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Neurotransmitters and putative neuromodulators in the gut of *Anguilla anguilla* (L.). Localizations in the enteric nervous and endocrine systems

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SUMMARY

The gut of silver eels (*Anguilla anguilla* L.) was investigated in order to describe both the cholinergic and adrenergic intramural innervations, and the localization of possible accessory neuromediators. Histochemical reactions for the demonstration of nicotinamide adenine dinucleotide phosphate, reduced form-(NADPH-)diaphorase and acetylcholinesterese (AChEase) were performed, as well as the immunohistochemical testing of tyrosine hydroxylase, met-enkephalin, substance P, calcitonin gene-related peptide (CGRP), bombesin, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), somatostatin, cholecystokinin-octapeptide (CCK-8), serotonin, cholineacetyltransferase. The results evidenced a different pattern in comparison with other vertebrates, namely mammals, and with other fish. Both NADPH-diaphorase and AChEase activities were histochemically detected all along the gut in the myenteric plexus, the inner musculature and the propria-submucosa. Tyrosine hydroxylase immunoreactivity was observed in the intestinal tract only, both in the myenteric plexus and in the inner musculature. Several neuropeptides (met-enkephalin, CGRP, bombesin, substance P, VIP, NPY, somatostatin) were, in addition, detected in the intramural innervation; some of them also in epithelial cells of the diffuse endocrine system (met-enkephalin, substance P, NPY, somatostatin). Serotonin was only present in endocrine cells. Tyrosine hydroxylase immunoreactivity was present in localizations to those of similar NADPH-diaphorase-reactivity, and in the same nerve bundles in which substance P- and CGRP-like-immunoreactivities were detectable in the intestinal tract. In addition, NADPH-diaphorase-reactive neurons showed an anatomical relationship with AChEase-reactive nerve terminals, and a similar relationship existed between the latter and substance P-like immunoreactivity.

INTRODUCTION

The enteric nervous system (ENS) is reputed to be the third division of the autonomic nervous system as it can function independently from the cen-

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tral nervous system (CNS) (Furness and Costa, 1987). The ENS of most species of fish lacks a submucosal plexus, whereas the myenteric plexus contains, as in higher vertebrates, nerve cell bodies and terminals (Kirtisinghe, 1940; Nicol, 1952; Smith, 1989; Domeneghini, 1995). In addition, the fish autonomic nervous system is differently composed and arranged in comparison with that of higher vertebrates, above all in its gut cephalic components (Burnstock, 1969). In fish too, as in mammals, the ENS co-operates with the epithelial cells of the diffuse endocrine system (DES) (Barrenechea *et al*., 1994; Kiliaan *et al*., 1996; Reinecke *et al*., 1997) to regulate and coordinate motility, secretion and absorption, as well as blood flow. Even if knowledge about the distribution, origin and functional roles of different messengers in the fish gastrointestinal tract is meager as compared with mammals, a lot of mammalian substances have been identified in the neuroendocrine system of the fish gut (Bjenning and Holmgren, 1988; Killian *et al*., 1992; Li and Furness, 1993; Jensen and Holmgren, 1994; Domeneghini *et al*., 1999).

The micro-anatomy of the alimentary canal of the eel is well known (Yamamoto and Hirano, 1978; Clarke and Witcomb, 1980; Kurokawa *et al*., 1995). This morphological interest probably resides in the eel life cycle, during which changes in environmental salinity occur and cause morpho-functional adaptations of the gut. Some knowledge on the presence of neuropeptides in the eel gut also exists (Uesaka *et al*., 1996; Loretz *et al*., 1997). However, sufficient information about the distributional features of neurotransmitters and possible accessory neuromediators in the European eel gut, so as to be able to obtain in good detail an anatomical description of the intramural innervation both in its extrinsic and intrinsic components, as well as of endocrine cell populations, is lacking. The analyses on the eel gut has thus been performed on this basis, using histochemical and immunohistochemical methods to visualize cholinergic and adrenergic components, as well as peptidergic and nitrergic ones.

Nitric oxide (NO) has in recent years been reputed to be involved in digestive functions in fish, as in mammals (Olsson and Holmgren, 1997; Shleiffer and Raul, 1997). The possible anatomical relationships between neurotransmitters and neuromodulators have also been investigated utilizing successive sections. We have chosen to study two putative accessory mediators which in mammals are reputed to be cholinergic co-mediators, such as substance P and CGRP, in addition to nitric oxide, as visualized by nicotinamide adenine dinucleotide phosphate, reduced form- (NADPH-)diaphorase histochemistry.

The aim of this work was to obtain the anatomical basis for understanding the control systems that regulate and modulate the various gastrointestinal functions in the European eel, comparing the information obtained with those known for other fish species, and for other higher vertebrates.

MATERIAL AND METHODS

Adult *Anguilla anguilla* fish of both sexes, obtained from Maricoltura Italia fish hatchery (Monfalcone, Italy), were used for this study. Body weight was about 400 g ("silver eel" stage). Fish were killed by an overdose of MS222 (Sandoz, Italy) anaesthesia at 10 a.m. and gut specimens were collected immediately afterwards. Several samples of the oesophagus (proximal, medium and distal), stomach (cardiac, fundic and pyloric zones) and intestine (proximal, medium and distal) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.4 for 4-5 h at 4°C, rinsed overnight in PBS, then in 20% sucrose in the same buffer for 24 h at 4°C, finally snap-frozen in liquid nitrogen-cooled isopentane. In parallel, other specimens were frozen without fixation. Successive cryostat sections of both fixed and unfixed specimens were processed as follows.

Histochemistry

NADPH-diaphorase - Cryostat sections (20 μm) from fixed specimens were picked up on gelatin-coated glass slides and incubated for 1 h at 37°C in 0.1 M PBS, pH 7.4, containing 0.15 mg/ml nitroblue tetrazolium (Sigma, Italy), 0.1% Triton X-100 and 1 mg/ml NADPH (Sigma), according to Scherer-Singler *et al*. (1983). The sections were then rinsed in PBS, dehydrated and mounted in Eukitt. The specificity of this stain was verified by excluding NADPH from the incubating medium, which abolished all activity. Positive controls included mammalian (horse, bovine, dog) gut samples.

Acetylcholinesterase - Cryostat sections (20 μm) from unfixed specimens were stained for

AChEase according to Karnovsky and Roots (1964) and Filipe and Lake (1983). Differently from other vertebrates, AChEase may be considered a marker of cholinergic neurons and nerve terminals of fish, where unspecific cholinesterases are not present (Pecot-Dechavassine, 1961). The specificity of the stain was verified by excluding acetylthiocholine iodide from the incubating medium, which abolished the activity. Positive controls included mammalian (see above) gut and skeletal muscle samples.

Immunostaining

Cryostat sections (10 µm) from both fixed and unfixed specimens were picked up on gelatin-coated glass slides. After inhibition of endogenous peroxidases by a 0.5% solution of H_2O_2 in methanol, sections were incubated with the primary antisera indicated in Table 1.

The incubation with the primary antisera was carried out overnight at 4°C in a moist chamber.

The antigen-antibody complexes were visualized using biotinylated swine anti-rabbit immunoglobulins (Dako, Italy) as secondary serum (dilution 1:600 for 30 min at room temperature), followed by the StreptABComplex/HRP (horseradish peroxidase) (Dako) for 30 min at room temperature. Tris-buffered saline pH 7.6 (TBS: 0.05 M Tris/HCl, 0.15 M NaCl) was used for dilutions and rinses throughout both procedures. 3-amino-9-ethylcarbazole (AEC, Dako) was employed as a chromogen to mark the sites of reaction. Sections were mounted using an aqueous mounting medium (Glycergel, Dako) and examined under an Olympus BX50 photomicroscope.

The specificity of peptide immunostaining was verified: 1) by incubating sections with normal rabbit serum instead of specific antisera and 2) by incubating sections with antiserum preabsorbed with the respective antigen (10-100 µg/ml). The preabsorption procedures were carried out overnight at 4°C. Peptides and choline acetyltransferase were purchased from Sigma. As positive controls, fish (seabream, sturgeon) and mammalian (horse, bovine) gut samples were used, as well as rat skeletal muscle.

RESULTS

Histochemistry

NADPH-diaphorase was present all along the gut in the myenteric plexus, both in neurons and nerve terminals, in nerve terminals running in the circular musculature, and in small nerve bundles localized in the tunica propria (Fig. 1a). Reactive nerve cell bodies were especially numerous in the myenteric plexus of the oesophagus. Nerve terminals of the tunica muscularis were seen running either parallel to the smooth muscle cells or oblique or transverse (Fig. 1b). Limited to the oesophagus and stomach, subtle bundles of reactive nerves were seen in the subserous connective tissue and in the longitudinal musculature. These nerve terminals were sometimes seen in continuity with the

Table I
Primary antisera tested, all raised in rabbits

Primary antisera tested	source	code	working dilution
anti-tyrosinehydroxylase	Chemicon, USA	AB151	1:1000
anti-synthetic methionine-enkephalin	Amersham, UK	RPN 1562	1:600
anti-substance P	Peninsula, UK	IHC 7451	1:600
anti-human calcitonin gene-related peptide (CGRP)	Peninsula, UK	RAS 6009 N	1:500
anti-rat calcitonin gene-related peptide (CGRP)	Peninsula, UK	RIN 6006	1:500
anti-bombesin	Peninsula, UK	IHC 7113	1:600
anti-human, porcine and rat vasoactive intestinal peptide (VIP)	Peninsula, UK	IHC 7161	1:600
anti-synthetic porcine neuropeptide Y (NPY)	Amersham, UK	RPN 1702	1:600
anti-somatostatin	Amersham, UK	RPN 1612	1:600
anti-cholecystokinin-octapeptide (CCK-8)	Amersham, UK	RPN 1592	1:600
anti-serotonin (5-HT)	Peninsula, UK	61066	1:1000
anti-cholineacetyltransferase	Chemicon, USA	AB143	1:100

Fig. 1 – a) NADPH-diaphorase histochemistry in the proximal intestine. The reaction is present in the myenteric plexus (*asterisks*) and in subtle nerve fibres (*arrow*) in the inner musculature (*im*). (*om* outer musculature). *Scale bar* 320µm. **b)** Distal intestine. NADPH-diaphorase reactivity is localized in both nerve cell bodies (*arrowheads*) and fibres (*curved arrows*) in the myenteric plexus, and in nerve fibres (*arrows*) of the inner circular musculature. *Scale bar* 100µm. **c)** AChEase histochemistry in the medium intestine. Nerve cell bodies are strongly reactive. *Scale bar* 20µm. **d)** AChEase histochemistry in the stomach (gastric proper gland zone). Reactivity is evident in nerve fibres of the mucosa, near the gastric glands (*thin arrows*), and of the propria-submucosa (*thick arrows*), as well as in the inner circular musculature (*asterisks*). A strong reactivity is present in both nerve cell bodies and fibres of the myenteric plexus (*arrowheads*). *Scale bar* 200µm. **e)** AChEase histochemistry in the striated musculature of the oesophagus. Reactive nerve terminals (*arrowheads*) in the form of motor endplates contact some muscle fibres. *Scale bar* 40µm.

voluminous ganglia which were apposed to the periphery of the organs (Fig. 4g).

AChEase activity was histochemically detected all along the gut in the myenteric plexus, both in nerve cell bodies (Fig. 1c) and nerve terminals, and in small nerve bundles localized in the lamina propria (Fig. 1d). The latter were limited to the stomach. In the fundic zone of the stomach, nerve terminals were especially numerous in contiguity with the gastric glands. In the oesophagus, where

Fig. 2 – Medium intestine. **a** Tyrosine-hydroxylase immunoreactivity is evident in nerve fibres of the inner circular musculature (*arrows*) and in nerves of the myenteric plexus (*asterisks*). (*im* inner musculature; *om* outer musculature). *Scale bar* 55μm. **b** Met-enkephalin-like immunoreactivity is present in thin nerve bundles located in the propria-submucosa (*asterisks*), in nerve terminals running in the inner circular musculature (*thin arrows*) and in the myenteric plexus, both in nerve cell bodies and nerves (*thick arrows*). Reactivity is also present in endocrine cells of the epithelial layer (*arrowheads*). *Scale bar* 110μm. **c** CGRP-like immunoreactivity is present in rare subtle nerve terminals running in the inner circular musculature (*thick arrows*) and in both nerve cell bodies and fibres of the myenteric plexus (*thin arrows*). *Scale bar* 125μm. Insert: positive nerve cell bodies are visible in the myenteric plexus at a higher magnification. *Scale bar* 70μm. **d** Substance P-like immunoreactivity is evident in rare subtle nerve terminals running in the inner circular musculature (*thin arrows*) and in both nerve cell bodies and nerves of the myenteric plexus (*thick arrows*). *Scale bar* 80μm. **e** A particular at higher magnification showing the SP-like immunoreactivity in nerve terminals of the myenteric plexus. *Scale bar* 20μm.

the musculature was made by striated muscle fibres, reactive nerve terminals were seen in contact with striated muscle fibres, in the form of motor endplates (MEPs: Fig. 1e).

Immunohistochemistry

The preabsorption procedures resulted in the absence of reactions.

Tyrosine hydroxylase immunoreactivity was detected in nerve terminals of the myenteric plexus of intestinal tracts (Fig. 2a). In addition, nerve terminals were seen running in the intestinal circular musculature (Fig. 2a). In the oesophagus and stomach, tyrosine hydroxylase immunoreactivity was observed in ganglia which were peripherally located in the organs and in large nerve bundles which penetrated the tunica serosa and longitudinal musculature.

Met-enkephalin-like-immunoreactivity (Fig. 2b) was observed all along the gut in the myenteric plexus, both in nerve cell bodies and fibres, in nerve terminals running in the circular musculature, and in small nerve bundles located in the propria-submucosa. In addition, the intestinal epithelial layer of the mucosa was furnished by numerous immunoreactive endocrine cells.

CGRP-like- (Fig. 2c) and bombesin-like-immunoreactivities were detected all along the gut in the myenteric plexus, both in nerve cell bodies and nerves, and in rare nerve terminals running in the circular musculature.

Substance P-like-immunoreactivity (Fig. 2d, e) was heavy and detected all along the gut in neuronal localizations similar to those of the two above-mentioned peptides. It was, in addition, detected in intestinal endocrine cells.

VIP-like-immunoreactivity was observed all along the gut in nerve terminals of the myenteric plexus and the circular musculature as well as in small nerve fibre bundles of the tunica propria-submucosa (Figs. 3a, b). In the myenteric plexus, it was usually found that nerve terminals were apposed to negative neuronal bodies (Fig. 3b).

NPY-like-immunoreactivity was present only in proximal and distal intestine, in nerve terminals of the myenteric plexus, and in occasional epithelial endocrine cells, basally located in the intestinal glands.

Somatostatin-like-immunoreactivity was present all along the gut in nerve fibres of the myenteric plexus, as well as in gastric endocrine cells.

5-HT-immunoreactivity was only detected in epithelial endocrine cells which were present from the oesophagus to the distal intestine (Fig. 3c).

CCK 8-like-immunoreactivity was never detected in any localization.

Immunohistochemistry for choline acetyltransferase failed to give a reaction on gut samples of *A.anguilla* although it stained a number of motor endplates in mammalian skeletal muscle, and nerve cell bodies and terminals in mammalian gut. As immunohistochemistry for choline acetyltransferase fails to give a reaction on gut and skeletal muscle samples of several fish species (unpublished observations), our choice of the histochemical method for acetylcholinesterase is, at present, the most valid to identify fish cholinergic neurons.

Immunohistochemical and histochemical reactivities in consecutive sections

In consecutive sections of the intestinal tracts tyrosine hydroxylase immunoreactive nerve terminals of the myenteric plexus (Fig. 4a) were noticed in the same localization as the NADPH diaphorase-reactive (Fig. 4b) nerve terminals. Moreover, tyrosine hydroxylase-immunoreactive nerve terminals (Fig. 4c) showed the same localization as substance P-like- (Fig. 4d), as well as CGRP-like immunoreactive nerves.

Substance P-like immunoreactive (Fig. 4e) and AChEase-reactive (Fig. 4f) nerve terminals were localized similarly. Both ran in the myenteric plexus of the oesophagus, stomach and intestine, but the AChEase-reactive nerves were apposed to the SP-like immunoreactive nerve cell bodies and terminals.

Similarly, in the myenteric plexus of the intestinal tracts, AChEase-reactive nerves were apposed to NADPH diaphorase-reactive nerve cell bodies and terminals. In the oesophagus and stomach, the same reciprocal pattern of NADPH-diaphorase- and AChEase-reactivity was present in ganglia located in the periphery of the organs, in a subserous location (Figs. 4g, h).

CONCLUSIONS

This work has shown that in the eel gut, as in most fish species, the myenteric plexus is present and predominant in the ENS, and contains nerve

Fig. 3 – a) VIP-like immunoreactivity in the distal intestine. The reactivity is present in small nerve fibre bundles of the tunica propria-submucosa (*asterisks*) as well as in nerve terminals of the inner circular musculature (*thin arrows*) and of the myenteric plexus (*thick arrows*). (*im* inner musculature; *om* outer musculature. *Scale bar* 100µm. **b)** VIP-like immunoreactivity in the medium intestine. Reactivity is present in small nerve fibre bundles of the inner circular musculature (*thin arrows*). In the myenteric plexus, nerve terminals were apposed to negative neuronal bodies (*arrowheads*). *Scale bar* 25µm. **c** 5-HT immunoreactivity in epithelial endocrine cells (*asterisks*) of the proximal intestine. *Scale bar* 100µm.

Fig. 4 – a, b) Two consecutive sections of the medium intestine, respectively, processed for tyrosine hydroxylase-immunohistochemistry and NADPH-diaphorase histochemistry. In the myenteric plexus, nerve components similarly localized (*asterisks*) are reactive for either tyrosine hydroxylase (a) or NADPH-diaphorase (b) and both reactivities involve the same nervous structures. (*im* inner musculature; *om* outer musculature). *Scale bar* 35µm. **c, d)** Two consecutive sections of the medium intestine, respectively incubated with anti-tyrosine hydroxylase and anti-substance P-like sera. In the myenteric plexus, tyrosine hydroxylase (c) immunoreactive nerve fibre bundles show the same localization (*asterisks*) as substance P-like immunoreactive components (d). *Scale bar* 45µm. **e, f)** Two consecutive sections of the medium intestine, respectively processed for substance P-like immunohistochemistry and AChEase histochemistry. In the myenteric plexus, substance P-like (e) immunoreactive nerve terminals (*asterisks*) are similarly localized as AChEase-reactivity (f) *(thin arrows)*, without exactly involving the same nervous structures. *Scale bar* 45µm. **g, h)** Two consecutive sections of pyloric stomach, incubated respectively for NADPH-diaphorase (g) and AChEase (h) histochemistry. In a subserous ganglion, numerous NADPH-diaphorase reactive nerve cell bodies (*asterisks*) are surrounded by subtle AChEase reactive nerve terminals (*thin arrows*). Note the AChEase unreactivity of the nerve cell bodies. (*ts* tunica serosa). *Scale bar* 50µm.

cell bodies in addition to nerve terminals. Numerous nerve terminals were seen running in the inner circular musculature, whereas the outer musculature was sporadically innervated. A submucosal plexus may be present, but lacks nerve cell bodies and shows only the presence of subtle nerve bundles or solitary nerves. In addition, voluminous ganglia are present in a subserous localization, limited to the oesophagus and stomach. The diffuse endocrine system is present above all in the epithelial layer of the intestinal mucosa. The classical neurotransmitters and a number of possible accessory neuromodulators are identifiable in the intramural innervation, in some instances in possible mutual relationships. The large number of chemically different neuromodulators histochemically found in intramural nerve components may suggest a functional complexity of the fish ENS similar to that shown in mammalian species, despite the morphologically simple arrangement of the fish gut innervation as compared with that of mammals.

The cholinergic component of the gut intramural innervation, as evidenced by the AChEase-reactivity, is identifiable from the oesophagus to the distal intestine. Nerve cell bodies are shown all along the gut in the myenteric plexus. The further observation of reactive nerve terminals contacting oesophageal striated muscle fibres and gastric glands of the fundic stomach suggests a direct role of local cholinergic neurons upon target structures, both muscular and epithelial. In the sturgeon, too (Domeneghini *et al.* 1999), the cholinergic component of the intramural innervation is present from the oesophagus to the rectum, even if its pattern of distribution is in part different.

The possible adrenergic component of the intramural innervation, as evidenced by tyrosine hydroxylase immunoreactivity, is present in the myenteric plexus of the intestinal tracts in the form of nerve terminals, which penetrate the inner circular musculature. These results are in agreement with the previous ones obtained by Read and Burnstock (1968; 1969) on histochemical grounds. The myenteric plexus of the oesophagus and stomach lacks tyrosine hydroxylase immunoreactivity, which is, on the contrary, present in nervous ganglia peripherally distributed in these organs.

Among the putative accessory neuromodulators, a prominent functional role may be assigned to nitric oxide which is a gaseous mediator largely observed in the fish ENS (Li and Furness, 1993; Olsson and Karila, 1995; Olsson and Holmgren, 1997; Schleiffer and Raul, 1997). There is now strong evidence that nitric oxide sustains the physiology of the fish alimentary canal (Karila and Holmgren, 1995), as of that of mammals and other vertebrates. In mammalian species, nitrergic neurons are reputed to be the non-adrenergic non-cholinergic (NANC) inhibitory neurons (Sanders and Ward, 1992). In the eel ENS, the presence and release of nitric oxide may be related to the NADPH-diaphorase histochemical reactivity, because in fish, as in numerous mammalian species, this enzyme, as histochemically revealed, is co-localized with NOS (nitric oxide synthase immunoreactivity, and thus it is reputed to be a marker of nitric oxide synthesis (Li and Furness, 1993). NADPH-diaphorase reactivity is detectable in nerve cell bodies and terminals of the myenteric plexus all along the gut and in nerve terminals running in the inner circular musculature and in the propria-submucosa. In the same species, we have recently shown that NADPH-diaphorase is present in terminals which distribute to skeletal muscle fibres of the *adductor mandibulae* complex (Radaelli *et al*., 1998). Patterns of gut distribution similar to those described in this paper were found in the rainbow trout (Li and Furness, 1993). These localizations, in part, recall those of the AChEase-reactivity, and their examination may suggest a functional relationship between the neurotransmitter acetylcholine and the fish putative neuromodulator nitric oxide. On the other hand, a further relationship may be suggested between NO and the possible adrenergic component of the intramural innervation, because NADPH-diaphorase is shown in those subserous ganglia of the oesophagus and stomach in which a tyrosine hydroxylase immunoreactivity is also present.

Putative peptide neuromodulators are, in addition, immunohistochemically identifiable in the intramural innervation of the eel gut. Most of them (met-enkephalin-, substance P-, CGRP-, bombesin-, VIP-, and somatostatin-like peptides) are present all along the gut. Met-enkephalin-, substance P-, CGRP-, bombesin-like peptides are detectable both in nerve terminals and cell bodies of the myenteric plexus, as well as in nerve terminals of the inner musculature and propria-submucosa. On the contrary, VIP- and somatostatin-like

peptides are only observed in nerve terminals which were apposing to immuno-unreactive neurons, thus suggesting that the reactive fibres are not of local origin, and that they have a possible modulatory role upon other neuronal families. Conlon *et al*. (1988) showed that somatostatin-14 peptide of *A. anguilla* gut is identical to mammalian somatostatin-14. An NPY-like peptide, present in proximal and distal intestine, is similarly immunohistochemically observed in nerve terminals, but not in nerve cell bodies. CCK-8-like immunoreactivity was never detected.

These results show some interspecies differences in the presence and localization of neuropeptides within the intramural innervation of the fish gut (Bjenning and Holmgren, 1988; Kiliaan *et al*., 1992; Visus *et al*., 1996), possibly of taxonomic significance. The intramural innervation of the eel gut does not show the presence of 5-HT immunoreactive nerve components, whereas serotonergic neurons were observed in the gut of other fish (Watson, 1979; Anderson, 1983; Karila *et al*., 1998). On the other hand, 5-HT is immunohistochemically identifiable in eel oesophageal, gastric and intestinal endocrine cells, and it is conceivable that its generally-assigned inhibitory role upon the musculature may be ruled only by the biogenic amine synthesized and released by the endocrine cells. In addition, the diffuse endocrine system shows the presence of met-enkephalin-, substance P-, NPY-like peptides in intestinal endocrine cells, and a somatostatin-like peptide in gastric endocrine cells.

With the aim to get a deeper insight upon the anatomical and possible functional relationships between neurotransmitters and putative accessory neuromediators, a sequential analysis on the presence of different substances has been made on successive sections. In the intestinal tracts, NADPH-diaphorase-reactivity has been observed in the same localizations as the tyrosine hydroxylase immunoreactivity in numerous nerve terminals of the myenteric plexus. In addition, the tyrosine hydroxylase immunoreactivity showed anatomical relationships with substance P-like- and CGRP-like-immunoreactive nerve terminals of the myenteric plexus. It may thus be hypothesized that, in the eel intestine the adrenergic components of the autonomic system utilize nitric oxide, substance P and CGRP as possible accessory neuromodulators. These peptides are, in turn, possibly modulated in their release by the cholinergic innervation, whose presence is histochemically demonstrated in relation with the neuropeptide-immunoreactive neurons in successive sections. By contrast, none of the possible mutual relationships of these substances has been demonstrated in the myenteric plexus of the oesophagus and stomach. Only nitric oxide and acetylcholine have been observed to possibly influence one another within the large nervous ganglia which are peripherally located in these organs.

These latter features may be interpreted considering the different anatomical arrangement and functional relationship between the cholinergic and adrenergic compartments of the autonomic innervation in the oesophagus and stomach versus intestine in the fish gut. From this point of view, the additional presence of accessory neuromodulators may be different in the two segments, and they are more numerous in the intestinal tract, where the adrenergic compartment may be anatomically predominant over the cholinergic one, as in most fish (Burnstock, 1969). Moreover, the presence of a larger number of possible neuromodulators in the eel intestine than in oesophageal-gastric tracts, might functionally sustain the well known intestinal osmoregulatory role (Baldisserotto and Mimura, 1995; Lionetto *et al*., 1996; Trischitta *et al*., 1996).

These results, even if merely anatomical, demonstrate the neurochemical heterogeneity of intramural neurons in the eel alimentary canal, and enable us to postulate special anatomical and functional relationships among the different components of its intramural innervation. This, in turn, possibly differentiates this from other fish species and provides indirect evidence for the complexity of the gastrointestinal innervation of this class of vertebrates.

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