

# Glucagon-like peptide-1 is co-localized with neurotensin in the chicken ileum

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**Abstract** Glucagon-like peptide (GLP)-1 and neurotensin (NT) are distributed throughout the chicken ileum. Here, we attempt to determine if GLP-1 and NT co-localize in the chicken ileum by using immunofluorescence, immunocytochemistry and in situ hybridization techniques. Three types of enteroendocrine cells, GLP-1<sup>+</sup>/NT<sup>+</sup>, GLP-1<sup>+</sup>/NT<sup>-</sup> and GLP-1<sup>-</sup>/NT<sup>+</sup> cells, were detected in the mucosal epithelium by the double immunofluorescence method. The ratio of GLP-1<sup>+</sup>/NT<sup>+</sup> cells at the crypts in the distal ileum was significantly higher than that in the proximal ileum. The ratios of the three cell types were similar along the crypt–villous axis in the proximal ileum but the percentage of GLP-1<sup>+</sup>/NT<sup>+</sup> cells significantly decreased at the middle part of villi relative to crypts and the bottom part of villi in the distal ileum. Enteroendocrine cells that were immunoreactive to both GLP-1 and NT peptides and showed both proglucagon and NT precursor mRNA signals were found in the crypts of the distal ileum but not in the villous epithelium. The results from performing an immunocytochemical method with colloidal gold indicated that the GLP-1 content within GLP-1<sup>+</sup>/NT<sup>+</sup> cell secretory granules decreased stepwise from the crypt to the middle part of the villus but the NT content in these granules

increased in this direction. These findings reveal that the cells producing both GLP-1 and NT are mainly localized in the crypts of the chicken ileum but these endocrine cells specialize in NT-producing cells at the villous epithelium of the distal ileum.

**Keywords** Chicken · Co-localization · Glucagon-like peptide-1 · Ileum · Neurotensin

## Introduction

Various kinds of enteroendocrine cells have been identified in the mucosal epithelium of both the mammalian and avian alimentary tracts by using immunohistochemical staining and ultrastructural observation (Buchan and Polack 1980; Fujita and Kobayashi 1977; Larsson 1979; Rawdon and Andrew 1999). These cell types have been named according to their secretory products and the morphology of their secretory granules. Glucagon-like peptide (GLP)-1 and neurotensin (NT) are major intestinal hormones and are secreted from L and N cells, respectively. Recently, however, complex patterns of colocalization of gastroenteropancreatic hormones have been indicated by some investigators (Cho et al. 2014, 2015; Grunddal et al. 2016; Martínez et al. 2000; Svendsen et al. 2015). The old one cell–one hormone theory has collapsed and a new nomenclature for enteroendocrine cells has been proposed (Helander and Fändriks 2012).

GLP-1 is a 30-amino acid peptide derived from proglucagon (PG) and is released from enteroendocrine L cells in response to food ingestion (Baggio and Drucker 2007; Karhunen et al. 2008). It is generally accepted that GLP-1 stimulates insulin secretion from pancreatic B cells and reduces the blood glucose level via stimulation of insulin release in mammalian species (Holst 2007). This peptide also

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inhibits food intake in birds (Furuse et al. 1997; Tachibana et al. 2003). Immunohistochemical studies have shown that enteroendocrine cells exhibiting GLP-1 immunoreactivity are distributed in both small and large intestines of mammals (Calingasan et al. 1984; Cho et al. 2015; Damholt et al. 1999; Eissele et al. 1992). In contrast, chicken GLP-1-immunoreactive cells are mainly observed in the whole jejunum and ileum and show the highest frequency in the distal ileum (Hiramatsu et al. 2003, 2005).

NT is a tridecapeptide originally isolated from the bovine hypothalamus that is present in the highest amount within enteroendocrine N cells of mammalian and avian intestines (Carraway and Ferris 1983; Carraway and Leeman 1973). This peptide has multiple functions as a neurotransmitter and as a circulating hormone. In the gastrointestinal tract, NT is involved in the regulation of gastric and intestinal motility (Andersson et al. 1977; Thor and Rosell 1986), gastric and pancreatic secretion (Wood et al. 1988) and mucosal growth (Chung et al. 1992; Evers 2006). Avian NT stimulates motility of the chicken ileum and cecum (Rawson et al. 1990). Mammalian N cells are found scattered in the mucosal epithelium of jejunum; a few N cells are also found in the ileum but they are very scarce in the duodenum (Helmstaedter et al. 1977; Sundler et al. 1977a). In contrast to mammals, chicken N cells are distributed along the entire intestinal tract (Atoji et al. 1994; Sundler et al. 1977b) and their frequency is relatively higher in the ileum (our unpublished data).

These two types of enteroendocrine cells, L and N cells, show similar frequencies and distribution patterns in the chicken ileum. Recently, Svendsen et al. (2015) reported that GLP-1 and NT co-localize within the same endocrine cells of mammalian intestines. Thus, it seems likely that GLP-1 and NT are co-localized in the chicken ileum.

Here, we aim to clarify the co-localization pattern of GLP-1 and NT in the chicken ileum as well as the expression of PG and neurotensin precursor (NTP) mRNA by using double immunofluorescence and in situ hybridization methods. Moreover, we attempt to quantitatively evaluate the GLP-1 and NT contents in secretory granules by means of an immunocytochemical method with colloidal gold.

## Materials and methods

### Experimental birds and tissue samples

Seven-day-old, male White Leghorn chicks ( $n = 5$ ), each weighing about 97 g, were used in this study. Chicks were sacrificed by decapitation under anesthesia with diethyl ether. The proximal and distal parts of the ileum were dissected out from each bird, washed with a 0.75% NaCl solution and subsequently used as tissue samples in a variety of protocols. All procedures and animal treatments performed for this study

were reviewed and approved by the Institutional Animal Care and Use Committee of Shinshu University (approval number: 270071).

### Double immunofluorescence method for GLP-1 and NT

Tissue samples were immersed in Bouin's fluid (Catalogue No. 16045; Polyscience, Warrington, PA, USA) at 4 °C overnight and embedded in paraffin wax. The paraffin sections were cut at 5  $\mu\text{m}$  and mounted onto silane-coated glass slides (Matunami, Tokyo, Japan). An immunofluorescence technique was performed as previously described to detect GLP-1 and NT localization (Nishimura et al. 2013). A mouse monoclonal antibody against synthetic human GLP-1 (aa 7–36) (1:2,000; A6104.1; Immunodiagnostik, Bensheim, Germany) and a rabbit anti-NT serum (1:500; AB5496; Millipore, Temecula, CA, USA) were used as primary antibodies in this study. A cocktail of DyLight 488-labeled donkey anti-mouse IgG (1:300; 611–741-127; Rockland Immunochemicals, Gillbertsville, PA, USA) and DyLight 549-labeled donkey anti-rabbit IgG (1:300; 611-700-127; Rockland Immunochemicals) was used as the secondary antibody. Sections were covered with a coverslip using aqueous mounting medium (PermaFluor; Thermo Fisher Scientific, Fremont, CA, USA) and then observed and photographed under a fluorescence microscope (AxioImagerA1; Zeiss, Göttingen, Germany). All incubations were carried out in a moisture chamber at room temperature. The specificities of the primary antibodies used in this study were determined by pre-absorption test. Chicken/common turkey GLP-1 (7-36) amide (H-5824; Bachem, Bubendorf, Switzerland) and synthesized chicken NT (GenScript, Piscataway, NJ, USA) were used as peptides for this test. The primary antibodies against GLP-1 and NT were pre-absorbed with either appropriate or inappropriate peptide. Working dilutions of antibodies were incubated in 10  $\mu\text{g}/\text{ml}$  of GLP-1 or NT peptide at 4 °C for 24 h and used as the primary antibodies for the immunofluorescence method.

### In situ hybridization for proglucagon mRNA and NT precursor mRNA

“Mirror sections” were created by taking two consecutive 5- $\mu\text{m}$ -thick paraffin sections that were made from the tissue blocks described above and mounting them on silane-coated glass slides with the cut surface facing upward. The following procedures all used mirror sections. An in situ hybridization technique was performed to detect PG mRNA and NTP mRNA in chicken ileums as previously described (Watanabe et al. 2014). The oligonucleotide probes used in this study were designed based on the PG sequence (Richards and McMurtry 2008) and the NTP sequence (Tanaka et al. 2013) and were synthesized commercially (BEX, Tokyo, Japan).

The sequences of the PG antisense and sense probes were 5'-GCTGTAGTCACTGGTGAATGTGCCTTGTGAATGACGCTTTA-3' and

5'-TAAAGCGTCATTCAACAAGGCACATTCACCAGTGACTACAGC-3', respectively and the sequences of the NTP antisense and sense probes were

5'-TCAAATGCGTCTTGCTGAAGTAACTCCCAGTGCTGAAAGG-3' and

5'-CCTTTCAGCACTGGGAGTTACTTCAGCAAGACGCATTTGA-3', respectively.

One section from each mirror section pair was used for in situ hybridization for PG mRNA and was continuously incubated with rabbit anti-NT serum at room temperature for 24 h followed by incubation with DyLight 549-labeled donkey anti-rabbit IgG at room temperature for 3 h. The remaining section from each mirror section pair was used for in situ hybridization for NTP mRNA and was continuously incubated with mouse monoclonal antibody against synthetic human GLP-1 (aa 7–36) at room temperature for 24 h, followed by an incubation with DyLight 488-labeled donkey anti-mouse IgG at room temperature for 3 h. Sections were covered with a coverslip using aqueous mounting medium, then observed and photographed under a microscope (AxioImagerA1; Zeiss).

#### Double immunocytochemical staining for GLP-1 and NT at the electron microscopic level

A double immunocytochemical method using colloidal gold was performed for the detection of GLP-1 co-localization with NT at the electron microscopic level as previously described (Nishimura et al. 2013). Tissue samples were immersed in Zamboni's solution containing 0.01% glutaraldehyde at 4 °C overnight and embedded in epoxy resin (Quetol 812; Nisshin EM) as previously described. Ultrathin sections were made with an ultramicrotome (Super Nova; Reichert-Jung, Vienna, Austria) and mounted on 200-mesh nickel grids (Nisshin EM). Ultrathin sections pretreated with a 1% sodium periodate solution were sequentially treated with 1% normal donkey serum (IHR-8135; ImmunoBioScience, Mukilteo, WA, USA) and 1% normal goat serum (50-062Z; Invitrogen, UK) and then incubated with the mouse monoclonal antibody against GLP-1 (1:2,000) at room temperature overnight. After washing them several times with phosphate-buffered saline, the ultrathin sections were incubated with rabbit anti-NT serum (1:2,000) followed by incubation with a mixture of colloidal gold (12 nm in diameter)-labeled donkey anti-mouse IgG (1:200; 711-205-152; Jackson Immuno Research) and colloidal gold (20 nm in diameter)-labeled goat anti-rabbit IgG (1:200; EM GAR20; BB International) at room temperature for 2 h. After electron staining with TI blue (Nisshin EM) and 2% lead citrate, the sections were observed under a transmission electron

microscope (JEM-1400; JEOL, Tokyo, Japan). All incubations were carried out in a moisture chamber at room temperature. The specificities of the colloidal gold-labeled secondary antisera were controlled by the omission of the primary antibody. No cross-reactivity of the secondary antisera was observed.

#### Quantificational evaluation

The mucosal epithelium of the ileum was divided into three parts: crypts and the bottom and middle parts of the villi. To evaluate the amount of GLP-1 and NT co-localization in enteroendocrine cells, 40 areas were observed in each epithelial part from each of five birds, for a total of 200 areas for each epithelial part, according to a previously described procedure (Svendsen et al. 2015). The number of GLP-1- and NT-positive gold particles per a secretory granule in GLP-1<sup>+</sup>/NT<sup>+</sup> cells was counted on photomicrographs at a magnification of ×20,000 to estimate the granular contents of GLP-1 and NT. Twenty GLP-1<sup>+</sup>/NT<sup>+</sup> cells were observed in each epithelial part in each of five birds, for a total of 100 GLP-1<sup>+</sup>/NT<sup>+</sup> cells for each epithelial part.

Minimum and maximum diameters of all secretory granules in GLP-1-immunoreactive cells were measured using an image analyzer (KS400; Zeiss). Secretory granules were measured in 20 GLP-1<sup>+</sup>/NT<sup>+</sup> cells in each epithelial part from each of five birds, for a total of 100 GLP-1<sup>+</sup>/NT<sup>+</sup> cells for each epithelial part.

#### Statistical analyses

Average ratios of each cell type were calculated at each epithelial part and are shown as mean ± SD. The relationship between the ratio and the localization of each cell type was analyzed using the Chi-square test at a significance level of  $p < 0.05$ . Data analysis was performed using js-STAR for Windows statistics software v.2.9.9 J. Statistical analyses for the number of gold particles per a secretory granule and the diameter of secretory granules were conducted using Steel–Dwass tests for multiple comparisons. A  $p$  value of  $< 0.01$  was regarded as indicating statistical significance.

## Results

### Co-localization of GLP-1 and NT

Double immunofluorescence staining was performed in the mucous epithelium of the chicken ileum to investigate the potential co-localization of GLP-1 and NT. Three types of enteroendocrine cells were identified based on their immunoreactivity for GLP-1 and NT. Specifically, endocrine cells showing immunoreactivity for only GLP-1 (GLP-1<sup>+</sup>/NT<sup>-</sup>),

both GLP-1 and NT (GLP-1<sup>+</sup>/NT<sup>+</sup>), or only NT (GLP-1<sup>-</sup>/NT<sup>+</sup>) were found scattered in the mucosal epithelium (Fig. 1). These cells were observed throughout the whole ileum. NT-immunoreactive cells tended to show a stronger fluorescent intensity in the villi than they did in the crypts.

The numbers of GLP-1<sup>+</sup>/NT<sup>+</sup>, GLP-1<sup>+</sup>/NT<sup>-</sup> and GLP-1<sup>-</sup>/NT<sup>+</sup> cells were counted in the proximal and distal ileum and the ratios of these cell types are summarized in Fig. 2. GLP-1<sup>+</sup>/NT<sup>+</sup> cells were observed in both the proximal and distal parts of the ileum. The percentage of this cell type was significantly higher than the other two cell types in every epithelial part ( $p < 0.0001$ ) except the middle part of villi of the distal ileum.

The relationship between the ratio of each cell type at each epithelial part and the intestinal regions was analyzed using the Chi-square test. The ratio of GLP-1<sup>-</sup>/NT<sup>+</sup> cells was significantly higher in the middle part of villi of the distal ileum ( $39.9 \pm 16.0$ ) than that of the proximal ileum ( $19.3 \pm 6.2$ ) ( $p = 0.0048$ ). The ratio of GLP-1<sup>+</sup>/NT<sup>+</sup> cells was significantly higher in the middle part of villi of the proximal ileum ( $62.1 \pm 8.4$ ) than that of the distal ileum ( $47.2 \pm 15.0$ ) ( $p = 0.0048$ ). Ratios of this cell type in the crypts and the middle of villi were relatively higher in the distal ileum than the proximal ileum. Ratios of GLP-1<sup>+</sup>/NT<sup>-</sup> cells at each epithelial part were relatively lower in the distal ileum than those in the proximal ileum. Thus, the ratios of the three cell types showed a gradient along the proximal–distal axis in the ileum.

The numbers of GLP-1<sup>+</sup>/NT<sup>+</sup>, GLP-1<sup>+</sup>/NT<sup>-</sup> and GLP-1<sup>-</sup>/NT<sup>+</sup> cells were then counted along the crypt–villous axis in the proximal and distal ileum and the ratios of these cell types are summarized in Fig. 2. The relationship between the ratio and epithelial parts of each cell type in the proximal and distal ileum was analyzed using the Chi-square test. The cell type ratios were similar among the three epithelial parts in the proximal ileum (Fig. 2, upper panel) ( $p = 0.5047$ ) but were different in the distal ileum (Fig. 2, lower panel). There was a significant decrease in the percentage of GLP-1<sup>+</sup>/NT<sup>+</sup> cells in the middle part of the villi of the distal ileum ( $47.2 \pm 15.0$ ) relative to that in the bottom part of the villi ( $73.0 \pm 9.1$ )

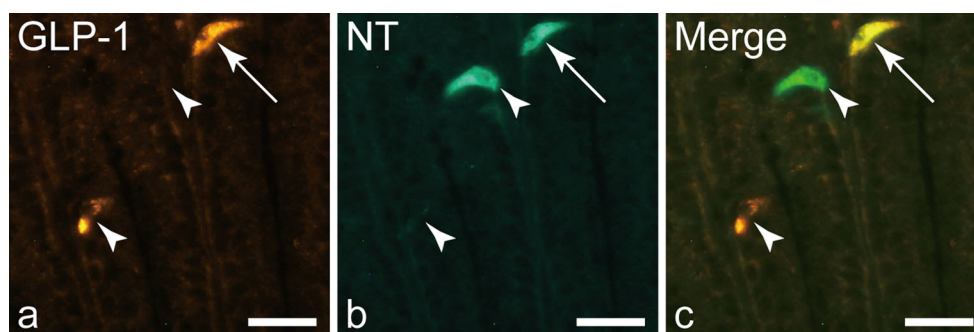
( $p < 0.0001$ ). In contrast, the percentage of GLP-1<sup>-</sup>/NT<sup>+</sup> cells in the middle part of the distal ileum ( $39.9 \pm 16.0$ ) was significantly increased relative to that in the bottom part of the villi ( $15.0 \pm 4.8$ ) and the crypts ( $13.3 \pm 4.2$ ) ( $p < 0.001$ ). Thus, the ratios of these three cell types showed a gradient along the crypt–villous axis in the distal ileum.

### Expression of proglucagon mRNA and NT precursor mRNA

The in situ hybridization technique was performed to clarify the expression of PG and NTP mRNA signals in the chicken ileum. In the proximal ileum, endocrine cells expressing a PG mRNA signal were mainly distributed in the crypts and the bottom part of villi but a few of these cells were found in the middle part of the villi. In the distal ileum, these endocrine cells were distributed from the crypts to the bottom part of the villi but were never found in the middle part of the villi. In contrast, endocrine cells expressing a NTP mRNA signal were observed from the crypts to the upper part of villi throughout the ileum. GLP-1-immunoreactive cells lacking a PG mRNA signal were detected in the villous epithelium (Fig. 3, arrowheads). In the crypts and villous epithelium, NTP mRNA signals and immunoreactivity for NT peptide were detected within the same cells. Endocrine cells expressing mRNA signals of both PG and NTP and having immunoreactivity for both NT and GLP-1 peptides were often found in the crypts (Fig. 3, arrows) but not in the villous epithelium.

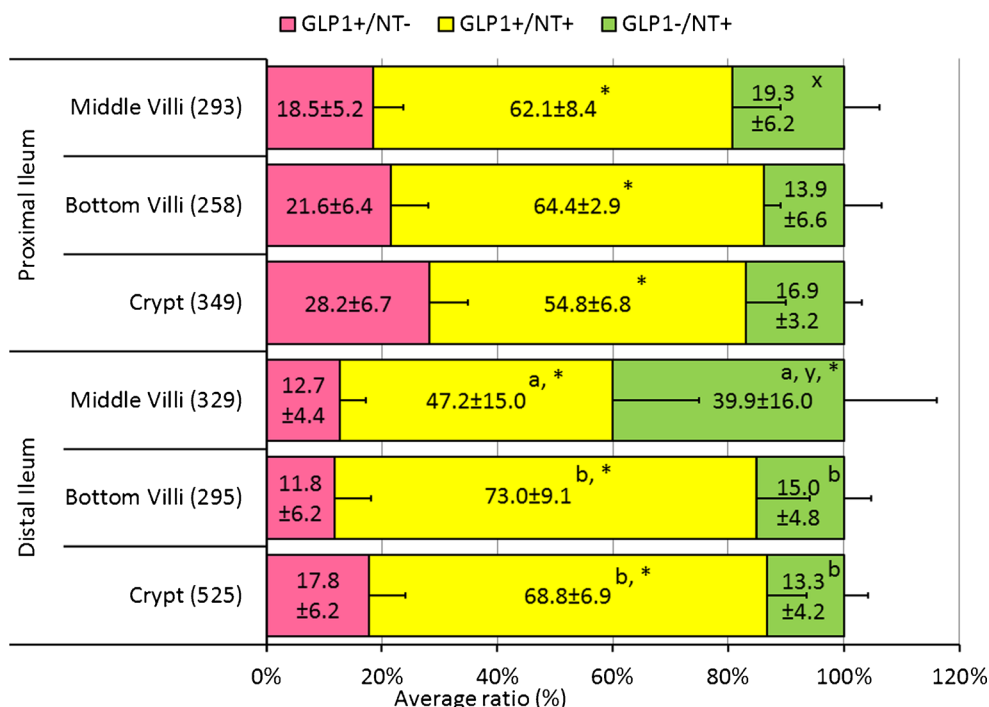
### Double immunocytochemical staining for GLP-1 and NT at the electron microscopic level

Double immunocytochemical staining with colloidal gold was performed to evaluate the granular contents of GLP-1 and NT in the GLP-1<sup>+</sup>/NT<sup>+</sup> cells. Cells showing immunoreactivity for both GLP-1 and NT were observed at the electron microscopic level. Many secretory granules in these cells were round to oval in shape and showed a moderate electron density. The



**Fig. 1** Distribution in the chicken distal ileum of three types of enteroendocrine cells. Enteroendocrine cell types were identified by a double immunofluorescence technique for glucagon-like peptide-1 (GLP-1) and neurotensin (NT). *Arrows* indicate cells showing

immunoreactivity for both GLP-1 and NT (GLP-1<sup>+</sup>/NT<sup>+</sup>). *Arrowheads* indicate cells containing either GLP-1 (GLP-1<sup>+</sup>/NT<sup>-</sup>) or NT (GLP-1<sup>-</sup>/NT<sup>+</sup>). *Bars* 20  $\mu$ m



**Fig. 2** Average ratios of three enteroendocrine cell types in the three epithelial parts, the middle (*Middle*) and the bottom (*Bottom*) parts of villi and the crypts (*Crypt*) of the proximal and distal ileum. Cells were identified by a double immunofluorescent technique for their expression of glucagon-like peptide-1 (*GLP-1*) and neurotensin (*NT*) along the crypt–villous axis in the proximal (*top panel*) and distal (*bottom panel*) ileum. *Red*, *yellow* and *green* columns indicate percentages of cells showing immunoreactivity for only *GLP-1* (*GLP-1<sup>+</sup>/NT<sup>-</sup>*), both *GLP-1* and *NT* (*GLP-1<sup>+</sup>/NT<sup>+</sup>*) and only *NT* (*GLP-1<sup>-</sup>/NT<sup>+</sup>*), respectively. The number in parenthesis indicates the total number of counted cells at each epithelial

part. The *number* in each *column* indicates the average percentage of each cell type and standard deviation (mean ± SD). *x*, *y* Values with different *superscripts* show a significant difference between the proximal and distal ileum in the ratio of *GLP-1<sup>-</sup>/NT<sup>+</sup>* cells at the middle part of villi ( $p = 0.0048$ ). *a*, *b* Values with different *superscripts* indicate a significant difference among three epithelial parts in the ratio of the same cell type at the same intestinal region ( $p < 0.0001$ ). Asterisk (\*) shows a significant difference in the ratio of the cell type relative to that without asterisk at the same epithelial part of the proximal and distal ileum ( $p < 0.0001$ ). *Bars* indicate standard deviation.

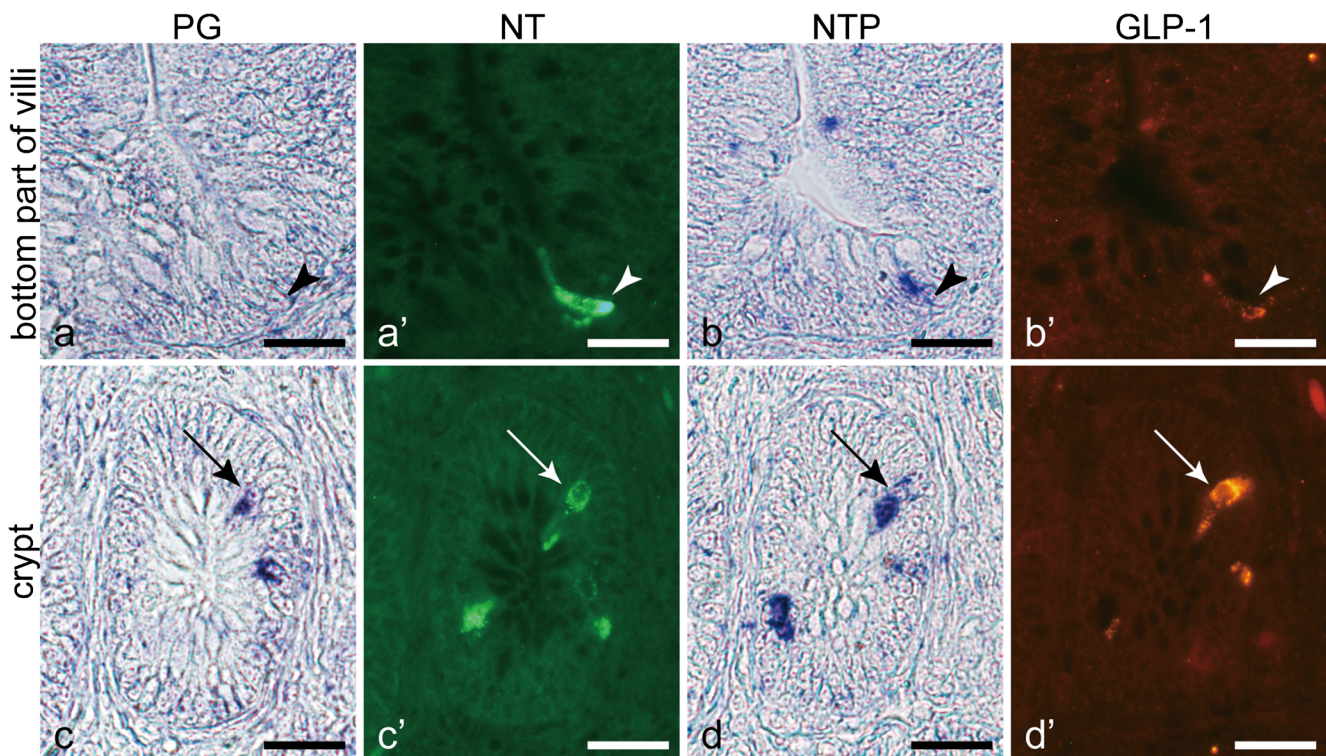
minimum and maximum recorded diameters of the secretory granules in *GLP-1<sup>+</sup>/NT<sup>+</sup>* cells from the three epithelial parts are summarized in Table 1. The secretory granule minimum and maximum diameters were largest in the cells located in the crypts and were smallest in those located in the middle part of the villi. Significant differences in the average secretory granule diameters were observed among cells from the three epithelial parts ( $p < 0.01$ ).

Our immunocytochemical technique using two kinds of gold particles with different diameters identified four types of secretory granules (Fig. 4a–d). Type 1 granules contained both *GLP-1* and *NT* (Fig. 4a), while type 2 granules contained neither *GLP-1* nor *NT* (Fig. 4b). Type 3 and type 4 granules contained only *GLP-1* and only *NT*, respectively (Fig. 4c, d). The cells that exhibited immunoreactivity for both *GLP-1* and *NT* contained many type 1 granules with other types (Fig. 4e) and some of them were observed to contain all four secretory granule types. Granules classified as types 1 or 3 were often seen in the *GLP-1<sup>+</sup>/NT<sup>+</sup>* cells in the crypts. Type 4 granules were abundant in the *GLP-1<sup>+</sup>/NT<sup>+</sup>* cells of the villous epithelium. There were fewer type 2 granules than any other

granule type in each of the epithelial parts. Notably, there was no obvious difference in the granule electron density among the four granule types. However, the numbers of each gold particle type per secretory granule varied along the crypt–villous axis. Progressing up to the villi, the amount of *GLP-1*-positive particles decreased while the number of *NT*-positive particles increased (Table 2). There were significant differences in the average number of gold particles per secretory granule among the three epithelial parts of the distal ileum ( $p < 0.01$ ).

**Specificities of the primary antibodies and ISH probes**

Preabsorption tests of the primary antibodies incubated with appropriate peptides abolished specific immunoreactivity (Fig. 5a, c). Conversely, preabsorption of the primary antibodies with inappropriate peptides showed no reduction of staining intensity and specific immunostaining (Fig. 5b, d). While sections incubated with antisense probe showed signals of *PG* mRNA and *NTP* mRNA (Fig. 6a, c), sections incubated with the sense probe showed no signals of either *PG* mRNA or *NTP* mRNA (Fig. 6b, d).



**Fig. 3** Photomicrographs showing mRNA signals of proglucagon (*PG*) (**a**, **c**) and neurotensin precursor (*NTP*) (**b**, **d**). Cells expressing *PG* and *NTP* mRNA signals in the crypts (*bottom panels*) and the bottom part of villi (*top panels*) of the distal ileum were identified by in situ hybridization and those with immunoreactivity for neurotensin (*NT*) (**a'**, **c'**) and glucagon-like peptide-1 (*GLP-1*) (**b'**, **d'**) were identified by an indirect immunofluorescence method. The photomicrographs showing

*NTP* mRNA (**b**, **d**) and *GLP-1* peptide (**b'**, **d'**) are digitally reversed. *Arrows* in the *bottom panels* indicate a cell showing *PG* and *NTP* mRNA signals as well as immunoreactivity for *NT* and *GLP-1*. *Arrowheads* in the *top panels* indicate a cell showing a *NTP* mRNA signal but not a *PG* mRNA signal that has immunoreactivity for both *NT* and *GLP-1*. *Bars* 20  $\mu$ m

## Discussion

The present study demonstrates that three types of enteroendocrine cells,  $GLP-1^+/NT^+$ ,  $GLP-1^+/NT^-$  and  $GLP-1^-/NT^+$  cells, exist in the chicken ileum. In chickens, previous immunohistochemical studies showed that *GLP-1*-immunoreactive cells were primarily observed in the whole jejunum and ileum (Hiramatsu et al. 2003), while *NT*-immunoreactive cells were distributed throughout the intestine (Atoji et al. 1994). Relatively higher numbers of *GLP-1*-immunoreactive cells were observed in the mucosa of ileum

(Hiramatsu et al. 2003, 2005) and these cells were mainly observed in the lower part of the villi and the crypts of the ileum (Hiramatsu et al. 2005). In contrast, *NT*-immunoreactive cells were found scattered throughout the epithelium, from the crypts to the upper part of the villi in the ileum (Atoji et al. 1994). These data indicate that *GLP-1*- and *NT*-immunoreactive cells show similar distribution patterns and form major enteroendocrine cell subpopulations in the chicken ileum, so it seemed likely that *GLP-1* and *NT* co-localize in the same cells. Here, we used double immunofluorescence and immunocytochemical techniques to confirm that *GLP-1* co-localizes with *NT* in the chicken ileum.

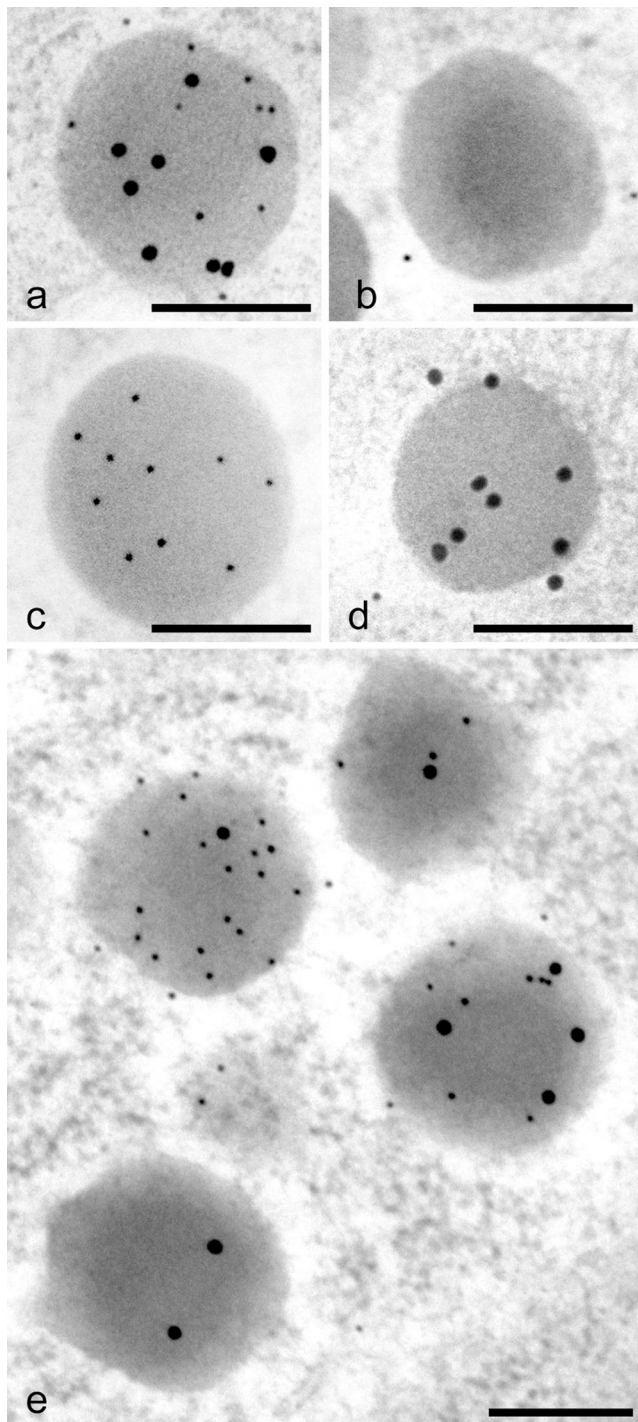
The one hormone per cell concept has been long accepted as the dogma in enteroendocrinology. Recent studies, however, have shown the co-localization of more than one hormone in the same enteroendocrine cell, suggesting that these cells in both mammals and chickens might have the potential to express multiple hormones (Cho et al. 2014; Grunddal et al. 2016; Martínez et al. 2000). For example, in cholecystokinin (*CCK*)-eGFP transgenic mice, *CCK*-eGFP-positive cells in the duodenum were also stained immunohistochemically with antibodies against secretin, glucose-dependent insulinotropic polypeptide (*GIP*), *GLP-1*, *NT* and peptide *YY* (*PYY*)

**Table 1** Minimum and maximum diameters of secretory granules in  $GLP-1^+/NT^+$  cells

Parts	Maximum diameter (nm)	Minimum diameter (nm)
Middle part of villi	249 $\pm$ 70 a	202 $\pm$ 55 a
Bottom part of villi	281 $\pm$ 63 b	224 $\pm$ 49 b
Crypt	290 $\pm$ 57 c	229 $\pm$ 43 c

Values are shown as the mean  $\pm$  SD

Values with different lowercase letters are significantly different from one another ( $p < 0.01$ )



**Fig. 4** Electron micrographs of secretory granules observed in enteroendocrine cells in the distal ileum. Four types of secretory granules can be identified by an immunocytochemical technique with colloidal gold. Type 1 granules (a) contain both GLP-1 (identified by 12-nm particles) and NT (identified by 20-nm particles). Type 2 granules (b) contain neither GLP-1 nor NT. Type 3 (c) and type 4 (d) granules contain only GLP-1 and only NT, respectively. The cell that exhibits immunoreactivity for both GLP-1 and NT contains mainly type 1 granules with other types of granules (e). Bars 100 nm

(Egerod et al. 2012). Our previous study revealed that GLP-1 and GLP-2 were co-localized in the same secretory granules

**Table 2** Average number of gold particles per secretory granule in GLP-1<sup>+</sup>/NT<sup>+</sup> cells

Parts	GLP-1	NT
Middle part of villi	1.8 ± 2.5 a	2.7 ± 2.2 a
Bottom part of villi	2.9 ± 3.0 b	2.3 ± 2.0 a
Crypt	4.8 ± 3.7 c	1.9 ± 1.4 b

Values are shown as the mean ± SD

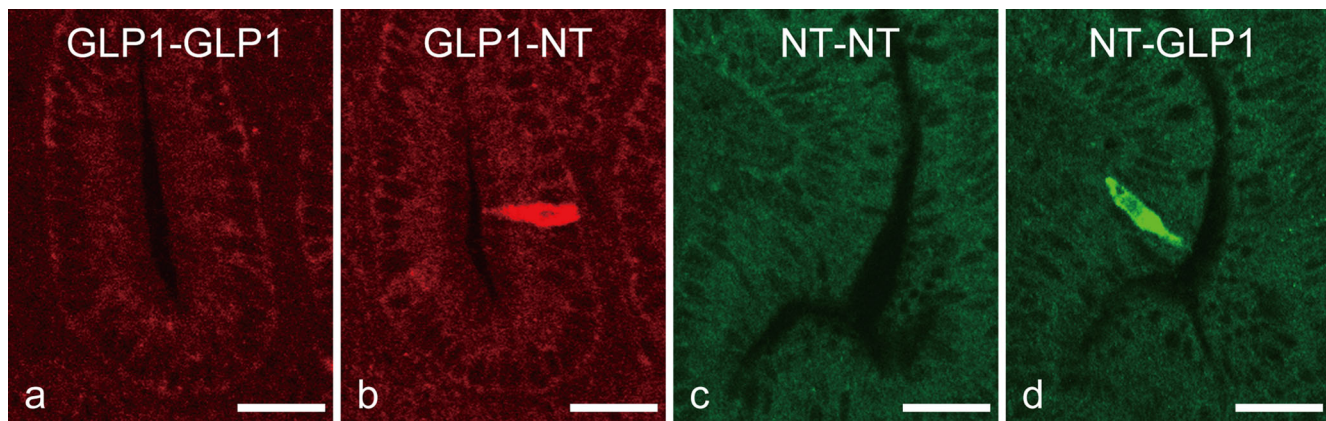
Values with different lowercase letters are significantly different from one another ( $p < 0.01$ )

of L cells in the chicken ileum (Nishimura et al. 2013). Thus, endocrine cells that can produce and secrete at least three hormones, GLP-1, GLP-2 and NT, exist in the chicken ileum.

Our study reveals three different types of endocrine cells based on their GLP-1 and NT immunoreactivity. The main cell type is the one demonstrating immunoreactivity for both peptides (GLP-1<sup>+</sup>/NT<sup>+</sup>). Grunddal et al. (2016) reported that there was a large degree of co-expression of GLP-1, PYY and NT within single cells in the distal ileum of mice. Overlap between NT-immunoreactive and GLP-1-immunoreactive cells was observed in approximately 15% of cells in mouse small intestines (Svendsen et al. 2015). Cell lineage with GLP-1 and NT is predominantly found in the mammalian ileum (Schonhoff et al. 2004). We found that about 47–73% of the enteroendocrine cells counted in the proximal and distal ileum were GLP-1<sup>+</sup>/NT<sup>+</sup> cells. Therefore, it might be characteristic of chickens that GLP-1 co-localizes with NT in a high proportion of enteroendocrine cells.

Schmidt et al. (2014) reported that co-infusion of GLP-1 and PYY 3–36 decreased ad libitum food intake in healthy overweight men compared with mono-infusions of these hormones. Additionally, Grunddal et al. (2016) revealed that NT, GLP-1 and PYY act synergistically as inhibitors of gastric emptying. Our immunocytochemical observation in this study demonstrates the co-storing of GLP-1 and NT in the same secretory granules. GLP-1 controls intestinal motility in mammals (Holst, 2007) and NT controls the motility of the alimentary tract in chickens (Rawson et al. 1990). Our findings suggest that GLP-1 and NT co-released from the same enteroendocrine cells may act as synergistic modulators of intestinal motility in chickens.

GLP-1<sup>+</sup>/NT<sup>+</sup> cells exist at a high frequency in the crypts of the chicken distal ileum but these cells only synthesize and secrete NT in the villous epithelium. Our previous studies in chicken ileums revealed that GLP-1-immunoreactive cells are distributed from the crypts to the lower part of the villi (Hiramatsu et al. 2003, 2005); in contrast, NT-immunoreactive cells are distributed from the crypts to the upper part of villi (Atoji et al. 1994). We found that NT-immunoreactive cells exhibited stronger fluorescence intensity in the villi than they did in the crypts. Additionally, we did



**Fig. 5** Adjacent serial sections (**a** and **b**, **c** and **d**) immunohistochemically stained with preabsorbed primary antibodies. **a** Incubation with monoclonal antibody against glucagon-like peptide (GLP)-1 preabsorbed with chicken/common turkey GLP-1 abolishes specific immunoreactivity. **b** Incubation with monoclonal antibody against GLP-1 preabsorbed with synthesized

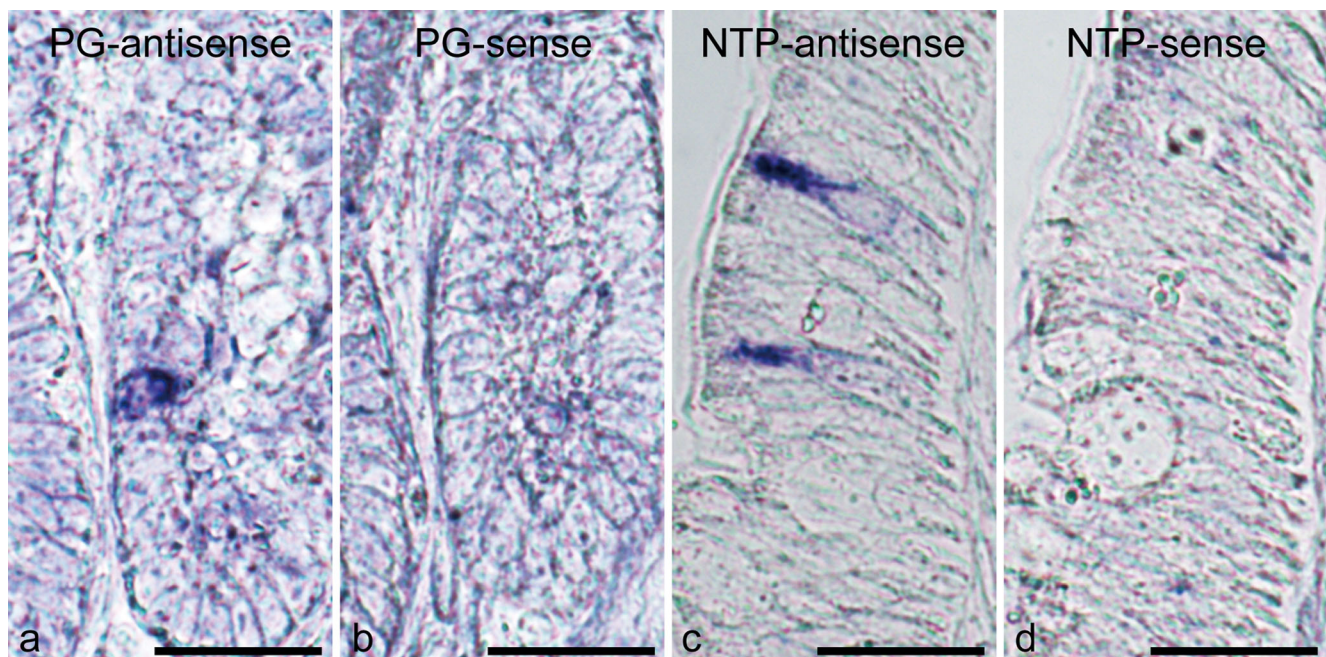
chicken neurotensin (NT) shows immunoreactive enteroendocrine cell. **c** Incubation with anti-NT serum preabsorbed with synthesized chicken NT abolishes specific immunoreactivity. **d** Incubation with anti-NT serum preabsorbed with chicken/common turkey GLP-1 shows NT-immunoreactive enteroendocrine cell. Bars 20  $\mu$ m

not detect any PG mRNA signal in the villous epithelium of chicken ileum but we observed NTP mRNA signals even in the epithelium of the higher part of the villi. These findings explain why the ratio of GLP-1<sup>+</sup>/NT<sup>+</sup> cells is decreased along the crypt–villous axis in the distal ileum.

This distributional pattern of GLP-1<sup>+</sup>/NT<sup>+</sup> cells along the crypt–villous axis in the chicken ileum is different from that in the mammalian intestine. Previous immunohistochemical analyses in mammals have shown that the amount of cells co-expressing GLP-1 and NT increases along the crypt–villous axis (Egerod et al. 2012; Grunddal et al. 2016).

Therefore, the declining gradient in the frequency of GLP-1<sup>+</sup>/NT<sup>+</sup> cells might also be characteristic of chickens.

The results of our immunocytochemical studies indicate that GLP-1 and NT can be co-stored in the same secretory granule. The granular content of GLP-1, however, is decreased along the crypt–villous axis, while that of NT is increased in this direction. Recently, we reported that L cells mature and complete GLP-1 production in the crypts of chicken ileum (Nishimura et al. 2016). These data suggest that GLP-1<sup>+</sup>/NT<sup>+</sup> cells in crypts specialize in NT-production along the crypt–villous axis of the chicken distal ileum. Secretory



**Fig. 6** Adjacent serial sections (**a** and **b**, **c** and **d**) stained by in situ hybridization with antisense (**a**, **c**) and sense (**b**, **d**) probes. **a** Incubation with proglucagon (PG) antisense-oligoprobe shows the specific mRNA signal. **b** Incubation with PG sense-oligoprobe abolishes the mRNA

signal. **c** Incubation with neurotensin precursor (NTP) antisense-oligoprobe shows the specific mRNA signal. **d** Incubation with NTP sense-oligoprobe abolishes the mRNA signal. Bars 20  $\mu$ m



granules containing either GLP-1 or NT (type 3 and 4 secretory granules) were also found in GLP-1<sup>+</sup>/NT<sup>+</sup> cells. It is likely that these types of secretory granules contain both peptide hormones, because immunocytochemical staining has the limit of its detection. More sensitive techniques are necessary for this problem.

There may be a close relationship between the size and the content of secretory granules. Our immunocytochemical results show that the minimum and maximum diameters of the secretory granules in GLP-1<sup>+</sup>/NT<sup>+</sup> cells were largest in the crypts and got progressively smaller from the bottom to the upper part of the villi. Martínez et al. (1991) reported that, in the chicken proventriculus, the secretory granules in enteroendocrine cells immunoreactive for only NT were 200 nm in diameter, while those showing immunoreactivity for glucagon, GLP-1 and NT were 280 nm in diameter. These values are very similar to those that we obtained from the endocrine cells in the middle part of villi ( $249 \pm 70 \times 202 \pm 55$  nm) and crypts ( $290 \pm 57 \times 229 \pm 43$  nm). Moreover, there was a significant difference in the size of the secretory granules in the middle part of the villi and those in the crypts. Therefore, secretory granules containing GLP-1 are larger than those containing only NT.

We were able to observe endocrine cells containing either only GLP-1 or only NT by using a double immunofluorescence method. In addition to GLP-1 and NT, many peptide and monoamine hormones, such as somatostatin, PYY, glucagon, secretin, CCK, substance P and serotonin, have been previously identified in the avian gastrointestinal tract (Rawdon and Andrew 1999). Grunddal et al. (2016) showed that mature GLP-1-immunoreactive cells in the villous epithelium also possess immunoreactivity for other peptides, like NT and PYY. Therefore, although the cells in the villi that showed immunoreactivity for only NT do not contain GLP-1, NT may co-localize with other hormones.

In conclusion, GLP-1 and NT co-localize in the same enteroendocrine cells, which are found at a high frequency in the chicken ileum and these cells specialize in NT-production along the crypt–villous axis in the distal ileum.

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