GPR41/FFAR3 and GPR43/FFAR2 as Cosensors for Short-Chain Fatty Acids in Enteroendocrine Cells vs FFAR3 in Enteric Neurons and FFAR2 in Enteric Leukocytes

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The expression of short-chain fatty acid receptors GPR41/FFAR3 and GPR43/ free fatty acid receptor 2 (FFAR2) was studied in the gastrointestinal tract of transgenic monomeric red fluorescent protein (mRFP) reporter mice. In the stomach free fatty acid receptor 3 (FFAR3)-mRFP was expressed in a subpopulation of ghrelin and gastrin cells. In contrast, strong expression of FFAR3-mRFP was observed in all cholecystokinin, glucose-dependent insulinotropic peptide (GIP), and secretin cells of the proximal small intestine and in all glucagon-like peptide-1 (GLP-1), peptide YY, and neurotensin cells of the distal small intestine. Throughout the colon and rectum, FFAR3-mRFP was strongly expressed in the large population of peptide YY and GLP-1 cells and in the neurotensin cells of the proximal colon. A gradient of expression of FFAR3-mRFP was observed in the somatostatin cells from less than 5% in the stomach to more than 95% in the rectum. Substance P-containing enterochromaffin cells displayed a similar gradient of FFAR3-mRFP expression throughout the small intestine. Surprisingly, FFAR3-mRFP was also expressed in the neuronal cells of the submucosal and myenteric ganglia. Quantitative PCR analysis of fluorescence-activated cell sorting (FACS) purified FFAR3-mRFP positive cells confirmed the coexpression with the various peptide hormones as well as key neuronal marker proteins. The FFAR2-mRFP reporter was strongly expressed in a large population of leukocytes in the lamina propria of in particular the small intestine but surprisingly only weakly in a subpopulation of enteroendocrine cells. Nevertheless, synthetic ligands specific for either FFAR3 or FFAR2 each released GLP-1 from colonic crypt cultures and the FFAR2 agonist mobilized intracellular Ca^{2+} in FFAR2 positive enteroendocrine cells. It is concluded that FFAR3-mRFP serves as a useful marker for the majority of enteroendocrine cells of the small and large intestine and that FFAR3 and FFAR2 both act as sensors for short-chain fatty acids in enteroendocrine cells, whereas FFAR3 apparently has this role alone in enteric neurons and FFAR2 in enteric leukocytes. **(*Endocrinology* 154: 3552–3564, 2013)**

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Abbreviations: AR420626, N-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide; CCK, cholecystokinin; CFMB, (S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-yl)butamide; FACS, fluorescence-activated cell sorting; FFAR2, free fatty acid receptor 2 (GPR43); FFAR3, free fatty acid receptor 3 (GPR41); GI, gastrointestinal; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide 1; mRFP, monomeric RFP; NMC, neuromedin C; PYY, peptide YY; qPCR, quantitative PCR; RFP, red fluorescent protein; SCFA, short-chain fatty acid; VGF, nerve growth factor inducible; VIP, vasoactive intestinal polypeptide.

The enteroendocrine system is the largest and most diversified endocrine organ of the body with its many different types of hormone producing cells scattered among the enterocytes along the epithelium throughout the gastrointestinal (GI) tract (1). Enteroendocrine cells are generally flask shaped with an apical, microvillus-decorated sensory extension reaching the gut lumen and with dense-core secretory granules at the base of the cell from which the gut peptide hormones are released (2, 3). Often the enteroendocrine cells have basolateral extensions, which may serve both sensory and targeted, paracrine secretory functions (4, 5). The prevailing dogma has been that each of the approximately 15 different enteroendocrine cell types express and release peptides only from a single peptide hormone precursor, ie, except for the glucagon-like peptide-1 (GLP-1)/peptide YY (PYY) cells of the distal small intestine and colon and the ghrelin/motilin cells of the proximal small intestine (1, 6, 7). However, recently it became clear that cells expressing the functionally related peptides, cholecystokinin (CCK), secretin, glucose-dependent insulinotropic peptide (GIP), GLP-1, PYY, and neurotensin, are more closely related than generally appreciated and that they each have the capacity to express other peptide hormones from this group of peptides (8, 9). The functional consequence of this relatively broad peptide coexpression is still unclear. However, together with the fact that the enteroendocrine cells are renewed approximately every week, it does indicate that the enteroendocrine system could be subject to a larger degree of plasticity that potentially could be targeted for the treatment of obesity and type 2 diabetes (9).

An essential function of the individual enteroendocrine cell is to sense the luminal content of the gut and report on this to the central nervous system and to other endocrine and metabolic organs of the body through neuronal, endocrine, and paracrine pathways, which are activated by the peptide hormones (3, 6). In addition to glucose, most nutrients or food components are sensed through their metabolites by chemosensors, ie, dedicated seven-transmembrane (7TM) G protein-coupled receptors located in the cell membrane (3). For example, triglycerides are degraded by pancreatic lipase and the metabolites, and long-chain free fatty acids and monoacyl-glycerol, are sensed by GPR40/GPR120 and by GPR119, respectively (10).

Short-chain fatty acids (SCFAs), ie, mainly acetate, butyrate, and propionate, are generated by the gut microbiota through the digestion of complex carbohydrates, ie, mainly dietary fibers (11, 12). The SCFAs, which can reach concentrations around 100 mM in the lumen of the colon, are readily absorbed and constitute 6%-9% of our daily energy requirement (13). The absorbed SCFAs are also readily taken up by the liver, and concentrations in peripheral plasma are consequently generally low compared with the gut lumen but are still approximately 10^{-5} M (14). However, SCFAs not only serve as an important energy source, but they also act as chemical messengers or signaling molecules. In the intestine SCFAs are known to affect, for example, smooth muscle contractions, electrolyte secretion, and cell growth (15–17). Recently SCFAs have been shown to increase proglucagon and pro-PYY gene expression and to increase plasma levels of GLP-1 and PYY and inhibit ghrelin secretion (18). Moreover, ingestion of complex carbohydrates such as oligofructose and inulin has been reported to up-regulate GLP-1 and PYY (17, 19). SCFAs are also well known to affect leukocyte function (20–23), and it was recently shown that receptors for SCFAs are essential for the recruitment of neutrophils during intestinal inflammation and that the regulation of inflammatory responses by gut microbiota is mediated through SCFA receptors (24, 25)

In 2003 several groups reported that the 2 closely related, orphan seven-transmembrane or G protein coupled receptors, FFAR3 (GPR41) and free fatty acid receptor 2 (FFAR2; GPR43) were receptors for SCFAs (26–28). The 2 SCFA receptor subtypes are encoded in tandem at a single chromosomal locus, and their selectivity for SCFAs is rather similar because they both are activated by 2 of the dominating SCFAs, propionate and butyrate, with EC_{50} values approximately 10^{-5} M. However, whereas acetate has a similar potency on FFAR2 as propionate and butyrate, it is 100-fold less potent on free fatty acid receptor 2 (FFAR3), at least in man (26). In contrast, the ketone body, β-hydroxybutyrate, produced by the liver during starvation, has been reported to act on FFAR3 as opposed to FFAR2, suggesting that FFAR3 may play a more fundamental role in the integration of external and internal metabolic signals (29). Both FFAR2 and FFAR3 couple through Gi/o pathways and both receptors inhibit cAMP production, whereas FFAR2 as opposed to FFAR3 also couples efficiently through Gq (26). There has been some confusion about the expression pattern of FFAR2 and FFAR3. However, it is generally agreed that FFAR2 is highly expressed on leukocytes, which is underlined by the fact that FFAR2 was recloned in the mouse purely based on its function as a leukocyte specific 7TM receptor (26–28, 30). Importantly, it has recently become increasingly clear that both FFAR2 and FFAR3 are expressed on enteroendocrine cells (5, 18, 31–36).

In the present study, we use transgenic reporter mice expressing monomeric red fluorescent protein (mRFP) (37) under the control of promoter elements for FFAR2 and for FFAR3, respectively, to identify SCFA receptor-expressing cells in the GI tract. The expression of FFAR3-mRFP in enteroendocrine cells was extensive and intense

throughout the GI tract and was characterized in detail by immunohistochemical costaining and by analysis of fluorescence-activated cell sorting (FACS) purified cells. Surprisingly, FFAR3-mRFP was found to be expressed also in enteric neurons, ie, in both submucosal and myenteric ganglia. The expression of FFAR2-mRFP was particularly intense in the leukocytes of the lamina propria but was also found in enteroendocrine cells but more sporadically. Our functional studies indicated that both FFAR2 and FFAR3 are important for hormone release from the enteroendocrine cells.

Materials and Methods

Animals

To generate the FFAR2 and FFAR3 reporter mice, we used bacterial artificial chromosomes in which the coding sequence of Ffar3 or Ffar2 was replaced by a cassette consisting of mRFP followed by a polyadenylation signal from bovine GH and a selection gene (β-lactamase) flanked by flippase (FLP) recombinase target sites using a RecE/RecT recombination, as described previously (38, 39). After verification and fragment length polymorphism-mediated excision of the β-lactamase gene, transgenic founder lines were generated via pronucleus injection into FvB/N oocytes. At least 3 different founders were used to generate FFAR2 and FFAR3 reporter lines, which all showed consistent expression patterns for mRFP. Animals were kept on a C57BL/6 background. A full description of the generation of FFAR3-mRFP and FFAR2-mRFP transgenic mice and characterization of their expression pattern of the reporter in other tissues such as adipose tissue will be published elsewhere.

Only male mice were used in this study, and they were housed with ad libitum access to regular chow and water under a 12-hour light, 12-hour dark cycle. Animals aged 8–10 weeks were used and euthanized by cervical dislocation. Animal procedures were conducted in accordance with the Danish Animal Research authorities (personal animal license 2012–15-2934–00221 issued by the Danish Committee for Animal Research).

Compounds

Sodium propionate was purchased from Sigma Aldrich. The FFAR2 selective agonist (S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-yl)butamide (CFMB) was purchased from Calbiochem. The FFAR3 selective agonist N-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (AR420626) was synthesized as described elsewhere (40).

Immunohistochemistry

Tissue samples were rinsed in PBS, fixed in ice-cold freshly prepared 4% paraformaldehyde for 24 hours followed by cryoprotection (20% sucrose, PBS) at 4°C for 48 hours, and rinsed in cold PBS before being embedded and snap frozen in optimal cutting temperature compound (Tissue-Tek; Sakura). Sections, 8 μm were cut (CM3050 S cryostat; Leica), dried for 1 hour at room temperature, washed (3 × 5 min) in cold PBS and incubated with blocking buffer (2% BSA in PBS) at room temperature before being incubated overnight at 4°C with primary antibodies (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) diluted in blocking buffer. Sections were washed (3 × 5 min) in PBS before incubated 1 hour at room temperature with secondary antibodies diluted 1:200 and washed in PBS (3 × 5 min). Tissue sections were coverslipped with fluorescent mounting medium (S3023; Dako). Tissue sections for enteric nerve evaluation were mounted in medium containing 4′,6′-diamino-2-phenylindole (DAPI, ProLong Gold reagent; Molecular Probes). Sections were analyzed with an IX70 Olympus microscope and a XM10 Olympus camera (Olympus). Pseudocolor application and picture merging were done by Adobe Photoshop.

Single-cell suspension and FACS purification

The distal 10 cm of the small intestine (ileum) was harvested from and pooled from 3 transgenic animals. Segments were inverted, inflated, and digested for 20 minutes with 0.13 Wünsch units Liberase (Roche) in DMEM 1885 while being slowly shaken at 37°C. Every fifth minute, the tissue was shaken vigorously for 5 seconds. This procedure was repeated 3 times. Before sorting, the cells were slowly shaken for a second period of 20 minutes at 37°C, passed through a 70-μm pore diameter cell strainer, pelleted at 1500 rpm for 5 minutes, and resuspended in DMEM 1885 with 10% fetal bovine serum. Red fluorescent protein (RFP)-positive cells were purified by FACS using a MoFlo XDP (Beckman Coulter) and stored at −80°C (9).

Quantitative RT-PCR analysis

Total cellular RNA was extracted using NucleoSpin RNA XS kits (Macherey-Nagel), and RT-PCR was performed using SuperScript III reverse transcriptase (Invitrogen). Custom-designed StellARray quantitative PCR (qPCR) arrays (Lonza) covering 93 selected peptide hormones and neuropeptides were used according to manufacturer's instructions by SYBR Premix Ex Taq (Takara). Primers were designed within single exons; consequently, a DNA sample can function to serve as a calibrator. Quantitative RT-PCR was performed using a LightCycler480 (Roche). A relative copy number was calculated for each gene using genomic DNA as a calibrator and further normalized to the reference gene *YWHAZ* as described (41).

GLP-1 secretion from primary colonic crypt cultures

Colonic crypts were prepared from male C57BL/6 mice by collagenase digestion as described by Reimann et al (42) and seeded into 24-well plates coated with Matrigel (BD Biosciences). The following day, cells were washed and incubated for 3 hours with ligands (quadruplicates) in standard solution (42) containing 0.1% fatty acid-free BSA (Sigma-Aldrich) and 10 mM glucose. GLP-1 was measured according to the protocol Total GLP-1 version 2 from Meso Scale Discovery (model number K150JVC-1).

Ca^{2+} imaging in FFAR2-mRFP-positive cells

Crypts from FFAR2-mRFP reporter mice were prepared as described above, plated on matrigel-coated glass coverslips (Thermo Scientific) in growth medium [DMEM-F12 with 10% fetal bovine serum, 30 ng/L murine epidermal growth factor, 0.1 mg/L gentamicin (Sigma-Aldrich), 2 mM L-glutamine, penicillin, and streptomycin]. Ca^{2+} imaging experiments were performed

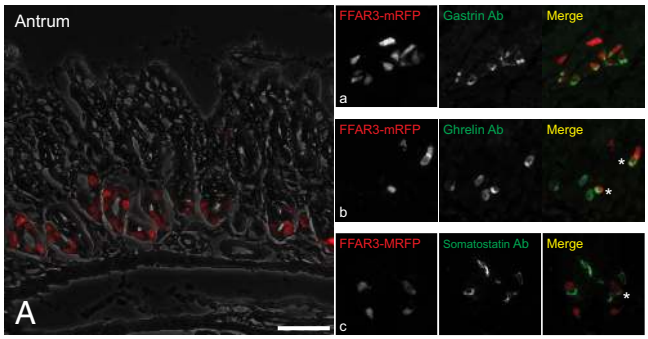

Figure 1. Immunohistochemical colocalization of selected gastric peptide hormones with FFAR3-mRFP in the gastric mucosa from FFAR3-mRFP transgenic reporter mice. A, Overview of the localization of FFAR3-mRFP in the gastric mucosa. Aa, Colocalization of FFAR3-mRFP (red in left panel) with gastrin (green in middle panel). Ab, Colocalization of FFAR3-mRFP (red in left panel) with ghrelin (green in middle panel) in 2 of 5 ghrelin-immunoreactive cells indicated with asterisks in the right panel. Ac, Colocalization of FFAR3-mRFP (red in left panel) with somatostatin (green in middle panel) in 1 of 8 somatostatin-immunoreactive cells indicated with an asterisk in the right panel. Dark-field image was used to visualize the background of the tissue. Bar, 50 μm.

after 3–8 days when cells were loaded for 45 minutes at 37°C with 4 μM fluo-4 AM (Invitrogen), 2.5 mmol probenicid (Invitrogen), 300 μM eserine (Sigma-Aldrich), and 10 μL power load (Invitrogen) in growth medium. Hoechst 33342 (Thermo Scientific) was added for the last 5 minutes, and the coverslips with cells were placed in standard solution (42) with 10 mM glucose. Live cell imaging was performed using a Zeiss Inverted LSM 780 microscope with a ×40 water-immersion objective in an incubation chamber at 37°C. Fluo-4 was excited at 488 nm, Hoechst 33342 at 405 nm, and mRFP at 543 nm using an argon, diode, and HeNe laser, respectively, controlled by ZEN software. The FFAR2 agonist CFMB was added at a final concentration of 1 μM, and the fluo-4 fluorescence measurements were recorded every 1.5 seconds.

FFAR3 signaling through inositol phosphate accumulation

Receptor cDNAs were cloned into pCMV-Tag(2B) (Stratagene). COS-7 cells were grown in DMEM-1885 with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were cotransfected through calcium phosphate precipitation with the receptor and the chimeric G protein, $G\alpha_{qi4myr}$, enabling the $G\alpha_i$-coupled signal to be measured through the $G\alpha_q$ pathway (43).The cells were incubated with various concentrations of propionate (SUPELCO) and AR420626, followed by inositol phosphate turnover measurements (44).

Results

In the transgenic line expressing mRFP under the control of the FFAR3 promoter (FFAR3-mRFP), red fluorescence was observed in enteroendocrine-like cells throughout the entire GI tract, ie, from the stomach to the rectum. However, in the following, the expression of mRFP was detected by immunohistochemistry using an RFP antibody.

In the gastric mucosa, FFAR3-mRFP is expressed in gastrin and ghrelin-positive cells

FFAR3-mRFP-expressing cells were relatively scarce in the mucosa of the body of the stomach but were found abundantly in a relatively narrow band along the bottom of the gastric glands of the antrum (Figure 1). Immunohistochemical analysis demonstrated that more than 90% of the gastrin immunoreactive cells of the antrum expressed FFAR3-mRFP (Figure 1A and Table 1). In addition, approximately 50% of the ghrelin cells were clearly FFAR3-mRFP positive (Table 1). However, a relatively large fraction of the rest of the ghrelin cells also appeared to express FFAR3-mRFP but only weakly. In contrast, the somatostatin-immunoreactive cells, which were scattered evenly throughout the gastric mucosa, very rarely (<5%) expressed FFAR3-mRFP (Figure 1C and Table 1).

In the small intestine, FFAR3-mRFP is expressed mainly in the CCK-secretin-GIP-GLP-1-PYY-neurotensin lineage of enteroendocrine cells

The FFAR3-mRFP-positive enteroendocrine-like cells were abundant throughout the small intestine scattered rather evenly along the crypt-villus axis (Figure 2, A–C).

Table 1. Quantitative Expression of GPR41-RFP by the Enteroendocrine Cells

Peptide (Cell Type)	Stomach	Duodenum	Jejunum	Ileum	P. Colon	M. Colon	Rectum
Ghrelin	50%	–	–	–	–	–	–
Gastrin	>90%	–	–	–	–	–	–
CCK	–	100%	–	–	–	–	–
Secretin	–	100%	–	–	–	–	–
GIP	–	100%	100%	–	–	–	–
Substance P	–	30%	50%	90%	–	–	–
Neurotensin	–	–	–	100%	100%	–	–
GLP-1	–	–	–	100%	100%	100%	100%
PYY	-	–	–	100%	100%	100%	100%
Somatostatin	<5%	5–10%	5–10%	50%	80%	90%	>95%

Abbreviations: M. Colon, middle colon; P. Colon, proximal colon; –, not tested.

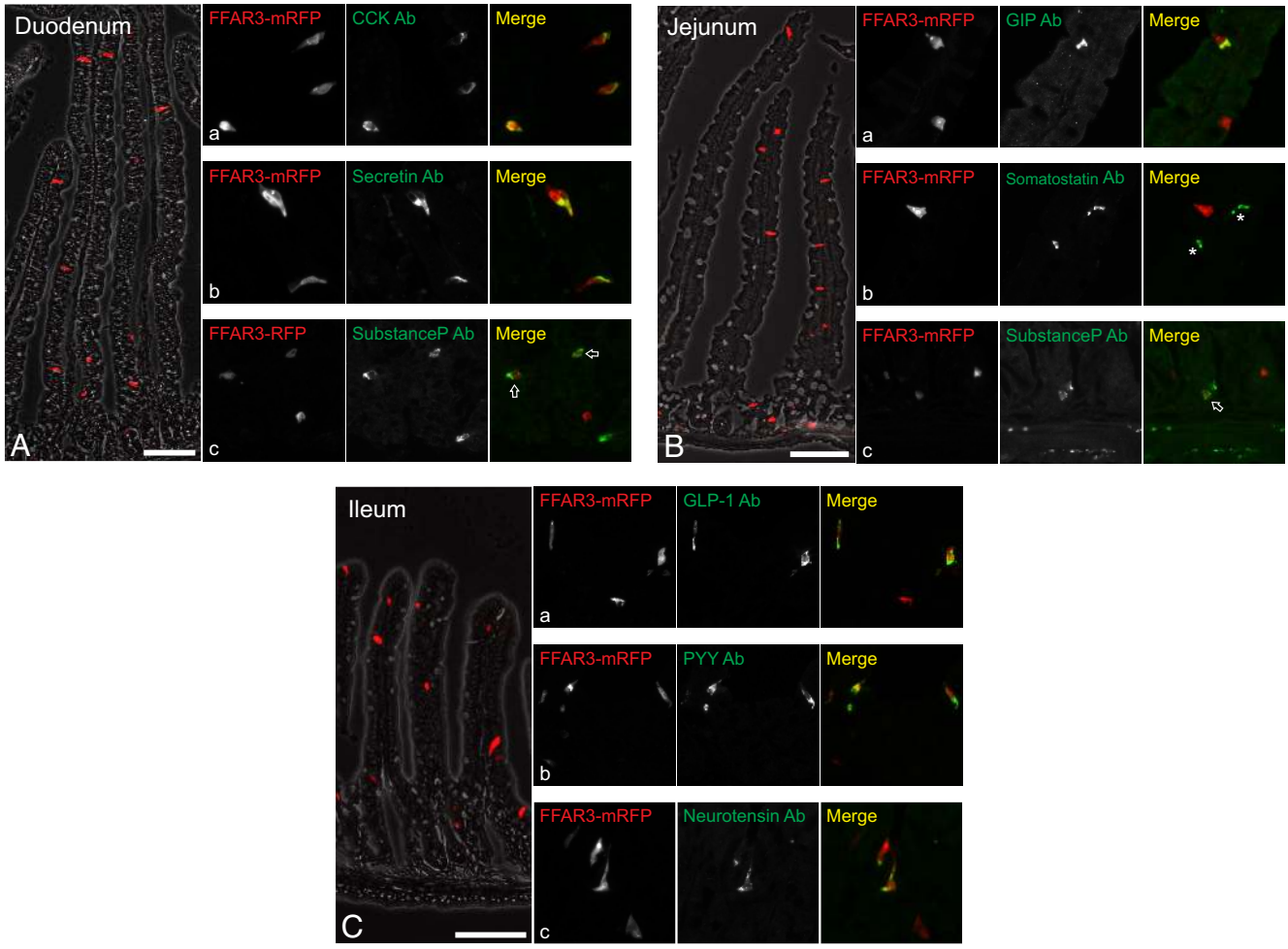

Figure 2. Immunohistochemical colocalization of selected peptide hormones with FFAR3-mRFP in the mucosa from duodenum, jejunum, and ileum from FFAR3-mRFP transgenic reporter mice. A, Overview of the localization of FFAR3-mRFP in the duodenal mucosa. Aa, Colocalization of FFAR3-mRFP (red in left panel) with CCK (green in middle panel) in 3 of 3 CCK-immunoreactive cells. Ab, Colocalization of FFAR3-mRFP (red in left panel) with secretin (green in middle panel) in 2 of 2 secretin-immunoreactive cells. Panels Ac, Colocalization of FFAR3-mRFP (red in left panel) with substance P (green in middle panel) in 2 of 3 substance P-immunoreactive cells. B, Overview of the localization of FFAR3-mRFP in the jejunal mucosa. Ba, Colocalization of FFAR3-mRFP (red in left panel) with GIP (green in middle panel) in a GIP-immunoreactive cells. Bb, Lack of colocalization of FFAR3-mRFP (red in left panel) with somatostatin (green in middle panel) in 2 somatostatin-immunoreactive cells (asterisks). Bc, Colocalization of FFAR3-mRFP (red in left panel) with substance P (green in middle panel) in 1 (asterisk) of 2 substance P-immunoreactive cells. C, Overview of the localization of FFAR3-mRFP in the ileal mucosa. Ca, Colocalization of FFAR3-mRFP (red in left panel) with GLP-1 (green in middle panel) in 2 of 2 GLP-1-immunoreactive cells. Cb, Colocalization of FFAR3-mRFP (red in left panel) with PYY (green in middle panel) in 2 of 2 secretin-immunoreactive cells. Cc, Colocalization of FFAR3-mRFP (red in left panel) with neurotensin (green in middle panel) in 3 of 3 neurotensin-immunoreactive cells. Dark-field image was used to visualize the background of the tissue. Bar, 100 μm.

CCK immunoreactive cells, which mainly were found in the proximal part of the small intestine, basically all expressed FFAR3-mRFP as shown for duodenal CCK cells in Figure 2Ab. Similarly, all immunoreactive secretin cells, which almost exclusively were found in the duodenum, expressed FFAR3-mRFP (Figure 2Aa). GIP-immunoreactive cells were relatively abundant in the jejunum but were also found in the duodenum, and like the 2 other main hormones of the proximal small intestine, CCK and secretin, all GIP cells expressed FFAR3-mRFP (shown for jejeunal GIP cells in Figure 2Ba).

In the distal part of the small intestine, basically all enteroendocrine GLP-1 immunoreactive cells, which mostly were located in the crypts and lower part of the villi, expressed FFAR3-mRFP (Figure 2Ca and Table 1). This was also the case for the PYY-immunoreactive cells, which were located along the whole crypt-villus axis (Figure 2Cb) and for the neurotensin-immunoreactive cells, which mainly were located in the villi (Figure 2Cc; GLP-1, PYY, and neurotensin are often expressed in the same cell either 2 and 2 or all 3 together; however, this issue is not further addressed in the present study because basically all of the cells expressing one or more of the hormones also expressed FFAR3-mRFP). Very rarely a single GLP-1 or PYY cell did not express FFAR3-mRFP, and in all cases these cells were located at the very bottom of the crypts.

Enteroendocrine cells containing substance P immunoreactivity were exclusively located in the crypts and found throughout the small intestine. The fraction of substance P-positive cells expressing FFAR3-mRFP increased toward the distal part of the small intestine (Table 1). Thus, only approximately 30% of the substance P-positive cells in the duodenum expressed FFAR3-mRFP (Figure 2Ac); however, this fraction increased to approximately 50% in the jejunum (Figure 2Bc) and to approximately 90% in the ileum (Table 1).

Enteroendocrine somatostatin cells were found with a relatively equal density throughout the small intestine, but in contrast to substance P-positive cells, the somatostatin cells were scattered along the entire crypt-villus axis. As in the stomach, only a small fraction (~5%-10%) of the somatostatin cells of the duodenum and jejunum expressed FFAR3-mRFP, which, however, increased to approximately 50% of in the ileum (Figure 2Cb and Table 1).

In conclusion, in the small intestine, FFAR3-mRFP is expressed in basically all the enteroendocrine cells derived from the CCK-secretin-GIP-GLP-1-PYY-neurotensin lineage (9) but only in a fraction of the substance P and somatostatin cells, respectively. However, this fraction increases toward the distal part of the small intestine.

Dense expression of FFAR3-mRFP in enteroendocrine cells of the colon

The number and density of FFAR3-mRFP-expressing cells in the mucosa of, in particular the proximal colon, were remarkable (Figure 3A). Red fluorescent enteroendocrine cells were found densely scattered from the bottom to the top of the crypts as well as along the epithelium between the crypts.

The PYY cells, which clearly constituted the dominating population of enteroendocrine cells in the proximal colon, were found along the entire depth of the crypts and at the surface epithelium, and all PYY cells expressed FFAR3-mRFP (Figure 3Ab and Table 1). Similarly, all GLP-1 cells, which mainly were found within the crypts, expressed FFAR3-mRFP. In the proximal colon, neurotensin immunoreactivity was detected in a population of the enteroendocrine cells, which were found in the upper part of the crypts and in particular along the epithelium between the crypts. These neurotensin cells also all expressed FFAR3-mRFP (Figure 3Ac and Table 1).

In the proximal colon, somatostatin immunoreactivity was found in a relatively small population of enteroendocrine cells but also in a rather rich network of somatostatin-immunoreactive nerve fibers located just below the epithelium. Although the number of somatostatin-positive cells decreased from the small intestine to the colon, an increasing fraction of these displayed FFAR3-mRFP expression, ie, approximately 80% (Table 1).

In the middle colon, the density of FFAR3-mRFP cells was lower as compared with the proximal colon (Figure 3, A and B), and the red fluorescent cells were mainly found in the middle and lower part of the crypts and were in particular scarce or in many places, even totally absent from the epithelium between the crypts (Figure 3B). As in the proximal colon, basically all GLP-1 and PYY cells expressed FFAR3-mRFP in the middle colon (Fig. 3, Ba and Bb). Surprisingly, neurotensin cells, which in the proximal colon were found mainly in the epithelium between the crypts, were very scarce or often totally missing in the middle part of the colon.

Somatostatin-immunoreactive cells were as scarce in the middle colon as in the proximal colon; however, the fraction of these expressing FFAR3-mRFP increased even further to approximately 90% (Figure 3Bc and Table 1).

In the rectum, the occurrence of FFAR3-mRFP-positive cells was rather similar to that of the middle colon, and here all the GLP-1 and all the PYY immunoreactive cells also expressed FFAR3-mRFP. In the rectum almost all of the somatostatin cells also expressed FFAR3-mRFP (Table 1).

SCFA induced hormone release

In view of the abundance of FFAR3-mRFP-positive cells in the colon, we studied the effect of the prototypic SCFA propionate on GLP-1 release from murine colonic crypt cultures. Propionate (1 and 10 mM) increased GLP-1 secretion almost as efficiently as neuromedin C (NMC), ie, 1.47 ± 0.08- and 1.55 ± 0.14-fold (mean ± SEM), respectively, vs 1.63 ± 0.11-fold for NMC. AR420626, which is a novel, synthetic selective FFAR3 agonist having a potency of 2.7×10^{-7} M in transfected COS-7 cells (Fig. 3Cc), also stimulated GLP-1 release from the colonic crypt cultures, albeit only 1.26 ± 0.06-fold in response to 10 μM AR420626 (Fig. 3Cb). GLP-1 secretion was also increased by the FFAR2 selective ligand CFMB, although this did not reach statistical significance (Figure 3Cb).

Purified FFAR3-mRFP cells express a number of peptide transcripts

FFAR3-mRFP-positive cells were isolated from ileal mucosa by enzymatic digestion, purified by FACS and analyzed by qPCR for expression of peptide messengers and granins (Figure 4). All the expected transcripts corresponding to precursors for peptide hormones identified by the immunohistochemical analysis in FFAR3-mRFP cells were found among the most highly expressed and highly enriched genes in the FACS-purified cells. Of these,

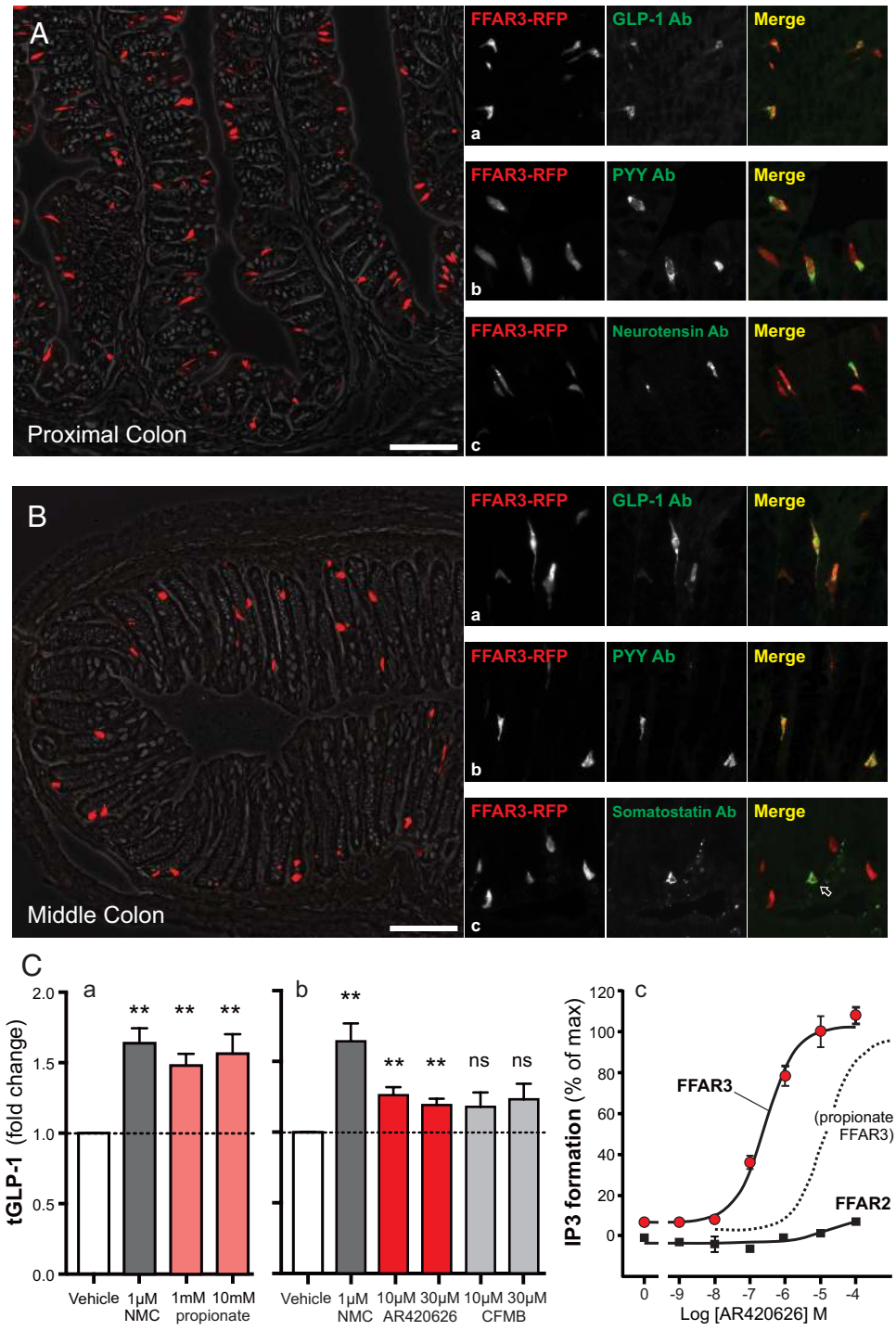

Figure 3. Immunohistochemical colocalization of selected peptide hormones with FFAR3-mRFP in the mucosa of the colon and effect of SCFAs, propionate and FFAR2 and FFAR3 selective ligands, on GLP-1 release from colonic crypt cultures. A, Overview of the localization of FFAR3-MRFP in the proximal colon. Aa, Colocalization of FFAR3-mRFP (red in left panel) with GLP-1 (green in middle panel) in 3 of 3 GLP-1-immunoreactive cells. Ab, Colocalization of FFAR3-mRFP (red in left panel) with PYY (green in middle panel) in 3 of 3 PYY-immunoreactive cells. Ac, Colocalization of FFAR3-mRFP (red in left panel) with neurotensin (green in middle panel) in 2 of 2 immunoreactive cells. B, Overview of the localization of FFAR3-mRFP in the middle colon. Ba, Colocalization of FFAR3-mRFP (red in left panel) with GLP-1 (green in middle panel) in 2 of 2 GLP-1-immunoreactive cells. Bb, Colocalization of FFAR3-mRFP (red in left panel) with PYY (green in middle panel) in 2 of 2 PYY-immunoreactive cells. Bc, Colocalization of FFAR3-mRFP (red in left panel) with somatostatin (green in middle panel) in 1 of 2 immunoreactive cells (arrow). Dark-field image was used to visualize the background of the tissue. Bar, 100 μm. Ca and Cb, GLP-1 release from colonic crypt cultures in response to NMC vs propionate (Ca) and NMC vs the FFAR3 selective agonist AR420626 and the FFAR2 selective agonist CFMB (n = 5 for each ligand in quadruplicates) (Cb). Cc, Selectivity of AR420626 determined by inositol 1,4,5-triphosphate (IP3) accumulation in COS-7 cells transfected with either FFAR3 ($EC_{50} = 2.7 \times 10^{-7}$ M) or FFAR2 ($EC_{50} > 10^{-4}$ M) and in both cases cotransfected with Gaqi4myr. The dose-response curve for propionate on FFAR3 is indicated in the dashed line ($EC_{50} = 1.3 \times 10^{-5}$ M).

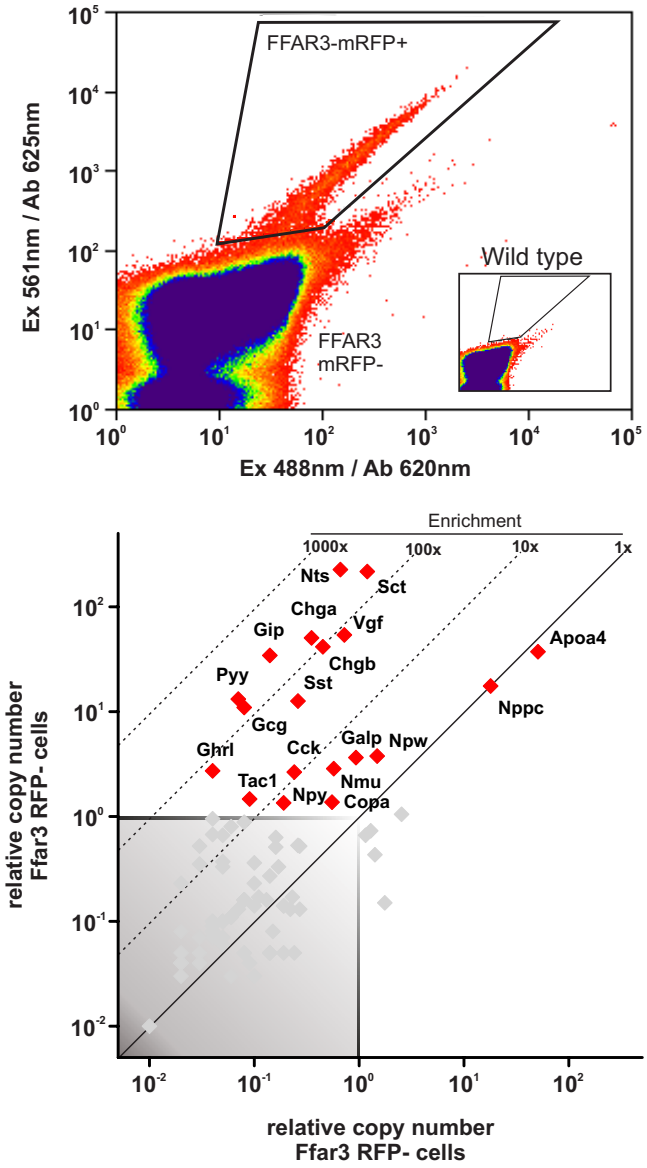

Figure 4. qPCR analysis of RNAs encoding peptide precursors and chromogranins in FACS-purified FFAR3-mRFP-positive cells isolated from ileal mucosa from transgenic FFAR3-MRFP reporter mice. Upper panel, A representative FACS diagram showing the gate (black line surrounding red cells) used for sorting of the FFAR3-mRFP-positive cells based on the fluorescence from mRFP vs the autofluorescence. Insert indicates a similar FACS diagram from wild-type animals. Lower panel, Scattergram of the expression of 93 peptide hormones and chromogranins expressed in relative copy number (see *Materials and Methods* for calculation details) in the FFAR3-mRFP-positive cells (y-axis) vs the FFAR3-mRFP-negative cells (x-axis). A solid line is drawn to indicate equal expression in mRFP-positive vs -negative cells, and dotted lines indicate 10-fold, 100-fold, and 1000-fold enrichment of expression in FFAR3-mRFP cells. In gray is indicated the noise area for the analysis (~cycle threshold = 35). Mean relative copy numbers of genes above noise in the Ffar3-RFP-positive cells are given in Supplemental Table 2.

Nts (proneurotensin) and *Sct* (prosecretin) were the most highly expressed peptide precursors at the RNA level, followed by *Gip* (pro-GIP), *Pyy* (pro-PYY), *Gcg* (proglucagon, GLP-1), and *Sst* (prosomatostatin) and with Tac1 (prosubstance P) being just above the detection limit (Figure 4B). Transcripts for chromogranin A and B were also both highly expressed and enriched in the FFAR3-mRFP-positive cells (Figure 4B).

Surprisingly, the transcript for nerve growth factor inducible (VGF), which is a precursor for the TLQP-21 peptide, was also highly expressed and enriched in the FFAR3-mRFP-positive cells (Figure 4B). Because VGF is normally expressed in neuronal tissue, its presence in FFAR3-mRFP-positive cells could therefore indicate either that FFAR3-mRFP is expressed in neuronal tissue in the gut mucosa or that VGF is expressed in some of the enteroendocrine cells in which FFAR3-mRFP is also expressed. However, a number of other neuropeptide precursors such as GalP (progalanin), Npw (proneuropeptide W), Nmu (proneuromedin U), and NPY (proneuropeptide Y) were all also expressed in FFAR3-mRFP-positive cells, albeit at relatively low levels but nevertheless supporting the notion that FFAR3 might be expressed in nerve cells.

FFAR3-mRFP is expressed in submucosal and myenteric ganglial cells

When focusing on the submucosa and muscle layers of the small intestine of the FFAR3-mRFP reporter mice, relatively weak red fluorescence was detected in cell bodies in the submucosal ganglia. However, immunohistochemical analysis using anti-mRFP antibodies clearly identified FFAR3-mRFP in cell bodies in the submucosal ganglia (Figure 5). Counterstaining with antibodies against the neuropeptide vasoactive intestinal polypeptide (VIP) demonstrated colocalization of VIP and FFAR3-mRFP (Figure 5A). However, a number of FFAR3-mRFP-positive ganglia cells did not show VIP immunoreactivity, indicating that FFAR3-mRFP is expressed in a broader population of neurons of the submucosal ganglia than the VIP-expressing secretomotor neurons. FFAR3-mRFP could be identified only in the soma of the ganglia cells and could not be detected in nerve fibers in the submucosa, even using mRFP antibody (data not shown).

FFAR3-mRFP was also expressed in cell bodies of myenteric ganglia located between the circular and the longitudinal muscles as visualized by the neuronal marker PGP9.5 (45). However, the FFAR3-mRFP-positive cells of the myenteric ganglia were fewer in number and also weaker in fluorescence than observed in the submucosal ganglia (Figure 5B).

FFAR2-mRFP is expressed in leukocytes in lamina propria and in enteroendocrine cells of the small intestine

The initial histological characterization of the GI tract from the FFAR2-mRFP mice demonstrated that the

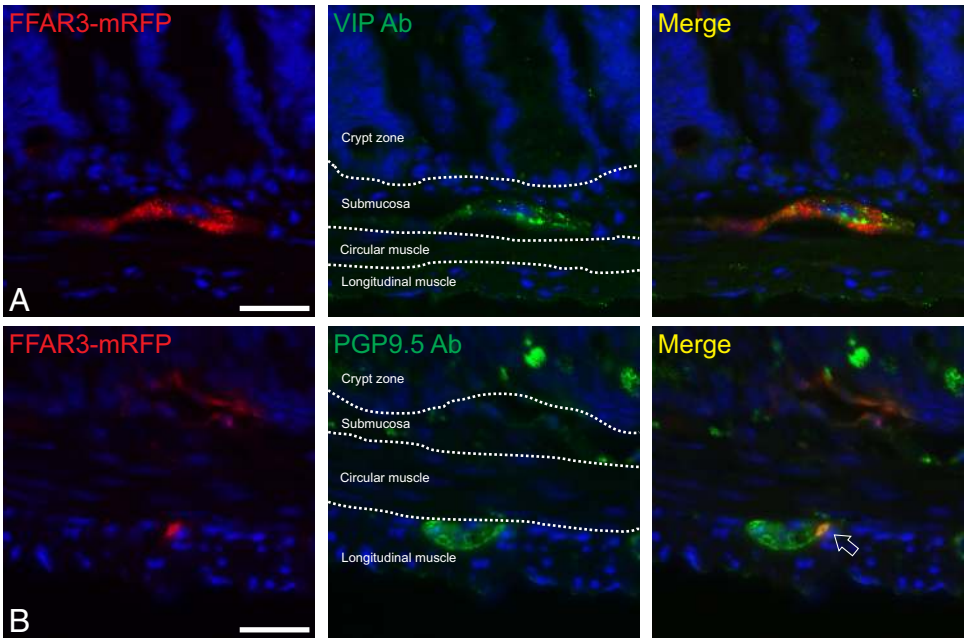

Figure 5. Expression of FFAR3-mRFP in cells of submucosal and myeteric ganglia of the small intestine from transgenic FFAR3-mRFP mice. A, FFAR3-mRFP expression in VIP-immunoreactive cells of a submucosal ganglion. Dotted lines indicate the border between the cryptal zone, the submucosa, the circular muscle layer, and the longitudinal muscle layer. B, FFAR3-mRFP expression in a PGP9.5 (a general marker for enteric neurons)-immunoreactive cell in a myenteric ganglion indicated by the arrow on the merged panel to the right. 4′,6′-Diamino-2-phenylindole (DAPI) counterstain was used as background. Bar, 25 μm.

FFAR2-mRFP was strongly expressed in leukocytes in the lamina propria as well as in scattered enteroendocrine cells (Figure 6A). FFAR2 has previously been reported to be expressed in leukocytes/ macrophages (26–28); however, the number and density of FFAR2-mRFP cells in the lamina propria of the small intestine of the FFAR2-mRFP mice was astonishing, as shown for the ileum in Figure 6.

In the present FFAR2-mRFP reporter line of mice, the mRFP was expressed in only a fraction of the enteroendocrine cells, and the expression was much weaker than the immune cells of the lamina propria. Thus, approximately 40% of the PYY and GLP-1cells and approximately 20% of the neurotensin cells expressed FFAR2-mRFP (Figure 6). In view of the fact that FFAR2-mRFP was only weakly and apparently partially expressed in enteroendocrine cells, which previously have been reported to express FFAR2 rather strongly (5, 31, 36), we did not here characterize the enteroendocrine expression of the FFAR2-mRFP further in this line of mice.

FFAR2 agonist-induced calcium response in FFAR2-mRFP-positive cells

Due to the fact that the FFAR2 receptor is Gq coupled, we loaded colonic crypt cultures from FFAR2-mRFP mice with Fluo-4 AM as described in *Materials and Methods* and identified FFAR2-mRFP-positive cells, which had taken up the Fluo-4 and recorded from these during exposure to ligands. As shown in Figure 6, although the enteroendocrine cells expressed the FFAR2-mRFP only rather weakly (Figure 6Aa–c), the FFAR2 selective agonist CFMB triggered a strong calcium response in the FFAR-mRFP-positive cells (Figure 6B).

Discussion

In the present study, the expression of mRFP was used as a reporter for the expression of the 2 receptors for SCFAs, FFAR2 and FFAR3, in enteroendocrine cells, enteric leukocytes, and enteric nerves throughout the GI tract (Figure 7). The study provides a novel, much broader picture than expected of how and where the host senses the metabolites, which are generated from dietary complex carbohydrates (fibers) by the gut microbiota. But the study also raises some methodological questions.

Does mRFP expression diligently reflect expression of FFAR3 and FFAR2?

Expression of GFP or RFP analogs driven by the transcriptional control elements has been used extensively as a tool to identify cells expressing proteins of interest in particular in the central nervous system (www.gensat.org) (46). Although the positive result, ie, expression of the fluorescent reporter protein, generally is considered to be a trustworthy indication of expression of the protein of interest, the negative result, ie, lack of expression of GFP

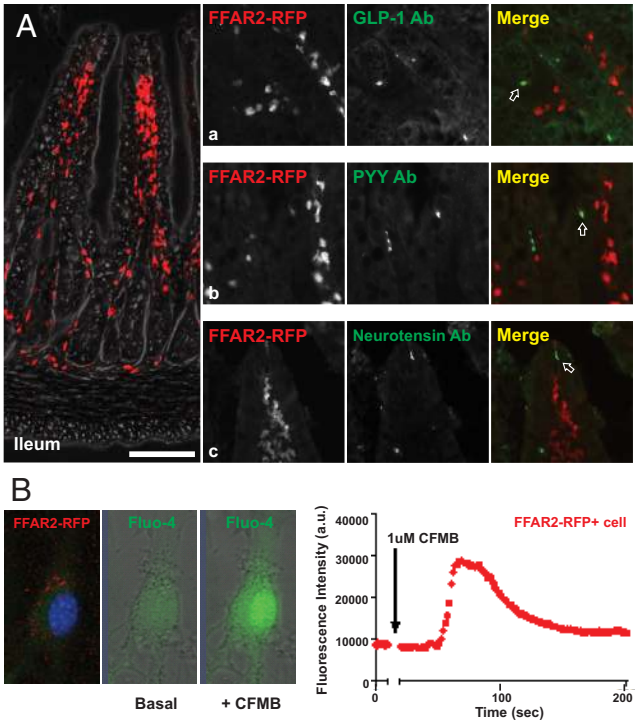

Figure 6. Expression of FFAR2-mRFP in the mucosa of the small intestine from transgenic FFAR2-mRFP mice. A, FFAR2-mRFP expression in leukocytes of the lamina propria of the ileum. Aa, Weak expression of FFAR2-mRFP in a GLP-1-immunoreactive cells of the ileal mucosa (arrow) among mRFP-positive leukocytes. Ab, Weak expression of FFAR2-mRFP in a PYY-immunoreactive cells of the ileal mucosa (arrow). Ac, Weak expression of FFAR2-mRFP in a GLP-1-immunoreactive cells of the ileal mucosa (arrow). Dark-field image was used to visualize the background of the tissue. Bar, 100 μm. B, Increase in intracellular calcium of FFAR2-mRFP-positive cells in colonic crypt cultures in response to the selective FFAR2 agonist CFMB. Ba, A FFAR2-mRFP-positive cell (pseudocolored red). Bb and Bc, Fluo-4 AM fluorescence (pseudocolored green) in the FFAR2-mRFP-positive cell before (Bb) and 80 seconds after addition of CFMB (Bc). Bd, The dynamics of the increase in intracellular Ca^{2+} in a FFAR2-mRFP-positive cell in a colonic crypt culture as determined by increase in Fluo-4 AM fluorescence intensity in response to CFMB.

or RFP, is more problematic. For example, using a similar construct targeting the ghrelin cells humanized renilla reniformis green fluorescent protein is expressed in basically all ghrelin cells of the stomach but only in very few of the ghrelin cells of the small intestine (47). Thus, despite the fact that the enteroendocrine cells of the stomach and the small intestine express the same proghrelin precursor protein from the same gene, the bacterial artificial chromosomes construct comprising a very large DNA segment surrounding the ghrelin gene directs expression of the GFP reporter protein only in the ghrelin cells of the stomach.

In the case of the FFAR3-mRFP mouse strain used in the present study, mRFP expression is strong in basically all enteroendocrine cells of the small intestine but is not detectable in the surrounding cells of the mucosa, even using mRFP antibodies. However, qPCR analysis of, for example, FACS-purified GFP-CCK cells indicates that although FFAR3 is highly expressed in the enteroendocrine CCK cells, it is also expressed in the neighboring, GFP-negative cells isolated from the mucosa. In accordance with this, qPCR analysis of the FACS-purified FFAR3-mRFP cells in the present study showed only a rather limited, 7-fold enrichment of the FFAR3 RNA transcript (data not shown). Nevertheless, it has previously been reported that FFAR3 RNA is enriched approximately 50- to 100-fold in enteroendocrine cells of the small intestine (32, 34, 36).

Importantly, the selective expression of the FFAR3-mRFP reporter in enteroendocrine cells of the intestinal mucosa is very useful as a marker of these cells. FFAR3-mRFP is expressed in basically all CCK, secretin, and GIP cells of the proximal small intestine and in all GLP-1, PYY, and neurotensin cells of the distal small intestine. Recently we described that the CCK, secretin, GIP, GLP-1, PYY, and neurotensin cells constitute a common lineage of enteroendocrine cells distinct from, for example, the somatostatin and substance P cells (9). Thus, even the mature cells of this lineage have the capacity to express several of the peptides as demonstrated by, for example, cell ablation studies based on the expression of the diphtheria toxin receptor driven by the promoter for proglucagon (9). Moreover, single-cell qPCR analysis of FACS sorted CCK-GFP-positive cells demonstrated that approximately half of the cells express 2 of the peptides and approximately 15% express 3 peptide hormones (9). Interestingly, FFAR3-mRFP is expressed in basically all of the enteroendocrine cells of this lineage. In fact, in the small intestine, FFAR3-mRFP is a more reliable marker for enteroendocrine cells than a chromogranin A-enhanced green fluorescent protein mouse we have generated in which high-resolution green fluorescent protein is expressed, for example, in all cells of the adrenal medulla and the endocrine pancreas but only in a relatively small fraction of the enteroendocrine cells of the small intestine (data not shown).

Concerning the FFAR2-mRFP reporter, we find it to be strongly expressed in enteric leukocytes in agreement with the fact that FFAR2 previously has been shown to be expressed in leukocytes (26–28). However, although FFAR2 by classical immunohistochemistry and by qPCR analysis of FACS purified cells has been reported to be expressed and enriched in, for example, GLP-1 cells (5, 31, 36), the FFAR2-mRFP reporter is expressed only rather weakly and only in a fraction of the enteroendocrine cells. Nevertheless, the cells that only weakly express the reporter gene FFAR2-mRFP do express functional FFAR2 receptor as demonstrated by the strong intracellular calcium response triggered by the selective FFAR2 agonist in FFAR2-mRFP-positive cells (Figure 6B).

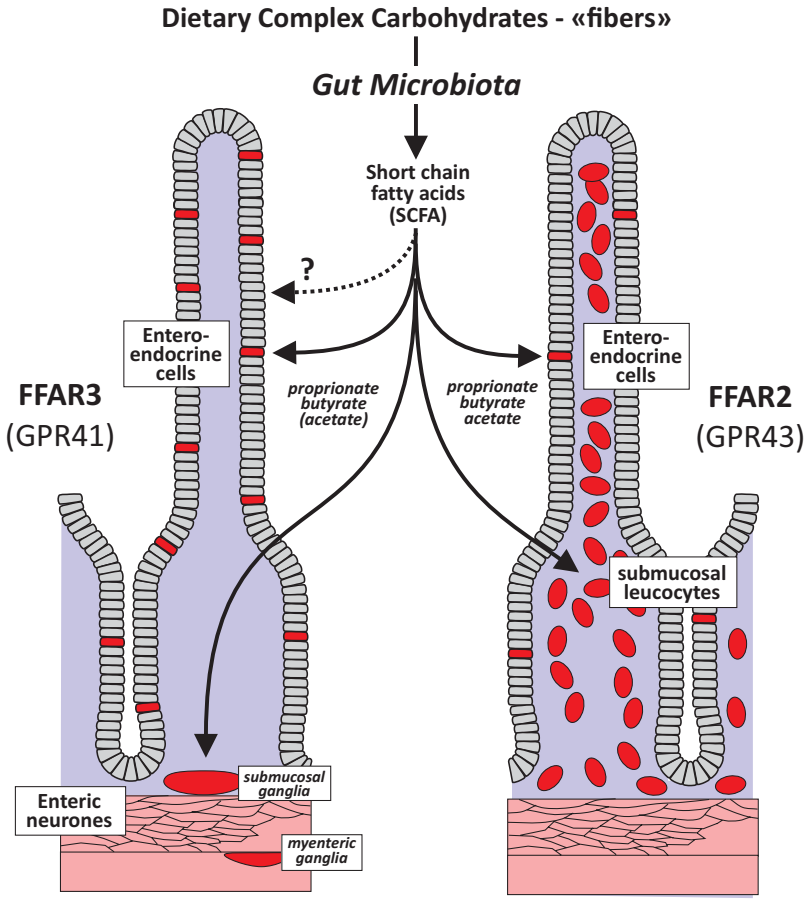

Figure 7. Schematic overview of the expression and function of FFAR3/GPR41 and FFAR2/GPR43 in the intestine based on the analysis of the FFAR3-mRFP and FFAR2-mRFP reporter mice of the present study and information available in the literature. FFAR3 (GPR41) and FFAR2 (GPR43) appear to be coexpressed and cofunction as sensors for SCFAs in the enteroendocrine cells (here indicted in the small intestine), whereas FFAR3 is expressed alone in enteric neurons of both the submucosal and myenteric ganglia and FFAR2 is expressed alone in enteric leukocytes.

Thus, it is concluded that in the mouse strains of the present study, the FFAR3-mRFP is an excellent reporter for FFAR3 expression in enteroendocrine cells and enteric neuronal cells. Similarly, the FFAR2-mRFP is particularly good for the detection of FFAR2 expression in enteric leukocytes but apparently has only limited value as a reporter of FFAR2 expression in enteroendocrine cells.

Coexpression and function of FFAR3 and FFAR2 in enteroendocrine cells

It has previously been reported that FFAR2 is expressed in, for example, GLP-1 cells of the intestine and that SCFAs stimulate GLP-1 secretion from colonic crypt cultures (36, 48). Interestingly, Tolhurst et al (36) found that deletion of either FFAR3 or FFAR2 had rather similar effects on the SCFA-induced GLP-1 secretion in both the ex vivo and in vivo setting and that deletion of each of the 2 receptors impaired the glucose tolerance to almost the same degree. However, based on the observation that the effect of deleting FFAR2 was slightly larger than deleting FFAR3 and the fact that only FFAR2 signals through Gq, they argued that FFAR2 and not FFAR3 is the main sensor for SCFAs in the regulation of hormone secretion from enteroendocrine cells (36). Lin et al (18) reached a similar conclusion through analysis of FFAR3-deficient mice because they found that although butyrate and propionate protect against diet-induced obesity and stimulate the secretion of a number of gut hormones, most of these effects were apparently not mediated through FFAR3. Interestingly, in both FFAR3 and FFAR2-deficient mice, a decreased expression of the other SCFA receptor was observed instead of a compensatory up-regulation (36). Thus, it appears that there is a close coupling and potential cooperation between the 2 SCFA receptors that are coexpressed in the enteroendocrine cells. In particular, the physiological role of the highly expressed FFAR3 is still rather unclear. However, we find that a selective FFAR3 agonist is at least as efficacious as an FFAR2 selective agonist in triggering GLP-1 secretion (Figure 3C).

SCFA ligand availability

The SCFA ligands are produced in large amounts by the gut microbiota, in particular in the colon, in which concentrations in the lumen can be in the order of 100 mM (5, 13). This is several orders of magnitude above the EC_{50} of both FFAR3 and FFAR2, which means that these receptors probably cannot function as sensors of luminal SCFAs. However, it is likely that nutrient receptors in general are expressed not only at the apical, luminal tip of the enteroendocrine cells but also at the basolateral membrane as has been described, at least for FFAR2 (3, 5). Thus, it could be argued that the enteroendocrine cells, at least in the colon, probably sense or monitor the considerably lower concentration of SCFAs found in the intracellular space between the mucosal cells and at the basal membrane.

In the small intestine, the function of FFAR3 and FFAR2 in enteroendocrine cells is, however, more unclear. However, FFAR3 and FFAR2 at the basolateral membrane of the enteroendocrine cells in the small intestine could simply be sensing the absorbed SCFAs in plasma.

Moreover, although not generally appreciated, there is in fact a rather large and diversified microbiota also in the small intestine (12). Thus, the SCFA receptors on the enteroendocrine cells in the small intestine could also be monitoring SCFA being generated by bacteria in the lumen of the small intestine (Figure 7).

In the case of the FFAR3-expressing gastrin and ghrelin cells of the stomach, there is probably rather limited local, luminal production of SCFAs, for example, in man. However, in ruminants these enteroendocrine cells will be exposed to large amounts of SCFAs being produced in the rumen, which is located more proximally to the ordinary stomach.

FFAR3 expression in enteric neurons

FFAR3-mRFP was surprisingly found to be expressed also in enteric nerves, ie, in ganglia cells of both the submucosal and myenteric ganglia (Figure 7). In the submucosal ganglia, some of these cells costain with VIP, indicating that SCFAs are sensed by FFAR3 on secretomotor neurons, which are involved in the control of secretion of water and salt from the enterocytes. However, FFAR3-mRFP was also found in non-VIP neurons, which need to be further characterized. Previously it has been reported that FFAR3 is expressed in sympathetic ganglia, and it was shown that SCFAs could stimulate sympathetic neuronal firing rate (49). Thus, it appears that FFAR3 functions as a SCFA sensor on various neurons of the body.

Some conclusions

In the present study, we find the FFAR3-mRFP reporter to be strongly expressed in the main, large population of enteroendocrine cells throughout the GI tract but surprisingly also in neurons of both submucosal and myenteric ganglia. In contrast, FFAR2-mRFP was expressed only in a subpopulation of the enteroendocrine cells but very strongly in a large population of leukocytes in the lamina propria throughout the small intestine. Thus, apparently FFAR2 and FFAR3 cofunction as sensors of short chain fatty acid signals in the enteroendocrine cells, whereas FFAR3 alone is responsible for the direct neuronal sensing and FFAR2 alone for leukocyte sensing of SCFAs generated from complex carbohydrates by the gut microbiota (Figure 7). Thereby our study provides a novel, more complex and refined picture of how the metabolism of the gut microbiota is recorded by the host through SCFA metabolites and conceivably how the microbiota thereby controls the metabolism of the host via their SCFA metabolites being sensed not only through enteroendocrine but also neuronal and immunological routes. However, we need to understand the complex physiological interplay of FFAR2 and FFAR3 in the enteroendocrine cells at the molecular and cell biological level much better to exploit this system for pharmacotherapy. Nevertheless, the FFAR3-mRFP reporter is in this study introduced as a novel, convenient general marker for the major population of enteroendocrine cells.

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