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Distribution and function of biogenic amines in the heart of *Nautilus pompilius* L. (Cephalopoda, Tetrabranchiata)

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Summary

Biogenic amines (serotonin and catecholamines), play an important role in the control of the blood flow not only in vertebrates, but also in invertebrates such as cephalopods. In contrast to the well investigated hearts of the 'modern,' coleoid cephalopods, the innervation of the heart of the archaic *Nautilus pompilius* L. has not been studied in detail. In this study the distribution and effects of biogenic amines in the *Nautilus* heart were investigated. Serotonin and catecholamines were visualised by the glyxoylic acid induced fluorescence. High performance liquid chromatotography analysis was performed to discriminate between the catecholamines, which showed a high content of noradrenaline in the 4 auricles, the aorta and the ventricle, whereas the ventricle showed a high dopamine content. Adrenaline was found at a very low concentration in the ventricle. Serotonin and dopamine were also immunohistochemically localised to larger nerves and throughout the heart, respectively. In organ bath experiments, the auricles showed little spontaneous activity. After adding serotonin, they displayed rhythmical contractions, which were accelerated dose-dependently by noradrenaline. In summary, these data suggest an important role for biogenic amines in the control of the heart of *Nautilus pompilius* L., with serotonin possibly stimulating excitatory nerve fibres, whereas noradrenaline is likely to influence the muscle contraction itself.

Introduction

Serotonin as well as noradrenaline and dopamine have a widespread distribution in the central nervous system (CNS) of coleoid cephalopods and may act as neurotransmitters, while adrenaline is absent in the CNS of these animals (Juorio 1971, Kime & Messenger 1990). In the circulatory system, the presence of catecholamines has been shown by glyoxylic acid induced fluorescence (GIF) in the systemic heart (Kling 1986, Kling & Schipp 1987, Versen *et al.* 1999), branchial hearts (Fiedler & Schipp 1990) and vessels (Andrews & Tansey 1983) of *Sepia*. In the heart, noradrenaline causes a significant increase of amplitude and frequency, while dopamine and adrenaline are less potent (Kling & Schipp 1987). Contrary to the systemic heart, dopamine, noradrenaline and adrenaline induce an increase in amplitude but not frequency in the branchial hearts (Fiedler & Schipp 1990). In both compartments application of phentolamine leads to a blockade of the effects (Kling & Schipp 1987, Fiedler & Schipp 1990). In *Sepia*, a HPLC-analysis of the central heart showed a high dopamine-content, whereas the vena cava and the branchial heart mainly showed noradrenaline (Fiedler *et al.* 1992). Taken together these studies lead to the assumption that catecholamines are widespread in the circulatory systems of coleoid cephalopods and play an important role in the regulation of the vascular tone and cardiac output. In contrast to the thoroughly investigated circulatory system of the 'modern' coleoid cephalopods, little is known about the distribution and function of biogenic amines in *Nautilus*. While there is some evidence for a catecholaminergic innervation of the gut and mantle (Westermann *et al.* 1997, Westermann *et al.* 2002) and aorta (Kleemann & Schipp 1996), no studies have addressed the innervation with catecholamines and serotonin as well as their physiological effects in the systemic heart of *Nautilus* so far. In the aorta, noradrenaline also seems to be the predominant catecholamine inducing vasoconstriction (Schipp 1994, unpublished results), yet dopamine and small amounts of adrenaline were also found in the aorta (Kleemann & Schipp 1996).

The aim of this study was the localisation of serotonin and catecholamines in the central heart of *Nautilus pompilius* L. and the evaluation of their physiological effects on the isolated auricle.

Material and methods

Animals

The investigations were carried out on the hearts of 4 semi-adult of either sex (shell diameter 7.2–11 cm, net body weight 205–330 g) and 4 juvenile (shell diameter 7–8 cm, net body weight 143–240 g) *Nautilus pompilius* L. The animals were captured in Philippine coastal waters, purchased from a local dealer (Bachmann, Homburg/Limbach, Germany) and kept in seawater tanks for up to 4 months before sacrificing. During that time they were fed with dead shrimps every second day. The animals were anaesthetised with 2% ethanol in seawater as approved by the animal committee.

Tissue preparation

The hearts were fixed in Bouin's solution or an osmium–zinciodide solution after Maillet (1963). Osmium–zinciodide solution fixed tissue was embedded in paraffin and serial sections with a thickness of 7 μm were prepared. The tissue for immunohistochemical investigations was subjected to cryoprotection and embedded in OCT compound. Cross sections of the auricles and the ventricle were cut with a cryostat (Leica) with a thickness of 10 μm. Tissue for HPLC and histochemistry was snap frozen in liquid nitrogen.

Silver staining of nervous tissue after Bodian (1936)

Paraffin sections of the Bouin-fixed hearts were re-hydrated in a graded series of ethanols. To remove traces of picric acid, the 70% ethanol contained a small amount of NH_3. The sections were placed in 3% acetic acid for 4–6 h before transferring them into a 2% protargol (Merck, Darmstadt, Germany) solution for 20 h. After placing the sections in a hydrochinone (Sigma, Deisenhofen, Germany) solution for 15 min, they were again incubated in 2% protargol for 24 h. The reaction was visualised by placing the sections in goldchloride (Sigma) for 4 min, oxal acid (Merck) for 8 min, and finally in sodiumthiosulphate (Sigma) for 8 min. Following the incubation, the sections were de-hydrated in a graded series of alcohols and mounted in Rotihistokit (Roth, Karlsruhe, Germany).

Immunohistochemistry

Re-hydrated paraffin sections were blocked with 5% BSA (Sigma), 5% normal goat serum (DAKO, Hamburg, Germany), and 0.1% cold water fish skin gelatine (Plano, Wetzlar, Germany) and incubated with primary antibodies raised against serotonin (Sigma) and dopamine (Sigma) overnight. Serotonin immunoreactivity was visualised with diaminobenzidine (DAB, Sigma) and compared to the distribution of nervous tissue by Bodian silver stain in parallel sections, while the dopamine immunoreactivity was visualised with a Cy3 secondary antiserum (Jackson Immuno Research, Baltimore, USA).

Glyoxylic acid induced fluorescence after Barber (1982)

Cryosections (10 μm) of the auricles and ventricle were incubated with glyoxylic acid (18.5 g sucrose, 0.46 g HEPES, 1 g glyoxylic acid [Merck] ad. 50 ml water) for 7 min at 4 °C and air-dried for 30 min. The sections were heated to 85 °C for 5 min before mounting them in paraffin oil. Control sections were incubated without glyoxylic acid.

High performance liquid chromatography (HPLC)

The heart was rapidly removed from an anaesthetised animal (2% ethanol) and the auricles, the ventricle and the cephalic aorta were weighted and snap frozen. The tissues samples were mechanically homogenised in 0.1 M perchloric acid containing 2.7 mmol EDTA (1 ml per 100 mg of tissue), centrifuged and the supernatant stored at −80 °C. The eluted samples (40 μl) were transferred into reaction tubes containing 1 ml of Tris buffer (1 mol/l, pH 8.6) and 50 μl of an internal standard (dihydroxybenzylamine, DHBA). Catecholamines were extracted by absorption onto Al_2O_3 and elution with acetic acid. Reverse-phase HPLC was performed on a catecholamine analysis system from Millipore-Waters (Eschborn, Germany) consisting of the M510 pump, the autosampler WISP 712, the electrochemical detector M 540 and the integrator DATD MODUL 746. A Resolve C_{18} 5 μm column (3.9 mm×150 mm) and a mobile phase from Recipe Chemicals were used for separation. The flow rate was 1 ml/min, the pressure 2000 psi. The sensitivity of the detector was 0.5 nA and the basic current of the ECD 1.5–2 nA. The internal recovery rate was determined to be 73%.

Histochemical detection of MAO after Glenner et al. *(1957)*

Snap frozen hearts were cryosectioned at 16 μm and incubated in 15 ml phosphate buffer containing 20 mg tryptamine and 0.05% tetranitro BT for 30 min at 37 °C control sections were incubated with ipronazid (all Sigma) or without substrate. Following the incubation the sections were fixed in 4% formaldehyde in seawater at room temperature and mounted in Kaiser's glycerol gelatine (Merck).

Pharmacological experiments

Three auricles were separated from the efferent branchial vessels at the auricular valves and approx. 1 mm of the atrio-ventricular junction. The auricles were

mounted on stainless steel clamps and isometrically suspended in a 50 ml water jacketed organbath containing filtered, iso-osmotic seawater with 0.17% glucose (w/v) at pH 8.2. The organbath was aerated and kept at 18 °C during the experiments. The auricles were precontracted with 0.5 cN and contractions were recorded with an F-10 transducer (Hugo Sachs Electronics [HSE], Germany), the signals amplified with the HSE-plugsys system and the signals recorded with a BD100 plotter (Kipp und Zonen, TECHLAB, Erkerode, Germany).

Serotonin was added cumulatively starting from 10^{-8} mol/l to a maximum of 10^{-5} mol/l. After a washout period of 45 min, norepinephrine was also added cumulatively (10^{-9}–10^{-5} mol/l). The effects of each concentration were recorded for 10 min.

Results

Distribution of nerve fibres

The largest branches of the visceral nerve enter the ventricle in the middle of the ligament (for details see Griffin 1900). They run parallel to the arteria septalis to the dorso-caudal side and can be located close to the epicardium (Figure 1a). In their course, smaller nerves branch off into the periphery and towards the inner muscle fibres (Figure 1b). Nervous elements can also be located in the atrio-ventricular region (Figure 1c). The auricles show large nervous elements not only close to the epicardium, but also within the myocardium and even close to the endocardium (Figure 1d). Overall, the auricles displayed a higher density of nerve fibres visualised by the osmium–zinciodide method as compared to the ventricle.

Glyoxylic acid induced fluorescence and HPLC

Using the GIF method we could demonstrate catecholamines (emission maxima at 480–490 nm) containing nerves within the ventricle (Figure 2a) and auricle (Figure 2b) wall, the larger ones in the ventricle being close to the ligament, decreasing in size towards the more luminal muscle layers. In accordance with the distribution of nerves analysed by osmium–zinciodide fixation, the auricle displayed large catecholaminergic nerve fibres in all layers. Serotonin could not be identified with certainty using this method due to background fluorescence. The tissue showed yellow autofluorescences (Figure 2) with emission maxima (510–520 nm), whose wavelength overlapped with the emission maxima of serotonin (520–530 nm).

In order to differentiate between the 3 catecholamines, a HPLC analysis was performed (Table 1), indicating that noradrenaline, adrenalin and dopamine are present in the central cardiovascular system of *Nautilus*. A high noradrenaline content was found in the auricles, whereas no noradrenaline was present in the samples of the ventricle. Adrenaline was only found in the ventricle at a very low concentration. Dopamine was found in all samples, both auricles and ventricle, and its content was higher in the auricles.

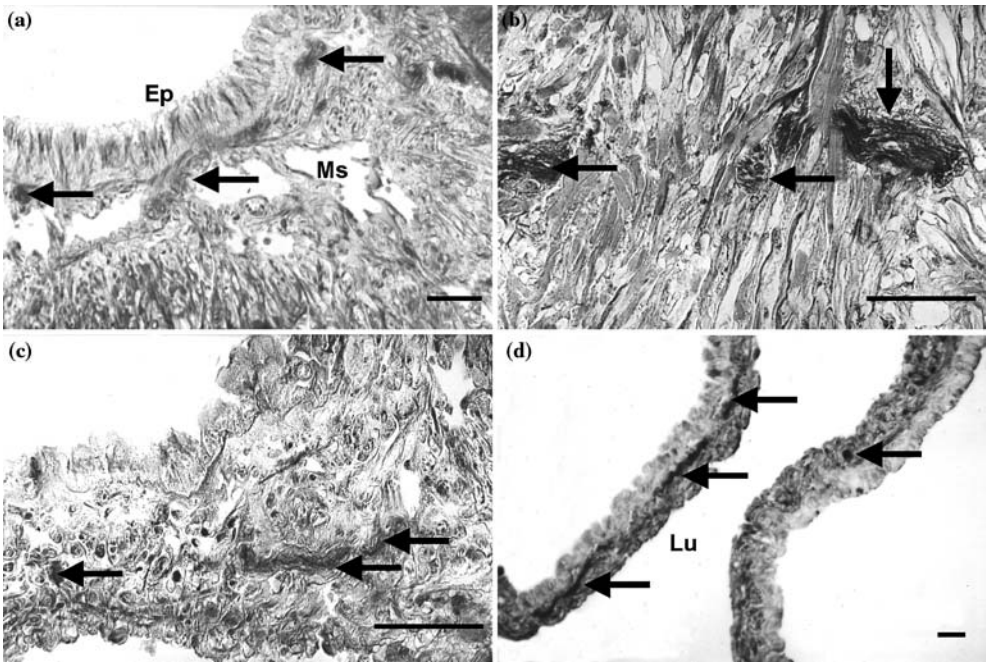

Figure 1. Distribution of polyaxonal nerve fibres (arrows) adjoining the pericardium (a), in the ventricle (b) the atrioventricular area (c) and auricle (d) localised by osmium–zinciodide fixation. Ep: epicardium; Ms: marginal sinus; Lu: lumen; scale bar: 50 μm.

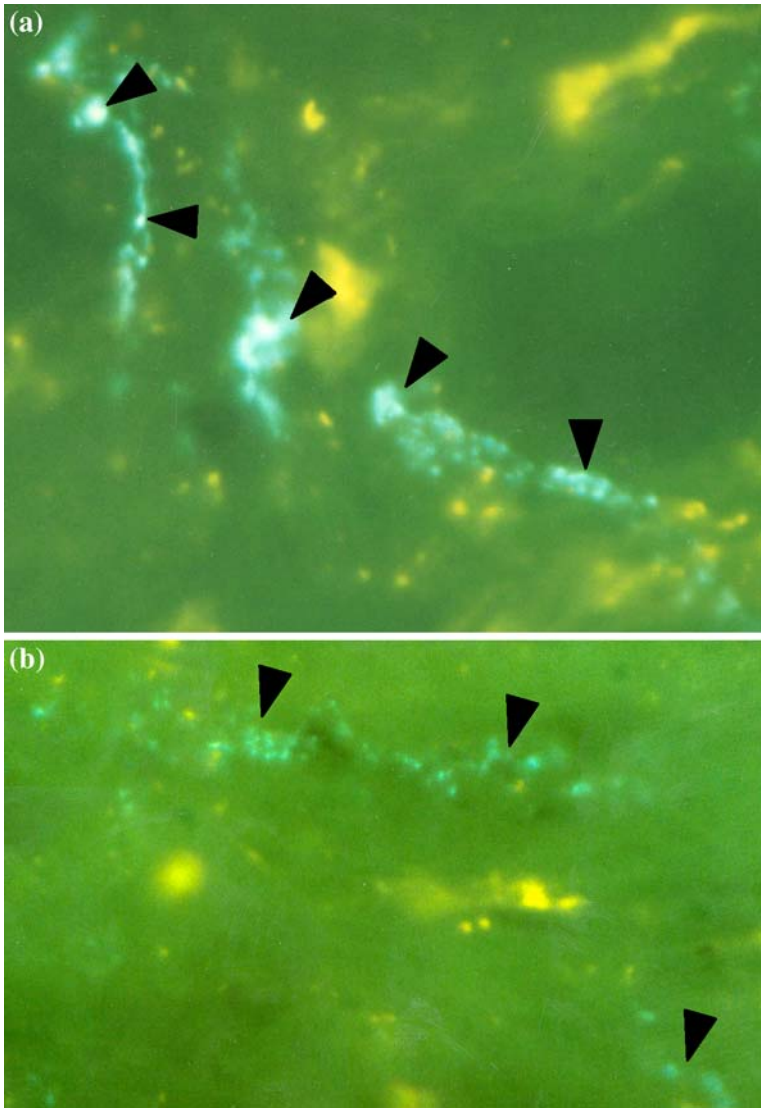

Figure 2. Localisation of catecholaminergic nerve fibres (arrow heads) by GIF in the ventricle (a) and auricles (b); arrowheads: blue fluorescence indicating catecholaminergic varicosities. Serotonin could not be localised by GIF, as the relevant wavelength overlaps with autofluorescences. Scale bar: 10 μm.

Table 1. HPLC-analysis for catecholamines of heart components of *Nautilus*.

Organ	Sample	Noradrenalin	Adrenaline	Dopamine
Ventricle	Ligamentum area	0	0.19	5.36
	Close to aorta	0	0	1.01
	Left ventricle half	0	0	0.96
	Right ventricle half	0	0	0.77
Auricle	Left auricles	83.94	0	66.18
	Right auricles	1.43	0	49.46

Immunohistochemical localisation of serotonin and dopamine

Serotonin-like immunoreactivity was detected in larger nerves in the ventricle close to the ligament using a polyclonal antibody, which was visualised by PAP method with diaminobenzidine (Figure 3a and b). No immunoreactivity was observed in the more luminal regions. There was also very weak staining in the auricles (not shown).

A polyclonal antiserum raised against dopamine was used to further investigate the overall distribution of catecholaminergic innervation of the heart, which was visualised with Cy3 immunofluorescence (Figure 4). All regions of the ventricle including the trabecular inner muscle layer (Figure 4a) and the semilunar muscular

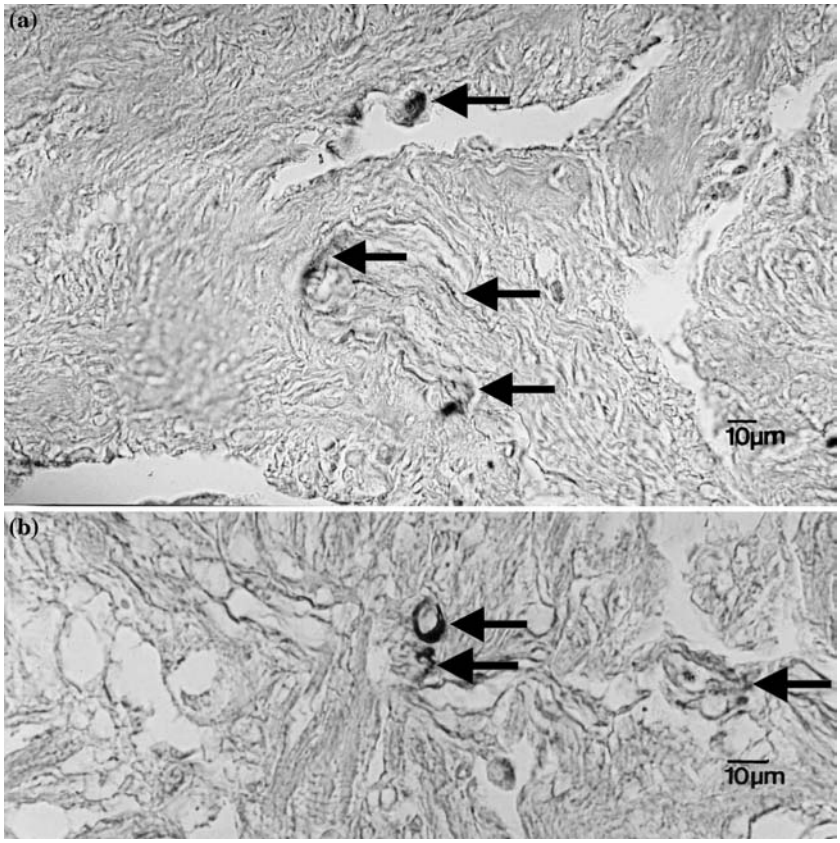

Figure 3. Localisation of serotonin in the ventricle by the PAP-method using DAB as substrate. Arrows: immunoprecipitation, scale bar: 10 µm.

atrioventricular valves (Figure 4b) displayed immunoreactive nerve fibres. Surprisingly, weak staining was observed in the auricles (Figure 4c), although the relative innervation density and, therefore, the dopamine content is higher than in the ventricle.

Histochemical detection of monoaminoxidase (MAO)

MAO activity was detected in the epicardium of the ventricle and auricles. In contrast to the epicardium, the myocardium of both heart compartments generally displayed only weak MAO activity, with locally defined strong reaction products, which were located closely to the arteria septalis and the ligament. Control experiments with ipronazide or without substrate were negative (not shown) (see Figure 5).

Isolated auricles in bioassay

Of the three auricles subjected to organbath experiments, only one showed spontaneous contractions for a short time at a tension of 0.5 cN. After stimulating the auricles with 10^{-6} mol/l serotonin all three displayed rhythmical contractions (Figure 6a). Increasing doses of serotonin lead to a decreased amplitude and frequency of the heartbeat (Figure 6a). After a wash out period of 45 min, the auricles displayed constant contractions and were subsequently used to study the effect of noradrenaline, which dose-dependently lead to positive chronotropic and inotrophic effects (Figure 6b).

Discussion

Catecholamines and serotonin play an important role in the regulation of the cardiac output and the vasotonus of not only vertebrates, but also of the highly evolved cephalopods (Messenger 1996). While much is known about the distribution and the physiological role of these substances in the coleoid cephalopods, little was known about the innervation of the ancient *Nautilus*.

The results of this study show that the heart of *Nautilus pompilius* L. is innervated by aminergic nerves, which are likely to be an excitatory system and which include serotonin, noradrenaline, dopamine and to a lesser extent adrenaline.

In accordance with the systemic heart of *Sepia* and *Octopus* (Messenger 1996) serotonergic innervation of the *Nautilus* heart was sparse and located only in large nerves close to the entrance of the nervus cardiacus, whereas a dense serotonergic innervation of hearts of several mussel species has been described (Ono *et al.* 1992). In contrast to histochemical results in the auricles of *Sepia* (Versen *et al.* 1999), serotonin could not be demonstrated by the GIF-Method due to background fluorescence, which had the same emission maxima as

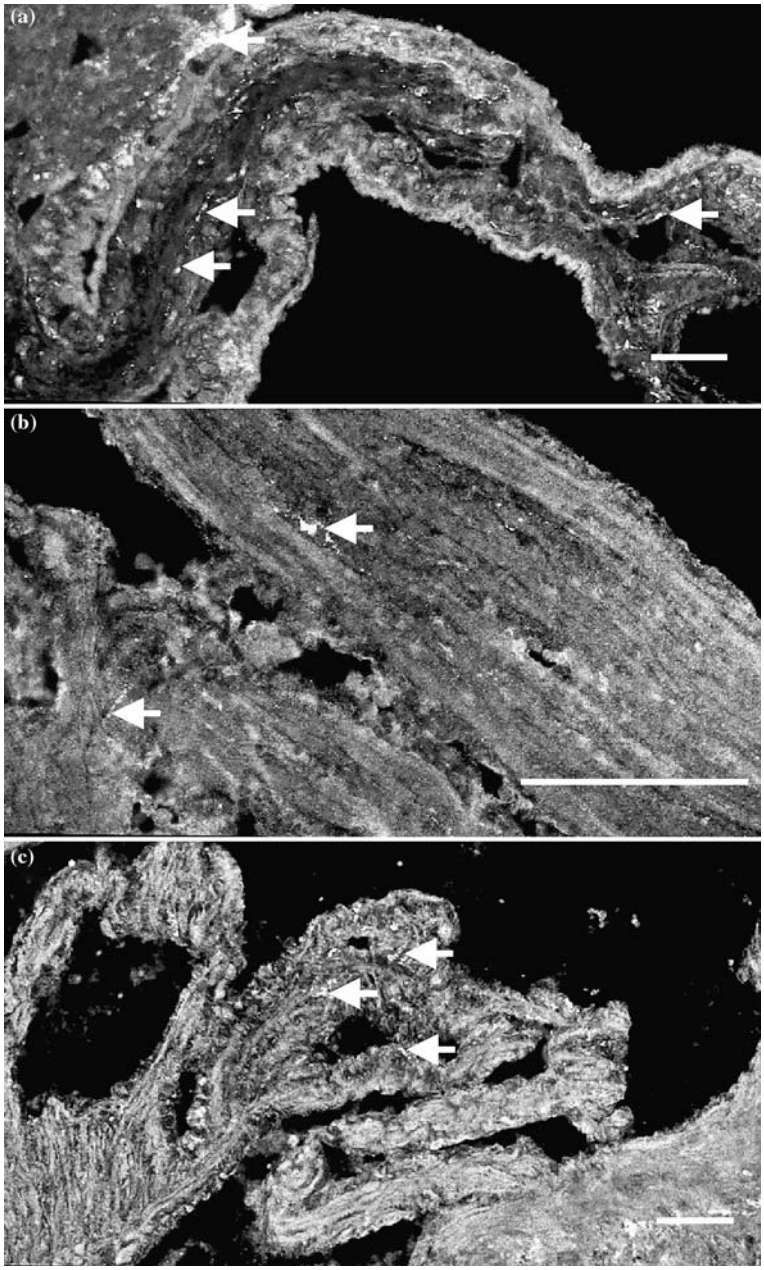

Figure 4. Dopamine immunoreactivity (arrows) in the inner, trabecular muscle layer of the ventricle (a), the semilunar muscular atrioventricular valves (b) and the auricle (c). Scale bar: 50 μm.

serotonin induced fluorescence. The same method also failed to show serotonin within the arteries of *Nautilus* (Kleemann & Schipp 1996, 1997), contrary to the intestine where it has been located by this method (Westermann *et al*. 1997).

Physiologically, up to 10^{-6} M serotonin induced rhythmical contractions in the isometrically suspended auricle of *Nautilus*, whereas higher doses lead to a decrease in amplitude and frequency of the heartbeat, suggesting either a stimulation of excitatory nerve endings by serotonin, which may have run empty during the experiments, or a reversed effect of serotonin by desentitation of the receptors or pre-synaptic inhibition of other nerve fibres, which has been described for the guinea pig (Adler-Graschinsky *et al*. 1989). Physiological and histochemical studies in *Sepia officinalis* suggest a highly differentiated 5-HT receptor system in the auricles, which involves at least two subtypes, 5-HT_1- and 5-HT_2-like receptors (Lehr & Schipp 2004a, b).

While serotonin could not be conclusively demonstrated by GIF, the catecholamines were clearly demonstrated within the myocardium of the auricles and the ventricle. The fluorescent varicosities are likely to be identical with the dense cored vesicles of the polyaxonal nerve fibres, that are also found in the systemic heart of coleoid cephalopods (Schipp & Schafer 1969a, b,

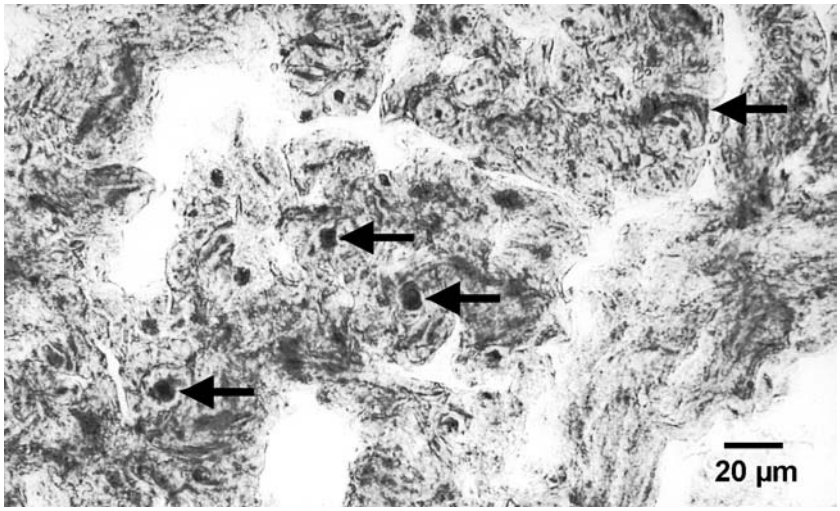

Figure 5. Histochemical localisation of the monoaminoxidase (MAO, arrows) in the ventricle myocardium. Scale bar: 20 µm.

Ducros & Arluison 1977, Kling & Schipp 1987, Fiedler & Schipp 1991). The occurrence of catecholamines has also been demonstrated by GIF in the systemic heart of *Eledone moschata* (Kling 1984) and *Sepia officinalis* (Kling 1986).

Since the different catecholamines cannot be differentially displayed or quantified by the GIF-method, a HPLC-analysis was performed, which showed dopamine in both auricles and ventricle, whereas adrenaline and noradrenaline were found only in the ventricle and auricles, respectively. Interestingly, noradrenaline is also the predominant catecholamine in the aorta of *Nautilus*, although small amounts of adrenaline were also found (Kleemann & Schipp 1996). In *Sepia*, HPLC-analysis of the central heart also showed mainly dopamine, whereas the vena cava and the branchial hearts mainly showed noradrenaline (Fiedler *et al*. 1992).

As both compartments of the *Nautilus* heart displayed high dopamine contents, the distribution of dopaminergic nerve fibres were investigated by immunohistochemistry. Positive nerve fibres were found throughout the entire heart, including the muscular semilunar heart valves, suggesting an important role of dopamine or related catecholamines in the neuronal control of the heart. The presence of dopamine supports two assumptions, (1) dopamine is released from nerve fibres and is acting as a neurotransmitter and (2) dopamine is further processed to adrenaline or noradrenaline. The low content of the latter two catecholamines found in the ventricle may be due to an increased activity of noradrenergic neurons due to the ethanol-anaesthesia. The catecholaminergic innervation of the auriculo-ventricular valves suggests a participation of the regulation of the heart output by modulation of the valve-tonus.

In pharmacological experiments, the effect of noradrenaline was investigated in the auricles, where it leads to a positive inotropic and chronotropic effect, that is dose-dependent. These results are consistent with data from earlier studies on *Sepia* (Versen *et al*. 1999).

Taken together the results presented in this study indicate that catecholamines and serotonin play an important role in the regulation of the cardiac output. The unequal distribution of noradrenaline may imply that due to the spongious tissue of the heart, the adequate transmitter for one heart compartment may be washed out into the haemolymph and thereby carried into the next compartment, e.g., noradrenaline released in the auricles is washed into the ventricle, where due to the possible lack of receptors, it would not have any effects. This statement may be supported by the fact that the major degrading enzyme of catecholamines, the monamine oxidase, was mainly located to the epicardium, meaning that the transmitter is very likely not degraded in the myocardium, but is washed out and degraded in the marginal sinus, which collects the hemolymphe that has passed through the myocardium. Furthermore, in the next compartment, the aorta, high levels of noradrenaline have been demonstrated (Kleemann & Schipp 1996, 1997).

On the other hand, the results can be interpreted that a strong release of noradrenaline in the auricles may result in a wash out that will activate specific receptors in the ventricle. A similar mechanism has been proposed for serotonin that shows a 100-fold higher affinity to the receptors expressed in the ventricle as compared to the auricular receptor type (Kling & Schipp 1987, Versen *et al*. 1999). Without the tools to study the distribution of receptor for the different biogenic amines, this question cannot be conclusively answered.

In summary, this study shows that the *Nautilus* heart is innervated by both serotonergic and catecholaminergic nerve fibres, which may play an important role in the chronotropic and inotropic regulation of the myogenic heart.

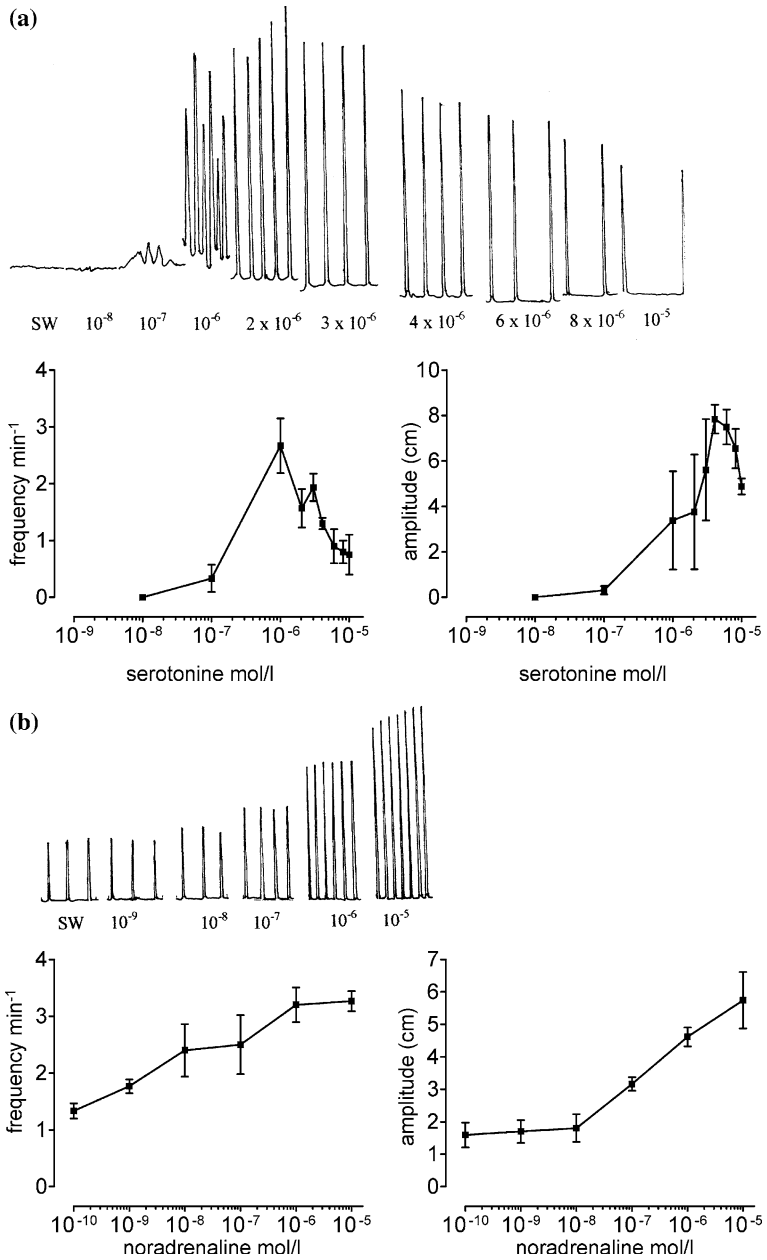

Figure 6. Pharmacological effects of serotonin (a) and noradrenalin (b). Increasing concentrations of serotonin reduced amplitude and frequency of the heartbeat, while increasing concentrations of noradrenaline had positive chronotropic and inotropic effects.

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