Somatostatin receptor 2 knockout/lacZ knockin mice show impaired motor coordination and reveal sites of somatostatin action within the striatum

ARTICLE *in* EUROPEAN JOURNAL OF NEUROSCIENCE · JUNE 2003

Impact Factor: 3.18 · DOI: 10.1046/j.1460-9568.2003.02629.x · Source: PubMed

9 AUTHORS, INCLUDING:

Gareth J Hathway
University of Nottingham
27 PUBLICATIONS **671** CITATIONS
SEE PROFILE

Neil J Clarke
GlaxoSmithKline plc.
13 PUBLICATIONS **465** CITATIONS
SEE PROFILE

Keith M Kendrick
University of Electronic Science and Techn…
220 PUBLICATIONS **8,603** CITATIONS
SEE PROFILE

THE JOURNAL OF COMPARATIVE NEUROLOGY 454:329–340 (2002)

Identification of Cells Expressing Somatostatin Receptor 2 in the Gastrointestinal Tract of *Sstr2* Knockout/*lacZ* Knockin Mice

JEREMY P. ALLEN,[1,2] ALISON J. CANTY,[3] STEFAN SCHULZ,[4] PATRICK P.A. HUMPHREY,[2] PIERS C. EMSON,[1] AND HEATHER M. YOUNG[3]*
[1]Department of Neurobiology, The Babraham Institute, Babraham, Cambridge, CB2 4AT, United Kingdom
[2]Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ, United Kingdom
[3]Department of Anatomy and Cell Biology, University of Melbourne, 3010, VIC, Australia
[4]Department of Pharmacology and Toxicology, Otto-von-Guericke University, 39120 Magdeburg, Germany

ABSTRACT

Somatostatin is found in neurons and endocrine cells in the gastrointestinal tract. The actions of somatostatin are mediated by a family of G-protein–coupled receptors that compose five subtypes (SSTR1–5), each of which is encoded by a separate gene. *lacZ* "knockin" mice, in which the reporter gene *lacZ* was engineered into the genomic locus of *Sstr2* by gene targeting, were used to examine the expression pattern of *Sstr2* and identify potential targets for neurally released and hormonal somatostatin in the gastrointestinal tract. In the body of the stomach, a large proportion of epithelial cells and subpopulations of myenteric neurons expressed *Sstr2*. Double- or triple-labeling with antisera to H^+K^+ATPase (to identify parietal cells) and/or histidine decarboxylase (to identify enterochromaffin-like [ECL] cells) combined with β-galactosidase staining revealed that both parietal cells and ECL cells expressed *Sstr2*, and these two cell types accounted for almost all of the *Sstr2*-expressing epithelial cells. Somatostatin inhibits gastric acid secretion. The presence of SSTR2 on both parietal and ECL cells suggests that somatostatin acting on SSTR2 may reduce acid secretion by both acting directly on parietal cells and by reducing histamine release from ECL cells. In the small and large intestine, subpopulations of neurons in the myenteric and submucosal plexuses expressed *Sstr2*, and many of the *Sstr2*-expressing myenteric neurons also showed SSTR2(a) immunostaining. Most of *Sstr2*-expressing neurons in the myenteric plexus showed nitric oxide synthase (NOS) immunoreactivity. Previous studies have shown that NOS neurons are descending interneurons and anally projecting, inhibitory motor neurons. Thus, somatostatin acting at SSTR2 receptors on NOS neurons might modulate descending relaxation. J. Comp. Neurol. 454:329–340, 2002.

Indexing terms: parietal cell; enterochromaffin-like cell; enteric neuron; nitric oxide synthase; motility

Grant sponsor: European Contract; Grant number: QLG3-CT-1999-00908; Grant sponsor: GlaxoSmithkline; Grant sponsor: National Health and Medical Research Council of Australia.

Dr. Humphrey's present address is Advanced Medicine, 901 Gateway Blvd., South San Francisco, CA 94080.

*Correspondence to: Heather M. Young, Department of Anatomy and Cell Biology, University of Melbourne, 3010, VIC, Australia.
E-mail: h.young@unimelb.edu.au

Received 12 September 2001; Revised 25 July 2002; Accepted 23 August 2002

DOI 10.1002/cne.10466

Published online the week of October 28, 2002 in Wiley InterScience (www.interscience.wiley.com).

Somatostatin is present in both endocrine cells and neurons in the gastrointestinal tract of a range of mammals. Somatostatin is found in D-type endocrine cells of the gastric mucosa and the mucosa of the small intestine (Hökfelt et al., 1975; Larsson et al., 1979; Track et al., 1979). In the small and large intestine of most species examined, somatostatin-containing nerve cell bodies are found in both the myenteric and submucosal plexuses and somatostatin-containing nerve terminals innervate the mucosa and the enteric plexuses but are sparse in the external muscle (Hökfelt et al., 1975; Costa et al., 1980; Furness et al., 1980; Schultzberg et al., 1980; Keast et al., 1984; Ekblad et al., 1987, 1988). Thus, somatostatin-containing enteric neurons are not external muscle motor neurons, but are likely to include interneurons and secretomotor neurons (Furness, 2000).

The actions of somatostatin in the gastrointestinal tract are extensive. A variety of physiological, pharmacologic, and anatomic studies have identified cell types that are likely to be targets for both neurally released and hormonal somatostatin. Somatostatin released from endocrine cells is a potent inhibitor of the gastrin-induced acid secretion in the stomach (Park et al., 1987); thus, parietal cells are very likely to be one of the targets of somatostatin. Histamine released from enterochromaffin-like (ECL) cells stimulates gastric acid secretion, and as somatostatin inhibits histamine release from gastric ECL cells, somatostatin may also indirectly reduce acid secretion by acting on ECL cells (Prinz et al., 1993). Somatostatin can also inhibit or excite myenteric neurons (Katayama and North, 1980), but it appears to have little direct effect on the muscle (Furness and Costa, 1979). A further way to identify potential targets of somatostatin is to localize cells that express somatostatin receptors.

Five different somatostatin receptor genes have been cloned; the receptors encoded by these genes, SSTR1 to SSTR5 (The gene and protein nomenclature used follows the guidelines of The Human Genome Organisation nomenclature committee, http:www.gene.ucl.ac.uk/nomenclature/), are all G-protein–coupled receptors with seven transmembrane domains (see Hoyer et al., 1995). In addition, two separate isoforms of SSTR2 exist, SSTR2(a) and SSTR2(b), as a result of alternative splicing (Vanetti et al., 1992; Schindler et al., 1998). Previous studies using in situ hybridization (Krempels et al., 1997) or immunohistochemistry (Sternini et al., 1997; Schindler and Humphrey, 1999) have identified some of the somatostatin receptor-expressing cells in the gastrointestinal tract of the rat. In the present study, cells that endogenously express *Sstr2* in the gastrointestinal tract were identified using gene targeted "knockin" mice in which the *Sstr2* coding sequence was replaced with the reporter gene, *lacZ*. This strategy places *lacZ* under the exclusive control of the entire *Sstr2* locus resulting in *lacZ* expression that exactly recapitulates the expression pattern of *Sstr2*.

MATERIALS AND METHODS

Animals

All animal use procedures were in strict accordance with U.K. Home Office guidelines and specifically licensed under the Animals (Scientific Procedures) Act 1986. We generated a *Sstr2* knockout/*lacZ* knockin allele, $Sstr2^{lacZ}$, by gene targeting in embryonic stem cells (Allen et al., 2002). Briefly, a gene targeting construct was engineered in a *Sstr2* genomic clone by replacing the *Sstr2* coding sequence with a promoterless *lacZ* reporter gene. This construct was electroporated into the 129/Ola embryonic stem cell line HM-1. Correctly targeted clones were identified by G418 resistance and Southern blot analysis. Chimeric mice were generated by blastocyst injection and germline transmission achieved by breeding chimeras to C57BL/6 (Babraham Institute Small Animal Facility). The $Sstr2^{lacZ}$ allele was then backcrossed onto the C57BL/6 background for a further five generations by using male and female $Sstr2^{+/lacZ}$ mice alternately. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis demonstrated a complete lack of *Sstr2* expression in $Sstr2^{lacZ/lacZ}$ mice, whereas *lacZ* expression was present in $Sstr2^{+/lacZ}$ and $Sstr2^{lacZ/lacZ}$ mice.

Mice of both genders, between 16 and 20 weeks of age, were used. Mice were housed with littermates under a 12-hour light-dark cycle and had free access to food and water at all times. Animals were killed by cervical dislocation.

Histochemical and immunohistochemical staining. Segments of esophagus, the ileum, and colon and the entire stomach were removed and fixed in 2% paraformaldehyde in 0.01 M phosphate buffered saline (PBS), pH 7.3, or in Zamboni's fixative (2% paraformaldehyde plus 15% saturated picric acid in 0.01 M PBS), pH 7.3, for 4 hours. The tissue was then washed in 0.1 M PBS and incubated overnight at 4°C in a solution of 30% sucrose in 0.1 M PBS, and then 20 μm frozen sections were cut on a cryostat. Whole-mount preparations of myenteric plexus/longitudinal muscle from the ileum and colon were also dissected.

To detect β-galactosidase activity, tissues were washed twice for 10 minutes in 0.1 M PBS and then transferred to β-galactosidase reaction mixture (0.1 M PBS, pH 7.3, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P40, 1 mg/ml X-gal [5-bromo-4-chloro-3-indolyl β-D-galactopyranoside], Melford, Whaley Bridge, UK). Tissues were incubated at 37°C for either 3 hours or overnight, rinsed twice in 0.1 M PBS and then once in distilled water. Tissue was mounted by using Mowiol mounting medium (Bernex and Tiret, 1997). Preliminary experiments detected β-galactosidase activity in the mucosal layer of wild-type tissues. Sections, therefore, were post-fixed at room temperature for 5 minutes in 3% paraformaldehyde in 0.1 M PBS. In tissues from wild-type mice, this treatment eliminated detectable β-galactosidase activity after staining in X-gal reaction mixture at 37°C for 3 hours, although after overnight incubation, faint β-galactosidase activity could still be detected. Consequently, all of the X-gal histochemical reactions were performed for 3 hours, and any β-galactosidase staining can be assumed to be due to the expression of *lacZ*.

After staining for β-galactosidase activity, tissues were processed for immunohistochemistry as described previously (Young et al., 1998) by using the primary and secondary antisera shown in Table 1. Initial studies demonstrated a high level of background staining in stomach sections using antibodies against H^+K^+ATPase and histidine decarboxylase (HDC). This background was significantly reduced by blocking endogenous avidin and biotin sites by using an avidin/biotin blocking kit, according to manufacturers instructions (Vector laboratories, Burlin-

TABLE 1. Tissue Processing for Immunohistochemistry[1]

Primary antisera	Species in which primary antiserum was raised	Dilution	Source or reference	Secondary antisera	Dilution	Source
Calbindin	Rabbit	1:1600	Furness et al., 1988	Goat anti-rabbit Alexa 490	1:200	Molecular Probes, Eugene, OR, USA
Calretinin	Rabbit	1:1000	SWANT Swiss Antibodies, Bellizona, Switzerland	Goat anti-rabbit Alexa 488	1:200	Molecular Probes
Gastrin	Rabbit	1:100	ICN, Costa Mesa, CA, USA	Goat biotinylated anti-rabbit followed by streptavidin-Alexa 488	1:200 1:200	Vector Laboratories, Burlingame, CA, USA Molecular Probes
H^+K^+ ATPase	Mouse	1:4000	Beck et al., 1999	Horse anti-mouse FITC or goat biotinylated anti-mouse followed by streptavidin-Alexa 488	1:200 1:200	Vector Molecular Probes
Histidine decarboxylase (HDC)	Rabbit	1:500	ICN, Aurora, OH, USA	Goat biotinylated anti-rabbit followed by streptavidin-Alexa 488 or streptavidin-Cy3	1:200 1:200 1:200	Vector Molecular Probes Amersham Pharmacia, Little Chalfont, UK
Kit	Rabbit	1:50–1:200	Calbiochem	Goat biotinylated anti-rabbit followed by streptavidin-Alexa 488	1:200 1:200	Vector Molecular Probes
Neuronal NOS	Sheep	1:2000	Norris et al., 1995	Donkey anti-sheep FITC	1:250	Jackson Immunoresearch, West Grove PA, USA
Somatostatin	Rabbit	1:200	Peninsula, Belmont, CA, USA	Biotinylated donkey anti-rabbit followed by streptavidin Alexa 594	1:100 1:200	Jackson Molecular Probes
SSTR2(a)-6291	Rabbit	1:500–1:1000	Schulz et al., 1998	Goat anti-rabbit Alexa 488 or biotinylated donkey anti-rabbit followed by streptavidin Alexa 594	1:200 1:100 1:200	Molecular Probes Jackson Molecular Probes
SSTR2(a), antiserum #9431	Rabbit	1:2000–1:5000	Sternini et al., 1997	Goat anti-rabbit Alexa 488 or biotinylated donkey anti-rabbit followed by streptavidin Alexa 594	1:100 1:200	Jackson Molecular Probes
SSTR2(b)	Rabbit	1:100–1:500	Schindler and Humphrey, 1999	Biotinylated donkey anti-rabbit followed by streptavidin Alexa 594	1:100 1:200	Jackson Molecular Probes

[1]NOS, nitric oxide synthase; FITC, fluorescein isothiocyanate.

game, CA). Furthermore, the anti-HDC antibody required sections to be blocked with 5% normal serum. Preliminary studies showed that very few somatostatin-immunoreactive nerve terminals could be detected in whole-mount preparations of myenteric plexus fixed in 2% or 4% paraformaldehyde. Somatostatin-immunoreactive nerve terminals could be observed after fixation in Zamboni's fixative, but only after overnight fixation. To examine the presence and distribution of somatostatin-containing nerve terminals, segments of gut were fixed in Zamboni's overnight, washed in dimethyl sulfoxide and then phosphate buffer, and then processed for immunohistochemistry.

For counts of the percentage of different neuron types that showed β-galactosidase staining, a minimum of 100 nitric oxide synthase (NOS) or calretinin neurons or 50 calbindin neurons were analyzed from each preparation. For each antibody, three preparations from different animals were analyzed from each genotype. Video images were recorded by using an ImagePoint cooled charge-coupled device camera (Photometrics, Tucson, AZ) and V for Windows imaging software (Digital Optics, Auckland, New Zealand), or by using a Spot 35 digital color camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Confocal images were obtained by using a Bio-Rad 1024 confocal microscope.

RESULTS

Cells that expressed the $Sstr2^{lacZ}$ allele were recognized by histochemical staining for β-galactosidase (β-gal), the gene product of *lacZ*. The expression of *lacZ* was examined in frozen sections through the esophagus, stomach, ileum, and colon of $Sstr2^{+/+}$, $Sstr2^{+/lacZ}$, and $Sstr2^{lacZ/lacZ}$ mice that had been incubated for 3 hours in the X-gal reaction mixture. Apart from cells in the circular muscle layer of the colon (see below), the cell types showing β-gal staining and their abundance were not obviously different between $Sstr2^{+/lacZ}$ and $Sstr2^{lacZ/lacZ}$ mice.

Regional distribution of *Sstr2*-expressing cells

In frozen, transverse sections through the esophagus and the fundus, the only β-gal+ cells observed were a subpopulation of myenteric neurons located between the two layers of external muscle (Fig. 1A–C). In the body of the stomach, many epithelial cells and myenteric neurons were β-gal+ (Fig. 1D–F). The β-gal+ epithelial cells occurred at a very high density in the middle portion of the gastric glands (Fig. 1D,E); stained cells were sparse close to or at the luminal surface of the mucosa and occurred at lower density at the bases of the gastric glands than in the middle region of the mucosa (Fig. 1D). By using differential interference contrast optics at high magnification, al-

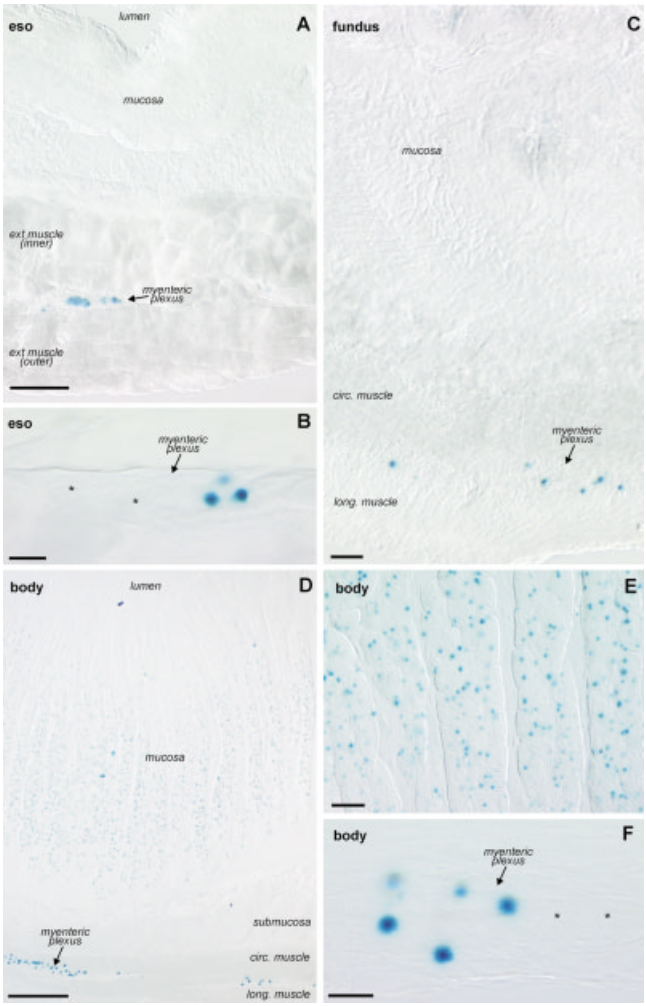

Fig. 1. Frozen transverse sections through the esophagus and stomach of *Sstr2*$^{lacZ/lacZ}$ mice processed for β-galactosidase (β-gal) histochemistry and viewed using differential interference contrast optics. **A:** The only β-gal+ cells in the esophagus (eso) are located in the myenteric plexus. **B:** Higher magnification view of a myenteric ganglion in the esophagus showing that both β-gal+ and β-gal– negative (asterisks) cells are present in the ganglion. **C:** The only β-gal+ cells in the fundus are located in the myenteric plexus; none of the mucosal cells show any β-gal staining. **D:** Low-magnification image of a section through the body of the stomach. β-gal+ cells are present in the myenteric plexus and in the middle part of the mucosa. **E:** Higher magnification image of the β-gal+ cells in the middle of the mucosa. The vast majority of the epithelial cells in this region show some β-gal staining. **F:** Higher magnification view of a myenteric ganglion in the body of the stomach showing that both β-gal+ and β-gal–negative (asterisks) cells are present in the ganglion. ext, external; long, longitudinal; circ., circular. Scale bars = 100 μm in A,D, 25 μm in B,E, 50 μm in C, 10 μm in F.

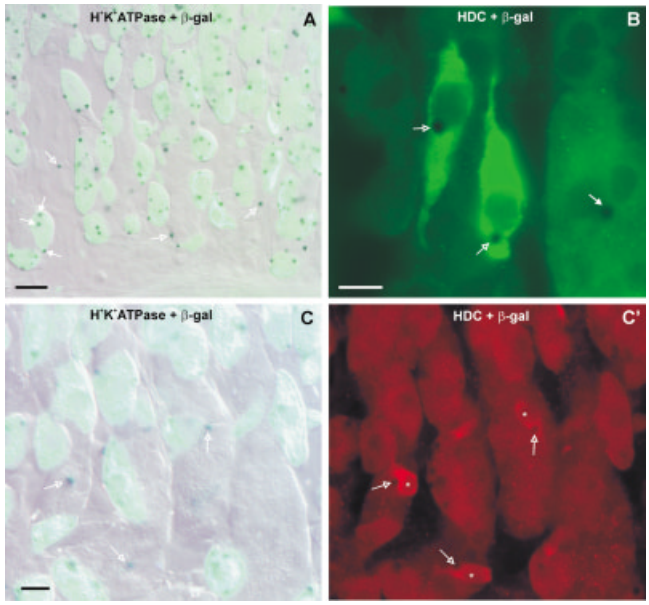

Fig. 2. Frozen transverse sections through the body of *Sstr2*$^{+/lacZ}$ mice that were processed for β-galactosidase (β-gal) histochemistry combined with H^+K^+ATPase immunohistochemistry (A), histidine decarboxylase (HDC) immunohistochemistry (B), or both H^+K^+ATPase and HDC immunohistochemistry (C,C′). In A and C, the sections were viewed by using a fluorescence microscope but also with white light (differential interference contrast) at low levels of illumination. **A:** All of the H^+K^+ATPase-immunoreactive cells (parietal cells), which appear pale green, contain multiple β-galactosidase reaction products (filled arrows). However, there is a small amount of β-galactosidase staining (open arrows) that is not associated with parietal cells (open arrows). **B:** High-magnification, fluorescence photomicrograph of two HDC-immunoreactive cells, both of which contain β-galactosidase reaction products (open arrows). There are also some β-galactosidase reaction products (closed arrows) that are not associated with the HDC-immunoreactive cells. **C,C′:** Paired photomicrographs of the same field of view showing β-galactosidase staining (blue dots) and H^+K^+ATPase-immunostaining (pale green, C) plus HDC immunostaining (C′). There are some β-galactosidase reaction products (open arrows, C) that are not associated with parietal cells; these reaction products are within ECL (HDC-immunoreactive) cells (C′). Scale bar = 25 μm in A, 10 μm in B,C,C′.

most all of the cells in the middle region of the mucosa were observed to show some β-gal staining (Fig. 1E). The β-gal+ epithelial cells were round in shape, and based on their shape and abundance in the middle parts of the mucosa, it appeared likely that many of the *Sstr2*-expressing epithelial cells were parietal cells. To identify the β-gal+ epithelial cells in the body, frozen sections from *Sstr2*$^{+/lacZ}$ mice were processed for β-gal histochemistry and immunohistochemistry using antisera to H^+K^+ATPase (to identify parietal cells) and/or histidine decarboxylase (HDC; to identify ECL cells). There was a high degree of colocalization of β-gal activity and H^+K^+ATPase immunoreactivity (Fig. 2A). However, some cells in the basal third of the mucosa showed β-gal activity but no H^+K^+ATPase immunoreactivity (Fig. 2A), and some cells near the luminal surface showed H^+K^+ATPase immunoreactivity but no β-gal activity. The HDC-immunoreactive cells were found in the basal third of the mucosa and most of them also showed β-gal activity (Fig. 2B), but HDC-immunoreactive cells composed only a small percentage of all β-gal+ cells. In tissue triple labeled for β-gal histochemistry and H^+K^+ATPase and HDC immunohistochemistry, the vast majority of β-gal+ cells showed either H^+K^+ATPase or HDC immunoreactivity (Fig. 2C,C′). However, a small number of β-gal+ cells, that were located near the base of the mucosa, did not display either H^+K^+ATPase or HDC immunoreactivity. In the pylorus, a subpopulation of myenteric neurons was β-gal+ and a subpopulation of epithelial cells in the

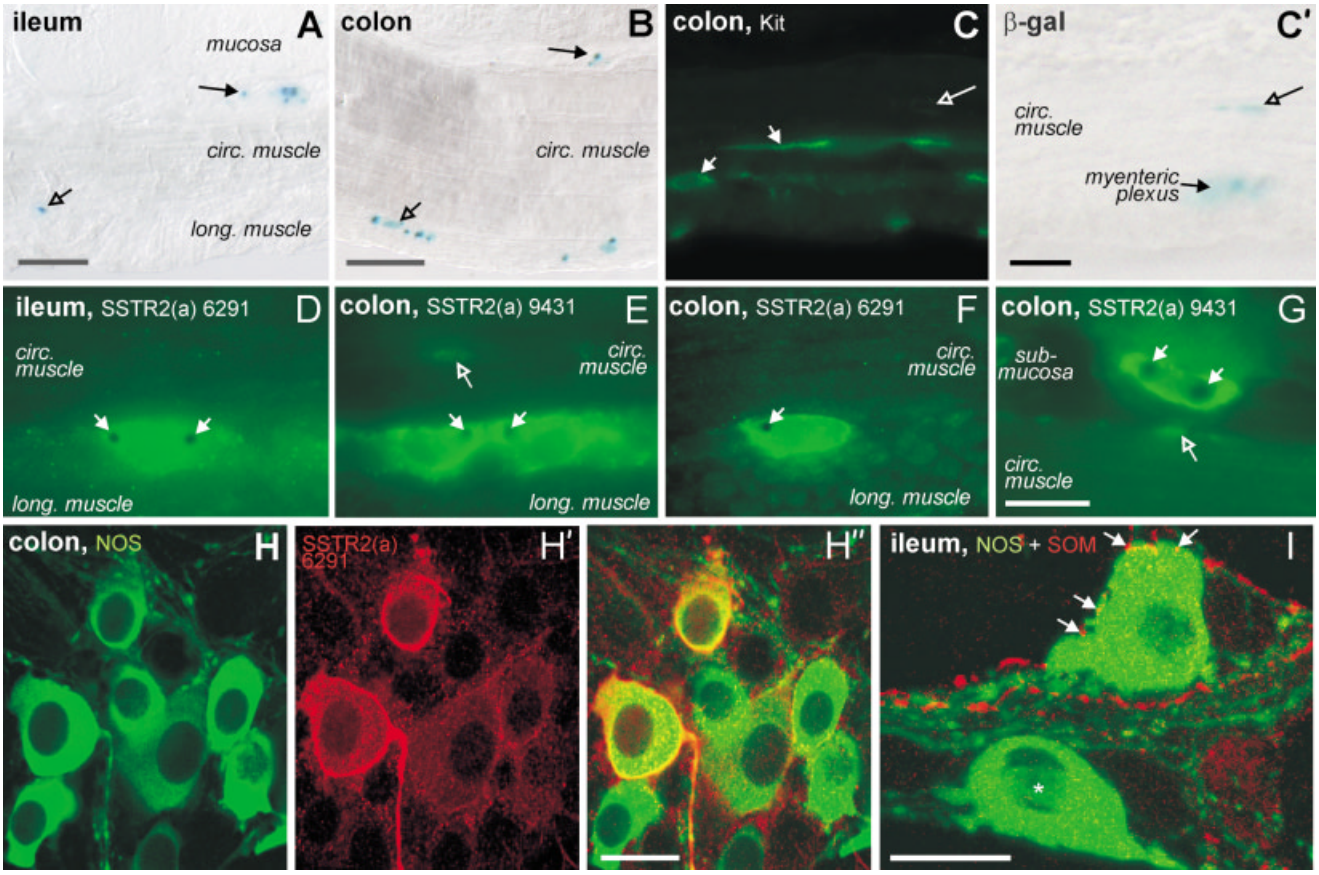

Fig. 3. **A,B:** Frozen sections through the ileum (A) and colon (B) of $Sstr2^{lacZ/lacZ}$ mice processed for β-galactosidase (β-gal) histochemistry and viewed using differential interference contrast optics. **A:** In the ileum, the only β-gal+ cells are in the myenteric (open arrow) and submucosal (filled arrow) ganglia. **B:** In the colon, myenteric (open arrow) and submucosal (filled arrow) neurons show β-gal staining. **C,C′:** Paired photomicrographs of the same field of view showing the fluorescence immunohistochemical localization of Kit (C) and histochemical localization of β-gal (C′) in $Sstr2^{lacZ/lacZ}$ mice. Kit+ interstitial cells of Cajal (ICC; small arrows, C) are present in the circular muscle and at the level of the myenteric plexus, but a β-gal+ cell in the circular muscle (open arrow, C′) is not Kit+. **D–G:** Immunohistochemical localization of SSTR2(a) in frozen sections of ileum (D) and colon (E–G) of $Sstr2^{+/lacZ}$ mice that had also been processed for β-gal histochemistry. By using either SSTR2(a) antibody 6291 (D,F) or antibody 9431 (E,G), a subpopulation of neurons in the myenteric (D–F) and submucosal (G) ganglia show SSTR2(a) immunostaining. The immunostained neurons also exhibit β-gal+ reaction products (filled arrows). Stained ICC-like cells in the circular muscle (open arrows in E,G) are also present after processing using SSTR2(a) antibody but not with SSTR2(a) antibody 6291 (D,F). **H–H′′:** Confocal microscope images of whole-mount preparations of myenteric plexus of colon from a $Sstr2^{+/+}$ mouse after double immunohistochemical labeling for NOS (H,H′′) and SSTR2(a) antibody 6291 (H′,H′′). The SSTR2(a)+ neurons form a subpopulation of the NOS neurons. **I:** Confocal microscope image of a whole-mount preparation of myenteric plexus from the ileum of a $Sstr2^{+/+}$ mouse after fixation in Zamboni's fixative and double-label immunohistochemistry using antisera to NOS and somatostatin. Somatostatin-immunoreactive nerve terminals (red, arrows) are present around one of the NOS cell bodies (green) in the field of view, but a second NOS cell body (asterisk) does not have any somatostatin-immunoreactive nerve terminals in close apposition. circ., circular; long., longitudinal. Scale bar = 25 μm in A,B, in C (applies to C,C′), in G (applies to D–G), in H′′ (applies to H,H′′), in I.

middle and base of the mucosa also showed β-gal staining. Many of the β-gal+ epithelial cells in the pylorus showed HDC immunoreactivity but none showed gastrin immunoreactivity (not illustrated).

In the ileum, the only *Sstr2*-expressing cells were subpopulations of myenteric and submucosal neurons (Fig. 3A). In the colon, subpopulations of myenteric and submucosal neurons were β-gal+ (Fig. 3B). In addition, β-gal staining was also observed in spindle-shaped cells within different levels of the circular muscle layer of the colon (Fig. 3C′). Unlike the β-gal staining in neurons, which was punctate (Fig. 3A,B), the β-gal staining in cells in the circular muscle layer was weak and diffuse (Fig. 3C′). The number of β-gal+ cells in the circular muscle layer of the colon was variable between animals. β-gal+ cells were common in the circular muscle in two of six $Sstr2^{lacZ/lacZ}$ mice, were sparse or rare in four of six $Sstr2^{lacZ/lacZ}$ and four of seven $Sstr2^{+/lacZ}$ mice, and were not observed in three of seven $Sstr2^{+/lacZ}$ mice. To determine whether the β-gal+ cells in the circular muscle were interstitial cells of Cajal (ICC), β-gal histochemistry was combined with Kit immunohistochemistry. There was no overlap between β-gal staining and Kit immunostaining (Fig. 3C,C′), and Kit+ ICC vastly outnumbered β-gal+ cells in all preparations examined (n = 3 $Sstr2^{lacZ/lacZ}$ mice, and n = 3 $Sstr2^{+/lacZ}$ mice).

Immunohistochemical localization of SSTR2 and correlation with β-gal staining

Two separate isoforms of SSTR2 exist, SSTR2(a) and SSTR2(b), as a result of alternative splicing. We obtained two different antisera to SSTR2(a). One antiserum was generated by using a peptide corresponding to residues 355-369 of the C-terminal tail of SSTR2(a) (Schulz et al., 1998, antiserum 6291). and the other antiserum was generated to residues 361-369 (Sternini et al., 1997, antiserum 9431). To our knowledge, only one SSTR2(b) antiserum has been used in published immunohistochemical studies; this antiserum was used to examine the localization of SSTR2(b) in the brain and stomach of the rat (Schindler et al., 1998; Schindler and Humphrey, 1999). Immunohistochemistry was performed on the stomach (body), ileum, and colon only. The SSTR2(b) antibody showed no immunostaining in any region examined; hence, the correlation between β-gal staining and SSTR2(b) immunoreactivity could not be examined. The SSTR2(b) antibody was raised against a rat peptide sequence that is 33% different from the orthologous mouse sequence (Schindler et al., 1998); therefore, it is not surprising that it showed no immunolabeling in the mouse.

SSTR2(a) immunolocalization in gastric epithelial cells

No specific SSTR2(a) immunostaining was observed in the gastric mucosa using either SSTR2(a) antisera 6291 or 9431.

SSTR2(a) immunolocalization and correlation with β-gal staining in enteric neurons

By using either SSTR2(a) antisera 6291 or 9431, immunostained neurons were present in the myenteric plexus of the stomach and in the myenteric and submucosal plexuses of the ileum and colon of $Sstr2^{+/+}$ mice (Fig. 4). The intensity of immunostaining of enteric neurons seen using both SSTR2(a) antisera varied in different regions of the gastrointestinal tract, with the strongest staining seen in the colon (Fig. 4E), weaker staining in the ileum (Fig. 4D) and only very weak staining in the stomach (Fig. 4A). In contrast, tissue from different regions of the gastrointestinal tract that had been double-labeled for both SSTR2(a) and NOS immunohistochemistry (see below) showed no variation in the intensity of NOS immunostaining along the gastrointestinal tract. This finding suggests that the regional differences in intensity of SSTR2(a) immunostaining observed were not due to technical problems with preparing tissue from different gut regions for immunohistochemistry, but are most likely due to regional differences in the levels of SSTR2(a) protein.

In the stomach, only very weak SSTR2(a) immunostaining was observed in myenteric neurons of $Sstr2^{+/+}$ mice (Fig. 4A), and in $Sstr2^{+/lacZ}$ mice, no specific immunostaining was discernible using either SSTR2(a) antiserum. Therefore, we could not examine the correlation between β-gal staining and SSTR2(a) immunostaining in myenteric neurons in the stomach.

In the ileum and colon of $Sstr2^{+/+}$ and $Sstr2^{+/lacZ}$ mice, SSTR2(a)-immunoreactive nerve cell bodies were observed in both the myenteric or submucosal plexuses (Fig. 4B–E), but no immunostained neurons were observed in

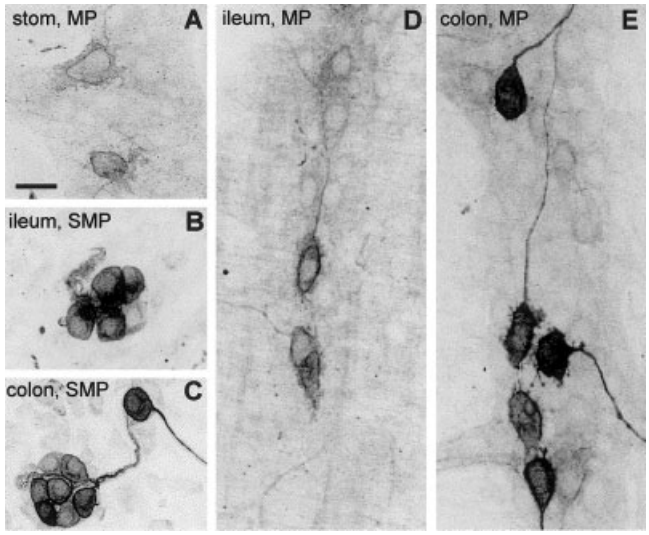

Fig. 4. Inverted, confocal microscope images of whole-mount preparations of myenteric (MP, **A,D,E**) and submucous (SMP, **B,C**) plexus from $Sstr2^{+/+}$ mice processed for immunohistochemistry by using SSTR2(a) antibody 6291. Immunoreactive neurons are present in the myenteric plexus of the stomach (stom, A) and in the MP and SMP of the ileum (B,D) and colon (C,E). The neurons in the myenteric plexus of the colon (E) show stronger immunostaining than those in the ileum (D) and stomach (A). Scale bar = 25 μm in A (applies to A–E).

$Sstr2^{lacZ/lacZ}$ mice using either SSTR2(a) antibody. There was a higher density of SSTR2(a)-immunostained neurons in both the myenteric and submucosal plexuses of the colon than in the ileum. Immunoreactive nerve terminals were not observed in the external muscle (Fig. 3D–G, 5D) or in the mucosa. The immunostained neurons showed a variety of staining intensities—some neurons showed very strong staining whereas others were only weakly immunostained (Fig. 5A,D). Whole-mount preparations of myenteric plexus revealed that, in addition to the cell bodies, the proximal parts of the axons were stained (Fig. 5A,B); hence, the projection patterns of individual stained neurons could be observed. The axons of the SSTR2(a)-immunostained myenteric neurons in both the ileum (Fig. 5B) and colon (Fig. 5A) projected anally. Most of the stained nerve fibers within myenteric ganglia appeared to be the proximal parts of axons (Figs. 4D,E, 5A,B). The immunostaining of myenteric neurons was largely associated with the cell membranes (Fig. 5C,D), whereas in submucosal neurons, both the cell membranes and some perinuclear organelles were stained (Fig. 4B,C). Because the cell membranes were well stained, the morphology of the immunoreactive cell bodies was revealed; in both the ileum and colon, the vast majority of cells were uniaxonal with lamellar dendrites and, hence, had Dogiel type-I morphology (Fig. 5C).

The correlation between SSTR2(a) immunostaining and β-gal histochemistry was examined in the ileum and colon of $Sstr2^{+/lacZ}$ mice. By using either SSTR2(a) antiserum, all of the SSTR2(a)-immunostained neurons in the myenteric and submucosal neurons were also β-gal+ (Fig. 3D–G), but there were some β-gal+ neurons that did not show detectable SSTR2(a) immunostaining.

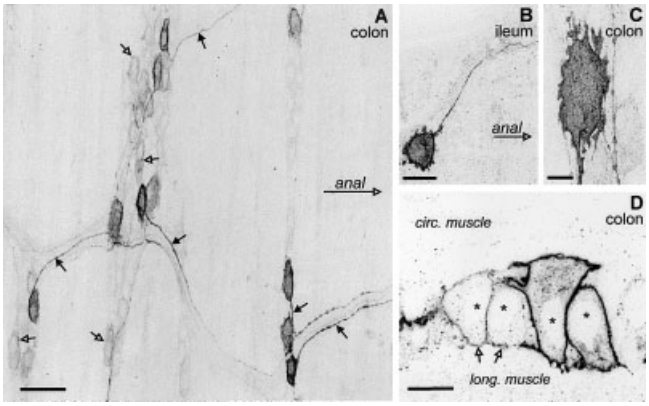

Fig. 5. Inverted, confocal microscope images of the myenteric plexus of the ileum (B) and colon (A,C,D) of $Sstr2^{+/+}$ mice after processing for immunohistochemistry using antisera to SSTR2(a) antibody 6291 (A–C) or SSTR2(a) antibody 9431 (D). **A,B:** Projected z-series of whole-mount preparations of colon (A) and ileum (B) in which oral is to the left and anal to the right of the field of view. The cell bodies and initial parts of the axons (arrows) of neurons are immunostained; therefore, the direction in which the axons project can be ascertained. In both the ileum and colon, the SSTR2(a)+ neurons project anally. Some SSTR2(a)+ neurons (A, open arrows) show only very weak immunoreactivity. **C:** High-magnification, projected z-series of a SSTR2(a)+ cell body in a whole-mount preparation of the myenteric plexus of the colon. The neuron is uniaxonal with lamellar dendrites and, therefore, has Dogiel type-I morphology. The varicose nerve fiber to the right of the cell body is the proximal part of an axon of a neighbouring neuron. **D:** Single optical section through a myenteric ganglion in a frozen (transverse) section of the colon. In the SSTR2(a)-immunostained neurons in this field of view, the immunostaining is predominantly on the cell membrane. There is no immunostaining of the nuclei (asterisks) and only a small amount of immunostaining in the cytoplasm. Two of the neurons in the field of view (open arrows) show only weak immunolabeling. circ., circular; long., longitudinal. Scale bars = 50 μm in A, 25 μm in B, 10 μm in C,D.

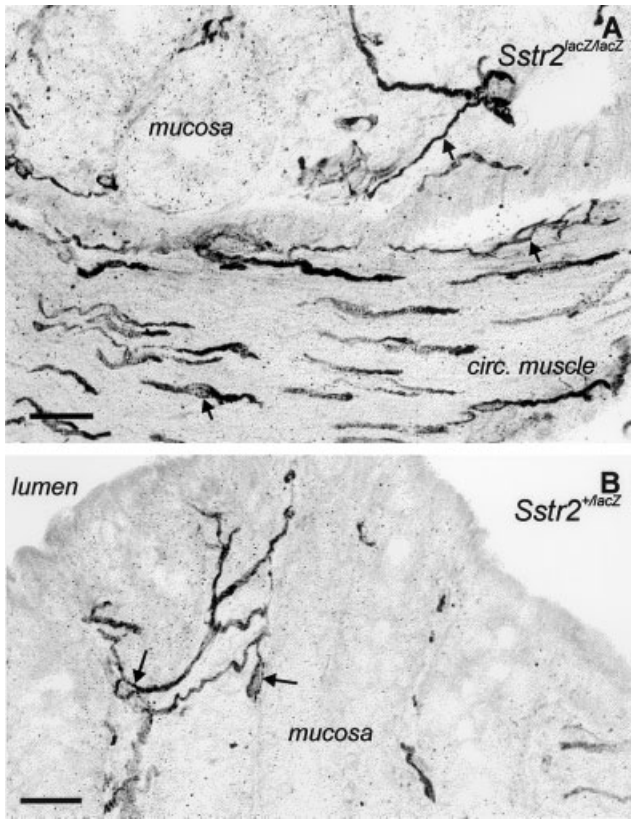

Fig. 6. Inverted, confocal microscope images of transverse sections of colon processed for immunohistochemistry using SSTR2(a) antibody 9431. **A:** Cells resembling interstitial cells of Cajal (ICC, arrows) are present in the external muscle and mucosa of *Sstr2* knockout mice. **B:** The immunostained structures (arrows) present in the lamina propria appear to be cell bodies and their processes. The intensity of immunostaining of the ICC-like cells is similar in $Sstr2^{-/-}$ (A) and $Sstr2^{lacZ/+}$ (B) mice. circ., circular. Scale bars = 25 μm in A,B.

SSTR2(a) immunolocalization and correlation with β-gal staining in non-neuronal cells in the external muscle

The only stained cells observed using SSTR2(a) antiserum 6291 were submucosal and myenteric neurons. However, in addition to revealing neurons, SSTR2(a) antiserum 9431 also revealed immunostained, non-neuronal cells in the lamina propria and external muscle of the stomach, ileum, and colon (Fig. 6A,B). The stained cells in the external muscle resembled ICC in shape, location, and density. However, their identity as ICC could not be confirmed by using immunohistochemistry as both SSTR2(a) antiserum 9431 and the Kit antiserum were from the same species (see Table 1). The immunostained structures present in the lamina propria were cellular (Fig. 6A,B), not nerve fibers, and resembled the ICC-like cells present in the external muscle. However, there are no Kit+ ICC in the mucosa (Maeda et al., 1992; Burns et al., 1997). Cajal did, however, report interstitial cells in the mucosa (see Fig. 2 of Takayama et al., 2002), and the mucosal cells revealed using SSTR2(a) antiserum 9431 resembled the cells depicted in Cajal's drawings. The immunostained ICC-like cells observed in the external muscle and lamina propria after staining with SSTR2(a) antiserum 9431 were observed in $Sstr2^{+/+}$, $Sstr2^{+/lacZ}$ (Fig. 6B), and $Sstr2^{lacZ/lacZ}$ mice (Fig. 6A) but were absent in control sections in which incubation in the primary antiserum was omitted. Furthermore, in $Sstr2^{+/lacZ}$ mice, the ICC-like cells did not show any β-gal staining. Together these data strongly suggest that SSTR2(a) antiserum 9431 immunoreactivity in ICC-like cells is nonspecific.

Identification of *Sstr2*-expressing neurons in the myenteric plexus of the ileum and their relationship to somatostatin-containing nerve terminals

Because the neurochemistry and projections of different classes of myenteric neurons in the mouse ileum have been examined previously (Sang and Young, 1996, 1998; Sang et al., 1997), the classes of myenteric neurons expressing *Sstr2* in this region were examined in detail. The nitric oxide synthetic enzyme nitric oxide synthase (NOS) and the calcium binding proteins calretinin and calbindin identify different classes of myenteric neurons (Sang and Young, 1996). NOS neurons project anally to innervate the circular muscle and myenteric and submucosal ganglia, most calretinin neurons are cholinergic neurons that project locally and orally to innervate the circular muscle, and calbindin neurons are anally projecting interneurons

or sensory neurons. Whole-mount preparations of myenteric plexus from *Sstr2*$^{lacZ/lacZ}$ mice that had been processed for immunohistochemistry by using antisera to NOS, calretinin, or calbindin were qualitatively indistinguishable from preparations from *Sstr2*$^{+/+}$ and *Sstr2*$^{+/lacZ}$ mice; the ganglia appeared to be of similar size and the abundance of the different subpopulations of neurons was not obviously different in the three genotypes. The great majority of calbindin- immunoreactive (Fig. 7A,A′) or calretinin-immunoreactive neurons did not show β-gal staining (Fig. 8). In contrast, approximately one third of the NOS neurons were β-gal+ (Figs. 7B, 8). The proportions of different cell types showing β-gal staining were similar in *Sstr2*$^{+/lacZ}$ and *Sstr2*$^{lacZ/lacZ}$ mice (Fig. 8). NOS neurons composed 79 ± 7% (mean ± SEM, n = 4) of β-gal+ myenteric neurons.

In the colon, many of the β-gal+ cells also showed NOS immunoreactivity (Fig. 7C). Double-labeling by using SSTR2(a) and NOS antisera in both the ileum and colon also revealed that the vast majority of SSTR2(a)-immunostained myenteric neurons were also NOS-immunoreactive, but SSTR2(a)+ neurons formed only a subpopulation of NOS+ myenteric neurons (Fig. 3H–H′′). There were also some SSTR2(a)-immunoreactive neurons that did not show NOS immunoreactivity.

Somatostatin-immunoreactive nerve cell bodies were present in the myenteric and submucosal ganglia, and somatostatin nerve terminals were present in the mucosa and myenteric and submucosal ganglia but not in the external muscle of the ileum. The fixative conditions required for optimal immunostaining of the somatostatin nerve terminals was not compatible with β-gal histochemistry; therefore, the relationship between *Sstr2*-expressing cells and somatostatin-immunoreactive nerve fibers could not be examined directly. We, therefore, examined the relationship between somatostatin-immunoreactive nerve terminals and NOS-immunoreactive cell bodies in the myenteric plexus, because approximately one third of NOS cell bodies express *Sstr2*^{lacZ}. Some, but not all, NOS+ cell bodies had somatostatin nerve terminals in close apposition (Fig. 3I). Somatostatin-immunoreactive nerve terminals also formed close appositions with neurons that did not contain NOS.

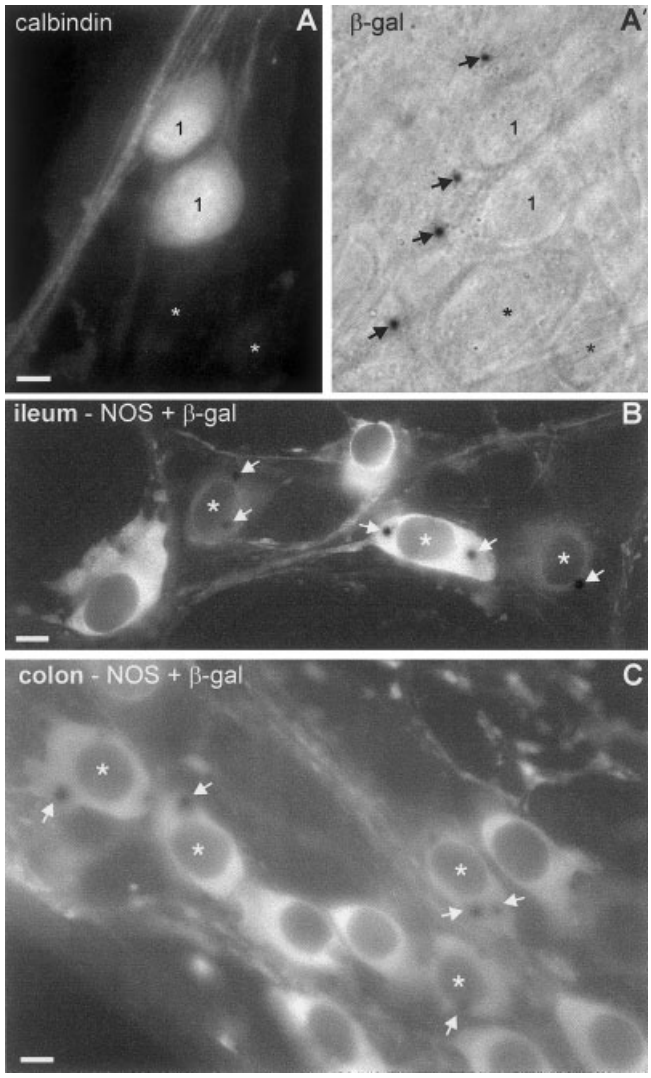

Fig. 7. **A,A′:** Paired photomicrographs of the same field of view of a whole-mount preparation of myenteric plexus from the ileum of *Sstr2*$^{lacZ/lacZ}$ mice processed for calbindin immunohistochemistry (A, viewed using a fluorescence microscope) and β-galactosidase (β-gal) histochemistry (A′, viewed using white light and differential interference contrast optics). Two calbindin-immunoreactive neurons (1) do not show any β-gal staining (A′), and conversely, neurons showing β-gal staining (arrows) are not calbindin-immunoreactive. Some neurons (asterisks) show neither calbindin immunoreactivity nor β-gal staining. **B:** Whole-mount preparation of the myenteric plexus of the ileum of a *Sstr2*$^{lacZ/lacZ}$ mouse processed for nitric oxide synthase (NOS) immunohistochemistry and β-gal histochemistry. The preparation was viewed using a fluorescence microscope but also with white light at low levels of illumination. In this field of view, some NOS+ neurons do not show any β-gal staining but other NOS neurons (asterisks) show both NOS immunostaining and β-gal staining (arrows). **C:** Whole-mount preparation of the myenteric plexus of the colon of an *Sstr2*$^{+/lacZ}$ mouse processed for NOS immunohistochemistry and β-gal histochemistry. Some NOS+ neurons (asterisks) show β-gal staining (arrows). Scale bars = 10 μm in A (applies to A,A′), in B,C.

DISCUSSION

Sstr2 expression in the stomach

In the rat stomach, sparse somatostatin-containing nerve fibers are found in the myenteric ganglia and the smooth muscle but they are not found in the mucosa (see Ekblad et al., 2000). Somatostatin is also found in D-type endocrine cells of the gastric mucosa, and somatostatin, released from these cells, is a potent inhibitor of the gastrin-induced acid secretion from parietal cells (Park et al., 1987). ECL cells stimulate gastric acid secretion by releasing histamine. Somatostatin is also a potent inhibitor of histamine release from gastric ECL cells (Prinz et al., 1993). Thus, in the rat, somatostatin inhibits gastric acid secretion not only by acting directly on parietal cells but also by reducing histamine release from ECL cells. The results from both in vitro and in vivo experiments in rats have suggested that the inhibitory effect of somatostatin on acid release is mediated by means of SSTR2 (Lloyd et al., 1995; Wyatt et al., 1996; Zaki et al., 1996). Moreover, a molecular and pharmacologic study showed that SSTR2 is the predominant somatostatin receptor subtype on ECL cells in the rat stomach (Prinz et al.,

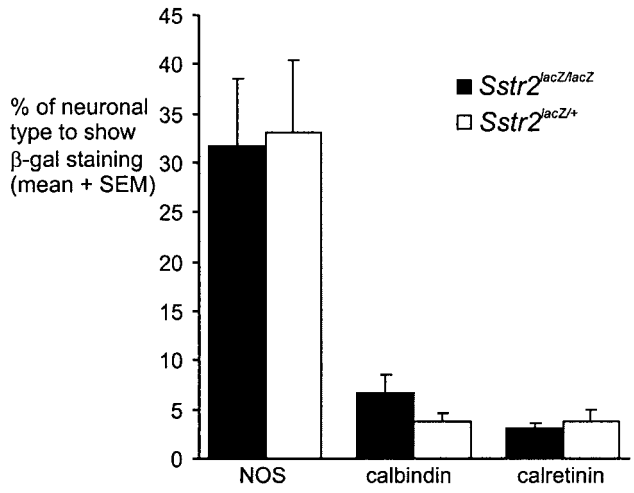

Fig. 8. The percentage (mean ± SD) of nitric oxide synthase (NOS), calbindin, and calretinin neurons in the myenteric plexus of the ileum that show β-gal staining. The data are from three animals of each genotype.

1994). Studies localizing *Sstr2* mRNA or protein in the rat have shown that *Sstr2* is expressed by both epithelial cells and enteric neurons. For example, an in situ hybridization study showed that *Sstr2* is expressed by epithelial cells of the gastric mucosa, as well as the external muscle and enteric ganglia of the rat stomach (Krempels et al., 1997). In addition, antisera to SSTR2(a) and SSTR2(b) proteins have been used to demonstrate that a subpopulation of parietal cells show SSTR2(b) immunoreactivity, and ECL cells and nerve fibers show SSTR2(a) immunoreactivity (Sternini et al., 1997; Schindler and Humphrey, 1999). It has yet to be determined whether the SSTR2(a)-immunoreactive nerve fibers arise from intrinsic and/or extrinsic neurons.

Somatostatin is also present in a subpopulation of endocrine cells in the stomach and small intestine of the mouse, and somatostatin-immunoreactive neurons are present in the mouse stomach and small and large intestines (Rombout et al., 1986; Burrell et al., 1996). Although few physiological studies have been performed on mice, somatostatin has been shown to decrease basal acid secretion from the mouse stomach (Schubert et al., 1989). Furthermore, transgenic mice lacking *Sstr2* show elevated basal levels of gastric acid, demonstrating that, as in rats, somatostatin inhibits acid release by acting on SSTR2 (Martinez et al., 1998).

In the current study, the vast majority of epithelial cells in the middle portion of the body of the mouse stomach expressed *Sstr2*; most of these cells were parietal cells as they showed H^+K^+ATPase immunoreactivity. Hence, somatostatin may inhibit acid secretion by acting on SSTR2 on parietal cells. Many of the ECL (HDC-immunoreactive) cells in the body and pylorus of the mouse also expressed *Sstr2*. Thus, in the mouse, it is likely that somatostatin inhibits histamine secretion from gastric ECL cells by acting on SSTR2, as occurs in the rat. The high basal gastric acid secretion in *Sstr2* knockout mice (Martinez et al., 1998; see above) is likely, therefore, to result both from a reduction or lack of somatostatin-induced inhibition of acid secretion from parietal cells and from a reduction or lack of somatostatin-induced inhibition of histamine release from ECL cells. There was also a small population of *Sstr2*-expressing epithelial cells in the mouse stomach that were not parietal or ECL cells and whose identity remains to be determined. Gastrin cells did not express *Sstr2*.

Two separate isoforms of SSTR2 exist, SSTR2(a) and SSTR2(b), as a result of alternative splicing. Neither the *Sstr2*-expressing parietal cells nor the ECL cells in the gastric mucosa showed SSTR2(a) immunoreactivity; therefore, we assume parietal cells and ECL cells in the mouse synthesize SSTR2(b). However, this assumption could not be examined directly as the only SSTR2(b) antiserum to which we had access did not recognize mouse SSTR2(b). In the rat, parietal cells show SSTR2(b) immunoreactivity, but ECL cells show SSTR2(a) immunoreactivity (Sternini et al., 1997; Schindler and Humphrey, 1999). Thus, the subtype of SSTR2 receptor present on ECL cells appears to differ between species.

Sstr2 was also expressed by a subpopulation of myenteric neurons throughout the stomach, although the functional types of neurons expressing the receptors were not examined. Hence, in addition to regulating acid secretion, somatostatin acting at SSTR2 may also regulate gastric motility in the mouse.

Sstr2 expression by enteric neurons in the small and large intestine

In the current study, subpopulations of both myenteric and submucosal neurons in the mouse ileum and colon expressed *Sstr2*. Many of the *Sstr2*-expressing myenteric and submucosal neurons also showed SSTR2(a) immunoreactivity. Most of the myenteric *Sstr2*-expressing and SSTR2(a) immunopositive neurons were NOS-immunoreactive and projected anally. The localization of the SSTR2(a) has been examined in the rat small intestine and large intestine using immunohistochemistry (Sternini et al., 1997), and consistent with the data obtained in the current study, SSTR2(a) immunoreactivity was also observed in subpopulations of neurons in the myenteric and submucosal plexuses in the rat. The only SSTR2(b) antibody available to us did not recognize SSTR2(b) in mouse tissue; therefore, we could not examine directly the localization of SSTR2(b). Some of the β-gal+ neurons did not show SSTR2(a) immunoreactivity. The β-gal+/SSTR2(a)-immunonegative neurons probably contain SSTR2(b), although it is possible that the levels of SSTR2(a) protein in some enteric neurons in $Sstr2^{+/lacZ}$ mice may be below the threshold of detection for the SSTR2(a) antisera. It is also possible that both SSTR2(a) and SSTR2(b) are produced by some enteric neurons.

In the guinea-pig ileum and colon, somatostatin at low concentrations excites inhibitory myenteric neurons and inhibits the release of acetylcholine, but it has no direct effect on the muscle (Guillemin, 1976; Furness and Costa, 1979). In the rat colon, somatostatin enhances release of vasoactive intestinal peptide (VIP) and causes relaxation of the muscle on the anal side (Grider et al., 1987). Recent studies have examined the role of somatostatin in motility in the mouse intestine. In the mouse colon, the descending relaxation induced by mucosal stroking is inhibited by a blocking antiserum to somatostatin and augmented by a SSTR2 agonist (J.R. Grider, personal communication). In both the rat and mouse small intestine, the regular patterns of contractile activity (migrating motor complexes)

induced by luminal distension are inhibited by somatostatin (Abdu et al., 2002). By using SSTR2 agonists and antagonists, Abdu et al. (2002) found that, in the rat small intestine, the inhibitory effects of somatostatin on the motor complexes were mediated largely by SSTR2. However, experiments using SSTR2 agonists and antagonists in both wild-type and *Sstr2* knockout mice (the same mice that were used in the current study) showed that the inhibitory effects of somatostatin on motor complexes in the mouse small intestine involve both SSTR2 and non-SSTR2 components. As there are no somatostatin-containing nerve terminals within the muscle, it is likely that any actions of endogenous somatostatin are likely to be at neuro-neuronal synapses within the enteric nervous system, rather than at neuromuscular junctions. In summary, experiments in a variety of laboratory animals, including the mouse, suggest that somatostatin appears to excite enteric neurons mediating relaxation and to inhibit neurons mediating contraction of the external muscle.

In all species in which it has been examined, the anally projecting inhibitory motor neurons mediating descending relaxation contain both NOS and VIP (Costa et al., 1992; Brookes, 1993; Ward et al., 1994; Sang and Young, 1996; Sang et al., 1997). The current study showed that, within the myenteric plexus of the mouse ileum and colon, (1) *Sstr2* and SSTR2(a) immunoreactivity was expressed predominantly by anally projecting NOS neurons. Previous studies have shown that, as in other laboratory mammals, the NOS neurons in the small and large intestine of mice also contain VIP and are anally projecting interneurons and inhibitory motor neurons (Costa et al., 1992; Sang and Young, 1996; Sang et al., 1997). Likewise, in the rat intestine, many of the SSTR2(a)-immunostained neurons contain VIP (Sternini et al., 1997). (2) Many, but not all NOS neurons have somatostatin nerve terminals in close apposition. Thus, as in the guinea pig ileum (Mann et al., 1997), somatostatin-containing nerve terminals appear to provide inputs to many NOS/VIP neurons. It is likely that somatostatin acting on SSTR2 on NOS/VIP neurons modulates descending relaxation. Only approximately one third of the NOS neurons in the mouse ileum expressed *Sstr2*. NOS neurons in the mouse ileum include anally projecting circular muscle neurons, longitudinal muscle motor neurons, and anally projecting interneurons (Sang and Young, 1996). It is likely that the subpopulation of NOS neurons that expresses *Sstr2* correspond to one of these functional groups.

In addition to exciting the NOS/VIP/ATP neurons mediating relaxation, several functional studies have also found that somatostatin inhibits neurons mediating contraction of the circular muscle (see above). In the mouse ileum, the excitatory motor neurons to the circular muscle can be identified by the presence of calretinin (Sang and Young, 1996, 1998). We found very few calretinin-containing neurons that expressed *Sstr2*. Therefore, it appears that either the inhibitory effects of somatostatin on excitatory neurons are mediated by a SSTR other than SSTR2, or, the effects of somatostatin on excitatory neurotransmission may be mediated by SSTR2, but are indirect. In the mouse colon, there is ongoing release of nitric oxide and other inhibitory neurotransmitters that maintain the circular muscle under tonic inhibition (Spencer et al., 1998). The apparent inhibitory effect of somatostatin on contractile activity may be due to a decrease in the excitability of the circular muscle caused by a somatostatin-induced increase in the ongoing activity of the inhibitory neurons. In the rat small intestine, a recent functional study has shown that the somatostatin-mediated inhibition of migrating motor complexes involves NOS neurons, as inhibition of NOS completely blocked the inhibitory effects of somatostatin on the motor complexes (Abdu et al., 2002). In contrast, in the mouse small intestine, NOS inhibitors had no effect on the somatostatin-mediated inhibition of migrating motor complexes (Abdu et al., 2002). However, in addition to NOS, the inhibitory motor neurons in the mouse and other species also contain VIP and probably ATP, and the contribution of nitric oxide, VIP, and ATP to neuron-mediated relaxation varies between different gastrointestinal regions and between species (Costa et al., 1986; Mashimo et al., 1996). Thus, although the inhibitory effects of somatostatin on the migratory motor complexes in the mouse small intestine are not blocked by NOS inhibition, it is possible they will be affected by inhibition of VIP and/or ATP.

SSTR2 in the external muscle layer of the gastrointestinal tract

In addition to subpopulations of enteric neurons, interstitial cells of Cajal (ICC) in the external muscle of the rat intestine were reported to show SSTR2(a) immunoreactivity (Sternini et al., 1997), although their identity as ICC was not confirmed using antisera to Kit. In the current study, we used Kit immunohistochemistry to identify ICC, and we did not find any Kit+ cells that also showed β-gal staining, indicating that ICC in the mouse intestine do not express *Sstr2*. We also used two different antibodies to SSTR2(a) (antiserum 6291 and antiserum 9431) to localize SSTR2(a)-containing cells; one of the antisera, SSTR2(a) antiserum 9431, was the same as that used by Sternini et al. (1997). The two different antisera showed similar patterns of staining except that SSTR2(a) antiserum 9431 revealed ICC-like cells in the external muscle and in the mucosa that were not observed by using SSTR2(a) antiserum 6291. However, the immunostained ICC-like cells (1) were observed in mice lacking SSTR2(a) ($Sstr2^{lacZ/lacZ}$ mice), (2) did not show β-gal staining in $Sstr2^{+/lacZ}$ mice, and (3) were not observed using SSTR2(a) antiserum 6291. The experiments reported by Sternini et al. (1997) show undoubtedly that SSTR2(a) antiserum 9431 does bind to SSTR2(a), and in the current study, antiserum 9431 showed the same pattern of enteric neuron staining as SSTR2(a) antibody 6291. However, our data suggest that, in the mouse intestine, SSTR2(a) antiserum 9431 also appears to bind to a molecule on ICC-like cells that is not SSTR2(a).

In the current study, sparse *Sstr2*-expressing cells were observed in the circular muscle of the colon; these cells were Kit-negative (and, hence, not ICC). As there are no somatostatin-containing nerve terminals in the muscle layers, the functional relevance of very sparse *Sstr2*-expressing cells in the circular muscle of the colon is unclear.

lacZ knockin approach as a method for localizing *Sstr2* expression

This study has demonstrated that the *lacZ* knockin approach is a sensitive and specific method for localizing *Sstr2* expression within the gut. The sites of $Sstr2^{lacZ}$ expression that were observed are consistent with previ-

ous functional and immunohistochemical studies. In all cells in which SSTR2-specific immunoreactivity was detected, $Sstr2^{lacZ}$ expression was also observed. However, $Sstr2^{lacZ}$ expression was also seen in some cells that did not show SSTR2 immunoreactivity. This finding may, in part, reflect cells that express the SSTR2(b) isoform. However, the intensity of SSTR2(a) immunoreactivity that was observed in myenteric and submucosal neurons of the stomach, ileum, and colon varied from strong to barely detectable. Hence, it is likely that a proportion of cells that express $Sstr2^{lacZ}$, which were readily detected by β-galactosidase histochemistry, also express SSTR2(a) but at levels that were below the threshold of immunohistochemical detection. This likelihood is consistent with previous studies using *lacZ* knockin mice that have revealed novel sites of gene expression that had not previously been detected by in situ hybridisation or immunohistochemistry studies (e.g., Ben-Arie et al., 2000; Allen et al., 2002).

The current study has also demonstrated that immunohistochemistry is not always specific, and one of the SSTR2(a) antisera used in this study appeared to bind to a molecule on ICC-like cells that is not SSTR2(a). However, there are also advantages in using immunohistochemistry rather than the *lacZ* knockin approach for localizing SSTR2. First, it is not possible to determine whether the β-gal staining in $Sstr2^{+/lacZ}$ or $Sstr2^{lacZ/lacZ}$ mice is due to SSTR2(a) or SSTR2(b) or both. Second, β-gal staining is only observed in the cell bodies of cells expressing the $Sstr2^{lacZ}$ allele, and the cellular localization of SSTR2 proteins cannot be ascertained. The current study showed that SSTR2(a) is predominantly localized to the cell bodies, dendrites, and proximal parts of the axons of neurons and not on the nerve terminals.

ACKNOWLEDGMENTS

We thank Professors Nicholas Brecha and Catia Sternini and the UCLA CURE Center Antibody Core (grant DK41301) for generously providing SSTR2(a) antiserum 9431. We also thank Professor John Furness for comments on the article and insightful discussion, and Dr. Gareth Hicks for comments on the article.

LITERATURE CITED

Abdu F, Hicks GA, Hennig G, Allen JP, Grundy D. 2002. Somatostatin sst(2) receptors inhibit peristalsis in the rat and mouse jejunum. Am J Physiol Gastrointest Liver Physiol 282:G624–G633.

Allen JP, Hathway GJ, Clarke NJ, Jowett MI, Topps S, Wilkinson LS, Kendrick KM, Humphrey PPA, Emson PC. 2002. Somatostatin receptor 2 knockout/lacZ knockin mice show impaired motor coordination and reveal novel sites of somatostatin action within the striatum. Submitted.

Beck F, Chawengsaksophak K, Waring P, Playford RJ, Furness JB. 1999. Reprogramming of intestinal differentiation and intercalary regeneration in Cdx2 mutant mice. Proc Natl Acad Sci U S A 96:7318–7323.

Ben-Arie N, Hassan BA, Bermingham NA, Malicki DM, Armstrong D, Matzuk M, Bellen HJ, Zoghbi HY. 2000. Functional conservation of atonal and Math1 in the CNS and PNS. Development 127:1039–1048.

Bernex F, Tiret L. 1997. Aqueous mounting preserves b-galactosidase activity on histological sections. Technical tips online. Available at: http://research.bmn.com/tto 1:T40023.

Brookes SJ. 1993. Neuronal nitric oxide in the gut. J Gastroenterol Hepatol 8:590–603.

Burns AJ, Herbert TM, Ward SM, Sanders KM. 1997. Interstitial cells of Cajal in the guinea-pig gastrointestinal tract as revealed by c-Kit immunohistochemistry. Cell Tissue Res 290:11–20.

Burrell MA, Montuenga LM, Garcia M, Villaro AC. 1996. Detection of nitric oxide synthase (NOS) in somatostatin-producing cells of human and murine stomach and pancreas. J Histochem Cytochem 44:339–346.

Costa M, Furness JB, Smith IJ, Davies B, Oliver J. 1980. An immunohistochemical study of the projections of somatostatin-containing neurons in the guinea-pig intestine. Neuroscience 5:841–852.

Costa M, Furness JB, Humphreys CM. 1986. Apamin distinguishes two types of relaxation mediated by enteric nerves in the guinea-pig gastrointestinal tract. Naunyn Schmiedebergs Arch Pharmacol 332:79–88.

Costa M, Furness JB, Pompolo S, Brookes SJ, Bornstein JC, Bredt DS, Snyder SH. 1992. Projections and chemical coding of neurons with immunoreactivity for nitric oxide synthase in the guinea-pig small intestine. Neurosci Lett 148:121–125.

Ekblad E, Winther C, Ekman R, Hakanson R, Sundler F. 1987. Projections of peptide-containing neurons in rat small intestine. Neuroscience 20: 169–188.

Ekblad E, Ekman R, Hakanson R, Sundler F. 1988. Projections of peptide-containing neurons in rat colon. Neuroscience 27:655–674.

Ekblad E, Mei Q, Sundler F. 2000. Innervation of the gastric mucosa. Microsc Res Tech 48:241–257.

Furness JB, Costa M. 1979. Actions of somatostatin on excitatory and inhibitory nerves in the intestine. Eur J Pharmacol 56:69–74.

Furness JB, Costa M, Franco R, Llewellyn-Smith IJ. 1980. Neuronal peptides in the intestine: distribution and possible functions. In: Costa E, Trabucchi M, Costa E, Trabucchi MCosta E, Trabucchi MS, editors. Neural peptides and neuronal communication. New York: Raven Press. p 601–617.

Furness JB, Keast JR, Pompolo S, Bornstein JC, Costa M, Emson PC, Lawson DE. 1988. Immunohistochemical evidence for the presence of calcium-binding proteins in enteric neurons. Cell Tissue Res 252:79–87.

Furness JB. 2000. Types of neurons in the enteric nervous system. J Auton Nerv Syst 81:87–96.

Grider JR, Arimura A, Makhlouf GM. 1987. Role of somatostatin neurons in intestinal peristalsis: facilitatory interneurons in descending pathways. Am J Physiol 253:G434–G438.

Guillemin R. 1976. Somatostatin inhibits the release of acetylcholine induced electrically in the myenteric plexus. Endocrinology 99:1653–1654.

Hökfelt T, Johansson O, Efendic S, Luft R, Arimura A. 1975. Are there somatostatin-containing nerves in the rat gut? Immunohistochemical evidence for a new type of peripheral nerves. Experientia 31:852–854.

Hoyer D, Bell GI, Berelowitz M, Epelbaum J, Feniuk W, Humphrey PP, O'Carroll AM, Patel YC, Schonbrunn A, Taylor JE. 1995. Classification and nomenclature of somatostatin receptors. Trends Pharmacol Sci 16:86–88.

Katayama Y, North RA. 1980. The action of somatostatin on neurones of the myenteric plexus of the guinea-pig ileum. J Physiol (Lond) 303: 315–323.

Keast JR, Furness JB, Costa M. 1984. Somatostatin in human enteric nerves. Distribution and characterization. Cell Tissue Res 237:299–308.

Krempels K, Hunyady B, O'Carroll AM, Mezey E. 1997. Distribution of somatostatin receptor messenger RNAs in the rat gastrointestinal tract. Gastroenterology 112:1948–1960.

Larsson LI, Goltermann N, de Magistris L, Rehfeld JF, Schwartz TW. 1979. Somatostatin cell processes as pathways for paracrine secretion. Science 205:1393–1395.

Lloyd KC, Wang J, Aurang K, Gronhed P, Coy DH, Walsh JH. 1995. Activation of somatostatin receptor subtype 2 inhibits acid secretion in rats. Am J Physiol 268:G102–G106.

Maeda H, Yamagata A, Nishikawa S, Yoshinaga K, Kobayashi S, Nishi K. 1992. Requirement of c-kit for development of intestinal pacemaker system. Development 116:369–375.

Mann PT, Southwell BR, Young HM, Furness JB. 1997. Appositions made by axons of descending interneurons in the guinea-pig small intestine, investigated by confocal microscopy. J Chem Neuroanat 12:151–164.

Martinez V, Curi AP, Torkian B, Schaeffer JM, Wilkinson HA, Walsh JH, Tache Y. 1998. High basal gastric acid secretion in somatostatin receptor subtype 2 knockout mice. Gastroenterology 114:1125–1132.

Mashimo H, He XD, Huang PL, Fishman MC, Goyal RK. 1996. Neuronal constitutive nitric oxide synthase is involved in murine enteric inhibitory neurotransmission. J Clin Invest 98:8–13.

Norris PJ, Charles IG, Scorer CA, Emson PC. 1995. Studies on the localization and expression of nitric oxide synthase using histochemical techniques. Histochem J 27:745–756.

Park J, Chiba T, Yamada T. 1987. Mechanisms for direct inhibition of canine gastric parietal cells by somatostatin. J Biol Chem 262:14190–14196.

Prinz C, Kajimura M, Scott DR, Mercier F, Helander HF, Sachs G. 1993. Histamine secretion from rat enterochromaffin-like cells. Gastroenterology 105:449–461.

Prinz C, Sachs G, Walsh JH, Coy DH, Wu SV. 1994. The somatostatin receptor subtype on rat enterochromaffin-like cells. Gastroenterology 107:1067–1074.

Rombout JH, van der Grinten CP, Binkhorst FM, Taverne-Thiele JJ, Schooneveld H. 1986. Immunocytochemical identification and localization of peptide hormones in the gastro-entero-pancreatic (GEP) endocrine system of the mouse and a stomachless fish, *Barbus conchonius*. Histochemistry 84:471–483.

Sang Q, Young HM. 1996. Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. Cell Tissue Res 284:39–53.

Sang Q, Young HM. 1998. The identification and chemical coding of cholinergic neurons in the small and large intestine of the mouse. Anat Rec 251:185–199.

Sang Q, Williamson S, Young HM. 1997. Projections of chemically identified myenteric neurons of the small and large intestine of the mouse. J Anat 190:209–222.

Schindler M, Humphrey PP. 1999. Differential distribution of somatostatin sst2 receptor splice variants in rat gastric mucosa. Cell Tissue Res 297:163–168.

Schindler M, Kidd EJ, Carruthers AM, Wyatt MA, Jarvie EM, Sellers LA, Feniuk W, Humphrey PP. 1998. Molecular cloning and functional characterization of a rat somatostatin sst2(b) receptor splice variant. Br J Pharmacol 125:209–217.

Schubert ML, Hightower J, Makhlouf GM. 1989. Linkage between somatostatin and acid secretion: evidence from use of pertussis toxin. Am J Physiol 256:G418–G422.

Schultzberg M, Hökfelt T, Olson L, Alund M, Nilsson G, Terenius L, Elde R, Goldstein M, Said S. 1980. Substance P, VIP, enkephalin and somatostatin immunoreactive neurons in intestinal tissue transplanted to the anterior eye chamber. J Auton Nerv Syst 1:291–303.

Schulz S, Schreff M, Schmidt H, Handel M, Przewlocki R, Hollt V. 1998. Immunocytochemical localization of somatostatin receptor sst2A in the rat spinal cord and dorsal root ganglia. Eur J Neurosci 10:3700–3708.

Spencer NJ, Bywater RA, Taylor GS. 1998. Disinhibition during myoelectric complexes in the mouse colon. J Auton Nerv Syst 71:37–47.

Sternini C, Wong H, Wu SV, de Giorgio R, Yang M, Reeve J Jr, Brecha NC, Walsh JH. 1997. Somatostatin 2A receptor is expressed by enteric neurons, and by interstitial cells of Cajal and enterochromaffin-like cells of the gastrointestinal tract. J Comp Neurol 386:396–408.

Takayama I, Horiguchi K, Daigo Y, Mine T, Fujino MA, Ohno S. 2002. The interstitial cells of Cajal and a gastroenteric pacemaker system. Arch Histol Cytol 65:1–26.

Track NS, Creutzfeldt C, Litzenberger J, Neuhoff C, Arnold R, Creutzfeldt W. 1979. Appearance of gastrin and somatostatin in the human fetal stomach, duodenum and pancreas. Digestion 19:292–306.

Vanetti M, Kouba M, Wang X, Vogt G, Hollt V. 1992. Cloning and expression of a novel mouse somatostatin receptor (SSTR2B). FEBS Lett 311:290–294.

Ward SM, Xue C, Sanders KM. 1994. Localization of nitric oxide synthase in canine ileocolonic and pyloric sphincters. Cell Tissue Res 275:513–527.

Young HM, Torihashi S, Ciampoli D, Sanders KM. 1998. Identification of neurons that express stem cell factor in the mouse small intestine. Gastroenterology 115:898–908.