THE JOURNAL OF COMPARATIVE NEUROLOGY 459:90–111 (2003)

Chemical Coding of the Human Gastrointestinal Nervous System: Cholinergic, VIPergic, and Catecholaminergic Phenotypes

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ABSTRACT

The aim of this investigation was to identify the proportional neurochemical codes of enteric neurons and to determine the specific terminal fields of chemically defined nerve fibers in all parts of the human gastrointestinal (GI) tract. For this purpose, antibodies against the vesicular monoamine transporters (VMAT1/2), the vesicular acetylcholine transporter (VAChT), tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), serotonin (5-HT), vasoactive intestinal peptide (VIP), and protein gene product 9.5 (PGP 9.5) were used. For in situ hybridization ^{35}S-labeled VMAT1, VMAT2, and VAChT riboprobes were used. In all regions of the human GI tract, 50–70% of the neurons were cholinergic, as judged by staining for VAChT. The human gut unlike the rodent gut exhibits a cholinergic innervation, which is characterized by an extensive overlap with VIPergic innervation. Neurons containing VMAT2 constituted 14–20% of all intrinsic neurons in the upper GI tract, and there was an equal number of TH-positive neurons. In contrast, DBH was absent from intrinsic neurons. Cholinergic and monoaminergic phenotypes proved to be completely distinct phenotypes. In conclusion, the chemical coding of human enteric neurons reveals some similarities with that of other mammalian species, but also significant differences. VIP is a cholinergic cotransmitter in the intrinsic innervation of the human gut. The substantial overlap between VMAT2 and TH in enteric neurons indicates that the intrinsic catecholaminergic innervation is a stable component of the human GI tract throughout life. The absence of DBH from intrinsic catecholaminergic neurons indicates that these neurons have a dopaminergic phenotype. J. Comp. Neurol. 459:90–111, 2003.

Indexing terms: dopamine; neurotransmitters; tyrosine hydroxylase; vasoactive intestinal peptide; vesicular acetylcholine transporter; vesicular monoamine transporters

In the gastrointestinal tract, motility and secretion are controlled by both intrinsic and extrinsic innervation. Extrinsic innervation includes both sensory and sympathetic inputs to the gastrointestinal (GI) tract and vagal cholinergic preganglionic parasympathetic neurons. The intrinsic innervation of the gut consists of the ganglia of the submucous and myenteric plexus. The input of preganglionic vagal fibers to the intrinsic gut ganglia is very limited. Thus, the intrinsic enteric nervous system is thought to function largely independently of preganglionic vagal or sacral parasympathetic control (Gershon et al., 1994; Furness et al., 1999).

Grant sponsor: Volkswagen-Stiftung; Grant number: I/71450; Grant number: I/75184.

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Received 28 May 2002; Revised 27 August 2002; Accepted 13 December 2002

DOI 10.1002/cne.10599

Published online the week of March 3, 2003 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. List of Primary Antibodies[1]

Antigen	Code	Source/reference	Dilution	Species
hVAChT	80153	Schäfer et al. 1995	1:5,000/1:600 (F)	Rabbit polyclonal
hVAChT	1624	L.E. Eiden, NIH, Bethesda, MD, USA	1:6,000/1:1000 (F)	Goat polyclonal
hVMAT2	80182	Erickson et al. 1996	1:4,000/1:400 (F)	Rabbit polyclonal
hVMAT1	VMAT1/10	Erickson et al. 1996	1:4,000/1:300 (F)	Rabbit polyclonal
TH	2/40/15	Boehringer, Mannheim, Germany	1:80/1:10 (F)	Mouse monoclonal
TH	21060595	Chemicon, Temecula, CA, USA	1:4,000/1:400 (F)	Sheep polyclonal
DBH	DBH 1537	Chemicon, Temecula, CA, USA	1:2,000/1:300 (F)	Sheep polyclonal
DBH	DBH MAB 308/01	Chemicon, Temecula, CA, USA	1:3,000	Mouse monoclonal
Serotonin	5-HT H209	DAKO, Hamburg, Germany	1:20/1:2 (F)	Mouse monoclonal
Serotonin	5 HT 43H37R	INC/IBL, Hamburg, Germany	1:60,000/1:4000 (F)	Rabbit polyclonal
VIP	VIP-Y010	Yanaihara, Shizuoka, Japan	1:10,000/1:1000 (F)	Rabbit polyclonal
VIP	20020304	Chemicon, Temecula, CA, USA	1:15,000/1:1000 (F)	Sheep polyclonal
PGP 9.5	RA95101	Ultraclone, Isle of Wright, UK	1:80,00/1:1000 (F)	Rabbit polyclonal
Gastrin	A568	DAKO, Hamburg, Germany	1:30,000	Rabbit, polyclonal
CgA	LK2H10	Boehringer, Mannheim, Germany	1:150/1:20 (F)	Mouse monoclonal

[1](F), dilution for immunofluorescence. hVAChT, human vesicular acetylcholine transporter; 5-HT serotonin; hVMAT, human vesicular monoamine transporter; TH, tyrosine hydroxylase; DBH, dopamine β-hydroxylase; VIP, vasoactive intestinal peptide; PGP 9.5, protein gene product 9.5; CgA, chromogranin A.

TABLE 2. Proportions of Cholinergic (VAChT+), VIPergic, Catecholaminergic (TH+), Monoaminergic (VMAT2+), and NANC Neurons in the Enteric Nervous System[1]

	VAChT	VIP	VMAT2	TH	NANC
Submucous plexus					
Stomach (n = 4)	70% ± 3% (144)	65% ± 2% (124)	18% ± 4% (128)	20% ± 3% (80)	10%
Duodenum (n = 3)	62% ± 1% (236)	53% ± 2% (278)	14% ± 3% (286)	15% ± 4% (276)	23%
Jejunum (n = 3)	60% ± 1% (210)	47% ± 2% (246)	14% ± 1% (186)	15% ± 1% (226)	25%
Ileum term. (n = 2)	53% ± 2% (238)	50% ± 2% (270)	4% ± 0% (212)	2% ± 0% (238)	45%
Colon (n = 2)	51% ± 2% (240)	79% ± 5% (226)	2% ± 0% (228)	0% ± 0% (230)	49%
Myenteric plexus					
Stomach (n = 4)	72% ± 4% (220)	59% ± 4% (218)	20% ± 4% (202)	20% ± 4% (226)	8%
Duodenum (n = 3)	66% ± 3% (218)	14% ± 2% (272)	16% ± 2% (214)	18% ± 2% (222)	16%
Jejunum (n = 3)	64% ± 1% (204)	12% ± 2% (250)	14% ± 2% (194)	13% ± 2% (230)	23%
Ileum term. (n = 2)	62% ± 2% (216)	11% ± 3% (256)	6% ± 1% (226)	6% ± 1% (222)	32%
Colon (n = 2)	65% ± 2% (196)	13% ± 1% (210)	6% ± 0% (174)	4% ± 0% (186)	31%

[1]Neuron counts are are expressed as means ± SEM. Figures in parentheses represent the absolute number of neurons positive for the pan-neuronal marker PGP 9.5. The population of nonadrenergic/noncholinergic (NANC) neurons is given in approximate percentages and was calculated by subtracting the number of TH- and VAChT-positive neurons from the number of PGP 9.5–positive neurons. n represents the number of patients from which sections were taken. For abbreviations, see Table 1.

It has been reported that there are minor populations of monoaminergic intrinsic neurons in the mammalian GI tract. These have been designated as serotoninergic, based on the presence of serotonin (5-HT) and tryptophan hydroxylase immunoreactivity, the uptake of exogenous [^{3}H]5-HT, the presence of serotonin binding protein (SBP), and the expression of serotonin membrane transporter (SERT) in either rodent or human gastrointestinal tissue (Gershon et al., 1965, 1977, 1983, 1989, 1994; Jonakiat et al., 1977; Tamir and Gershon, 1981; Costa et al., 1982; Furness and Costa, 1982; Griffith and Burnstock, 1983; Kurian et al., 1983; Costa et al., 1996; Meedeniya et al., 1998; Fiorica-Howells et al., 2000). Serotoninergic neurons are distinct from the serotonin-positive, vesicular monoamine transporter 1 (VMAT1) -positive enterochromaffin (EC) cells of the intestinal epithelium, which are involved in the hormonal and paracrine regulation of gastrointestinal function (Sjolund et al., 1983; Gershon et al., 1994; Weihe et al., 1994; Erickson et al., 1996). Because in the rat and mouse tyrosine hydroxylase (TH) immunoreactivity was detectable during development but not in adulthood, the concept of transient catecholaminergic innervation of the gut has been proposed. According to this concept, putative serotoninergic neurons of the enteric nervous system arise from the sacral neural crest by means of transient catecholaminergic progenitor cells that colonize the bowel and ultimately differentiate into serotoninergic neurons without a catecholaminergic phenotype (Cochard et al., 1978; Baetge and Gershon, 1989; Baetge et al., 1990; Gershon et al., 1993, 1994). On the other hand, a persistent population of intrinsic catecholamine-containing neurons was identified in the proximal colon of the guinea pig (Costa and Furness, 1971; Furness and Costa, 1971).

The presence of acetylcholine and vasoactive intestinal peptide (VIP) in the intrinsic innervation of the gut of mammals is well-documented (Furness and Costa, 1979; Jessen et al., 1980; Furness et al., 1984; Brookes et al., 1991; Steele et al., 1991; Crowe et al., 1992; Schemann et al., 1993, 1995; Schäfer et al., 1995, 1998; Costa et al., 1996; Porter et al., 1996, 1997). Estimates of the percentage of intrinsic neurons that express the cholinergic phenotype in humans have been steadily revised upward, as improved immunohistochemical methods for their detection have become available. For example, by using a monoclonal antibody to human choline acetyltransferase (ChAT), Porter and colleagues (1996) estimated that 52–55% of all submucous neurons and 63–65% of all myenteric neurons of the human small and large intestine are cholinergic. It has been shown that, in the GI tract of guinea pigs and other mammalian species, cholinergic and VIP-containing neurons are mostly separated. Thus, the neuropeptide VIP was considered to be primarily a nonadrenergic, noncholinergic (NANC) neurotransmitter candidate (Furness et al., 1984, 1995; Jacobson et al., 1994; Schemann et al., 1995; Costa et al., 1996; Vanden Berghe et al., 1999; Lomax and Furness, 2000). In contrast to these observations, studies performed in the primate lung and the human rectum have shown that a major population of intrinsic neurons were copositive for ChAT and VIP (Fischer et al., 1995; Nohr et al., 1995; Schneider et al., 2001).

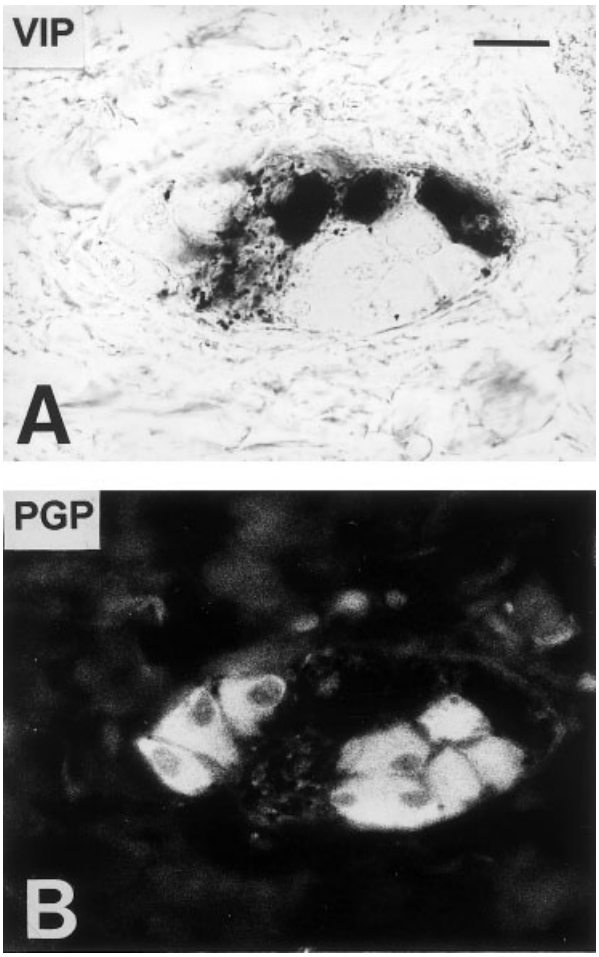

Fig. 1. Example of the costaining method used to quantify the proportion of phenotype-identified neuronal cell bodies in relation to the total number of ganglionic cells. **A:** Vasoactive intestinal peptide (VIP) immunoreactivity in a subpopulation of neurons of a submucous ganglion of the duodenum visualized in a first step as nickel-enhanced diaminobenzidine-peroxidase reaction product. **B:** Cy3 immunofluorescence for the pan-neuronal marker protein PGP 9.5 (protein gene product 9.5) visualized in a second step on the identical section. The VIP-negative but not the VIP-positive neuronal cell bodies in A are PGP-positive in B. Note quenching of the immunofluorescence signal for PGP by the optically dense VIP immunoreaction product. The total number of ganglionic cells in the ganglion is the sum of the VIP-positive cell bodies in A and PGP-positive/VIP negative cell bodies in B. Scale bars = 20 μm in A,B.

Enteric neurons can be classified according to their shape, their projections, their electrophysiological properties and their content of established or putative neurotransmitters (Gershon et al., 1994). The neurochemical characterization of distinct enteric neurons and their terminal fields is an important aspect in the understanding of their function (Bornstein and Furness, 1988; Costa et al., 1992; Gershon et al., 1994). The nature of the chemical coding of biogenic amine neurons and nerve fibers in the gastrointestinal tract has been studied most intensively in the guinea pig, rat, and mouse (Cochard et al., 1978; Costa et al., 1982, 1996; Furness and Costa, 1982; Keast et al., 1984; Erde et al., 1985; Baetge et al., 1990; Carnahan et al., 1990; Brookes et al., 1991; Steele et al., 1991; Schemann et al., 1993, 1995; Gershon et al., 1994; Furness et al., 1995, 1999; Gershon, 1997; Li and Furness, 1998; Reiche and Schemann, 1998; Sharkey et al., 1998; Lomax et al., 1999; Mann et al., 1999; Vanden Berghe et al., 1999; Lomax and Furness, 2000; Reiche et al., 2000). However, the chemical "coding" proved not to be strictly conserved between various species (Gershon et al., 1994). Despite the clinical significance of cholinergic and catecholaminergic neurotransmission in the human GI tract, little is known about the region-specific anatomic organization of cholinergic and monoaminergic neurons and nerve fibers in the healthy and diseased human gut (Kurian et al., 1983; Llewellyn-Smith et al., 1984; Wakabayashi et al., 1989; Crowe et al., 1992; Edwards et al., 1993; Singaram et al., 1995; Porter et al., 1996, 1997; Belai et al., 1997; Schneider et al., 2001). By combining 5-hydroxydopamine loading with fluorescence histochemistry and ultrastructural investigations presumed noradrenergic terminals were localized around ganglionic cells in the myenteric plexus and around blood vessels in the human small intestine (Llewellyn-Smith et al., 1984).

Previous immunohistochemical studies demonstrated that vesicular acetylcholine transporter (VAChT) and VMAT2 are excellent markers for cholinergic and monoaminergic neurons and nerve fibers in the peripheral nervous system (Weihe et al., 1994; Peter et al., 1995; Schäfer et al., 1995, 1998; Erickson et al., 1996; Arvidsson et al., 1997; Schütz et al., 1998). VMAT2 is contained in all monoamine-sequestering neurons examined to date, including histaminergic, serotoninergic, and catecholaminergic neurons (Weihe and Eiden, 2001). Thus, by using VMAT2 in conjunction with TH, dopamine β-hydroxylase (DBH), and 5-HT, catecholamine- vs. non–catecholamine-containing neurons can be identified with high specificity and sensitivity.

The aim of this investigation was to examine the chemical coding of enteric neurons in distinct parts of the human GI tract with these new markers. The expression patterns of VAChT, VMAT1, VMAT2, TH, DBH, 5-HT, and VIP in intrinsic neurons were analyzed. The target relations and terminal fields of cholinergic, peptidergic, and monoaminergic nerves are described. The ability to distinguish between intrinsic and extrinsic innervation of the human gut based on the differential expression of markers that are unique to cell bodies of enteric intrinsic versus extrinsic sensory, sympathetic, or vagal extrinsic inputs to the gut is considered.

MATERIAL AND METHODS

Tissue collection and preparation

Stomach and small and large bowel samples were collected during abdominal surgery from 12 patients suffering from neoplastic disease. The patients consisted of six men and six women with an average age of 61 years and an age range of 36–79. Surgical procedures included gastrectomy in two cases, classic Whipple resection in three cases, partial ileectomy in one case, ileocaecal resection in one case, right hemicolectomy in one case, left hemicolectomy in one case, sigmoidectomy in one case, and proctectomy in one case. For immunohistochemistry and in situ hybridization the paratumorous, normal tissue of the following regions was analyzed: corpus region of the stomach (n = 5), duodenum (n = 3), first jejunal loop (n = 3),

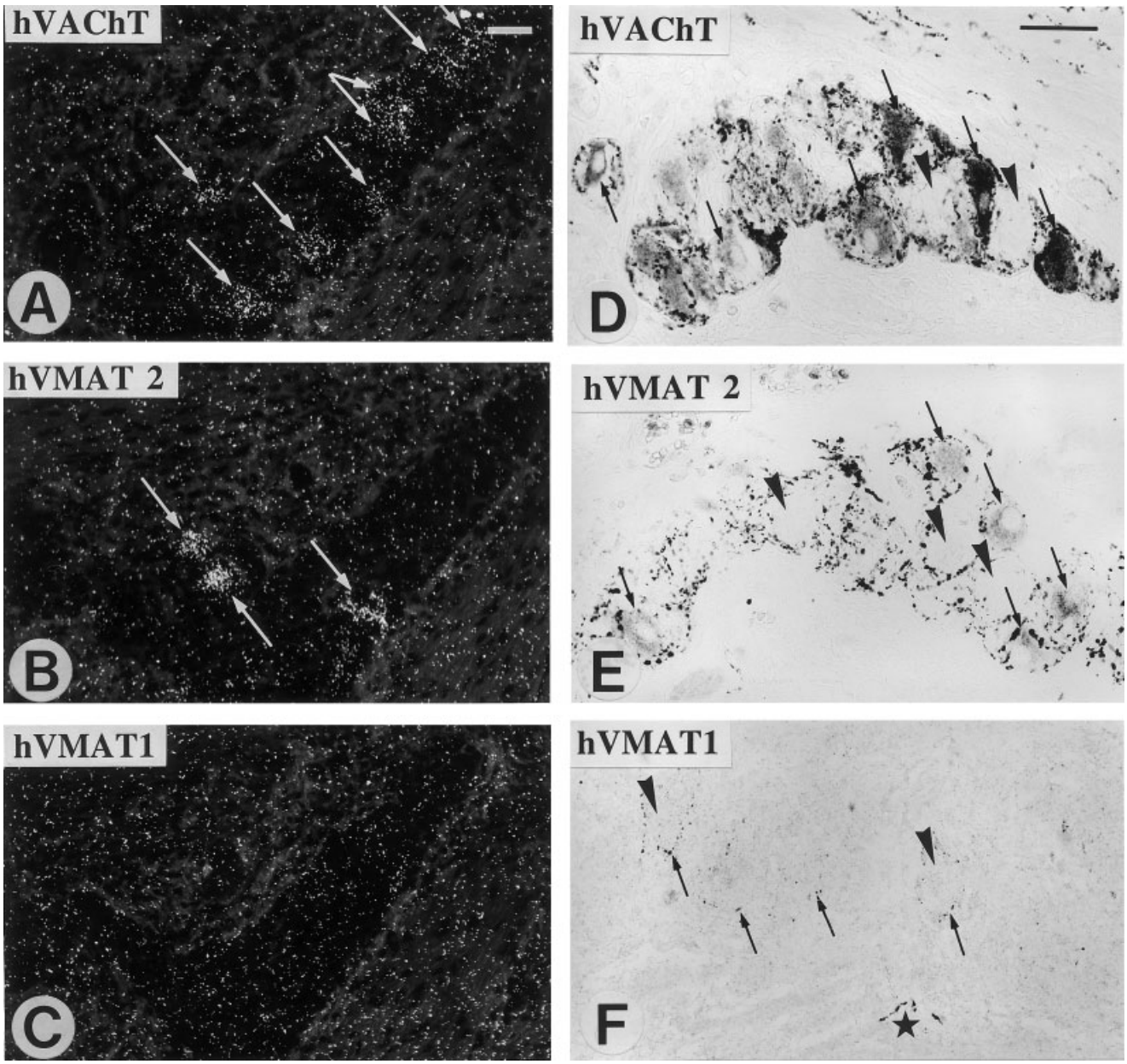

Fig. 2. Distribution pattern of cholinergic (vesicular acetylcholine transporter [VAChT]) and monoaminergic (vesicular monoamine transporter 2 [VMAT2]) neurons in gastric and intestinal myenteric ganglia, as revealed by in situ hybridization and immunohistochemistry. **A–C:** Stomach, myenteric plexus, in situ hybridization. Darkfield photomicrographs of adjacent sections. VAChT mRNA-positive neurons (arrows in A). VMAT2 mRNA-positive ganglionic cells (arrows in B), segregated from VAChT mRNA-positive ganglionic cells. VMAT1 mRNA is absent from intrinsic neurons (C). **D,E:** Upper intestine, myenteric plexus, immunohistochemistry. High-power brightfield photomicrographs on serial sections demonstrating VAChT protein in the majority of neuronal cell bodies (arrows in D) and expression of VMAT2 in a considerable subpopulation of ganglionic cells (arrows in E). Some neurons are negative for VAChT and VMAT2 (arrowheads in D and E). Note the dense innervation of ganglionic cells by numerous strongly stained VMAT2- and VAChT-positive varicose fibers (A, B). VMAT1 is absent from intrinsic neurons (arrowheads in F) but present in a minor proportion of nerve fibers surrounding VMAT1 negative ganglionic cells (arrows in F) and single fibers innervating the longitudinal smooth muscle layer (asterisk in F). Scale bars = 50 μm in A,B, 20 μm in C,D.

terminal ileum (n = 2), and distinct parts of the large intestine (caecum, n = 1; ascending colon, n = 1; transverse colon, n = 2; descending colon, n = 1; sigmoid colon, n = 2). Various tissues of two rhesus monkeys and adrenal gland tissue from two patients with unilateral adrenalectomy, one suffering from pheochromocytoma (63-year-old man) and one from Conn's syndrome (70-year-old woman), were used as control tissues.

For the immunohistochemical analysis, all human tissues were removed rapidly during surgery and subse-

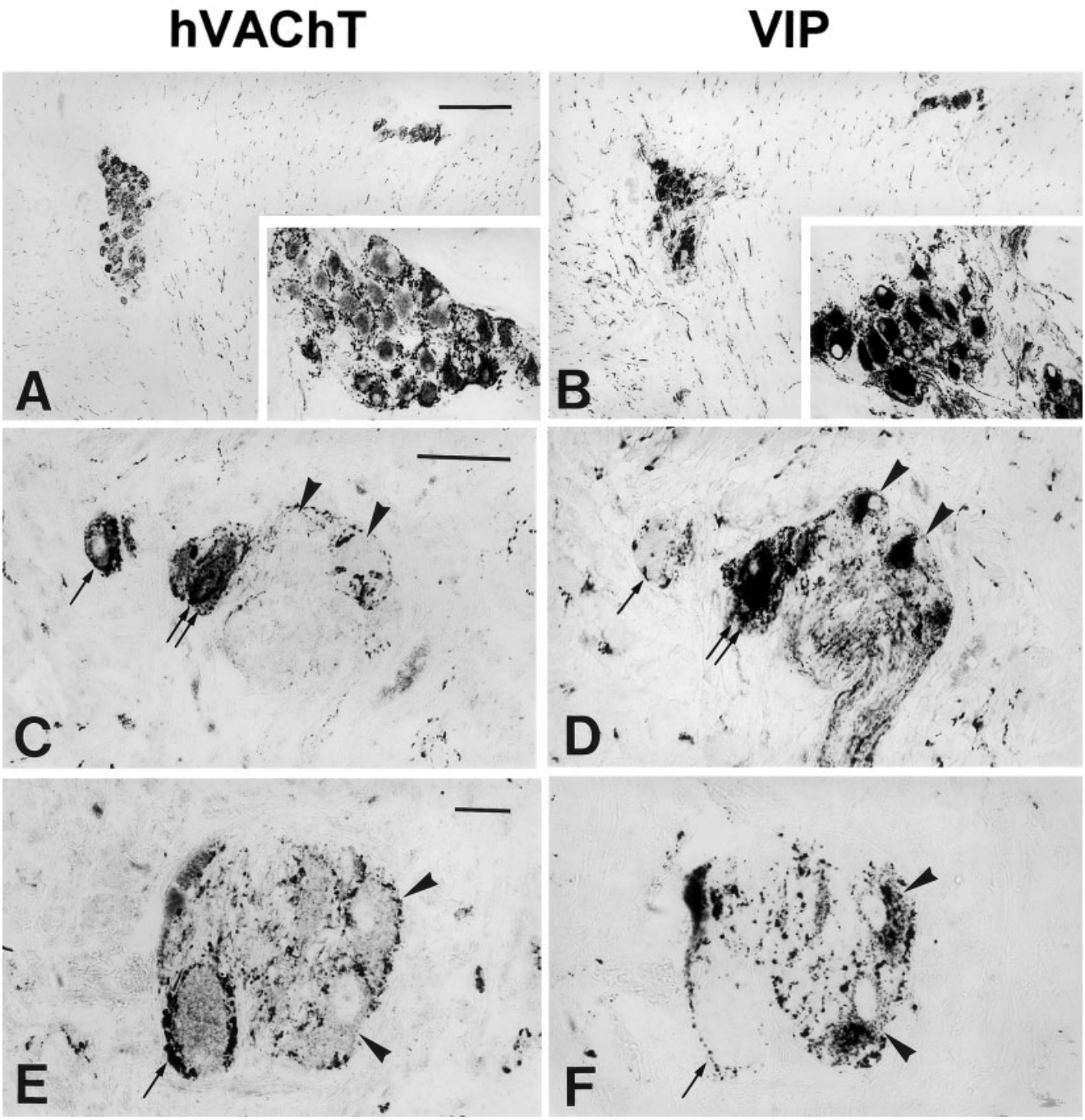

Fig. 3. Coexistence, segregation, and regional variation of vesicular acetylcholine transporter (VAChT) and vasoactive intestinal peptide (VIP) expression in neurons of the myenteric plexus revealed by immunohistochemistry. **A,B:** Stomach. Adjacent sections demonstrating strong VAChT and VIP immunoreactivity in neuronal cell bodies. Insets showing numerous strongly stained neuronal cell profiles for VAChT and VIP. Note the dense innervation of smooth muscle layers by VAChT- and VIP-immunoreactive nerve fibers. **C,D:** Stomach. Adjacent semithin section showing three neuronal phenotypes: VAChT+/VIP+ (double arrow), VAChT+/VIP- (arrow), and VAChT-/VIP+ (arrowhead). **E,F:** Duodenum. Adjacent semithin sections revealing coexistence of VAChT and VIP immunoreactivity in two neuronal cell bodies (arrowheads) and 1 VAChT+/VIP- neuronal perikaryon (arrow). Note the granular appearance of VAChT immunoreactivity in the less strongly stained ganglionic cells in E. Scale bars = 200 μm in A,B, 20 μm in C,D, 10 μm in E,F, 40 μm in G,H.

quently fixed for 48 hours in Bouin-Hollande fixative. None of the tissues examined in this study had been exposed to colchicine or other pharmacologic treatment for the enhancement of 5-HT or neuropeptide immunoreactivity. After dehydration in a graded series of 2-propanol solution, the tissues were embedded in Paraplast Plus

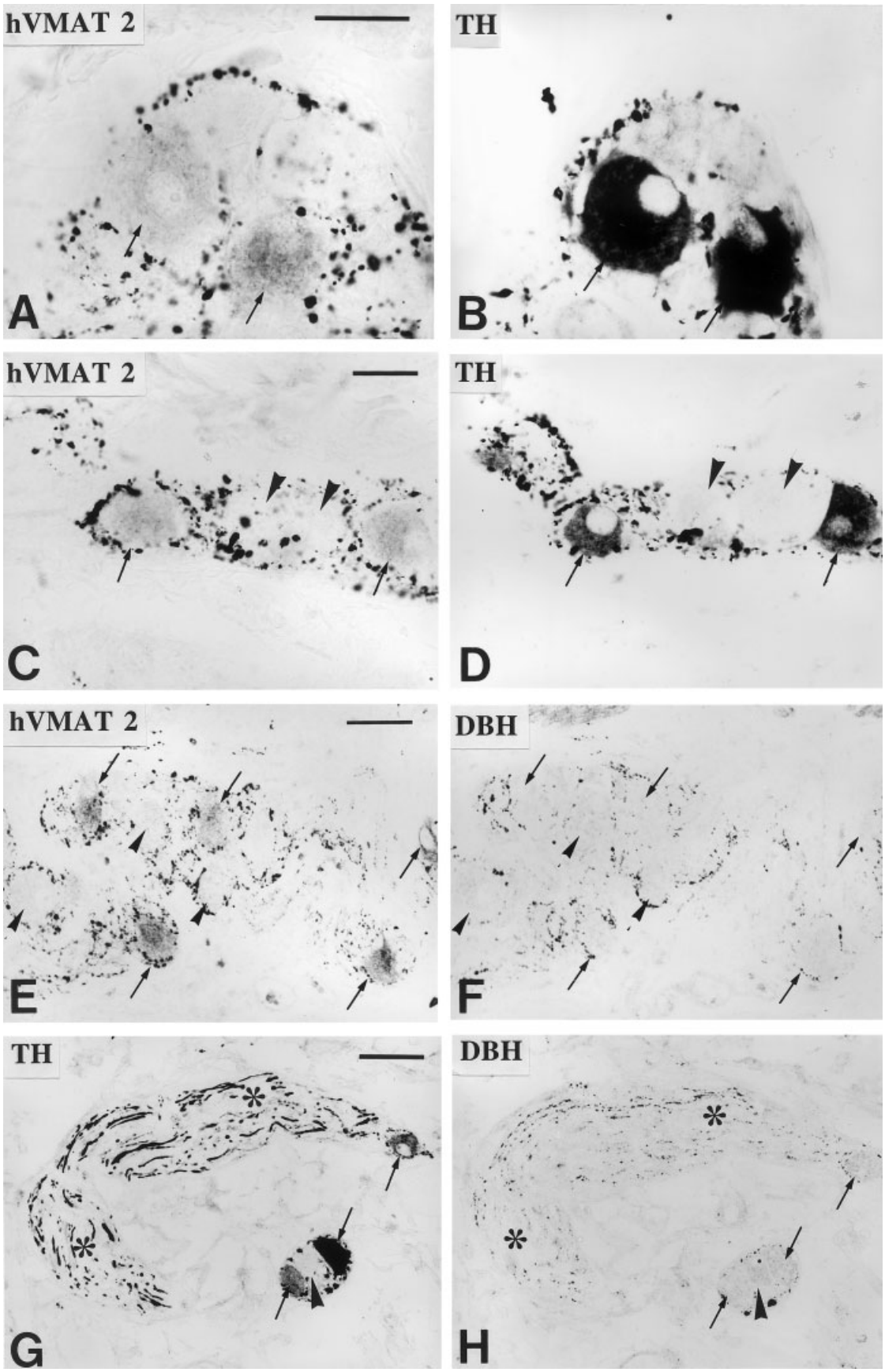

Figure 4

(Merck, Darmstadt, Germany). Adjacent sections (3-μm or 7-μm thick) were cut and deparaffinized. Antigen retrieval to increase the sensitivity of immunodetection was performed by heating the sections at 92°–95°C for 15 minutes in 0.01 M citrate buffer (pH 6.0) according to the DAKO protocol (Hamburg, Germany). Nonspecific binding sites were blocked with 5% bovine serum albumin (BSA, Serva, Heidelberg) in phosphate buffered saline (PBS, pH 7.45) followed by an avidin-biotin blocking step (avidin-biotin blocking kit, Boehringer Ingelheim, Germany).

Tissue for in situ hybridization was immediately frozen on dry ice and stored at −80° C. Cryosections (14–16 μm thick) were placed on presilanized glass slides, fixed in 4% phosphate-buffered formaldehyde for 60 minutes followed by three 10-minute washes in 50 mM PBS (pH 7.4). The slides were then briefly rinsed in distilled water, incubated in 0.1 M triethanolamine (pH 8.0) for 1 minute and for 10 minutes in the same solution containing 0.25% vol/vol acetic anhydride under rapid stirring. They were then quickly rinsed in 2× standard saline citrate (SSC), dehydrated in 50% and 70% ethanol, and air-dried.

Immunohistochemistry and quantitative analysis

Rabbit antiserum no. 80153 and goat antiserum no. 1624 raised against the ultimate C-terminal dodecapeptide predicted from the open reading frame of the human VAChT cDNA sequence, antiserum no. 80182 raised against the C-terminal sequence of human VMAT2, and antiserum no. VMAT1/10 raised against the C-terminal sequence of human VMAT1 were used for the immunohistochemical examination (Table 1). Several antisera against TH, DBH, 5-HT, VIP, protein gene product 9.5 (PGP 9.5), gastrin, and chromogranin A were used. They are summarized in Table 1.

The tissue sections were incubated with the primary antibodies overnight at 18° C (diluted as shown in Table 1) and further incubated for 2 hours at 37°C. They were then washed in distilled water and in 50 mM PBS and were then incubated with species-specific biotinylated secondary antibodies (Dianova, Hamburg, Germany) for 45 minutes at 37°C, washed several times, and incubated for 30 minutes with the ABC reagents (Vectastatin Elite ABC kit, Boehringer Ingelheim, Germany). Immunoreactions were visualized with 3-3′-diaminobenzidine (DAB, Sigma, Deisenhofen, Germany) enhanced by the addition of 0.08% ammonium nickel sulfate (Fluka, Buchs, Switzerland), resulting in dark blue staining. No binding was detected in the absence of the primary antibody. For preabsorption of VAChT, VMAT1 and VMAT2, the antisera were incubated at 4°C overnight with 25 μmol of the corresponding peptide sequence and then examined immunocytochemically.

The percentages of neurons of the myenteric and submucous plexus of the stomach, duodenum, proximal jejunum, terminal ileum, and transverse colon expressing VAChT, VIP, VMAT2, and TH were determined by using peroxidase labeling with antibodies to the above antigens and subsequent fluorescent labeling of the same sections with the neuronal marker PGP 9.5. Only ganglionic cells with a visible nuclear profile were evaluated. Immunoreactivity was quantified on the basis of the number of neurons positive for each marker in relation to the total number of PGP 9.5–positive neurons. For statistical purposes, the number of patients from which sections were taken was given as the value of n. The percentage of neurons that were immunoreactive for a particular marker was calculated and expressed as mean ± the standard error of mean (SEM) (Table 2). Figure 1 illustrates neuronal cell counts in the submucous plexus of the duodenum, double-labeled for VIP and PGP 9.5.

The density of nerve fibers in the tissue compartments was assessed by a subjective rating system. The global frequency of nerve fibers was estimated on sections stained with the pan-neuronal marker PGP 9.5. The relative frequency of subpopulations of VAChT-, VMAT1-, VMAT2-, TH-, DBH-, 5-HT-, and VIP-immunoreactive nerve fibers was compared with the density of PGP 9.5–immunoreactive nerve fibers and scored on a scale from 1+ (extremely sparse) to 4+ (abundant).

In situ hybridization

For VMAT2 mRNA detection a 269-bp-long DNA fragment of the hVMAT2 cDNA (Erickson and Eiden, 1993) corresponding to nucleotides (nt.) 244-512 was subcloned into pCDNAI (Invitrogen, Leek, Netherlands). To generate specific probes for the localization of VMAT1 mRNA, a 247-bp-long DNA restriction fragment of the hVMAT1 cDNA (Erickson et al., 1996) corresponding to nt. 436-682 was subcloned into Bluescript II KS+ (Stratagene, Heidelberg, Germany). For VAChT mRNA detection, an about 800-bp-long DNA fragment of the hVAChT cDNA (Erickson et al., 1994) was subcloned into pcDNA (Invitrogen). Inserted sequences were verified by double-stranded DNA sequencing. In vitro transcription of the vector constructs by using ^{35}S-UTP-labeled nucleotide yielded antisense riboprobes for hVMAT1 after linearization with *Xho*I and incubation with T7 RNA polymerase, for hVMAT2 after linearization with *Xba*I and incubation with SP6 RNA polymerase, and for hVAChT after linearization with *Xho*I and incubation with T7 RNA polymerase. To increase the tissue penetration of probes, the generated transcripts were reduced to approximately 200 nucleotide fragments by limited alkaline hydrolysis as described by

Fig. 4 (Overleaf). Vesicular monoamine transporter 2 (VMAT2) and tyrosine hydroxylase (TH) coexpression and absence of dopamine β-hydroxylase (DBH) in a subpopulation of neurons of the submucous and the myenteric plexus of the stomach and upper intestine, as revealed by immunohistochemistry. **A,B:** Stomach, corpus, myenteric plexus. Adjacent sections demonstrating coexistence of VMAT2 and TH immunoreactivity in two profiles of neuronal cell bodies (arrows). Note the presence of VMAT2 and TH in intraganglionic terminals targeting both VMAT2- and TH-copositive neuronal cell bodies. **C,D:** First jejunal loop, submucous plexus. Adjacent sections demonstrating coexistence of VMAT2 and TH immunoreactivity in two profiles of neuronal cell bodies (arrows). Note the presence of VMAT2 and TH in intraganglionic terminals and absence of VMAT2 and TH from two neuronal bodies (arrowheads). **E,F:** Stomach, corpus, myenteric plexus. Adjacent sections showing absence of DBH in 5 VMAT2 immunoreactive ganglionic cells (arrows). Some VMAT2-negative neurons targeted by DBH- and VMAT2-positive varicose fibers are labeled by arrowheads. **G,H:** First jejunal loop, submucous plexus. Adjacent sections demonstrating the presence of TH (A) in three ganglionic cells (arrows) and a large nerve trunk (asterisk). DBH (B) is present in the same nerve trunk (asterisk) but absent from TH-positive intrinsic neurons (arrows). One neuron is negative for TH and DBH (arrowhead). Note TH and DBH-positive terminal fibers in close proximity to ganglionic cells. Scale bars = 20 μm in A–F, 40 μm in G,H.

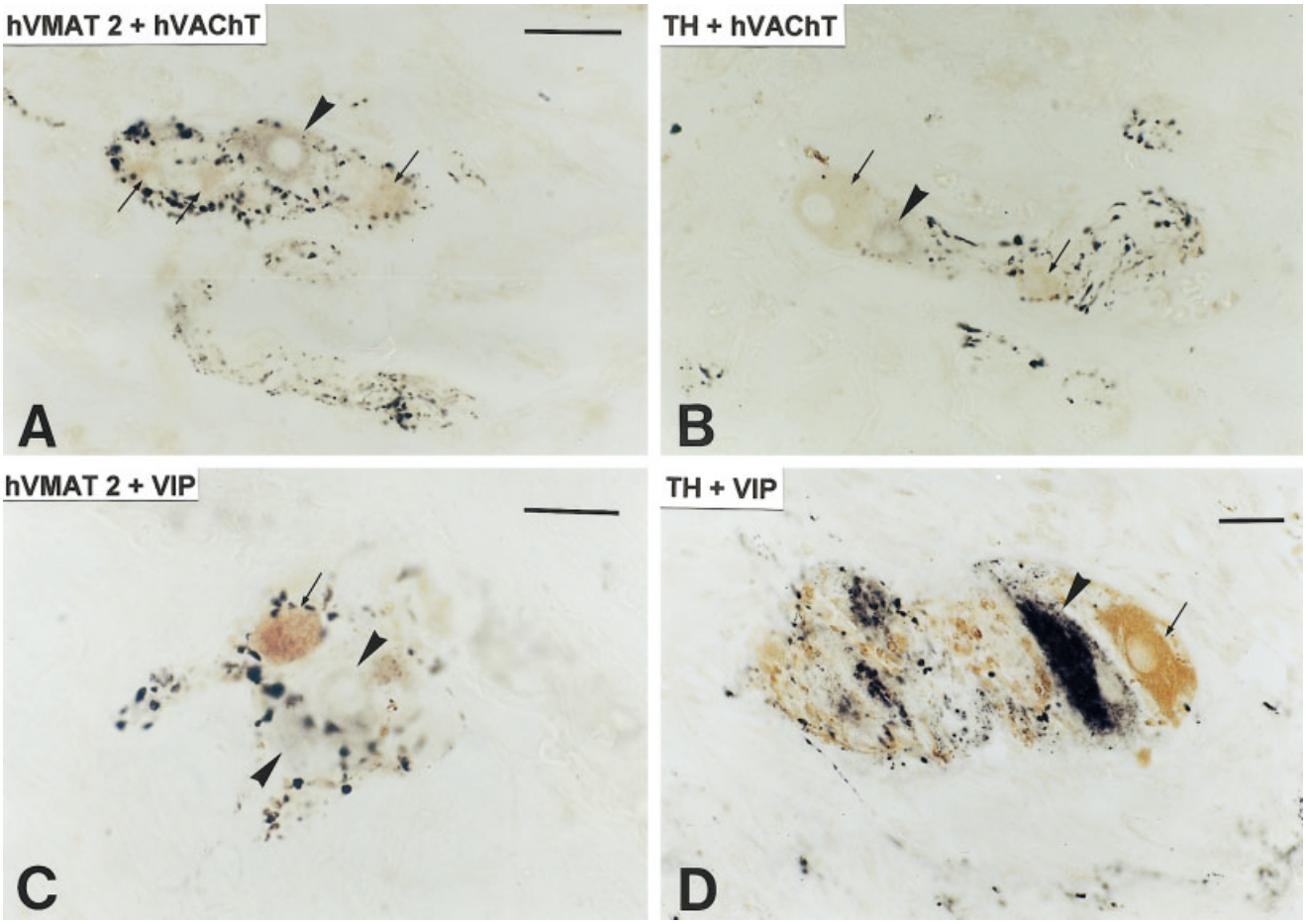

Fig. 5. Segregation of cholinergic/vasoactive intestinal peptide (VIP)ergic and catecholaminergic vesicular monoamine transporter 2/tyrosine hydroxylase (VMAT2/TH) phenotypes in submucous and myenteric plexus revealed by sequential dual color immunohistochemistry. **A,B:** Duodenal submucous plexus. VMAT2 and TH immunoreactivity (strong black reaction products in A,B) in a neuronal cell body (arrowhead) is segregated from the vesicular acetylcholine transporter (VAChT) immunoreactivity (light brown reaction product) present in three neuronal cell bodies in A and in two neuronal cell bodies in B (arrows). Note the black VMAT2- and TH-positive terminal fibers. VAChT terminal fibers are below detection limit. **C:** Jejunal submucous plexus. VMAT2 immunoreactivity (weak to moderate black reaction product) in two neuronal cell bodies (arrowheads) is segregated from the VIP immunoreactivity (strong brown reaction product) present in a neuronal cell body profile (arrow). Note black VMAT2 terminal fibers surrounding the VIP-positive cell body and the VMAT2-positive cell bodies. VIP terminal fibers are only weakly stained. Note their intermingling with and close apposition to VMAT2-positive varicosities and fiber profiles. **D:** Gastric myenteric plexus. Segregation of a TH-positive cell body (arrowhead) from a VIP-positive neuronal cell body (arrow). Note the intermingled and cluster-like segregated varicosities and fiber profiles staining for TH (black) or VIP (brown to dark brown). Scale bars = 50 μm in A,B, 20 μm in C,D

Angerer et al. (1984). To each section, hybridization buffer (3× SSC, 50 mM $NaPO_4$ pH 7.4, 1× Denhardt's solution, 0.25 mg/ml yeast tRNA, 10% dextran sulfate, 50% formamide, 10 mM dithiothreitol) was applied. The hybridization mix contained 50,000 dpm/ml of ^{35}S-labeled RNA probes. The coverslips were removed in 2× SSC. The sections were then incubated in moist chambers at 60°C for 16 hours, after which they were subjected to the following posthybridization steps: RNAse treatment (20 μg/ml Rnase A an 1 U/ml Rnase T1 in 10 mM Tris, pH 8.0, 0.5 M NaCl, 1 mM EDTA) for 60 minutes at 37°C and successive washes in decreasing salt concentrations (2×, 1×, 0.5×, and 0.2× SSC) for 10 minutes each, followed by incubation in 0.2× SSC at 60°C for 60 minutes. For autoradiography, the slides were dipped in NTB-2 nuclear emulsion (Eastman Kodak, NY) and exposed for 3 weeks. The developed sections were stained with hematoxylin and eosin and analyzed and photographed with an Olympus AX 70 microscope (Hamburg, Germany).

Colocalization studies

To study the colocalization of VAChT, VIP, VMAT2, TH, and DBH immunoreactivity in ganglionic cells and nerve fibers, four strategies were used: (1) alternate staining of adjacent semithin sections (2–3 μm thick), (2) the two-color immunoperoxidase technique (Hancock, 1984), (3) a combination of enzyme and fluorochrome enhanced immunohistochemistry (Anlauf et al., 2000), and (4) double-fluorescence labeling (Hörsch et al., 1993).

For adjacent section analysis, randomly selected pairs of semithin sections of all gut regions were stained for

TABLE 3. Region and Compartment-Specific Density of Terminal Field Innervation in the Gut Wall[1]

	VMAT2	TH	DBH	VMAT1	5-HT	VAChT	VIP
Stomach							
Mucosa	++	++	+	−	−	+++	+++
Mucosal muscle	+++	+++	(+)	−	−	++	+++
Subm. ganglia	++++	++++	+	−	−	++++	++++
Myent. ganglia	++++	++++	++	+	(+)	++++	++++
Outer muscle layers	+++	+++	(+)	(−)	−	++++	++++
Arteries	++++	++++	++++			+	+
Upper small intestine							
Mucosa	+	+	−	−	−	++++	++++
Mucosal muscle	+++	+++	(+)	−	−	++	+++
Subm. ganglia	++++	++++	+	−	−	++++	+++
Myent. ganglia	++++	++++	++	+	(+)	++++	++
Circ. muscle	+++	+++	(+)	(+)	−	++++	++++
Long. muscle	+	+	(+)	−	−	+	+
Arteries	++++	++++	++++	−	−	+	+
Ileum term.							
Mucosa	(+)	(+)	−	−	−	++	++++
Mucosal muscle	(+)	+	(+)	−	−	++	+++
Subm. ganglia	+	+	+	−	−	++++	+++
Myent. ganglia	++	++	+	+	(+)	++++	++
Circ. muscle	++	++	(+)	(+)	−	++++	++++
Long. muscle	(+)	(+)	(+)	−	−	+	+
Arteries	++++	++++	++++	−	−	+	+
Colon							
Mucosa	(+)	(+)	−	−	−	+	++++
Mucosal muscle	(+)	(+)	(+)	−	−	++	++++
Subm. ganglia	+	+	+	−	−	+++	+++
Myent. ganglia	++	++	++	+	(+)	++++	++
Circ. muscle	+	+	(+)	(+)	−	+++	++++
Long. muscle	(+)	(+)	(+)	−	−	+	+
Arteries	++++	++++	++++	−	−	+	+

[1]−, absent; (+), extremely sparse; +, low density; ++, medium density; +++, high density; ++++, abundant. Subm., submucous; Myent., myenteric; Circ., circular; Long., longitudinal; term. terminal. For other abbreviations, see Table 1.

each marker combination, as described above. A minimum of 100 ganglionic cells per region and per plexus was analyzed for each marker combination. Only ganglionic cells with the same, clearly visible cytoplasmic profile were evaluated. Coexistence patterns were classified into one of five categories: full overlap, major coexistence, minor coexistence, rare coexistence, or no coexistence. For the two-color peroxidase technique, the first primary antibody was detected with the nickel-enhanced DAB procedure. After dehydration through a graded series of 2-propanol and one passage through xylene, the sections were rehydrated in a graded series of 2-propanol and treated with BSA and the avidin-biotin reagents to block potential nonspecific binding of the second avidin-biotin-peroxidase complex. The second primary antibody was then visualized with the DAB/peroxidase reaction without nickel enhancement, resulting in a brown staining product, or by CY3 fluorochrome labeling (Dianova, Hamburg, Germany). In control sections, the primary antibodies were omitted. Double immunofluorescence detection was performed by covering the sections with a mixture of the two different primary antibodies in appropriate dilutions (Table 1) and by subsequent labeling with the species-specific secondary antibodies bearing the fluorochromes Cy3 or DTAF (Dianova). The sections were analyzed with an AX 70 microscope (Olympus, Hamburg, Germany) or a confocal laser scanning microscope BX 50WI (Olympus) equipped with the appropriate filter cubes for discriminating between Cy3 and DTAF. For photomicrographic production of the confocal laser scanning figures, the software of the Olympus Fluoview system and the Olympus Camedia digital color printer were used. All color figures were scanned with a Nikon CS 100 film scanner with 2,700-dpi resolution. Brightness and contrast were then adjusted in Adobe Photoshop.

Ethics

The procurement of human material during surgery was approved by the Ethics Committee of the Medical Faculty of the University of Marburg. Oral informed consent was obtained from each patient before surgery.

RESULTS

General remarks

VAChT, VMAT2, TH, and VIP stained neuronal perikarya. In contrast, VMAT1, 5-HT, and DBH immunoreactivity was absent from neuronal perikarya but present in nerve fibers. Immunohistochemical staining for VAChT and VMAT2 revealed a weak and clearly distinguishable granular cytoplasmic pattern in neurons. Varicose nerve fibers, which contain a high density of synaptic

Fig. 6. Distribution pattern of vesicular monoamine transporter 2 (VMAT2), tyrosine hydroxylase (TH), and dopamine β-hydroxylase (DBH) nerve fibers in human stomach. **A–C:** Oxyntic mucosa, adjacent sections. Dense innervation of the oxyntic mucosa by VMAT2- and TH-positive nerve fibers (arrows). DBH-positive terminals innervating the epithelium are sparse (arrows). Note the close association between VMAT2-positive fibers and enterochromaffin-like (ECL) cells visualized by VMAT2 immunoreactivity in A. **D–F:** Submucous blood vessel, adjacent sections. Strong and equal density of VMAT2, TH, and DBH nerve fibers in the perivascular plexus of a small artery. **G–I:** Submucous large nerve fiber bundle, adjacent sections. Strong and copositive VMAT2, TH, and DBH immunoreactivity in many nerve fibers. **J–L:** Smooth muscle, interval sections. High density of VMAT2- and TH-positive nerve fibers (arrows). The DBH innervation of smooth muscle layers is extremely sparse (arrows). Scale bars = 20 μm in A–C,J–L, 50 μm in D–I.

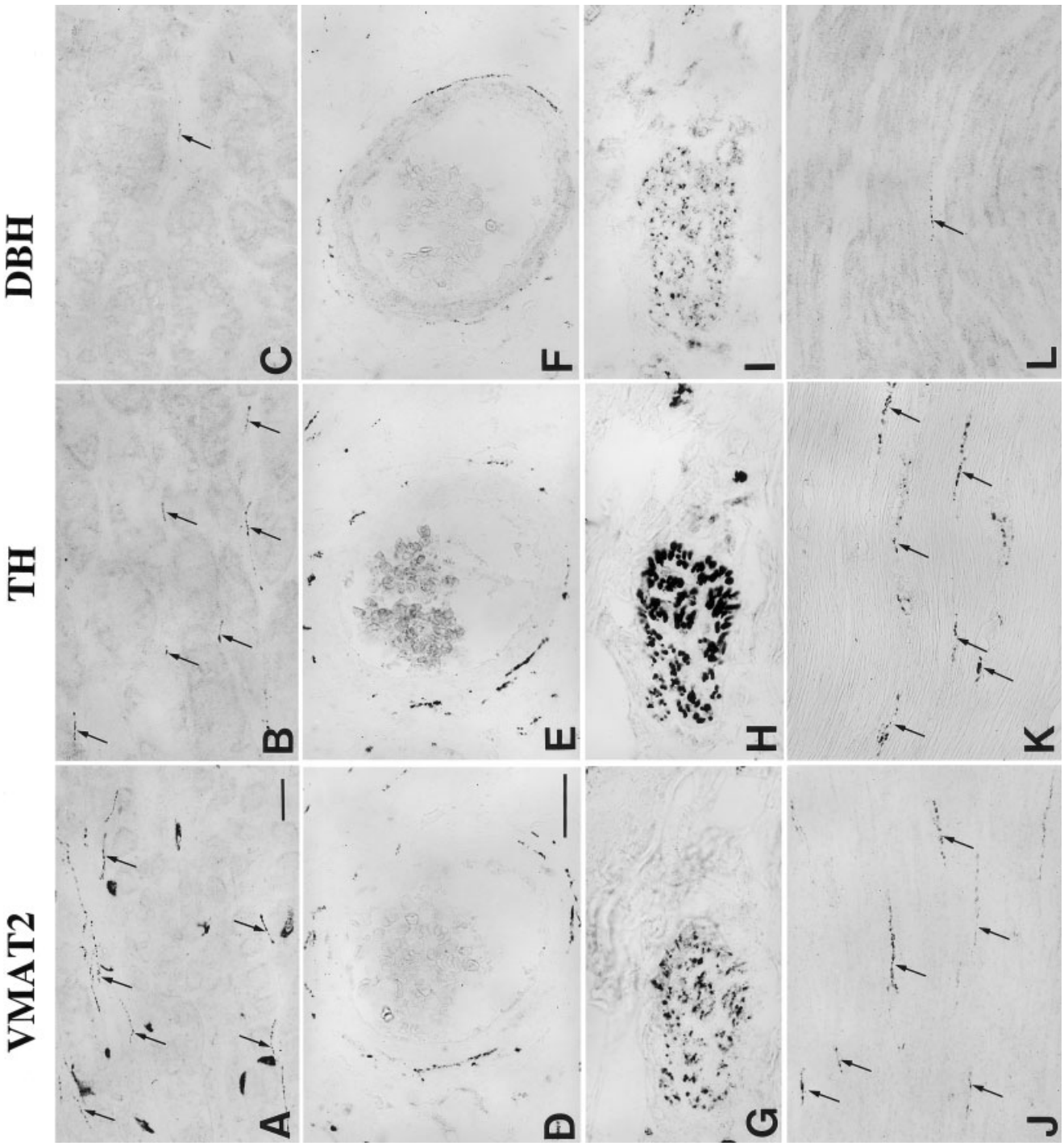

Figure 6

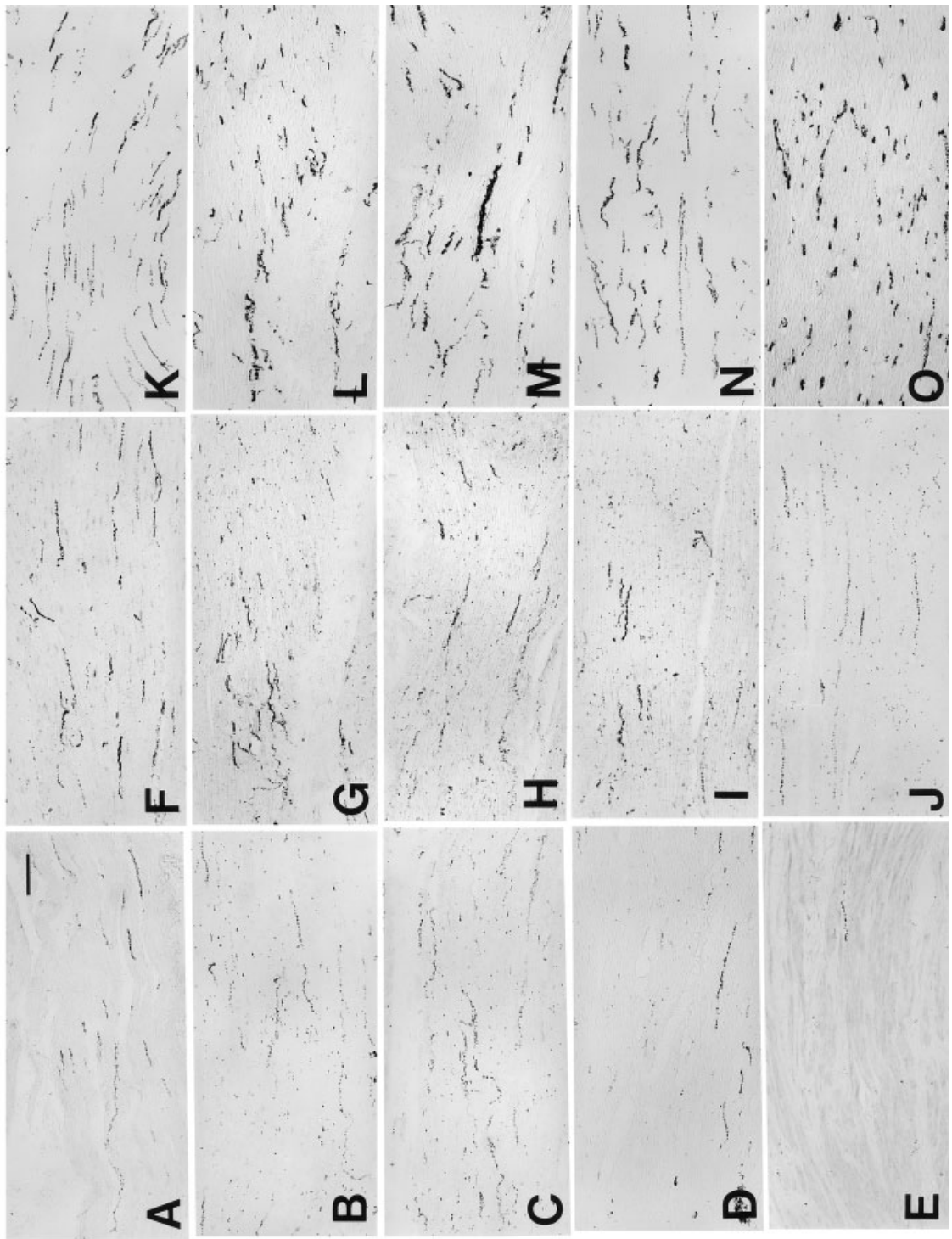
hVMAT 2
hVAChT
VIP
Stomach
Duodenum
Jejunum
Ileum
Colon
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 7

vesicles bearing the vesicular amine transporters, stained much more strongly.

The specificity of VAChT, VMAT1, and VMAT2 detection was demonstrated by preabsorption of the antisera with 25 μmol of the respective C-terminal human peptide, which resulted in complete abolition of staining of gastrointestinal neurons and nerve fibers. VMAT2 staining of enterochromaffin-like (ECL) cells of the oxyntic mucosa, VMAT1 staining of EC cells, and VMAT1 and VMAT2 staining of chromaffin cells of the human adrenal medulla was successfully abolished by homologous preabsorption.

We tested the specificity and the sensitivity of the DBH antisera in various central nervous system regions of the rhesus monkey brain and spinal cord as well as in various peripheral organs of rhesus monkey and the human adrenal medulla, nota bene locus coeruleus, paravertebral sympathetic ganglia, celiac ganglion, blood vessels of skin, kidney, spleen, lung, and adrenal gland. In the latter, the DBH antiserum nicely stained the noradrenergic cell populations without staining the PNMT-positive adrenergic cell populations.

Apparent VAChT immunoreactivity in endocrine cells of small and large intestine was not abolished after homologous preabsorption, indicating false-positive staining of these cells. VAChT mRNA was never detected in endocrine cells of the human gut.

Distribution and quantitative analysis of chemically defined intrinsic neurons

The pan-neuronal antibody PGP 9.5 was used throughout this study to label all nerve cell bodies in the myenteric and submucous plexus as basis for further quantification (Hörsch et al., 1993; Schemann et al., 1995; Costa et al., 1996; Fig. 1). With this immunohistochemical method, neuronal cell bodies were easily distinguishable from each other (Fig. 1). For the quantitative analysis, randomly selected sections from each GI region from two to four patients were used (Table 2). With the exception of the submucous plexus of the stomach, where ganglionic cells were sparsely present, approximately 200 PGP 9.5–positive ganglionic cells per region and per plexus were analyzed for each marker (Table 2). A total of 8,438 PGP 9.5–immunoreactive neurons were counted. In each section, the absolute number of neurons was counted, rather than the number of neurons per ganglion, because it was often difficult to define the boundary between individual ganglia. The proportions of VAChT-, VIP-, and VMAT2-immunostained cell somata were similar to those detected by in situ hybridization (Fig. 2).

The majority of the intrinsic neurons at all levels of the human GI tract was found to be cholinergic. In each part of the gut, 51–70% of all ganglionic cells stained for VAChT (Fig. 2, Table 2). Numerous VIP-immunoreactive neurons were seen in the submucous plexus of all GI regions (47–79%; Table 2). The highest proportion of VIP-positive ganglionic cells was found in the submucous plexus of the stomach and the large intestine (65% and 79%, respectively; Table 2). The myenteric plexus of the stomach revealed a larger number of VIP-immunoreactive neurons (59%) than the myenteric plexus of the small and large intestine (11–14%; Fig. 3; Table 2).

A considerable subpopulation of ganglionic cells of both plexus in the upper GI tract exhibited a VMAT2-positive phenotype (14–20%), as revealed by in situ hybridization and immunohistochemistry, although the proportion of VMAT2-positive ganglionic cells decreased in the lower small intestine and large intestine (1–6%; Figs. 2, 4; Table 2). TH-positive neurons were visualized in approximately identical proportions in the myenteric and submucous plexus of the stomach and the upper small intestine (13–20%; Fig. 4; Table 2). The proportion of TH-positive neurons decreased in the lower small intestine and the large intestine (0–6%; Table 2). VMAT1, 5-HT, and DBH were not seen in intrinsic neurons, although VMAT1-, 5-HT–, and DBH-positive nerve fibers surrounding ganglionic cells were seen throughout the gut (Figs. 2, 4).

Coexistence patterns of myenteric and submucous neurons

For the evaluation of coexistence patterns, adjacent sections and the two-color immunoperoxidase technique were used. The large diameter of human enteric neurons made it easily possible to identify identical neuronal cell bodies in adjacent sections. The double fluorescence analysis of neuronal perikarya was successful for TH and VIP but not for VMAT2 and VAChT.

There was an extensive overlap between VIP and VAChT in neuronal cell bodies of the gut at all levels. The majority of VIP-positive myenteric neurons of all GI regions also stained for VAChT (Fig. 3). With the exception of the submucous plexus of the large intestine, purely VIP-expressing neurons (VIP+/VAChT-) were rare. In the submucous plexus of the large intestine, a considerable population of ganglionic cells was positive for VIP but negative for VAChT.

The analysis of adjacent sections revealed coexpression of VMAT2 and TH in enteric neurons (Fig. 4). In contrast, immunoreactivity for VMAT1, DBH, TH, and 5-HT was absent from neuronal somata throughout the gut (Fig. 4). VMAT2- and TH-copositive neurons were completely distinct from cholinergic and VIP-positive neurons (Fig. 5).

A minor population of intrinsic neurons of the human upper GI tract was neither cholinergic nor catecholaminergic (NANC neurons), as judged by the lack of expression of VMAT2, TH, or VAChT. The population of NANC neurons was calculated for each region by subtracting the sum of TH- and VAChT-positive neurons from the number of PGP 9.5–positive neurons and expressed as an approximate percentage (Table 2). This calculation of the number of NANC neurons was confirmed for the stomach and the upper intestine by dual color immunohistochemistry for VAChT and TH/VMAT2 and subsequent immunofluorescence detection of PGP 9.5 (data not shown).

Fig. 7. **A–O:** Distribution pattern and oroanal variations of monoaminergic, cholinergic and vasoactive intestinal peptide (VIP)ergic terminal fields in the circular muscle. The density of vesicular acetylcholine transporter (VAChT) and VIP-immunoreactive terminals is higher than that of vesicular monoamine transporter 2 (VMAT2) terminals throughout all gastrointestinal segments. Note the decrease in VMAT2 innervation in the ileum and the marked loss of VMAT2 innervation in the colon, compared with gastric and duodenojejunal segments. Note the similar homogeneous density of VAChT and VIP terminals from stomach to ileum and a slight reduction of VAChT in the colon. Scale bar = 50 μm in A–O.

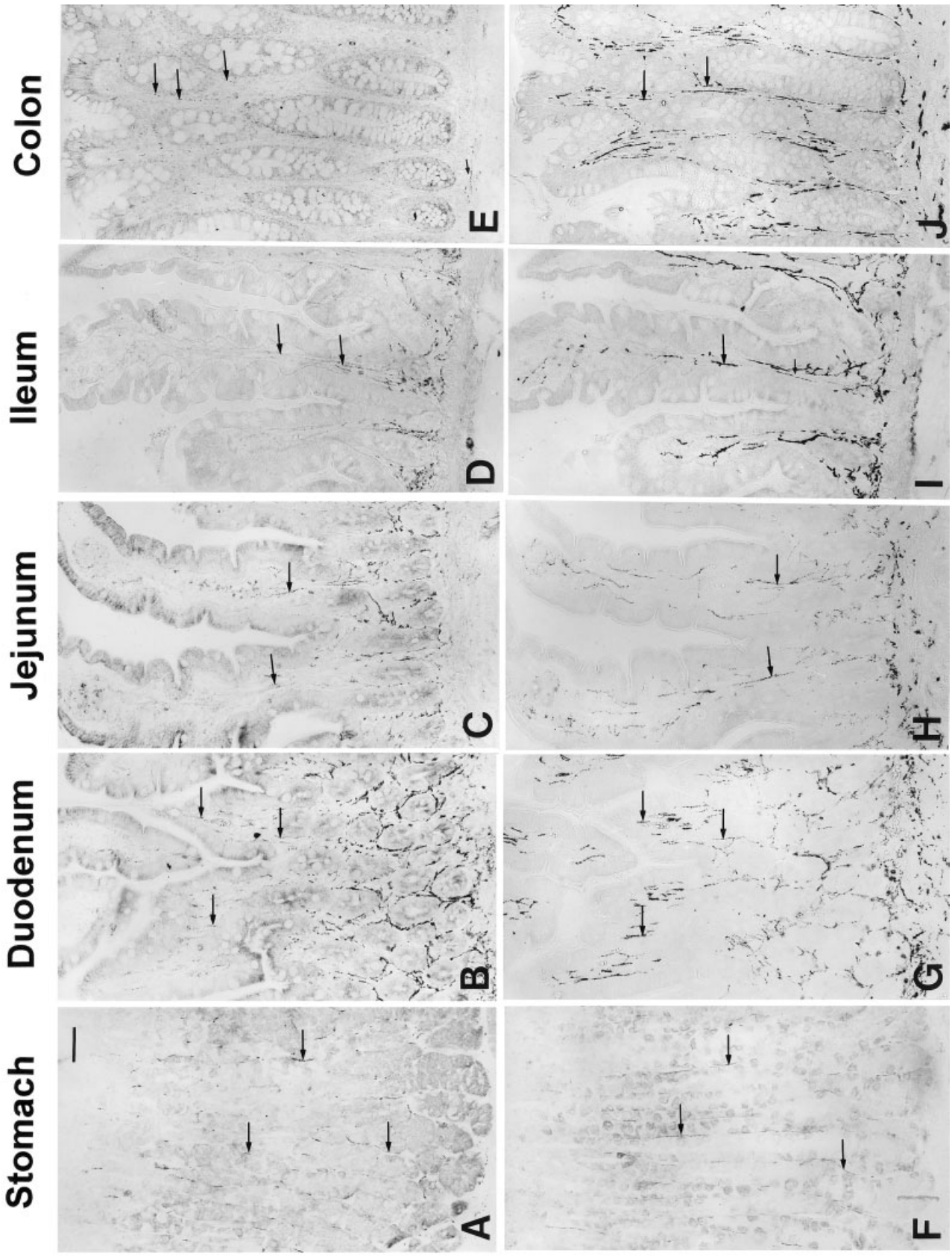
Stomach
Duodenum
Jejunum
Ileum
Colon
A
B
C
D
E
F
G
H
I
J
h V A C h T
V I P

Figure 8

Region-specific distribution of chemically defined nerve fibers

At all gut levels, the patterns of innervation by VMAT2- and TH-immunoreactive fibers revealed a high density of fibers around blood vessels and around enteric ganglia, whereas the VMAT2- and TH-positive terminal density in the mucosal and muscle layers was diminished in the upper GI tract, compared with the ileum and large intestine (Table 3). Many VMAT2- and TH-positive fibers were present in the mucosa and the smooth muscle layers of the stomach, duodenum, and upper jejunum, whereas the mucosal and the smooth muscle layers of the terminal ileum and large intestine revealed sparse VMAT2- and TH-positive nerve fibers (Figs. 6, 7; Table 3). VMAT2 and TH fibers were equally dense at all GI-levels. DBH fibers were as dense as TH and VMAT2 fibers in the perivascular plexus but less dense in other tissue compartments (Fig. 6; Table 3). VMAT1 was sparse in nerve fibers of the circular muscle layer and in fibers surrounding myenteric ganglionic cells (Fig. 2; Table 3). A few 5-HT fibers were seen in association with ganglionic cells of the myenteric plexus and in the outer smooth muscle layers but not in other tissue compartments of the gut wall (Table 3).

Patterns of innervation by VIP-positive and cholinergic (VAChT+) fibers suggest that VIPergic innervation was of relatively constant density in the oroanal direction, whereas the VAChT-positive terminal density and intensity were diminished in large compared with small bowel in the mucosal though not the muscular layer (Figs. 7, 8; Table 3). There was a high density of VIP- and VAChT-positive terminal fibers in the smooth muscle layers throughout all GI regions (Fig. 7; Table 3). In the longitudinal muscle, cholinergic and VIP-positive nerve fibers were less dense than in the circular muscle layer (Table 3). Mucosal VAChT fibers were abundant in the mucosa of the stomach, duodenum, and jejunum but less dense in the ileum. The mucosa of the large intestine revealed only scattered VAChT-positive fibers, which were less dense and less strongly stained than VIP terminals (Fig. 8; Table 3). In contrast, VIP-positive mucosal innervation was of constant density throughout all mucosal regions examined (Fig. 8; Table 3).

Dual color immunohistochemistry and Giemsa staining revealed close apposition of mucosal VAChT, VIP, VMAT2, and TH-immunoreactive fibers to mast cells, smooth muscle cells of the villi, and endocrine cells. The close association of chemically defined nerve fibers to endocrine cells of the stomach and the intestine is shown in Figure 9. Blood vessels of all layers were innervated by cholinergic and VIP-containing nerve fibers, which were outnumbered by noradrenergic (VMAT2+/TH+/DBH+) fibers (Fig. 10; Table 3).

Tissue compartment-specific coexistence patterns of nerve fibers

Double fluorescence revealed target-specific coexistence patterns in nerve fibers. In detail, the following patterns were seen: in nerve fibers of all layers, VAChT and VIP showed a substantial overlap with minor populations of purely VIP-positive or purely cholinergic (VAChT+) nerve fibers (Fig. 11; Table 4). Cholinergic/VIPergic (VAChT+/VIP+) and monoaminergic (VMAT2/TH/VMAT1 or DBH-positive) fibers were strictly distinct (Fig. 11; Table 4). VMAT2- and TH-positive nerve fibers were visualized in approximately identical proportions in all layers. However, some VMAT2-positive fibers in the muscle and mucosal layers appeared to lack TH expression. They may correspond to projections of intrinsic serotoninergic neurons. There was a full overlap between VMAT2, TH, and DBH in the perivascular plexus (Table 4). In contrast, DBH-positive fibers of the muscular and mucosal layers comprised a minor portion of VMAT2- and TH-copositive fibers (Fig. 11; Table 4). VMAT2, TH, and DBH-positive fibers were strictly separated from VAChT- and VIP-immunoreactive nerve fibers (Table 4). VMAT1 immunoreactivity was exclusively seen in TH/DBH-positive fibers and, therefore, comprised a minor population within the individual TH- and DBH-immunoreactive fibers (Fig. 11; Table 4). Immunofluorescence labeling failed to reveal any immunoreactivity for 5-HT in nerve fibers.

DISCUSSION

The present study provides for the first time a full account of cholinergic and monoaminergic phenotypes of the innervation throughout the human gut. The results of our examination indicate that each region of the human GI tract is supplied differentially by enteric neurons and nerves containing specific sets of neurochemical markers. In the following sections, we shall discuss the classes of neurons and the patterns of terminal fields characterized in our study. Figure 12 summarizes our attempt to classify chemically defined intrinsic and extrinsic phenotypes of neurons and putative projections of intrinsic neurons.

Cholinergic phenotypes

We have shown that, in all regions of the human GI tract, the majority of intrinsic neurons are cholinergic. VAChT is a reliable marker for cholinergic neurons and nerve fibers (Schäfer et al., 1994, 1995, 1998; Weihe et al., 1996; Arvidsson et al., 1997; Schütz et al., 1998; Weihe and Eiden, 2000). It is, therefore, likely that the identified VAChT-positive neurons are able to synthesize and release acetylcholine (ACh). Studies of cholinergic neurons and fibers in the human gut have been performed previously with the enzyme marker ChAT in the terminal ileum, the colon, and the submucous plexus of rectum (Porter et al., 1996; Schneider et al., 2001). No comparable data on cholinergic neurons and their terminal fields in other regions of the human gut have been published.

Fig. 8. **A–J:** Distribution and oroanal variations of cholinergic and vasoactive intestinal peptide (VIP)ergic terminal fields in the mucosa. Delicately stained vesicular acetylcholine transporter (VAChT) -positive terminals and more broadly stained VIP-positive terminals are present in the lamina propria throughout all gut segments. Sparse to moderate density of VAChT and VIP terminals (arrows in A,B) in the lamina propria of the stomach. VAChT terminal field density in the lamina propria and mucosal muscle layer is highest in the duodenum (B) and decreases toward the colon (E). Note the presence of VAChT (B,C) and VIP terminals (G,H) up to apical regions of duodenojejunal villi. VAChT but not VIP immunoreactivity is reduced in the apex of ileal villi (arrows in D, I) compared with duodenum and jejunum. In the colon, VAChT terminal fields in the lamina propria appear to be less dense and less strongly stained than VIP terminals. Note the presence of strongly staining VIP terminals (H–J) in the mucosal muscle layer, where VAChT terminals are only faintly stained (C–E with arrow in E). Scale bar = 50 μm in A–F.

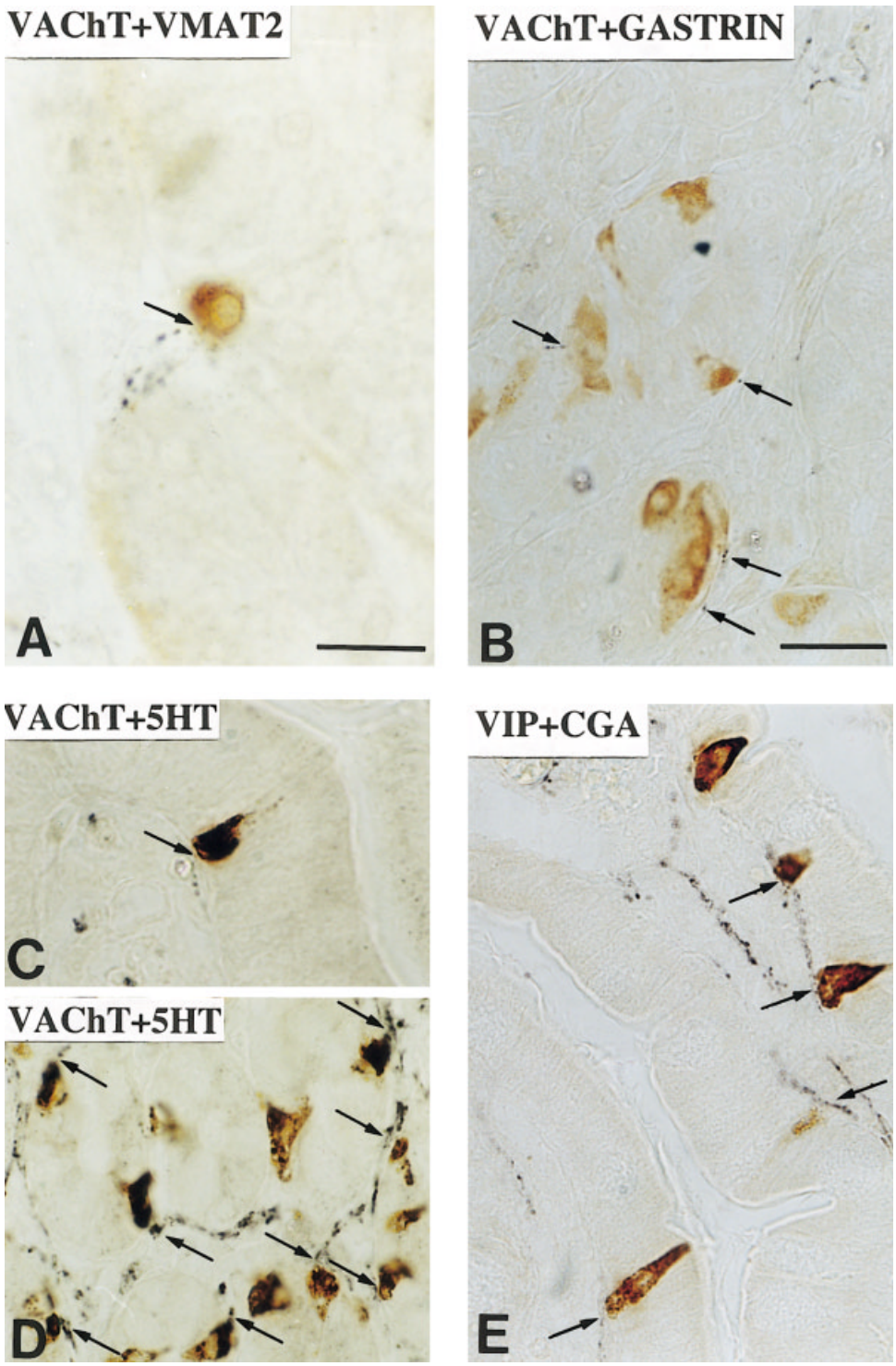

Fig. 9. Close association of nerve fibers to neuroendocrine cells of the gut. Dual color immunohistochemistry revealing close association of chemically defined nerve terminals to endocrine cells of the gastrointestinal mucosa. **A:** vesicular acetylcholine transporter (VAChT) -positive terminals (black) and vesicular monoamine transporter 2 (VMAT2) -positive enterochromaffin-like (ECL) cell of the oxyntic mucosa (brown). **B:** VAChT-positive terminals (black) and gastrin cells of the antral mucosa (brown). **C,D:** VAChT-positive nerve fibers (black) and serotonin (5-HT) -positive EC cells of the villus (C) and the crypts (D) of the duodenum (brown). **E:** Vasoactive intestinal peptide (VIP) -positive fibers (black) and chromogranin A (CgA) -positive endocrine cells of the duodenum (brown). Scale bars = 15 μm in A,C–E, 20 μm in B.

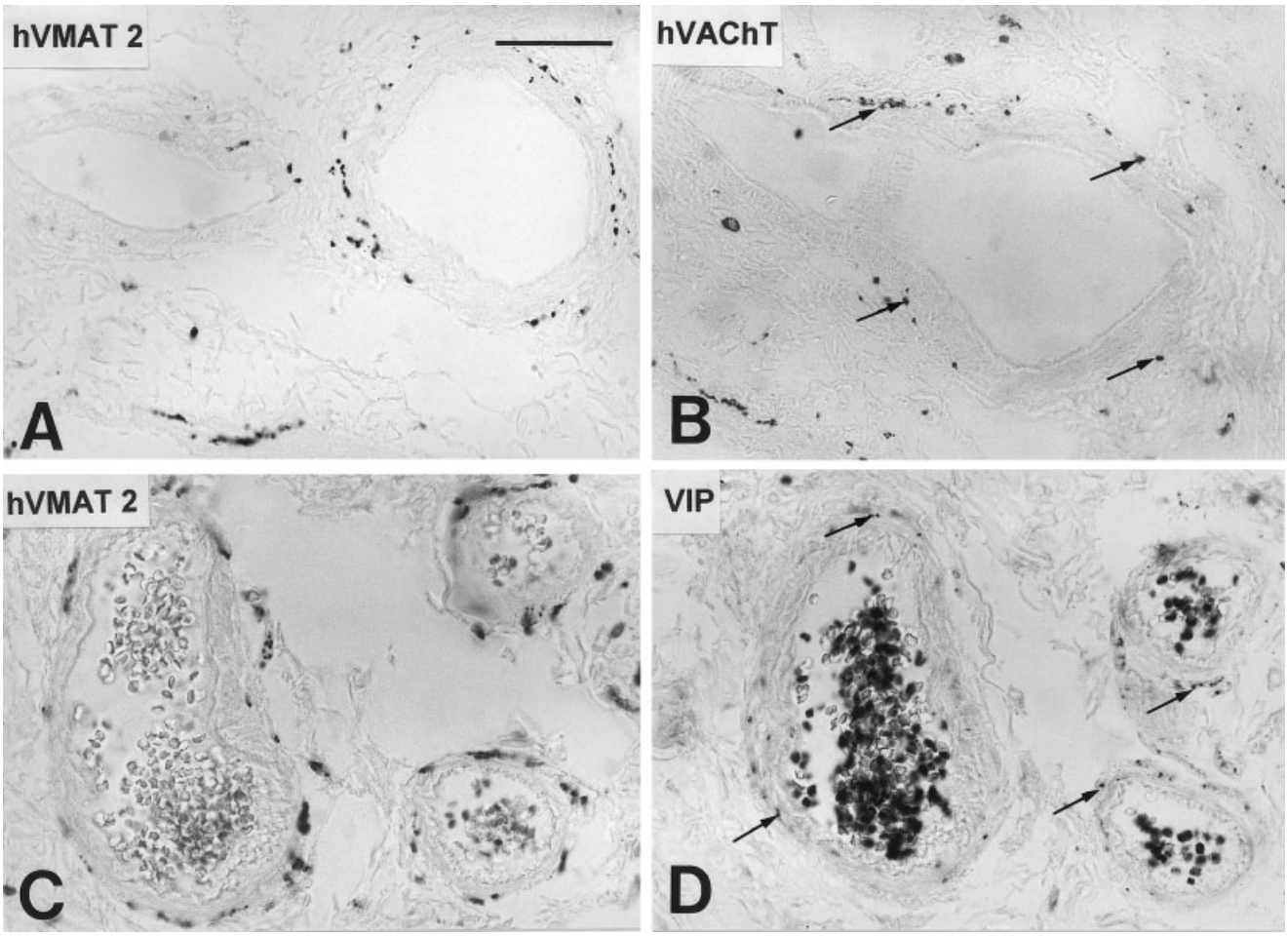

Fig. 10. Catecholaminergic and cholinergic/vasoactive intestinal peptide (VIP)ergic coinnervation of submucosal blood vessels. **A,B:** Duodenum. Adjacent sections demonstrating innervation of the same submucous blood vessel with numerous differentially distributed vesicular monoamine transporter 2 (VMAT2) and vesicular acetylcholine transporter (VAChT) -positive terminals. **C,D:** Jejunum. Adjacent sections demonstrating innervation of the same submucous blood vessel with numerous differentially distributed VMAT2- and VIP-positive terminals. Scale bar = 30 μm in A–D.

The detection of cholinergic nerve fibers with VAChT reveals terminal fields that are far more extensive than previously described in humans (Porter et al., 1996). As we demonstrated, the mucosa of the upper GI tract is supplied by numerous VAChT-positive fibers and the density of mucosal cholinergic fibers is decreasing in the anal direction. This observation suggests that cholinergic innervation has a region-specific influence on mucosal functions. The high density of VAChT fibers in the outer muscle layers of all GI regions is consistent with earlier observations that ACh released from cholinergic motor neurons is involved in the contraction of the gut (Benett, 1965; Porter et al., 1996). The close association between cholinergic varicose fibers and mast cells, immunocytes, and neuroendocrine cells indicates that ACh is involved in neuroimmune and neuroendocrine functions of the gut.

In contrast to earlier observations indicating the existence of a specific cholinergic (ChAT-immunoreactive) endocrine cell (McMurray et al., 1993; Porter et al., 1996), VAChT in situ hybridization and immunohistochemistry provided no evidence for the existence of a specific cholinergic endocrine cell. Our results show that detection and quantification of cholinergic neurons with VAChT offers the advantage to analyze the enteric cholinergic neurons in paraffin-embedded tissue with high sensitivity and high resolution and reveal terminal fields far more extensive than previously described in human.

VIP and VAChT coexistence patterns

VIP/ACh coexistence is very common in the parasympathetic nervous system. However, in the enteric nervous system of guinea pig and other mammalian species, VIP is considered to be primarily a NANC transmitter. Numerous studies have shown that, in the guinea pig, most enteric cholinergic neurons were devoid of VIP (Furness et al., 1984, 1995; Brookes et al., 1991; Jacobson et al., 1994; Schemann et al., 1995; Costa et al., 1996; Vanden Berghe et al., 1999; Lomax and Furness, 2000; Furness and Lomax, 2000). VIP and ChAT are strictly distinct in myenteric motor neurons and nerve fibers innervating the outer smooth muscle layers and in myenteric primary afferent

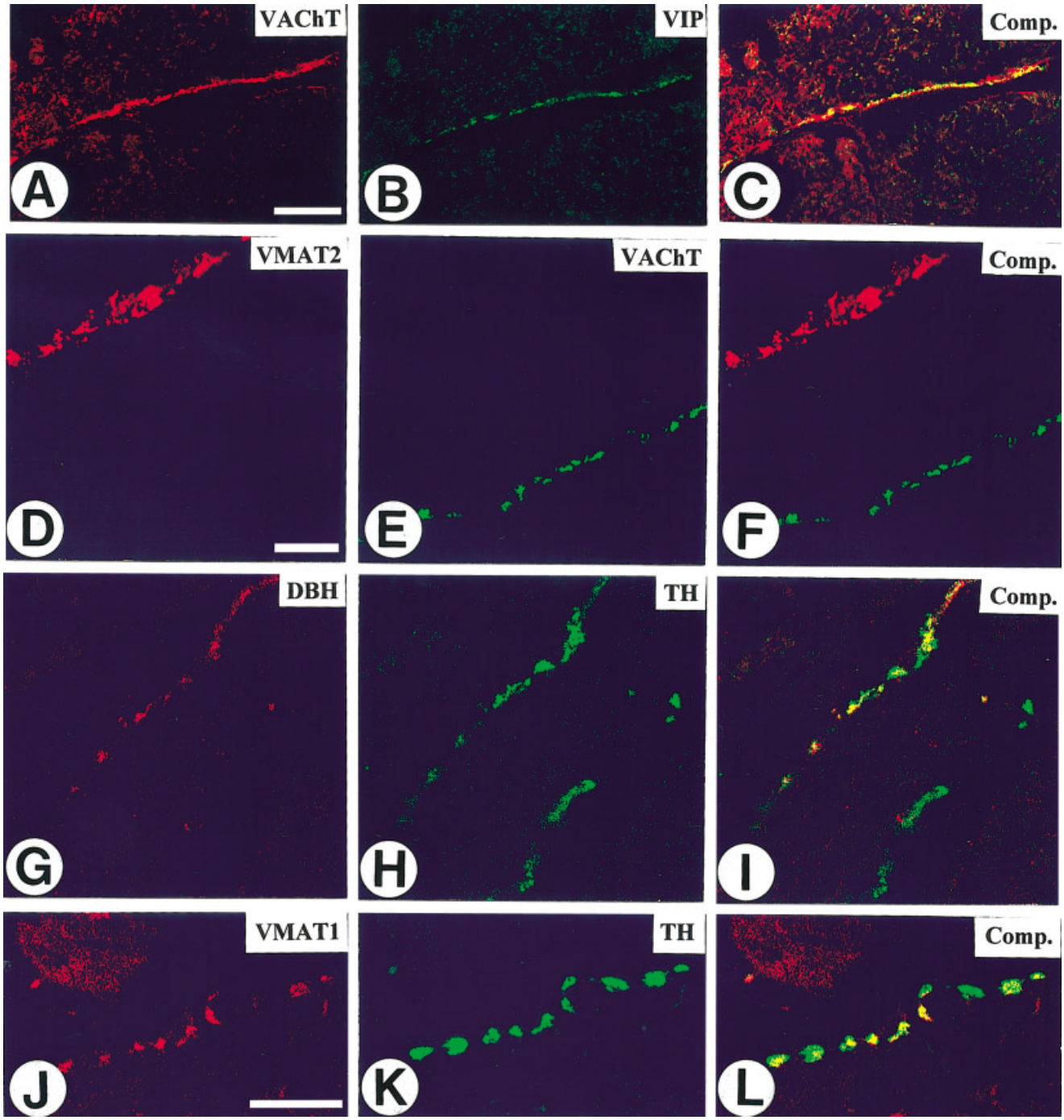

Fig. 11. Coexistence patterns of nerve fibers. The high-magnification confocal laser scanning microscopy of nerve fibers supplying the mucosa and the smooth muscle layers of the stomach reveals completely overlapping vesicular acetylcholine transporter (VAChT) and vasoactive intestinal peptide (VIP) immunoreactivity (**A–C**), strict segregation of vesicular monoamine transporter 2 (VMAT2) and VAChT (**D–F**), presence of dopamine β-hydroxylase (DBH) in a subpopulation of tyrosine hydroxylase (TH) -positive fibers (**G–I**) and the presence of VMAT1 in TH-positive fibers (**J–L**). Scale bars = 30 μm in A–C, 15 μm in D–I, 10 μm in J–L.

neurons. Rare ChAT/VIP-copositive neurons in the myenteric plexus of the guinea pig and mouse were classified as ascending, descending, or intestinofugal neurons (Furness et al., 1990, 1995; Brookes et al., 1991; Messenger and Furness, 1991; Mann et al., 1995; Schemann et al., 1995; Costa et al., 1996; Sang and Young, 1998; Sharkey et al., 1998; Lomax and Furness, 2000). A colocalization of VIP and ChAT was never observed in the submucous plexus of guinea pig (Furness et al., 1984; Costa et al., 1996; Neunlist and Schemann, 1998; Lomax and Furness, 2000).

TABLE 4. Coexistence Patterns in Nerve Fibers and Their Specific Target Relations (Double-Fluorescence Analysis)[1]

Antigen	Myenteric plexus	Submucous plexus	Smooth muscle	Mucosa	Blood vessels
VAChT/VIP	+/+	+/+	+/+	+/+	+/+
VAChT/VMAT2	−	−	−	−	−
VAChT/TH	−	−	−	−	−
VAChT/DBH	−	−	−	−	−
VAChT/VMAT1	−	−	−	−	−
VMAT2/VIP	−	−	−	−	−
VMAT2/TH	+/−	+/−	+/−	+/−	+
VMAT2/DBH	+/−	+/−	+/(−)	+/(−)	+
VMAT1/VAChT	−	−	−	−	−
VMAT1/VIP	−	−	−	−	−
VMAT1/TH	−/+	abs/+	(−)/+	abs/+	abs/+
VMAT1/DBH	−/+	abs/+	(−)/+	abs/+	abs/+

[1]+, complete colocalization, neither antigen found without the other; +/−, colocalized, but first antigen as listed in antigen column also found alone; −/+, colocalized, but second antigen as listed in antigen column also found alone; +/+, colocalized, but both antigens also found alone; −, no colocalization, but presence of both antigens in separate fibers; +/abs., presence of first antigen, but absence of second antigen; abs./+, absence of first antigen, but presence of second antigen. Parentheses indicate extremely sparse presence of the respective antigens. For abbreviations, see Table 1.

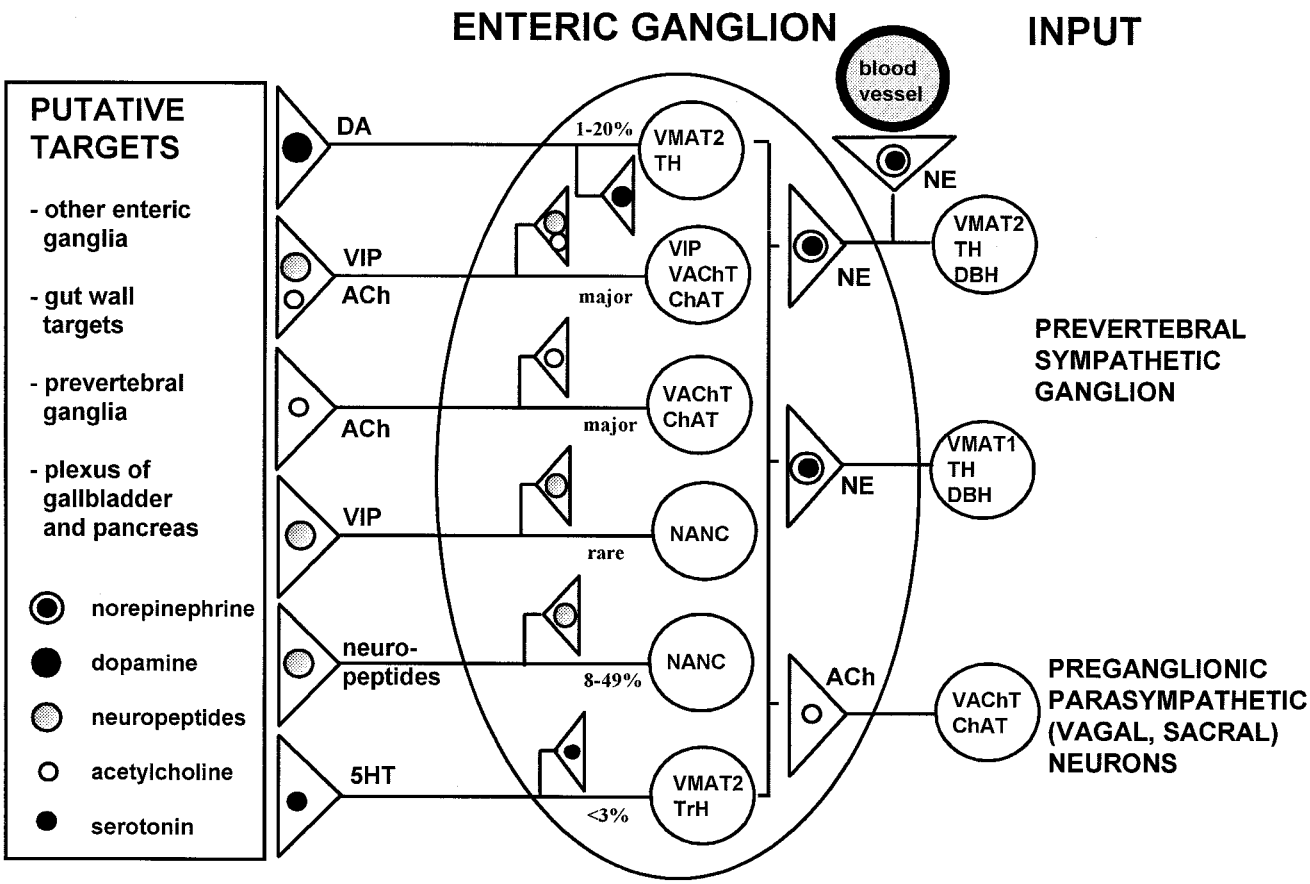

Fig. 12. Principle categories of neurotransmitter phenotypes in intrinsic and extrinsic innervation of the human gut. The figure summarizes the chemical phenotypes of the sympathetic and parasympathetic extrinsic innervation of the human gut (Input) and the chemical phenotypes of intrinsic gut neurons (Enteric ganglion) with respect to cholinergic and catecholaminergic phenotypes based on our observations and previously published data. Furthermore, major putative targets of intrinsic neurons are summarized. Prevertebral sympathetic neurons are characterized by the presence of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) and are extrinsic-noradrenergic. Both vesicular monoamine transporter 1 (VMAT1) and VMAT2 were found in separate sympathetic neurons of the prevertebral ganglia. The efferent vagal cholinergic innervation of the gut is sparse and is characterized by the presence of vesicular acetylcholine transporter (VAChT) and ChAT. Connections to the dorsal root ganglia (DRG), the sympathetic ganglia, and vagal afferences are not shown. The enteric nervous system consists of a large number of intrinsic neurons and mainly functions autonomously. Intrinsic monoaminergic neurons of the gut are characterized by their content of VMAT2 and TH but absence of DBH and are, therefore, presumably dopaminergic. Acetylcholine and vasoactive intestinal peptide (VIP) are copresent in a considerable population of enteric neurons. Populations of purely cholinergic (VAChT- and ChAT-positive) and purely VIPergic (nonadrenergic, noncholinergic [NANC]) neurons are also present. NANC neurons are characterized by their content of VIP or other neuropeptides. A minor population of VMAT2-positive neurons is serotoninergic, as characterized by their content of TrH (tryptophan hydroxylase).The putative target relationships of intrinsic neurons included other enteric ganglionic cells and cell body free parts of the plexus, diverse targets of the gut wall with special differences for different compartments, as well as extraintestinal targets of enteric neurons such as projections to the prevertebral ganglia and the plexus of gallbladder and pancreas.

In contrast to these observations in guinea pigs, our study demonstrated that, in humans, with the exception of the submucous plexus of the colon, VIP and VAChT are mainly colocalized in neurons and nerve fibers. VIP is a regular cholinergic cotransmitter candidate in the intrinsic innervation of the human gut. Our results are in accordance with earlier studies on other parts of the primate autonomic nervous system. For example, studies on the lung of humans and rhesus monkeys revealed coexpression of VIP and ChAT in virtually all neuronal cell bodies of intrinsic peribronchial ganglia, whereas in the lung of guinea pig VIP and ChAT were strictly separated (Fischer et al., 1995; Maggi et al., 1995; Nohr et al., 1995). Our results are in accordance with those of Schneider et al. (2001), who showed that, in the human rectum, a major proportion of submucous ganglionic neurons costained for VIP and VAChT.

VIP is a potent mediator of smooth muscle relaxation and stimulates intestinal secretion of fluid and electrolytes (Grider, 1989; Grider et al., 1992; Binder and Sandle, 1994; Jacobson et al., 1994). VIP has been demonstrated in human myenteric inhibitory motor neurons and in interneurons (Furness and Costa, 1979; Wattchow et al., 1997). It has been suggested that VIP enhances the secretory response evoked by electrical stimulation of cholinergic neurons (Cooke et al., 1987; Cooke and Reddix, 1994). Our results in the human gut suggest a regulatory influence of both transmitters released from identical neurons. A major role of VIP in intrinsic cholinergic neurons might be the modulation of cholinergic neurotransmission.

The presence of a considerable population of purely VIP-positive (noncholinergic) neurons in the submucous plexus and the high density of purely VIP-containing mucosal fibers in the human colon indicates that they function independent of cholinergic neurotransmission. Of interest, VIP has been localized to sensory nerves of human large intestine and can be released from enteric nerves by capsaicin treatment (Maggi et al., 1989, 1990; Jacobson et al., 1994). Capsaicin releases neurotransmitters from primary sensory neurons. Therefore, it is conceivable that, among the numerous VIPergic (noncholinergic) terminals in the human colon, there is a population of primary sensory fibers projecting from dorsal root ganglia.

Catecholaminergic phenotypes

We identified a population of intrinsic biogenic monoaminergic neurons and nerve fibers in all regions of the human gut that were immunoreactive for VMAT2 and TH but negative for DBH and 5-HT. Monoaminergic neurons of the mammalian gut were previously designated as purely serotoninergic. The synthesis, presence, release on adequate stimuli, and physiological action of neuronal 5-HT in the gut are well documented (Gershon et al., 1965, 1977, 1983, 1994; Tamir and Gershon, 1981; Costa et al., 1982, 1996; Furness and Costa, 1982; Griffith and Burnstock, 1983; Kurian et al., 1983; Meedeniya et al., 1998). Immunohistochemical studies demonstrated 5-HT in less than 5% of myenteric neurons (Costa et al., 1982; Furness and Costa, 1982; Griffith and Burstock, 1983; Schemann et al., 1995; Costa et al., 1996; Vanden Berghe et al., 1999). We found a few 5-HT–positive fibers in the myenteric plexus and the outer smooth muscle layer but no serotonin-positive cell bodies. Rare VMAT2-positive myenteric cell bodies that appeared in adjacent sections to lack TH and DBH may correspond to intrinsic serotoninergic neurons. Our failure to demonstrate 5-HT–immunoreactive neuronal cell bodies may be because pharmacologic treatment is necessary to visualize serotoninergic cell bodies. It has been shown that, in freshly fixed tissue, the levels of 5-HT in enteric neurons are very low and have to be increased for immunohistochemical detection by preloading the tissue with 5-HT or its analogues, by administration of monoamine oxidase (MAO) inhibitors or by treatment with reserpine (Robinson and Gershon, 1971; Costa and Furness, 1979; Costa et al., 1982; Furness and Costa, 1982; Kurian et al., 1983; Erde et al., 1985; Mawe and Gershon, 1989; Schemann et al., 1995; Meedeniya et al., 1998; Vanden Berghe et al., 1999; Lomax and Furness, 2000). In untreated tissue, 5-HT was weak or absent from neuronal cell bodies (Robinson and Gershon, 1971; Dubois and Jacobowitz, 1974; Dreyfus et al., 1977; Costa et al., 1982; Keast et al., 1984; Hörsch et al., 1993; Belai et al., 1997).

To our surprise, immunohistochemistry and in situ hybridization revealed a large number of VMAT2-positive neurons (14–20%) in both plexus of the stomach and upper intestine. In contrast, VMAT2-positive neurons were rare in the ileum and large intestine (1–6%). These VMAT2-positve neurons as well as a major population of nerve fibers innervating the mucosal and muscular layers of the upper GI tract were regularly copositive for TH, but negative for DBH, and are, therefore, catecholaminergic. It has been well documented in rodents that catecholaminergic neurons appear during development in the gut (Cochard et al., 1978; Teitelman et al., 1978). In rodents, intrinsic catecholaminergic innervation of the gut is present during development, but it is lost upon gut maturation and, therefore, is transient. These vagal crest–derived TH-positive neurons were defined as transient catecholaminergic cells (TC cells), and it was suggested that they are precursors of noncatecholaminergic neurons in the adult (Teitelman et al., 1981; Gershon et al., 1984; Baetge et al., 1990). On the other hand, there is some evidence for the presence of TH in enteric neurons of the adult human gut and for a down-regulation of TH along with reduction of gut dopamine levels in Parkinson patients (Eaker et al., 1988; Wakabayashi et al., 1989, Singaram et al., 1995), which is in accordance with our results.

The presence of VMAT1 in the nervous system has not been reported previously. Because both VMAT1 and VMAT2 were found in sympathetic neurons of the coeliac ganglion (unpublished observations), sparsely present VMAT1+/TH+/DBH+ fibers in the human gut can be characterized as extrinsic, postganglionic, and noradrenergic. The minor VMAT1 system in the adult postganglionic sympathetic system may represent a developmental vestige of VMAT1-positive neurons, which were abundant during embryogenic development in the rodents (Schütz et al., 1998). The human gut may be characterized by the persistence of VMAT1 neuronal phenotypes into adulthood, something that is not seen in rodents.

CONCLUSIONS

Our study of the human enteric nervous system indicates that there are some similarities between primates and other mammalian species but that there are also fundamental differences. The anatomic organization of

the cholinergic nervous system is similar to other mammalian species. In the human gut, VIP is a cholinergic cotransmitter in intrinsic gut neurons. The intrinsic catecholaminergic phenotype that is lost upon gut maturation in rodents is a stable component of the human gastrointestinal tract throughout life. TH and VMAT2 coexist in intrinsic neurons and do not contain DBH. Hence, these neurons of the human gut are dopaminergic. In this context, it is interesting to note the statement by Burks (1995), that "Dopaminergic neurotransmission in the enteric nervous system regulates motility and perhaps other gastrointestinal function." These findings reviewed by Burks are based on ample pharmacologic and clinical evidence. However, the possibility that there is a partial aminergic phenotype as observed in other species and locations (Furness and Costa, 1978; Morris and Gibbins, 1987; Kummer et al., 1990, 1993; Elfvin et al., 1997; Schütz et al., 1998; Weihe and Eiden, 2000) has to be also taken into consideration.

As TH and VMAT2 never costain for VAChT, this system is clearly noncholinergic. Thus, the intrinsic innervation of the human gut can be divided into three major components with respect to cholinergic and catecholaminergic phenotypes: (1) cholinergic, (2) catecholaminergic (dopaminergic), and (3) noncholinergic, nonadrenergic (NANC). The NANC system in the human GI tract represents a minor population of ganglionic cells (with segmental variations) and is characterized by neuropeptides that we have not yet examined. The DBH+/TH+ innervation is purely extrinsic, as DBH was never found in intrinsic neurons of the human gut. Therefore, VMAT2+/TH+/DBH+ nerve fibers represent the extrinsic, postganglionic, noradrenergic sympathetic innervation of the human.

It will be now possible to further classify these three major components in human with respect to various peptidergic phenotypes and co-phenotypes known to comprise distinct functional subsets in various species (Gershon et al., 1994; Furness et al., 1999). Given the data available in the literature for various species, complicated subset patterns of various peptide coexistences are to be expected (for review, see Gershon et al., 1994; Furness et al., 1999). Further characterization of these newly categorized three main components of the human enteric nervous system with respect to their specific peptidergic subsets is certainly in order for future investigations. In addition, it will be necessary to characterize the neuropeptide spectrum of the human enteric NANC system and of the minor VIPergic and serotoninergic intrinsic neurons. It should be mentioned here that the possibility will have to be considered that the enteric nervous system contains also inhibitory or excitatory transmitters, which can be now investigated by using newly established specific markers like VIAAT (the vesicular inhibitory amino acid transporter for GABA and glycine; Eiden, 2000) and the vesicular glutamate transporter isoforms VGLUT1, VGLUT2, and VGLUT3 (Varoqui et al., 2002).

Future studies have to demonstrate adequate mechanisms for dopamine-uptake, dopamine-release on nerve stimulation and dopamine-inactivation in the human enteric nervous system to further characterize dopamine as neurotransmitter of the human gut. Intrinsic dopaminergic neurons in the human GI-tract may become an important matter for investigations of pathophysiology and treatment of acute and chronic inflammation and Parkinson's disease.

ACKNOWLEDGMENTS

We thank Petra Lattermann, Heidí Hlawaty, Petra Sack, Elke Rodenberg-Frank, Marion Zibuschka, and Christian Fiebiger for the excellent technical assistance, and Heidemarie Schneider and Waldemar Strauss for the brilliant photo documentation. We also thank Prof. M. Rothmund, Dr. C. Nies, and Dr. B. Stinner, Department of General Surgery, University of Marburg, for providing human tissue and K. Dege for critically reading the article.

LITERATURE CITED

Angerer LM, Stoler MH, Angerer RC. 1984. In situ hybridization with RNA-probes: an annoted recipe. In: Valentino L, Eberwine I, Barchas D, editors. In situ hybridization: application to neurobiology. New York: Oxford University Press. p 42–70.

Anlauf M, Weihe E, Hartschuh W, Hamscher G, Feurle G. 2000. Localization of xenin-immunoreactive cells in the duodenal mucosa of humans and various mammals. J Histochem Cytochem 48:1617–1626.

Arvidsson U, Riedl M, Elde R, Meister B. 1997. Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous system. J Comp Neurol 378:454–467.

Baetge G, Gershon MD. 1989. Transient catecholaminergic (TC) cells in the vagus nerves and bowel of fetal mice: relationship to the development of enteric neurons. Dev Biol 132:189–211.

Baetge G, Pintar JE, Gershon MD. 1990. Transiently catecholaminergic (TC) cells in bowel of the fetal rat: precursors of noncatecholaminergic enteric neurons. Dev Biol 141:353–380.

Belai A, Boulos PB, Robson T, Burnstock G. 1997. Neurochemical coding in the small intestine of patients with Crohn's disease. Gut 40:767–774.

Benett A. A pharmacological investigation of human isolated ileum. Nature 208:1289–1291.

Binder HJ, Sandle GI. 1994. Electrolyte transport in the mammalian colon. In: Johnson LR, Alpers DH, Christensen J, Jacobson ED, Walsh JH, editors. Physiology of the gastrointestinal tract. New York: Raven press. p 2133–2171.

Bornstein JC, Furness JB. 1988. Correlated electrophysiological and histochemical studies of submucous neurons and their contribution to understanding enteric neural circuits. J Auton Nerv Sys 25:1–13.

Brookes SJ, Steele PA, Costa M. 1991. Identification and immunohistochemistry of cholinergic and noncholinergic circular muscle motor neurons in the guinea pig small intestine. Neuroscience 42:863–878.

Burks TF. 1995. Neurotransmission and neurotransmitters. In: Johnson LR, Alpers DH, Christensen J, Jacobson ED, Walsh JH, editors. Physiology of the gastrointestinal tract. New York: Raven press. p 211–242.

Carnahan JF, Anderson DJ, Patterson PH. 1990. Evidence that enteric neurons may derive from the sympathoadrenal lineage. Dev Biol 148: 552–561.

Cochard P, Goldstein M, Black IB. 1978. Ontogenetic appearance and disappearance of tyrosine hydroxylase and catecholamines in the rat embryo. Proc Natl Acad Sci U S A 6:2986–2990.

Cooke HJ, Reddix RA. 1994. Neural regulation of intestinal electrolyte transport. In: Johnson LR, Alpers DH, Christensen J, Jacobson ED, Walsh JH, editors. Physiology of the gastrointestinal tract. New York: Raven press. p 2083–2132.

Cooke HJ, Zafirova M, Carey HV, Walsh HJ, Grider J. 1987. Vasoactive intestinal polypeptide actions on the guinea pig intestinal mucosa during neural stimulation. Gastroenterology 92:361–370.

Costa M, Furness JB. 1971. Storage, uptake and synthesis of catecholamines in the intrinsic adrenergic neurones in the proximal colon of the guinea-pig. Z Zellforsch Mikrosk Anat 120:364–385.

Costa M, Furness JB. 1979. On the possibility that an indolamine is a neurotransmitter in the gastrointestinal tract. Biochem Pharmacol 28:565–571.

Costa M, Furness JB, Cuello AC, Verhofstad AA, Steinbusch HW, Elde RP. 1982. Neurons with 5-hydroxytryptamin-like immunoreactivity in the

enteric nervous system: their visualization and reactions to drug treatment. Neuroscience 7:351–363.

Costa M, Brookes SJH, Waterman S, Mayo S. 1992. Enteric neuronal circuitry and transmitters controlling intestinal motor function. In: Holle G, Wood JD, editors. Advances in the innervation of the gastrointestinal tract. Amsterdam: Elsevier. p 115–121.

Costa M, Brookes SJH, Steele PA, Gibbins I, Burcher E, Kandiah CJ. 1996. Neurochemical classification of myenteric neurons in the guinea-pig ileum. Neuroscience 75:949–967.

Crowe R, Kamm MA, Burnstock G, Lennard-Jones JE. 1992. Peptide-containing neurons in different regions of the submucous plexus of human sigmoid colon. Gastroenterology 102:461–467.

Dubois A, Jacobowitz DM. 1974. Failure to demonstrate serotoninergic neurons in the myenteric plexus of the rat. Cell Tissue Res 150:493–496.

Dreyfus CF, Bornstein MB, Gershon MD. 1977. Synthesis of serotonin by neurons of the myenteric plexus in situ and in organotypic tissue culture. Brain Res 128:125–139.

Eaker EY, Bixler GB, Dunn AJ, Moreshead WV, Mathias JR. 1988. Dopamine and norepinephrine in the gastrointestinal tract of mice and effects of neurotoxins. J Pharmacol Exp Ther 244:438–442.

Edwards L, Quigley EM, Hofman R, Pfeiffer RF. 1993. Gastrointestinal symptoms in Parkinson disease: 18-month follow-up study. Mov Disord 8:83–86.

Eiden LE. 2000. The vesicular monoamine transporters: current perspectives and future prospects. FASEB J 14:2396–2400.

Elfvin LG, Holmberg K, Emson P, Schemann M, Hokfelt T. 1997. Nitric oxide synthase, choline acetyltransferase, catecholamine enzymes and neuropeptides and their colocalization in the anterior pelvic ganglion, the interior mesenteric ganglion and the hypogastric nerve of the male guinea pig. J Chem Neuroanat 14:33–49.

Erde SM, Sherman D, Gershon MD. 1985. Morphology and serotoninergic innervation of physiologically identified cells of the guinea pig's myenteric plexus. J Neurosci 5:617–633.

Erickson JD, Eiden LE. 1993. Functional identification and molecular cloning of a human brain vesicle monoamine transporter. J Neurochem 61:2314–2317.

Erickson JD, Varoqui H, Schafer MKH, Modi W, Diebler MF, Weihe E, Rand J, Eiden LE, Bonner TI, Usdin TB. 1994. Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus. J Biol Chem 269:21929–21932.

Erickson JD, Schäfer MK, Bonner TI, Eiden LE, Weihe E. 1996. Distinct pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human vesicular monoamine transporter. Proc Natl Acad Sci U S A 93:5166–5171.

Fiorica-Howells E, Maroteaux L, Gershon MD. 2000. Serotonin and the 5-HT(2B) receptor in the development of enteric neurons. J Neurosci 20:294–305.

Fischer A, Canning BJ, Kummer W. 1995. Correlation of vasoactive intestinal peptide and nitric oxide synthase with choline acetyltransferase in the airway innervation. Ann N Y Acad Sci 805:717–722.

Furness JB, Costa M. 1971. Morphology and distribution of intrinsic adrenergic neurons in the proximal colon of the guinea-pig. Z Zellforsch Mikrok Anat 120:346–363.

Furness JB, Costa M. 1978. Distribution of intrinsic nerve cell bodies and axons which take up aromatic amines and their precursors in the small intestine of the guinea-pig. Cell Tissue Res 188:527–543.

Furness JB, Costa M. 1979. Projections of intestinal neurons showing immunoreactivity for vasoactive intestinal polypeptide are consistent with these neurons being enteric inhibitory neurons. Neurosci Lett 15:199–204.

Furness JB, Costa M. 1982. Neurons with 5-hydroxytryptamine-like immunoreactivity in the enteric nervous system: their projections in the guinea pig small intestine. Neuroscience 7:341–350.

Furness JB, Costa M, Keast JR. 1984. Choline acetyltransferase- and peptide immunoreactivity of submucous neurons in the small intestine of the guinea pig. Cell Tissue Res 237:329–336.

Furness JB, Kuramoto H, Messenger JP. 1990. Morphological and chemical identification of neurons that project from the colon to the inferior mesenteric ganglia in the guinea pig. J Auton Nerv Syst 31:203–210.

Furness JB, Heather MY, Pompolo S, Bornstein JC, Kunze WAA, Mc Conalogue K. 1995. Plurichemical transmission and chemical coding of neurons in the digestive tract. Gastroenterology 108:554–563.

Furness JB, Bornstein JC, Kunze WAA, Clerc N. 1999. The enteric nervous system and its extrinsic connections. In: Yamada T, Alpers DH, Laine L, Owyang C, Powell DW, editors. Textbook of gastroenterology. Vol. 1. Philadelphia: Lippincott, Williams and Wilkins. p 11–35.

Gershon MD. 1997. Genes and lineages in the formation of the enteric nervous system. Curr Opin Neurobiol 7:101–109.

Gershon MD, Drakontides AB, Ross LL. 1965. Serotonin: synthesis and release from the myenteric plexus of mouse intestine. Science 149:197–199.

Gershon MD, Dreyfus CF, Pickel VM, Joh TH, Reis DJ. 1977. Serotoninergic neurons in the peripheral nervous system: identification in gut by immunohistochemical localization of tryptophan hydroxylase. Proc Natl Acad Sci U S A 74:3086–3089.

Gershon MD, Liu KP, Karpiak K, Tamir H. 1983. Storage of serotonin in vivo as a complex with serotonin-binding protein in central and peripheral serotoninergic neurons. J Neurosci 3:1901–1911.

Gershon MD, Rothman TP, Teitelman GN. 1984. Transient and differential expression of aspects of the catecholaminergic phenotype during development of the fetal bowel of rats and mice. J Neurosci 4:2269–2280.

Gershon MD, Mawe GM, Branchek TA. 1989. 5-Hydroxytryptamine and enteric neurones. In: Fozard JR, editor. Peripheral actions of 5-hydroxytryptamine. Oxford: Oxford University Press. p 247–264.

Gershon MD, Chalazonitis A, Rothman TP. 1993. From neural crest to bowel: development of the enteric nervous system. J Neurobiol 24:199–214.

Gershon MD, Kirchgessner AL, Wade PR. 1994. Functional anatomy of the enteric nervous system. In: Johnson LR, Alpers DH, Christensen J, Jacobson ED, Walsh JH, editors. Physiology of the gastrointestinal tract. New York: Raven press. p 381–422.

Grider JR. 1989. Identification of neurotransmitters regulating intestinal peristaltic reflex in humans. Gastroenterology 97:1414–1419.

Grider JR, Murthy KS, Makhlouf GM. 1992. Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. Am J Physiol 262:774–778.

Griffith SG, Burnstock G. 1983. Serotoninergic neurons in human fetal intestine: an immunohistochemical study. Gastroenterology 85:929–937.

Hancock MB. 1984. Two color immuno-peroxidase staining: visualization of anatomic relationships between immunoreactive neural elements. Am J Anat 175:343–352.

Hörsch D, Fink T, Büchler M, Weihe E. 1993. Regional specificities in the distribution, chemical phentotypes, and coexistence patterns of neuropeptide containing nerve fibers in the human anal canal. J Comp Neurol 335:381–401.

Jacobson ED, Berguer R, Pawlik WW, Hottenstein OD. 1994. Mesenteric purinergic and peptidergic vasodilatators. In: Johnson LR, Alpers DH, Christensen J, Jacobson ED, Walsh JH, editors. Physiology of the gastrointestinal tract. New York: Raven press. p 1647–1667.

Jonakait GM, Tamir H, Rapport MM, Gershon MD. 1977. Detection of a soluble serotonin-binding protein in the mammalian myenteric plexus and other peripheral sites of serotonin storage. J Neurochem 28:277–284.

Jessen KR, Saffrey MJ, Van Noorden S, Bloom SR, Polak JM, Burnstock G. 1980. Immunohistochemical studies of the enteric nervous system in tissue culture and in situ: localization of vasoactive intestinal polypeptide (VIP), substance-P and encephalin immunoreactive nerves in the guinea pig gut. Neuroscience 5:1717–1735.

Keast JR, Furness JB, Costa M. 1984. Origins of peptide and norepinephrine nerves in the mucosa of the guinea pig small intestine. Gastroenterology 86:637–644.

Kummer W, Bachmann S, Neuhuber WL, Hänze J, Lang RE. 1993. Tyrosine-hydroxylase-containing vagal afferent neurons in the rat nodose ganglion are independent from neuropeptide-Y-containing populations and project to esophagus and stomach. Cell Tissue Res 188:527–543.

Kummer W, Gibbins IL, Stefan P, Kapoor V. 1990. Catecholamines and catecholamine-synthesizing enzymes in guinea-pig sensory ganglia. Cell Tissue Res 261:595–606.

Kurian SS, Feri GL, Demeg J, Polak M. 1983. Immunohistochemistry of serotonin-containing nerves in human gut. Histochemistry 78:523–529.

Li ZS, Furness JB. 1998. Immunohistochemical localization of cholinergic markers in putative intrinsic primary afferent neurons of the guinea pig small intestine. Cell Tissue Res 294:35–43.

Lomax AE, Furness JB. 2000. Neurochemical classification of enteric neurons in the guinea pig distal colon. Cell Tissue Res 302:59–72.

Lomax AEG, Sharkey KA, Bertrand PP, Bornstein JC, Furness JB. 1999. Correlation of morphology, electrophysiology and chemistry of neurons in the myenteric plexus of guinea pig distal colon. J Auton Nerv Syst 76:45–61.

Llewellyn-Smith IJ, Furness JB, O'Brian PE, Costa M. 1984. Noradrenergic nerves in human small intestine. Distribution and ultrastructure. Gastroenterology 87:513–529.

Maggi CA, Giuliani S, Santicioli P, Patacchini R, Said SI, Theodorsson E, Turine D, Barbanti G, Giachetti A, Meli A. 1990. Direct evidence for the involvement of vasoactive intestinal polypeptide in the motor response of the human isolated ileum to capsaicin. Eur J Pharmacol 185:169–178.

Maggi CA, Giachetti A, Dey RD, Said SI. 1995. Neuropeptides as regulators of airway Function: vasoactive intestinal peptide and the tachykinins. Physiol Rev 75:277–322.

Maggi CA, Santicioli P, Del Bianco E, Geppetti P, Barbanti G, Turini D, Meli A. 1989. Release of VIP- but not CGRP-like immunoreactivity by capsaicin from the human isolated small intestine. Neurosci Lett 98: 317–320.

Mann PT, Furness JB, Pompolo S, Mäder M. 1995. Chemical coding of neurons that project from different regions of intestine to the coeliac ganglion of the guinea pig. J Auton Nerv Syst 56:15–25.

Mann PT, Furness JB, Southwell BR. 1999. Choline acetyltransferase immunoreactivity of putative intrinsic primary afferent neurons in the rat ileum. Cell Tissue Res 297:241–248.

Mawe GM, Gershon MD. 1989. Structure, afferent innervation and transmitter content of ganglia of the guinea pig gallbladder: relationship to the enteric nervous system. J Comp Neurol 283:374–390.

McMurray G, Shaw C, Johnston CF, Halton DW. 1993. Choline acetyltransferase (ChAT) immunoreactivity in a sub-population of mammalian intestinal endocrine cells. Comp Biochem Physiol 106:509–515.

Meedeniya ACB, Brookes SJH, Henng GW, Costa M. 1998. The projections of 5 hydroxytryptamine-accumulating neurons in the myenteric plexus of the small intestine of the guinea pig. Cell Tissue Res 291:375–384.

Messanger JP, Furness JB. 1991. Calbindin-immunoreactive nerve terminals in the guinea pig coeliac ganglion originate from colonic nerve cells. J Auton Nerv Syst 35:133–142.

Morris JL, Gibbins IL. 1987. Neuronal colocalization of peptides, catecholamines, and catecholamine-synthesizing enzymes in guinea pig paracervical ganglia. J Neurosci 7:3117–3130.

Neunlist M, Schemann M. 1998. Polarized innervation pattern of the mucosa of the guinea pig distal colon. Neurosci Lett 246:161–164.

Nohr D, Eiden LE, Weihe E. 1995. Coexpression of vasoactive intestinal peptide, calcitonin gene-related peptide and substance P immunoreactivity in parasympathetic neurons of the rhesus monkey lung. Neurosci Lett 199:25–28.

Peter D, Liu Y, Sternini C, de Giorgio R, Brecha N, Edwards R. 1995. Differential expression of two vesicular monoamine transporters. J Neurosci 15:6179–6188.

Porter AJ, Wattchow DA, Brookes SJ, Schemann M, Costa M. 1996. Choline acetyltransferase immunoreactivity in the human small and large intestine. Gastroenterology 111:401–408.

Porter AJ, Wattchow DA, Brookes SJ, Costa M. 1997. The neurochemical coding and projections of circular muscle motor neurons in the human colon. Gastroenterology 113:1916–1923.

Reiche D, Schemann M. 1998. Ascending choline acetyltransferase and descending nitric oxide synthase immunoreactive neurons of the myenteric plexus project to the mucosa of the guinea pig gastric corpus. Neurosci Lett 241:61–64.

Reiche D, Michel K, Pfannkuche H, Schemann M. 2000. Projections and neurochemistry of interneurons in the myenteric plexus of the guinea pig gastric corpus. Neurosci Lett 295:109–112.

Robinson R, Gershon MD. 1971. Synthesis and uptake of 5-hydroxytryptamine by the myenteric plexus of the small intestine of the guinea pig. J Pharmacol Exp Ther 178:311–324.

Sang Q, Young HM. 1998. The identification and chemical coding of cholinergic neurons in the small and large intestine of the mouse. J Neurosci 7:3117–3130.

Schäfer MKH, Weihe E, Varoqui H, Eiden E, Erickson JD. 1994. Distribution of the Vesicular acetylcholine transporter (VAChT) in the central and peripheral nervous system of the rat. J Mol Neurosci 5:1–26.

Schäfer MKH, Weihe E, Erickson JD, Eiden LE. 1995. Human and monkey cholinergic neurons visualized in paraffin-embedded tissues by immunoreactivity for VAChT, the vesicular acetylcholine transporter. J Mol Neurosci 6:225–235.

Schäfer MKH, Eiden LE, Weihe E. 1998. Cholinergic neurons and terminal fields revealed by immunohistochemistry fort the vesicular acetylcholine transporter. II. The peripheral nervous system. Neuroscience 84: 361–376.

Schemann M, Sann H, Schaaf C, Mader M. 1993. Identification of cholinergic neurons in enteric nervous system by antibodies against choline acetyltransferase. Am J Physiol 265:1005–1009.

Schemann M, Schaaf C, Mäder M. 1995. Neurochemical coding of enteric neurons in the guinea pig stomach. J Comp Neurol 353:161–178.

Schneider J, Jehle EC, Stralinger MJ, Neunlist M, Michel K, Hoppe S, Schemann M. 2001. Neurotransmitter coding of enteric neurones in the submucous plexus is changed in non-inflamed rectum of patients with Crohn's disease. Neurogastroenterol Motil 13:255–264.

Schütz B, Schäfer MKH, Eiden LE, Weihe E. 1998. Vesicular amine transporter expression and isoform selection in developing brain, peripheral nervous system and gut. Dev Brain Res 106:181–204.

Sharkey KA, Lomax AEG, Bertrand PP, Furness JB. 1998. Electrophysiology, shape and chemistry of neurons that project from guinea pig colon to inferior mesenteric ganglia. Gastroenterology 115:909–918.

Singaram C, Ashraf W, Gaumnitz EA, Torbey C, Sengupta A, Pfeiffer R, Quigley EM. 1995. Dopaminergic defect of enteric nervous system in Parkinson's disease patients with chronic constipation. Lancet 346: 861–864.

Sjolund K, Sanden G, Hankanson R, Sundler F. 1983. Endocrine cells in human intestine: an immunocytochemical study. Gastroenterology 85: 1120–1230.

Steele PA, Brookes SJ, Costa M. 1991. Immunohistochemical identification of cholinergic neurons in the myenteric plexus of guinea pig small intestine. Neuroscience 45:227–239.

Tamir H, Gershon MD. 1981. Intracellular proteins that bind serotonin in neurons, paraneurons and platelets. J Physiol 77:283–286.

Teitelman G, Joh TH, Reis DJ. 1978. Transient expression of a noradrenergic phenotype in cells of the rat embryogenic gut. Brain Res 158:229–234.

Teitelman G, Gershon MD, Rothman TP, Joh TH, Reis DJ. 1981. Proliferation and distribution of cells that transiently express a catecholaminergic phenotype during development in mice and rats. Dev Biol 86:348–355.

Vanden Berghe P, Coulie B, Tack J, Mawe GM, Schemann M, Janssens J. 1999. Neurochemical coding of myenteric neurons in the guinea pig antrum. Cell Tissue Res 297:81–90.

Varoqui H, Schafer MKH, Zhu H, Weihe E, Erickson JD. 2002. Identification of the differentiation-associated Na+/PI transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. J Neurosci 22:142–155.

Wakabayashi K, Takahashi H, Ohama E, Ikuta F. 1989. Tyrosine hydroxylase-immunoreactive intrinsic neurons in the Auerbach's and Meissner's plexus of humans. Neurosci Lett 96:259–263.

Wattchow DA, Porter AJ, Brookes SJH, Costa M. 1997. The polarity of neurochemically defined myenteric neurons in the human colon. Gastroenterology 113:497–506.

Weihe E, Schäfer MKH, Hartschuh W, Eiden LE. 1994. Localization of vesicular monoamine transporter isoforms (VMAT1 and VMAT2) to endocrine cells and neurons in rat. J Mol Neurosci 5:149–164.

Weihe E, Tao-Cheng JH, Schäfer MKH, Erickson JD, Eiden LE. 1996. Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicles. Proc Natl Acad Sci U S A 93:3547–3552.

Weihe E, Eiden LE. 2000. Chemical neuroanatomy of the vesicular amine transporters. FASEB J 14:2435–2449.