

Neurotensin Is Coexpressed, Coreleased, and Acts Together With GLP-1 and PYY in Enteroendocrine Control of Metabolism

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The 2 gut hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) are well known to be coexpressed, costored, and released together to coact in the control of key metabolic target organs. However, recently, it became clear that several other gut hormones can be coexpressed in the intestinal-specific lineage of enteroendocrine cells. Here, we focus on the anatomical and functional consequences of the coexpression of neurotensin with GLP-1 and PYY in the distal small intestine. Fluorescence-activated cell sorting analysis, laser capture, and triple staining demonstrated that GLP-1 cells in the crypts become increasingly multihormonal, ie, coexpressing PYY and neurotensin as they move up the villus. Proglucagon promoter and pertussis toxin receptor-driven cell ablation and reappearance studies indicated that although all the cells die, the GLP-1 cells reappear more quickly than PYY- and neurotensin-positive cells. High-resolution confocal fluorescence microscopy demonstrated that neurotensin is stored in secretory granules distinct from GLP-1 and PYY storing granules. Nevertheless, the 3 peptides were cosecreted from both perfused small intestines and colonic crypt cultures in response to a series of metabolite, neuropeptide, and hormonal stimuli. Importantly, neurotensin acts synergistically, ie, more than additively together with GLP-1 and PYY to decrease palatable food intake and inhibit gastric emptying, but affects glucose homeostasis in a more complex manner. Thus, neurotensin is a major gut hormone deeply integrated with GLP-1 and PYY, which should be taken into account when exploiting the enteroendocrine regulation of metabolism pharmacologically. (*Endocrinology* 157: 176–194, 2016)

The gut hormone-secreting enteroendocrine cells are scattered along the epithelium throughout the gastrointestinal (GI) tract. Although they comprise less than 1%

of the epithelial cells, the enteroendocrine cells constitute the largest endocrine organ of the body (1). The gut hormones regulate secretory and motility functions in the GI

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Abbreviations: Alpi, alkaline phosphatase; DTX, diphtheria toxin; DVC, dorsal vagal complex; FACS, fluorescence-activated cell sorting; Gcg, glucagon; GI, gastrointestinal; GIP, glucose-dependent insulinotropic peptide; GLP-1, Gcg-like peptide-1; GTT, glucose tolerance test; hDTR, human DTX receptor; IPGTT, ip GTT; Lyz1, lysozyme; NMC, neuromedin C; OGTT, oral GTT; PYY, peptide YY; qPCR, quantitative polymerase chain reaction; RFP, red fluorescent protein; SIM, structured illumination microscopy; SR-SIM, superresolution SIM.

tract. Furthermore, they control appetite and energy expenditure, ie, mainly via the gut-brain axis, as well as glucose homeostasis through effects on pancreatic hormone secretion (2–5). The physiological understanding of the gut hormones has led to the development of 2 classes of antidiabetes drugs, glucagon (Gcg)-like peptide-1 (GLP-1) mimetics and dipeptidyl peptidase-4 inhibitors, of which the former has recently been approved for the treatment also of obesity (6–10). Although the role of individual hormones has been challenged (11), it is generally believed that the impressive curative effect of bariatric surgery on diabetes and obesity is largely mediated through the combined effects of a number of changes in gut hormone signals (12–14).

The enteroendocrine cells are constantly renewed with a lifespan of approximately a week as they are generated from stem cells located in the crypts of the mucosa. Gradually, they move up along the villus, to eventually be extruded at the tip (15). It is generally believed that the cells are terminally differentiated in respect of which peptide precursor they “choose” to express when they leave the crypt (15–19) although certain older studies indicated that the expression of peptides may change along the crypt-villus axis (20, 21). It was also generally believed that enteroendocrine cells only express and release peptides from a single peptide precursor, with the exception of the well-established coexpression of GLP-1 and peptide YY (PYY) in the lower intestine. However, we recently discovered a surprisingly broad coexpression of 6 different, functionally related peptides: cholecystokinin, secretin, glucose-dependent insulinotropic peptide (GIP), GLP-1, PYY, and neurotensin, but importantly, not somatostatin and substance P (22, 23). Gribble and coworkers observed comparable coexpression of peptides, whereas Gradwohl and coworkers reached a very similar conclusion based on genetic analysis of the role played by key transcription factors (24, 25). Importantly, liquid chromatography–mass spectrometry proteomics analysis confirmed that this coexpression also occurs at the peptide level. Immunohistochemical analysis demonstrated that this was also the case for human enteroendocrine cells (22). However, single-cell quantitative polymerase chain reaction (qPCR) analysis revealed that not all of the peptides are expressed simultaneously in all of the cells (22).

In the present study, we focus on the coexpression of neurotensin with GLP-1 and PYY in enteroendocrine cells of the distal small intestine. Neurotensin was originally discovered by Caraway and Leeman in 1973 as a vasodilator in a side fraction obtained during the purification of substance P (26). Much attention was at that time and still is devoted to the function of neurotensin as a neuropeptide being involved in the central control of metabolism where

neurotensin and the neurotensin 1 receptor recently have been identified as important regulators of the dopaminergic system, food reward, and leptin-induced regulation of food reward (27–29). However, although neurotensin at an early stage was identified also as a gut hormone (30), this part of the neurotensin biology has to a large degree been ignored in the last 2–3 decades despite the fact that the population of neurotensin cells is similar in size to those of GLP-1 and PYY cells (22). Moreover, in addition to its effects on gastric motility, neurotensin has also been implicated as a bona fide incretin, stimulating insulin secretion (31–33).

Based on observations in a transgenic reporter mouse for the short-chain fatty acid receptor GPR41 free fatty acid receptor 3, we describe how neurotensin is closely connected with GLP-1 and PYY in the enteroendocrine system. We find that, although they are not stored in the same secretory granules, the 3 peptide hormones are not only coexpressed but also cosecreted. Furthermore, we find that neurotensin acts synergistically together with GLP-1 and PYY in the control of metabolic target organs.

Materials and Methods

Compounds

Neuromedin C (NMC) was purchased from Bachem. The GPR40 agonist AM-5262 and the GPR119 agonist AR231453 were synthesized as published (34, 35). The structure of the TGR5 (G protein-coupled bile acid receptor 1) agonist “Merck-TGR5-A” (36) is shown in [Supplemental Figure 1](#). The EC50 of Merck-TGR5-A on the human TGR5 receptor is 7nM and 25nM on the mouse TGR5 receptor.

Animals

All rodents were allowed to acclimatize for at least 1 week before experiments were conducted. Rodents were housed in a temperature- and humidity-controlled environment under a 12-hour light, 12-hour dark cycle with free access to water and a chow diet unless otherwise stated. All animal experiments were approved by the Danish Animal Inspectorate and performed according to institutional guidelines and the Gothenburg Animal Ethics committee.

Fluorescence-activated cell sorting (FACS) purification of crypt and villus fractions

Male GPR41-red fluorescent protein (RFP) transgenic mice were euthanized and ileum was excised, inverted, inflated, and digested for 20 minutes with 0.13 Wünsch units of Liberase (Roche) in DMEM (low glucose) while being shaken in a water bath at 37°C. During this period, the tube with the tissue was inverted 10 times every 5 minutes. Cells liberated during this first enzyme digestion were used for villus fraction. Over the course of the next 35 minutes, the remaining tissue was shaken vigorously for 5 seconds every fifth minute, and cells liberated were discarded. A crypt fraction was yielded through a final incuba-

tion with fresh enzyme solution. Before sorting, the cells were slowly shaken in enzyme solution for a second period of 20 minutes at 37°C, passed through a 70- μ m pore diameter cell strainer (catalog number 352350; BD Falcon), pelleted at 340 relative centrifugal force for 5 minutes, and resuspended in DMEM (low glucose) with 5mM MgCl₂ and 2- μ L DNase (D5319; Sigma-Aldrich). Cells were sorted on dry ice as RFP-positive or RFP-negative using a MoFlo XDP.

RNA extraction and quantitative PCR analysis

RNA was extracted from FACS-purified cells using QIAzol Lysis Reagent (QIAGEN) according to the manufacturer's protocol. The purified RNA was treated with DNase (TURBO DNA-free; Ambion, Thermo Fischer Scientific) to remove potential genomic DNA contamination. cDNA was synthesized using Superscript III (Invitrogen, Thermo Fischer Scientific). Quantitative PCR analysis was performed with SYBR Premix Ex Taq (TaKaRa) and the next primers: alkaline phosphatase (Alpi), 5'-CTGCCAAGAAGCTGCAGCCCA-3' and 5'-GGCTAGGGGTGTCTCCGGTCC-3'; lysozyme (Lyz1), 5'-AGCTGGCTGACTGGGTGTGT-3' and 5'-CGGTGCTTCGGTCTCCA CGG-3'; neurotensin, 5'-CTGGTGTGCTGACTCTCCT-3' and 5'-TCACATCTTCTTCTGAATCTGAGC-3'; preproglucagon, 5'-GGTTGATGAACACCAAGAGGA-3' and 5'-CAGCATGCCTCTCAAATTC-3'; and ppy, 5'-GCAGCGGTA TGGAAAAGAG-3' and 5'-GTCGCTGTCGTCTGTGAA GA-3'. The qPCR was run on a LightCycler480 (Roche).

Laser capture microdissection

Female C57BL/6J (Taconic) mice were used at 12 weeks of age. Laser capture microdissection was performed as described previously (37). SYBR Green Master Mix buffer (1 \times ; Bio-Rad Laboratories) was used for quantitative real-time PCR at final reaction volumes of 25 μ L. Gene-specific results were normalized to the ribosomal protein L32 mRNA. Primer sequences were L32, 5'-CCTCTGGTGAAGCCCAAGATC-3' and 5'-TCTGGTTTCCGCCAGTTT-3'; neurotensin, 5'-CTGGTGTGCTGACTCTCCT-3' and 5'-TCACATCTTCTTCTGAATCTGAGC-3'; Gcg, 5'-AGGGACCTTACCAGTGATGT-3' and

5'-GCGAATGGCGACTTCTTCTGGGAA-3'; and ppy, 5'-ACGGTGCAGCAATGCTGCTAAT-3' and 5'-GACATCTCTTTTCCATACCGCT-3'. Assays were performed in a CFX96 Real-Time System (Bio-Rad Laboratories). The reactions were analyzed with the $\Delta\Delta$ CT analysis method.

Immunohistochemistry

Immunohistochemical staining of the GPR41-RFP reporter mouse

Twenty-week-old male transgenic GPR41-RFP mice were euthanized by cervical dislocation. The distal ileum was excised, rinsed in PBS and fixated in ice-cold, freshly made 4% formaldehyde in PBS for 24 hours at 4°C, cryoprotected for 24 hours (20% sucrose PBS) at 4°C, and embedded in mounting medium for cryotomy (361603E; VWR Chemicals) and plunge-frozen in dry ice-cooled isopentane and subsequently stored at -80°C. Sections (8 μ m) were cut in a cryostat (CM3050 S; Leica), air-dried for 1 hour at room temperature, washed in PBS (3 \times 2 min), and incubated with blocking buffer (2% bovine serum albumin, 0.1% Triton X-100, PBS) for 10 minutes at room temperature, before being incubated with primary antibodies (Table 1) overnight at 4°C. Sections were then washed and incubated with secondary antibodies (Table 1), after mounting of coverslips with mounting medium (S3023; DAKO).

Diphtheria toxin (DTX) receptor-mediated cell ablation

Twenty week-old male Gcg-human DTX receptor (hDTR) transgenic and wild-type mice received ip injections of DTX (D0564; Sigma-Aldrich) dissolved in 0.1% BSA, PBS (100 ng/g body weight). Before injection and 1, 2, 4, and 7 days after, mice were euthanized by cervical dislocation, and distal ileum was excised, rinsed in 1 \times PBS, and fixated in ice-cold, freshly made 4% formaldehyde in PBS for 24 hours at 4°C, stored in 70% alcohol and paraffin embedded with a Shandon excelsior (Thermo Fisher Scientific). Sections (5 μ m) were cut using a microtome (RM2155; Leica), mounted on superfrost plus slides (60°C for 1 h), and stored at 4°C. From each distal ileum, 3–5 cross-sections (50- μ m interval) were collected from 4 different

Table 1. Antibody Table

Peptide/Protein Target	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species	Dilution: Cell-Ablation Studies	Dilution: GPR41-RFP Studies	Dilution: Triple Staining
GLP-1	2135-8	Rabbit	1:8000	1:6000	
GLP-1	Abcam, ab26278	Mouse			1:200
Neurotensin	3844-7	Rabbit	1:10 000	1:3200	
Neurotensin	Santa Cruz Biotechnology, Inc, cc-7592	Goat			1:1000
PYY	Biogenesis, 7260-5104	Rabbit	1:3200	1:6000	1:4000
RFP	Chromotek, 5F8	Rat		1:400	
Somatostatin	Santa Cruz Biotechnology, Inc, sc-7819	Goat	1:600		
AF 488 antirabbit IgG	Life Technologies, A21206	Donkey		1:200	
AF 594 antirat IgG	Life Technologies, A21209	Donkey		1:200	
AF 350 antirabbit IgG	Life Technologies, A10039	Donkey			1:200
AF 488 antimouse IgG	Life Technologies, A21202	Donkey			1:200
AF 568 antigoat IgG	Life Technologies, A11057	Donkey			1:200
Biotinylated antigoat IgG	Dako, E0466	Rabbit	1:200		
Biotinylated antirabbit IgG	Vector Labs, BA-1000	Goat	1:200		

areas (5-mm interval). Sections were then dewaxed and rehydrated using xylene and graded dilutions of alcohol, boiled in 0.01M citrate buffer (pH 6.0) for 15 minutes, and allowed to cool for 30 minutes. Sections were blocked and incubated with primary antibodies (Table 1) overnight at 4°C after incubation with biotinylated secondary antibodies (Table 1) for 1 hour at room temperature, washed, and peroxidase blocked with PBS 3% H₂O₂ for 8 minutes. Sections were incubated with vectastain reagents (Vectastain ABC kit PK 4000; Vector Laboratories) for 30 minutes at room temperature. Stains were developed with 3,3'-Diaminobenzidine solution (catalog number 4170; Kementec Diagnostics) for 15 minutes and enhanced with aqueous solution of CuSO₄ in Tris-NaCl-Tween buffer for 1 minute before counterstaining with Mayer's hematoxylin (Ampliqon), dehydration in graded solutions of alcohol, and mounting of coverslips with pertex (catalog number 00801; Histolab). Cross-sections were evaluated in a bright-field microscope (IX70; Olympus Corp) and positive stains in the intestinal epithelium were quantified.

Triple-labeling immunohistochemistry

Five male C57BL/6N (Taconic) mice were euthanized and the distal ileum was excised, fixed, and embedded in paraffin as described above. Sections were then incubated with combinations of anti-GLP, anti-PYY, and antineurotensin (Table 1) overnight at 4°C. Sections were then washed and incubated with secondary antibodies (Table 1) in blocking buffer for 1 hour at room temperature, followed by washing and mounting of coverslip with mounting medium (S3023; DAKO Corp). For quantification purposes, pictures were captured with a black/white camera (XM10; Olympus Corp) on a fluorescence microscope (IX70; Olympus Corp). Application of pseudocolor and merging of pictures were performed in adobe photoshop CS5.

For quantification, 2 distal ileum cross-sections (>4 mm apart) were stained, and pictures from 8 positions were collected per animal. Each merged picture represent an area analyzed in all 3 channels, enteroendocrine cells located in the intestinal epithelium were quantified as being mono-, di-, or triple-labeled. Furthermore, the position of the enteroendocrine cells along the crypt-villus axis was classified as crypt, lower villus, or top villus. Calculation of cells/mm² was done by measuring the area of interest in Image J and multiplying by the number of counted cell in the same area.

Primary antibody specificity was tested by antigen-saturation. Anti-GLP-1, anti-PYY, and antineurotensin were preincubated with 5- to 10-fold excess (by weight) of peptide GLP-1 (H-6795.1000; Bachem), PYY (H-9180.0500; Bachem), and neurotensin (sc-7592 P; Santa Cruz Biotechnology, Inc), respectively, for 2 hours at room temperature, before being applied in immunohistochemical staining as described above. Control studies revealed no unspecific labeling of the secondary antibodies.

Structured illumination microscopy (SIM)

Mouse distal ileum and archival human ileum of untraceable origin was prepared as described in the section above. Fluorescence was visualized by superresolution SIM (SR-SIM) using an ELYRA PS.1 (Zeiss) microscope equipped with a 488- and 561-nm diode lasers and a 63x/1.40 NA PlanApo oil immersion objective. SI images were captured with a z-distance of 100 nm

with 25 raw SI images per plane (5 phases, 5 angles). Images were then computationally reconstructed with the Zen software (Zeiss), and channels were aligned using parameters obtained from calibration measurements with 100-nm diameter Tetraspeck fluorescent beads (Life Technologies).

Rat intestine perfusion study

Male Wistar rats (Taconic) weighing 250–300 g were used for cosecretion studies. Cosecretion of neurotensin, GLP-1, and PYY was assessed in the perfused rat small intestine as previously described (38).

Hormone secretion from primary colonic crypts

Male C57BL/6N mice (Taconic) were euthanized by cervical dislocation, colons excised, washed with PBS, opened, and cut into 1-mm² pieces. Tissue pieces were washed with PBS 3 times (pipetting and sedimentation) and digested in 10-mL DMEM with 0.4-mg/mL collagenase (Sigma-Aldrich) for 10 minutes and the sedimented crypts were digested for 15 minutes 3 more times. Crypts from digestion 3 and 4 were collected with centrifugation and seeded into 24-well plates coated with Matrigel (200- μ L Matrigel; BD Biosciences; diluted 1:100 in DMEM-F12 and aspirated after at least 1 h) in 700- μ L DMEM-F12 with 10% fetal bovine serum, 0.01-mg/mL penicillin/streptomycin and 2mM glutamine. The next day, cells were incubated with ligands (triplicates) in 250- μ L standard solution (39) containing 0.1% fatty acid free BSA (Sigma-Aldrich) and 10mM glucose (and 0.1% dimethyl sulfoxide from the ligand solution) for 2.5 hours. The standard solution was then transferred to micro tubes and centrifuged for 5 minutes at 3000 relative centrifugal force at 4°C. The supernatant was collected and stored at –80°C until hormone measurement. GLP-1 was measured with Total GLP-1 version 2 from Meso Scale Discovery. Neurotensin and PYY were measured with RIAs as previously described (38).

In vivo testing of coaction of neurotensin with GLP-1 and PYY

In the next experiments, male C57BL/6N mice (Taconic) weighing 25 g upon arrival were used.

Glucose tolerance tests (GTTs) and liquid phase gastric emptying

Mice (n = 7–10 per group) were fasted overnight and experiments were performed the next morning. The GLP-1 receptor agonist liraglutide (30 μ g/kg; Novo Nordisk) was given sc 1 hour before oral or ip administration of glucose (2 g/kg; Sigma-Aldrich) for GTTs, or oral administration of acetaminophen (100 mg/kg; Sigma-Aldrich) for gastric emptying experiments. Neurotensin (6 mg/kg; Polypeptide Laboratories) and PYY3–36 (50 μ g/kg; Alta Bioscience) were given ip 15 minutes before glucose or acetaminophen administration. For GTTs, blood glucose was measured basally, and at time points 0, 15, 30, 60, and 120 minutes after glucose administration using a glucometer (Bayer-Contour; Bayer) drawing blood from a tail vein puncture. Blood samples were also drawn from the retroorbital sinus basally and 15 and 120 minutes after glucose administration, plasma was prepared, and insulin levels determined in duplicates using the Mouse/Rat insulin kit (Meso Scale Discovery). For gastric emptying experiments, blood samples were drawn from the retroor-

bital sinus basally and at 15, 30, and 60 minutes after acetaminophen administration. Plasma was prepared and acetaminophen concentrations were determined in duplicates using an acetaminophen kit with colorimetric detection (MULTIGENT, B2K996; Abbott Laboratories). Acetaminophen can be used as a measure of gastric emptying rate, as it is not absorbed in the stomach but readily absorbed when it reaches the upper small intestine. Thus, gastric emptying rate can be determined from the plasma acetaminophen levels over time.

Palatable food intake studies

Nutridrink is a highly palatable liquid diet containing 1.5 kcal/mL (16% protein, 35% fat, and 49% carbohydrate; Nutricia). Mice ($n = 7–8$ per group) were habituated to Nutridrink diet, TSE cages and single-housing for 5 days before being placed in a TSE indirect calorimeter system (Phenomaster; TSE Systems), in which precise food and liquid intake measurements can be made over time without human disturbance and intervention. On the experimental day, the Nutridrink diet was removed at 10 AM, and liraglutide (30 $\mu\text{g}/\text{kg}$, sc) was given at 4 PM, whereas neurotensin (6 mg/kg, ip) was administered just before lights out at 6 PM. At lights out, Nutridrink was returned to the cages, and intake was measured.

Statistics

Results were analyzed using unpaired 2-tailed t tests, one-way ANOVA with Tukey post hoc test, and two-way ANOVA repeated measures with Tukey post hoc test as indicated in figure legends.

Results

Crypt vs villus expression of GLP-1, PYY, and neurotensin

Using a reporter mouse in which RFP was expressed under the control of the promoter for the short-chain fatty acid receptor GPR41, we recently showed that all GLP-1, PYY, and neurotensin cells express the reporter protein RFP (23, 40, 41). As shown in Figure 1A, the GPR41-RFP cells are evenly distributed along the crypt-villus axis. However, cells costaining for GLP-1 appear to be more abundant in the crypts and lower villi, whereas cells costaining for neurotensin are most abundant in the villi. Two different methods were used to differentiate between crypt and villus expression: FACS and laser-capture technology.

Enteroendocrine cells can be isolated and FACS-purified selectively from the villi vs the crypts using time-resolved enzymatic liberation of cells (Figure 1, B and C) (22). As shown in Figure 1C, the expression of the marker proteins, *Alpi* for enterocytes and *Lyz1* for paneth cells, demonstrated a highly efficient separation of cells from villi vs crypts from the ileum of the GPR41-RFP reporter mice. Quantitative PCR analysis revealed a 5-fold enrichment of the transcripts for the common GLP-1/Gcg precursor in the GPR41-RFP-

positive cells from the crypts compared with cells from the villi (Figure 1C). In contrast, GPR41-RFP positive cells from the villi displayed a 10-fold enrichment of the Pyy transcript and a 28-fold enrichment in the neurotensin transcript relative to the cells from the crypts (Figure 1C).

By laser-capture technology, we harvested cell populations selectively from the tip of the villi and from the crypts from ileal sections and from the surface epithelium and the crypts from colonic sections from normal C57B16/J female mice (Figure 1D). Quantitative PCR analysis demonstrated that also in the laser-captured tissue samples, the transcript for Gcg was preferentially expressed in the crypts of the ileum, ie, 46-fold enriched in crypts vs the villi, whereas Pyy and neurotensin preferentially were expressed in the villi, 162- and 491-fold enriched, respectively (Figure 1D). A similar pattern was observed in the colon, with an 8-fold enrichment of Gcg in the crypts and an 11- and 117-fold enrichment of Pyy and neurotensin, respectively, in the surface epithelium (Figure 1E).

We conclude that, at the mRNA level, GLP-1 is preferentially expressed in the crypts, whereas neurotensin is preferentially expressed in the villi of the ileum and at the surface epithelium of the colon. Concerning PYY, the immunohistochemistry indicates a rather even distribution of PYY cells along the crypt-villus axis, whereas the qPCR analysis shows a clear accumulation of Pyy mRNA mainly in the villi.

Differential reappearance of GLP-1, PYY, and neurotensin cells after cell ablation

Proglucagon promoter-driven expression of the hDTR, followed by treatment with DTX, efficiently and selectively ablates all members of the intestinal-selective enteroendocrine cell lineage without affecting the pan-GI tract enteroendocrine somatostatin- and 5-HT-containing cells (22). Here, we use this technology to study the rate of reappearance of enteroendocrine cells in the distal ileum, based on the hypothesis that preferentially crypt-localized GLP-1 cells would reappear earlier than the preferentially villus-localized PYY and, in particular, neurotensin cells.

As previously observed, already 24 hours after DTX treatment, 89% of the GLP-1 cells, 84% of the PYY cells, and 77% of the neurotensin cells were ablated, whereas the somatostatin cells were not affected at all (Figure 2). However, from 24 to 48 hours after toxin treatment, the number of GLP-1 cells already started to increase, whereas the few remaining PYY and neurotensin cells continued to die and were almost totally eradicated at 48 hours (Figure 2). During the next days, all 3 cell types increased in number. On day 7, the number of GLP-1-positive cells was approaching pretreatment level, whereas the number of

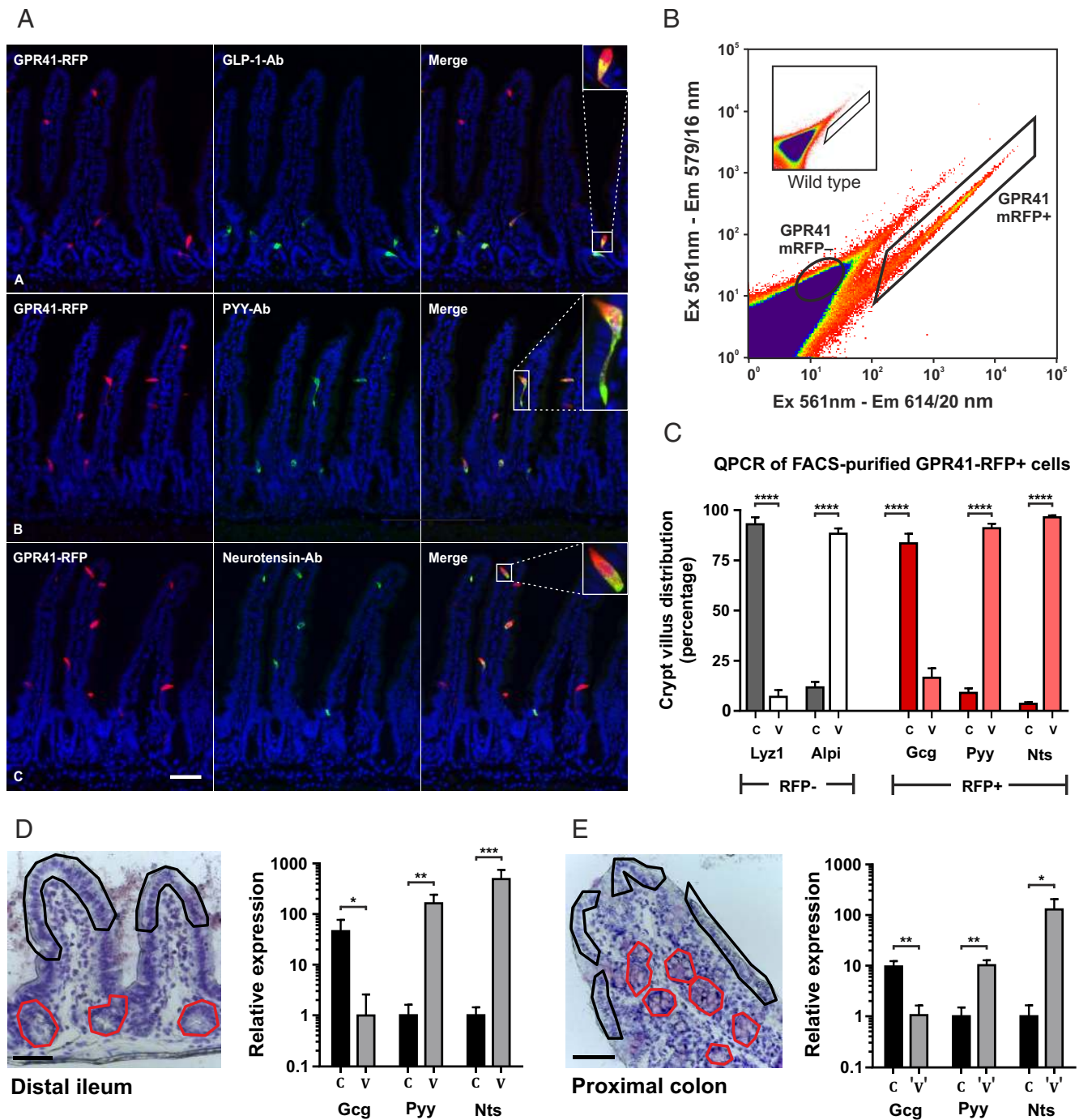


Figure 1. Crypt vs villus hormonal expression in the Gpr41-RFP-positive cells. A, Immunohistochemical staining for GLP-1, PYY, and neurotensin (NTS) in distal ileum of Gpr41-RFP reporter mice. From right to left, red-fluorescent GPR41-expressing cells, antibody-mediated hormone staining and a merged picture. GLP-1 cells are mainly found in crypt and lower part of villi, whereas NTS is mainly found on villi and PYY more evenly distributed. Scale bar, 50 μ m. B, Representative FACS diagram showing the gate (trapezoid surrounding red dots) used for sorting GPR41-RFP-positive cells based on red emission at 614 and 579 nm after excitation at 561 nm. C, Quantitative PCR analysis of FACS-purified GPR41-RFP-positive cells in crypt (C) vs villus (V). Lyz1 and Alpi were used as positive controls for crypt and villus, respectively. Gcg is primarily expressed in crypt fractions, whereas PYY (Pyy) and NTS are primarily found in villus fractions. Data tested with 2-tailed unpaired *t* test. D and E, Quantitative PCR analysis of laser-captured crypt and villus tip. Picture displays areas in mouse distal ileum (left) and proximal colon (right), from which crypts (red circles) and villus tip (black circles) have been isolated by laser capture and used for qPCR. In both ileum and colon, Gcg is primarily expressed in crypts, whereas PYY and NTS are primarily expressed in the villus tips. Data tested with 2-tailed unpaired *t* test. Error bars represent SEM.

PYY- and neurotensin-positive cells only had reached approximately 50% of pretreatment level (Figure 2).

Thus, although the proglucagon promoter-driven cell ablation is highly efficient in eliminating not only GLP-1

cells but also all PYY and neurotensin cells, these latter cell types reappear more slowly than the GLP-1 cells. This is in agreement with their predominant expression higher up in the villus, ie, in more mature enteroendocrine cells.

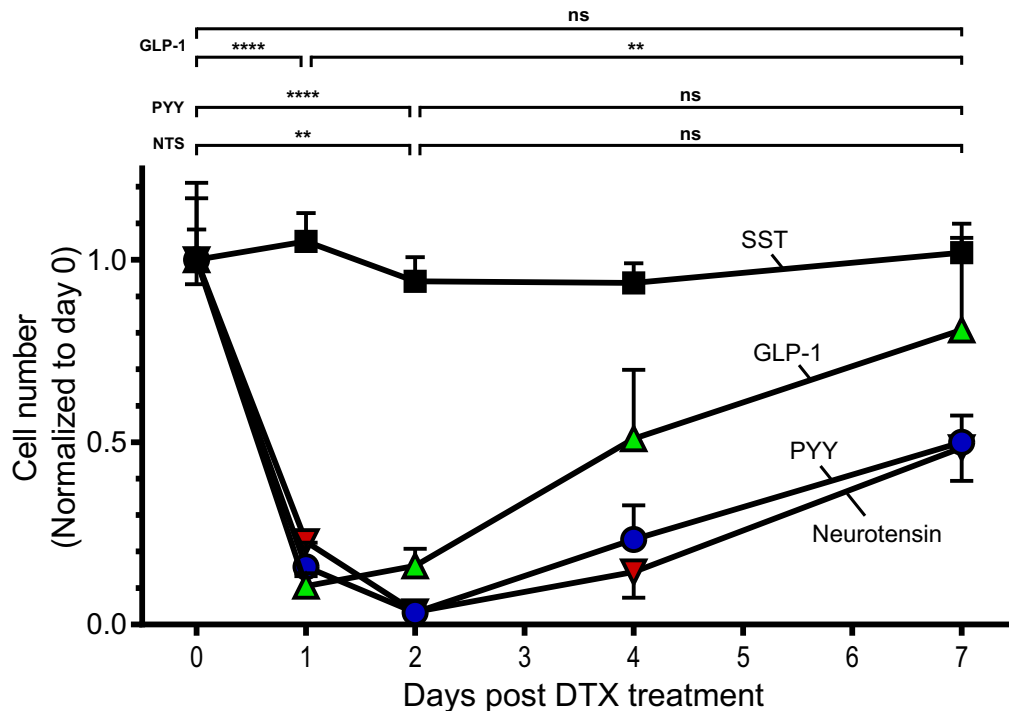


Figure 2. Quantification of enteroendocrine cells in the distal ileum upon hDTR-mediated GLP-1 cell ablation. Somatostatin (SST), GLP-1, PYY, and neurotensin cell populations were quantified before and 1, 2, 4, and 7 days after DTX treatment. The data for each hormone is normalized to the cell count before DTX treatment (normalized to d 0). Before injection, the average cell numbers per tg cross-section were as follows: GLP-1 cell, 30 cells; PYY cells, 32; neurotensin cells, 22; and SST cells, 8. GLP-1 as well as PYY and neurotensin cells were ablated within 24 hours of DTX injection. GLP-1 cells started to recover on day 2, whereas the PYY and neurotensin cell populations continued to decrease. GLP-1 cells continued to recover faster than PYY and neurotensin cells. The SST control cells were completely unharmed by the DTX treatment. Cell counts derive from 10–20 cross-sections per mouse; $n = 5$ mice per time point. Data tested with two-way ANOVA with Tukey post hoc test. Error bars represent SEM.

Coexpression of GLP-1, PYY, and neurotensin in enteroendocrine cells

In order to study and quantify the putative coexpression of GLP-1, PYY, and neurotensin along the crypt-villus axis at the single cell protein level, we performed triple immunohistochemical labeling using specific antisera against the 3 hormones. Cells that were mono-, double-, or triple-labeled for each of the 3 peptide hormones were counted in the crypts and in the villi, which were arbitrarily divided into 2 parts: “top villus” and “lower villus” (Figure 3, A and B).

The quantitative immunohistochemical analysis demonstrated that, overall, GLP-1-positive cells were enriched approximately 2-fold in the crypts vs the top villus, whereas neurotensin cells were enriched by approximately 2-fold in the top villus vs the crypts. PYY-positive cells were rather evenly distributed along the crypt villus axis, with a tendency to be more numerous in the middle, ie, the lower villus (Figure 4, left panels).

Concerning coexpression, a large degree of double- or triple-labeling was observed among the 3 hormones, as mono-labeled cells constituted only a minor fraction of the total number of cells (blue in the right panels of Figure 4). Nevertheless, for both GLP-1 and neurotensin, approxi-

mately 40% of the positive cells in the crypts were mono-labeled for either one or the other hormone. Strikingly, for GLP-1, this fraction dropped to less than 10% in the lower villus, and no mono-labeled GLP-1 cells were observed in the top villus, in which 60% of the GLP-1-positive cells were in fact triple-labeled. In other words, in the top villus, GLP-1-positive cells always also store either PYY (14%) or neurotensin (26%) or both (60%), ie, they are triple-labeled (Figure 4, right upper panel).

For PYY, the most striking observation was that mono-labeled cells in general were very scarce and only amounted to 13% in the villi, where they were most abundant. In the crypts, mono-labeled PYY cells were almost absent; 80% of the PYY cells were double-labeled with GLP-1, corresponding to classical so-called “L cells” (red in the middle right panel of Figure 4).

Neurotensin was the only one of the 3 hormones found in mono-labeled cells in a sizeable fraction along the whole crypt-villus axis, ie, from 45% in the crypts to just below 30% in the villus (blue in the right lower panel of Figure 4). Otherwise, neurotensin was frequently coexpressed with GLP-1 either in double- or triple-labeled cells and only rather infrequently coexpressed with PYY only, ie, less than 10% double-labeled neurotensin-PYY cells in

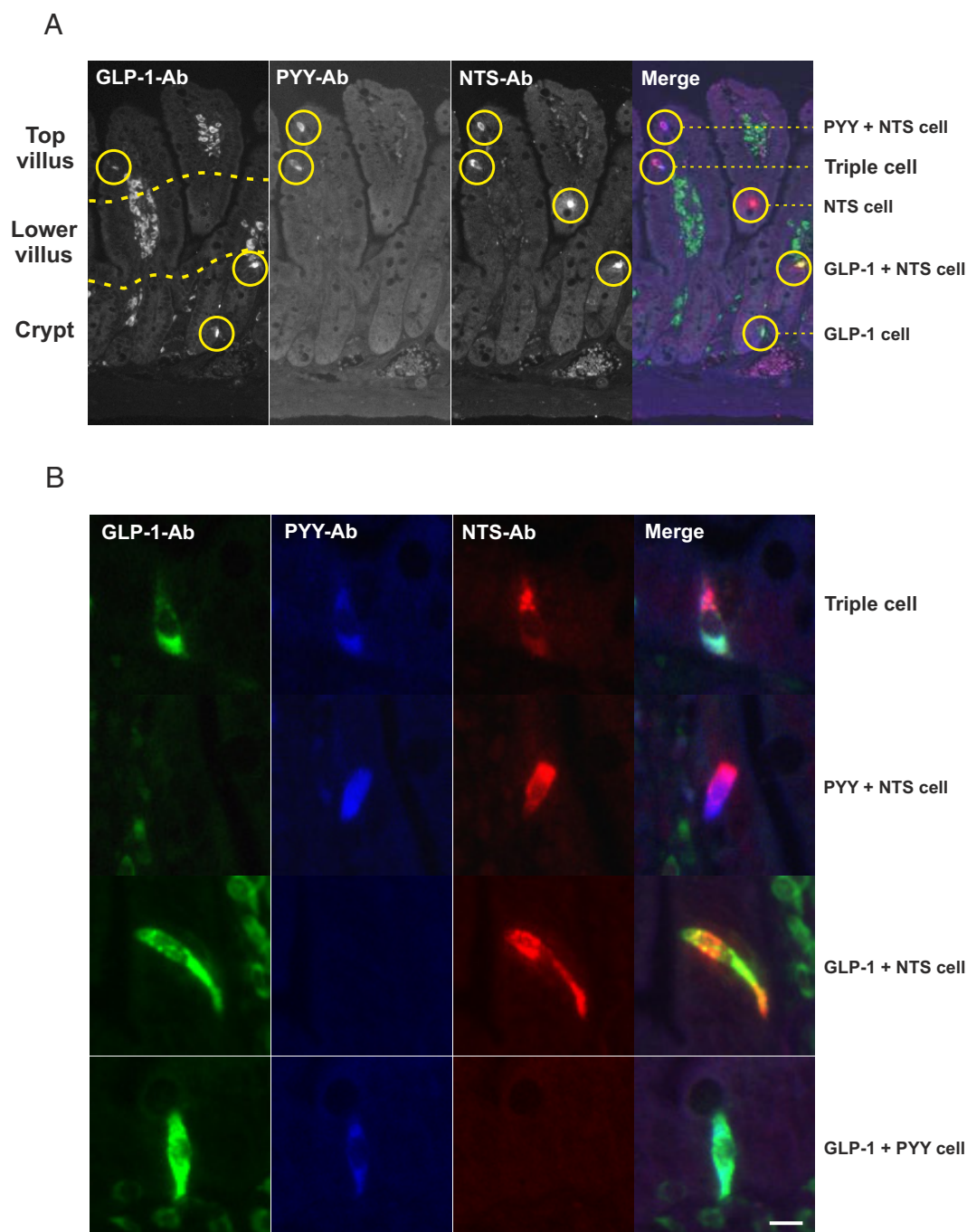


Figure 3. Immunohistochemical triple staining analysis of mouse distal ileum for GLP-1, PYY, and neurotensin (NTS). A, Representative ileal mucosal cross-section with enteroendocrine cells immunohistochemically stained for GLP-1, PYY, and NTS shown in the left 3 subpanels and with the merged picture shown in the right panel. In the GLP-1 subpanel are indicated the 3 regions, "crypt," lower villus, and top villus used for the quantification of the mono-, double-, and triple-stained cell types presented in Figure 5. B, Examples of triple-labeled cell, double-labeled PYY and NTS, and GLP-1 and PYY cells all shown in larger magnification. Scale bar, 10 μ m.

both the crypt and lower villus (green in the right lower panel in Figure 4).

Thus, the immunohistochemical analysis demonstrated a large degree of coexpression of GLP-1, PYY, and neurotensin at the single-cell protein level with, for example mature GLP-1 cells in the villus always storing either PYY or neurotensin or all 3 hormones, and triple-labeled

cells being the dominating cell population in the upper part of the villus.

Subcellular, secretory vesicle localization of GLP-1, PYY, and neurotensin

The dogma that GLP-1 and PYY are costored in common large, dense-core vesicles of the enteroendocrine cells

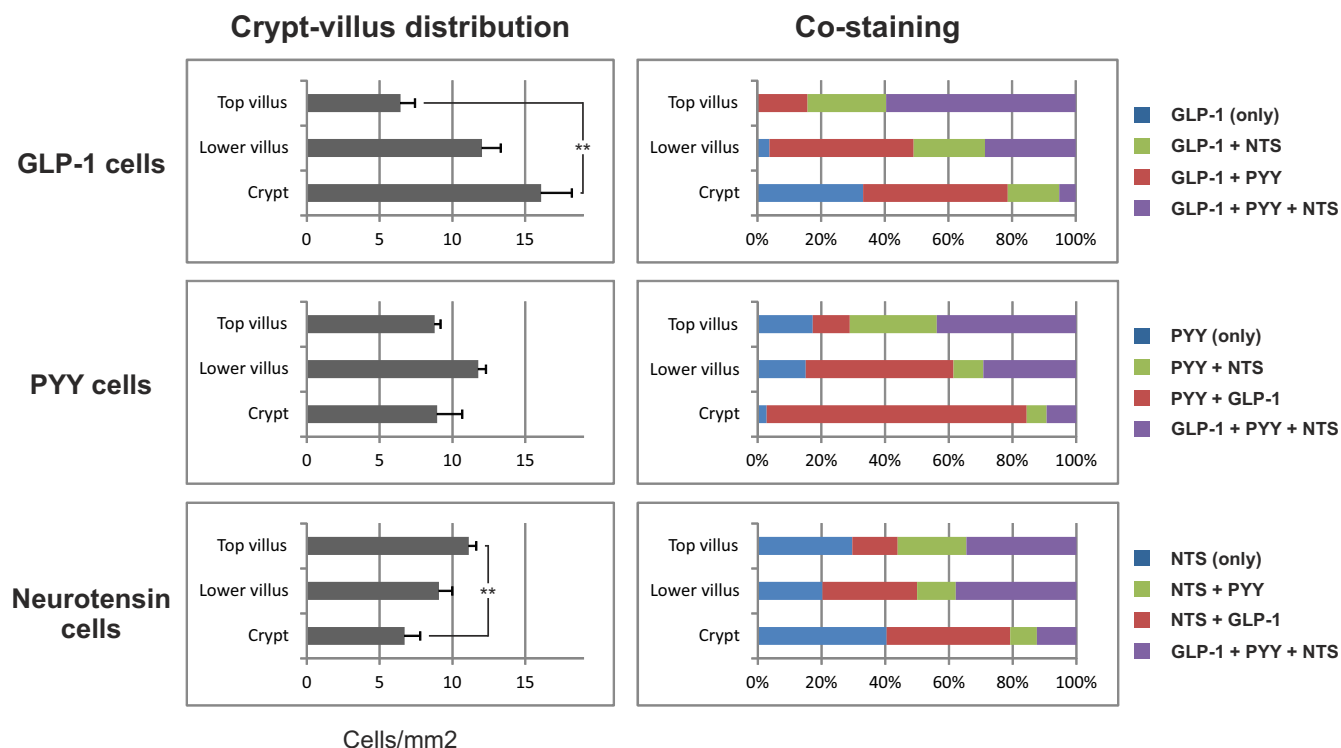


Figure 4. Quantitative immunohistochemical analysis of enteroendocrine cells being stained for GLP-1, PYY, and neurotensin (NTS) in crypt, vs lower villus vs top villus of cross-sections of mouse distal ileum. Left panels, The number of GLP-1 (upper)-, PYY (middle)-, and neurotensin (lower)-positive cells/mm² in the crypt, lower villus, and top villus (see A). Data tested with one-way ANOVA with Tukey post hoc test. Cell counts derive from 2 cross-sections per mouse; n = 5 mice. Bars represent SEM. Right panels, The occurrence of mono-, double-, and triple-labeled enteroendocrine cells color coded as indicated to the right of each panel in the crypt vs lower villus vs top villus presented as percentage of the total number of GLP-1-positive cells (upper), PYY-positive cells (middle), and neurotensin-positive cells (lower).

(42) was recently challenged by high-resolution confocal fluorescence microscopy studies, indicating that GLP-1 and PYY are, in fact, stored in different granules (43). Here, we applied double-labeling immunohistochemistry to mouse and human ileum and visualized the labeling with SR-SIM.

Concerning GLP-1 and neurotensin, a rather clear separation of granules storing either one or the other peptide was observed in the human small intestine. There was no indication of costaining of secretory granules, although granules staining for either one of the 2 peptides were found mixed between each other at the base of the cells (Figure 5B). In the mouse ileum, a similar picture was observed; however, cells were frequently found to contain neurotensin-staining vesicles localized solely in the apical region of the endocrine cells, ie, “above” the nucleus, whereas the GLP-1 staining vesicles, were found mainly “below” the nucleus at the base of the usually flask-shaped cells (Figure 5A). This “compartmentalization” phenomenon was not observed in human ileum (Figure 5B).

As observed for GLP-1 and neurotensin, PYY and neurotensin were clearly localized in separate secretory granules both in the human and murine enteroendocrine cells (Figure 5B). However, for GLP-1 and PYY, the picture

was less clear. Although a majority of distinct secretory vesicles labeled for either GLP-1 or PYY were observed in coexpressing cells, indications of overlapping yellow staining were also detected in particular in subcellular regions with many vesicles. Unfortunately, The SR-SIM technology applied in this study could not distinguish between whether this was colocalization in the same vesicles or labeling of very closely associated vesicles.

We conclude that when GLP-1, PYY, and neurotensin are coexpressed in enteroendocrine cells, the 3 peptides apparently are stored in separate secretory granules. However, with the employed techniques, we cannot rule out that GLP-1 and PYY can be costored in some secretory vesicles, whereas neurotensin clearly is located in separate vesicles.

Cosecretion of GLP-1, PYY, and neurotensin from ex vivo gut preparations

In the perfused rat small intestine, the BB2 neuropeptide receptor agonist, NMC, induced an abrupt and large release of GLP-1 and a parallel release of not only PYY, but also neurotensin (Figure 6A). Similarly, GIP, which is an efficient GLP-1 secretagogue (44) induced a quick and sustained parallel release of neurotensin together with

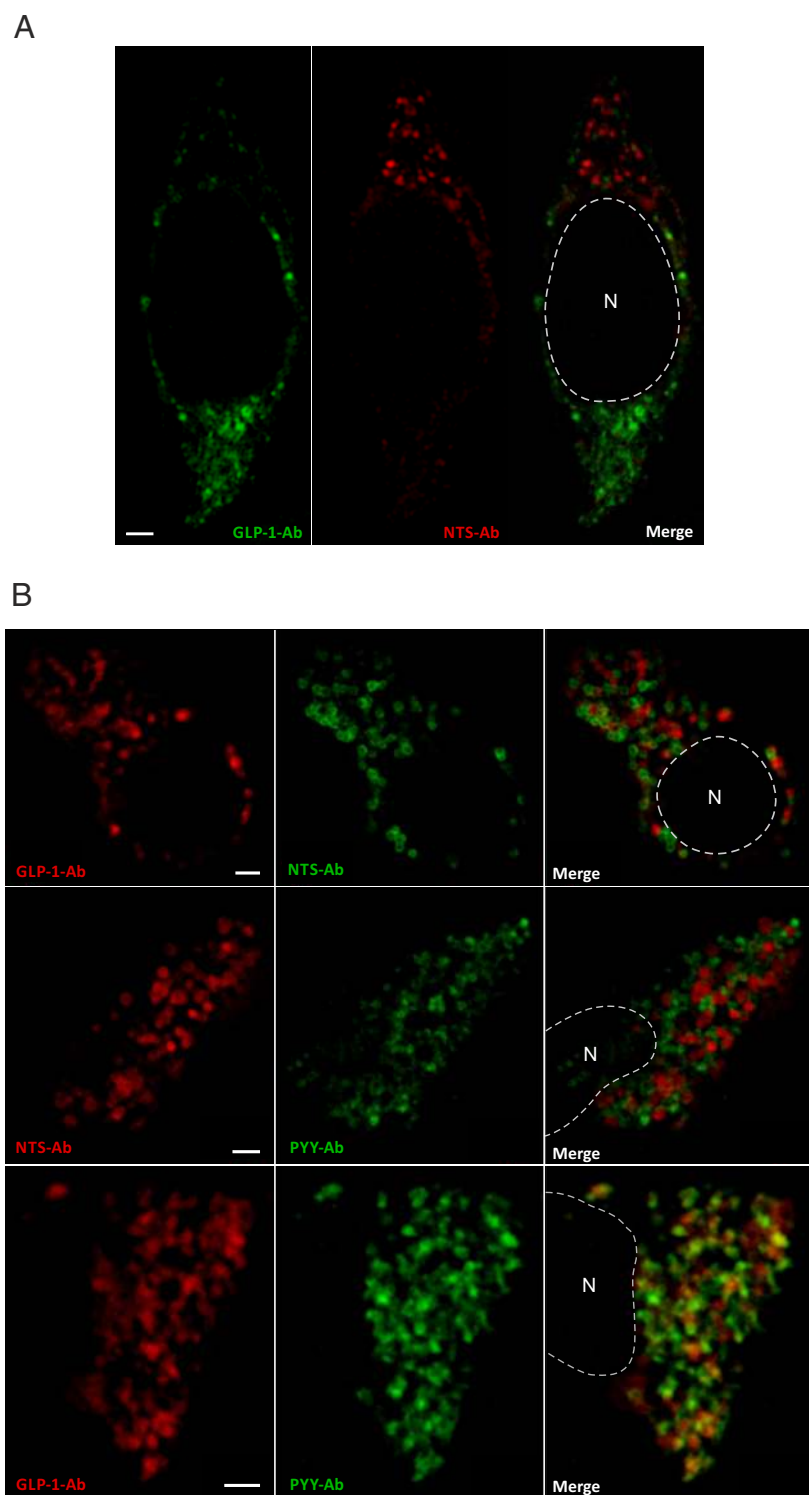


Figure 5. SR-SIM images of double-labeled enteroendocrine cells. A, Mouse distal ileum enteroendocrine cell double-labeled with α -GLP-1 (green) and α -NTS (red) and a merged picture. In this example, the apical part of the cell faces the lumen and the basolateral side faces the lamina propria. GLP-1 and NTS appear to be stored in separate vesicles. Furthermore, GLP-1 vesicles are primarily present in the basolateral part of the cells, whereas NTS vesicles often localize the apical part of the cells. B, Basolateral area of a human ileum enteroendocrine cells double-labeled with α -GLP-1, α -PYY, and α -NTS. Although closely associated, GLP-1 and NTS, and PYY and NTS appear to be stored in separate vesicles, whereas it is difficult to determine whether GLP-1 and PYY are stored in the same vesicles. N, nucleus. Scale bars, 1 μ m.

PYY and GLP-1 from the perfused rat small intestine (Figure 6C). The release pattern of neurotensin, PYY, and GLP-1 was very similar. However, on a molar basis the amount of released neurotensin was larger than that of GLP-1 and PYY in response to NMC, and larger than that of PYY in response to GIP (Figure 6, B and D).

In murine colonic crypt cultures, a maximal stimulatory dose (10^{-6} M) of NMC and similar doses of agonists for the 2-monoacylglycerol receptor GPR119 (AR231453), the bile acid receptor TGR5 (Merck V), and the long-chain fatty acid receptor GPR40 (AM-5262) all stimulated the secretion of all 3 hormones: GLP-1, PYY, and neurotensin (Figure 6E). Although the efficacy of the secretagogues varied, the rank order or the relative efficacy of the 4 secretagogues in respect of stimulating the secretion of each of the 3 hormones was very similar. That is, NMC was the least efficacious secretagogue stimulating GLP-1, PYY, and neurotensin secretion only 1.5- to 2-fold, whereas the TGR5 agonist was the most efficacious compound stimulating the release of all 3 hormones almost 6-fold, whereas the agonists for GPR119 and GPR40 both stimulated hormone secretion 2.5- to 4-fold (Figure 6E). Thus, we conclude that GLP-1, PYY, and neurotensin are co-secreted in response to a rather broad repertoire of neuronal, hormonal and metabolite secretagogues.

Coaction of neurotensin with GLP-1

GTTs were performed in order to observe potential additive or synergistic effects of neurotensin with GLP-1. We administered a low dose (30 μ g/kg) of the long-acting GLP-1 mimetic, liraglutide, which was shown in a dose-response experiment to induce only small, insignificant effects on glucose and insulin during an oral

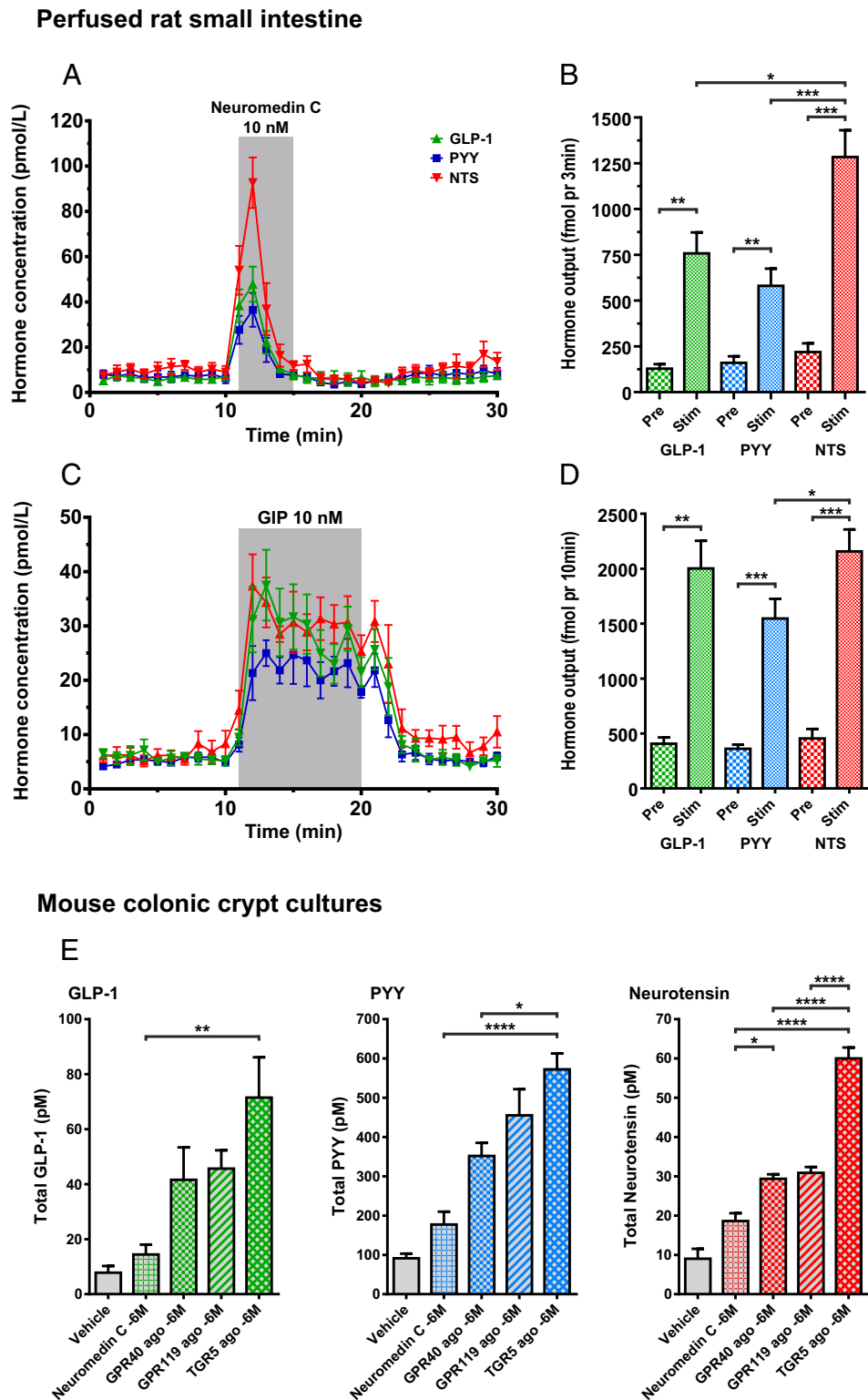


Figure 6. Cosecretion of GLP-1, PYY, and neurotensin from perfused rat intestine and from mouse colonic crypt cultures. A, Concentrations of GLP-1 (green), PYY (blue), and neurotensin (red) in the perfusate from isolated perfused rat distal small intestine during 5 minutes stimulation with 10nM neuromedin C. B, Calculated release of hormones before and during the first 3 minutes of neuromedin C stimulation. C, As A but during 10 minutes of stimulation with 10nM GIP. D, Calculated release of hormones before and during the first 10 minutes of GIP stimulation. E, Release from mouse colonic crypt cultures of GLP-1 (green, left panel), PYY (blue, middle panel), and neurotensin (red, right panel) in response to neuromedin C (10⁻⁶M), the Gq+Gs GPR40 agonist AM-5262 (10⁻⁶M), the GPR119 agonist AR231453 (10⁻⁶M), or the bile acid receptor TGR5 agonist, Merck-V (10⁻⁶M). DMSO was used as vehicle for all agents. Data tested with 2-tailed unpaired *t* test B and D. Error bars represent SEM.

GTT (OGTT) (Supplemental Figure 2, A–D). The neurotensin dose (6 mg/kg) was chosen based on a previous publication (45). As a result of the low dose administered, liraglutide-treated animals showed only a trend towards lower glucose levels at the early time points. Glucose levels did, however, reach statistical significance at the 60-minute time point during the OGTT ($P = .007$ vs saline) (Figure 7A). In contrast, neurotensin delayed the glucose excursions, significantly lowering the glucose level at the 15-minute time point ($P = .008$ vs saline), whereas a significantly higher glucose level was observed at the 60-minute time point ($P = .006$ vs saline) (Figure 7A). However, the combined effect of neurotensin and liraglutide was rather similar to that observed with liraglutide administration alone but with a trend towards a delay in the glucose excursions (Figure 7A; for integrated values see Figure 7B). Insulin levels were similar for all groups during the OGTT, with the exception of the neurotensin-treated animals, in which lower insulin levels were observed at the 15-minute time point (Figure 7C; for integrated values see Figure 7D). Thus, the main effect of neurotensin appeared to be delayed gastric emptying. Accordingly, neurotensin-treated animals showed a trend towards lower glucose levels at all time points when compared with saline-treated animals in ip GTTs (IPGTTs), in which GI tract motility is not a factor (Figure 7E). Liraglutide decreased blood glucose significantly at the time points from 15 to 60 minutes (Figure 7E). In animals receiving both liraglutide and neurotensin, the glucose excursions during IPGTT were in between those observed in response to administration of each of the hormones separately. At the 15-minute time point, the blood glucose was similar to that observed with neurotensin administration alone. Thus, it appears that neurotensin had a counteractive rather than an additive action on the effects of liraglutide (Figure 7E; for integrated values see Figure 7F). All groups had similar insulin levels during the IPGTT (Figure 7G; for integrated values see Figure 7H). Based on the OGTT and IPGTT studies in animals treated with the GLP-1 mimetic, liraglutide, we conclude that neurotensin does not have an additive effect with GLP-1 with respect to glucose metabolism. In fact, the results indicate that neurotensin may even counteract the effects of GLP-1.

Gastric emptying was studied directly in 3 separate experiments. In all 3 experiments, neurotensin delayed gastric emptying, as observed by a reduction in plasma acetaminophen concentrations (Figure 8B). In the experiment shown in Figure 8A, the acetaminophen concentrations were $58 \pm 3 \mu\text{g/mL}$ in saline-treated animals compared with $44 \pm 7 \mu\text{g/mL}$ in neurotensin-treated animals at the 15-minute time point ($P = .012$). The low dose of liraglu-

tide on its own did not have any effect on gastric emptying. However, when neurotensin and liraglutide were administered together, their combined inhibitory effect on gastric emptying was stronger than that observed with neurotensin alone ($P = .0012$) (Figure 8, A and B). Based on these results, we conclude that neurotensin and GLP-1 act synergistically to inhibit gastric emptying.

Palatable food intake was tested by measuring intake of Nutridrink in mice. In this case, neurotensin did not show any effect on its own, whereas liraglutide had a small but significant inhibitory effect on intake of the palatable Nutridrink (Figure 8C). Importantly, when neurotensin and liraglutide were administered together a larger inhibitory effect on food intake was observed than with liraglutide alone ($P = .048$) (Figure 8C). Thus, we conclude that neurotensin and GLP-1, tested using liraglutide, act synergistically to inhibit palatable food intake.

Coactions of neurotensin with PYY

PYY is known to inhibit gastric emptying (46–48). We used a dose-response study to identify a suitable low dose of PYY (50 $\mu\text{g/kg}$) to be used in combination studies with neurotensin (Supplemental Figure 2E). Three identical coadministration experiments were performed, which overall demonstrated an additive inhibitory effect of PYY and neurotensin on gastric emptying (Figure 8E). In one of the 3 experiments, PYY did not produce any inhibition on its own. Importantly, however, we observed a clear synergistic effect between neurotensin and PYY on gastric emptying in this experiment (Figure 8D). The combined effect of neurotensin and PYY was larger than that observed with neurotensin alone ($P = .011$) (Figure 8D). We hereby conclude that neurotensin and PYY coact at least additively and probably synergistically with respect to inhibition of gastric emptying.

Discussion

In the present study, we “reintroduce” neurotensin as a major gut hormone closely integrated with the 2 well-established hormones, GLP-1 and PYY. These 3 peptides are expressed, stored, and released together. Furthermore, they act together in the regulation of peripheral and central metabolic targets. By virtue of their differential expression along the crypt-villus axis, their storage in different secretory granules, and their joint but differentiated actions on different target organs, these 3 hormones are emerging as part of a, not only redundant, but also highly sophisticated and multifaceted, endocrine system.

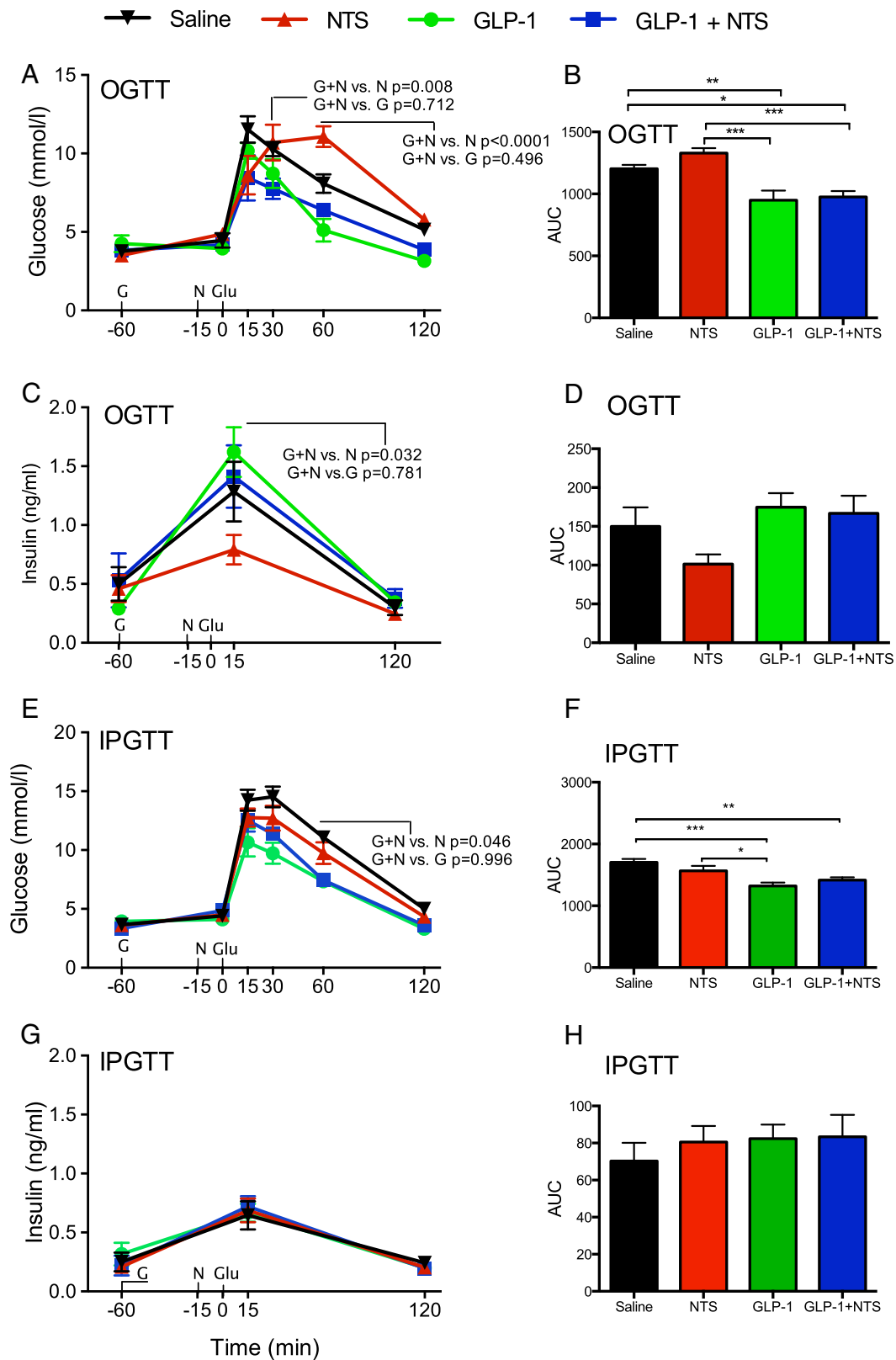


Figure 7. Coactions between neurotensin (NTS) and Glp-1 during GTTs. A, Glucose during OGTT after NTS and GLP-1 administration. B, Integrated values from A. C, Insulin during OGTT after NTS and GLP-1 administration. D, Integrated values from C. E, Glucose during IPGTT after NTS and GLP-1 administration. F, Integrated values from E. G, Insulin during IPGTT after NTS and GLP-1 administration. H, Integrated values from G. Data tested with two-way ANOVA repeated measurements with Tukey post hoc test (A, C, E, and G) and with one-way ANOVA with Tukey post hoc test (B, D, F, and H); n = 8 per group. Error bars represent SEM. G, GLP-1; N, NTS; Glu, glucose.

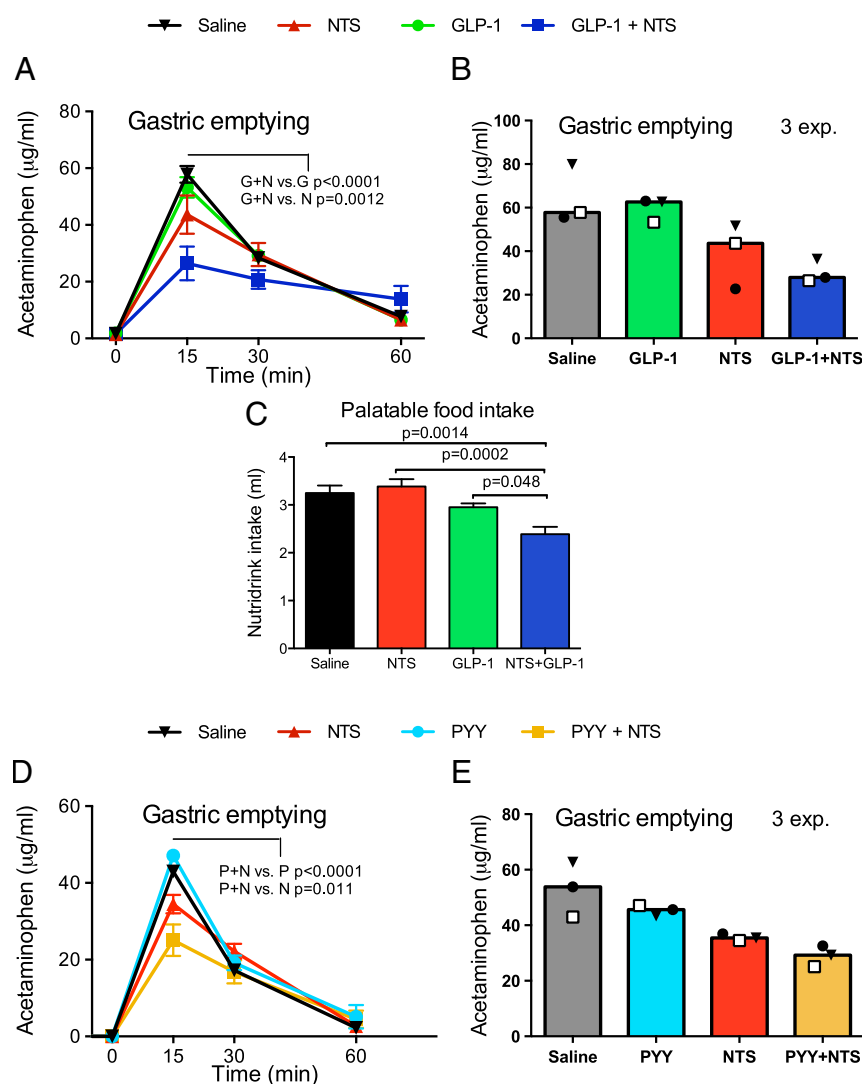


Figure 8. Coactions between neurotensin (NTS), GLP-1, and PYY during gastric emptying tests and in inhibition of palatable food intake. A and B, Gastric emptying after NTS and GLP-1 administration. Representative experiment (A) and median values of the 3 identical experiments performed to time point 15 minutes (B). C, Cumulative intake of a palatable liquid diet after NTS and GLP-1 administration over 3 hours. D and E, Gastric emptying after NTS and PYY administration. Experiment where PYY did not have an effect by itself and synergism was observed (D) and median values of the 3 identical experiments performed at time point 15 minutes (E). Data tested with two-way ANOVA repeated measurements with Tukey post hoc test (A and D) and with one-way ANOVA with Tukey post hoc test (C); $n = 7-10$ per group. Error bars represent SEM. G, GLP-1; N, NTS; Glu, glucose.

Coexpression of GLP-1, PYY, and neurotensin

It is generally assumed that enteroendocrine cells are terminally differentiated and have “chosen” which peptide precursor to express when they leave the crypts, where they all are formed from a common progenitor stem cell (15–19). However, early studies by Gordon and coworkers (49, 50) indicated that enteroendocrine cells often express more than one type of peptide hormone, as recently confirmed in several studies (22, 25, 38). Furthermore, their studies indicated that the expression of the peptides

also differs along the crypt-villus axis (49, 50). Here, we have characterized this phenomenon by FACS purification, laser-capture technology, and triple labeling immunohistochemistry, as well as cell ablation-reappearance techniques focusing on GLP-1, PYY, and neurotensin.

The coexpression of these 3 peptides is not absolute, but increases along the crypt-villus axis. For example, the mature GLP-1 cells in the villus apparently always store either PYY or neurotensin or both, in addition to GLP-1. In fact, 60% of GLP-1 cells in the upper part of the villus, which is conceivably the region that is most exposed to the luminal sensing of nutrients, store all 3 peptides. Interestingly, the difference in expression for the 3 hormones changes more dramatically along the crypt-villus axis at the transcriptional level than it does at the protein level, as observed by immunohistochemistry.

The ablation studies, using the expression of DTX receptor under the control of the Gcg promoter, clearly indicate that the proglucagon transcript is expressed in practically all PYY and neurotensin cells of the mucosa (Figure 2) (22). Thus, the apparent differences in the number of cells detected by immunohistochemistry are probably mainly a reflection of differences in the level of expression of peptides at the protein level. Furthermore, the fact that the immunohistochemical method has a poor detection limit compared with the

DTX receptor ablation method may account for some of these differences.

The faster reappearance pattern of GLP-1-positive cells compared with PYY- and neurotensin-positive cells after DTX-mediated ablation supports the notion that most intestine-specific enteroendocrine cells in the ileum are conceived as GLP-1 cells, which gradually begin to also express PYY and neurotensin, at the detectable protein level - as they move up along the crypt-villus axis. Nevertheless, cells only expressing neurotensin at the detectable protein level are present even in the crypts. However, these cells

also do express Gcg, at least at the transcriptional level, as demonstrated by the efficient ablation of nearly all neurotensin cells in the DTX ablation studies (Figure 2).

Thus, the apparent transdifferentiation process is conceivably more of a quantitative phenomenon, reflecting a change in level of expression controlled by transcriptional factors, which are still rather ill-characterized (51, 52).

Storage of GLP-1, PYY, and neurotensin in separate vs common vesicles

In the eighties, immunogold-electron microscopy studies indicated that proglucagon-derived peptides were co-stored with PYY in the secretory granules (42). This dogma has since remained. However, recent superresolution fluorescence microscopy studies carried out by Furness and coworkers cast a doubt on this notion, as they observed that GLP-1 and PYY clearly were stored in separate secretory vesicles although they concluded that in subcellular regions with many secretory vesicles, separate storage could not be resolved with the technique used (43). In the present study, we also find a mixed picture with both separate and apparently overlapping staining for GLP-1 and PYY in granules from cells coexpressing these 2 peptides. Importantly, in both mouse and man, we observe a clear separation of granules storing neurotensin from those storing either GLP-1 or PYY in cells in which neurotensin was coexpressed with either of the 2 other peptides. In the mouse, neurotensin-containing vesicles were often even segregated in the apical side of the nucleus (Figure 5). The molecular basis for sorting of proteins into large, dense core secretory vesicles is rather poorly understood. Proposed mechanisms for this phenomenon include aggregation-mediated and receptor-mediated sorting, which could be responsible for the sorting of different proteins into different granules (53).

Corelease of GLP-1, PYY, and neurotensin

The 3 different peptides are apparently released in parallel from the enteroendocrine cells in response to a series of different physiological metabolite, neuropeptide, and hormonal stimuli as judged from our experiments in both primary cell cultures and perfused intestines (Figure 6). It should in this context be noted that with the methods used in the present study we are addressing the issue of cosecretion at the physiological level and not addressing the cell biological issue of potential separate release of peptides from separate granules because the 3 different peptides in the tissue preparations used conceivably are released from both mono-, di-, and triple-storing cells (Figure 3).

In the colonic crypt cultures, there were relatively large differences in the efficacies of the different stimuli. Importantly,

however, the rank order for the different stimuli was identical for all 3 hormones; the TGR5 agonist was the most efficacious stimulus, whereas neuromedin C was the least efficacious stimulus (Figure 6).

Notably, although the 3 peptides are coreleased from the distal small intestine, this is not the case from the proximal small intestine. In the proximal small intestine, unlike GLP-1 and neurotensin, PYY is poorly expressed (38), which is also shown in other species ie, dog, monkey, and human (54, 55). Thus, it could be argued that GLP-1 and neurotensin may be more closely associated than GLP-1 and PYY.

Coaction of neurotensin with GLP-1 and PYY

Additive or synergistic effects have previously been described for PYY and GLP-1 (56–59); however, to the best of our knowledge, the present study is the first to demonstrate synergism, ie, more than additive effects of neurotensin with GLP-1 and PYY.

At the molecular level, the GLP-1 receptor and the neurotensin receptor 1, which is dominating in the periphery, are coupled to Gs and Gq/11, respectively, which are signaling pathways that are well established to act synergistically (60, 61). In contrast, PYY most likely exerts its metabolic effects through the Y2 receptor, which is a Gi-coupled receptor. However, activation of Gi-coupled receptors affect other signaling pathways, for example, via $\beta\gamma$ activation, which could act synergistically with Gq activation in the cell (61). Moreover, the synergism we observe in the present study in the whole organism does not necessarily occur at the same cellular target. Rather, the effects could be produced via activation of different physiological pathways acting synergistically on the same target organ.

Effect on gastric emptying and food intake

By decreasing the doses of GLP-1 and PYY, we were able to observe synergistic effects between both of these 2 hormones and neurotensin with respect to inhibition of gastric emptying, and between GLP-1 and neurotensin in relation to food-intake inhibition (Figure 7). The dorsal vagal complex (DVC) in the brainstem and the hypothalamus are central targets for gut hormone control of food intake (62), whereas the DVC and the vago-vagal reflex circuitry are essential for the regulation of gastric emptying. Gut hormones can signal to the DVC either through afferent vagal nerves or directly through the blood circulation to the area postrema, in which the blood brain barrier is incomplete. The GLP-1 receptor, the Y2 receptor and the neurotensin receptor 1 are all present in the nodose ganglion and/or on vagal afferent terminals (63–65) and PYY and GLP-1 can modulate vagal afferent firing (64,

66, 67), suggesting that vagal afferents are important mediators of gut hormone signaling. This seems particularly plausible for gut hormones such as GLP-1 and neurotensin, which have very short half-lives in the circulation (68, 69). Whether neurotensin reaches concentrations in the blood that can exert physiological effects has been a matter of debate (70, 71). Thus, gut hormones could act in a paracrine manner in higher concentrations on vagal afferents close to their release site.

There is, however, also evidence for gut hormones acting directly in the DVC and hypothalamus to regulate feeding and gastric emptying. Receptors for GLP-1, PYY, and neurotensin are present in the DVC and hypothalamus (63, 72–76). Furthermore, microinjections of GLP-1 and PYY into the DVC can directly modulate vagal efferents, which in turn modulate gastric emptying (77–79), and central administration of PYY and GLP-1 can inhibit feeding (62), supporting a direct role of these hormones in the brain. Several studies have tried to establish the relative contribution of the vagal afferent projections in mediating the effects of GLP-1 and PYY on food intake and gastric emptying via surgical or chemical ablation of the vagus nerve, but with discrepant results (64, 80–83). Whether neurotensin acts on vagal afferents in the gut or directly in the brain is not known. Theoretically, it could act through both pathways, as neurotensin receptors are present in the nodose ganglion, DVC and hypothalamus (63, 72, 84). Thus, neurotensin, PYY, and GLP-1 likely act through similar mechanisms involving both the vagus nerve and a direct humoral action in the brain in the control of food intake and gastric emptying.

Effect on glucose homeostasis

The best described action of GLP-1 is as an incretin hormone releasing insulin in a glucose-dependent manner (85). Neurotensin was initially also proposed to stimulate insulin release (31–33). More detailed work has revealed a dual role of neurotensin in glucose homeostasis, as it stimulates insulin release at low glucose concentrations but inhibits glucose-mediated insulin release (86, 87). Thus, in OGTTs during which the glucose concentration is high, it is not surprising that neurotensin seems to counteract the effects of GLP-1. Despite its apparent hyperglycemic effects, neurotensin has been proposed to exert a protective effect on the pancreas independent of hormone secretion. Neurotensin increases pancreatic weight, as well as its DNA and protein content (88, 89) while protecting β -cells from apoptosis in response to cytotoxic agents (86, 90). Thus, neurotensin may act in a similar fashion to GLP-1 in protecting β -cells from cell death in diabetic patients.

Perspectives

The present study demonstrates that, under physiological circumstances, neurotensin is a major coplayer with GLP-1 and PYY in the control of key metabolic functions. The great success of GLP-1 mimetics has demonstrated that a single gut hormone can be exploited pharmacologically to treat diabetes and obesity (6, 7, 9, 10). Our results indicate that even better effects could perhaps be obtained using neurotensin in combination with GLP-1 mimetics. The problem related to the very short half-life of neurotensin could rather easily be solved through generation of prolonged-acting analogs or sustained-release formulations and the development of tachyphylaxis does not appear to be a major problem, at least not for stable NT analogues (91, 92). However, our study indicates that an even better approach may be to stimulate the cosecretion of the full mixture of endogenous gut hormones, including neurotensin, to act in symphony. This could be done, for example, by using an appropriate synthetic agonist for a metabolite receptor, such as a combined Gs- and Gq-signaling agonists for the long-chain fatty acid receptor, GPR40 (60). The observation that all of the neurotensin and PYY cells of the intestine do in fact express the transcript for the peptide precursor for GLP-1 (Figure 2) indicates that it should be possible to increase the number and capacity of the enteroendocrine cells to store and secrete all 3 hormones by affecting the differentiation during their weekly renewal process rather than increasing their proliferation. Proof-of-concept for this approach has recently been achieved in intestinal organoids by using short-chain fatty acids or a notch signaling pathway inhibitor (93, 94). Thus, a dual approach using combinations of compounds that increase the expression and the secretion of the beneficial gut hormones might be the optimal approach.

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