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Cellular and subcellular localization of cholecystokinin (CCK)-1 receptors in the pancreas, gallbladder, and stomach of mice

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Abstract

Information concerning the cellular localization of cholecystokinin (CCK)-1 receptors has been discrepant and remained scanty at ultrastructural levels. The present immunohistochemical study at light and electron microscopic levels revealed the distinct localization of CCK1 receptors in visceral organs. Immunohistochemistry by use of a purified antibody against mouse CCK1 receptor was applied to fixed tissue sections of the pancreas, gallbladder, stomach, and intestine of mice. A silver-intensified immunogold method revealed the subcellular localization under electron microscope. The immunoreactivity for CCK1 receptors was selectively found in the basolateral membrane of pancreatic acinar cells and gastric chief cells but was absent in pancreatic islets and gastric D cells. Another intense expression in the gut was seen in the myenteric nerve plexus of the antro-duodenal region and some populations of c-Kit-expressing pacemaker cells in the duodenal musculature. The gallbladder contained smooth muscle fibers with an intense immunoreactivity of CCK1 receptors on cell surfaces. The restricted localization of CCK1 receptors on the basolateral membrane of pancreatic acinar cells and gastric chief cells, along with their absence in the islets of Langerhans and gastric D cells, provides definitive information concerning the regulatory mechanism by circulating CCK. Especially, the subcellular localization in the acinar cells completes the investigation for the detection of circulating CCK by the basolateral membrane.

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Introduction

Gut hormone cholecystokinin (CCK), secreted from endocrine cells (I cells) dispersed in the epithelium of the upper small intestine, exerts various functions as a hormone and paracrine signal (Williams 1982). Intravenous infusions of CCK-8 or its larger molecular forms at a physiological dose induce pancreatic enzyme secretion and gallbladder contraction (Walsh 1987). Besides the direct stimulation by CCK, indirect pathways via the vagal nerve have been proposed for the regulation of the pancreas and gallbladder (Mawe 1991; Owyang and Logsdon 2004; Singer and Niebergall-Roth 2009). CCK also plays a role in the intestinal phase in the control of gastric functions that negatively regulate meal-stimulated gastric acid secretion and gastrin release (Lloyd et al. 1992a, b), possibly via somatostatin release (Schmidt et al. 1994). The hindbrain is a direct or indirect target of peripherally released CCK which is related to the regulation of feeding behavior and satiety. However, as compared with the apparent involvement of CCK in various phenomena, the cellular and subcellular localization of CCK receptors is still controversial.

CCK receptors are G protein-coupled receptors (GPCRs) and have been classified as CCK1 and CCK2 receptors on the basis of their affinity for CCK and related peptides. The CCK1 receptor has a 1000-fold higher affinity for CCK than for gastrin and is expressed abundantly in the pancreas, gallbladder, and stomach—which are the major targets of CCK (Wank 1995). The CCK2 receptor has an equal affinity to gastrin and CCK-8 of sulphated and non-sulphated types and may be involved in the proliferation of normal and tumor tissues rather than the direct stimulation of gastric acid secretion (Rozengurt and Walsh 2001). Expression sites of the CCK-specific receptor (CCK1 receptor) have been investigated morphologically by binding studies of radioligands on tissue sections, immunohistochemistry, and in situ hybridization methods. However, findings have been inconsistent for the expression sites—even in the pancreas. Some histochemical studies reported the localization of CCK1 receptors in acinar cells of the pancreas (Bourassa et al. 1999; Ohlsson et al. 2000), while other histochemical studies of the pancreas documented the localization of CCK1 receptors in islet cells in the pancreas of several mammalian species (Kageyama et al. 2005; Karlsson et al. 1998; Morisset et al. 2003; Schweiger et al.

2000); some of these appeared to detect no immunoreactivity for CCK1 receptors in acinar cells. Cell types of islets expressing the CCK1 receptor are also controversial among researchers, possibly due to species difference, varied immunohistochemical procedures, and the specificity of the antisera used. On the other hand, Northern blot, RT-PCR, and in situ hybridization methods (de Weerth et al. 1993; Ji et al. 2001) failed to detect any mRNA expression of the CCK1 receptor in the adult human pancreas, thereby agreeing with the lack of any acinar cell response to CCK agonists (Ji et al. 2001). More recently, a study which paid special attention to quick samplings revealed that fresh tissues of the human pancreas responded to CCK without the involvement of neuronal elements, confirming the increase of enzyme secretion by hormonal CCK in such samples (Murphy et al. 2008).

Mechanisms of stimulus-secretion coupling have been actively studied using pancreatic acinar cells. However, physiological studies using isolated acini have proposed different localizations of receptors for CCK at a cellular level. Early Ca^{2+} imaging studies indicated that GPCRs for CCK were expressed in the basolateral region of rat acinar cells (Habara and Kanno 1991). Subsequent studies recognized the initiation of GPCR-evoked Ca^{2+} waves at the apical pole and their propagation to the basal pole of mouse, rat, and human pancreatic acini (Criddle et al. 2009; Li et al. 2004; Murphy et al. 2008; Shin et al. 2001). In accordance, the immunostaining of isolated rat pancreatic acini demonstrated a localization of the CCK1 receptor *at the apical pole of lateral membrane* at the light microscopic level (Li et al. 2004). Based on the fact that CCK is conveyed via the blood circulation from the duodenum, the receptors should be localized at the basolateral membrane of acinar cells. This intriguing finding should be provable by the immunohistochemistry of fixed tissues at an electron microscopic level and co-localization of signal molecules. The present study using an antibody specific for the murine CCK1 receptor reports on the cellular and subcellular localization of the CCK1 receptor in the pancreas, gallbladder, stomach, and intestine of mice.

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Materials and Methods

Antibody for CCK1 receptor and specificity

A polyclonal antibody to mouse CCK1 receptor was raised against the C-terminal amino acid residues 422–436 (GeneBank Accession number: NM_009827). The pentadeca peptide was expressed as glutathione S-transferase (GST) fusion proteins using the pGEX4T-2 vector (GE Healthcare Biosciences, Uppsala, Sweden) and BL21 cells (Takara Bio, Tokyo, Japan). The fusion protein was purified with glutathione-Sepharose 4B (GE Healthcare Biosciences), emulsified with Freund's complete or incomplete adjuvant (Difco, Detroit, MI), and injected subcutaneously into a female New Zealand White rabbit and a Hartley guinea pig (Japan SLC, Shizuoka, Japan) at intervals of 2 weeks. Ten days after the fifth injection, affinity-purified antibodies were prepared from serum, first using Protein G-Sepharose (GE Healthcare Biosciences) and then antigen peptides coupled to CNBr-activated Sepharose 4B (GE Healthcare Biosciences). For preparation of the affinity media, antigen peptides free from GST were obtained by the elution of cleaved polypeptides after the in-column digestion by thrombin (Sigma, St. Louis, MO).

The specificity of the CCK1 receptor antibody was checked by immunocytochemistry using a human embryonic kidney (HEK293T) cell line transfected with pEF-BOS mammalian expression vectors (Mizushima and Nagata, 1990) encoding CCK1 or CCK2 receptor cDNA. The cells were fixed with 4% formaldehyde/0.1 M phosphate buffer for 10 min at room temperature and then incubated successively with 10% donkey normal serum for 20 min, primary antibodies for 2 h, and species-specific secondary antibodies conjugated with Cy3 for 1 h (Jackson ImmunoResearch, West Grove, PA). Counter staining was performed with TOTO-3 (Invitrogen, Eugene, Oregon). Phosphate-buffered saline (PBS) containing 0.1% Tween20 was used for washing and dilution buffers. Fluorescent images were taken with a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan).

In the immunoblotting with the CCK1 receptor antibody, CCK1 receptor- or CCK2 receptor-transfected HEK cells and the mouse pancreas were homogenized using a Potter homogenizer in an ice-cold homogenization buffer (0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid, 10 mM Tris-HCl, pH 7.2, and 0.4 mM phenylmethylsulfonyl fluoride). The resultant homogenate was subjected

to centrifugation at 1000 × g for 10 min to remove nuclei and debris. Homogenates were mixed with an equal volume of 2 × sodium dodecyl sulfate (SDS) sampling buffer (63 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.002% bromophenol blue), and denatured with 50 mM (±)-dithiothreitol at 55°C for 30 min. Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis, and electroblotted onto an Immobilon-P Transfer Membrane (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk for 30 min, blotted membranes were incubated with primary antibody (1 μg/ml) for 1 h and then with peroxidase-conjugated secondary antibodies for 1 h (Jackson ImmunoResearch; 1 : 10 000). A Tris-buffered saline (10 mM Tris-HCl, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20 was used as the dilution and washing buffer. Immunoreactions were visualized with the ECL chemiluminescence detection system (GE Healthcare, Little Chalfont, UK), and captured using an ImageQuant LAS 500 (GE Healthcare).

Furthermore, the specificity was confirmed by disappearance of the immunoreactivities with use of both antigen-preabsorbed antibodies and tissue sections from CCK1 receptor-deficient mice (Suzuki et al. 2001).

Tissue sampling

Eight-week-old male ddY and Balb/c mice were supplied by Japan SLC. For immunohistochemistry at the light and electron microscopic levels, deeply anesthetized mice were perfused via the aorta with a physiological saline, followed with 4% formaldehyde plus 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The pancreas, stomach, small intestine, and liver with the gallbladder were removed and immersed in the same fixative for an additional 6 h at 4°C. All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

Immunohistochemistry

The formaldehyde-fixed tissues were dipped in 30% sucrose solution overnight at 4°C, embedded in OCT

compound (Sakura Finetek, Tokyo, Japan), and quickly frozen in liquid nitrogen. Frozen sections, about 10 μm in thickness, were mounted on poly-L-lysine-coated glass slides and stained by the indirect immunofluorescence method using the rabbit anti-CCK1 receptor antibody. After pretreatment with 0.3% Triton X-100-containing PBS (pH 7.2) and normal donkey serum, the sections were incubated with the anti-mouse CCK1 receptor antibody at a concentration of 1 μg/ml. The sites of the antigen-antibody reaction were detected by incubation with Cy3-labeled anti-rabbit IgG (Jackson ImmunoResearch) or Alexa Fluor 488-labeled anti-rabbit IgG (Invitrogen). Finally, the sections were counterstained with TOTO-3 or SYTOX green (Invitrogen). The stained sections were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (FV1000; Olympus).

For double immunostaining, the stained sections were further incubated with either the goat anti-Gαq11 antibody (sc-3921; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat anti-somatostatin (sc-7819; Santa Cruz Biotechnology, Inc.), goat anti-SCF receptor (c-Kit) antibody (AF1356; R&D Systems, Inc., Minneapolis, MN), guinea pig anti-insulin antibody (18-0067; ZYMED/Invitrogen), or guinea pig anti-PGP9.5 antibody (RA-95101; Ultraclone, Isle of Wight, UK). The antigen sites in the second immunostaining were visualized with Alexa Fluor 488-labeled anti-goat IgG or anti-guinea pig IgG (Invitrogen).

Silver-intensified immunogold method for electron microscopy

The formaldehyde-fixed tissues were dipped in 30% sucrose solution overnight at 4°C, embedded in OCT compound, and quickly frozen in liquid nitrogen. Frozen sections of 15 μm in thickness were mounted on poly-L-lysine-coated glass slides, incubated with the rabbit anti-CCK1 receptor antibody (1 μg/ml) overnight, and subsequently reacted with goat anti-rabbit IgG covalently linked with 1-nm gold particles (1: 200 in dilution; Nanoprobes, Yaphank, NY). Following silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmificated, dehydrated, and directly embedded in Epon (Nisshin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with both uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan). The specificity of the immunoreactions was confirmed by the disappearance of immunolabeling when the antibody was

pre-incubated with the antigen.

Fluorescent in situ hybridization (FISH) technique

For FISH, we prepared frozen sections, about 30 μm in thickness. Mouse cDNA fragments of the CCK1 receptor (nucleotides 348–1658bp; GenBank accession number, NM_009827), pepsinogen C (1–1352bp; NM_025973.3), and c-Kit (37–1320bp; BC052457.1) were subcloned into the pBluescript II plasmid vector. Digoxigenin (DIG)- or fluorescein-labeled cRNA probes were transcribed in vitro for FISH analysis (Yamasaki et al. 2010). The fragmentation of riboprobes by alkaline digestion was omitted in order to increase the sensitivity and specificity. After the inactivation of residual peroxidase activity by dipping sections in 1% H_2O_2 for 30 min, the second detection was performed by incubating sections in a DIG-labeled cRNA probe, followed by peroxidase-conjugated anti-DIG antibody (1:1000; Roche Diagnostics, Mannheim, Germany) and the Cy3-TSA plus amplification kit (PerkinElmer, Waltham, MA). Sections were counterstained with TOTO-3 (1:50 in PBS; Invitrogen). Images were taken with a confocal laser-scanning microscope (FV1000; Olympus) equipped with a HeNe/Ar laser.

Results

Antibody characterization

In the immunoblotting, the CCK1 receptor antibody detected multiple bands at 37, 75, 140, and 250 kDa in HEK293T cells transfected with CCK1 receptor cDNA and at 75, 110, and 140 kDa in the mouse pancreas with the major band both at 75 kDa (Fig. 1A).   The specificity of the CCK1 receptor antibody was checked by immunocytochemistry using HEK293T cells transfected with mammalian expression vectors encoding either the CCK1 receptor or CCK2 receptor cDNA.   Immunostaining detected a cell membrane-bound immunoreactivity in the HEK293T cells transfected with the CCK1 receptor cDNA but not the CCK2 receptor cDNA (Fig. 1B, C).   In an absorption test using HEK293T cells and tissue sections, the immunoreactivities were completely abolished using the antibodies preabsorbed with the corresponding antigen at 1–10 μg/ml diluted antibody.   Furthermore, the specificity was confirmed by disappearance of the immunoreactivities in the pancreas, gallbladder, stomach, and duodenum with the use of CCK1 receptor-deficient mice (Fig. 1D, E and Supplemental Figure-1).

Pancreas

Immunostaining using the antibody against the CCK1 receptor intensely labeled the plasma membrane of pancreatic acinar cells but failed to label any of islets or duct cells (Fig. 2A).   It was clear at the light microscopic level that the basolateral membrane of acinar cells was intensely positive in reaction; noteworthily, the immunoreactivity tended to be more intense in the lateral cell membrane than the basal membrane (Fig. 2B).   Many dot-like structures with an immunoreactivity for the CCK1 receptor were found on the basolateral membrane, as was more clearly shown in images superposed on the Z-axis (Fig. 2C); they were more noticeable in the basal membrane than the lateral membrane.   When the same materials were stained using an anti-heterotrimeric G protein subunit (Gαq11) antibody, an identical staining pattern appeared along the basolateral membrane (Supplementary Figure-2).   The silver-intensified immunogold method for electron microscopy revealed a subcellular localization of the CCK1 receptor immunoreactiviy that was restricted to the basolateral membrane and completely absent

on the luminal side (Fig. 2D). Gold particles on the lateral membrane were heavily distributed next to the tight junction but never beyond it. On the basal membrane, aggregations of gold particles were found in spotted areas covered with short microvilli (Fig. 2E) which corresponded to the dot-like features captured under the light microscope (Fig. 2C). The immunoreactivity for Gαq11 also accumulated in the microvillous domains on the basal membrane (Fig. 2F).

Gallbladder

Although another major target of CCK is the gallbladder, no previous studies have directly demonstrated the localization of CCK1 receptors in smooth muscles of this organ. In the current staining, all smooth muscle fibers of the gallbladder were immunolabeled by the CCK1 receptor antibody, with an intense immunoreactivity on the cell surface (Fig. 3A). Under the electron microscope, the localization of the CCK1 receptor was examined by the silver-intensified immunogold method (Fig. 3B). Gold particles showing the existence of immunoreactivity appeared predominantly on the plasma membrane along the entire length of smooth muscle cells. The pancreatic duct in the mouse has a thin muscle coat but does not develop any sphincter at the orifice to the duodenum; no immunoreactivity for the CCK1 receptor was found in the smooth muscles associated with the pancreatic duct.

Stomach and duodenum

Mucosa of the gastric corpus displayed an intense immunoreactivity for the CCK1 receptor. The immunoreactivity was restricted to the bottom region of the fundic glands, where the basolateral membrane of the chief cells was selectively labeled (Fig. 3C, D). The basolateral membrane of the chief cells also expressed Gαq11 (Supplementary Figure-2), like pancreatic acinar cells. Under the electron microscope, immunogold particles for the CCK1 receptor appeared distributed along the basolateral membrane of chief cells; again the plasma membrane on the luminal side completely lacked the immunoreactivity (Fig. 3E). Unlike pancreatic acinar cells, gastric chief cells did not develop distinct microvillous domains with accumulations of immunogold particles. A small number of immunoreactive

cells with the same staining pattern occurred at the very bottom of pyloric glands, though only in the region close to the acid-secreting area (data not shown). Somatostatin-secreting D cells in the gastric corpus and antrum avoided immunolabeling with the CCK1 receptor antibody in double immunostaining for the CCK1 receptor and somatostatin (Fig. 3F). Smooth muscle layers in the stomach and intestine were judged to be immunonegative for the CCK1 receptor, as compared with the stainability of the gallbladder smooth muscle. Another intense immunoreactivity in the gastrointestinal tract was found in the interstitial cells of Cajal (ICC) in the muscle layer; these were identified by double staining with the c-Kit antibody (Fig. 4A). The distribution of CCK1 receptor-immunoreactive ICCs was restricted to the beginning of the duodenum, which largely corresponded to the region of the duodenal gland. Some populations of c-Kit-positive ICCs in this region were completely immunonegative for the CCK1 receptor, suggesting that ICCs are not a homogeneous population for the expression of CCK1 receptors.

Myenteric nerve plexuses—but not submucous nerve plexuses—in the antro-duodenum exhibited a positive reactivity with a dot-like appearance on neuronal cell bodies and fibers (Fig. 4B, C), as clearly shown in whole mount preparations (Fig. 4D). Stainability of the intramural nerve plexuses with the CCK1 receptor antibody remarkably decreased in intensity in the gastric corpus, jejunum, and ileum. In the myenteric nerve plexus of the duodenum, only a small number of neuronal cell bodies was immunoreactive for the CCK1 receptor (Supplementary Figure-3). The mucosal layer of the stomach and intestine absolutely lacked CCK1 receptor-immunoreactive nerve fibers.

FISH analysis of CCK1 receptor mRNA

Signals for CCK1 receptor mRNA in the pancreas were found evenly in the exocrine glands but not in the duct system or islets (Fig. 5A). The gallbaldder displayed a restricted distribution of the signals in the muscle coat (Fig. 5B). In the glandular stomach, an intense expression was localized at the bottom region of the fundic glands (Fig. 5C); double detection of the CCK1 receptor and pepsinogen C mRNAs indicated selective expressions of CCK1 receptors in the chief cells (Fig. 5D). Scattered cells in the intermuscular layer of the duodenum exihibited the CCK1 receptor mRNA, and they also expressed c-Kit mRNA (Fig. 5E). These cellular localizations of mRNA perfectly coincided with the distribution of

CCK1 receptor immunoreactivities.

Discussion

This study revealed the cellular and subcellular localization of CCK1 receptors in generally accepted target organs of CCK: the pancreas, gallbladder, stomach, and intestine.   The basolateral membrane of pancreatic acinar cells and gastric chief cells held the immunoreactivity for CCK1 receptors, but the luminal membrane of both cell types was free of the immunoreactivity.   Pancreatic acinar cells provided microvillous pockets dispersed on the basolateral membrane, possibly as a special site for sensing CCK. In contrast to previous studies, the present immunostaining completely excluded pancreatic islet cells and gastric D cells for targets specific to CCK.   These immunoreactivities were all confirmed at a mRNA level by in situ hybridization analysis.

CCK1 receptor in pancreatic acinar cells

The present study clearly showed the localization of CCK1 receptors along the basolateral membrane of pancreatic acinar cells but did not detect any immunoreactivity within the islets, in contrast to previous immunohistochemical studies (Kageyama et al. 2005; Morisset et al. 2003; Schweiger et al. 2000).   The selective localization of CCK1 receptors in acinar cells holds true in the pancreas of other mammals, including the guinea pig and rat (Supplementary Figure-4).   In agreement with the current study, two previous immunohistochemical studies for the CCK1 receptor have reported a positive immunoreaction in the plasma membrane of pancreatic acinar cells in the mouse and rat (Bourassa et al. 1999; Ohlsson et al. 2000).   Unlike our investigation, however, those studies simultaneously detected the immunoreaction for CCK1 receptors in pancreatic islets.   Although exact reasons for the discrepant findings of immunohistochemistry are unknown, they may be caused by different animal species examined, specificities of antibodies, and different processing of tissue sections (paraffin sections, unfixed frozen sections, or frozen sections from fixed tissues).   Our staining results were sufficiently certificated by use of CCK1 receptor-deficient mice and in situ hybridization method.

This is the first demonstration of the subcellular localization of the CCK1 receptor in fixed samples of the pancreas under an electron microscope.   Unexpectedly, the immunoreactivity was more intense in

the lateral plasma membrane than the basal membrane.   Instead, the basal membrane developed microvillous pockets with a condensed expression of CCK1 receptors.   The surface view of the microvillous pockets was three-dimensionally captured by scanning electron microscopy (Supplemental Figure-5).   Although the functional significance of the microvillous domains remains to be elucidated, we demonstrated the accumulated localization of Gαq11 there, suggesting their important role in the reception of CCK and subsequent signal transduction.   The basolateral localization of the CCK1 receptor and G protein contrasts with a series of Ca^{2+} imaging and immunohistochemical studies using isolated acini of rodent (Criddle et al. 2009; Li et al. 2004; Shin et al. 2001) and human pancreas (de Weerth et al. 1993).   Namely, expressions of the GPCR are highly enriched in the apical pole of acinar cells, and CCK-induced Ca^{2+} waves initiate on the apical side—though the same studies also paid attention to the lateral membrane just underneath the tight junction.   The discrepancy for the immunohistochemical localizations of the CCK1 receptor may be due to the very narrow luminal surface of acinar cells, occupying only a small part (~5%) of the overall surface membrane (Bolender 1974).   In such a sample, the immuno-negative luminal surface might be partially superimposed onto an intensely labeled lateral membrane at light microscopic observation of isolated acini.   Another reason for the discrepancy may be the loss of CCK receptors during the preparation of isolated acini by enzymatic treatments because we noticed that the stainability of CCK1 receptors decreased in the basal membrane compared with the lateral membrane during the processing of isolated acini.

CCK1 receptors in gallbladder and gastric mucosa

One of the main functions of duodenal CCK is the stimulation of gallbladder contraction at the entrance of food to the duodenum.   In vitro binding assays have suggested a rich occurrence of CCK1 receptors in the muscle layer of the human gallbladder (Reubi et al. 1997; Schjoldager et al. 1989; Tang et al. 1996). However, Mawe (1991) reported previously that neural elements expressing CCK1 receptors might be involved in the response of the gallbladder to hormonal CCK in guinea pigs.   The present study confirmed the abundant localization of CCK1 receptors along the plasma membrane of smooth muscle fibers in the mouse gallbladder.   The muscle coat along the pancreatic duct did not express CCK1

receptors up to the junction with the duodenum.

CCK stimulates the secretion of pepsinogen as well as the inhibition of gastric acid secretion. This CCK-induced pepsinogen secretion is regulated mainly via the CCK1 receptor on the chief cells of the fundic glands in rats, guinea pigs, and rabbits (Blandizzi et al. 1999; Lin et al. 1992; Tang et al. 1993). Autoradiographic studies using radiolabeled ligands have reported the existence of CCK1 receptors in the deeper region of the fundic mucosa in rats, dogs, and humans (Reubi et al. 1997; Mantyh et al. 1994). Immunohistochemically, only Schulz et al. (2005) detected an immunoreactivity for the CCK1 receptor on the surface of some chief cells in the human stomach. The present study confirmed a selective localization of CCK1 receptors along the basolateral plasma membrane of the chief cells under the electron microscope. Vasoactive intestinal polypeptide (VIP)-containing nerve fibers running in the lamina propria of rat gastric fundus were reported to be immunoreactive to an antibody against the CCK1 receptor (Sternini et al. 1999). However, we could not detect any specific immunoreactivity for the CCK1 receptor in neural elements of the fundic and antral mucosa, in agreement with a pharmacological study denying the involvement of nerve fibers in the basal secretion of pepsinogen in the rat (Blandizzi et al. 1999). Although the direct stimulation of pepsinogen secretion by duodenal CCK became clearly apparent, the functional significance of pepsinogen secretion after the entrance of nutrients into the duodenum remains unknown.

CCK and gastrin stimulate the release of somatostatin from gastric D cells in several mammals—though no data are available for the mouse. It is reported that CCK may act preferably on CCK1 receptors of D cells to trigger somatostatin release, resulting in the inhibition of gastric acid secretion in rats (Lloyd et al. 1992a), dogs (Lloyd et al. 1994), and humans (Buchan et al. 1993; Schmidt et al. 1994). One histochemical study was able to detect the expression of CCK1 receptors in some D cells of the human stomach (Schmitz et al. 2001) whereas another immunostaining reported that most of the gastric D cells in the dog and guinea pig were immunoreactive for the CCK2 receptor (Helander et al. 1997). In accordance with immunohistochemical findings in the rat stomach (Patterson et al. 2001; Sternini et al. 1999), we failed to find D cells or other endocrine cell types expressing CCK1 receptors in the gastric fundus and antrum of mice.

CCK1 receptors in the antro-duodenal region

It is generally accepted that exogenous and endogenous CCK reduces food intake via the CCK1 receptor. CCK peptides at a physiological dose cause contractions of smooth muscles in the gastro-duodenal junction (Scheurer et al. 1983), resulting in an inhibitory effect on gastric emptying. The smooth muscle contraction by CCK is modulated by neural and non-neural (myogenic) pathways. A study using isolated esophago-gastro-duodenal preparations of the rat showed that the CCK action on tonic contraction was neuronal while its action on phagic contraction was non-neuronal (Scheurer et al. 1983). The direct action of CCK upon the pyloric sphincter is supported by pharmacological studies and pylorectomy (Moran et al. 1990; Morgan et al. 1978; Murphy et al. 1987; Scheurer et al. 1983). An autoradiographic study using radiolabeled CCK-33 demonstrated a condensation of binding sites within the surfacemost layer of circular muscle in the pyloric sphincter in the rat (Smith et al. 1984). Patterson et al. (2001) reported that immunohistochemistry for the CCK1 receptor labeled smooth muscle fibers of the rat pyloric sphincter only with the use of higher antibody concentrations. However, we failed to stain any smooth muscle of the mouse pylorus in the same staining procedure which intensely stained the gallbladder smooth muscle—though the pyloric sphincter was heavily immunolabeled for the CCK2 receptor (our unpublished data). Thus, if a direct action of hormonal CCK occurs in the mouse, CCK can act on the pyloric sphincter only via the CCK2 receptor. Instead, we found ICC expressing CCK1 receptors in the duodenum, in agreement with immunohistochemical findings in rats and mice (Patterson et al. 2001). Characteristically, some populations of ICCs only at the beginning of the duodenum expressed CCK1 receptors. These results lead us to the idea that CCK contracts the smooth muscle of the gastro-duodenal junction via an ICC-dependent myogenic pathway and then leads to gastric distension, producing rapid and short-term satiety.

However, this action does not stop feeding immediately since the organisms continue a single period of feeding until an adequate caloric supply is attained. The satiation effects of CCK must be strengthened by the following two pathways to the hindbrain: regulation of the nucleus tractus solitarium (NTS) via vagal afferents (Raybould et al. 1988; Smith et al. 1981; 1989), and hormonal regulation of the

area postrema, another center of feeding behavior with a leaky blood-brain barrier in the hindbrain (Glatzle et al. 2001; Ladenheim et al. 1988; Moran et al. 1990). CCK1 receptor-immunoreactive nerve fibers were found in the present study to be absent in the mucosal layer but gather in the myenteric nerve plexus of the antro-duodenum; they may be projected to the hindbrain via the nodose ganglia. Actually, several cell bodies in the nodose ganglion were immunolabeled for the CCK1 receptor (Supplementary Figure-3). Furthermore, our parallel immunostaining analysis using the same antibody in the brain revealed expression profiles of CCK1 receptors in the NTS and area postrema (Konno K, unpublished data). Post-prandially released CCK may cause a total inhibition of feeding by three pathways—paracrine (ICC), hormonal (area postrema), and neural inputs (vagus-NTS), resulting in the cessation of feeding with different sensitivities and durations. The involvement of CCK and the CCK1 receptor in the satiety-associated neural circuit will be reported elsewhere.

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Figure legend

Fig. 1   In the immunoblotting (A), the CCK1 receptor (CCK1R) antibody detected multiple bands at 37, 75, 140, and 250 kDa in HEK293T cells transfected with CCK1R cDNA and at 75, 110, and 140 kDa in the mouse pancreas with the major band both at 75 kDa.   Immunofluorescence staining of the CCK1R in HEK293T cells transfected with the pBOS-CCK1R (B) or pBOS-CCK2 receptor (CCK2R) (C).   Only cells transfected with cDNA of the CCK1R show a positive immunoreactivity along the plasma membrane.   Nuclei are stained blue with TOTO-3.   Immunohistochemistry of the pancreas with the CCK1R antibody stains the exocrine pancreas of a wild type mouse (WT) but the immunoreactivity disappears in a CCK1R-knockout (KO) mouse (D and E).   I: the islet of pancreas. Bar, 20 μm (C), 100 μm (D, E)

Fig. 2   CCK1 receptor (CCK1R) immunoreactivities in the pancreas.   Double staining of CCK1R and insulin (A) displays a selective localization of CCK1R in acinar cells but not the islets of Langerhans (I). In pancreatic acini, the immunoreactivity for CCK1R is localized along the basolateral membrane (B). An image superposed on the Z-axis of a thick section displays dot-like structures with an intense immunoreactivity for CCK1R in the basal membrane of acinar cells (C).   Immunogold particles for CCK1R on the lateral membrane are distributed close to the tight junction (arrows) under the electron microscope (D).   Electron-microscopically, microvillous pockets (arrows) along the basal membrane hold an aggregation of immunogold particles for CCK1R (E) and Gαq11 (F) in a similar manner.   In Fig. 2B and C, nuclei are counterstained green with SYTOX green.   L: lumen, N: nucleus   Bar, 50 μm (A), 10 μm (B and C), 1 μm (D–F)

Fig. 3   CCK1 receptor (CCK1R) in the gallbladder and stomach.   Smooth muscle fibers in the gallbladder are immunoreactive for CCK1R with a predominant localization along the cell surface under light (A) and electron microscopes (B).   In the gastric corpus, CCK1R-immunoreactive cells gather at the deeper region of the mucosa, where the basolateral membrane of chief cells is immunolabeled (C, D), as confirmed by electron microscopy (E).   Double staining with somatostatin shows the lack of CCK1R in the somatostatin-containing D cells (F).   In Fig. 3A, C, and D, nuclei are counterstained green with

SYTOX green.   E: epithelium, L: lumen, N: nucleus, SM: smooth muscle   Bar, 20 μm (A, D and F), 1 μm (B and E), 100 μm (C)

Fig. 4   CCK1 receptor (CCK1R) in the muscle layer of the duodenum.   Double staining with c-Kit shows that a population of c-Kit-expressing cells in the muscle layer are immunoreactive for CCK1R (A) while other c-Kit-expressing cells (green-colored cells) are immunonegative for CCK1R.   Arrows indicate the entire thickness of the muscle layer.   The myenteric nerve plexus is largely labeled with the CCK1R antibody (B).   A closer view of the myenteric plexus shows a dotted immunoreaction for CCK1R in the myenteric nerve plexus (C).   Double staining with PGP9.5, a neuronal marker, of a whole mount preparation demonstrates a selective distribution of CCK1R-immunoreactive nerve fibers on the myenteric nerve plexus (D).   In Fig. 4B and C, nuclei are counterstained green with SYTOX green. Bar, 20 μm (A, B and C), 50 μm (D)

Fig. 5   In situ hybridization analysis of the CCK1 receptor mRNA.   In the pancreas, signals for the CCK1 receptor (CCK1R) mRNA are restricted to the acini of the exocrine pancreas (A).   The gallblader displays dot-like signals only in the muscle coat (B).   The glandular stomach contains selective signals of CCK1R mRNA at the bottom of fundic glands (C), where the signals colocalize with those of pepsinogen C mRNA (D1 and D2).   In the duodenum (E1 and E2), CCK1R mRNA is found in scattered cells expressing c-Kit mRNA.   I: pancreatic islet, E: endothelium of the gallbladder   Bar, 20 μm (A–E)
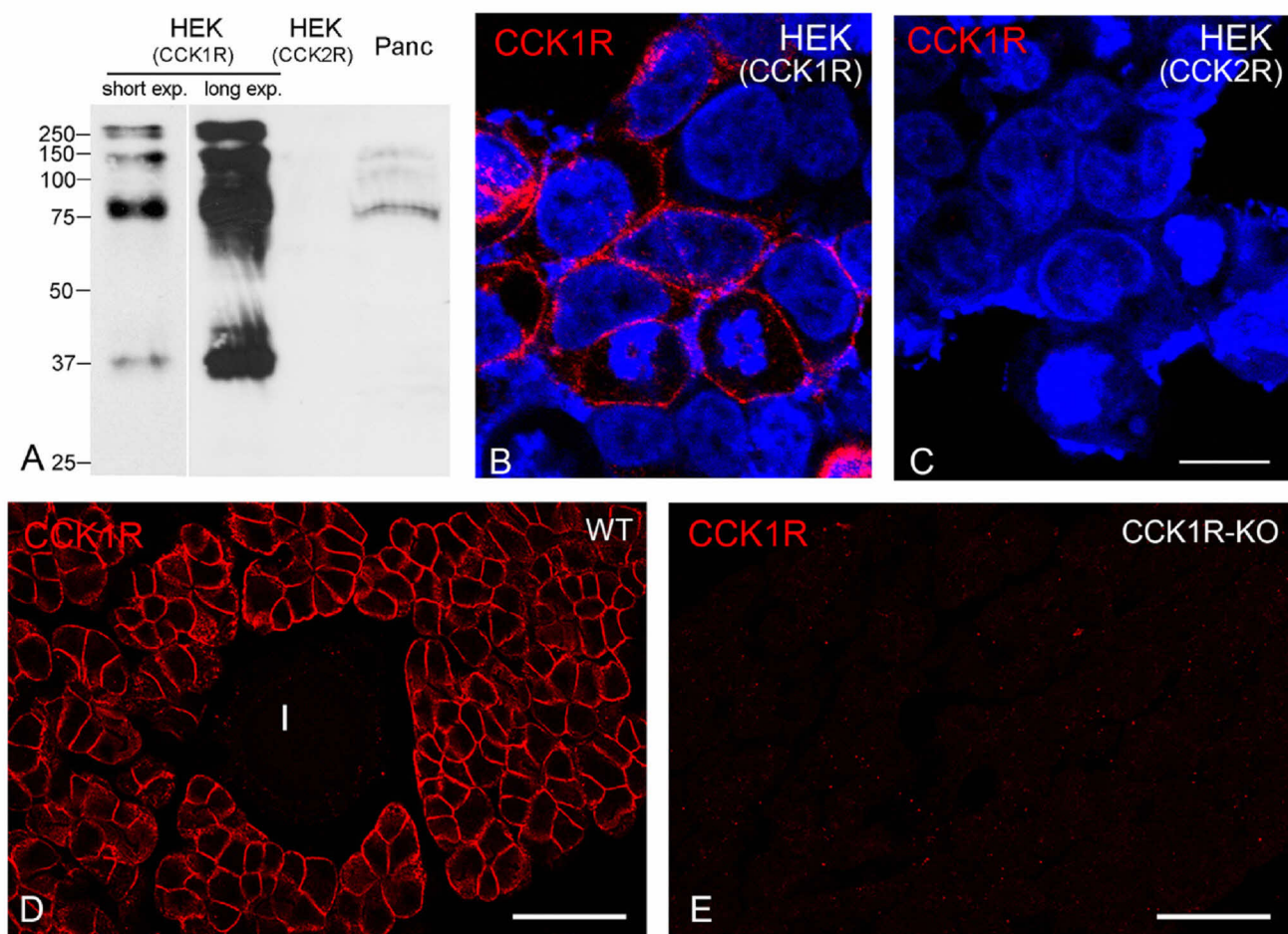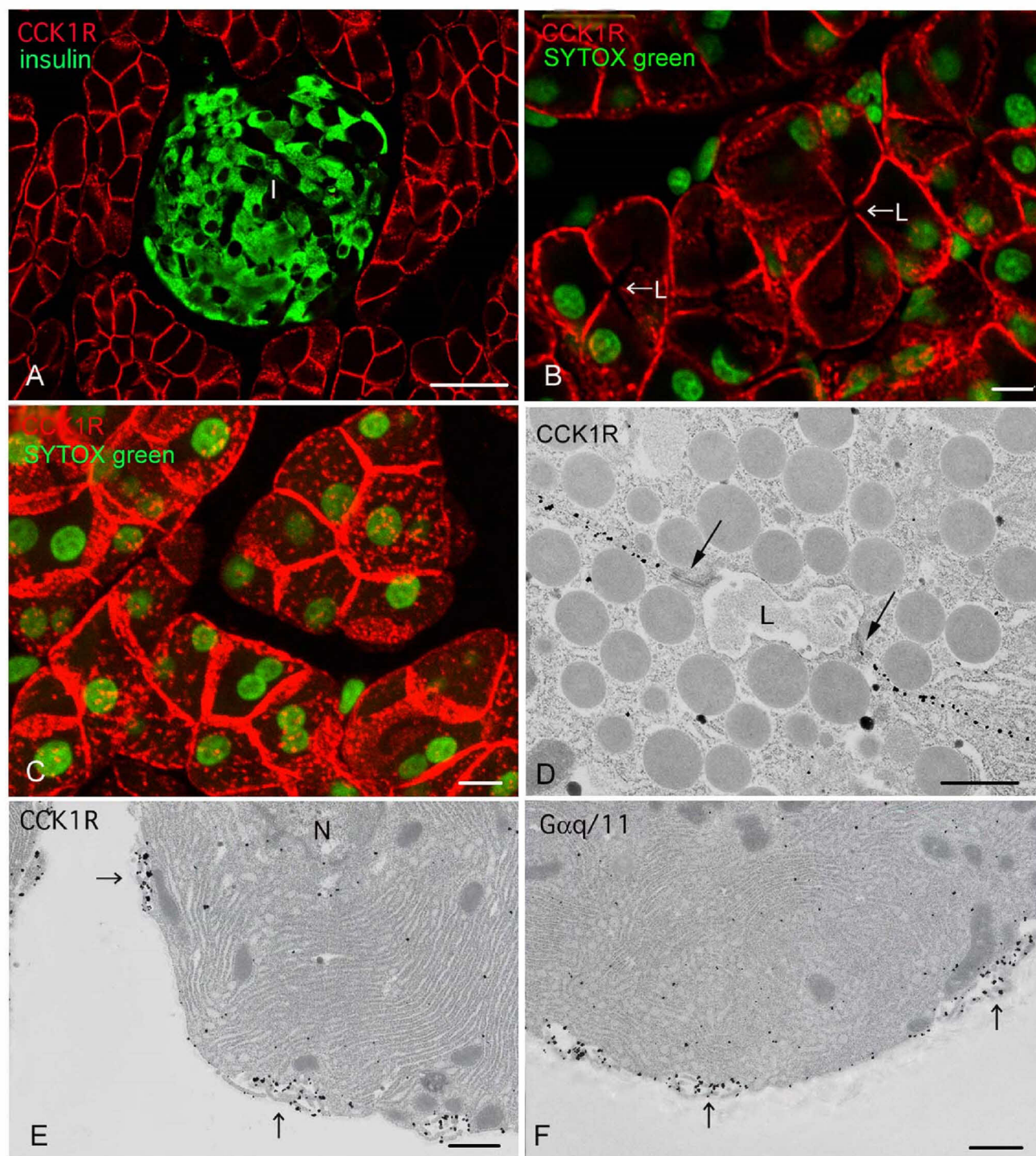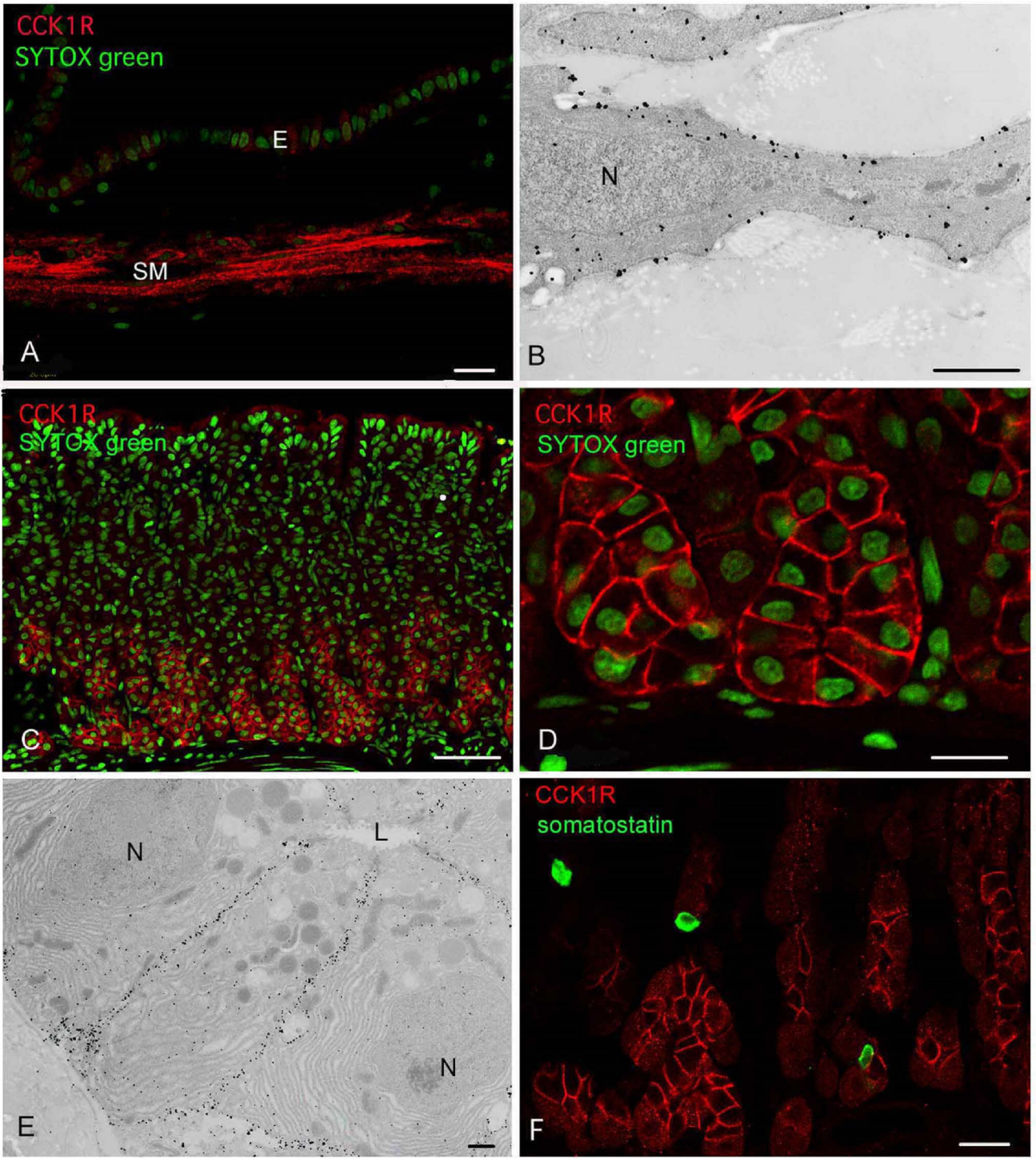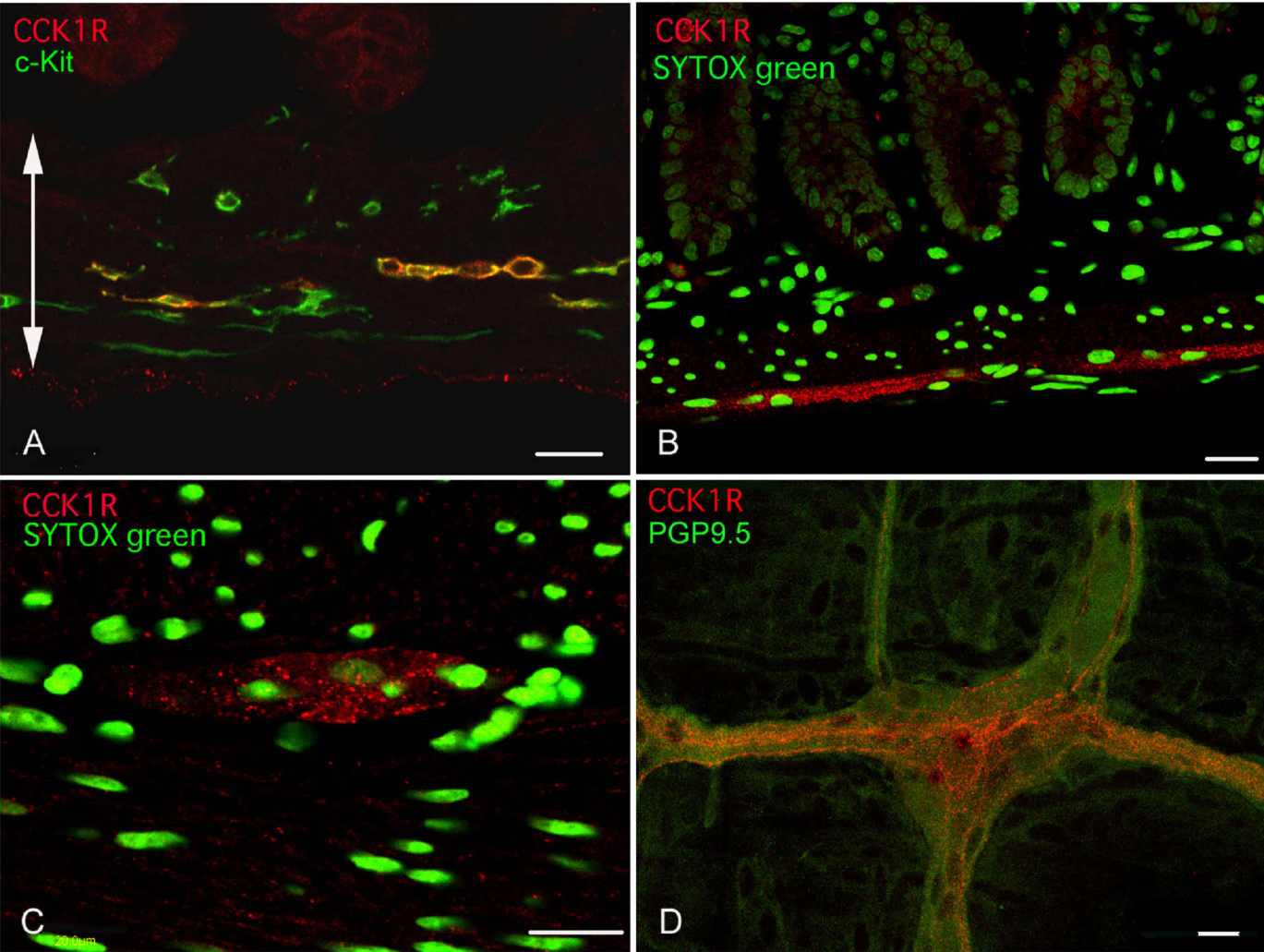
Figure 1

Figure 2

Figure 3

A

CCK1R
c-Kit

B

CCK1R
SYTOX green

C

CCK1R
SYTOX green

D

CCK1R
PGP9.5

Figure 4

Figure 5