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Catecholaminergic, cholinergic and peptidergic innervation of gut-associated lymphoid tissue in porcine jejunum and ileum

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Abstract With its abundance of neurons and immunocytes, the gut is a potentially important site for the study of the interaction between the nervous and immune systems. Using immunohistochemical techniques, we tested the hypothesis that gut-associated lymphoid tissue in the porcine small intestine might receive catecholaminergic, cholinergic and peptidergic innervation. Antibodies against protein gene product (PGP) 9.5 were employed to detect neuronal membranes; antibodies against tyrosine hydroxylase (TH), type 2 vesicular monoamine transporter (VMAT-2) and choline acetyltransferase (ChAT) were used to detect catecholaminergic and cholinergic neurons; and antibodies to neuromedin U-8 (NMU-8), substance P (SP) and vasoactive intestinal peptide (VIP) were also used. PGP9.5-immunoreactive nerve fibers were observed between jejunal Peyer's patch (PP) follicles and in submucosal ganglia localized at the base of continuous ileal PP. Many ChAT-positive and a few TH-/VMAT-2-immunoreactive neurons or axons adjacent to jejunal and ileal PP were observed. Neurons and fibers from ganglia situated between or at the base of PP follicles manifested robust immunoreactivities to VIP and NMU-8; relatively less SP immunoreactivity was observed at these locations. All neuromedin-U 8-positive neurons observed exhibited immunoreactivity to ChAT as did some VIP-positive neurons. The specific chemical coding of enteric neurons in close apposition to jejunal and ileal PP and the differential localization of neuropeptides within the jejunal and ileal PP are indicative of neuroimmunomodulation at these sites.

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Introduction

Because it comprises a vast surface area in contact with the external environment, the intestinal mucosa contains the largest amount of lymphoid tissue in the body, which functions in host defense against luminal pathogens. The gut-associated lymphoid tissue (GALT) consists of organized regions of lymphoid tissue, such as the Peyer's patches (PP) and diffuse populations of lymphocytes found in the lamina propria and epithelium of the wall of the gastrointestinal tract (Bienenstock and Befus 1985; Doe 1989; Mowat and Viney 1997). The PP of the small intestine can initiate local and systemic immune responses, as well as oral tolerance (Pabst 1987). In porcine small intestine, two types of PP occur: jejunal PP are organized, solitary follicles, whereas ileal PP are continuous lymphoid follicles (Binns and Licence 1985). In the jejunum, PP contain a germinal center overlain with a distinctive dome epithelium containing cuboidal microfold (M) cells. High numbers of T lymphocytes reside in the interfollicular areas of jejunal PP. The M cells endocytose luminal antigens and transport them to antigen-presenting cells and regulatory T cells lying beneath the PP dome to initiate mucosal immune responses (Stokes et al. 1994). In contrast, porcine ileal PP are covered by a villous epithelium devoid of M cells. PP in these two different locations also differ in lymphocyte trafficking and B-cell production. For example, lymphocyte homing to jejunal PP is greater than to ileal PP (Binns and Licence 1985). Moreover, jejunal PP possess comparatively more T cells and less B cells than ileal PP (Rothkötter and Pabst 1989). These differences may indicate that jejunal and ileal PP are subject to different regulatory influences.

The enteric nervous system (ENS) plays an important role in modulating and coordinating complex intestinal

functions such as propulsive motor activity, mucosal blood flow and active ion transport (Goyal and Hirano 1996). It may also regulate aspects of intestinal inflammation and immunity during pathological conditions (Theodorou et al. 1996). In general, there is a close relationship between the nervous and immune systems. For example, many lymphoid organs receive projections from adrenergic and cholinergic neurons (Felten et al. 1985). Moreover, lymphocytes, macrophages and other immune cells appear to express adrenoreceptors (Madden et al. 1995). Receptors for gut neuropeptides such as somatostatin, substance P (SP) and vasoactive intestinal peptide (VIP) are also expressed on rodent and human immunocytes; they may regulate aspects of cell proliferation and immunoglobulin synthesis (Stead et al. 1987).

The porcine small intestine contains three major ganglionated plexuses: a myenteric plexus and two submucosal plexuses; the submucosal plexus is further subdivided into the internal submucosal plexus (ISP) and external submucosal plexus (ESP; Timmermans et al. 1990; Balemba et al. 1998). The porcine ISP is structurally less complex than the myenteric plexus or ESP (Timmermans et al. 1997). In ISP, large neurons with Dogiel type 2 morphology and mini-neurons, respectively, coexpress immunoreactivities towards acetylcholine/SP/calcitonin gene-related peptide/(NMU)/(galanin) and galanin/VIP or NMU/SP (Timmermans et al. 1990, 1997). The ESP contains large neurons with calcitonin gene-related peptide immunoreactivity; multidendritic cells immunoreactive for 5-hydroxytryptamine/calbindin/enkephalin or nitric oxide synthase/calretinin/(neuropeptide Y or somatostatin); and mini-neurons with galanin/VIP immunoreactivity (Timmermans et al. 1990, 1997).

The porcine intestine possesses physiological and pathophysiological similarities with the human intestine and has been used increasingly as a model for intestinal function (Kararli 1995). Furthermore, it may be useful in small and large bowel allotransplantation. Despite a growing literature on porcine GALT, there have been few investigations of the neuromodulation of this extensive immunological compartment (Stead 1992). In the present report, we tested the hypothesis that porcine jejunal and ileal PP are differently innervated by submucosal catecholaminergic, cholinergic and peptidergic neurons.

Materials and methods

Five 5- to 7-week-old and three 6-month-old Yorkshire pigs of both sexes were obtained from the University of Minnesota swine facility. Pigs were anesthetized by intramuscular administration of ketamine (11 mg/kg) and acepromazine (0.4 mg/kg) and subsequently put to death with intravenous pentobarbital (65 mg/kg). Portions of jejunum and ileum containing PP were dissected out and immersed in ice-cold 2% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 2 h. The protocol for the use of all animals was approved by the University of Minnesota Institutional Animal Care and Use committee (protocol no. 9505006).

All tissues were cryoprotected in graded (10–30%) concentrations of sucrose in PBS, embedded in Tissue Tek OCT compound (Baxter Healthcare Co., McGaw Park, IL), and frozen. Coronal sections of jejunal and ileal PP (15 μm thickness) were thaw mounted onto Superfrost-Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at –20°C until use. To confirm patterns of immunoreactivity, some tissues were sectioned in the plane of the longitudinal muscle.

Antibodies were chosen to detect catecholaminergic, cholinergic and peptidergic neurons and neuronal processes in porcine GALT. These are described in detail in Table 1. Adjacent sections of small intestine containing jejunal and ileal PP were rehydrated in PBS (pH 7.4) for 15 min, then incubated in 0.4% Triton-X 100 (Sigma Chemical Co., St. Louis, MO) and 2% bovine serum albumin (BSA, Sigma) in PBS for 30 min to block non-specific binding. Sections were incubated with various antibodies diluted in 0.4% Triton-X100 and 2% BSA for 1 h at room temperature (Table 1). After three rinses in PBS, sections were further incubated with appropriate secondary antibodies (donkey anti-rabbit Cy3-conjugated IgG at 1:400 dilution; or donkey and goat FITC-conjugated IgG at 1:50 dilution; Jackson Immunoresearch Laboratories Inc., West Grove, PA) in PBS for 1 h in dark. After three rinses in PBS for 15 min, coverslips were mounted with Vectashield (Vector Laboratory, Burlingame, CA) and the edges sealed with nail polish. To specifically localize immunoreactivity for choline acetyltransferase (ChAT), sections were blocked and incubated at 4°C overnight in the solution containing goat anti-ChAT antibody; sections were subsequently processed as described above. In double-labeling experiments, sections were initially incubated in goat anti-ChAT antibody overnight followed by a second incubation in rabbit anti-VMAT-2, -NMU-8, -SP or -VIP antisera on adjacent sections respectively. To investigate the presence of catecholaminergic innervation, double-labeling experiments were performed in which sections were simultaneously incubated in a solution containing antibodies against TH and VMAT-2. After PBS rinses, the sections were simultaneously incubated in donkey anti-goat FITC-conjugated IgG (1:50 dilution in PBS; Jackson) and donkey anti-rabbit Cy3-conjugated IgG (1:400 dilution in PBS; Jackson) for 1 h at room temperature in the dark. Some sections were stained using the avidin-biotin peroxidase technique for localization of PGP9.5 (1:200 dilution) immunoreactivity. For this purpose, a Zymed Laboratories kit (South San Francisco, CA) was used and staining was performed according to the manufacturer's protocol.

Table 1 Description of antibodies used for immunohistochemistry (*ChAT* choline acetyltransferase, *NMU-8* neuromedin U-8, *PGP9.5* protein gene product 9.5, *SP* substance P, *TH* tyrosine hydroxylase, *VIP* vasoactive intestinal peptide, *VMAT-2* vesicular monoamine transporter, type 2)

Antigen	Host species	Dilution	Source
ChAT	Goat	1:20	Chemicon International Inc., Temecula, CA
ChAT	Rabbit	1:75	Biometra, Tampa, FL
NMU-8	Rabbit	1:1000	Accurate Chemical and Scientific Co., Westbury, NY
PGP9.5	Rabbit	1:100	Chemicon International Inc.
SP	Rabbit	1:600	Accurate Chemical and Scientific Co.
SP	Rabbit	1:600	DiaSorin, Stillwater, MN
TH	Goat	1:500	Santa Cruz Biotechnology Inc., Santa Cruz, CA
VIP	Rabbit	1:600	Accurate Chemical and Scientific Co.
VIP	Rabbit	1:600	Incstar, Stillwater, MN
VMAT-2	Rabbit	1:50	Chemicon International Inc.

Controls consisted of omission of primary antibody from the staining protocol or preabsorbing the primary antibodies against antigens obtained from various sources (ChAT and VIP, Sigma; NMU-8 and SP, Peninsula Laboratories, Belmont, CA; VMAT-2, Chemicon International, Temecula, CA). In all cases, the omission of primary antibodies resulted in an absence of immunoreactivity. In preabsorption experiments, a 10- to 30-fold excess concentration of VMAT-2; 1, 5 and 10 μM NMU-8, SP or VIP; or 1–2 μg/ml ChAT was incubated overnight at 4°C with the primary relevant antibody to preabsorb the antisera. After centrifugation, the supernatant was used in place of the primary antibody in the staining protocol. Preabsorption of anti-VMAT-2 antiserum with 30-fold excess of VMAT-2 peptide completely abolished VMAT-2 immunoreactivity in neuronal profiles. However, a non-specific punctate staining pattern of VMAT-2 immunoreactivity was observed in jejunal and ileal PP, and this persisted even in sections incubated only with the secondary antibody. ChAT immunoreactivity was substantially reduced when anti-ChAT antiserum was preincubated with 2 μg/ml ChAT antigen; however, a non-specific punctate staining pattern identical to that seen with VMAT-2 antibody persisted. After preincubation of anti-SP or NMU-8 antisera with 10 μM of the respective peptide, SP or NMU-8-like immunoreactivity in all regions above the circular muscle layer was completely absent, although weak immunoreactivity was still present in circular muscle. Preincubation of VIP antiserum with 10 μM VIP completely eliminated the ability of the antibody to detect immunoreactive elements in any intestinal subregion.

At least five PP-containing sections from individual pigs were used in determining the occurrence of immunoreactive neurons and fibers in porcine GALT. The number of neurons and fibers were counted in ESP and ISP as well as in the muscle coat in five different fields. In the case of ChAT and VIP immunoreactivity, the percentage of immunoreactive neurons and fibers was determined by counting the neurons and fibers in ESP as well as ISP in a minimum of five different fields from sections obtained from each animal. Sections were scanned using a BioRad confocal laser scanning microscope (CLSM; Model 1000), which was attached to a Nikon fluorescence microscope. Images were obtained using Comos software (version 6.05.8; Comos BioRad, Hercules, CA) and further processed employing NIH Image (version 1.59) and Adobe Photoshop (version 4.0). For brightfield photomicrography, an Olympus microscope attached to a Spot camera (Spot Camera Diagnostic Instruments, Ann Arbor, MI) was used with Spot software (version 2.1.2).

Results

In the porcine small intestine, jejunal and ileal PP exhibited distinct morphologic profiles. In the jejunum, solitary PP follicles were observed. In contrast, a continuous aggregate of PP follicles was observed in the ileum. The various subregions of jejunal and ileal PP and of the ENS are shown in Fig. 1A and Fig. 1B, respectively. For purposes of orientation, the ESP lies above the circular muscle and the ISP lies close to the abluminal side of the lamina muscularis mucosae. Because PP follicles intrude into the submucosa, interfollicular ganglia are considered to be a component of the ISP (Krammer and Kühnel 1993; Balemba et al. 1998).

Distribution of PGP9.5 immunoreactivity in jejunal and ileal Peyer's patches

PGP9.5-immunoreactive neurons and fibers were observed in the ganglionated ESP and ISP and in longitudinal muscle and circular smooth muscle layers in ileum and jejunum in close apposition to the PP. PGP9.5-immunoreactive fibers were observed in the jejunal PP follicle proper as well as at the margins of follicles; some PGP9.5 immunoreactivity exhibited a punctate staining pattern in the follicle and dome region. Moreover, PGP9.5-immunoreactive fibers were observed terminating near the epithelial cells in the dome epithelium. Large blood vessels at the base of the follicles as well as in the interfollicular region were innervated by PGP9.5-immunoreactive fibers in the jejunum and ileum.

Table 2 Distribution of catecholaminergic, cholinergic and peptidergic immunoreactivity in enteric neurons and fibers apposed to porcine jejunal and ileal Peyer's patches [*ChAT* choline acetyltransferase, *D* dome area, *ESP* external submucosal plexus, *F* follicle, *IF* interfollicular region, *ISP* internal submucosal plexus, *CM* circular muscle, *MP* myenteric plexus, *NA* not analyzed, *NMU-8* neuromedin U-8, *PGP9.5* protein gene product 9.5, *SP* substance P, *TH* tyrosine hydroxylase, *VIP* vasoactive intestinal peptide, *VMAT-2* vesicular monoamine transporter, type 2, + presence of immunoreactivity (+ represents few, ++ low, +++ moderate, ++++ high, >++++ a very high number of immunoreactive neuronal cell bodies and/or fibers/field), – absence of immunoreactivity]

	PGP9.5	TH	VMAT-2	ChAT	NMU-8	SP	VIP
Jejunum							
MP	+++++	NA	NA	NA	NA	NA	NA
CM	+++++	+	+	+	+	+++	++++
ESP	+++++++	+	+	++	++	+	+++++
ISP							
IF	++++	+	+	++	++	++	++
F	+	–	–	+	+	+	+
D	++	–	–	+	–	+	++
Ileum							
MP	+++++	NA	NA	NA	NA	NA	NA
CM	+++++	+	+	+	+	+++	++++
ESP	+++++++	++	++	+++	+++	++	++++++
ISP							
IF	++++	+	+	+++	+++	++	++++
F	–	–	–	–	–	–	–

Catecholaminergic, cholinergic and peptidergic innervation of the jejunal and ileal Peyer's patches

Catecholaminergic, cholinergic and peptidergic innervation of the jejunal and ileal PP was observed in the porcine small intestine. ChAT, VIP (Fig. 2) and VMAT-2 (Fig. 3A) and SP (Fig. 3B–D) immunoreactivities were localized in the jejunal PP. In the ileum, TH-immunoreactive (Fig. 3E,F',G) and VMAT-2-positive neurons (Fig. 4A) were observed; neurons expressing ChAT immunoreactivity (Figs. 4B, 5A,C,E) also displayed immunoreactivity towards NMU-8 (Fig. 4C) or VIP (Fig. 5B,D,F). The localization of catecholaminergic, cholinergic and peptidergic immunoreactivity in jejunal and ileal PP is summarized in Table 2.

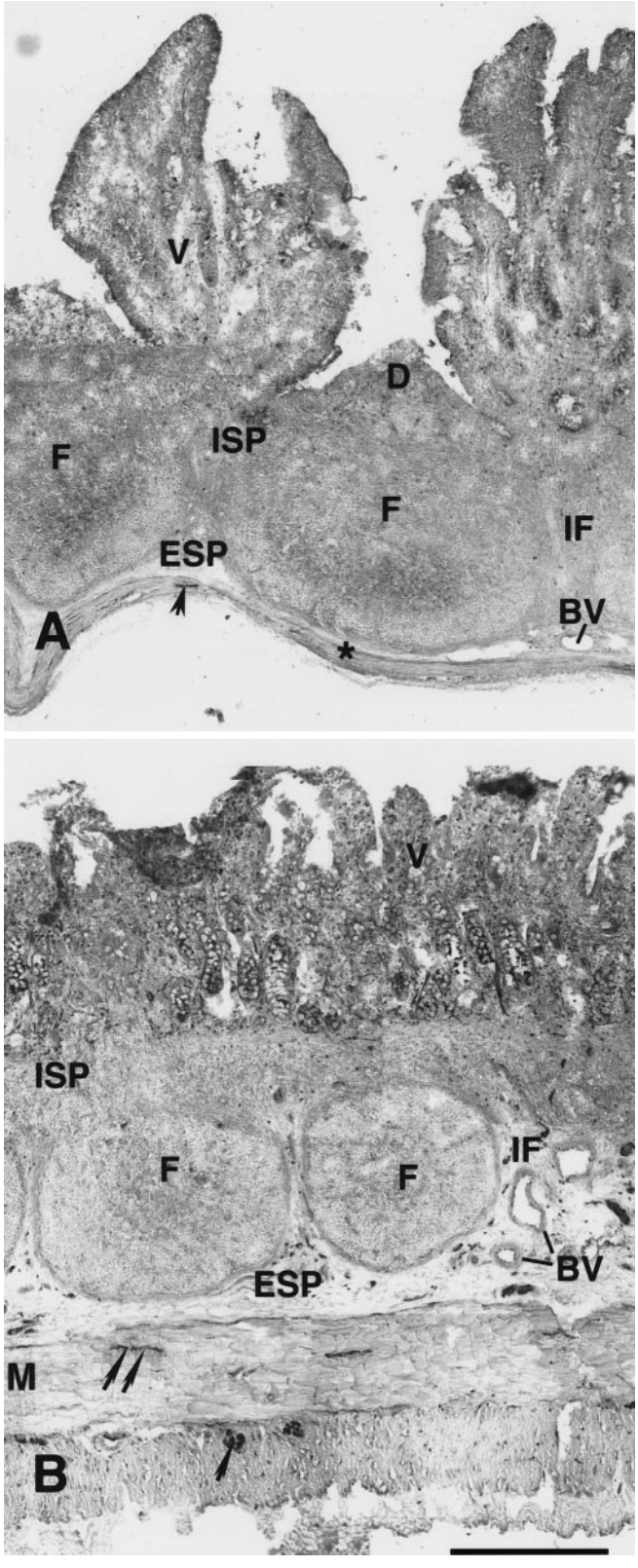

Fig. 1A,B Organization of jejunal and ileal Peyer's patches (PP) as seen in coronal sections with primary antibody to protein gene product 9.5 (PGP9.5), a general neuronal marker. **A** Porcine jejunal PP showing an isolated lymphoid follicle (*F*) and a thin muscle layer (*) overlain by the external submucosal plexus (*ESP*). Note that the muscle layer in jejunal PP is relatively much thinner. An immunoreactive fiber is seen in the muscle layer (*arrow*). A few blood vessels (*BV*) can be seen at the base of the follicle. The follicle is covered by a dome (*D*) lined with epithelium (*IF* interfollicular region, *ISP* internal submucosal plexus or interfollicular ganglion, *V* villous). **B** PP follicles in the porcine ileum. Note that the smooth muscle layer is thicker than in the preceding jejunal section; longitudinal and circular muscle (*M*) layers display a few PGP9.5-immunoreactive fibers (*double arrow*). The muscle coats are separated by a thin myenteric plexus which also exhibits PGP9.5-immunoreactive neurons (*single arrow*). *Bar* 250 µm **(A,B)**

Catecholaminergic innervation

In jejunum, VMAT-2-immunoreactive neurons and fibers were localized at the base of PP follicles (Fig. 3A). In interfollicular areas, single positive fibers could be seen, but neurons expressing VMAT-2 immunoreactivity could not be detected. VMAT-2-immunoreactive nerve fibers did not occur within PP follicles or near blood vessels. Double-staining of sections using antibodies to VMAT-2 and TH revealed that immunoreactivities to VMAT-2 and TH were colocalized in the smooth muscle layer, ESP and ISP.

There appeared to be a greater number of VMAT-2-immunoreactive neurons and fibers in the ileum. A few VMAT-2-positive fibers were observed coursing through the circular muscle and near the base of a follicle. Neurons in the external submucosal ganglia expressed VMAT-2 immunoreactivity. As in the jejunum, VMAT-2 immunoreactivity was colocalized with TH immunoreactivity (Fig. 3E,F',G) in muscle layer, ESP and ISP. A few fibers and neurons exhibiting VMAT-2 immunoreactivity were localized in interfollicular ganglia (Fig. 4A). Some fibers were seen above the follicle near the mucosal epithelium. As in the jejunum, VMAT-2-immunoreactive fibers did not occur within follicles or near blood vessels.

Cholinergic innervation

In the jejunum, ChAT-immunoreactive fibers were observed in the circular muscle. Several neurons exhibiting

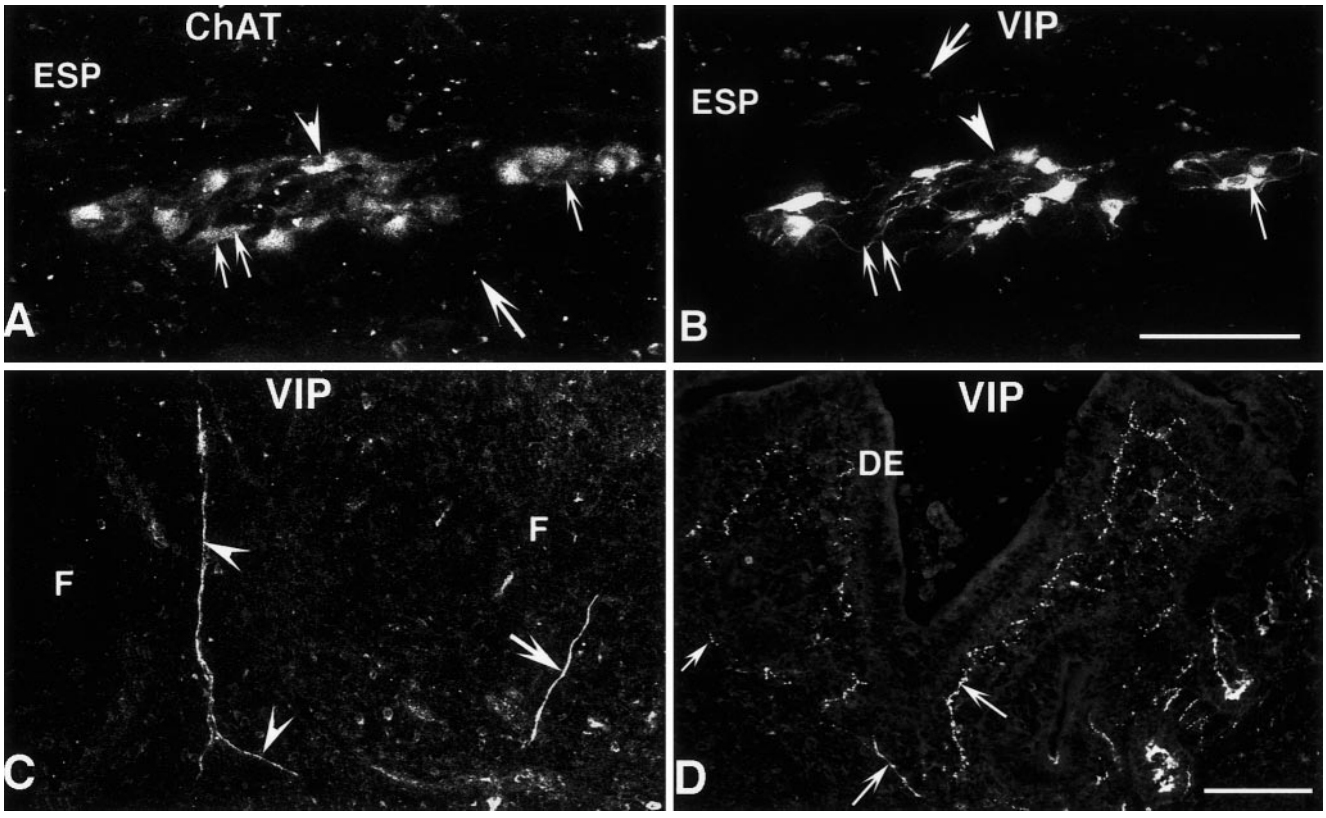

Fig. 2A–D Coronal sections of jejunal Peyer's patch showing the localization of immunoreactivities for choline acetyltransferase (*ChAT*) and vasoactive intestinal peptide (*VIP*). In **A** and **B**, non-specific staining (*large arrow*) persisted after preabsorbing anti-ChAT or anti-VIP antisera with their respective antigens or by omitting the primary antibody in the staining protocol. **A,B** Double staining of the section using anti-ChAT and -VIP antisera respectively. In **A** numerous large neurons exhibiting ChAT-like immunoreactivity (*large arrowhead*) are localized in the ganglionated external submucosal plexus (*ESP*). A few smaller neurons displaying weaker ChAT-like immunoreactivity are situated in close proximity to the larger neurons (*small arrow*). **B** Double staining with primary antibody against VIP. The large ChAT-immunoreactive neurons in **A** do not coexpress VIP immunoreactivity (*arrowhead*). Note that some smaller ChAT-immunoreactive neurons (*double arrow*, **A**) also do not display VIP-like immunoreactivity (*double arrow*). A small neuron resembling an enteric mini-neuron possesses VIP-like immunoreactivity (*single arrow*) and also expressed weak ChAT immunoreactivity as seen in **A**. **C** VIP-immunoreactive fiber (*arrowheads*) at the base of a follicle (*F*) courses through the interfollicular region. A single VIP-immunoreactive fiber is seen within the PP follicle (*arrow*). **D** Numerous VIP-immunoreactive fibers (*long arrows*) terminate near the base of the dome epithelium(*DE*). A punctate staining pattern (*short arrow*) of immunoreactivity is seen in the dome region which was completely absent in preabsorption controls. *Bars* 50 μm **(A,B)**, 100 μm **(C,D)**

ChAT immunoreactivity were observed at the base of PP follicles (Fig. 2A). Most of these neurons were large, although a few smaller neurons were also observed within a ganglion. ChAT-immunoreactive fibers could be observed at the margins of PP follicles. Although few fibers or neurons expressing ChAT immunoreactivity were observed in interfollicular ganglia, ChAT-immunoreactive fibers could occasionally be observed within PP follicles and domes. However, nerve fibers terminating near the base of the dome epithelium were never observed. Submucosal blood vessels situated at the base of the follicles were sparsely innervated with ChAT-immunoreactive fibers.

ChAT-immunoreactive fibers were also observed in the circular muscle of the ileum. ChAT-like immunoreactivity was visualized within several neurons in ganglia situated at the base of ileal PP follicles (Figs. 4D, 5A,C,E). Neurons exhibiting ChAT immunoreactivity were also localized in interfollicular ganglia (Fig. 5E). ChAT-positive neural elements were not observed within the follicle or in the vicinity of submucosal blood vessels.

Peptidergic innervation

Localization of neuromedin U-8-like immunoreactivity

In the jejunum, fibers in the circular muscle exhibited NMU-8 immunoreactivity. Large NMU-8-immunoreactive neurons were observed in ganglia at the base of PP follicles. Only isolated single neurons could occasionally be observed in interfollicular ganglia. Within a PP follicle, two or three NMU-8-immunoreactive fibers were observed. However, NMU-8-immunoreactive fibers were not observed near the PP dome. NMU-8-immunoreactive fibers did not appear to innervate submucosal blood vessels.

In the ileum, the pattern of NMU-8 immunoreactivity was generally similar to that observed in the jejunum. NMU-8-immunoreactive fibers were observed in the circu-

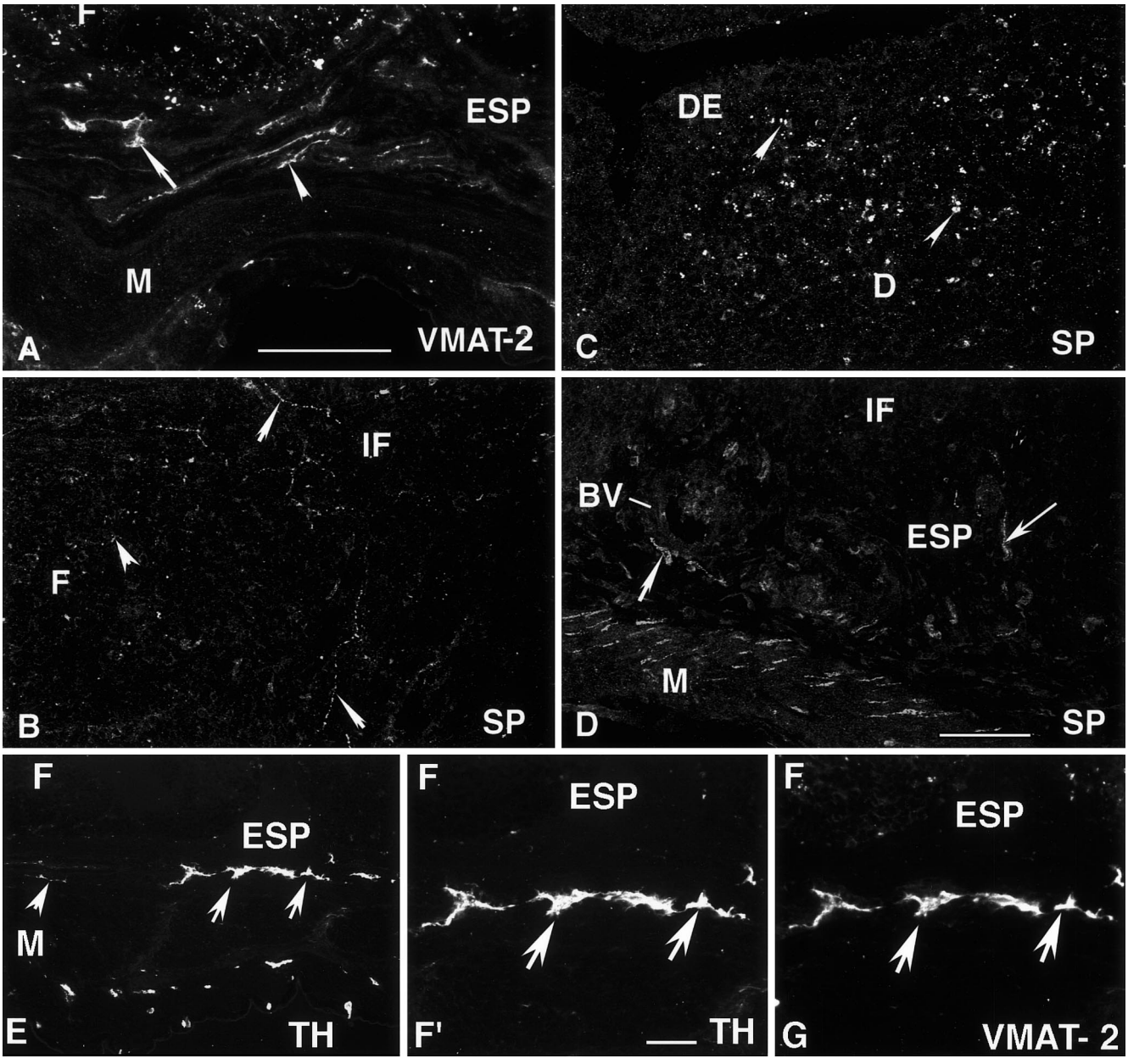

Fig. 3 A–D Coronal sections of jejunal Peyer's patch (PP) showing immunoreactivity towards vesicular monoamine transporter, type 2 (*VMAT-2*) and substance P (SP). **E,F** Coronal sections of an ileal Peyer's patch double-stained with antibodies against tyrosine hydroxylase (*TH*) and VMAT-2. **A** VMAT-2-immunoreactive fibers (*arrowhead*) and neurons (*arrow*) at the base of a follicle (*F*) in the external submucosal plexus (*ESP*). The punctate staining pattern observed within the follicle probably represents non-specific fluorescence since it persisted in omission and preabsorption controls (*M* circular muscle). **B** Varicose SP-immunoreactive fibers (*arrows*) in the interfollicular region (*IF*). Immunoreactivity is also observed in the follicle (*arrowhead*). **C** Punctate pattern of SP immunoreactivity (*arrowhead*), probably representing nerve fibers in cross section, in PP dome (*D*) region (*DE* dome epithelium). The punctate staining pattern was completely abolished in preabsorption controls. **D** SP-immunoreactive fibers in circular muscle (*M*), the interfollicular region (*IF, long arrow*) and innervating thick-walled submucosal blood vessels (*BV, short arrow*). **E** TH-immunoreactive fibers (*arrowhead*) and neurons (*arrows*) in the ESP in ileal Peyer's patch. **F'** Higher magnification of the neurons shown in **E** expressing TH immunoreactivity. **G** Double-staining of the section with antibodies against TH **(E,F')** and VMAT-2 **(G)** reveals colocalization of VMAT-2 with TH. *Bars* 100 μm **(A–E)**, 50 μm **(F',G)**

lar muscle, and several fibers were observed at the base of PP follicles. Large and small NMU-immunoreactive neurons were observed in submucosal ganglia. Most of the neurons were large, and occasionally a single process emanating from a cell body could be seen (Fig. 4C). Two to three fibers were observed at the basal margin of PP follicles. Only one or two neurons and fibers were observed in the interfollicular area. NMU-8-immunoreactive fibers were not observed within the follicles or innervating the blood vessels.

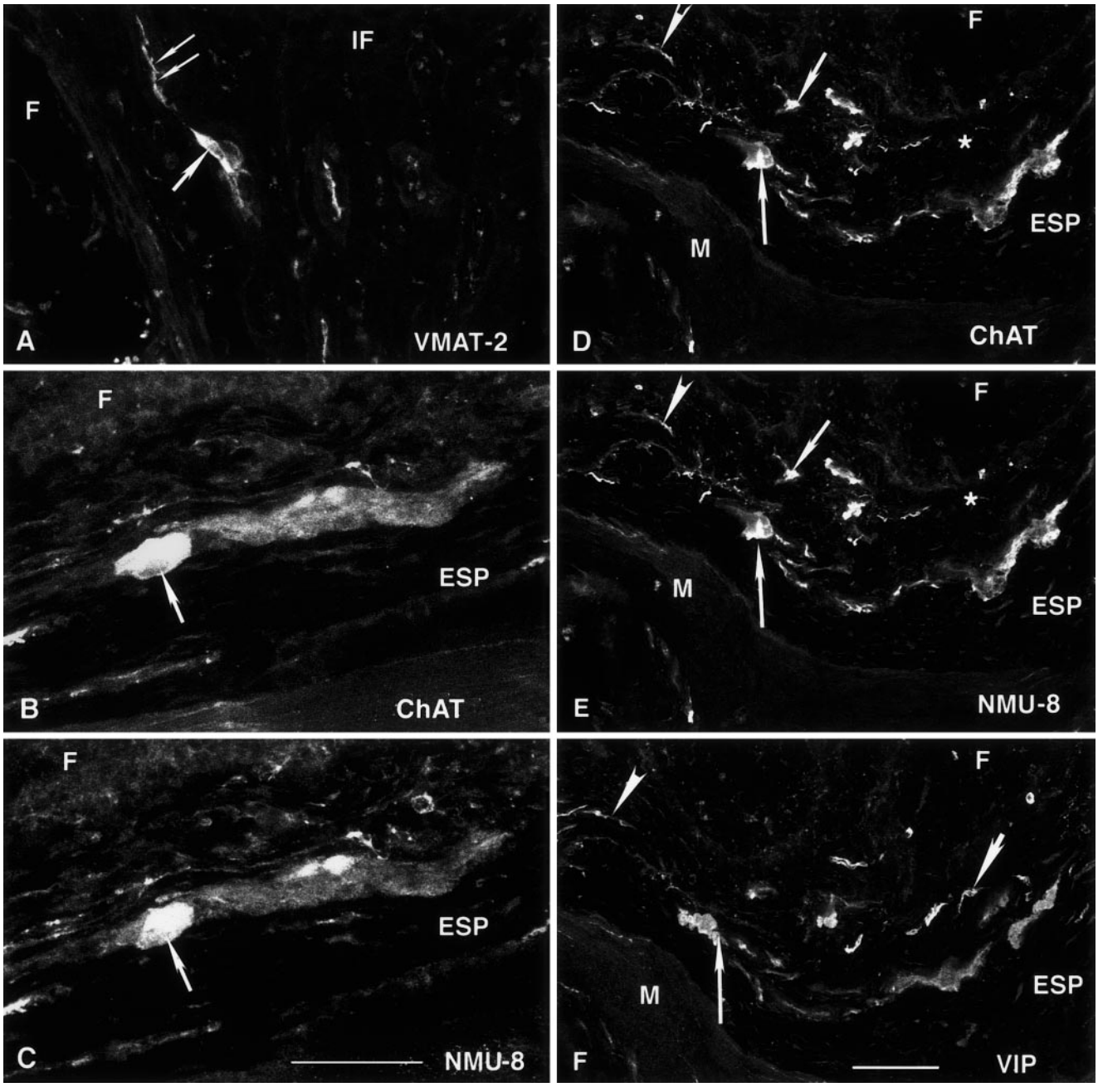

Fig. 4A–F Coronal sections of ileal Peyer's patch (PP) showing localization of immunoreactivity to the type 2 vesicular monoamine transporter (*VMAT-2*), choline acetyltransferase (*ChAT*), neuromedin U-8 (*NMU-8*) and vasoactive intestinal peptide (*VIP*). **A** VMAT-2-immunoreactive neuron (*arrow*) and fiber (*double arrow*) are seen in the interfollicular region (*IF*). **B** A large neuron (*arrow*) situated at the base of the follicle (*F*) in the external submucosal plexus (*ESP*) displays immunoreactivity to ChAT. **C** Identical neuron from **B** manifests NMU-8-like immunoreactivity (*arrow*). **D,E** Respective colocalization of ChAT **(D)** and NMU-8 **(E)** immunoreactivities in ileal neurons. Nerve fiber (*arrowhead*) situated at the base of PP follicle **(F)**, small neurons (*short arrow*) and a subset of larger neurons (*long arrow*) all coexpress ChAT and NMU-8 immunoreactivity. A small VIP-immunoreactive neuron shown in **F** (*short arrow*) is not immunoreactive for ChAT or NMU-8 (*) as seen in **D** and **E**. **F** Ileal section identical to those displayed in **D** and **E** that was incubated in anti-VIP antiserum. VIP-like immunoreactivity is observed in a fiber (*arrowhead*) that also expressed ChAT and NMU-8 immunoreactivity situated at the base of a follicle. A smaller VIP-positive neuron (*short arrow*) is negative for ChAT and NMU-8 (see * in **D** and **E** respectively). Other neurons also displayed VIP-like immunoreactivity (*long arrow*) (*M* circular muscle). *Bars* 50 μm **(A–C)**; 100 μm **(D–F)**

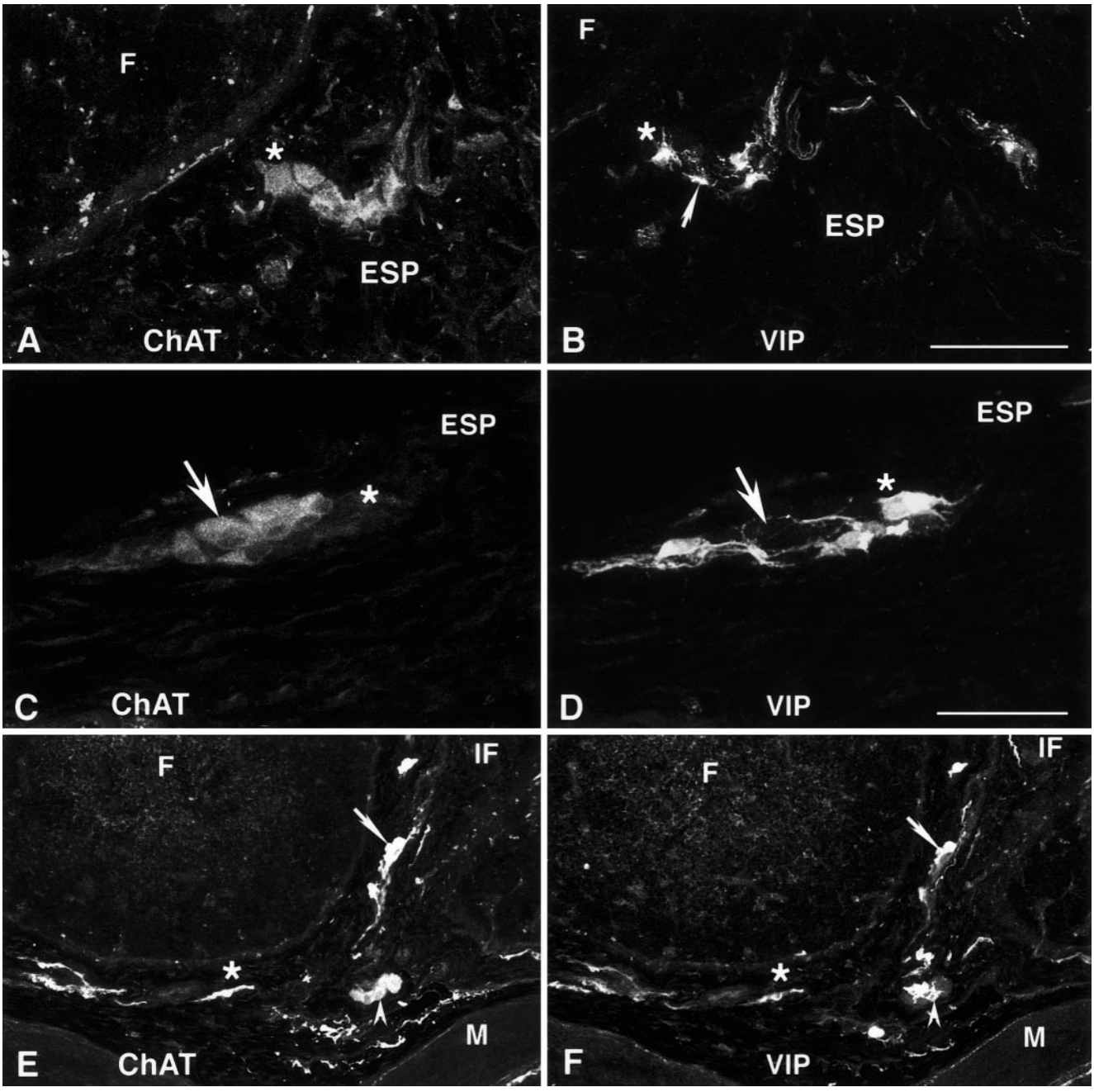

Fig. 5A–F Coronal sections of ileal Peyer's patch co-stained with antibodies against choline acetyltransferase (*ChAT*) and vasoactive intestinal peptide (*VIP*). **A** Cluster of ChAT-immunoreactive neurons in the external submucosal plexus (*ESP*). One large neuron (*) expressed ChAT immunoreactivity but not VIP **(B)** immunoreactivity (*). Note that the photomicrographic field in **B** is shifted to right as compared to that in **A** so as to show a smaller follicular area and to illustrate more VIP-immunoreactive neurons in the ESP. A small, VIP-immunoreactive neuron which resembles a mini-neuron (*arrow*) can be seen. Neurons expressing **(C)** ChAT-like and **(D)** VIP-like immunoreactivities in the ESP. Note a large neuron (*arrow in* **C**) that displays ChAT-like immunoreactivity is not immunoreactive for VIP (*arrow in* **D**). Another neuron that does not express ChAT immunoreactivity (* *in* **C**) is immunoreactive to VIP (* *in* **D**). **E,F** Colocalization of ChAT and VIP immunoreactivities in small neurons situated at the base of a follicle **(F)** in the ESP (*), and a few neurons in an interfollicular ganglion (*IF, arrow*). Only some of the larger neurons expressing ChAT immunoreactivity expressed VIP immunoreactivity (*arrowheads in* **E,F**) (*M* circular muscle). *Bars* 200 μm **(A,B,E,F)**; 50 μm **(C,D)**

Localization of SP-like immunoreactivity

SP-immunoreactive fibers in the jejunum were observed throughout the circular muscle and at the base of PP follicles. Thin, varicose fibers were also observed in the interfollicular area (Fig. 3B); these SP-immunoreactive fibers appeared in close proximity to blood vessels situated in the interfollicular area. SP-immunoreactive fibers were also found in PP domes (Fig. 3C). In cross sections of jejunal PP, these fibers resembled nerve terminations

in their profile and were similar in intensity and thickness to those observed in other areas and to those immunoreactive for PGP9.5. A specific punctate staining pattern of SP immunoreactivity was observed within PP follicles, which might represent severed nerve fibers; SP-immunoreactive fibers were also observed at similar locations in sections cut in the longitudinal plane. SP-immunoreactive fibers appeared to innervate the walls of the large blood vessels situated at the base of the follicles (Fig. 3D).

In the ileum, SP-immunoreactive fibers were observed at the base of PP follicles, but they were present at relatively lower numbers than in the jejunum. Varicose fibers were observed in the interfollicular area which were also present in fewer numbers than in the jejunum. In contrast to the jejunum, SP-immunoreactive fibers were not observed within the follicle or in the vicinity of submucosal blood vessels. The staining pattern of SP-immunoreactive fibers was similar for both antibodies. Although no immunoreactive neuronal perikarya could be observed with the use of one anti-SP antibody (Accurate), SP-immunoreactive neurons were observed in the ESP and ISP in both jejunum and ileum with the use of a second antibody (DiaSorin). The numbers of neurons were always greater in the ileum relative to the jejunum.

Localization of VIP-like immunoreactivity

In the jejunum, VIP-immunoreactive fibers were observed in the circular muscle. Varicose and non-varicose immunoreactive fibers and numerous neurons in submucosal ganglia were observed at the base of PP follicles (Fig. 2B). These VIP-immunoreactive neurons were small, and some of them were multidendritic. VIP-immunoreactive fibers were observed near the PP dome epithelium (Fig. 2D); a punctate pattern of immunoreactivity was observed in the dome which was completely absent in preabsorption controls. VIP-immunoreactive fibers were also observed in interfollicular areas and within individual follicles (Fig. 2E). Two to three VIP-immunoreactive fibers were observed innervating walls of the blood vessels situated at the base of a follicle (Fig. 2F).

Although VIP immunoreactivity was observed in a few large neurons in ileal ESP ganglia, most immunoreactive neurons were relatively small (Fig. 5B,D). As in the jejunum, VIP-immunoreactive fibers were observed in the ileal circular muscle. Numerous VIP-immunoreactive neurons were present in ganglia at the base of PP follicles and in interfollicular areas (Fig. 5F). Occasionally, VIP-immunoreactive fibers could be seen at the margins of PP follicles; however, immunoreactive fibers were not visualized within follicles. Immunoreactive nerve fibers were occasionally seen coursing towards the mucosal epithelium, and were rarely observed near submucosal blood vessels.

Colocalization of neurotransmitters

To examine the colocalization of transmitters in neurons innervating porcine GALT, double-labeling experiments were performed using anti-ChAT antisera in combination with antisera towards VMAT-2 and the three gut neuropeptides. In both jejunum and ileum, neurons or nerve fibers expressing ChAT immunoreactivity did not display immunoreactivity to VMAT-2. On the other hand, all NMU-8-positive neurons in jejunum and ileum manifested ChAT immunoreactivity. About 35% of VIP-immunoreactive neurons also displayed ChAT-like immunoreactivity in ESP and ISP in jejunum and ileum. In the jejunum, large neurons in submucosal ganglia expressed ChAT immunoreactivity, but not VIP immunoreactivity. However, some VIP-immunoreactive small neurons weakly expressed ChAT-like immunoreactivity (Fig. 2A,B). Staining of adjacent sections using antibodies against VIP and SP revealed that some SP-immunoreactive fibers in the circular muscle, near submucosal blood vessels and at the base of PP follicles were also immunoreactive for VIP. However, the majority of the fibers did not exhibit immunoreactivities for both VIP and SP. Indeed, VIP and SP immunoreactivity was not observed to be colocalized in neuronal perikarya in ESP or ISP of either jejunum or ileum.

In the ileum, large neurons lying at the base of ileal PP follicles exhibited immunoreactivity to both ChAT and NMU-8 (Fig. 4B,C). Sections incubated with antibodies to ChAT, NMU-8 and VIP revealed that NMU-8 was colocalized with ChAT- but not VIP-like immunoreactivity in both small and large neurons (Fig. 4D–F). Some NMU-8-immunoreactive fibers also displayed VIP immunoreactivity. VIP-/ChAT-immunoreactive neurons lying at the base of PP follicles were generally small, only a small number of the larger ChAT-immunoreactive neurons in the same location exhibiting VIP-like immunoreactivity (Fig. 5E,F).

Discussion

In the porcine jejunum and ileum, immunoreactivity towards PGP9.5 was detected in neurons or fibers in smooth muscle, as well as ESP, ISP and IF ganglia; fibers originating in the ISP often surrounded lymphoid follicles. These observations are in general support of a previous investigation of porcine PP which described a similar pattern of neural innervation using antisera towards neuron-specific enolase and PGP9.5 (Krammer and Kühnel 1993). In addition, PGP9.5-immunoreactive nerve fibers were observed to course through PP follicles in porcine jejunum, but not ileum. Nerve fibers have not been described previously within PP follicles (Krammer and Kühnel 1993; Balemba et al. 1998). This difference in the innervation of jejunal and ileal PP could arise from differences in tissue processing, the fixatives used, tissue sampling or differences in the structural organization of these two PP (see below).

Immunoreactivity towards VMAT-2 was observed in submucosal neurons and fibers in ESP and ISP as well as at the basolateral aspects of jejunal and ileal PP follicles. The colocalization of VMAT-2- and TH-like immunoreactivities suggests the presence of a catecholaminergic neuronal network in the porcine small intestine. A dense network of adrenergic fibers in the submucosal plexuses has been previously described in the porcine small intestine using the glyoxylic acid fluorescence method, but these fibers were not specifically observed near PP follicles (Scheuermann and Stach 1984). The possibility exists that different segments of the porcine small intestine were examined in the previous and present studies. Norepinephrine, acting through α-adrenoceptors, decreases spontaneous longitudinal contractions and alters active epithelial ion transport in the porcine ileum (Brown et al. 1990; Hildebrand and Brown 1990). The actions of dopamine have not been examined. It is possible that the VMAT-2-immunoreactive fibers observed in the present study subserve functional roles in the regulation of ileal motor function, mucosal transport and, possibly, immunity. In the rat intestine, VMAT-2-immunoreactive neurons have been identified in both myenteric and submucous plexuses; immunoreactive fibers have been observed terminating in smooth muscle and mucosa, and near submucosal blood vessels (De Giorgio et al. 1996). However, no VMAT-2-immunoreactive fibers were found near blood vessels. This could be due to interspecies difference or the use of different antibodies to VMAT-2. Glyoxylic-acid-fluorescence fibers were observed in internodular septa and interdomal regions of the PP in rabbit appendix (Felten et al. 1981) as well as in various lymphoid tissues, i.e., spleen, thymus and lymphoid tissue (Felten et al. 1985). The presence of these VMAT-2-immunoreactive fibers in the T-cell-rich interfollicular space is analogous to glyoxylic-acid-fluorescent fibers observed in rabbit appendix (Felten et al. 1981). VMAT-2 may modulate aspects of intestinal T-cell function, such as lymphocyte maturation or differentiation.

In the porcine small intestine, abundant ChAT-immunoreactive neurons were observed in both submucosal plexuses. Acetylcholine has previously been found to evoke active anion secretion in porcine jejunal and ileal epithelia after its direct application to mucosal sheets or its endogenous release by electrical stimulation of submucosal nerves (Chandan et al. 1991a, 1991b). Relatively large ChAT-immunoreactive neurons were found in the ESP, whereas smaller immunoreactive neurons were observed in the ISP. In the jejunum, ChAT-immunoreactive fibers were observed at the margin of PP follicles. ChAT-immunoreactive neurons have been observed in the myenteric and submucosal plexuses of the guinea pig small intestine, but there have been no previous reports of a juxtaposition of cholinergic neural elements with gut lymphoid tissue (Li and Furness 1998; Schemann et al. 1993).

NMU-8-immunoreactive nerves were found in circular muscle as well as in submucosal ganglia near jejunal and ileal PP. The localization of NMU-8 immunoreactivity in the submucosal plexuses is consistent with the previous findings of Timmermans et al. (1989, 1997), who found that immunoreactivities to ChAT, calcitonin gene-related peptide, SP, NMU and galanin were colocalized in large neurons of the ISP and that submucosal "mini-neurons" co-contain immunoreactivities to NMU and SP. Relatively fewer NMU-8-positive neuronal elements were observed in the myenteric plexus and smooth muscle (Timmermans et al. 1989). Large neurons identified as type II neurons in the former study resemble the large ESP neurons observed in the present study. No prominent differences in the presence of NMU-8 neurons between plexuses were noted in the present study, and relatively few NMU-8-positive fibers were observed in the circular muscle. At present, there has been no study of the possible actions of NMU on immune function. However, NMU increases active anion secretion in porcine small intestine, but does not alter smooth muscle contractility (Brown and Quito 1988).

In porcine small intestine, SP has been detected in the larger and relatively few type II neurons; fewer varicose and non-varicose fibers in the ISP express SP immunoreactivity (Timmermans et al. 1989). Varicose fibers were observed in both submucosal plexuses in the present study with the use of substance P antibodies from different sources. However, only one antibody could label SP-immunoreactive neuronal perikarya. This could be attributed to differences in the affinities of the two antibodies or differences in the peptide sequences used for the generation of each antibody. SP stimulates anion secretion in porcine jejunum through interactions with NK_1-neurokinin receptors that are expressed on both epithelial cells and submucosal neurons (Parsons et al. 1992). It also stimulates active ion transport in porcine ileal mucosa (Hildebrand and Brown 1990).

VIP-immunoreactive nerve fibers were observed in the jejunal and ileal PP as well as the circular muscle, ESP and ISP. A similar pattern of VIP immunoreactivity in porcine ENS has been reported by others (Balemba et al. 1998). As it does in intestinal mucosal preparations from a variety of species, VIP has a prosecretory action in the porcine small intestine and colon (Brown and O'Grady 1997). In earlier studies of porcine small intestine, VIP-immunoreactive fibers have been reported under PP domes but not within lymphoid follicles (Krammer and Kühnel 1993; Balemba et al. 1998). In the present study, however, VIP- and SP-immunoreactive fibers were localized in PP follicles as well as under domes. VIP and SP might regulate the uptake of luminal antigens by M cells or modulate aspects of antigen presentation. The areas under the PP dome contain dendritic cells which potently prime $CD4^+$ T lymphocytes (Mowat and Viney 1997). Receptors for SP and VIP appear to be expressed on several classes of immunocytes within lymphoid organs, and these gut peptides have been shown to modulate lymphocyte proliferation and immunoglobulin synthesis (Stead 1992; Stead et al. 1987; Chen and O'Dorisio 1993; Straub et al. 1998). VIP and

its homolog, pituitary adenylate cyclase-activating polypeptide, modulate cytokine expression in T lymphocytes (Martinez et al. 1996). Recently, SP has also been shown to modulate T-cell adhesion by acting through receptors expressed by T cells (Levite et al. 1998). In addition to gut lymphoid tissue, VIP- and SP-immunoreactive fibers appeared to terminate near blood vessels situated at the base of the follicles or in the interfollicular area. This pattern of distribution is in support of the actions of these peptides on intestinal blood flow (Chou and Alemayehu 1993). As lymphocyte trafficking has been shown to be affected by VIP, the presence of VIP-immunoreactive fibers in the vicinity of submucosal blood vessels could be of importance in immune responses to intestinal infection (Ottaway 1991). Lymphocytes migrate from blood throughout the follicle via postcapillary venules lined with high endothelial cells situated in the interfollicular area (Yamaguchi and Schoefl 1983; Bjerknes et al. 1986). In cat PP, VIP receptors are localized predominantly on extravascular T cells near high endothelial venules and lymphatic vessels and SP receptors are localized in T- and B-cell-enriched areas close to the margins of PP follicles (Ichikawa et al. 1994). In human PP, receptors for VIP were localized predominantly on T lymphocytes surrounding the follicle, and SP receptors were expressed on submucosal blood vessels (Reubi et al. 1998). The distribution of SP- and VIP-immunoreactive fibers terminating near blood vessels situated between, or at the base of, PP follicles suggests that these neurotransmitters regulate vascular tone and lymphocyte trafficking in PP.

ChAT, SP and VIP immunoreactivities were differently distributed in jejunal and ileal PP. In jejunal PP, ChAT, SP and VIP immunoreactivities were localized beneath the dome epithelium and ChAT, NMU, SP and VIP immunoreactivities were visualized within follicles. Moreover, SP- and VIP-immunoreactive fibers were observed to innervate jejunal blood vessels. In contrast, no immunoreactivity to VMAT-2, ChAT or gut peptides was observed within ileal PP follicles, although nerve fibers and ganglia resided at the margins of follicles. The differential innervation of jejunal and ileal PP may have morphological and functional significance (Binns and Licence 1985; Stokes et al. 1994). Ileal PP are lined by intestinal villi and do not possess the dome epithelium characteristic of discrete jejunal PP. Jejunal PP has more T cells than ileal PP and the ratio of T:B cells varies between jejunal and ileal PP (Binns and Licence 1985; Rothkötter and Pabst 1989). It is possible that the presence of immunoreactive fibers in jejunal PP dome and ileal villi may be related to differential antigen sampling by these two PP. Antigen must cross M cells in the dome of jejunal PP to initiate the antigen response, whereas in ileum antigen may be sampled by the villous epithelium (Stokes et al. 1994). It is interesting to note that following antigenic stimulation, antigen-specific B cells migrate to the margins of PP follicle and interact with T cells (Garside et al. 1998). The presence of VIP- and SP-immunoreactive fibers in the interfollicular area, as observed in the present study, might influence T lymphocyte function in jejunum and ileum. The occurrence of catecholaminergic, cholinergic and peptidergic nerves around or within jejunal and ileal PP suggests that several types of neurotransmitters could modulate different aspects of gut lymphoid function. The precise roles of these transmitters in the regulation of gut immunity await further examination.

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