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# Neuromedin U-immunoreactivity in the nervous system of the small intestine of the pig and its coexistence with substance P and CGRP

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**Summary.** In the small intestine of the pig, neuromedin U (NMU)-immunoreactivity was mainly confined to the nerve plexus of the inner submucosal and mucosal regions. After colchicine treatment, a high number of immunoreactive nerve cell bodies was observed in the plexus submucosus internus (Meissner), whereas only a low number was found in the plexus submucosus externus (Schabadasch). The plexus myentericus as well as the aganglionic nerve meshworks in the circular and longitudinal smooth muscle layers almost completely lacked NMU-immunoreactivity. Double-labeling experiments demonstrated the occurrence of distinct NMU-containing neuron populations in the plexus submucosus internus: (1) relatively large type-II neurons revealing immunoreactivity for NMU and calcitonin gene-related peptide (CGRP) and/or substance P (SP); (2) a group of small NMU- and SP-immunoreactive neurons; (3) a relatively low number of small neurons displaying immunoreactivity for NMU but not for SP. Based on its distributional pattern, it is concluded that NMU plays an important role in the regulation and control of mucosal functions.

**Key words:** Neuromedin U (NMU) – Substance P (SP) – Calcitonin gene-related peptide (CGRP) – Enteric nervous system – Nerve plexus – Small intestine – Pig

The functional role of NMU, the most recently discovered member of the neuromedin family, remains unclear. Two of its molecular forms, one consisting of 8 amino acids and the other of 25 (Minamino et al. 1985a, b), seem to be bioactive. Its unique C-terminal amide structure as well as its potent uterus-stimulating activity and hypertensive effect strongly suggest a specialized physiological role (Minamino et al. 1985a, b). Nevertheless, the present knowledge of NMU is still fragmentary. NMU has been found in considerable amounts in several parts of the central nervous system (Domin et al. 1986, 1987; Honzawa et al. 1987; Ballesta et al. 1988; Steel et al. 1988), the urogenital tract (Domin et al. 1987), and the gastrointestinal tract of the rat (Domin et al. 1987; Augood et al. 1988; Ballesta et al. 1988) and guinea-pig (Augood et al. 1988).

Previous reports have already pointed to important species differences with regard to the morphology and function of the different parts of the enteric nervous system, more particularly, the submucosal ganglionic nerve networks (Stach 1977a; Gabella 1979; Scheuermann et al. 1987a, b). Therefore, the principal aim of this study is to determine the occurrence and distributional pattern of NMU-immunoreactivity in the enteric nerve plexuses of the porcine small intestine using immunocytochemical techniques and, secondly, to study its possible coexistence with other neurotransmitters and/or neuromodulators in one neuron.

## Materials and methods

Six-week-old domestic pigs were killed by a blow on the head and decapitated 5 1/2 h after intraperitoneal injection of colchicine (10 mg/kg). In order to diminish the background staining on the whole-mounts due to endogenous peroxidase activity, the blood of the intestinal vessels was washed out by perfusion with Krebs-Ringer solution through the superior mesenteric artery. Then, jejunal and ileal segments were dissected out and an oxygenated Krebs' solution was circulated through these segments for 30 min at 37° C. The segments were then ligated, filled with and immersed in 4% paraformaldehyde containing 0.2% picric acid in phosphate buffer (pH = 7.2; 0.01 M). The remainder of the procedure has previously been described (Scheuermann et al. 1987c).

Aiming at better immunocytochemical conditions, the method initially described by Llewellyn-Smith et al. (1985) for electron-microscopical purposes, was successfully applied with slight modifications for improved light-microscopical immunocytochemistry.

Whole-mounts containing either the submucosal plexuses and part of the mucosal layer or the plexus myentericus adhered to the longitudinal muscle layer, were preincubated in 10% normal donkey serum followed by incubation in primary antiserum for 17 h at room temperature. A detailed description of the primary antisera used is given in Table 1. For single-labeling experiments, donkey anti-rabbit biotinylated IgG (diluted 1:100; RPN.1004, Amersham, Brussels, Belgium) was used for 2 h at room temperature followed by incubation in streptavidin-biotinylated peroxidase com-

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**Table 1.** Detailed description of the primary antisera used for immunocytochemistry

| Antigen | Host species | Dilution | Source |
|---|---|---|---|
| Synthetic SP (poly) | Rabbit | 1:100–400 | RPN.1572 Amersham, Brussels, Belgium |
| Synthetic SP (poly) | Rabbit | 1:400–800 | i675/002 UCB Bioproducts, Brussels, Belgium |
| Synthetic SP (mono) | Rat | 1:20–50 | MAS 035b Sera-lab, Sussex, UK |
| Synthetic rat α-CGRP (poly) | Rabbit | 1:400–800 | RPN.1842 Amersham, Brussels, Belgium |
| Synthetic rat α-CGRP (poly) | Rabbit | 1:400–800 | Hammersmith Hospital, London, UK |
| Neuromedin U | Rabbit | 1:200–400 | Hammersmith Hospital, London, UK |

plex (diluted 1:100, RPN.1051, Amersham) for 17 h at room temperature. The peroxidase reaction was visualized using 4-chloro-1-naphtol as chromogen. Double staining for SP and NMU was performed by simultaneous incubation in the primary antisera at room temperature for 17 h. SP- and NMU-immunoreactivities were visualized using fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (4 h; diluted 1:20; Nordic; Tilburg, The Netherlands) and tetramethylrhodamineisothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (4 h; diluted 1:20; Nordic), respectively. Double-labeling experiments for SP and CGRP were carried out in the same manner. For the co-localization of NMU and CGRP, a sequential immunostaining method involving the elution technique of Tramu et al. (1978) had to be used since both primary antisera were raised in the same host species. In the latter method, the first antigen was visualized with the biotin-streptavidin method as described above using diaminobenzidine as the chromogen. Then, the antibodies were eluted by immersion for 2 min with agitation in a mixture containing 2.5% $KMnO_4$ (50 ml), 5% $H_2SO_4$ (50 ml) and 7 ml distilled water. After rinsing in PBS, the tissue was decolorized in 0.5% $Na_2S_2O_5$ at room temperature for 5 min, again thoroughly rinsed in PBS several times, and then additionally immersed in 0.15% $NaCNBH_3$ for 30 min. Subsequently, the tissue was incubated in the second primary antiserum and immunoreactivity was visualized by means of the indirect fluorescence method using a FITC- or TRITC-conjugated secondary antibody. The tissue was then sequentially viewed with the light microscope in the normal transmitted light mode and in the specific fluorescence mode.

The specificity of the methods was controlled by omitting one or more steps in the indirect-labeling procedure or by replacing the primary antisera with non-immune serum. In the double-labeling experiments, no reactivity was found between primary antibodies raised in rabbits and secondary antibodies raised against rat IgG, whereas similarly the primary antibodies raised in rats did not react with the secondary antibodies raised against rabbit IgG. To test the efficiency of the elution procedure, whole mounts incubated in the corresponding FITC- or TRITC-conjugated antibody or a secondary antibody followed by a peroxidase anti-peroxidase complex after the elution, did not reveal any immunostaining. Similar staining patterns for the same antigen were observed after labeling with primary antisera obtained from different sources (see Table 1). The specificity of the NMU-antiserum was tested previously by absorption controls (Domin et al. 1986; Ballesta et al. 1988). Preabsorption of the NMU-antiserum with SP (UCB Bioproducts B570; 100 uM/ml antiserum) did not decrease immunostaining.

## Results

### *Neuromedin U in the plexus submucosus internus (Meissner)*

Immunoreactivity for NMU was mainly found in the inner layer of the submucosa and in the mucosa (Fig. 1). Although immunoreactive fibres were revealed in the plexus submucosus internus (Figs. 2, 3), an even larger amount was observed in the lamina muscularis mucosae (Fig. 4)

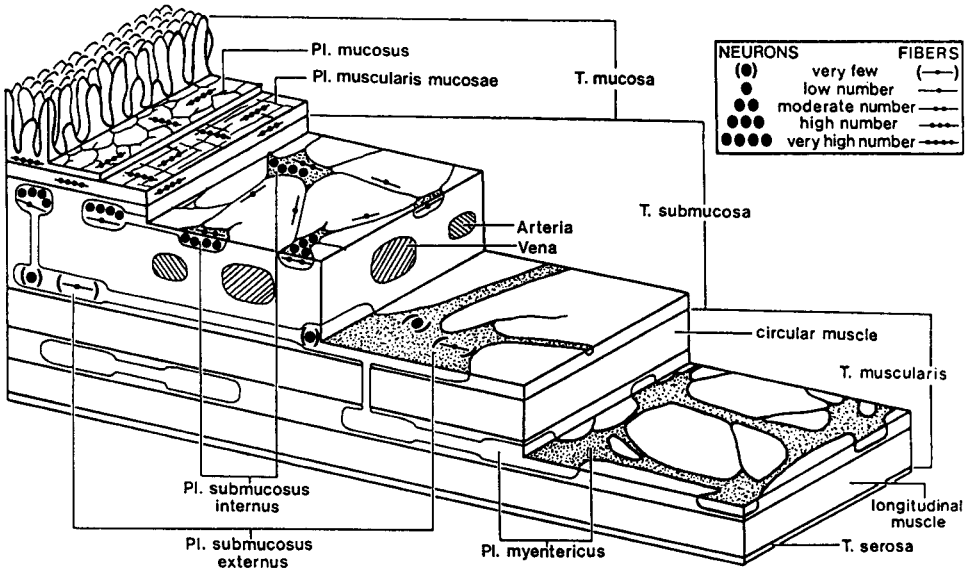



**Fig. 1.** Schematic representation of the occurrence and distribution of NMU in the intramural nerve plexuses in the small intestine of the pig

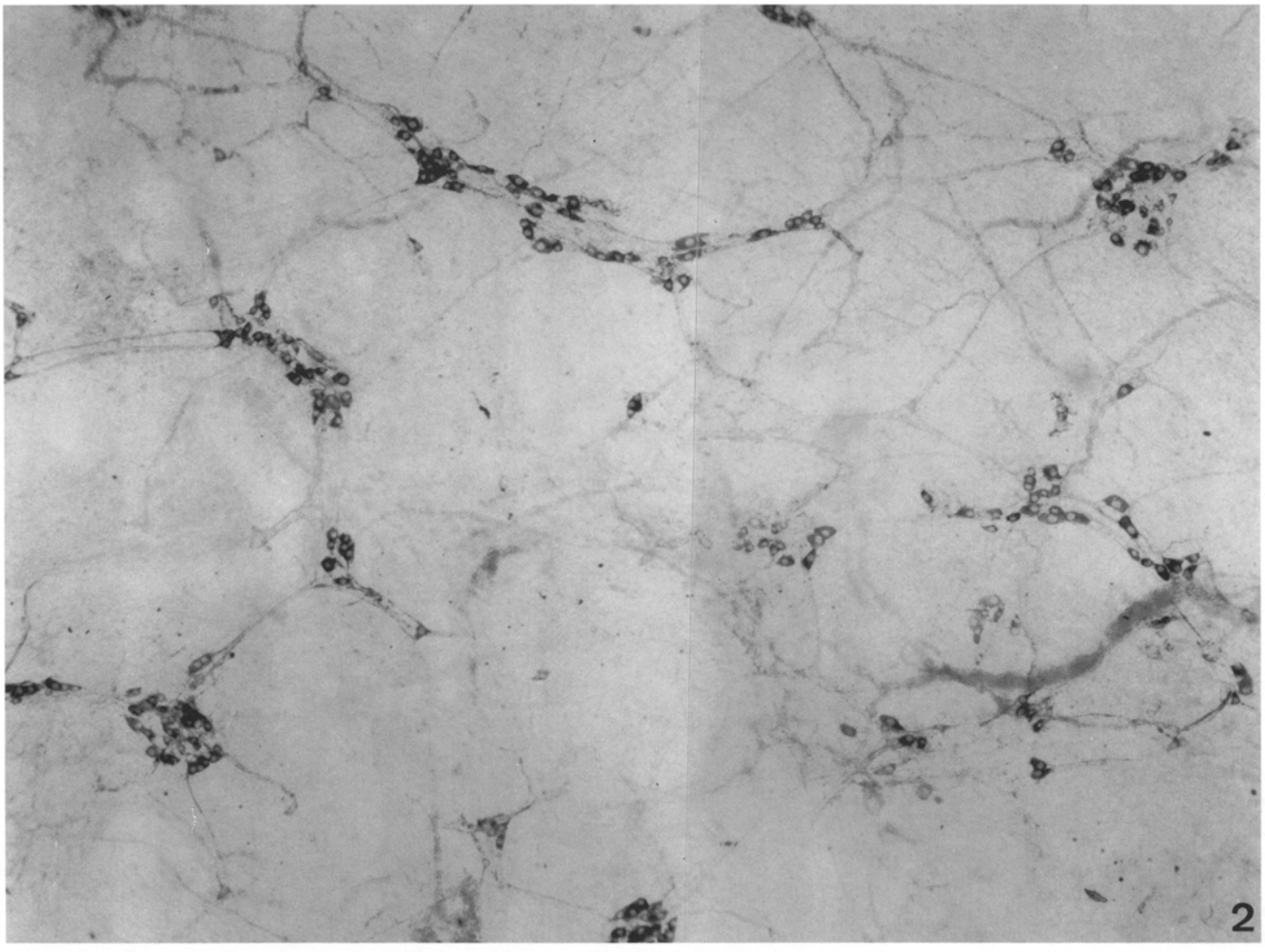



**Fig. 2.** Survey picture of the plexus submucosus internus (Meissner) in a whole-mount preparation after immunolabeling for NMU. ×110

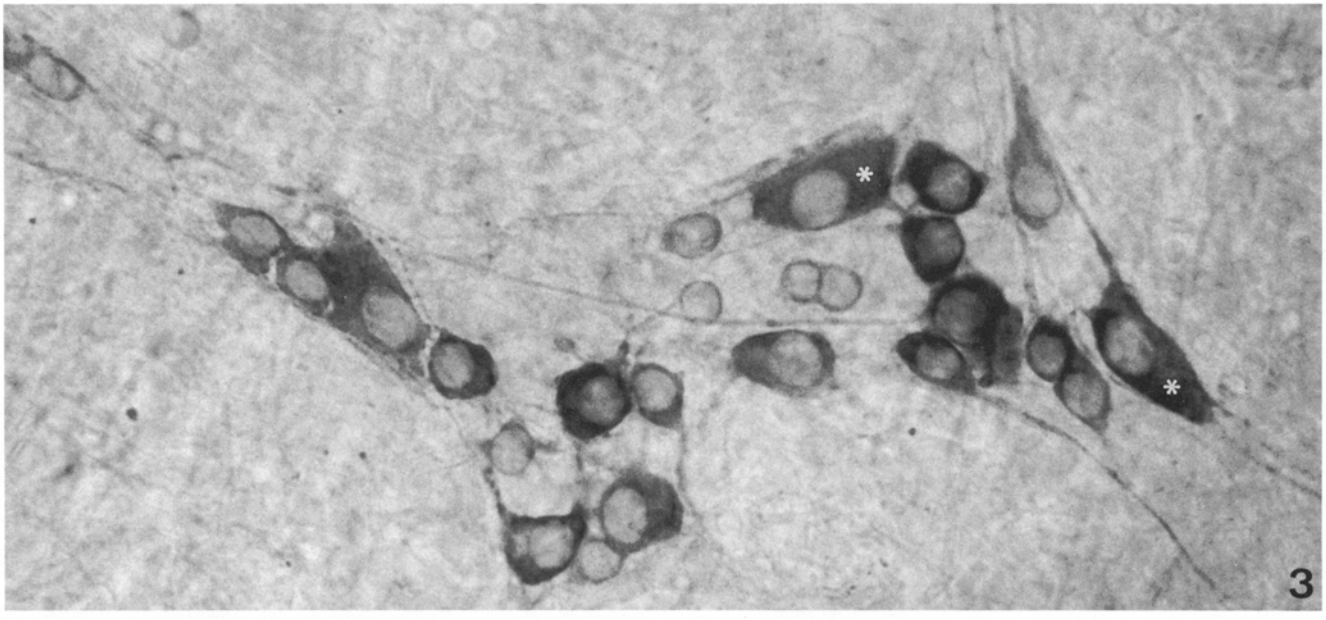
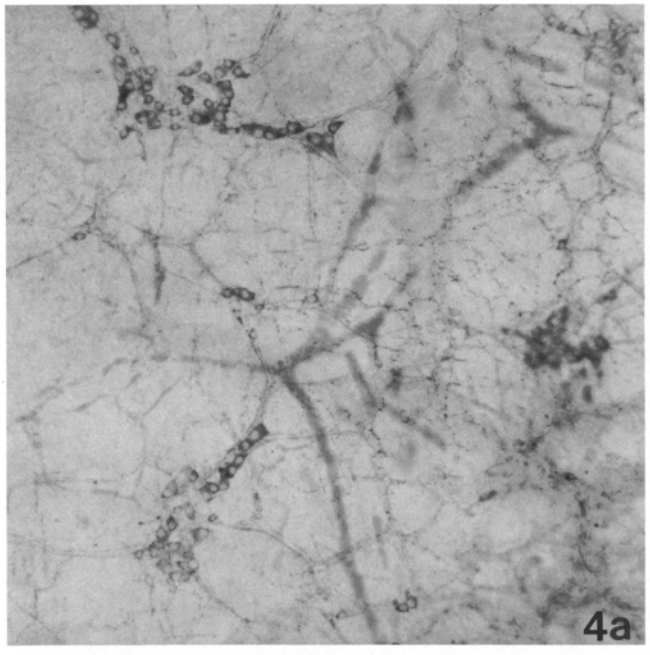
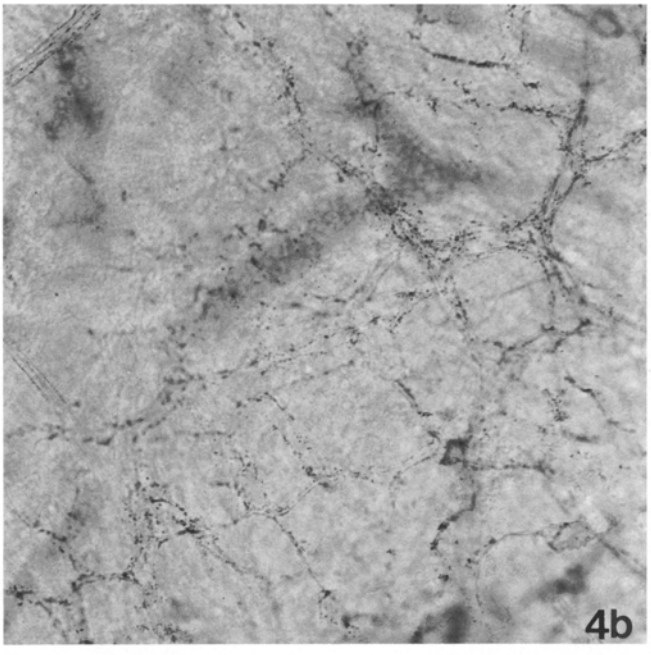
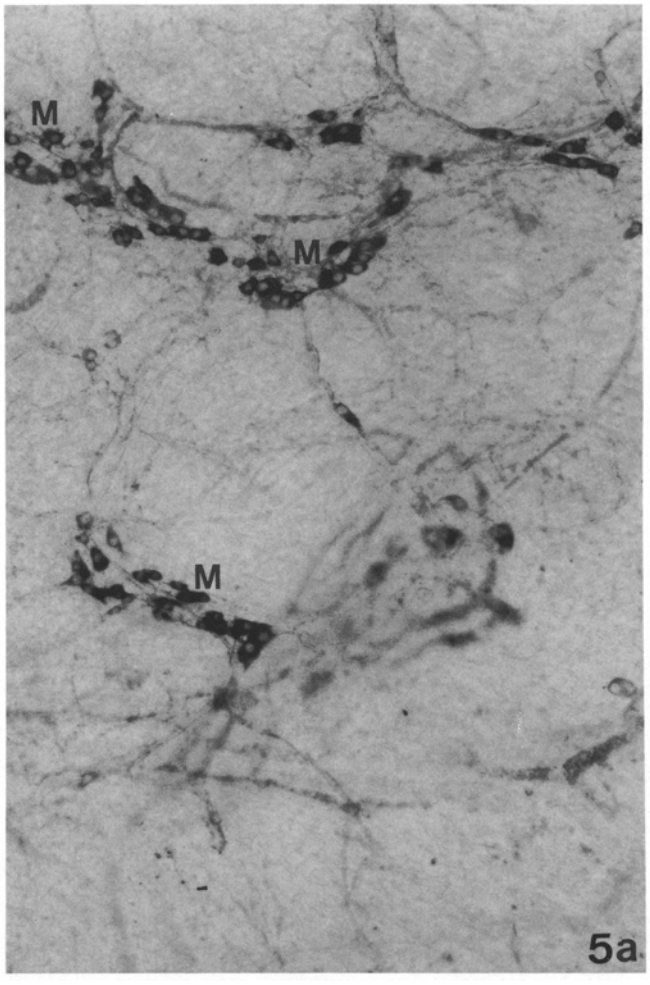



**Fig. 3.** Detail of a ganglion of the plexus submucosus internus showing NMU-immunoreactive neurons characterized by a small diameter. Note the presence of a few larger NMU-immunoreactive, adendritic pseudouni- or multiaxonal type-II neurons (*asterisk*). ×870



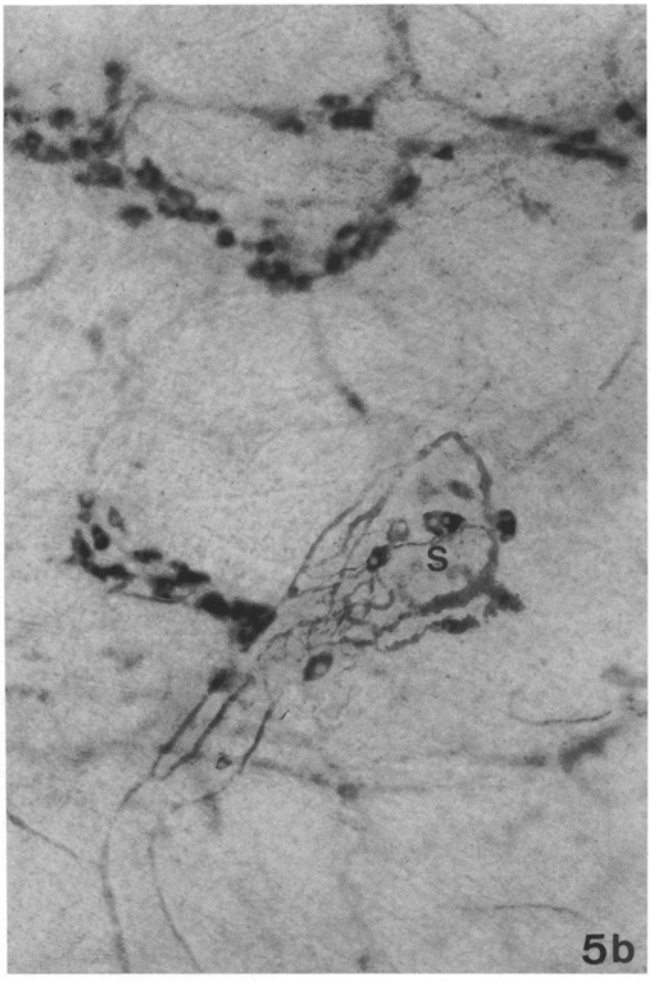
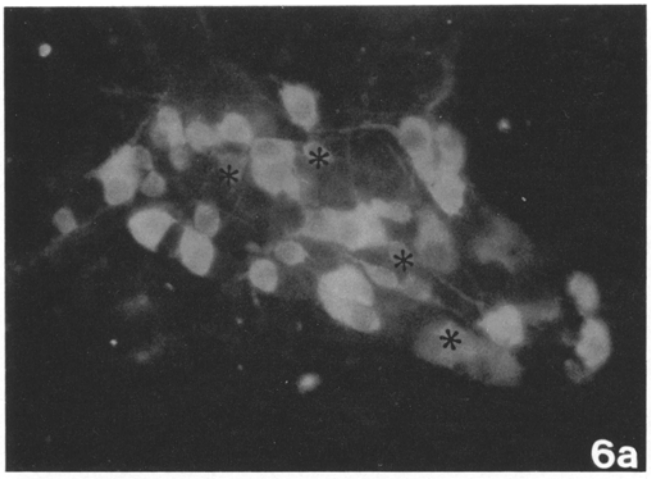
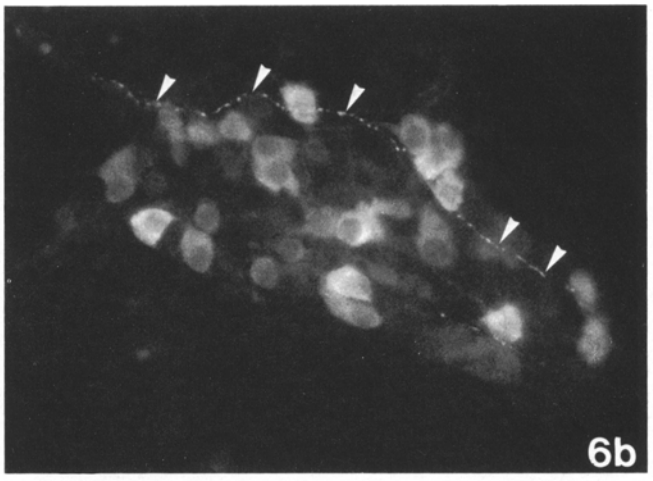
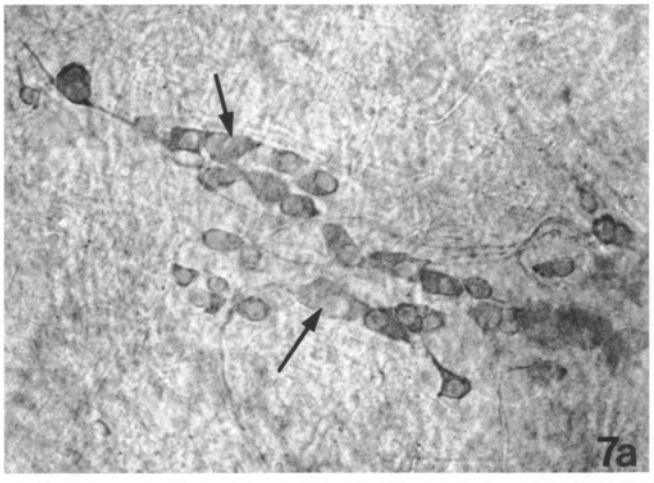



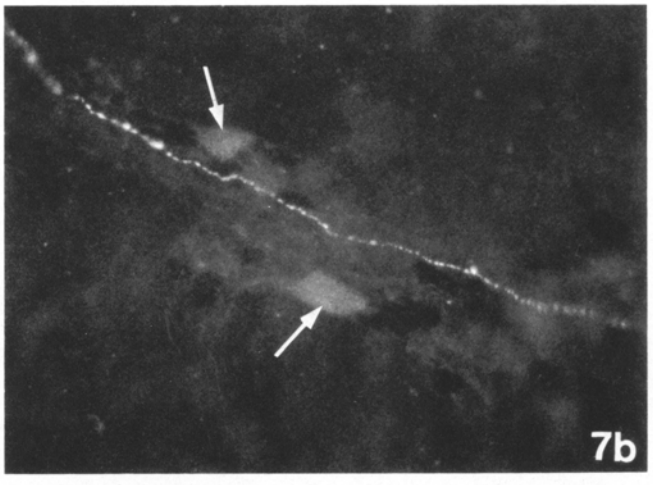

**Fig. 4. a** The plexus submucosus internus (Meissner) and the plexus muscularis mucosae visualized after immunolabeling for NMU; **b** detail of the dense NMU-immunopositive nerve meshwork traversing or innervating the lamina muscularis mucosae. **a** ×95, **b** ×240

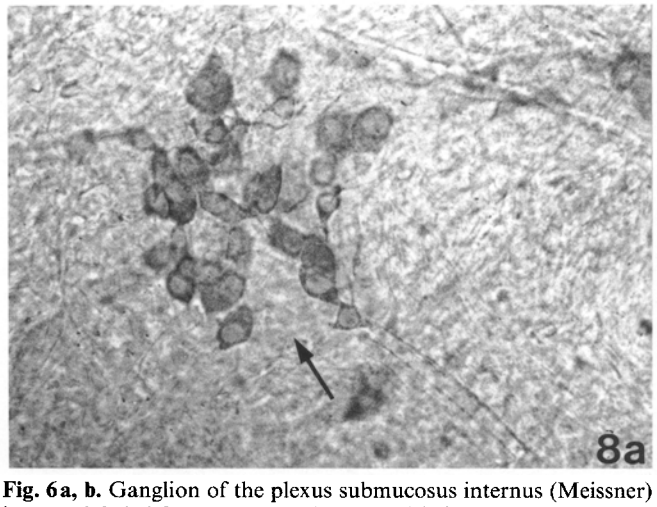
**Fig. 5a, b.** Part of the submucosal layer, immunolabeled for NMU, photographically focused on either **a** the plexus submucosus internus (Meissner) or **b** the plexus submucosus externus (Schabadasch). Only few NMU-immunoreactive neurons can be observed in the ganglion of the plexus submucosus externus (*S*) compared to the high number in the plexus submucosus internus (*M*). **a, b** ×120



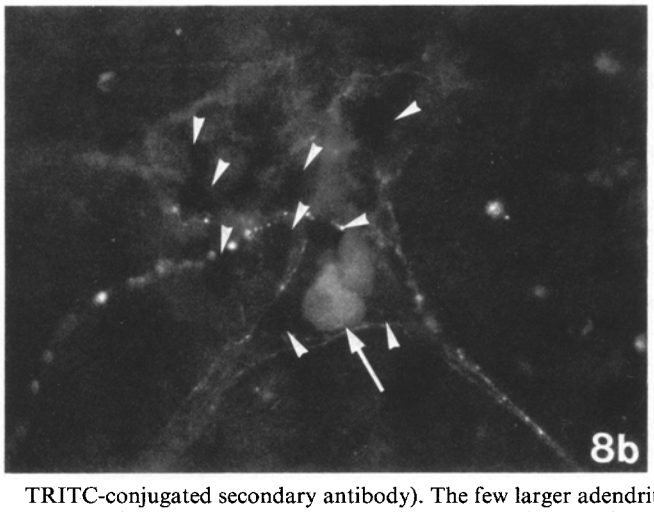




**Fig. 6a, b.** Ganglion of the plexus submucosus internus (Meissner) immunolabeled for **a** NMU and **b** SP. With few exceptions (*asterisk*), most of the NMU-immunopositive neurons also stain for SP. Note the presence of a varicose SP-positive nerve fibre (*arrowheads*), probably originating either from cell bodies located in the plexus submucosus externus or plexus myentericus or from extrinsic neurons; this fibre does not show any immunostaining for NMU. **a, b** × 275

**Fig. 7a, b.** Ganglion of the plexus submucosus internus (Meissner) immunolabeled for **a** NMU (visualized by the biotin-streptavidin method using diaminobenzidine) and **b** CGRP (visualized by a TRITC-conjugated secondary antibody). The few larger adendritic pseudouni- or multiaxonal type-II neurons reveal a coexistence of NMU and CGRP (*arrows*), whereas the small NMU-immunoreactive neurons are CGRP-immunonegative. **a, b** × 275

**Fig. 8a, b.** Ganglion of the plexus submucosus internus (Meissner) immunolabeled for **a** NMU and **b** CGRP. Some of the type-II neurons display immunoreactivity for CGRP and not for NMU (*arrow*). In **b**, NMU-immunoreactive neurons can be recognized by the dark, non-fluorescent contours of their cell bodies (*arrowheads*). **a, b** × 290

and in the aganglionic nerve network of the lamina propria. A high number of NMU-immunoreactive nerve cell bodies ($90 \pm 21$ cells/mm$^2$) was detected; most of them were relatively small in diameter (mean maximum diameter $45 \pm 13$ µm; $n = 100$). However, some of the larger smooth-contoured neurons (mean maximum diameter $94 \pm 20$ µm; $n = 30$), morphologically displaying all the features of the adendritic pseudouni-or multiaxonal type-II neurons, also appeared to be immunoreactive for NMU (Fig. 3). Compared to the high number of NMU-immunoreactive neuronal cell bodies, there were relatively few NMU-immunoreactive varicose nerve fibres in the ganglia and in the connecting nerve strands. Most of the nerve fibres within the ganglia appeared smooth-surfaced, resembling the proximal part of axons (Fig. 3). Varicose NMU-immunoreactive processes surrounding NMU-immunoreactive neurons and non-reactive nerve cell bodies were only scarcely seen.

*Neuromedin U in the plexus submucosus externus (Schabadasch)*

A few scattered individual immunoreactive fibres were observed in this plexus. Weakly immunoreactive neuronal perikarya occurred in low numbers individually or in small groups ($0.68 \pm 0.34$ cells/mm$^2$) (Figs. 1, 5). The latter were large in diameter, had a smooth-contoured appearance with one or more long processes and could therefore be identified as type-II neurons.

*Neuromedin U in the plexus myentericus (Auerbach)*

Very little immunoreactivity was observed in the plexus myentericus or in the plexus muscularis superficialis or profundus (Fig. 1). The number of NMU-immunoreactive neurons, morphologically similar to those of the plexus submucosus externus, was even less than 1 cell/2 cm$^2$ and can be considered as negligible with regard to the total number of intrinsic neurons.

*Neuromedin U in the vascular nerve plexus*

No NMU-immunoreactive nerve fibres were seen in close proximity to blood vessels.

*Coexistence of neuromedin U with substance P and calcitonin gene-related peptide*

Double-labeling experiments revealed the coexistence of NMU with SP (Fig. 6) and CGRP (Figs. 7, 8) in the plexus submucosus internus and in the aganglionic nerve meshworks of the mucosa. Almost all the neuronal cell bodies displaying immunoreactivity for NMU also revealed immunoreactivity for SP. The same observation holds true for the major part of the varicose NMU-immunoreactive nerve fibres in the lamina muscularis mucosae and in the lamina propria. A few nerve cell bodies showed immunoreactivity for NMU and not for SP (Fig. 6), whereas individual varicose nerve fibres were observed running through a ganglion of the plexus submucosus internus, displaying immunoreactivity for SP but not for NMU (Fig. 6). Only a portion of the CGRP-immunoreactive type-II neurons of the plexus submucosus internus proved to be reactive for NMU.

## Discussion

A number of similarities and differences exist between the pig and the rat regarding the NMU-immunoreactivity in the enteric nervous system. In the small intestine of the rat, NMU was confined to the neuronal elements in the plexus myentericus and submucosus and to the nerve fibres of the mucosa (Domin et al. 1987; Ballesta et al. 1988). In the porcine gut, the most dense NMU-innervation was also found at the luminal side. The absence of NMU-immunoreactive fibres in the circular and longitudinal smooth muscle layers as well as in the vascular plexus is similar to the findings in the rat gastrointestinal tract (Domin et al. 1987; Augood et al. 1988; Ballesta et al. 1988).

In the pig, however, the difference in NMU-immunoreactivity between the luminal and abluminal region is much more pronounced than in the rat because the plexus myentericus is almost completely devoid of NMU-immunoreactive neuronal cell bodies and nerve fibres. The difference in the occurrence and distribution of NMU between the plexus submucosus internus and the externus argues for the distinct functional entity of the morphologically and topographically different nerve networks (Stach 1977a, b; Scheuermann et al. 1989a, b; Timmermans et al. 1989). Moreover, a portion of the larger NMU-containing neuronal cell bodies in the plexus submucosus internus, more particularly some of the type-II neurons (Stach 1981), also contain CGRP. In the rat enteric nervous system, NMU was revealed to coexist with CGRP (Ballesta et al. 1988) but not with other neuropeptides.

An additional interesting observation was the resemblance between the staining patterns of the inner region of the porcine small intestine after NMU- and SP-immunohistochemistry. Most of the nerve cell bodies as well as the varicose and non-varicose nerve fibres in the plexus submucosus internus and in the mucosa also show SP-immunoreactivity. Thus, based on its neurotransmitter and/or neuromodular content, at least three distinct NMU-containing neuronal populations can be distinguished: (1) some larger type-II neurons reveal NMU and CGRP, (2) some small neurons only contain NMU, and (3) others show NMU and SP. Since previous reports (Scheuermann et al. 1987c; Stach et al. 1988a) already revealed the coexistence of CGRP and SP in the larger and relatively few type-II neurons present in the plexus submucosus internus, evidence is available that at least some of them contain NMU as well as CGRP and SP. The presence of a high number of NMU/SP-containing nerve cell bodies in the plexus submucosus internus contrasted with the relatively low density of the varicose-immunoreactive fibres. This may be related to the high number of NMU/SP-containing fibres observed in the lamina muscularis mucosae and lamina propria, which originate from the nerve cell bodies located in the plexus submucosus internus. The scanty, varicose SP-immunoreactive fibres that are NMU-immunonegative can be assumed to have an extrinsic source or to originate from cell bodies located in the plexus submucosus externus and myentericus.

In the present investigation, only small numbers of NMU-immunoreactive neuronal elements were seen in the plexus submucosus externus, and even less in the plexus myentericus and in the smooth muscle coat. On the other hand, abundant SP-immunoreactivity was observed in intrinsic nerve fibres and in multidendritic neurons of the plexus submucosus externus and myentericus as well as in nerve fibres of the plexus muscularis profundus (Stach et al. 1988b). Available evidence therefore indicates the existence of functionally distinct SP-containing neurons. The data

presented here argue for NMU playing an important motorial role in the mucosal absorptive and/or secretory processes. However, a detailed functional explanation of the working mechanism of this novel neuropeptide remains to be clarified.

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## References

Augood SJ, Keast JR, Emson PC (1988) Distribution and characterization of neuromedin U-like immunoreactivity in rat brain and intestine and in guinea pig intestine. Regul Pept 20:281–292

Ballesta J, Carlei F, Bishop AE, Steel JH, Gibson SJ, Fahey M, Hennessey R, Domin J, Bloom SR, Polak JM (1988) Occurrence and developmental pattern of neuromedin U-immunoreactive nerves in gastrointestinal tract and brain of the rat. Neuroscience 25:797–816

Domin J, Ghatei MA, Chohan P, Bloom SR (1986) Characterization of neuromedin U-like immunoreactivity in rat, porcine, guinea-pig and human tissue extracts using a specific radioimmunoassay. Biochem Biophys Res Commun 140:1127–1134

Domin J, Ghatei MA, Chohan P, Bloom SR (1987) Neuromedin U. A study of its distribution in the rat. Peptides 8:779–784

Gabella G (1979) Innervation of the gastrointestinal tract. Int Rev Cytol 59:129–193

Honzawa M, Sudoh T, Minamino N, Tohyama M, Matsuo H (1987) Topographic localization of neuromedin U-like structures in the rat brain: an immunohistochemical study. Neuroscience 23:1103–1122

Llewellyn-Smith IJ, Costa M, Furness JB (1985) Light- and electron microscopic immunocytochemistry of the same nerves from whole mount preparations. J Histochem Cytochem 33:857–866

Minamino N, Kangawa K, Matsuo H (1985a) Neuromedin U-8 and U-25: novel uterus stimulating and hypertensive peptides identified in porcine spinal cord. Biochem Biophys Res Commun 130:1078–1085

Minamino N, Sudoh T, Kangawa K, Matsuo H (1985b) Neuromedins: novel smooth-muscle stimulating peptides identified in porcine spinal cord. Peptides 6 [Suppl] 3:245–248

Scheuermann DW, Stach W, Timmermans J-P (1987a) Topography, architecture and structure of the plexus submucosus internus (Meissner) of the porcine small intestine in scanning electron microscopy. Acta Anat (Basel) 129:96–104

Scheuermann DW, Stach W, Timmermans J-P (1987b) Topography, architecture and structure of the plexus submucosus externus (Schabadasch) of the porcine small intestine in scanning electron microscopy. Acta Anat (Basel) 129:105–115

Scheuermann DW, Stach W, De Groodt-Lasseel MHA, Timmermans J-P (1987c) Calcitonin gene-related peptide in morphologically well-defined type-II neurons of the enteric nervous system in the porcine small intestine. Acta Anat (Basel) 129:325–328

Scheuermann DW, Stach W, Timmermans J-P, Adriaensen D (1989a) Immunohistochemistry provides evidence for the existence of two functionally different submucosal plexuses in the small intestine of the pig. Anat Anz (in press)

Scheuermann DW, Stach W, Timmermans J-P (1989b) Serotonin-immunoreactivity in the wall of the porcine small intestine. Verh Anat Ges 83 (in press)

Stach W (1977a) Der Plexus submucosus externus (Schabadasch) im Dünndarm des Schweins I. Form, Struktur und Verbindungen der Ganglien und Nervenzellen. Z Mikrosk Anat Forsch 91:737–755

Stach W (1977b) Neuronenstruktur und -architektur im Plexus submucosus externus (Schabadasch) des Duodenums. Verh Anat Ges 71:867–871

Stach W (1981) Zur neuronalen Organisation des Plexus myentericus (Auerbach) im Schweinedünndarm. II. Typ II-Neurone. Z Mikrosk Anat Forsch 95:161–182

Stach W, Scheuermann DW, Timmermans J-P (1988a) Morphology and immunohistochemistry of the intramural nervous system in the porcine small intestine. Part I. Acta Gastroenterol Belg 51:A1–A2

Stach W, Scheuermann DW, Timmermans J-P (1988b) Vorkommen und Verteilung von Substanz P in Neuronen und Axonen des Darmwandnervensystems im Schweinedünndarm. Verh Anat Ges 82 (in press)

Steel JH, Van Noorden S, Ballesta J, Gibson SJ, Ghatei MA, Burrin J, Leonhardt U, Domin J, Bloom SR, Polak JM (1988) Localization of 7B2, neuromedin B, and neuromedin U in specific cell types of rat, mouse, and human pituitary, in rat hypothalamus, and in 30 human pituitary and extrapituitary tumors. Endocrinology 122:270–282

Timmermans J-P, Scheuermann DW, Stach W, Adriaensen D (1989) The plexus submucosus externus (Schabadasch) and plexus submucosus internus (Meissner) in the small intestine of the pig: topography and structure with reference to the distribution of immunoreactivity for serotonin and some neuropeptides. Anat Rec 223:115A

Tramu G, Pillez A, Leonardelli J (1978) An efficient method of antibody elution for the successive or simultaneous localization of two antigens by immunocytochemistry. J Histochem Cytochem 26:322–324

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