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Serotonin receptor diversity in the human colon: Expression of serotonin type 3 receptor subunits 5-HT3C, 5-HT3D, and 5-HT3E

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Abstract

Since the first description of 5-HT_3 receptors more than 50 years ago, there has been speculation about the molecular basis of their receptor heterogeneity. We have cloned the genes encoding novel 5-HT3 subunits 5-HT3C, 5-HT3D, and 5-HT3E and have shown that these subunits are able to form functional heteromeric receptors when coexpressed with the 5-HT3A subunit. However, whether these subunits are actually expressed in human tissue remained to be confirmed. In the current study, we performed immunocytochemistry to locate the 5-HT3A as well as the 5-HT3C, 5-HT3D, and 5-HT3E subunits within the human colon. Western blot analysis was used to confirm subunit expression, and RT-PCR was employed to detect transcripts encoding 5-HT_3 receptor subunits in microdissected tissue samples. This investigation revealed, for the first time, that 5-HT3C, 5-HT3D, and 5-HT3E subunits are coexpressed with 5-HT3A in cell bodies of myenteric neurons. Furthermore, 5-HT3A and 5-HT3D were found to be expressed in submucosal plexus of the human large intestine. These data provide a strong basis for future studies of the roles that specific 5-HT_3 receptor subtypes play in the function of the enteric and central nervous systems and the contribution that specific 5-HT_3 receptors make to the pathophysiology of gastrointestinal disorders such as irritable bowel syndrome and dyspepsia.

Keywords

5-HT receptor; 5-HT_3; ligand-gated ion channel; antibody; gastrointestinal tract

Serotonin (5-hydroxytryptamine; 5-HT)-mediated signaling is important in bidirectional brain–gut interactions involved in cognition, emotions, and digestive function (Gershon and Tack, 2007). Most of the body's 5-HT (~95%) is produced and stored in enterochromaffin cells (EC) in the enteric mucosal epithelium; however, smaller stores of 5-HT are also present in the bowel in serotonergic neurons of the enteric nervous system (ENS), where 5-HT participates in both slow and fast neurotransmission (Gershon, 2004, 2005; Gershon and Tack, 2007). After its release from EC cells or neurons, 5-HT stimulates the 5-HT_1,[1] 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 subtypes of receptor, all of which are expressed in the gut. Animal studies have indicated that 5-HT activates submucosal intrinsic primary afferent neurons (IPANs) through 5-HT_{1P} receptors to initiate peristaltic reflexes (Pan and Gershon, 2000; Wade et al., 1994) and myenteric IPANs through 5-HT_3 receptors (Bertrand et al., 1997) to initiate giant migrating contractions (Karaus and Sarna, 1987; Nagakura et al., 2002; Sarna, 2007). 5-HT also stimulates 5-HT_4 receptors to increase secretion of acetylcholine and calcitonin gene-related peptide (CGRP; Grider, 2003). This 5-HT_4

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[1]5-HT3 subunits and 5-HT_3 receptors are termed according to the Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR; Peters et al., 2010).

receptor stimulation facilitates neurotransmission in prokinetic pathways (Liu et al., 2005; Pan and Galligan, 1994; Ren et al., 2008) and probably underlies the successful use of 5-HT_4 agonists to treat constipation (Camilleri et al., 2008; Muller-Lissner et al., 2001). More recently, in mice, the 5-HT_4 receptor has been shown to be neuroprotective and also to stimulate stem cells to give rise to new enteric neurons, even in adult animals (Liu et al., 2009). Finally, 5-HT activates the enteric processes of extrinsic sensory neurons in vagal and dorsal root ganglia to transmit nociceptive information to the central nervous system (CNS; Grundy and Schemann, 2006). Mucosal 5-HT is inactivated by serotonin transporter (SERT)-mediated uptake into enterocytes after it stimulates its receptors on IPANs and extrinsic sensory neurons (Gershon, 2003).

With the exception of 5-HT_3, which is a cys-loop family ligand-gated ion channel, 5-HT receptors are all G-protein coupled. 5-HT_3 receptors are oligomeric complexes composed of five subunits. Since the first description of 5-HT_3 receptors within the gut, the molecular basis of 5-HT_3 receptor diversity has been a subject of speculation (Fletcher and Barnes, 1998; Gaddum and Picarelli, 1957). Until 1999, only two 5-HT_3 receptor subunit genes, *HTR3A* and *HTR3B*, had been identified (Belelli et al., 1995; Davies et al., 1999; Miyake et al., 1995). To determine whether additional subunit genes exist, we explored the human genome and isolated three novel *HTR3* homologous genes: *HTR3C*, *HTR3D*, and *HTR3E*. Sequence analysis of the novel 5-HT3C, 5-HT3D, and 5-HT3E subunits showed that they share features common to all 5-HT3 subunits (Niesler et al., 2003). Comparative expression analysis by RT-PCR revealed that transcripts encoding *HTR3* genes are expressed almost ubiquitously; however, expression of *HTR3E* is restricted to the colon, intestine, and stomach (Karnovsky et al., 2003; Niesler et al., 2003). Subsequent molecular and functional characterization indicated that none of the novel subunits can form functional 5-HT_3 receptors on its own, but, upon coexpression with the 5-HT3A subunit, the subunits give rise to functional receptors that differ in maximal responses to 5-HT (Holbrook et al., 2009; Niesler et al., 2007). These data support the hypothesis that the novel 5-HT3 subunits combine with 5-HT3A subunits to modulate receptor function. Receptor subtypes comprising different combinations of subunits may thus differ in their sensitivity to 5-HT_3 agonists and antagonists; consequently, responses to gastrointestinal (GI) drugs that act on 5-HT_3 receptors could be dependent on the subunit composition of these receptors, which in turn might vary under different physiological circumstances. For example, knockout of SERT has been shown to alter the subunit composition of 5-HT_3 receptors in the murine enteric nervous system (ENS), with accompanying alterations in receptor sensitivity and susceptibility to desensitization (Liu et al., 2002).

Activation of 5-HT_3 receptors in both the central and the peripheral nervous systems has been implicated in a number of gastrointestinal diseases, including anorexia, bulimia, and irritable bowel syndrome (Barnes et al., 2009; Graeff, 1997; Thompson and Lummis, 2007). 5-HT_3 receptors play roles in the control of gastrointestinal motility, secretion, and sensation (Gershon and Tack, 2007). 5-HT_3 antagonists, moreover, are beneficial in the treatment of diarrhea-predominant irritable bowel syndrome (IBS-D; Gershon and Tack, 2007; Jones and Blackburn, 2002; Thompson and Lummis, 2007). Because of these observations, sequence variants of *HTR3* genes have been suspected to be involved in the etiology of functional gastrointestinal disorders (Humphrey et al., 1999; Jones and Blackburn, 2002). The likely importance of 5-HT_3 receptors in the pathophysiology of functional gastrointestinal disorders is highlighted by our recent finding that the *HTR3A* variant c.–42C>T and the *HTR3E* variant c.*76G>A are associated with IBS-D. Both of these variants lead to a significant up-regulation of the expression of receptor protein in vitro, which could make affected individuals more susceptible to IBS-D (Kapeller et al., 2008).

Most studies on 5-HT receptor expression in the past have been carried out with animals, and relatively limited data have been obtained about 5-HT_3 expression in the human GI tract (Gershon and Tack, 2007). Because prior studies of 5-HT_3 receptor expression have focused on the nervous system of rodents (Chameau and van Hooft, 2006), knowledge of 5-HT_3 receptor distribution in the human nervous system is poor. The genes *HTR3C*, *HTR3D*, and *HTR3E*, furthermore, seem to be absent from the rodent genome (Karnovsky et al., 2003). The distribution of 5-HT3 receptor subunits in human tissue must thus be determined to allow studies of the roles played by these receptors in the control of gut function and brain–gut cross-talk (Fletcher and Barnes, 1998; Hussy et al., 1994; Jackson and Yakel, 1995). Because the particular combination of subunits in specific 5-HT_3 receptors is likely to affect their function, it is necessary to identify the different combinations that are present in situ before function can be understood or even effectively investigated. The current study was thus designed to determine the locations and subunit combinations of 5-HT_3 receptors within the human GI system.

MATERIALS AND METHODS

Preparation of tissue sections

Six unaffected, normal colon tissue samples from four female and one male patient (55–78 years old; three colonic cancer patients and two patients with diverticulitis) were used for cryosections. Written consent was obtained from all individuals, and the study was approved by the ethical board of the University of Heidelberg. Frozen colon mucosa samples were cut into 8-μm-thick sections using a cryostat (Leica CM1850; Leica Microsystems, Nussloch, Germany) and, after drying at room temperature, stored at −80°C until immunolabeling experiments.

Antibody characterization

Detailed information on immunogens, dilutions, and manufacturers for all primary antibodies used in this study can be found in Table 1. Antibodies against 5-HT3 subunit-specific epitopes using KLH-coupled peptides were raised in rabbit by Pineda Antikörper Service (Berlin, Germany; for 5-HT3C, -D, and -E) and Eurogentec (Cologne, Germany; for 5-HT3A). The resulting 5-HT3C-, 5-HT3D-, and 5-HT3E-specific antibodies were confirmed to react with the individual subunits expressed in a variety of cell lines. For this purpose, HEK293, COS7, and U2OS cells were transfected by cDNA constructs expressing the respective subunits and analyzed by immunostaining (data not shown). In the present study, we confirmed the specificity of the anti-5-HT3C, anti-5-HT3D, and anti-5-HT3DE antibodies in Western blot experiments by detecting bands of the expected sizes using protein lysates of HEK293 cells transfected with the respective subunit expression construct (see Fig. 5). The specific signal was abolished after preadsorption with 100 μg of the immunogen (see Fig. 5). For the investigation of endogenously expressed subunits, we used the epithelial gastrointestinal cell lines Caco2 and Colo320 (data not shown). Immunocytochemical analyses in the present study confirmed the specificity of all 5-HT3 antibodies in GI tissue sections; all produced distinct patterns of immunofluorescence at expected sites. Preincubation with the peptide immunogen abolished the staining in immunofluorescence experiments (Supp. Info. Fig. 1).

Specificity of the anti-5-HT3A, anti-5-HT3C, and anti-5-HT3DE antibodies was previously shown in immunoprecipitation experiments using lysates of HEK293 cells expressing the respective 5-HT3 subunits. Probing of the immunoprecipitated proteins with the antibodies resulted in immunoreactive bands with the expected sizes of ~55 kDa for 5-HT3A, 5-HT3C, and 5-HT3Ea as well as ~35 kDa for 5-HT3D (Walstab et al., 2010a).

Because the immunogen for the anti-5-HT3A antibody shows sequence similarity to 5-HT3B, we performed immunofluorescence experiments on COS7 cells transfected with N-terminally tagged Myc-5-HT3A or Myc-5-HT3B expression constructs. This was in principle carried out as described by Walstab et al. (2010a); primary and secondary antibodies are listed in Tables 1 and 2. Immunoreactivity of the anti-5-HT3A antibody was detectable only in cells expressing the Myc-5-HT3A subunit. No signal was present in cells expressing the Myc-5-HT3B subunit, which shows that there is no cross-reactivity to the 5-HT3B subunit (Supp. Info. Fig. 2). In addition, the staining of the anti-5-HT3A antibody was not abolished after preadsorption with 100 μg of a 5-HT3B-specific peptide (KEVRPVYNWTKATTV), which represents the homologous region compared with the 5-HT3A immunogen (KGVRPVRDWRKPTTV; Supp. Info. Fig. 2).

The specificity of the commonly used anti-PGP9.5 and the antimast cell tryptase antibody has been shown in a variety of studies as summarized by Lennerz et al. (2008). In particular, the anti-PGP9.5 antibody showed one band at 26–28 kDa on Western blot (Krimm et al., 2006). Imunofluorescence experiments using the anti-PGP9.5 antibody resulted in a distribution and a morphology of labeled neuronal structures similar to those reported in previous studies (Sams et al., 1992; Simpson et al., 2009).

The staining pattern of biotin-conjugated HuC/D monoclonal antibody is identical to that of the unconjugated antibody. The anti-HuC/D-biotinylated antibody labeled neuronal cell nuclei and perikarya (Marusich et al., 1994). The staining pattern of cellular morphology and distribution is the same as previously described (Hoff et al., 2008; Lin et al., 2002; Michel et al., 2005; Murphy et al., 2007).

According to the manufacturer, the anti-Na^+-K^+-ATPase antibody recognizes a single ~100-kDa band corresponding to the estimated size of the protein on immunoblots (Abcam, Cambridge, United Kingdom). The antivillin antibody has been previously used as a brush border marker in immunostainings of epithelial cells LS174T (Lee et al., 2007). In the present study, both antibodies produced typical staining of the gut mucosa consistent with previous reports (Gill et al., 2008).

The anti-Myc antibody is a well-characterized, routinely used antibody that detects recombinant proteins containing the Myc epitope tag according to the manufacturer (Cell Signaling, Beverly, MA). In the present study, this antibody specifically stained COS7 cells that overexpressed Myc-tagged 5-HT3 subunits, and no signal was detectable in cells transfected with the empty pcDNA3 expression vector (Supp. Info. Fig. 2).

Antibody labeling

Antibodies were purified from rabbit anti-5-HT3A serum by using an immobilized protein A column according to the indications of the manufacturer (Pierce, Rockford, IL). Immunopurified antibodies directed against 5-HT3C, 5-HT3D, and 5-HT3E were subsequently concentrated by ultrafiltration using a Microcon 100 centrifugal concentrator (100-kDa molecular weight cutoff; Millipore, Schwalbach, Germany), dialyzed against 1,000 volumes of 10 mM sodium phosphate (pH 7.6), and treated with an IgG purification resin that efficiently binds non-IgG serum proteins (Melon gel; Pierce) according to the indications of the manufacturer to increase antibody concentration and remove salt and amino-containing buffer components and to eliminate bovine serum albumin (BSA), respectively. The antibody concentration of the solutions obtained ranged from 0.2 to 1 mg protein/ml.

For fluorophore conjugation, 10 μl of 1 M $NaHCO_3$ (pH 8.3) was added to 90 μl antibodies in 10 mM sodium phosphate (pH 7.6); 1 μl of a 1% (w/v) solution of fluorophore in

dimethylformamide was added, corresponding to a 5- to 10-fold molar excess. After incubation for either 2 hours at room temperature or overnight at 4°C, the labeled antibodies were separated from the free fluorophore by gel filtration chromatography on a NAP-25 column (GE Healthcare, Freiburg, Germany) equilibrated and eluted with phosphatebuffered saline (Sigma, Cologne, Germany). For storage, BSA and NaN_3 were added to 0.1 and 0.02% (w/v), respectively.

The fluorophores used were N-hydroxysuccinimide-activated: fluorescein (Fluka; Neu-Ulm, Germany; for 5-HT3C, 5-HT3D, and 5-HT3E), 5(6)-carboxytetramethylrhodamine (Sigma; for 5-HT3A), and Cy3B (GE Healthcare; for 5-HT3C, 5-HT3D, and 5-HT3E), except for fluorescein isothiocyanate (Sigma) used to label 5-HT3A. Labeling of both heavy and light chains of the antibodies was demonstrated after SDS-PAGE by visual inspection under white light or UV illumination.

Immunofluorescence analysis

Immunolabeling of 5-HT3A, 5-HT3C, 5-HT3D, and 5-HT3E subunits in human colon tissue sections was performed as follows: tissue sections were fixed by incubation in 4% paraformaldehyde for 20 minutes. Afterward, they were washed three times for 10 minutes each in 1× PBS at room temperature. Then slides were blocked in 4% goat serum/0.25% Triton X-100/PBS. The fluorochrome-labeled primary antibodies rabbit anti-5-HT3A, 5-HT3C, 5-HT3D, and 5-HT3E or anti-5-HT3DE (Table 1) were diluted 1:100 in blocking solution and applied overnight at room temperature. Tissue sections were washed for 3 × 10 minutes in 1× PBS at room temperature. In case of the marker detection anti-PGP9.5 (ubiquitin-C-terminal hydrolase 1; Chemicon, Nuremberg, Germany), anti-HuC/D-biotinylated (Molecular Probes, Invitrogen, Karlsruhe, Germany) anti-villin, and anti-Na^+-K^+-ATPase (the latter two from Abcam; Table 1), the sections were incubated in blocking solution containing the respective secondary AlexaFluor 488 or 546-labeled goat anti-rabbit, anti-guinea pig, or anti-mouse antibodies (Invitrogen; Table 2) or, in case of the biotinylated antibodies, AlexaFluor 594-conjugated streptavidin (Invitrogen) in a 1:100 dilution for 3 hours. From now on, every step was carried out light protected. After washing three times for 5 minutes each in 1× PBS, a nuclear counterstain with 4′, 6-diamidino-2-phenylindol (DAPI; 1:10,000 in 1× PBS) was carried out, followed by two washes in 1× PBS. Sections were mounted in Vectashield (Vector, Peterborough, United Kingdom) and stored at 4°C until microscopic investigation. Photomicrographs were produced using a Nikon 90i microscope equipped with a Nikon DS-1QM black-and-white CCD camera using the NIS-Elements Ar 3.1 software. Images were processed in ImageJ (http://rsbweb.nih.gov/ij/) to adjust size, brightness, and contrast.

Laser microdissection and pressure catapulting

Fresh frozen colon mucosa samples of one individual were cut into 18-µm-thick sections using a cryostat (Leica CM1850; Leica Microsystems) and processed as follows: the sections were mounted on membrane slides (PEN-membrane, 1 mm glass; Carl Zeiss MicroImaging GmbH, Jena, Germany) and incubated for 10 minutes at −20°C in RNAlater-ICE (Ambion, Darmstadt, Germany). For further preservation, samples were fixed in ethanol and stained in cresyl violet acetate [1% (w/v) in ACS-grade ethanol; Sigma] for 15 seconds. Subsequently, the slides were washed in ethanol and incubated for 5 minutes in xylene. After air drying, the slides were mounted on the stage of an inverse microscope, which is a component of a Microbeam LMPC System (Carl Zeiss MicroImaging GmbH). We employed the RoboLPC method to microdissect and capture the appropriate tissue fragments (~10 mm^2 epithelium or lamina propria cells, ~100,000–250,000; cells ~15 mm^2 MP or muscular cell layer, ~100,000–250,000 cells). For sample collection, we applied 0.5 ml AdhesiveCaps opaque (Carl Zeiss MicroImaging GmbH).

RNA isolation and RT-PCR of microdissected samples

Total RNA was isolated from each sample using the guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) according to the manufacturer's instructions (peqGOLD TriFast kit; PeqLab Biotechnologie GmbH, Erlangen, Germany). For precipitation, 2 µl of Pellet Paint Co-Precipitant (Novagen) per sample was used. After DNase treatment (DNA-free; Ambion), 200 ng of RNA was reverse transcribed by using the Accuscript High Fidelity 1st strand cDNA synthesis kit (Stratagene, La Jolla, CA). PCR was performed with HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Primer sequences are given in Table 3.

Western blot analysis of a human colon sample

A colon sample from one individual, 5-HT3-transfected HEK293 cells (positive controls), and untransfected cells (negative control) were each homogenized in a previously described lysis buffer (Boyd et al., 2002) using a Polytron PT1300D homogenizer (Kinematica AG, Luzerne, Switzerland), followed by three rounds of short (~5 seconds) ultrasonic treatment. Protein concentrations were measured by standard BCA assay. Lysates (30 µg protein for the colon sample, 10 µg protein for HEK293 cells) were separated on a 4–12% Bis-Tris NuPAGE gel (Invitrogen) and blotted onto PVDF membranes, followed by blocking with the Odyssey Blocking Buffer (Licor, Bad Homburg, Germany) as recommended by the manufacturer. The blot was then probed with anti-5-HT3C, -D, and -E antibodies (1:200 in blocking buffer with 0.2% Tween 20 at 4°C overnight). After four rounds of 5 minutes washing with 0.1% Tween 20/PBS at room temperature, the blot was incubated with the anti-rabbit IRDye 800CW secondary antibody (1:15,000 in blocking buffer with 0.2% Tween 20 and 0.01% SDS) for 1 hour at room temperature, followed by the same washing procedure. Bands were visualized using the Odyssey system as recommended by the manufacturer (Licor).

RESULTS

The current investigation was designed to provide insight into GI 5-HT_3 receptor diversity through an analysis of the expression of the novel 5-HT3 receptor subunits 5-HT3C, 5-HT3D, and 5-HT3E in the human gut.

Immunolocalization of 5-HT3A, 5-HT3C, 5-HT3D, and 5-HT3E subunits in sections of human colon

To determine whether the 5-HT3C, 5-HT3D, and 5-HT3E subunits are actually translated and to learn the composition of native 5-HT_3 receptors in vivo, epitope-specific antibodies to 5-HT3C, 5-HT3D, and 5-HT3E (Table 1) were raised in rabbits and characterized in detail as described in Materials and Methods. Sections of human colon from six individuals were doubly immunostained with tetramethylrhodamine (TMR)-labeled antibodies to the 5-HT3A subunit in conjunction with fluorescein-labeled antibodies to the 5-HT3C, 5-HT3D, or 5-HT3E subunits. The immunoreactivities of the 5-HT3C, 5-HT3D, and 5-HT3E subunits were coincident with the immunoreactivity of the 5-HT3A subunit in cell bodies of myenteric neurons (Fig. 1). Submucosal neurons also displayed coincident 5-HT3A and 5-HT3D immunoreactivity (Fig. 2). In addition, 5-HT3A, 5-HT3C, 5-HT3D, and 5-HT3E staining was observed in enterocytes of the colonic epithelium (Supp. Info. Fig. 3) and in immune cells such as macrophages as well as mast cells of the lamina propria (Supp. Info. Fig. 3; confirmed by tryptase coimmunostaining; not shown). The immunoreactivity of lymphocytes was not detected throughout every section. The specificity of the immunocytochemical detection of 5-HT3 subunits was supported by using RT-PCR to demonstrate the presence of corresponding transcripts in microdissected tissue.

RT-PCR confirmation of *HTR3A*, *HTR3C*, *HTR3D*, and *HTR3E* expression in microdissected layers of the human colon

The colonic mucosal epithelium and the muscularis externa were isolated by microdissection. The separated layers were analyzed by RT-PCR, verifying that transcripts encoded by the *HTR3A*, *HTR3C*, *HTR3D*, and *HTR3E* genes are transcribed in the epithelium and the lamina propria. The transcription in the lamina propria correlates with the detected staining in immunofluorescence experiments in immune cells such as macrophages and mast cells. For all *HTR3* genes, presence of messenger RNA was confirmed in myenteric plexus (MP); however, none but weak expression of *HTR3D* was detectable in the muscular layer (Fig. 3). The identity of microdissected tissue subregions was confirmed by RT-PCR analysis using specific primers for the respective markers. In particular, the identity of the epithelium was provedn by amplification of cytokeratin 20 (*CK20*) and of the lamina propria by amplification of the protein tyrosine phosphatase, receptor type C (*CD45*). Within the muscular layer, the MP was confirmed by *ELAVL13* (HuC/D) as a marker for nerve cells and actin gamma 2 (*ACTG2*) as a marker for muscular cells in the GI tract. The genes *HTR3C* and *HTR3E* turned out to be alternatively spliced in the epithelium of the colon and *HTR3D* in the myenteric plexus, indicated by additional bands (Fig. 3).

Coexpression analysis of HuC/D and PGP9.5 by immunofluorescence studies

To clarify the nature of the 5-HT3-positive cells, immunolabeling was performed with the particular 5-HT3-specific antibodies and the neuronal marker-specific antibodies recognizing HuC/D (Fig. 4) and PGP9.5 (data not shown). In agreement with the RT-PCR data, 5-HT3A, 5-HT3C, 5-HT3D, and 5-HT3E were shown to be expressed in HuC/D-positive cell somata of the myenteric plexus, confirming neuronal localization (Fig. 4). A nuclear staining of a few nonneuronal cells within the myenteric plexus is visible, most prominently for 5-HT3C and 5-HT3D.

Colon colocalization Na^+-K^+-ATPase and villin immunofluorescence analysis

To specify further the localization of 5-HT3A, 5-HT3C, 5-HT3D, and 5-HT3E expression in enterocytes of the human colon epithelium (red staining, Supp. Info. Fig. 4), expression of Na^+-K^+-ATPase or villin was assessed as well (green staining, Supp. Info. Fig. 4). Na^+-K^+-ATPase was used as a marker for the basolateral sites, whereas villin indicated the apical membrane (Supp. Info. Fig. 4). It turned out that all analyzed 5-HT3 subunits were expressed basolaterally and that only 5-HT3C and 5-HT3D subunits were in addition found apically at the surface of enterocytes (Supp. Info. Fig. 4). In the case of 5-HT3A subunit, a prominent staining was detected at the basis of the crypts within the mucosal cell layer.

Western blot analysis of colon tissue lysates

A human colon cell lysate was separated on a 4–12% Bis-Tris NuPAGE gel, blotted onto PVDF membranes, and probed with anti-5-HT3C, anti-5-HT3D, and anti-5-HT3E antibodies (Table 1). After incubation overnight, the secondary anti-rabbit-IRDye antibody 800CW (Table 2) was used, and bands were visualized with the Odyssey system as recommended by the manufacturer. Immunoreactive bands of the expected size of ~25 kDa for 5-HT3D and ~40–45 kDa for 5-HT3E, but 5-HT3C was not detectable in GI tissue lysate (Fig. 5). Specifity of all antibodies was confirmed by loading protein lysates of HEK293 cells transfected with the respective 5-HT3 expression constructs and untransfected cells as negative control. The staining for the anti-5-HT3C, anti-5-HT3D, and anti-5-HT3E antibodies was abolished after preincubation with 100 μg of the immunizing peptide (Fig. 5).

DISCUSSION

Despite 50 years of research, the molecular basis of 5-HT_3 receptor diversity is far from clear. Furthermore, the existence of the 5-HT3C, 5-HT3D, and 5-HT3E subunits in vivo was questioned and their role and function called into doubt. Previous RT-PCR studies revealed distinct expression of the 5-HT3D and 5-HT3E subunits in gastrointestinal tissue, pointing to a specific role in the bowel (Karnovsky et al., 2003; Niesler et al., 2003). Because of this restricted expression pattern, we investigated the molecular makeup of 5-HT_3 receptors within the human gut to gain insight into the basis of 5-HT_3 receptor diversity and the expression of the 5-HT3 subunits.

5-HT_3 receptor binding sites have been shown to be expressed in the myenteric plexus (MP) in humans (Sakurai-Yamashita et al., 2000). The present study showed expression of all tested 5-HT3 subunits in the MP. The expression in submucosal plexus (SMPs) was restricted to 5-HT3A and 5-HT3D. For the 5-HT3A subunit, this is in accordance with previous data (Bottner et al., 2009; Michel et al., 2005). In addition, animal studies in the rat and guinea pig have already described expression of 5-HT3A within the MP and the SMP (Johnson and Heinemann, 1995). Our data are also consistent with data from electrophysiological studies demonstrating the presence of 5-HT_3 receptors in submucosal and myenteric ganglia in guinea pig that are involved in secretion processes and gut motility (Galligan, 1996; Mawe et al., 1986; Tack et al., 1992; Wade et al., 1994). The role of 5-HT_3 receptors in the regulation of peristalsis of the gut is underscored by the fact that 5-HT_3 receptor antagonists alter GI motility (Gershon and Tack, 2007).

For the first time, we found expression in enterocytes of the epithelium of the mucosa. Not only are 5-HT3A and 5-HT3E expressed in the epithelium (Kapeller et al., 2008) but also 5-HT3C and 5-HT3D. This finding suggests that native 5-HT_3 receptors in colonic enterocytes may be composed of 5-HT3A, 5-HT3C, 5-HT3D, and 5-HT3E subunits. Enterocytic 5-HT_3 receptor expression suggests an involvement in mucosal secretion and absorption. Although 5-HT_3 receptors have already been shown to regulate secretion in animal studies (Day et al., 2005), other studies have not been able to confirm this in humans (Engelmann et al., 2006; Michel et al., 2005). Nevertheless, the 5-HT_3 receptor antagonist alosetron has been shown to inhibit basal secretion in the healthy human jejunum (Bearcroft et al., 1997). Further studies addressing the role of 5-HT_3 receptors in secretional processes of the human GI tract are therefore inevitable.

Its exclusive expression in the GI tract made *HTR3E* an excellent candidate for the study of GI diseases such as IBS. Recently, we found a functional variant within the 3′ untranslated region of the *HTR3E* gene to be associated with diarrhea-predominant IBS in female patients. Functional analysis of this variant revealed that it may lead to up-regulation of the 5-HT3E subunit on the protein level (Kapeller et al., 2008). This up-regulation might in turn have consequences for receptor composition and density within the GI tract. After these findings, subsequent investigation using the novel 5-HT3-specific antibodies of colon biopsy material from patients suffering from GI disorders will be performed to elucidate the molecular consequences of the associated variants. This may help to illuminate the functional differences and stoichiometry of the resulting 5-HT_3 receptors. The in vivo data in combination with screening data of the *HTR3* genotypes of patients with GI disorders may clarify the role of 5-HT_3 receptors in the GI tract as well as the disease-related pathomechanism. The outcome may have direct consequences on the diagnosis and therapy of GI disorders (Walstab et al., 2010b).

Furthermore, in some of the tissue sections, immunoreactivity of 5-HT3 subunits was detectable in immune cells in the lamina propria, which may reflect inflammation status of

the tissue. This is the first time that 5-HT_3 receptor expression has been described for immune cells within the gut, pointing to a putative role in immunological processes that has recently been described (Fiebich et al., 2004). In addition, we found nuclear staining of a few nonneuronal cells within the myenteric plexus, in particular for 5-HT3C and 5-HT3D. The nature of these cells and the relevance with regard to the 5-HT_3 receptor system must be addressed in future studies.

The subregional expression of the 5-HT3 subunits was complemented and confirmed by the RT-PCR data from microdissected tissue material. The existence of alternative splice variants in distinct cell populations adds to the complexity of the 5-HT_3 receptor system. In addition, Western blot analysis confirmed expression of the 5-HT3D and the 5-HT3E subunits with sizes of 25 kDa and 40–50 kDa, respectively, in a human colon sample. For the 5-HT3D subunit, this is in line with the expected size as detected in the positive control sample. In contrast, for the 5-HT3E subunit, a smaller molecular weight of 40–50 kDa was detected in the colon sample compared with the positive control. This points to a smaller 5-HT3E colon-specific isoform, which presumably is encoded by the previously described exon 2-deleted splice variant (Holbrook et al., 2009). In contrast to the immunostaining results, no specific 5-HT3C subunit band was detectable in the colon sample, which may be due to a low expression level, because the specifity of the anti-5-HT3C antibody was clearly demonstrated by cross-reactivity to the positive control and the abolished signal after preadsorption of the antibody.

In conclusion, we have performed the first correlated 5-HT3 subunit localization study on transcript and protein expression level within the human gut. We were able to show coexpression of 5-HT3 subunits in the myenteric and submucosal plexus in somata of neuronal cells (Fig. 6). Furthermore, we describe the existence of 5-HT3 subunits in enterocytes within the mucosal layer and in immune cells within the lamina propria (Fig. 6). Our work also included the generation of well-characterized antibodies that represent the basis for future analyses of additional GI tissues such as stomach, duodenum, jejunum, and ileum and tissues from the CNS. Taking into account the fluorescence signal intensity, one could assume equal expression levels of the subunits. The establishment of differentially fluorophore-labeled antibodies will allow an even more detailed look concerning the coexpression of all subunits within these cells in future. Until now, most of the functional data concerning human 5-HT_3 receptors has been based on in vitro studies. In these studies, transfected cell lines expressing 5-HT3A and one of the other subunits have been investigated so far. Other combinations of 5-HT3 subunits have not been analyzed because the molecular makeup of native receptor complexes is unknown and a multitude of possible stoichiometries exist. Therefore, it cannot be excluded that functional receptors lacking the 5-HT3A subunit exist in vivo. This has to be addressed in future studies. To obtain an idea of the functional role of 5-HT_3 receptors in different cell types, the chemical coding of positive cell populations has to be addressed. In addition, electrophysiological and neuroimaging analyses will complete the picture of what the specific role of different receptor subtypes composed of individual subunits might be. Future studies will thus help to elucidate functional and regulatory processes in the nature of the stained cells in the ENS, in particular the brain–gut axis- and 5-HT-mediated neurotransmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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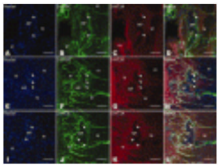

Figure 1.

Immunocytochemical analysis of the immunoreactivity of 5-HT3 subunits within the myenteric plexus of the human colon. The immunoreactivities of the 5-HT3C (**A–D**), 5-HT3D (**E–H**), and 5-HT3E (**I–L**) subunits (green fluorescence, fluorescein) are coincident (arrowheads) with the immunoreactivity of the 5-HT3A subunit (red fluorescence, tetramethylrhodamine) in the perikarya of neurons of the myenteric plexus (MP) within the muscular layer (M). Blue staining marks nuclei (DAPI counterstain). Scale bars = 100 μm.

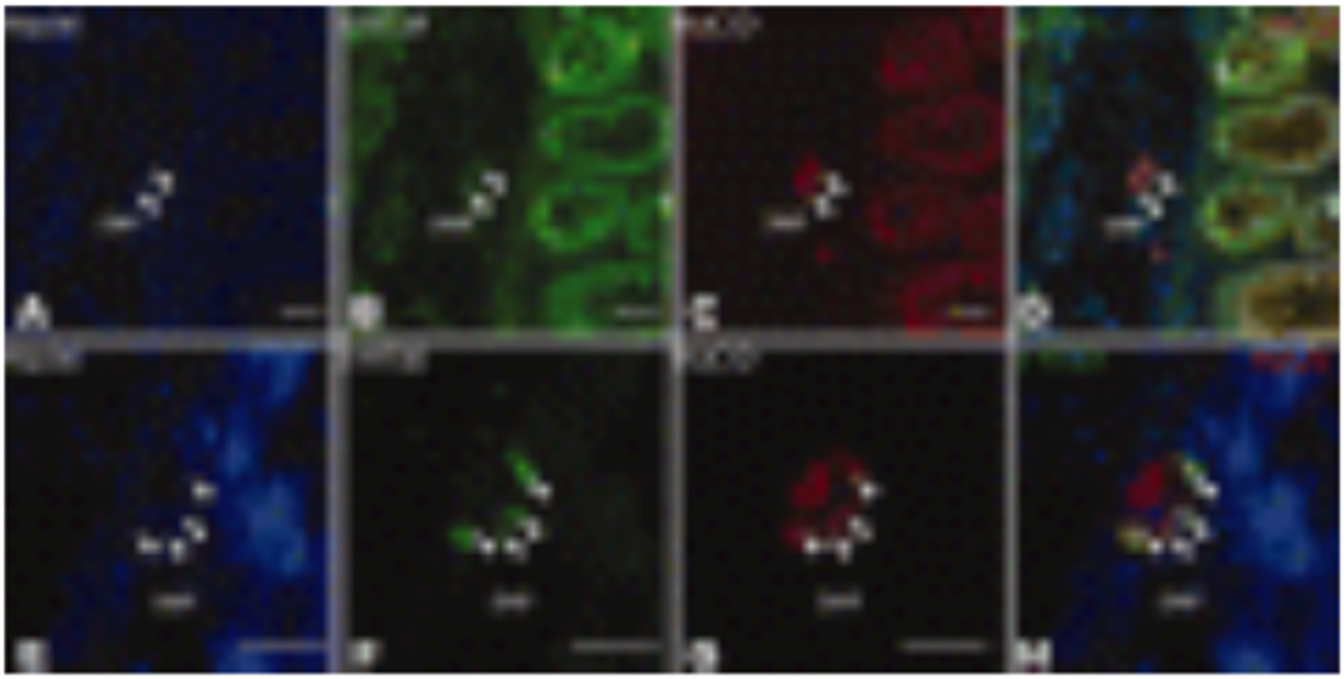

Figure 2.
Immunocytochemical analysis of the immunoreactivity of 5-HT3 subunits within the submucosal plexus of the human colon. The immunoreactivities of the 5-HT3A (**A–D**) and 5-HT3D (**E–H**) subunits (green fluorescence, fluorescein) are coincident (arrowheads) with the neuronal marker staining (HuC/D; red fluorescence) in the perikarya of neurons of the submucosal plexus (SMP). Blue staining marks nuclei (DAPI counterstain). Scale bars = 50 μm.

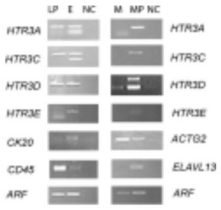

Figure 3.

Confirmation of *HTR3* genes expression by RT-PCR from microdissected colon tissue. Microdissected tissue of the mucosal cell layer of the epithelium and of the muscular layer of the human colon was analyzed by RT-PCR. All tested *HTR3* genes are expressed within enterocytes (E) as well as in the lamina propria (LP). In contrast, in the muscular layer (M), only a weak expression of *HTR3D* is detectable whereas there is expression of *HTR3A*, *HTR3C*, *HTR3D*, and *HTR3E* in myenteric plexus (MP). *CK20*, cytokeratin 20; *CD45*, protein tyrosine phosphatase, receptor type C; *ACTG2*, actin, gamma 2; *ELAVL13*, HuC/D; *ARF*, ADP-ribosylation factor; NC, negative control.

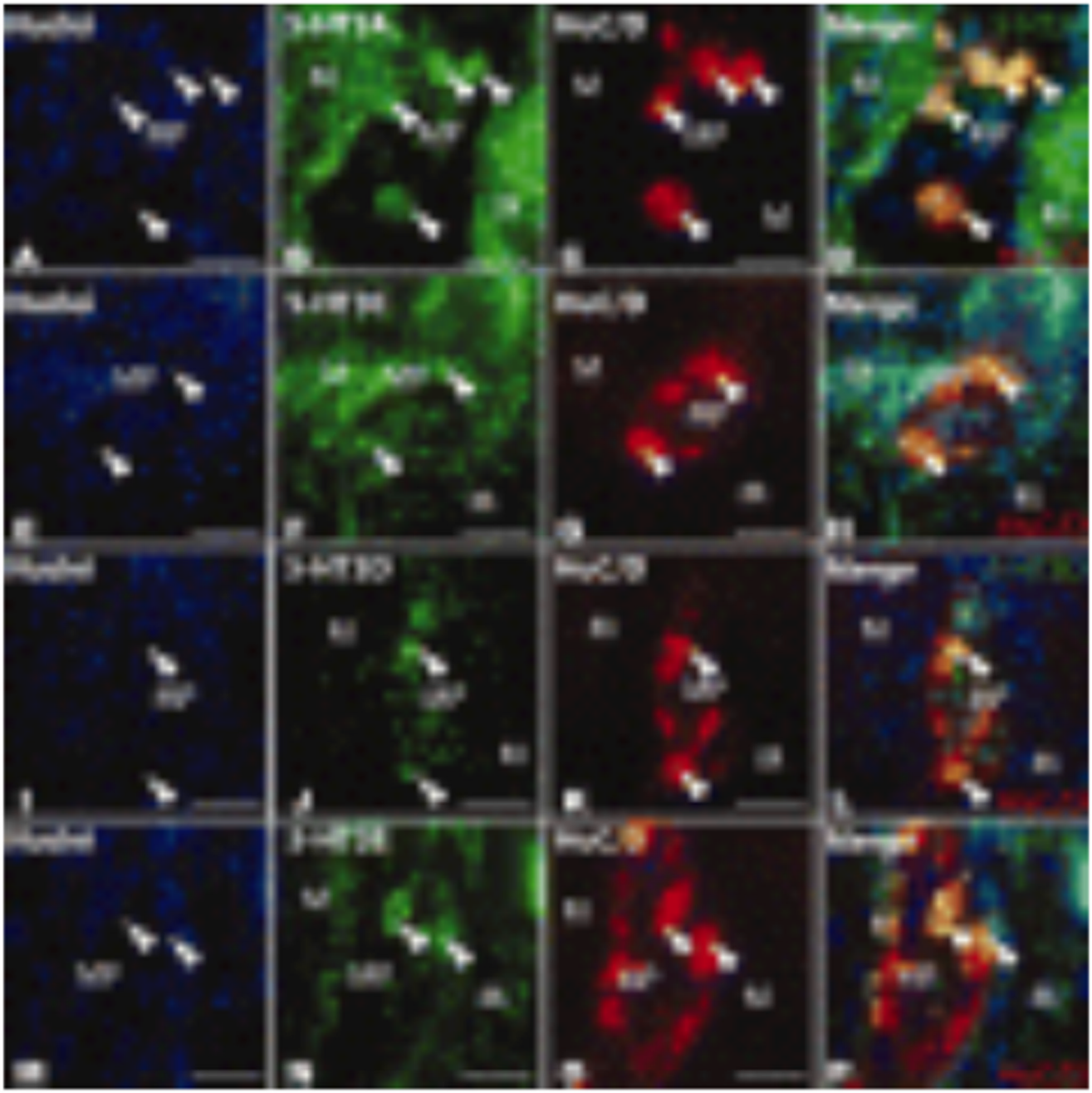

Figure 4.
Colon colocalization studies of 5-HT3A, 5-HT3C, 5-HT3D, and 5-HT3E with HuC/D. 5-HT3A (**A–D**), 5-HT3C (**E–H**), 5-HT3D (**I–L**), and 5-HT3E (**M–P**) are expressed in HuC/D-positive cell somata of neurons of the myenteric plexus (MP) in the muscular layer (M) of the colon. Coexpression is indicated by arrowheads. 5-HT3 subunits are labeled in green, and the neuronal marker HuC/D is stained in red. Blue staining marks nuclei (DAPI counterstain). Scale bars = 50 μm.

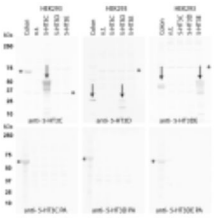

Figure 5.
Western blot analysis of a colon tissue lysate. Shown are results obtained with a colon cell lysate of one individual compared with lysates from HEK293 cells transiently transfected with the respective 5-HT3 subunit (positive control) and nontransfected cells (n.t., as negative control). Immunoreactive bands in the colon sample were detectable for 5-HT3D of ~25 kDa and for 5-HT3E of ~40–45 kDa (top). Specificity of antibodies was shown by preadsorption (PA) of the primary antibodies with the respective antigen peptide (bottom). Arrows indicate specific bands; asterisks indicate nonspecific background bands.

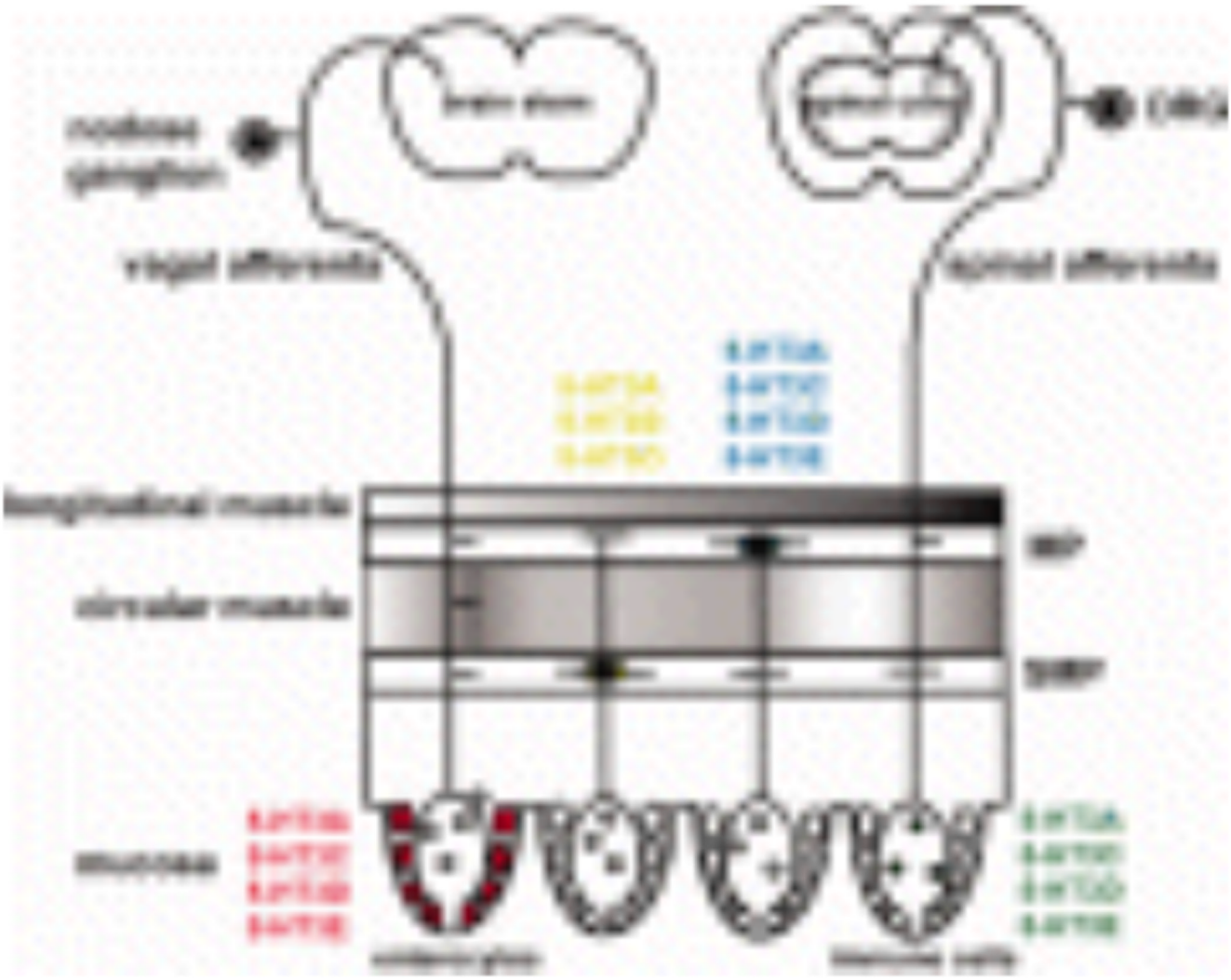

Figure 6.
Schematic illustration of 5-HT3 subunit expression in the human colon. Shown are the summarized results of this study as well as the previously described 5-HT3B expression within the SMP (Michel et al., 2005). Basic organization of this figure was adapted from Mawe et al. (1986). DRG, dorsal root ganglion; MP, myenteric plexus; SMP, submucosal plexus.

Table 1

Primary Antibodies[1]

Antibody (citation)	Immunogen	Manufacturer (catalog No.)	Host species	Dilution (for application)
Anti-5-HT3A (Walstab et al., 2010a)	KGVRPVRDWRKPTTV (5-HT3A)	Eurogentec	Rabbit polyclonal	1:100 (IF); 1:500 (IF*)
Anti-5-HT3C (Walstab et al., 2010a)	GELAGKKLGPRETEPD (5-HT3C)	Pineda	Rabbit polyclonal	1:200 (WB); 1:100 (IF)
Anti-5-HT3D	HASLVRPHPSRDQKR (5-HT3D)	Pineda	Rabbit polyclonal	1:200 (WB); 1:100 (IF)
Anti-5-HT3DE (Walstab et al., 2010a)	WTRAQREHEAQKQHS (5-HT3D, 5-HT3E)	Pineda	Rabbit polyclonal	1:200 (WB)
Anti-5-HT3E	VWEITDASRNILQTH (5-HT3C, 5-HT3E)	Pineda	Rabbit polyclonal	1:100 (IF)
Anti-PGP9.5 (Sams et al., 1992)	Amino acids 175–191 from soluble cytoplasmic human PGP9.5	Chemicon (AB5898)	Guinea pig polyclonal	1:500 (IF)
Anti-HuC/D-biotinylated (Marusich et al., 1994)	12-Residue synthetic peptide representing amino acids 240–251 from human HuD recognizing an epitope within the carboxy-terminal domain of HuD	Molecular Probes (A21272)	Mouse monoclonal (16A11)	1:100 (IF)
Anti-villin	Full-length human protein	Abcam (ab3304)	Mouse monoclonal (CWWB1)	1:100 (IF)
Anti-Na^+-K^+-ATPase	Full-length native protein (purified; rabbit renal outer medulla)	Abcam (ab7671)	Mouse monoclonal (464.6)	1:100 (IF)
Anti-mast cell tryptase (Lennerz et al., 2008)	Native human mast cell tryptase	Calbiochem (444905)	Mouse monoclonal (AA1)	1:1,000 (IF)
Anti-Myc	EQKLISEEDL	Cell Signaling (2276)	Mouse monoclonal (9B11)	1:500 (IF*)

[1] IF, immunofluorescence on tissue sections;

IF*, immunofluorescence on transfected cells (Supp. Info. Fig. 2); WB, Western blot.

Table 2

Secondary Antibodies[1]

Name	Conjugated to	Manufacturer (catalog No.)	Dilution (for application)
Goat anti	Alexa Fluor 488	Invitrogen (A-11070)	1:500 (IF)
Goat anti	Alexa Fluor 546	Invitrogen (A-11074)	1:500 (IF)
Goat anti	Alexa Fluor 488	Invitrogen (A-11029)	1:500 (IF)
Goat anti	Alexa Fluor 546	Invitrogen (A-11030)	1:500 (IF)
Donkey anti	Alexa Fluor 488	Invitrogen (A-21206)	1:500 (IF*)
Goat anti	Alexa Fluor 568	Invitrogen (A-11031)	1:500 (IF*)
Donkey anti	IREDye 800CW	Licor (926-32213)	1:15,000 (WB)

[1] IF, immunofluorescence on tissue sections;

IF*, immunofluorescence on transfected cells (Supp. Info. Fig. 2); WB, Western blot.

Table 3

Primers for RT-PCR Analysis[1]

Gene	5′-3′ Sequence for/rev	Annealing temp (°C)	Product size (bp)
HTR3A	CCTGGTTCTGGAGAGAATCG/GGGCTCTTCTCGAAGTCCTG	60	140
HTR3C	TCCCCAGAGAAGAGTCCAGA/TGGATTCCACGATGAAGATG	60	419
HTR3D	CTGGTGACATCGTTCCTGTG/TGGGAGCAAGTCATTCATCA	60	372
HTR3E	TGCTCCACTGCAACAGCCCG/CCCTGTCAGCTCTGCCTCCG	60	136
ACTG2	TGCCAACAATGTCCTCTCTG/AGGCTTGCTGATCCACATCT	60	199
ELAVL13	GCTGGACAATTTGCTCAACA/TTGTACACGAAGATGCACCAG	60	156
ARF	GCCAGTGTCCTTCCACCTGTC/CCTCGTTCACACGCTCTCTG	60	336
CD45	GGTTTCCACATTCGAGCAAT/GGGCACCAAGTGGATTAACA	60	170
CK20	GCGACTACAGTGCATATTACAG/CAGGACACACCGAGCATTT	60	87

[1] *ACTG2*, actin, gamma 2; *ELAVL13*, HuC/D; *ARF*, ADP-ribosylation factor; *CD45*, protein tyrosine phosphatase, receptor type C; *CK20*, cytokeratin 20; bp, base pairs.