HISTOCHEMICAL DETECTION OF DIFFERENT NEUROTRANSMITTERS IN THE DIGESTIVE TRACT OF NAUTILUS POMPILIUS L. (CEPHALOPODA)

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NAUTILUS POMPILIUS
DIGESTIVE TRACT
CATECHOLAMINES
ACETYLCHOLINESTERASE
FMRF-AMIDE

ABSTRACT. – In the organs of the digestive tract of *Nautilus pompilius* catecholamines and serotonin have been shown in the nerve endings by fluorescence histochemical methods. HPLC analysis confirm that noradrenaline and dopamine occur in all organs, while in the midgut gland only adrenaline is found. Using immune-histochemical methods against the tetrapeptide FMRF-amide and serotonin, positive reactions have been demonstrated within terminal nerves of the tunica muscularis of the oesophagus, crop, stomach, caecum, midgut and rectum. Additionally, histo- and cytochemical proof of acetylcholinesterase was found in the tunica muscularis of the oesophagus, crop, stomach, caecum, midgut, and rectum. The findings give evidence that – similar to the coleoids – an antagonistic cholinergic-aminergic neuroregulation, which seems to be modulated by FMRF-amide, also exists in the digestive tract of *N. pompilius.*

NAUTILUS POMPILIUS L.
ACÉTYLCHOLINESTÉRASE
FMRF-AMIDE
CATÉCHOLAMINES
FMRF-AMIDE

RÉSUMÉ. – La présence de catécholamines et de sérotonine dans les formations terminales nerveuses des organes digestifs de *Nautilus pompilius* est démontrée par des méthodes de fluorescence histochimique. La chromatographie liquide à haute pression (HPLC) confirme que la noradrénaline et la dopamine prédominent dans tous les organes, tandis que dans la glande digestive, seule l'adrénaline a été détectée. Des méthodes immuno-histochimiques, permettent de mettre en évidence des réactions positives contre le tétrapeptide FMRFamide et la sérotonine dans les nerfs terminaux de la tunique musculaire de l'oesophage, du jabot, de l'estomac, du caecum, de l'intestin et du rectum. Les réactions histochimiques de l'acétylcholinsetérase sont également positives dans la tunique musculaire de l'oesophage, du jabot, de l'estomac, du caecum, de l'intestin et du rectum. Il existe aussi – comme chez les Coleoidés – une neuro-régulation antagoniste-cholinerque-aminergique du système digestif de *N. pompilius* qui est probablement modulée par le FMRF-amide.

INTRODUCTION

Not many investigations were carried out on the innervation of the digestive tract of *Nautilus pompilius*. Only Young (1987) reports that the oesophagus of *Nautilus* is innervated by sympathic nerves which originate in the buccal ganglion, and there are no findings about the putative neurotransmitters within the terminal nerves of the digestive system of nautiloids. But the digestive tract of coleoid cephalopods was investigated by topographical (Fage and Racovitza 1913, Alexandrowicz 1928), histochemical, and pharmacological (Andrews and Tansey 1983) methods. These authors found catecholamine fluorescences in the gastric ganglion and in the nerves which innervate the oesophagus, crop and stomach. Furthermore investigations on the innervation of the posterior salivary glands of *Octopus* showed that ^{3}H-labelled serotonin is taken up into nerve endings of the posterior salivary glands, whereas ^{3}H-marked noradrenaline is not taken up (Martin and Barlow 1972). Chiba and Yaku (1979) also found histochemically in the nerve endings serotonin as well as dopamine fluorescences.

Our histological studies on *Nautilus pompilius* show that the digestive tract especially the foregut is densely innervated. For this reason the different organs of the digestive tract are investigated by histochemical methods and the high pressure liquid chromatography (HPLC), and the results are compared with those of the coleoids.

MATERIAL AND METHODS

Three adult *Nautilus pompilius* from the Celebes Sea (Southern Philippines) were used for this study. For the fluorescence and enzyme histochemical investigations tissue samples of the oesophagus, crop, stomach, caecum, and rectum were embedded in Tissue tec® and frozen in liquid nitrogen. The specific acetylcholinesterase (E.C. Nr. 3.1.1.7) has been localized according to the direct thiocholine method of Karnovsky and Roots (1964). For the fluorescence histochemical investigations the unfixed cryostat sections were stained according to Falck and Hillarp (1962) and Barber (1982).

For the immune histochemistry the digestive organs (except the midgut gland) were fixed in Bouin solution without acetic acid (immune reaction against FMRF-amide) and in 4 % saline formalin (immune reaction against serotonin), embedded in paraffin (melting point 51°-53°C), and stained according to Van Leeuwen (1986).

For the HPLC-analysis the tissues were portioned and frozen in liquid nitrogen. They were broken down by adding 0,1 M perchloric acid, in which 2,7 mmol EDTA was dissolved (1 000 ml perchloric acid/1 000 mg tissue) followed by ultrasounie treatment. The homogenate was centrifuged (12 000 g, 10 minutes at 4°C), and the supernatant was frozen (Kime and Messenger, 1990). Before preparation the samples were diluted 1 : 10 and 1 : 100 with 1,0 M Tris/HCl buffer, pH 8,6. The preparation of the samples and the eluation of the catecholamines was carried out according to a modified method of Adams *et al.* (1987).

For electron microscopy, small pieces of oesophagus, crop, stomach, caecum, midgut and rectum were fixed in 4 % glutaraldehyde in 0,1 M cacodylate buffer at pH 7,4 adjusted to seawater osmolarity (1 000 mOsm) with sodium chloride, postfixed in 1,5 % osmium tetroxide in the same buffer, dehydrated in ethanol, and embedded in Epon. Thin sections were stained in uranyl acetate and counterstained with lead citrate (Reynolds, 1963). For the immunocytological studies, small pieces (diameter 1 mm) of the above mentioned organs were fixed in 0,5 % glutaraldehyde, postfixed in 1,5 % osmium tetroxide, dehydrated, and embedded in LR-White (Griffond *et al.* 1986). The immuncytological analyses were carried out according to a modified method of Varndell *et al.* (1982) using immunogold as tracer. The cytochemical proof of the acetylcholinesterase was carried out according to Karnovsky (1964).

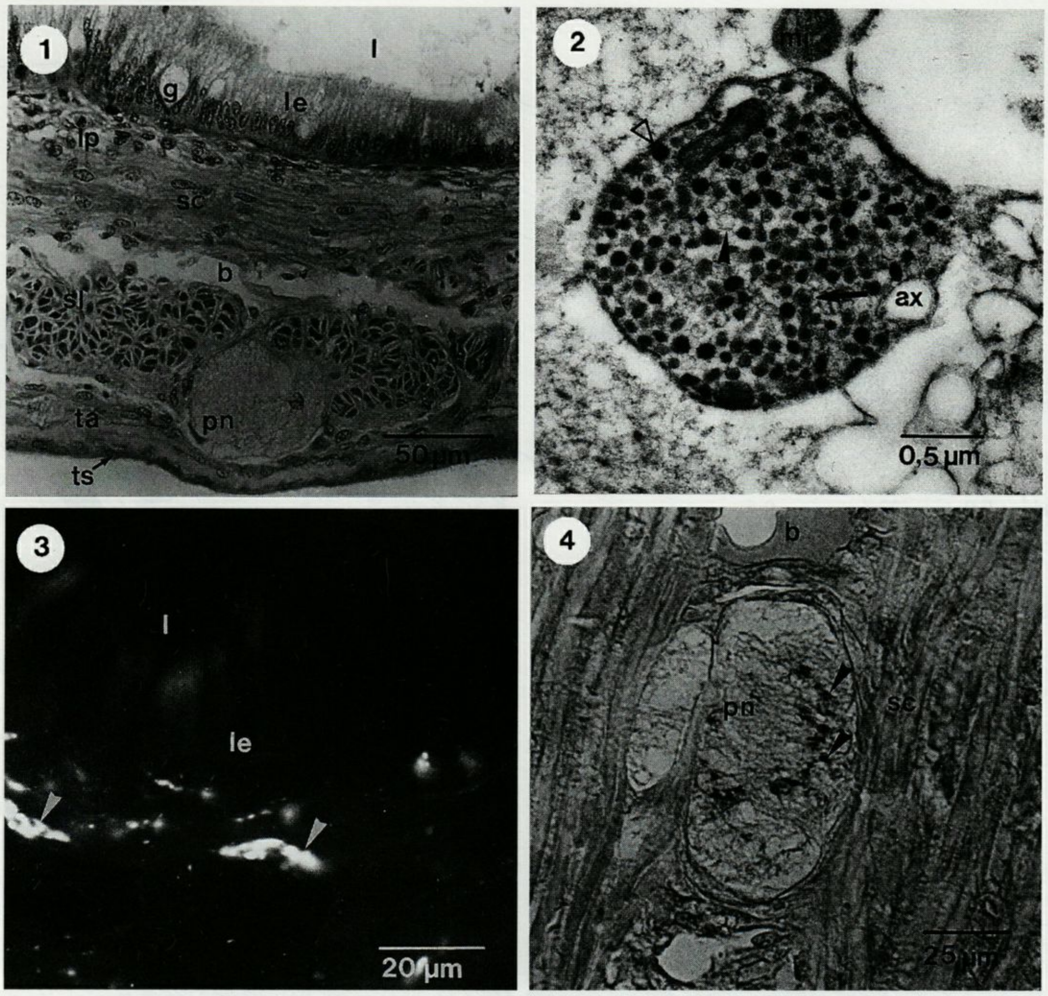
RESULTS

The digestive tract of *Nautilus* consits of the buccal mass, the foregut which is widened to a crop, the stomach, the vestibulum, the caecum, the midgut and the rectum. The caecum is connected with the midgut gland by the ductus hepatopancreas (Griffin 1900 ; Bidder 1966, 1976 ; Boucaud-Camou and Boucher-Rodoni 1983 ; Westermann and Schipp 1992). Light microscopical examinations show that the tunica mucosa is situated luminally and is subdivided into a lamina epithelialis mucosae, a lamina propria mucosae and a lamina muscularis mucosae. The lamina epithelialis mucosae of all organs of the digestive tract is a columnar epithelium containing goblet cells which secrete acid and neutral mucopolysaccharides and glycolipids. The tunica muscularis borders this layer peripherically and is followed by a tunica adventitia consisting of connective tissue. Stomach, midgut, and rectum are surrounded peripherically by an isoprismatic tunica serosa. A tela submucosa, as found in vertebrates, is lacking in nautiloida (Fig. 1). The electron microscopical investigations show a dense innervation of the tunica muscularis of the digestive organs. The terminal axons contain transparent (diameter 55-70 nm) as well as dense cored (diameter 80-100 nm) and osmiophilic vesicles (diameter approx. 130 nm, Fig. 2).

Glyoxylic-induced fluorescences with an emission maximum of 480 nm has been detected within the nerve endings of the lamina propria mucosae and the tunica muscularis in the digestive system as well as in the midgut gland, which indicates catecholamines as neurotransmitters (Fig. 3). In the midgut gland the catecholamine fluorescences could be observed only in the tunica muscularis of the ductus hepatopancreas. Additionally, fluorescences with an emission maximum of 520 nm could be demonstrated in the tunica muscularis, which suggests that serotonin there occurs as neurotransmitter, too. No fluorescences could be detected in the lamina epithelialis mucosae.

The HPLC-analysis showed that mainly dopamine and noradrenaline are found in the oesophagus, crop, and in the stomach, whereas adrenaline predominates in the midgut gland. In the caecum dopamine, adrenaline and noradrenaline were found in almost equal concentrations. In the midgut and rectum dopamine, and adrenaline predominate, while noradrenaline was only found in low concentration (Fig. 5a+b).

Using immune histochemical methods against serotonin (Fig. 4) and the family of the FMRF-amides (Fig. 6a), immune precipitations against both neurotransmitters could be found within the nerves of the tunica muscularis of the oesophagus, crop, stomach, caecum, midgut, and rectum. With the immunogold method, positive immune reac-



Pl. I. – Fig. 1 : Cross section of the midgut wall of *Nautilus pompilius*.
Fig. 2. – Terminal nerve fibre within the tunica muscularis of the stomach of Nautilus pompilius with (→) dense cored vesicles, (➤) transparent vesicles, (▷) osmiophilic vesicle.
Fig. 3. – Cross section of the crop wall of Nautilus pompilius; (➤) catecholamines fluorescences. tunica serosa.
Fig. 4. – Cross section of the tunica muscularis of the crop wall of Nautilus pompilius; immune reaction against serotonin within nerve fibres (➤).
Abbreviations : ax : axon; bl : blood sinus; g : goblet cell; l : lumen; le : lamina epithelialis mucosae; lp : lamina propria mucosae; mi : mitochondria; my : myofilaments; pn : polyaxonal nerve fibre; sc : stratum circulare; sl : stratum longitudinale; ta : tunica adventitia; tm : tunica muscularis; ts : tunica serosa.

tions against the tetrapeptide FMRF-amide have also been detected cytologically within the axons of the lamina propria mucosae and the tunica muscularis (Fig. 6b). Enzyme histo– and cytochemical reactions of the specific acetylcholinesterase yielded positive results in the tunica muscularis of all organs of the digestive tract of *N. pompilius* (Fig. 7a+b).

DISCUSSION

According to our findings in the neuromuscular systems of the digestive tract of *N. pompilius* probably an antagonistic cholinergic aminergic transmitter mechanism exists, in which FMRF-amide may play a modulating role.

Our examinations show that the catecholamines and serotonin found in all organs of the digestive tract, can probably be related to the dense cored vesicles with a diameter of 80 to 100 nm, detected in the lamina propria mucosae and in the tunica muscularis, and according to Dorsett (1986) dense cored vesicles of this size store monoamines, especially catecholamines. In the lamina epithelialis mucosae of the examined organs of the digestive tract of *N. pompilius*, however, fluorescences could not be observed. It can be assumed that there are no enterochromaffine cells which can be demonstrated in the posterior salivary glands of

5a

Organ	Noradre-naline	Adrena-line	Dopa-mine
Oesophagus	119,0	1,6	141,1
Crop	8,6	0,0	69,5
Stomach	4,2	0,2	8,0
Caecum	2,8	2,4	7,6
Midgutgland	0,0	22,4	0,5
Midgut	1,4	3,8	7,6
Rectum	1,6	8,8	36,3

ng/(g tissue)

5b

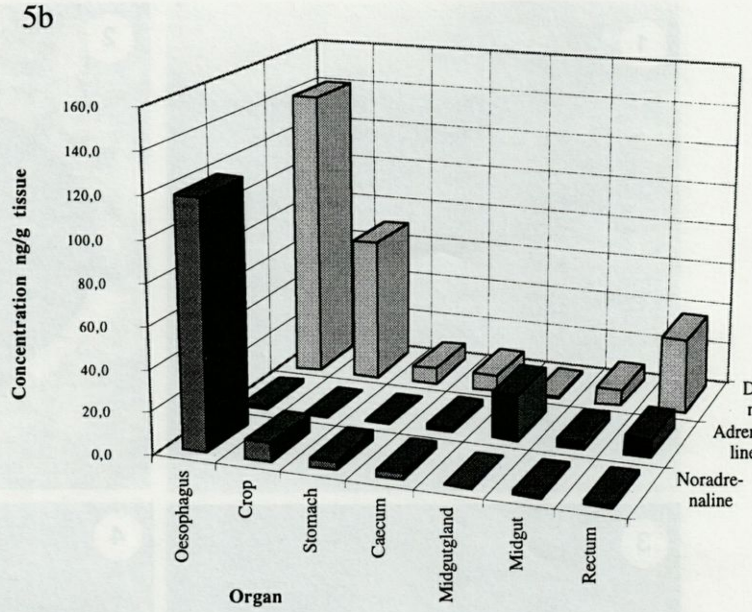
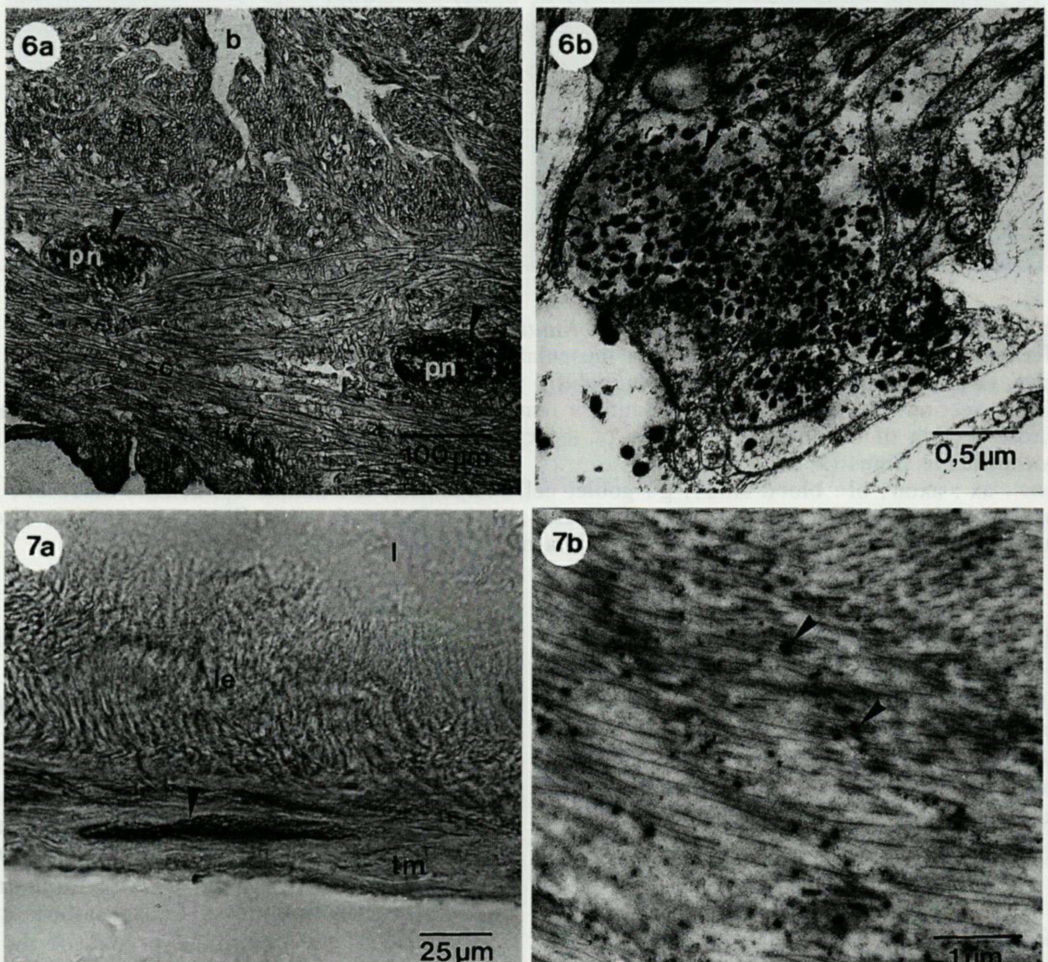


Fig. 5. a+b. – Table of catecholamine content in the digestive tract of *Nautilus pompilius*; b, diagram of the distribution of the catecholamines in the digestive tract of *N. pompilius*.



Pl. II. – Fig. 6. Immune reaction against FMRF-amide in the digestive tract of *Nautilus pompilius*. a, PAP method, (➤) immune precipitates within polyaxonal nerves in the tunica muscularis of the oesophagus. b, immunogold method, single axon in the tunica muscularis of the rectum with (→) dense cored, vesicles, (➤) transparent vesicles and (▷) immune precipitates.

Fig. 7 a+b. – Acetylcholinesterase activity (➤) in the tunica muscularis of a, the caecum (lightmicroscopical) and b, the rectum (electronmicroscopical).

the octopods (Chiba and Yaku 1979). These findings are in agreement with those of Andrews and Tansey (1983). They also found catecholamines in the gastric ganglion and in the nerves, which innervate the oesophagus, crop, and stomach of *Octopus vulgaris*, whereas none was found within the wall of the caecum.

Our HPLC-analysis showed that in the midgut gland adrenaline predominates (22,4 ng/g); scarce amounts of dopamine (0,5 ng/g) and noradrenaline have been found. The fluorescence histochemical examinations showed that catecholamine fluorescences only exist in the tunica muscularis of the ductus hepatopancreas, but not in the glandular tissue. So an adrenoendocrine function of the midgut gland can be ruled out.

In addition, the high content of noradrenaline (119 ng/g) and dopamine (141,1 ng/g) is conspicuous in the oesophagus. This confirms the high innervation density of this organ already shown by lightmicroscope. In all other organs dopamine is located in a higher concentration than noradrenaline and adrenaline.

The comparison of the digestive tracts of *Octopus vulgaris* (Juorio and Killick 1973) and *N. pompilius* shows that in both species dopamine and noradrenaline have the highest concentration in the oesophagus. In the oesophagus and crop of *O. vulgaris* the content of noradrenaline is three times higher than that of dopamine, while in the foregut of *N. pompilius* dopamine predominates. In all other organs of the digestive tract (stomach, caecum, and intestine) of both species dopamine and noradrenaline exist in approximately the same concentration.

So the HPLC-analyis confirms the fluorescence histochemical results that in the organs of the digestive tract of *N. pompilius* noradrenaline or dopamine act as a putative neurotransmitter.

Using immune-histochemical methods positive reactions against FMRF-amide and serotonin in the polyaxonal nerves of the tunica muscularis of the digestive tract of *N. pompilius* could be shown. In regard to the immune reaction against FMRF-amide, however, it has to be pointed out that this reaction is not only directed against this peptide, but also against all amides with the sequence FM at their c-terminal end. Also immune reactions against FMRF-amide in the axons of the polyaxonal nerves in the tunica muscularis of the digestive tract could be observed. Furthermore, the electron microscopic findings showed that osmiophilic vesicles (diameter approx. 130 nm) exist in the axons of the polyaxonal nerves of the lamina propria mucosae and the tunica muscularis. According to Dorsett (1986), these are peptide storing vesicles. Based on our findings it can be assumed that FMRF-amide as well as serotonin represent putative neurotransmitters in the nerve endings. Also in the nerve endings of the midgut gland (Ruth, 1993) and the tunica adventitia (Kleemann, 1994) of *Nautilus* immune reactions against FMRF-amide could be shown as well, whereas they could not locate serotonin in these organs.

Peptides also take part in the neuroregulation of the circulation of the coleoids. In the neurosecretorial nerve endings of the vena cava of *Octopus vulgaris* peptides of the FMRF-amide family were shown (Martin *et al.* 1981). By immune histochemical methods FMRF-amide could be located in the nerve fibres of the aorta cephalica (Schipp *et al.* 1991), the nervus cardiacus, the lobus visceralis, and the vena cava of *Sepia officinalis* (Jakobs 1991). Pharmacological investigations on the branchial hearts of *S. officinalis* show that FMRF-amide has any effect on the branchial heart beat when applied alone, but it counteracted the positive inotropic actions of noradrenaline (Fiedler 1992). On the smooth muscle of *Mytilus edulis* FMRF-amide (10-6 M) enhanced the contractions produced by doses of acetylcholine below the ED_{50}, but had no effect on contractions produced by acetylcholine at doses greater than the ED_{50} (Raffa and Bianchi 1986). Low concentrations of FMRF-amide (0,5 nM and above) induce rhythmic contractions of the tentacle retractor muscle of *Helix* (Cottrell *et al.* 1983). Cottrell *et al.* (1990) could also show that the neuropeptide FMRF-amide activates a ligand-gated ion channel in the neurons of *Helix* and they suppose that FMRF-amide is a depolarizing neurotransmitter.

Tansey (1980) describes acetylcholinesterase activity in the nerves of the digestive tract of *Octopus*. This enzyme could also be located in the tunica muscularis of the digestive tract of *Nautilus pompilius*, and transparent vesicles with a diameter of 50 to 70 nm were found by electron-microscopical examinations in the lamina propria mucosae and in the tunica muscularis of the digestive organs. According to Dorsett (1986) the transparent vesicles contain the transmitter acetylcholine. The proof of the acetylcholinesterase in the digestive tract of *N. pompilius* indicates that acetycholine acts as a neurotransmitter.

As these results base only on histochemical methods, pharmacological studies on the digestive tract of *N. pompilius* have to follow to get more detailed evidences about the effects of the different transmitters.

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