS precursors (Chalazonitis et al., 1998, 2012; Hearn et al., 1998; Heucheroth et al., 1998; Mwizerwa et al., 2011).

GFRα1 is completely lacking in ENS of all gastrointestinal regions of developing cat.

GFRα1 mRNA was detected in mouse (Golden et al., 1999) and zebrafish (Shepherd et al., 2004). These data could suggest that GFRα1 mRNA might not be stored in the cytoplasm, and/or stored at a level below the detection sensibility of our demonstration method. However, in the chick, GFRα1mRNA was lacking (Homma et al., 2000) and GFRα1 protein was detected (Schiltz et al., 1999).

The lack of GFRα1 in the cat ENS could suggest that GDNF may operate through RET receptor that binds GFRα1, supplied in a soluble or trans form (Trupp et al., 1997) or by alternative binding to other co-receptor (Homma et al., 2000).

GDNF is also distributed in epithelial cells of stomach first, and intestine later, from stage 14 onward. Positive cell distribution is higher in the gastric region, and lowers in the caudal tracts of gut. This pattern follows the cranio-caudal progression of gut development. From stage 14 onward, GFRα1 is also detected in cells of the gastric and gut epithelium, with similar localization and distribution to GDNF.

Interestingly, GDNF is co-localized with GFRα1 in epithelial cells. In addition, GDNF-GFRα1 cells are also chromogranin positive, and thus endocrine. These results are in agreement with data reported in a previous study on cat pancreas (Lucini et al.,

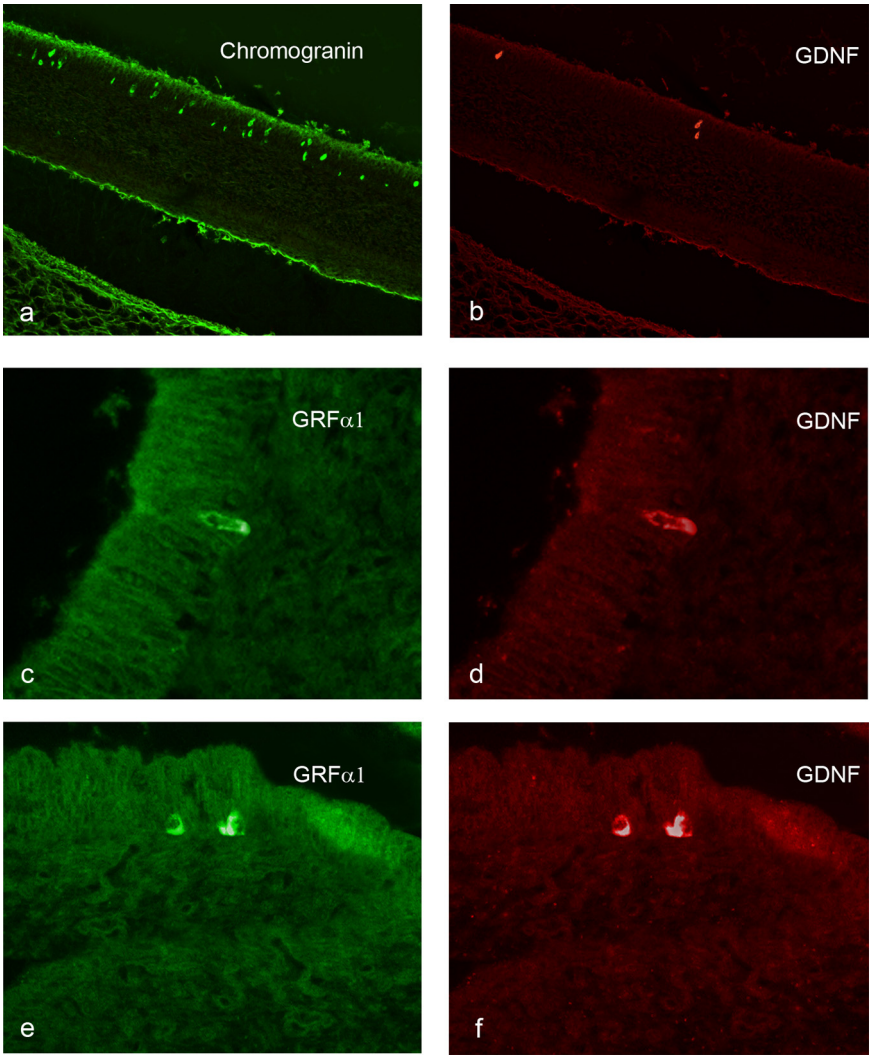

Fig. 5. Cat foetus stage 22. (a) and (b) double immunostaining chromogranin/GDNF in intestinal epithelium: chromogranin (green), GDNF (red); (c) and (d) double immunostaining GFRα1/GDNF in stomach epithelium: GFRα1 (green), GDNF (red); (e) and (f) double immunostaining GFRα1/GDNF in intestinal epithelium: GFRα1 (green), GDNF(red). Scale bars = 200 μ: (a) and (b); 50 μ: (c)–(f). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2008) showing GFRα1 in GDNF/chromogranin immunoreactive cells. Furthermore, the co-presence of GDNF and GFRα1 in cells of gastroenteric epithelium strengthens the hypothesis that GDNF signal could be transduced by co-receptor GFRα1, independently of RET receptor (Sariola and Saarma, 2003; Airaksinen et al., 2006).

The presence of endocrine cells containing GDNF is supported by previous findings on GDNF in adult gut of teleost (Lucini et al., 2005), and other neurotrophic factors in different vertebrate gut (Lucini et al., 2002, 2003; Maruccio et al., 2004), and suggests other roles aside from ENS development for neurotrophic factors.

Moreover the presence of neurotrophic factors in the gastro-entero-pancreatic endocrine cells may help to define the intestine as a kind of sensory organ as postulated by Furness et al. (1999).

In addition, the presence of GDNF and GFRα1 in endocrine cells suggests an autocrine role for GDNF as reported in cat pancreas development (Lucini et al., 2008) and in other mammalian tissues (Krieglstein et al., 1996; Belluardo et al., 1999; Lucini et al., 2003, 2005).

The onset of GDNF IR first in precursors of developing ENS and subsequently in gut endocrine cells seems to indicate a possible double origin of this neurotrophic factor in vagal crest cells and in epithelial gut cells. GDNF immunoreactive neurons and fibers in the ENS may arise from vagal crest cells, whereas GDNF positive endocrine cells may be of endodermic origin (Andrew et al., 1983, for review see Schonhoff et al., 2004).

Finally, these data complete the previous study on the cat gut development (Lucini et al., 2008, 2013), confirming the involvement of GDNF in ENS biology during gut development in this mammalian species also, as reported in other vertebrates (Shepherd et al., 2001).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aanat.2014.03.001.

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