Morphological, cytochemical and neuropharmacological evidence for the presence of catecholamines in hydrozoan planulae

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Summary
Planula larvae of Halocordyle disticha were examined for the presence of catecholamines using a multi-pronged approach. Transmission electron micrographs of planular sensory cells and ganglionic cells demonstrated dense-cored vesicles and electron-dense droplets in both cell types. These vesicles and droplets were similar in morphology to catecholamine-containing granules of vertebrates. Planulae processed with the SPG histofluorescence technique, specific only for catecholamines, exhibited blue-green fluorophores which were most prominent in the anterior ectoderm. Such fluorescence was associated with sensory cells, ganglionic cells and the neural plexus. Pretreatment of planulae with neuropharmacological agents which prevent reuptake (reserpine) or cause release (nicotine, ephedrine) of catecholamines caused a diminution of the fluorophores. Pretreatment of animals with 6-hydroxydopamine, which causes destruction of catecholamine-containing cells, prevented any fluorescent response. Ultrastructural examination of reserpine-treated planulae revealed a dramatic reduction in the populations of dense-cored vesicles and electron-dense droplets. Furthermore, many of the vesicles and droplets remaining in reserpinized animals appeared washed out, i.e. stained faintly. Exposure of planulae to exogenous norepinephrine caused premature, rapid metamorphosis and produced polyps with slightly stunted tentacles and pitted, irregular hypostomes. Exposure of planulae to nicotine caused similar effects. Rearing planulae in sea water containing alpha blockers, phentolamine and tolazoline, had no discernible effect on behaviour (motility, phototactic response) or gross morphology. However, planulae raised in sea water containing propranolol, a beta blocker, ceased all movement, became tact-shaped and died within 72 h. These results meet multiple criteria for the identification of catecholamines in hydrozoan planulae and suggest that such catecholamines may function as neurotransmitters, neurohormones or neuromodulators during larval development.

Key words: catecholamine, hydrozoan planula, fluorophore, reserpine, nicotine, ephedrine, neurotransmitter, cnidaria, fluorescence microscopy.

Introduction
Cnidarians possess a simple nervous system and most probably were the first animals to develop a nervous system during evolution. From ultrastructural studies, it has been shown that all classes of cnidarians possess dense-cored and electron-dense vesicles (Jha & Mackie, 1967; Spencer, 1979; Westfall, 1970, 1973a,b). Such vesicles are often seen in close association with pre- or postsynaptic membrane specializations, suggesting that synapses in cnidarians are chemical in nature. Physiological studies have demonstrated that neuronal activities in cnidarians can be inhibited by excess magnesium or by calcium depletion, thus further supporting the notion of chemical synapses (McFarlane, 1973; Satterlie, 1979; Spencer, 1978; Spencer & Arkett, 1984). Despite these morphological and physiological findings, no neurotransmitter has been conclusively identified in cnidarians (Martin & Spencer, 1983). In recent years Grimmelikhuijzen and associates, using immunocytochemistry and radioimmunoassays, have demonstrated several chemicals in the nervous systems of adult cnidarians, particularly that of Hydra, that are closely related to vertebrate and invertebrate neuropeptides (for a review see Grimmelikhuijzen, 1984). It has been postulated that such neuropeptide-like substances function as cnidarian neurotransmitters.
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(Grimmelikhuijzen & Graff, 1985). Grimmelikhuijzen and others have been unable to identify in cnidarians the 'classical' neurotransmitters such as catecholamines, serotonin and acetylcholine (Grimmelikhuijzen & Graff, 1985).

Despite the findings of Grimmelikhuijzen, evidence exists in the literature to suggest that catecholamines are present in cnidarians (Elöfsson et al. 1977). The Falck and Hillarp monoamine histofluorescence technique for the demonstration of catecholamines (Falck et al. 1962) has been used by Dahl and others (1963) on the anthozoans Metridium senile and Tealia felina and by Wood & Lentz (1964) on Hydra, Sagartia and Metridium. These studies demonstrated the presence of green fluorescing cells in these animals which was indicative of a positive response for catecholamines. The Falck and Hillarp method, however, has been criticized for being less specific and less sensitive than the newer glyoxylic acid methods for the identification of catecholamines (Martin & Spencer, 1983). The glyoxylic acid methods are reliable, stain only catecholamines, are extremely sensitive and work well with both sections and whole mounts (Furness et al. 1987). Glyoxylic acid treatment of tissues yields distinct dots of discontinuous fluorescence in adrenergic nerve varicosities, however, nerve cell bodies do not fluoresce strongly (Furness et al. 1987; Lindvall & Björklund, 1987).

Recently, Martin (1987) has demonstrated several neuropeptide-like substances in the nervous system of cnidian planula larvae. Furthermore, she has visualized at the fine-structural level dense-cored vesicles and electron-dense droplets in the neurites of planular nerve cells (Martin & Thomas, 1980; Martin, 1988). These vesicles and droplets resemble the catecholamine storage vesicles found in vertebrates (Hökfelt, 1968; Hervonen, 1971; Gibbins, 1987). The present study examines the planula of the marine hydrozoan Halocordyle disticha (formerly Pennaria tiarella) for the presence of catecholamines. The work is designed to meet multiple criteria for the identification of catecholamines, as outlined by Furness et al. (1987), and Lindvall & Björklund (1987) (after Werman, 1966). These criteria include: (1) specific cytochemical localization of catecholamines; (2) response of planulae to exogenous catecholamines; (3) evidence of release of catecholamines by appropriate stimulation and (4) predictable alteration in the behaviour of catecholamines due to exposure to pharmacological agents, such as receptor antagonists, reuptake blockers and toxins.

Materials and methods

Materials

Colonies of Halocordyle disticha were collected from pier pilings in Morehead City, NC. Fronds from male and female colonies were placed together in large glass finger bowls containing filtered sea water. The bowls were placed in the dark at 6.00 p.m. (EST) and at 9.00 p.m. early cleavage stages were collected, placed in small culture dishes of filtered seawater and reared at 23°C to the planula stage. Seawater in the culture dishes was changed twice daily.

Chemical treatments

At 26 h postfertilization a sample (40) of animals was exposed to 0.06 mm-exogenous norepinephrine (Sigma) in filtered seawater for 30 min. A group of animals was exposed to nicotine (Sigma) at 0.06 mm for 30 min. Another group of animals was exposed to 0.4 mm-reserpine for 30 min. After treatment these animals were washed, returned to filtered seawater, observed with a stereomicroscope, and some were fixed at selected time intervals for scanning electron microscopy (SEM). The number of planulae which attached and began metamorphosis was recorded (Table 1). Some treated animals were allowed to produce primary polyps which were subsequently fixed for SEM. Also at 26 h postfertilization, groups of animals were transferred to seawaters containing different adrenergic receptor blockers: 0.2 mm-phenotolamine (Regitine) (Ciba-Geigy), 0.2 mm-tolazoline (Sigma), or 0.2 mm-propranolol (Sigma). Animals were raised in these solutions for 96 h and observed with a stereomicroscope for behavioural effects on motility and phototactic response to a 10 foot-candle (ft-candle = 10^{-7} 639 lx) light from an American Optical Spot Illuminator and for gross morphological effects. At 96 h postfertilization planulae were exposed to the following neuropharmacological agents in sea water: 0.4 mm-reserpine (Sigma) for 30 min, 0.4 mm-reserpine plus 0.06 mm-nicotine for 30 min, 0.4 mm-reserpine plus 0.06 mm-mephedrine (Sigma) for 30 min, or 0.4 mm-6-hydroxydopamine (Sigma) for 2 h. After treatment these animals were processed for SPG fluorescence.

Electron microscopy

Control and chemically treated animals were processed for either transmission electron microscopy (TEM) or scanning electron microscopy (SEM). Samples were fixed for 1-5 h in 2.5% glutaraldehyde, pH 7.6, in 0.2 M-Millonig's phosphate buffer. Animals subsequently were rinsed three times for 15 min each in the buffer solution. Specimens were then postfixed for 1 h in 2% osmium tetroxide in 1.25% sodium bicarbonate buffer, pH 7.2, and rinsed three times in the buffer. Samples were then dehydrated in a graded series of ethanol. Specimens for TEM were infiltrated and embedded in Spurr's embedding media. Sections were cut on a Porter-Blum MT-2B ultramicrotome, placed on 200 mesh copper grids and stained with 5% ethanolic uranyl acetate followed by Reynolds lead citrate. Grids were examined and photographed with a Hitachi H-600 transmission electron microscope. Samples for SEM were critical-point-dried, sputter-coated with gold-palladium and examined with a JEOL JSM T-300 scanning electron microscope.

SPG fluorescence

Control and chemically treated planulae were dried to clean
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glass microscope slides, quickly dipped three times into sucrose–phosphate–glyoxylic acid (SPG) solution (10-2 g sucrose, 4-8 g potassium phosphate, and 1-5 g glyoxylic acid in 150 ml distilled water), placed in an air dryer and then transferred to an 80 ± 1°C oven for exactly 5 min (de la Torre & Surgeon, 1976). The slides were then coated with light mineral oil and observed with a Zeiss research microscope under epifluorescence using the Zeiss filter 487704 (exciter G405, barrier LP 495). The fluorescence response of animals was compared to the fluorescent staining of control slides of pure norepinephrine, pure dopamine, or pure octopamine solutions.

Statistics

Analysis of the means of percent metamorphosis from 26–50 h was carried out according to Sokal & Rohlf (1981) for single classification Anova with equal sample sizes (Model I). The probability of a larger F was obtained from Steel & Torrie (1980). Standard deviation of the mean was calculated by the method of Sokal & Rohlf (1981).

Results

The mature planula larva of Halocordyle disticha possesses two types of neural elements in the ectoderm: sensory cells and ganglionic cells (Figs 1 & 2).

Fig. 1. Sensory cell (ns) in the ectoderm of a 72 h control planula. The cell possesses an apical cilium (c) and a midbasal nucleus (nu). ×4100.

Fig. 2. Ganglionic cell at the base of the ectoderm of a 72 h control planula. The perikaryon contains a centrally located nucleus (nu) and segments of rough endoplasmic reticulum containing electron-dense material. As this electron-dense material passes from the rough endoplasmic reticulum to the Golgi (not visible in this micrograph), it becomes compacted and acquires the characteristic size and shape of the electron-dense droplets found in the neurites of the ganglionic plexus. n, neurite of the ganglionic plexus; d, electron-dense droplets; dc, dense-cored vesicles. ×14000.
of the animal (Fig. 2). A ganglionic cell forms neurites which radiate from both sides of the cell body. These neurites make contact with neurites from adjacent ganglionic cells thus forming a neural plexus adjacent to the mesoglea (Fig. 3). The cell body of the ganglionic cell contains some rough endoplasmic reticulum, a Golgi complex, numerous cytoplasmic microtubules, and electron-dense droplets and dense-cored vesicles. Mature electron-dense droplets (i.e. free in the cell cytoplasm and in the neurites) range from 90–120 nm in diameter, whereas, within the confines of the rough endoplasmic reticulum larger electron-dense droplets (most probably precursors of the smaller droplets) ranging in size from 210–290 nm are seen (Fig. 2). Neurites of ganglionic cells are rich in microtubules, mitochondria, and electron-dense droplets and dense-cored vesicles. Within the ganglionic plexus these droplets and vesicles are located in distinct clusters at certain locations along the neurites and are not homogeneously distributed throughout the neurites (Fig. 3). The ganglionic plexus, composed of sensory cell processes and ganglionic cell processes, extends in both a longitudinal and transverse direction along the length of the planula (Fig. 3).

Dishes of control, norepinephrine-treated, nicotine-treated and reserpine-treated planulae exhibited significant differences in the percentage of animals

![Fig. 3. Ganglionic plexus of a 72 h control planula. The plexus consists of neurites (n) from both sensory cells and ganglionic cells. Neurites (n) of the plexus are rich in microtubules, mitochondria, electron-dense droplets (d) and dense-cored (dc) vesicles. en, endoderm; mg, mesoglea. ×17200.](image-url)

![Fig. 4. Scanning electron micrograph (SEM) of control polyps of Halocordyle disticha. b, base; cn, crown; m, mouth; s, stalk. ×80.](image-url)

![Fig. 5. SEM of the head region of a control polyp. The polyp possesses an inner whorl of short capitate tentacles and an outer whorl of long filiform tentacles. The surface of the head is smooth and hilly. x370.](image-url)

![Fig. 6. SEM of a primary polyp formed from a planula treated with 0.06 mM-norepinephrine for 30 min at 26 h postfertilization. ×110.](image-url)

![Fig. 7. SEM of the head region of a polyp formed by a planula exposed to norepinephrine. Arrows denote deep pits in the polyp surface. ×370.](image-url)

![Fig. 8. SEM of a primary polyp formed from a planula treated with 0.06 mM-nicotine for 30 min at 26 h postfertilization. ×110.](image-url)

![Fig. 9. SEM of the head region of a polyp formed from a nicotine-treated planula. Arrows denote pits in the surface. ×370.](image-url)
that began metamorphosis from 26–50 h postfertilization (Table 1). Of the control animals 12.5% (± 5%) began metamorphosis, compared to 100% (± 0%) for norepinephrine-treated animals, 35% (± 5-8%) for nicotine-treated animals and 0% (± 0%) for reserpine-treated animals. The time from attachment to primary polyp formation in controls was approximately 30 h. Control polyps have a crown, stalk and base (Fig. 4). The crown region (hypostome) consists of an outer whorl of long filiform tentacles and an inner whorl of short capitate tentacles. A mouth is present at the tip of the crown, the stalk connects the crown to the base of the polyp. Stolons form from the basal region of the polyp. The surface of the hypostome of control polyps is relatively smooth, especially near the mouth (Fig. 5). The time from planular attachment to polyp formation in norepinephrine-treated animals decreased to less than 24 h. The polyps formed from norepinephrine-treated planulae possess slightly shortened filiform tentacles (Fig. 6) and the hypostome surfaces are severely pitted and irregular (Fig. 7). This pitted surface is most severe in the younger polyps. The time frame from planular attachment to polyp formation in nicotine-treated animals was approximately 24 h. Polyps formed from nicotine-treated planulae possess shortened filiform tentacles, and both capitate and filiform tentacles are often irregularly spaced (Figs 8 and 9). The hypostome surfaces of these polyps are pitted (Fig. 9) and resemble those polyps formed from norepinephrine-treated planulae.

Control planulae processed with SPG exhibit a strong blue–green fluorescence in the ectoderm which is particularly intense in the anterior end of the animal (Fig. 10). This blue–green fluorescence matches that of the pure norepinephrine control slide, but is distinctly different from the white–yellow fluorescence of pure dopamine and pure octopamine solutions. The endoderm of planulae exhibits a nonspecific yellow–orange fluorescence before and after processing with SPG. Planulae exposed to reserpine show a diminished blue–green fluorescent response when compared to control animals, though a distinct amount of fluorescence is still visible in the ectoderm of the anterior end (Fig. 11). Animals exposed to reserpine in combination with either nicotine or ephedrine show a still fainter blue–green fluorescent response, with only a dim fluorescence observed in the anterior ectoderm (Fig. 12). Planulae treated with 6-hydroxydopamine exhibit no blue–green fluorophores (Fig. 13). In the posterior regions of control planulae, blue–green fluorophores are more dispersed than in the head regions and can be resolved as distinct dots, smaller than whole cells, located just above the mesoglea in the region of the neural plexus and in some sensory cells (Fig. 14).

Planulae treated with reserpine exhibited a reduction in the populations of dense-cored vesicles and electron-dense droplets in the neurites (Fig. 15). Some droplets and vesicles were observed in the neurites, however, many of the droplets stained faintly and many of the vesicles appeared either empty or contained very small dense cores (Fig. 15). Phentolamine and tolazoline, blockers of alpha receptors (Weiner, 1985b) have no effect on planular motility, phototactic behaviour or gross morphology. Treated planulae are capable of metamorphosis. Animals exposed to propranolol, a beta blocker (Weiner, 1985b), cease all movement after 4 h of treatment, and after prolonged exposure (72 h), animals become tack-shaped and die.

![Figure 10](image1.png)
Control 96 h planula processed with SPG. Arrows denote catecholamine-specific blue–green fluorescence in the ectoderm at the anterior end of the planula. The core of endoderm (en) has a nonspecific yellow–orange fluorescence. x115.

![Figure 11](image2.png)
96 h planula treated with 0.4 mM-reserpine for 30 min prior to processing with SPG. Arrows denote blue–green fluorescence in the anterior end. Reserpine-treated animals show a reduction in the amount of blue–green fluorescence when compared to controls. en, endoderm. x220.

![Figure 12](image3.png)
96 h planula treated with 0.4 mM-reserpine and 0.06 mM-nicotine for 30 min prior to processing with SPG. Arrows denote small dim dots of ectodermal blue–green fluorescence. en, endoderm. x120.

![Figure 13](image4.png)
96 h planula treated with 0.4 mM-6-hydroxydopamine for 2 h prior to SPG processing. Only nonspecific yellow–orange fluorescence of the endoderm (en) is visible. There is no catecholamine-specific blue–green fluorescence. Arrow denotes external surface of the ectoderm. x160.
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Fig. 14. More posterior view of a 96 h control planula processed with SPG. Blue-green fluorophores, indicative of catecholamines, are present as small dots in the ectoderm. An upper level of dots (u) is in the region of sensory cells and their processes and a lower level of dots (g) is in the region of the ganglionic plexus. These dots most probably correspond to the clusters of droplets and vesicles visualized in neurites by transmission electron microscopy. en, endoderm; mg, mesoglea. ×270.

Fig. 15. Neurites in the ganglionic plexus of a 28 h planula exposed to 0-4 mM-reserpine for 30 min. Black arrows denote lightly staining faded droplets not seen in control animals. The white arrow shows an empty dense-cored vesicle. mg, mesoglea. ×28 900.

Discussion

Furness and others (1987) and Lindvall & Björklund (1987) (after Werman, 1966) proposed four criteria for the positive identification of catecholamines in an organism: (1) it should be possible to show the presence of catecholamines by specific stains, fluorescence or autoradiography; (2) the catecholamines should be released via appropriate stimulation; (3) exogenous application of a catecholamine should produce an appropriate response and (4) the behaviour of the catecholamines should be altered by pharmacological agents (agonists, antagonists or reuptake blockers) in a predictable manner. This study examines these criteria and provides evidence that catecholamines are present in cnidarian planulae and have neurophysiological and developmental functions in the larvae.

The first criterion is met by the results from both TEM studies and the SPG fluorescence method. The clusters of electron-dense droplets and dense-cored vesicles identified via TEM in sensory cells, ganglionic cells and the neural plexus of planulae closely resemble in size and morphology catecholamine-containing neurosecretory vesicles of mammals (Hökfelt, 1968; Hervonen, 1971; Gibbins, 1987). Hervonen (1971) identified electron-dense and dense-cored catecholamine-containing (CA) granules in the adrenal medulla and in the paraganglia of late human fetal stages. In the adrenal medulla, CA vesicles measure from 152–266 nm in diameter, whereas, in paraganglia they average from 109–271 nm in diameter. In planulae, electron-dense droplets and dense-cored vesicles measuring 90–120 nm in diameter are found in the cytosol of the cell body and in the cytosol
of the neurites of both sensory and ganglionic cells. Within the rough endoplasmic reticulum of young ganglionic cells large electron-dense droplets ranging from 210–290 nm in diameter are seen. These large granules fall within the range of the CA vesicles found in the adrenal medulla and most probably represent a precursor to the smaller granules found free in the planular cytosol. These large granules appear to decrease in size as they move from the rough endoplasmic reticulum to the Golgi. Control planulae exposed to SPG give a positive fluorescent response for catecholamines. They exhibit an intense blue-green fluorescence in their head regions and a scattered dot pattern more posteriorly. The colour of the planular fluorescence matches that of a pure norepinephrine solution slide, indicating a positive response for norepinephrine. The distribution of the planular fluorescence appears to correspond with the location of some sensory cells and with the entire ganglionic plexus. The ganglionic plexus association is especially visually than in the head region. Such distinct dots most probably represent the clusters of droplets and vesicles visualized by TEM in the neurites of the plexus. The more intense staining in the head region of the planula suggests an abundance of catecholamines in this area. However, there does not appear to be an accumulation of nerve cells in the head region. The ganglionic plexus of the head region is, nevertheless, quite extensive in this region (Martin, 1988). The glyoxylic acid methods are extremely sensitive and specific (de la Torre & Surgeon, 1976; Martin & Spencer, 1983; Lindvall & Björklund, 1987) and a positive staining response in planulae is unmistakable. Furthermore, Furness and others (1987) state that the only naturally occurring substances in peripheral nerves that produce a fluorescent product when reacted with glyoxylic acid are norepinephrine, epinephrine, dopamine and 5-hydroxytryptamine.

The results from the SPG fluorescence test of planulae treated with reserpine in combination with either nicotine or ephedrine provide evidence of satisfaction of the second criterion. Both nicotine and ephedrine cause release of catecholamines (Taylor, 1985; Weiner, 1985a) and reserpine prevents the reuptake of the released catecholamines (Weiner, 1985a; Gibbins, 1987). The diminution of the fluorescent response observed in treated planulae indicates that nicotine and ephedrine cause the release of the material stained by SPG, which, as established above, is catecholamines.

Exogenous application of norepinephrine to planulae results in premature, rapid metamorphosis and slightly abnormal polyps. These effects are similar to those produced by treating planulae with nicotine, which presumably caused planular release of endogenous norepinephrine. This similarity of effects between triggered endogenous release and exogenous exposure meets the third criterion and suggests that endogenous catecholamines are involved in metamorphosis of the planula, possibly acting as a neurotransmitter.

Several results satisfy the fourth criterion, the predictable alteration of catecholamines by pharmacological agents. Reserpine is the most widely used catecholamine-depleting drug. It is taken up by catecholamine-storage vesicles and causes their depletion by preventing both vesicular uptake and binding of catecholamines (Gibbins, 1987). Reserpine-treated planulae show a diminution of fluorescence when processed with SPG. This result is predicted if naturally released catecholamines are lost rather than taken up. Furthermore, transmission electron micrographs of reserpine-treated planulae indicate a reduction in the numbers of dense-cored vesicles and electron-dense droplets. Many of the remaining droplets which are observed in reserpinized planulae appear faded and stain only faintly, whereas some of the dense-cored vesicles appear either empty or contain very small cores. This faint staining and reduction in core content in planular droplets and vesicles would be expected if they contain catecholamines, and is consistent with findings for mammalian central and peripheral catecholamine neurones exposed to reserpine (Gibbins, 1987). 6-hydroxydopamine, a drug which causes selective autophagy of catecholamine-containing cells (Tranzer & Theonen, 1968), would be expected to greatly decrease, if not eliminate, any fluorescence produced by the SPG procedure. Such treated planulae, indeed, exhibit no fluorescence for catecholamines. Moreover, ultrastructural examination of these planulae demonstrate that 6-hydroxydopamine treatment causes a disruption of the plexus (K.J.S.K., unpublished observation). Results of the treatment of planulae with the beta blocker, propranolol, indicate that beta adrenergic receptors may be present in the planula and may be necessary for motility in the animal, indicating a possible neurotransmitter function for the catecholamines.

Both catecholamines and neuropeptide-like substances have been identified in the nervous system of the planula of Halocordyle disticha (Martin, 1987). The presence of catecholamines and peptides in planulae suggests that both are essential for the functioning of the primitive planular nervous system.

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References


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