The Distribution of FMRFamide-like Immunoreactivity in the Hydrozoan Nervous System.

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ABSTRACT

The distribution of FMRFamide-like immunoreactivity (Fa-IR) in the nervous system of four orders of hydromedusae and one species of hydroid was investigated using commercially produced polyclonal antisera to FMRFamide and whole mount immunocytochemistry. Representatives of the genera *Phialidium*, *Aequorea* (Leptomedusae), *Euphysa*, *Halitholus*, *Sarsia*, *Bougainvillia*, *Stomotoca* (Anthomedusae), *Gonionemus*, *Proboscidactyla* (Limnomedusae), *Aegina*, and *Solmissus* (Narcomedusae) were used. The hydroid studied was that of the Leptomedusa *Eirene viridula*.

In all hydromedusae Fa-IR was found to be restricted to a specific subset of nerves, those associated with smooth muscle and certain sensory structures, suggesting that a FMRFamide-like compound is a potential neurotransmitter or neuromodulator in hydrozoa. Two possible roles for the neuropeptide are suggested, one within a system modulating nematocyte discharge and one within a system mediating smooth muscle contraction. The extent of Fa-IR varied between different species being present in the manubrium of all but in the velum of only three. Fa-IR in the subumbrella and nerve rings also differed depending on the species.

In *Eirene viridula* Fa-IR was present in much of the nervous system, especially in sensory cells around the hypostome. Some morphological centralisation was

observed in the region of the tentacle bases where large multipolar immunoreactive cell bodies (fan cells) are present. These appear to be in a position to influence information transfer between the main body of the animal and the tentacles and hypostome. The FMRFamide-like compound in the hydroid may be functioning in similar systems to those suggested for the hydromedusae.

Since neuropeptides have previously been localised to dense-core vesicles an attempt was made to correlate presence of Fa-IR at the light microscope level with ultrastructual location in one hydromedusa *Phialidium* sp. using electron microscopy. Dense-core vesicles of neuropeptide-containing size (80-160 nm) were found to be present in a proportion of the neurons in all regions of the medusa. It was not possible to positively identify them as containing the FMRFamide-like compound. They are, however, a potential site of storage for a putative neurotransmitter or neuromodulator.

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INTRODUCTION

Studies of cnidarian neurobiology have a long history starting in the last century with the classic works of Romanes (1876, 1877, 1880) and Hertwig and Hertwig (1878). Since then much has been learned regarding the physiology of the cnidarian nervous system (for review of older work see Bullock and Horridge 1965; Spencer and Schwab 1982; Satterlie and Spencer 1983) but the pharmacological basis of nervous responses remains unclear. Some investigators have examined the effects of drugs or poisons on behaviour (Romanes 1877) and development (Spangenberg 1971; 1974) in medusae. In *Hydra* , effects of chemicals on nematocyst discharge (Lentz and Barrnett 1962) and regeneration (Lentz and Barrnett 1963) have been noted and in sea anemones, on muscular contraction (Ross 1960a; 1960b). However, evidence for the presence of any transmitters within the cnidarian nervous system is sparse and often contradictory (for review see Martin and Spencer 1983) and there has been little work on the comparative distribution of putative transmitters either within or between the three classes of cnidaria, Scyphozoa, Hydrozoa and Anthozoa (Grimmelikhuijzen 1983). Recent work has suggested the presence of compounds similar in structure to several vertebrate neuropeptides in the hydrozoan, *Hydra*. An undecapeptide responsible for head formation and located in neurosecretory granules (Schaller 1973; Schaller and Gierer 1973) has been shown to be conserved in structure from coelenterates to mammals (Bodenmüller and Schaller 1981). In addition, immunocytochemical

techniques have suggested that compounds similar to several vertebrate neuropeptides such as oxytocin, bombesin and substance P are present in *Hydra*, (Grimmelikhuijzen *et al.* 1980; 1981a; 1981b; 1981c; 1982; Grimmelikhuijzen 1985) but this is not a typical hydrozoan and can hardly be considered representative of the class. However this does suggest the intriguing possibility that the hydrozoans possess forms of neurotransmitters or neuromodulators that are related in terms of structure and maybe function to those found in higher animals and thus would be a good group of animals to study in order to expand our knowledge of these compounds. Furthermore, since cnidarians are the simplest animals possessing a recognisable nervous system, understanding their neuropharmacology may provide an insight into the role, mode of action and early evolution of neurotransmitters and neuromodulators. The proposal that peptides could function as potential neurotransmitters or neuromodulators is a relatively recent development but there is a growing body of evidence to suggest that they do indeed function in nervous transmission and can also act as hormones (for reviews see Haynes 1980; O'Shea and Schaffer 1985). They seem to be present in all phyla and in some cases to be conserved in structure between closely related phyla. In many cases 'families' of peptides occur, each member differing by only one or a few amino acids (*e.g.* the pancreatic polypeptides).

The hydromedusae are already well characterised in terms of neurophysiology and ultrastructure, are widely available (though seasonal) and have a nervous system composed of neurons resembling those of higher animals, comprising several physiological subsets yet relatively simply organised. An initial investigation of the presence of neuropeptide-like immunoreactivity in the

hydromedusae has not shown the same diversity of compounds to be present but antisera to a neuropeptide originally isolated from molluscs, phenylalanylmethionylarginylphenylalanylamide, abbreviated as FMRFamide, show consistent and intense immunoreactivity in components of the nervous system of the anthomedusan *Polyorchis penicillatus* (Grimmelikhuijzen and Spencer 1984), *Aglantha digitale* (Trachymedusae), *Proboscidactyla flavicirrata*, *Gonionemus vertens*, *Eperetmus typus* (Limnomedusae) and two Leptomedusae, *Phialidium gregarium* and *Aequorea victoria* (Mackie and Stell 1984; Mackie *et al.* 1985). Thus a FMRFamide-like molecule would appear to be a potential neurotransmitter or neuromodulator in hydrozoans and its presence in only a proportion of nerves represents a complexity within the nervous system, which had once been held to be physiologically and pharmacologically very simple (Pantin 1952). Since the late 1960's it has become apparent that this is not the case and that hydromedusae do indeed possess separate physiological systems (Mackie 1971; Mackie 1975; Spencer and Arkett 1984). Whether this is mirrored in the pharmacology remains to to be seen.

The tetrapeptide FMRFamide was originally isolated from the clam *Macrocallista nimbosa* (Price and Greenberg 1977) and has since been shown to have diverse effects on excitable tissue of molluscs, including cardiac and non-cardiac muscles, complex organs and neurons (Greenberg *et al.* 1983). It is associated with neurosecretory granules in *M. nimbosa* ganglion homogenates (Nagle 1981) and its release has been shown to be calcium-dependent (Nagle 1982) making it a strong candidate as a neurotransmitter or neuromodulator. FMRFamide or a similar molecule has also been shown to be present in other

invertebrate nervous systems such as *Limulus* (Watson and Groome 1984), the leech *Hirudo* (Kuhlman *et al.* 1985), *Aplysia* (Schaefer *et al.* 1985) and various insects (Walther *et al.* 1984; Carroll *et al.* 1986). In vertebrates FMRFamide-like immunoreactivity is present in goldfish (Stell *et al.* 1984; Muske *et al.* 1987), mice (Boer *et al.* 1980) and rats (O'Donohue *et al.* 1984; Chronwall *et al.* 1984). Within these groups the action of FMRFamide varies, being excitatory in some cells and inhibitory in others (Stone and Mayeri 1981; Painter and Greenberg 1982) and acting on both muscle and nerve preparations (Greenberg *et al.* 1983). Thus the FMRFamide 'family' of neuropeptides has a multitude of roles in a variety of tissues in a wide range of organisms. The actual form of the compound in hydrozoans is unknown but has been suggested to be arginylphenylalanylamide or RFamide (Grimmelikhuijzen 1985). The FMRFamide-like subtance has recently been extracted from two anthozoans and is also a tetrapeptide consisting of pyroglutamate, glycine and the RFamide moiety (Grimmelikhuijzen and Graff 1986; Grimmelikhuijzen and Groeger 1987). This carboxy-terminal arrangement has been shown to be necessary for biological activity and immunoreactivity (Painter *et al.* 1982; Walther *et al.* 1984) and is probably present in all forms of the neuropeptide. As yet, FMRFamide has not been localised to any neuronal ultrastructural components but immunoreactivty of antisera to other neuropeptides such as vasoactive intestinal polypeptide (VIP), neurophysin, vasopressin, substance P and serotonin has been localised in dense-core vesicles, 80-160nm in diameter, in vertebrate systems (Johansson and Lundberg 1981; Pelletier *et al.* 1981; van den Pol 1986) and it has been assumed that this is also their location in invertebrates. Thus it may be possible to tentatively identify

peptidergic neurons by the presence of the characteristic vesicles (Hökfelt *et al.* 1980).

The major objectives of this study are:

i) to determine the extent and nature of FMRFamide-like immunoreactivty (Fa-IR) in selected hydrozoa. If Fa-IR is present in only a part of the nervous system, is this a general feature in all species? If it is, is immunoreactivity restricted to the same components in all species and can these components be correlated with any previously identified systems in hydromedusae thus suggesting a possible role for the putative neurotransmitter? Alternatively, the association of Fa-IR with specific anatomical features may allow inferences as to possible function. Since the present state of our knowledge concerning transmitters in hydrozoans is incomplete and confused, a more comprehensive survey of the presence and distribution of one putative neurotransmitter will help to clarify the situation and may give an indication as to what is or is not representative within the class.

ii) to determine the ultrastructural location of the Fa-IR. In cnidarians the subcellular site of any putative neurotransmitter or neuromodulator has not been determined. Presence of Fa-IR in vesicles would be a strong indication that it is acting as a neurotransmitter. However, attempts at localising Fa-IR at the ultrastructural level using mainly immunogold but also peroxidase anti-peroxidase techniques proved difficult and results were inconclusive.

iii) to undertake an EM survey of the nervous system of a common and little studied Leptomedusan, *Phialidium*. Since immunostaining at the EM level failed, strictly structural evidence may suggest sites for the localisation of Fa-IR. Although *Phialidium* sp. cannot be regarded as being typical of all hydromedusae, an inference as to the subcellular location of Fa-IR and any possible correlations between presence of Fa-IR at the light microscope level and structures observed at the EM level may suggest a role for the putative neurotransmitter.

METHODS AND MATERIALS

Whole mount immunocytochemistry

Specimens of *Phialidium* sp., *Aequorea victoria*, (order Leptomedusae), *Euphysa* sp., *Bougainvillia* sp., *Stomotoca atra*, *Sarsia* sp., *Halitholus* sp., (order Anthomedusae) *Gonionemus vertens*, *Proboscidactyla flavicirrata*, (order Limnomedusae) *Aegina citrea* and *Solmissus* sp., (order Narcomedusae) were collected from the ocean around Oak Bay Marina, Victoria and Friday Harbor, Wash., USA. Animals were identified, at least to genus level, using system of Arai and Brinckmann-Voss (1980). They were maintained in the laboratory in recirculating seawater at 12°C for several days or processed immediately. Individual hydroids from a colony of *Eirene viridula* (order Leptomedusae) sent to me from Heidelberg, Germany by Dr. C.J.P. Grimmelikhuijzen and which I maintained in the lab were also studied. I anaesthetised animals prior to fixation by immersing them in a 1:1 mixture of SW and 0.7M $MgCl_2$ solution for approximately 30 minutes. Anaesthetised animals were then cut into pieces which were pinned out in Sylgard-lined dishes using cactus spines (*Opuntia* sp.). Regions of the bell were oriented either subumbrella or exumbrella uppermost. If possible I included the manubrium in the staining procedure. However in some cases this portion of the animal was too bulky or too strongly pigmented to facilitate successful whole mount immunocytochemistry. Hydroids were immunostained as whole animals. Tissue was fixed for 2.5-5 hours at room temperature in Zamboni's

fixative (Zamboni and DeMartino 1967). The pieces of tissue remained pinned in the same dishes throughout subsequent washes and incubations. Following fixation, tissue was washed in several changes of cold phosphate buffered saline (PBS), pH 7.2, until the yellow colour had been removed, usually overnight. The final PBS rinse was discarded and the tissue incubated in 50-100 µl of the primary antibody solution. This consisted of the stock antisera, anti-FMRFamide (Immunonuclear Corp., Stillwater, Mn., USA) diluted 1:100 in PBS containing 0.35% Triton-X to enhance antibody penetration and 0.03% NaN_3 to prevent bacterial and fungal growth in the solution. Goat serum was added at a concentration of 2% to block non-specific goat IgG binding sites. Controls using antisera preabsorbed with synthetic FMRFamide (100-500 µg FMRFamide/ml antisera) and primary antisera containing no anti-FMRFamide were included in all tests. Positive controls using tissue known to exhibit FMRFamide-like immunoreactivity (Fa-IR), usually *Aequorea victoria* or *Phialidium* sp., were included whenever possible. A roll of PBS-soaked absorbent paper was placed around the edge of the dish to maintain a humid atmosphere during the incubation which was carried out at room temperature for 24-48hrs. The pieces of tissue were then rinsed in PBS 3 times, 15-20 minutes each. The final rinse was discarded and the specimens incubated for 24-48 hrs at room temperature in affinity-purified fluorescein isothiocyanate labelled goat anti-rabbit gamma-globulin (FITC-GARGG; Sigma) diluted 1:20 in the same diluant as for the primary antibody solution. After this incubation the specimens were rinsed in PBS 3 times (15-20 mins. each) and mounted whole in 66% glycerol in distilled water with addition of 1.5% n-propyl-pyrogallate (Sigma) which retards photobleaching (Giloh

and Sedat 1982). To view specimens I used a Zeiss standard microscope equipped with phase contrast optics and with an epifluoresence UV lamp and accessories. For FITC labelled material an excitor filter with a 495 λ maximum transmission peak was used.

Electron Microscopy

Tissue was fixed for the transmission electron microscope (TEM) using 0.5% glutaraldehyde in Millonig's phosphate buffer at room temperature for 1-2 hours, rinsed in the same buffer and postfixed in 1% osmium tetroxide in phosphate buffer at 4°C followed by a distilled water rinse. Tissue was then carried through a graded series of alcohols and propylene oxide prior to embedding in Epon 812. Thick sections (0.5-1 μm) were cut with glass knives and stained with Richardson's stain (Richardson *et al.* 1960). Thin sections were cut with a diamond knife, mounted on grids and stained with uranyl acetate and lead citrate (Venable and Coggesgall 1965). The sections were then viewed with a Philips EM 300 T.E.M.

Tissue was fixed for the scanning electron microscope (SEM) in 0.5% glutaraldehyde as before then taken through a graded series of alcohols (70-100%), critical point dried and gold coated. The tissue was examined using JEOL 35 scanning electron microscope.

RESULTS

IMMUNOCYTOCHEMISTRY

FMRFamide-like immunoreactivity (Fa-IR) was observed in most of the nervous system of the hydroid *Eirene viridula* and in components of the nervous system of all hydromedusae investigated.

Controls using preabsorbed anti-FMRFamide (100-500 µg FMRFamide/ml antisera) reduced or eliminated Fa-IR. When anti-FMRFamide was omitted from the primary antibody solution no immunoreactivity was seen (fig. 21).

Leptomedusae

The leptomedusae studied showed consistent Fa-IR within the nervous system and were frequently used as positive controls. In both *Phialidium* sp. and *Aequorea victoria* the entire system innervating the manubrium, subumbrella and tentacles exhibits Fa-IR (figs. 1-4). However, in both species only a proportion of the total number of elements within the inner nerve ring (INR) and outer nerve ring (ONR) show Fa-IR (figs. 1-4). As in other hydromedusae, the subumbrella nerve plexus in *Phialidium* sp. and *A. victoria* lies within the ectodermal muscle and extends over the entire subumbrella surface. In both species, the plexus is approximately longitudinally oriented and merges with a manubrial plexus and

with elements of the INR (figs. 1f, 3a, b). In *A. victoria* the plexus is very strongly immunoreactive and was frequently visible from the exumbrella side. In both species, cell bodies are scattered throughout the plexus and these vary in diameter from 10 µm in *Phialidium* sp. to 16 µm in *A. victoria*. These are usually bipolar, however in *A. victoria*, additional short processes extend from some cell bodies. These appear to end blindly on the subumbrella muscle sheets and do not seem to be associated with any distinct structure (fig. 3a, b). In both species immunoreactive processes frequently branch to form separate elements or combine to form bundles (figs. 1f, 3a, b). The subumbrella plexus merges with the manubrial plexus (fig. 1a). In *A. victoria* further detail is obscured by autofluoresence.

In *Phialidium* sp. neurites extend down the manubrium and terminate in oval sensory cells along the edge of the manubrial lips (fig. 1b, c). Each Fa-IR sensory cell possesses a sensory process (1-2 µm). When the manubrial lips are viewed under phase contrast it appears that not all sensory cells possessing processes show Fa-IR. Around the margin of the bell, Fa-IR was restricted to approximately 5 neurites in the INR of *Phialidium* sp. (fig. 1h) and fewer than 8 in the ONR (fig. 1d). In *A. victoria* strong autofluoresence around the margin masks Fa-IR in the two nerve rings which also overlie each other. However there appear to be fewer than 5 distinct neurites in the ONR (fig. 3c, d). In both species, cell bodies of varied size and shape are present at irregular intervals around the nerve rings. In *Phialidium* sp. pear-shaped sensory cells are present at regular intervals along the margin between tentacles (fig. 1e). Neurites extend from these cells and appear to connect with the ONR although this is not clear. Similar cells are seen along

the outer edge of the tentacle bulb (fig. 1g, i). In both species a tentacle plexus extends from the ONR down the tentacle ectoderm. Many sensory cells extend out from this plexus along the length of the tentacle. These are bottle or pear-shaped and 10-15 µm long in *Phialidium* sp. and are oriented perpendicular to the axis of the tentacle (fig. 1j, k). Sensory hairs (1-2 µm) can be seen extending from some cells. In *A. victoria* cells tend to be round (10 µm diameter) with short sensory processes (1-2 µm) extending beyond the ectoderm (fig. 3e). No Fa-IR was observed in the velum of either species.

Fa-IR in the hydroid of the Leptomedusan *Eirene viridula* seems to be present in most of the ectodermal nerve net (figs. 5 and 6). Many strongly immunoreactive sensory cells are present around the hypostome (fig. 5a-c). An immunoreactive nerve plexus extends down the length of the hydroid (fig. 5) and disappears towards the base. There is no Fa-IR in the stolons. At the region where the tentacles join the stalk 8-15 large cells (11-32 µm in diameter) occur at regular intervals around the hydroid stem, either singly or in groups of 2 or 3 (fig. 5e, f). Fanning out from the top of these cells (which I named fan cells) are many neurites. These extend longitudinally towards the hypostome and up the tentacles. By contrast, few neurites (2-5) lead into the fan cells from below and there are no Fa-IR neurites between the fan cells. The function of these fan cells is unknown.

Anthomedusae

Patterns of Fa-IR varied between components of the nervous system in anthomedusae and between different species (figs. 7-15). Fa-IR in the nerve rings varied considerably. In *Euphysa* sp. and *Halitholus* sp. the ONR is not FMRFamide positive although up to six neurites in the INR are FMRFamide positive in both species (figs. 7a, 14b). In *Halitholus* sp. the immunoreactive fibres are divided into 2 distinct portions, consisting of 5-10 neurites each, which run directly above and below the ring canal. Neurites extend over the canal to connect the 2 portions at many locations (fig. 14b). By contrast, no Fa-IR was observed in the INR of *Bougainvillia* sp. but many elements in the ONR are strongly immunoreactive (fig. 10a). Neurites extend out from the ONR across the tentacle pad and down the tentacles to form the tentacle plexus. There does not appear to be any distinct Fa-IR in neurites innervating the ocelli, which are present at the base of each tentacle in *Bougainvillia* sp., *Halitholus* sp. and *Sarsia* sp. Ocelli are not present in any of the other species I studied.

Both the INR and ONR of *Sarsia* sp. and *S. atra* contain FMRFamide positive fibres. In *Sarsia* sp. there are about ten FMRFamide positive fibres in total and these seem to be distributed approximately equally between the two rings (fig. 8b) although it was not always possible to definitively identify each nerve ring since they lie close together above each other. A similar total number of immunoreactive processes are present in *S. atra* but a higher proportion are located in the ONR (fig. 12d). Many neurites extend out from the ONR in *S. atra* to innervate the marginal protrusions or tentacle buds (fig. 12b, d).

No Fa-IR was observed in the velum of any of the anthomedusae studied, in contrast to *Polyorchis penicillatus* (Grimmelikhuijzen and Spencer 1984), and all except *Sarsia* sp. showed similar Fa-IR in the subumbrella nerves which lie within the the radial muscle band overlying the radial canal. Some hydromedusae such as *S. atra* possess endodermal nerves in association with the canal system (Mackie and Singla 1975) but these were not immunoreactive. Fa-IR was not present on the subumbrella surface of *Sarsia* sp. (excepting the INR and manubrial plexus). In the other anthomedusae a FMRFamide-positive plexus extends from the INR up the radial canal (figs. 7b, 10b, 12e, 14c, e) and merges with the manubrial plexus which exhibits Fa-IR in all the species I studied. Cell bodies are scattered throughout the radial plexus but in *Bougainvillia* sp. they have a very characteristic appearance (fig. 10b, e) being star-shaped or multipolar and 20-25 µm in width. They are located at regular intervals along each edge of the radial canal and send short (40 µm) neurites out onto the subumbrella (fig. 10b, e). One neurite was observed which extended 1.4mm from a cell body onto the subumbrella but this seemed to be an exceptionally long process. As viewed with phase contrast microscopy these extensions did not seem to be associated with sensory cells on the subumbrella surface. In *Halitholus* sp. fibres extend out from the radial plexus onto the subumbrella for distances of up to 500 µm at irregular intervals up the radial canal and do not appear to be associated with any specialisations such as sensory cells.

In other anthomedusan species Fa-IR on the subumbrella was restricted to the radial plexus. This merges with the manubrial plexus which extends down the manubrium and terminates in clusters of sensory cells around the mouth. Many of

the cells were strongly FMRFamide-immunoreactive in all species I studied (figs. 8c, d, e, g, 10c, d, 12f, g, 14f, g). The manubrium of *Euphysa* sp. is covered with highly coloured and autofluorescent gonad tissue masking potential Fa-IR in the manubrial plexus but it seems likely that it is the same as in other anthomedusae. In all the anthomedusae studied the plexus innervating the tentacles was Fa-immunoreactive (figs. 8a, 10a, 12a, c, 14a). Cell bodies are scattered along it at intervals in all species but, in contrast to the leptomedusae, no distinct Fa-immunoreactive sensory cells were observed. No Fa-IR was seen in the tentacle bulbs of *Sarsia* sp., *Halitholus* sp. or *Euphysa* sp. although the thickness of the tissue may obscure detail of whether the tentacle plexus is weakly Fa-immunoreactive at this point (fig. 8f). Strong Fa-IR would probably be visible.

Limnomedusae

Fa-IR in the two limnomedusae I studied, *Gonionemus vertens* and *Proboscidactyla flavicirrata* , was similar although the 'layout' of the nervous system of each is different (figs. 16-19).

As in other medusae, Fa-IR was restricted to fewer than 5 neurites in the INR of both species (fig. 16a) and the ONR of *P. flavicirrata*. Strong pigmentation in the margin of *G. vertens* made it impossible to determine whether Fa-IR was present or not. Small sensory cells are present around the margin in *G. vertens* and appear to send projections into the INR although they are not clear (fig. 16a). In both species Fa-immunoreactive neurites extend from the INR up the ectodermal muscle sheets overlying the radial canals (figs. 16b, c, 18c). Cell bodies are present throughout the radial plexus in both species. Only in *G. vertens*

are there extensions from the radial canal out onto the subumbrella (fig. 16e, f) and these become more numerous as the radial canal nears the stomach. Neurites extend at right angles to the radial canal and cover the interradial surfaces merging with neurites from the neighbouring radial plexus. Cell bodies are occasionally present within the interradial plexus. Fa-IR is present in the manubrial plexus of both species although pigmentation in *G. vertens* obscures much detail. Fa-IR sensory cells are scattered over the manubrium of *G. vertens* (fig. 16d) however there was not the high density of Fa-IR sensory cells lining the manubrial lips that I observed in other orders and in *P. flavicirrata* where flask-shaped cells, similar in appearance to those in *Phialidium* sp., were densely packed along the manubrial lips (fig. 18a, b). *P. flavicirrata* also possesses clusters of nematocysts and sensory neurons on the exumbrella, known as cnidothylaces. Fa-IR is present in up 6 neurites in each cnidothylax (fig. 18d, e). Cell bodies, some with sensory processes, are also present along the cnidothylax stem. At the margin, neurites join the ONR merging with Fa-immunoreactive neurites in the ring. Neurites also extend out from the ONR onto the velum. In *G. vertens* Fa-IR neurites were present in the velum but I could not trace any connections to either nerve ring.

Narcomedusae

This order of hydromedusae comprises a group of oceanic, deep sea jellyfish which are usually fragile and easily damaged. They are not as well known as the previously mentioned orders and possess several structural differences which are worth noting. The tentacles leave the umbrella, which has a lobed appearance,

above the margin and are generally held upright. From the base of each tentacle deep clefts known as peronia run vertically down to the margin (see fig. 20). In *Aegina citrea* the peripheral canal system forms four loops each consisting of a canal running down one side of a peronium along the margin and up the side of the next peronium (Russell 1953). *Solmissus* sp. does not possess a peripheral canal system. The nerve rings run around the margin in both genera as in other hydromedusae.

The delicate nature of narcomedusan tissue makes successful immunocytochemistry difficult and the photographs I obtained are difficult to interpret individually. It was possible however to reconstruct a three-dimensional image of the Fa-IR in *Aegina citrea* and this is shown in fig. 20.

Fa-IR was evident in the tentacles of *Solmissus* sp. and in the tentacles and umbrella of *A. citrea* (fig. 20). Fa-IR cell bodies were scattered along the length of the tentacle plexus in *A. citrea* but I did not see any distinct Fa-IR sensory cells in either genus. The umbrella of *Solmissus* sp. is large (up to 60mm diameter) and thick making further whole mount immunocytochemistry impossible. The Fa-IR in *A. citrea* extended from the tentacle plexus onto the exumbrella and down either side of each peronium. Towards the margin the Fa-IR disappeared and I saw no immunoreactive elements along the margin edge on either side although there was substantial autofluoresence. Above the tentacle base on the subumbrella side a dense plexus extends over the stomach wall oriented radially to the mouth. Cell bodies were small and infrequently present and there were no Fa-IR sensory cells present around the mouth.

Short isolated Fa-IR neurites were observed in the velum of *A. citrea*.

Table 1 summarizes the distribution of Fa-IR in all hydromedusae investigated to date.

Key; +=Fa-IR present; -=Fa-IR absent; ?=unknown; (+)=Fa-IR present but detail obscured.

Man.=manubrium, Sub.=subumbrella, Tent.=tentacle

*=Grimmelikhuijzen and Spencer 1984

**=Mackie *et al.* 1985

Table 1: Summary of the distribution of Fa-IR in hydromedusae.

	INR.	ONR.	Man.	Sub.	Tent.	Velum
Leptomedusae						
Phialidium sp.	+	+	+	+	+	-
Aequorea victoria.	+	+	(+)	+	+	-
Anthomedusae						
Euphysa sp.	+	-	(+)	+	+	-
Halitholus sp.	+	-	+	+	+	-
Sarsia sp.	+	+	+	-	+	-
Bougainvillia sp.	-	+	+	+	+	-
Stomotoca atra.	+	+	+	+	+	-
*Polyorchis penicillatus.**	+	+	+	+	+	+
Limnomedusae						
Gonionemus vertens.	+	?	+	+	+	+
Proboscidactyla flavicirrata.	+	+	+	+	+	+
Trachymedusae						
*Aglantha digitale.***	+	+	+	+	+	-
Narcomedusae						
Aegina citrea.	?	?	+	+	+	+
Solmissus sp.	?	?	?	?	+	?

Key to symbols on previous page

ELECTRON MICROSCOPY

Sections of the bell margin, the umbrella and the tentacles of *Phialidium* sp. were studied using the TEM and SEM. The manubrium of *Phialidium* sp. is very delicate and insubstantial and the nerves tend to be very small and indistinct in sections so I looked at sections of *Bougainvillia* sp. to obtain a clearer impression of the innervation of the hydromedusan manubrium.

At the margin of the bell in all hydromedusae, the mesoglea of the velum meets the mesoglea of the subumbrella and exumbrella forming a distinctive triradius (fig. 22). On the subumbrella side of this lies the INR and on the exumbrella side lies the ONR (fig. 23). Connections between the two rings are common and small axon profiles are occasionally seen in the endoderm. The INR contains some large diameter cells (up to 9 μm) which are presumably the motor giants (Spencer and Schwab 1983). It also consists of many intermediate and smaller sized axon profiles, up to 5 μm in diameter (fig. 23A). In the region of the cell body an axon profile may be very large relative to other profiles thus axon diameter may not be a very good indication of function. Axo-axonal synapses are common in the INR (fig. 24A) and were all polarized. Dense-cored vesicles are present at these synapses and in approximately 50% of all profiles. They may occur either singly or in small groups but occasionally very dense accumulations are present (fig. 24B). The dense-cored vesicles range in diameter from 60-125nm. Clear vesicles of varying size are also present.

The ONR forms a ridge that runs around the outer margin of the bell (fig. 25A). It consists of a large number of small diameter (0.1-2 μm) axon profiles (fig. 23B). Dense-cored vesicles, 70-145nm in diameter, are present in many axon

profiles usually in small numbers but occasionally, as in the INR, in large accumulations (fig. 25B). These large accumulations may be representative of a section through or close to an axon terminal. Axo-axonal synapses are present.

The frequency distribution of dense-core vesicle sizes in the two nerve rings is shown in fig. 26. Although the range of vesicle sizes in the two nerve rings is similar the distributions are significantly different (Kolmogorov-Smirnov 2-sample test; $D=0.289$, $D_{.005}=0.208$, $p<0.05$; Sokal and Rohlf 1981). Though significant, the difference is small and can not be correlated with any difference in vesicle content in this study. However, if vesicle size is correlated with content (as has been suggested by many authors e.g. Hökfelt et al.1980) then it may be useful to pursue this sort of analysis to determine the relative distribution of neurotransmitters or neuropeptides in the nervous system of coelenterates. As yet this has not been attempted.

The velum of *Phialidium* sp. consists of two layers of musculoepithelial ectodermal cells separated by the velar mesoglea. I did not see any axon profiles on either side of the velum.

The main portion of the umbrella consists of the exumbrella ectoderm, a thin layer of vacuolated epithelial cells, the subumbrella endoderm which has a similar appearance and the subumbrella ectoderm which contains musculoepithelial cells and nerve bundles (fig. 27). The musculoepithelial cells contain blocks of circular striated muscle which lie at the base of each cell next to the mesoglea (fig. 27A, C). Overlying these cells is a more loosely arranged layer of cells which contain smooth radial muscle which is often very diffuse in appearance and difficult to distinguish. Nerve profiles are generally found between these two layers and

contain dense-core vesicles (60-120nm in diameter). These may be present singly (fig. 27B) or in clusters (fig. 27C). Nerve cells are usually found lying next to musculoepithelial cells but I did not see any synapses.

In *Phialidium* sp. tentacles are distributed around the margin at regular intervals and each extends from a tentacle bulb (fig. 28A). The tentacle bulb is a region of thickened ectoderm consisting of many epithelial cells which are electron-dense and densely packed with rough endoplasmic reticulum. This is expected since the tentacle bulb is a region of nematocyst formation (Westfall 1966) and thus active in protein synthesis. The tentacle ectoderm contains many nematocysts (fig. 28B) which have a cnidocil apparatus characteristic of hydrozoa. The cnidocil consists of a central flagellum with a dense core of microfilaments and is surrounded by nine stereocilia (see insets fig. 28). The whole apparatus is seated within a depression in the nematoblast. The nematocysts are present in a considerably higher density than sensory cells. Each sensory cell possesses a sensory hair or flagellum (1 μm in length) and is usually long, extending from the tentacle surface and tapering towards the mesoglea. Near the mesogloea small profiles of axons containing dense-core vesicles are often present lying next to the putative neurosensory cell (fig. 29A). Axon profiles may occur on their own or in small bundles (fig. 29B-D). No synapses were seen.

The manubrium of *Bougainvillia* sp. has a large electron-dense ectoderm. The endoderm consists of large vacuolated cells separated by endodermal spaces (fig. 30). Musculoepithelial cells containing large single blocks of circular striated muscle form an ectodermal layer which lies next to the mesoglea. Nematocysts are present in high density in the ectoderm and have the characteristic cnidocil

apparatus (fig. 30B). Sensory cells are also present (fig. 30A) and possess sensory hairs, 1.5-2 μm in length. Between the musculoepithelial cells, axon profiles can be seen (fig. 30C-D). These are generally small (0.2-0.7 μm) and sometimes contain dense-core vesicles (45-100nm) but I did not see any synapses.

Figure 1: Fa-IR in *Phialidium* sp.

a) Radially oriented manubrial plexus (scale: 100 µm).

b) Sensory cells along manubrial lips (scale: 50 µm).

c) Sensory cells with processes at edge of lips (scale: 20 µm).

d) Neurites within outer nerve ring (scale: 50 µm).

e) Subumbrella plexus and inner nerve ring. Marginal sensory cells (arrows) are present at regular intervals (scale: 100 µm).

f) Subumbrella nerve plexus (scale: 100 µm).

g) Tentacle bulb. Note no staining in velum (asterisk; scale: 100 µm).

h) Neurites within inner nerve ring (scale: 25 µm).

i) Sensory cells along edge of tentacle bulb (scale: 50 µm).

j) Tentacle nerve plexus and sensory cells (scale: 50 µm).

k) Cross-section through tentacle showing plexus lying within ectoderm (ec) and sensory cells oriented perpendicular to tentacle axis (en: endoderm; scale: 50 µm).

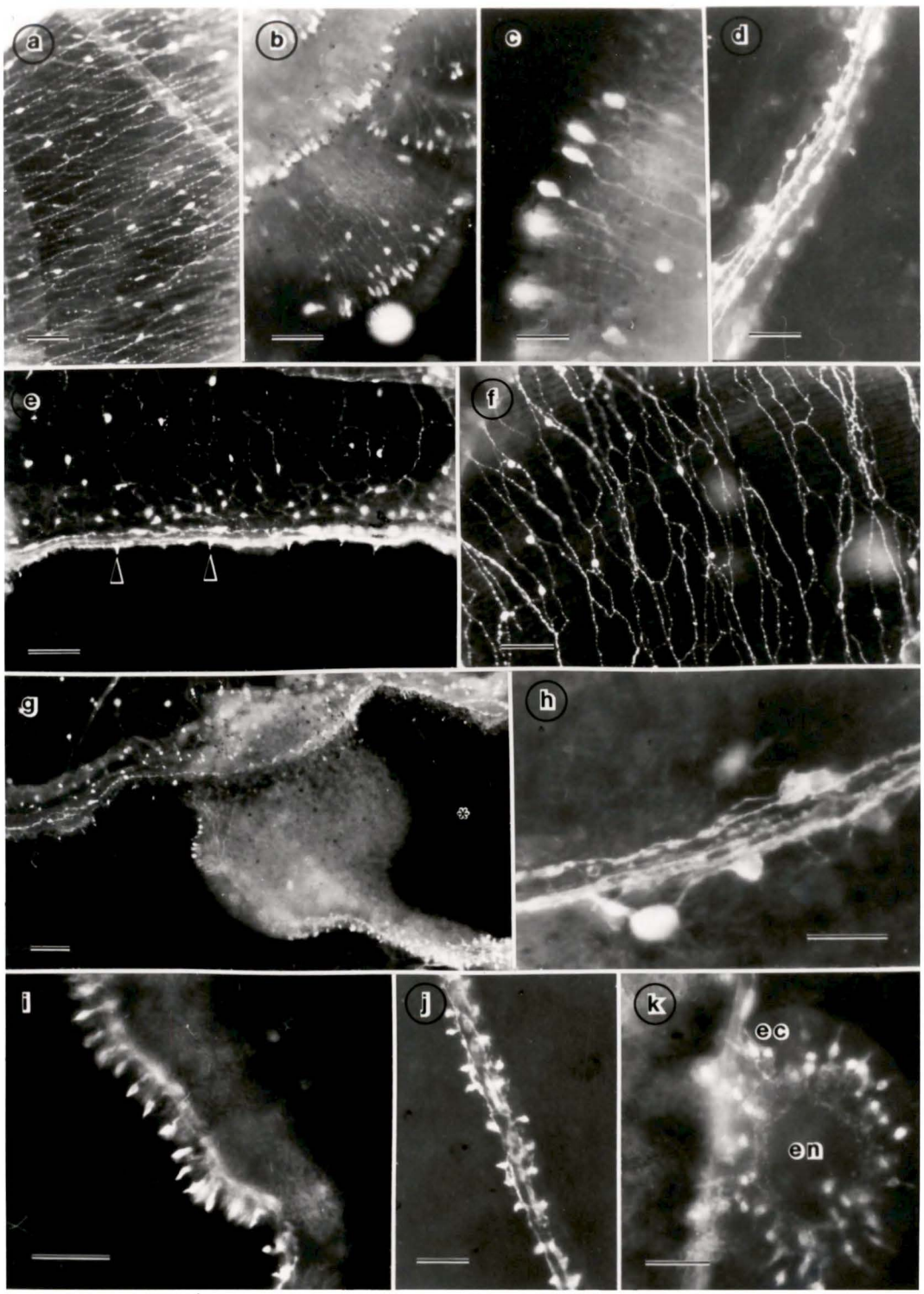

a
b
c
d
e
f
g
h
i
j
k
ec
en

Figure 2: Drawing showing the distribution of Fa-IR in *Phialidium* sp.

In this and the similar drawings of other medusae, the structures have been simplified and the proportions altered in order to allow a better representation of immunoreactive neurite distribution. For more realistic drawings and photographs of hydromedusae, see Arai and Brinckmann-Voss 1980.

An immunoreactive plexus extends across the subumbrella, over the manubrium and down the tentacles. Several elements within the inner and outer nerve rings show Fa-IR. Sensory cells are present around the lips of the manubrium, along the edges of the tentacle bulbs and along the rim of the bell between tentacles.

INR=inner nerve ring, ONR=outer nerve ring.

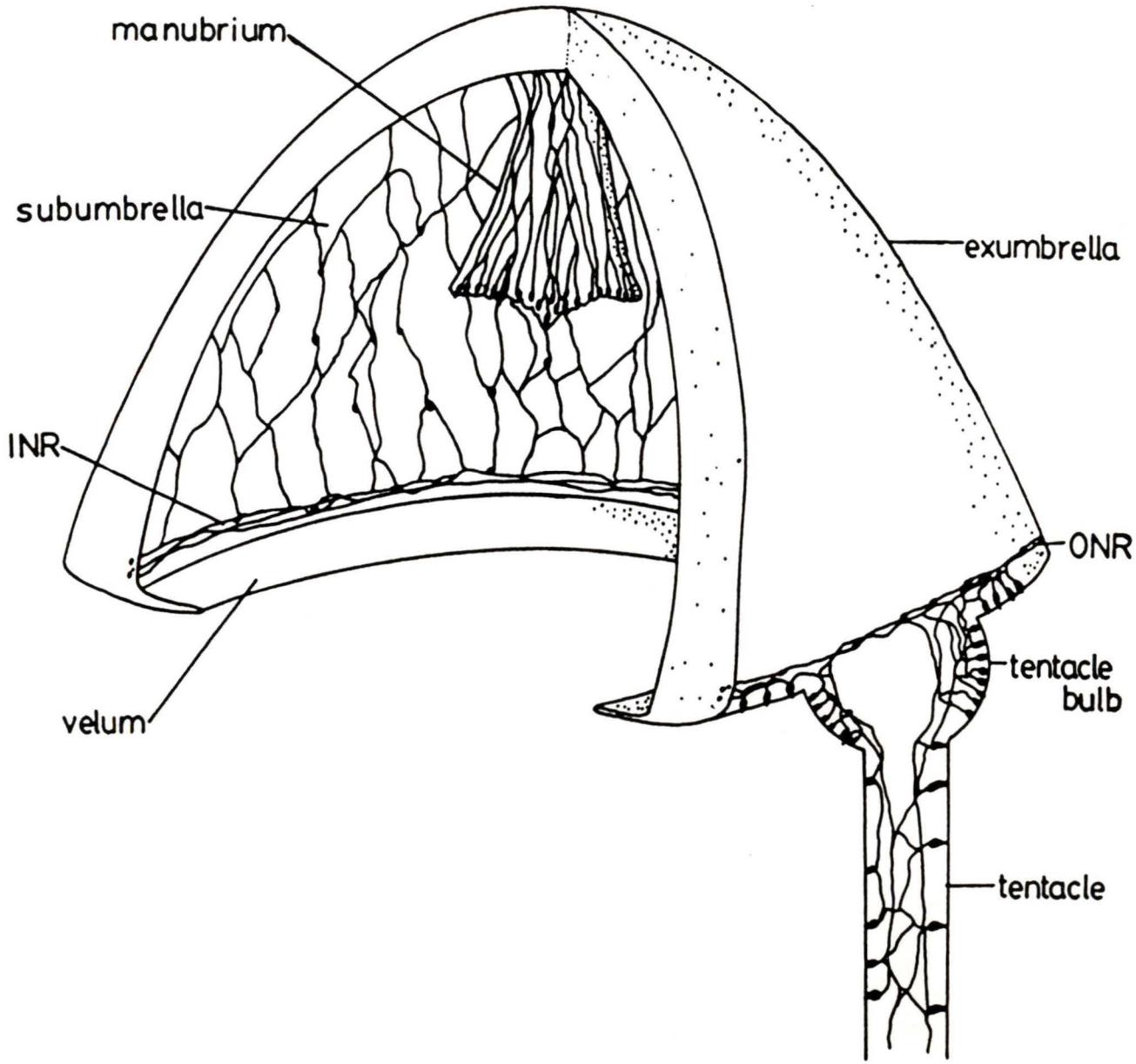

manubrium
subumbrella
exumbrella
INR
ONR
tentacle bulb
velum
tentacle

Figure 3: Fa-IR in *Aequorea victoria*

a) Subumbrella nerve plexus showing blind endings (arrows; scale: 25 µm).

b) Subumbrella nerve plexus. Individual elements combine to form bundles which often divide again (scale: 25 µm).

c) Neurites within the outer nerve ring (scale:25 µm).

d) Neurites extend from the outer nerve ring (ONR) to tentacle bulb (scale: 20 µm).

e) Sensory cells with processes (arrows) in tentacle (scale: 25 µm).

f) Tentacular nerve plexus (scale: 25 µm).

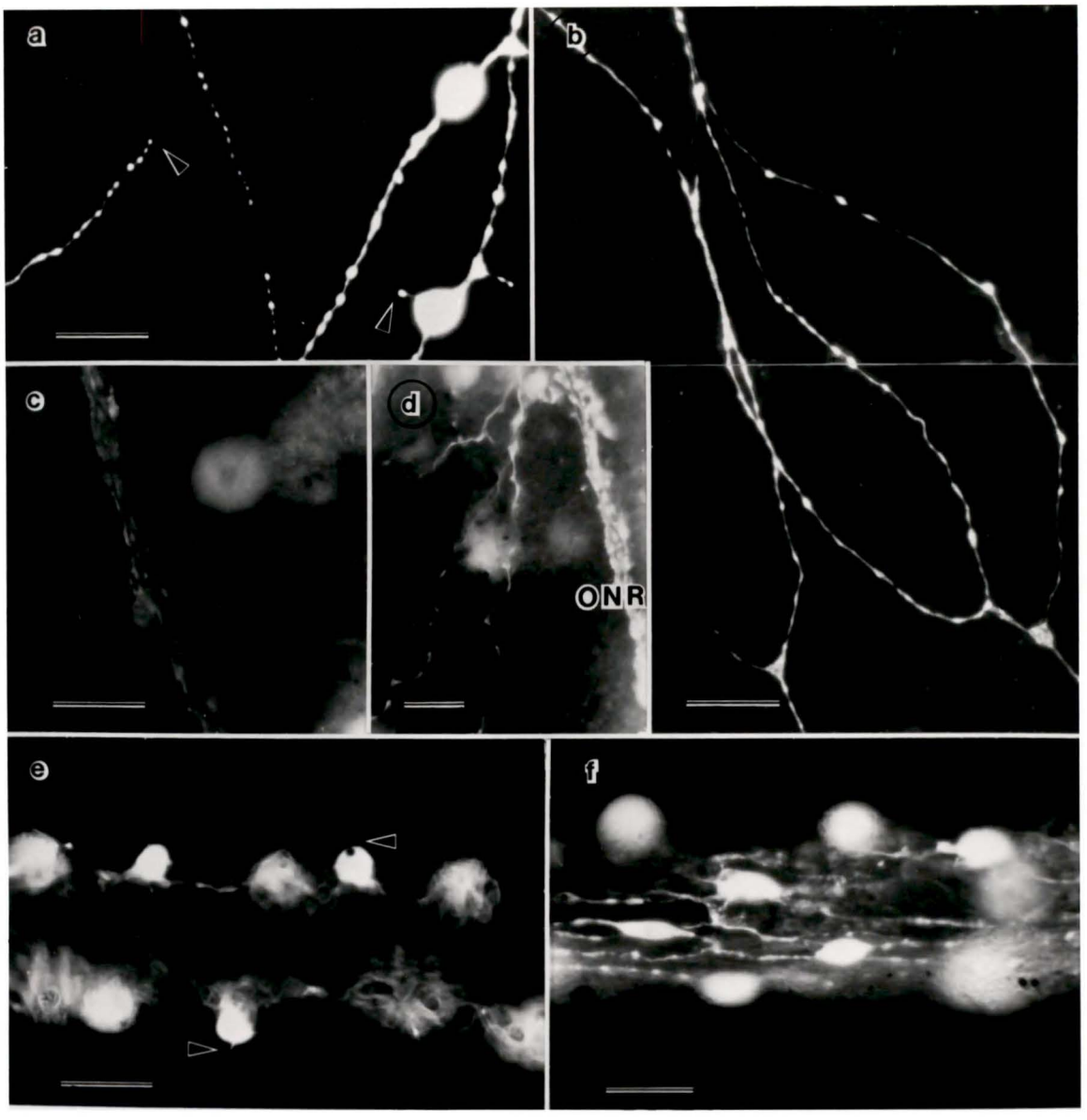

a
b
c
d
ONR
e
f

Figure 4: Drawing showing the distribution of Fa-IR in *Aequorea victoria*

The subumbrella plexus and tentacle plexus are both immunoreactive. Sensory cells showing Fa-IR are seen in the tentacles and many elements in the subumbrella plexus have blind endings onto the muscle sheet. There are several immunoreactive elements in the outer nerve ring but staining (if present) is obscured in the inner nerve ring.

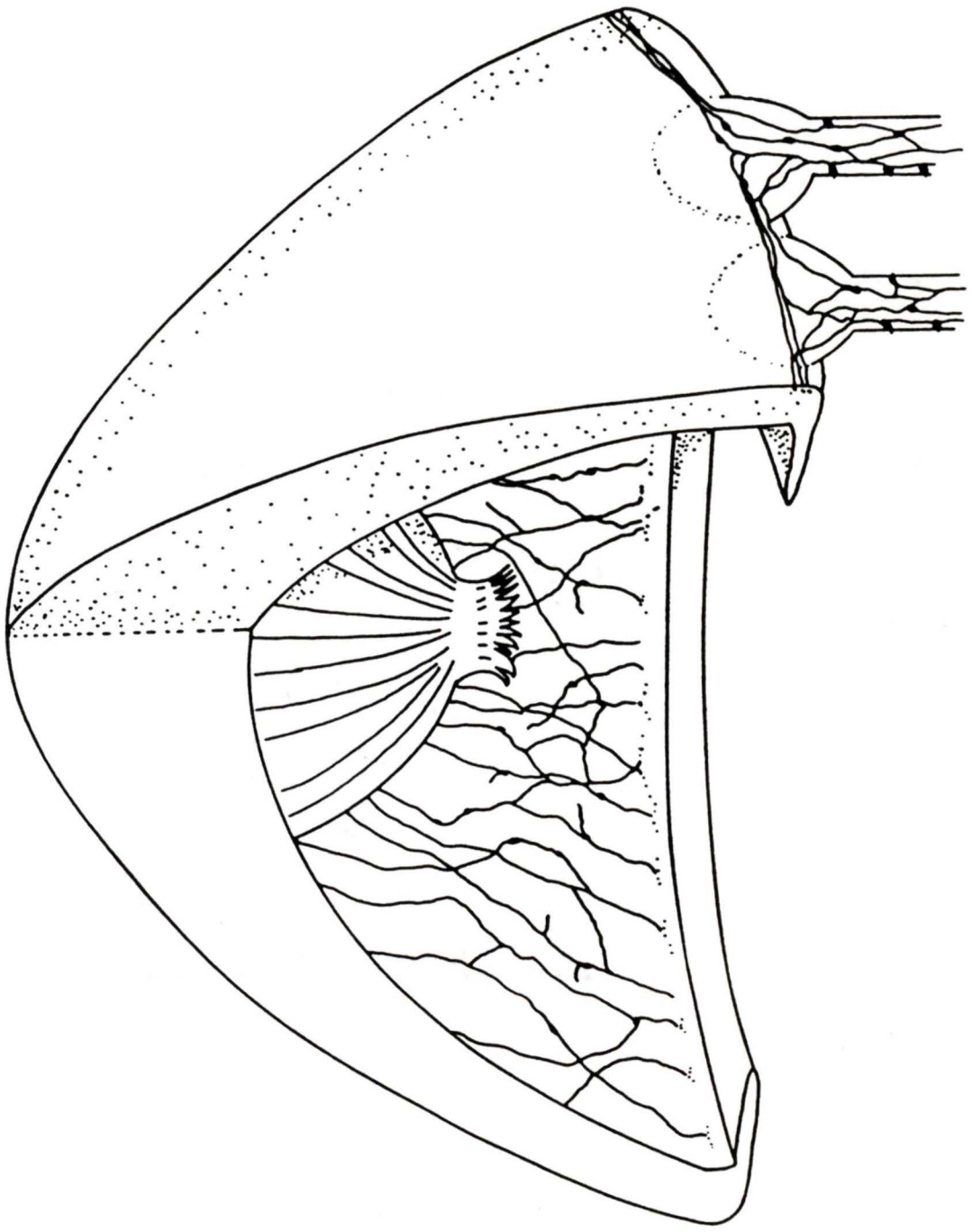


Figure 5: Fa-IR in *Eirene viridula*

a) Nerve plexus within tentacles (t) and hypostome (h), (scale: 100 µm).

b) Sensory cells concentrated around hypostome (scale: 20 µm).

c) Sensory cells around hypostome (scale: 20 µm).

d) Nerve plexus within hydroid stalk (scale: 50 µm).

e) Fan cells (arrows) within stalk. Neurites fan out towards hypostome (h). (scale: 100 µm).

f) Detail of fan cells (scale: 20 µm).

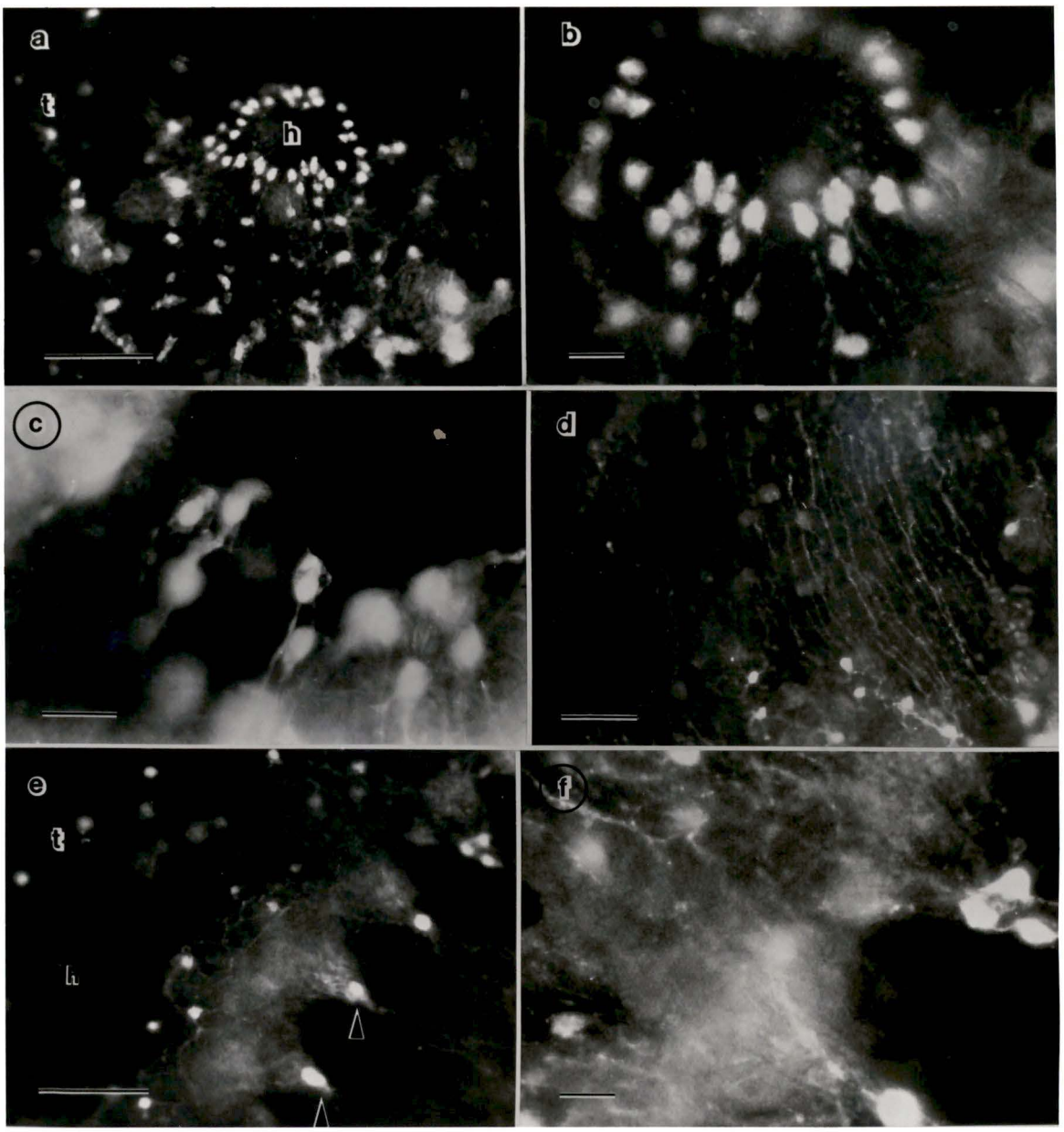

a
t
h
b
c
d
e
t
h
f

Figure 6: Drawing showing the distribution of Fa-IR in *Eirene viridula*

Structures have been simplified and proportions altered for simplicity.

Fa-IR is present in the ectoderm of both the tentacles and the main body of the animal. There is a concentration of immunoreactive sensory cells around the hypostome. Fan cells (either singly or in small groups) are present distributed around the main body of the animal near the tenatcle bases.

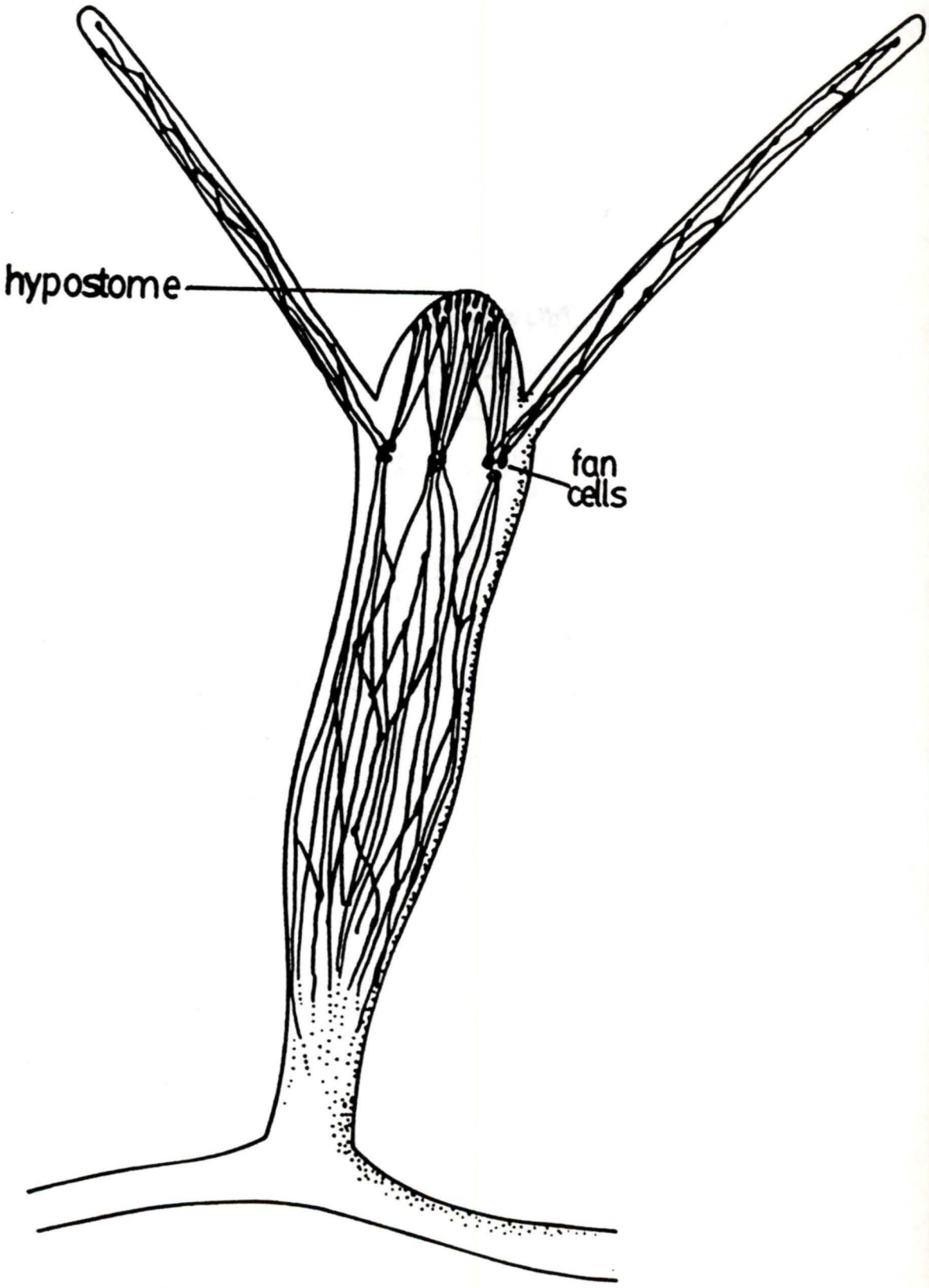

hypostome
fan
cells

Figure 7: Fa-IR in *Euphysa* sp

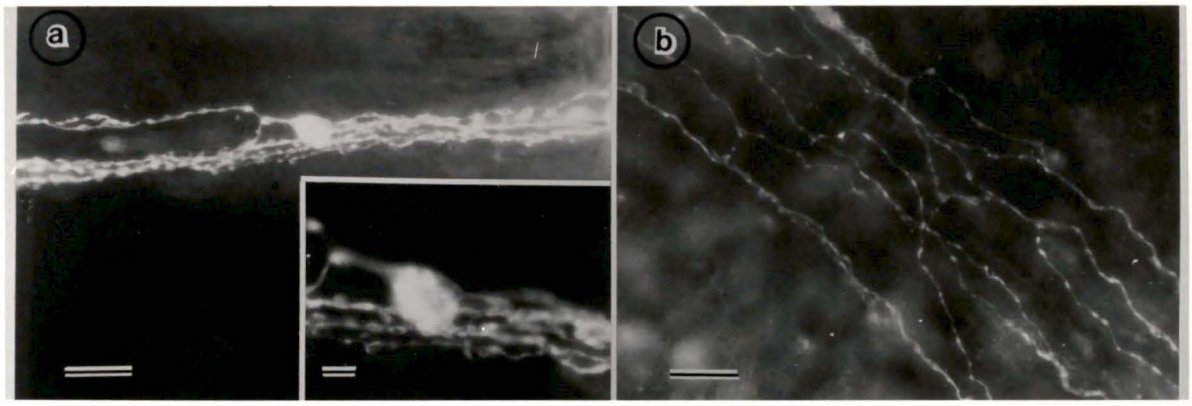
A). a) Neurites within inner nerve ring (scale: 100 µm). Inset: higher magnification of cell body (scale: 100 µm).

b) Nerve plexus overlying radial canal (scale:100 µm).

B) Drawing showing Fa-IR in *Euphysa* sp.

An immunoreactive plexus overlies each radial canal. Some elements connect with immunoreactive neurites in the inner nerve ring. There is no Fa-IR in the outer nerve ring but an immunoreactive tentacle plexus is present. Gonad tissue obscures staining (if present) in the manubrium but a few immunoreactive sensory cells can be seen around the manubrial opening.

A



B

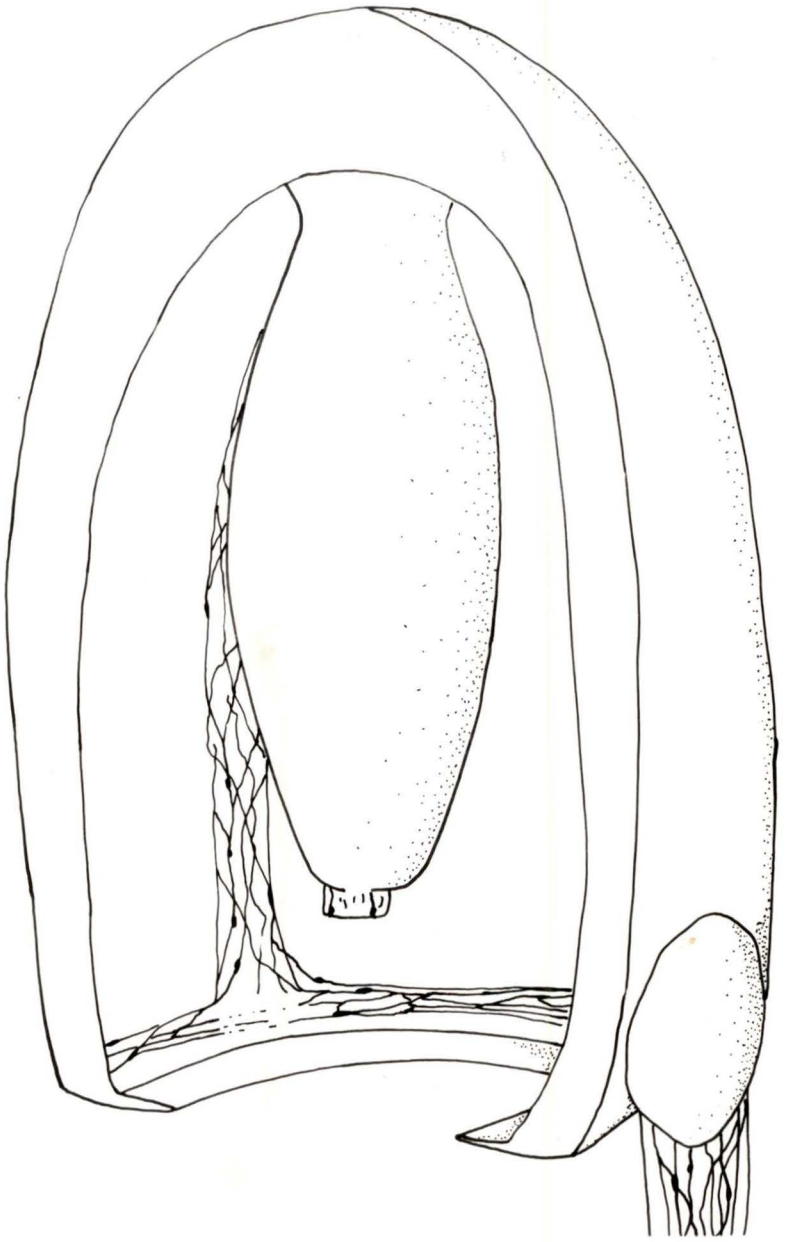


Figure 8: Fa-IR in *Sarsia* sp.

a) Two strongly immunoreactive neurites in the tentacle (scale: 1mm)

b) Neurites within the inner nerve ring (scale: 20 µm).

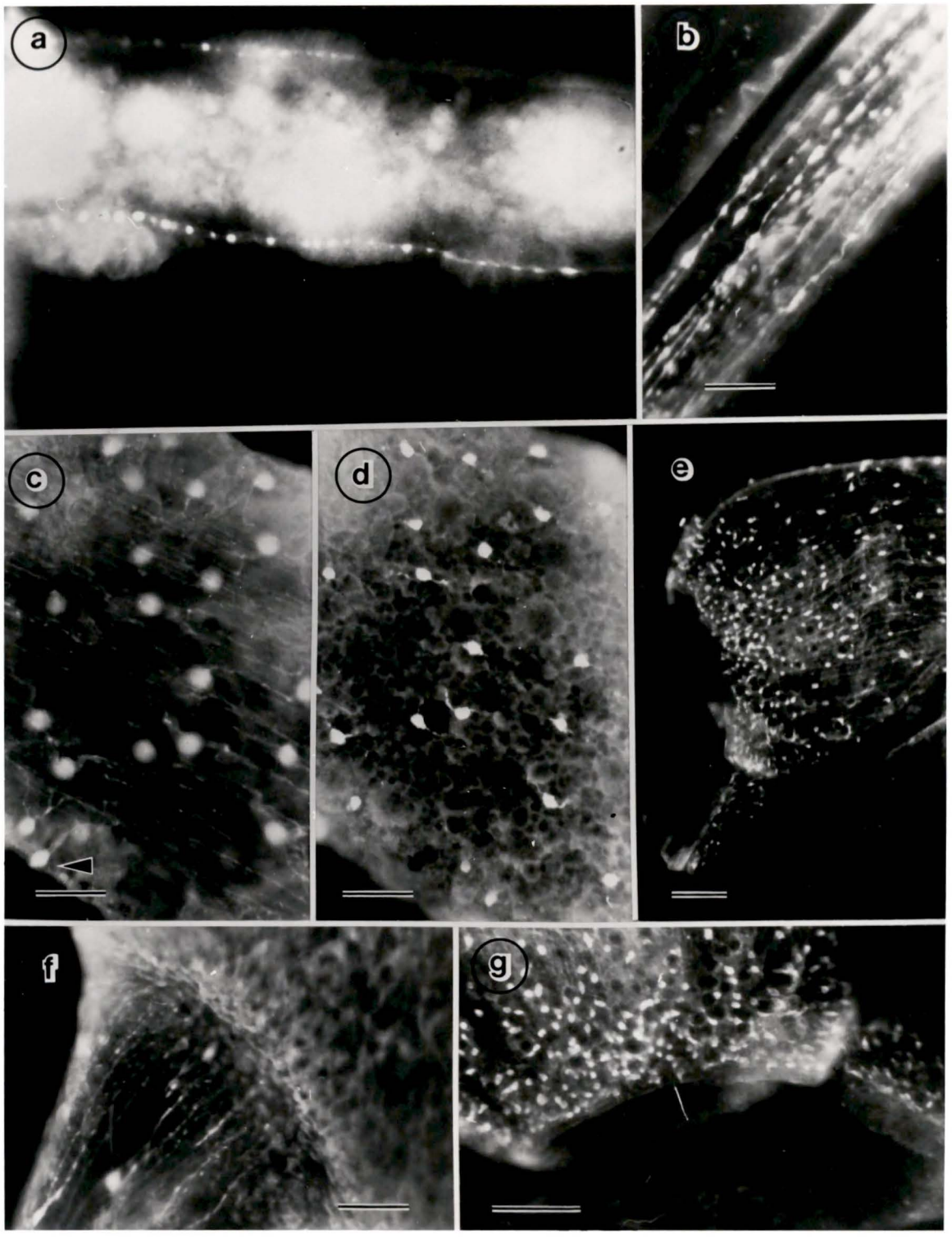
c) Manubrial nerve plexus. Note sensory cell close to surface (arrows; scale: 50 µm).

d) Same view as in c) but at a different level of focus showing only cell bodies (scale: 50 µm).

e) Higher density of sensory cells around opening of manubrium (scale: 100 µm).

f) Tentacle plexus obscured in region of ocellus (scale: 50 µm).

g) Higher magnification of manubrial opening (scale: 100 µm).


a
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g

Figure 9: Drawing showing the distribution of Fa-IR in *Sarsia* sp.

Fa-IR is present in some elements of the inner and outer nerve rings but not in the nerve plexus which overlies each radial canal. An immunoreactive plexus is present in both the manubrium and the tentacles. Immunoreactive sensory cells are present in the manubrium. The tentacle plexus disappears as it enters the tentacle bulb which is an area of thick tissue and thus staining (if present) may be obscured.

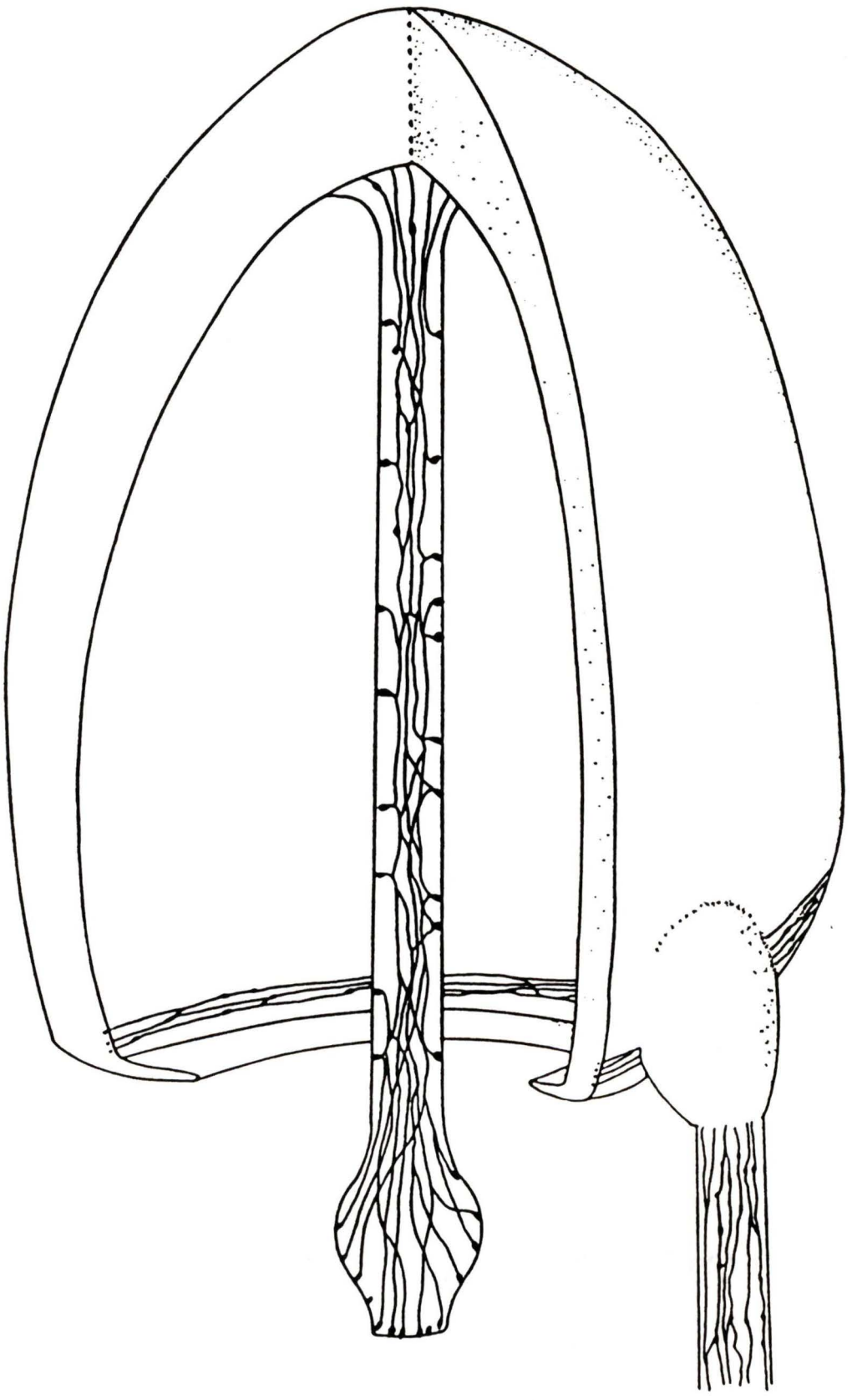


Figure 10: Fa-IR in *Bougainvillia* sp.

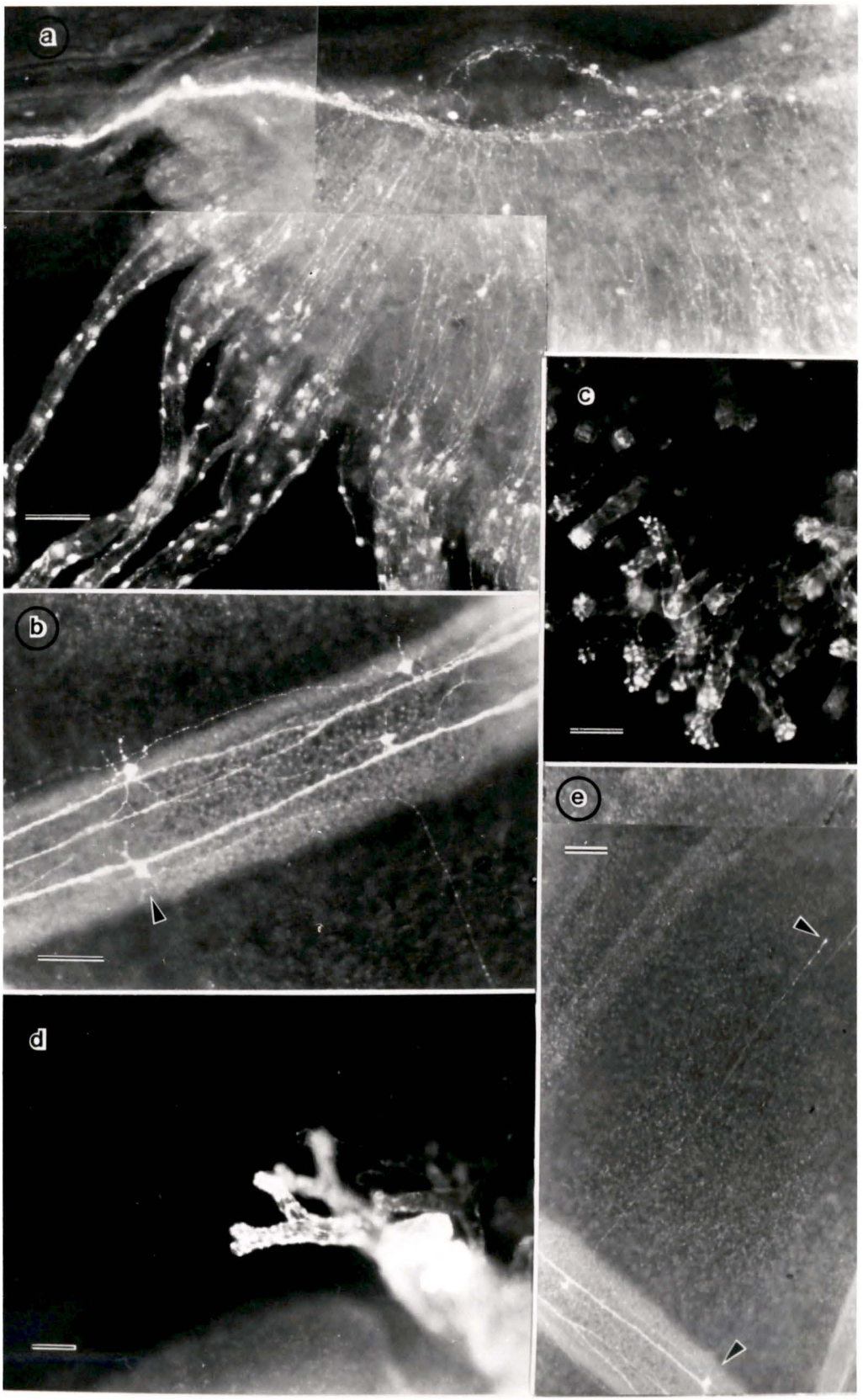
a) Tentacle pad showing nerve plexus and sensory cells in tentacle and neurites within the outer nerve ring (scale: 100 µm).

b) Plexus overlying radial canal. Multipolar cells (arrow) send processes onto the subumbrella which end blindly (scale: 100 µm).

c) Sensory cells within oral tentacles (scale: 250 µm).

d) Nerve plexus and sensory cells in oral tentacles (scale: 100 µm).

e) Single neurite extending from plexus overlying radial canal onto subumbrella (between arrows; scale: 100 µm).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d
e

Figure 11: Drawing showing the distribution of Fa-IR in *Bougainvillia* sp.

Fa-IR is present in a few elements of the outer nerve ring, in the plexus which overlies each radial canal and in the manubrium. A few elements extend from the radial canal plexus into the region of the inner nerve ring and also out from the plexus onto the interradial subumbrella muscle sheet. Fa-IR is present in the tentacles but not in the nerves innervating the ocelli in the tentacle pad.

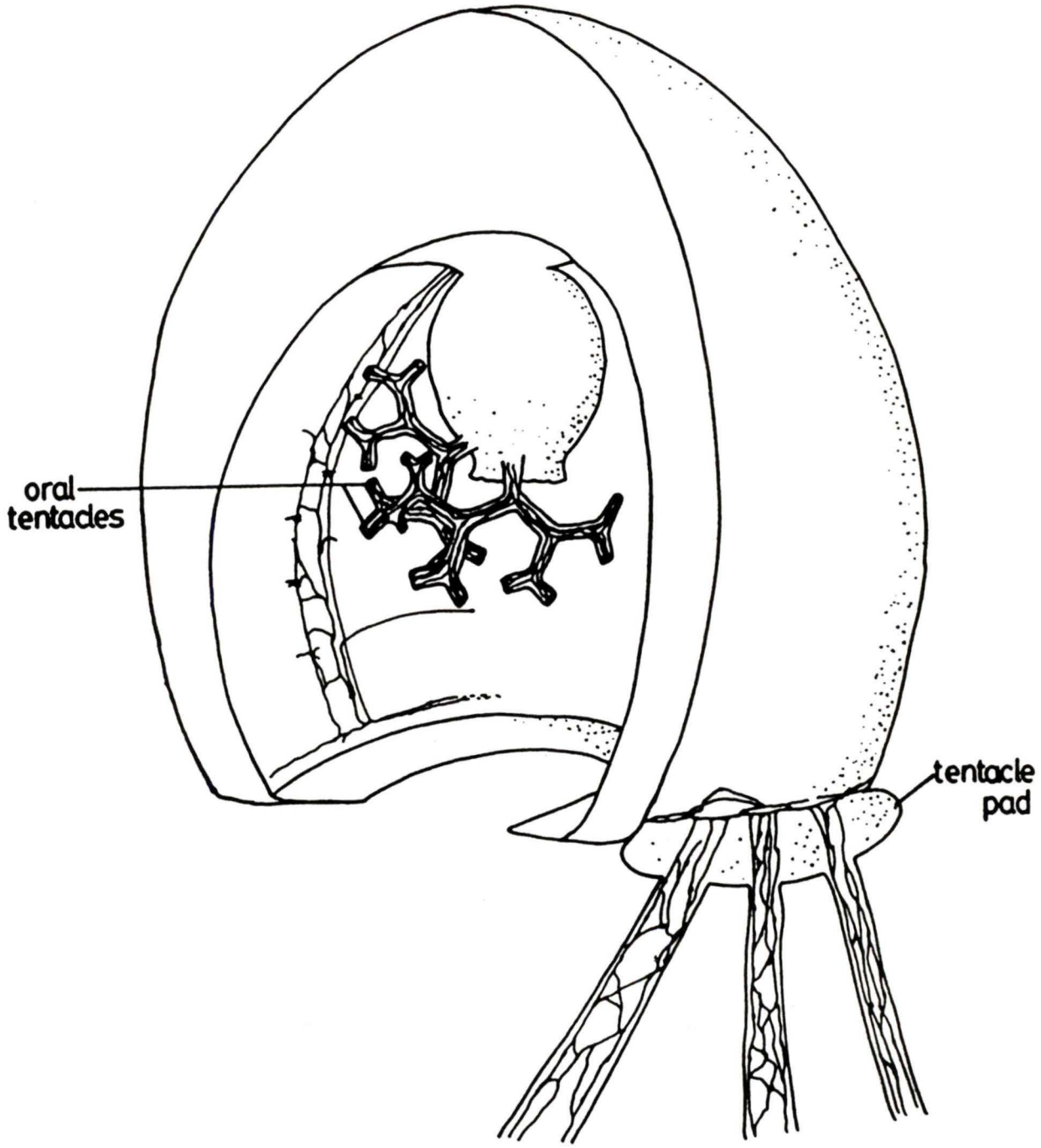
oral
tentacles
tentacle
pad

Figure 12: Fa-IR in *Stomotoca atra*

a) Tentacle nerve plexus (scale: 100 µm).

b) Plexus within tentacular bulb (scale: 100 µm).

c) Tentacle (scale: 100 µm).

d) Outer nerve ring and tentacular bulbs. Large cell bodies are visible in the bulb (scale: 50 µm).

e) Plexus overlying radial canal (scale: 20 µm).

f) Manubrial plexus and sensory cells lining lips (scale: 50 µm).

g) Manubrial plexus, radial orientation is evident (scale: 50 µm).

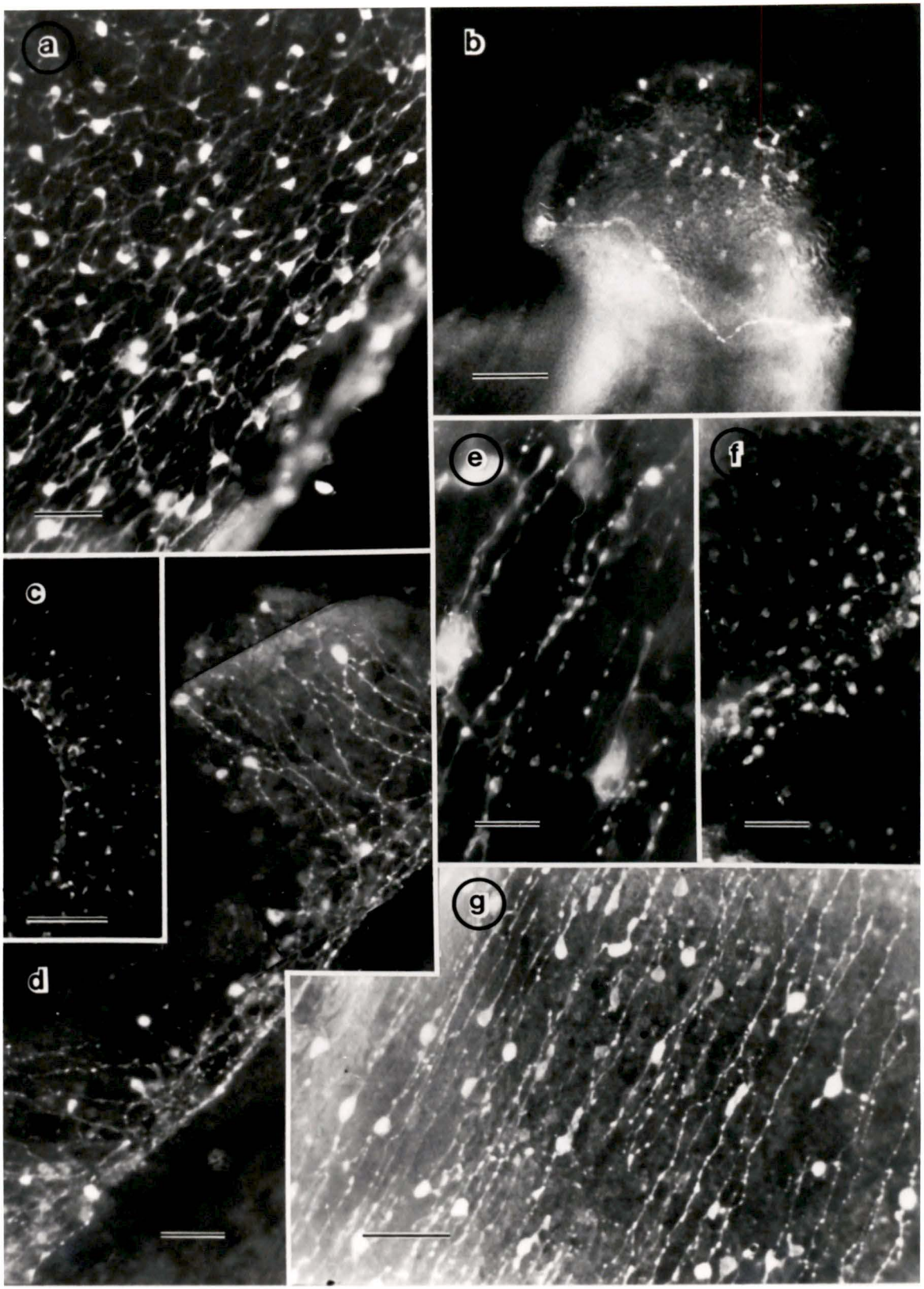
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Figure 13: Drawing showing the distribution of Fa-IR in *Stomotoca atra*

Fa-IR is present in elements of both the inner and outer nerve rings. An immunoreactive plexus extends up each radial canal. Pigmented gonad tissue obscures detail of staining (if present) in the stomach but an immunoreactive plexus is present in the lower regions of the manubrium. Immunoreactive sensory cells are present around the manubrial opening. Fa-IR is present in a tentacle plexus and in all tentacular bulbs and immunoreactive elements from these regions merge with the outer nerve ring.

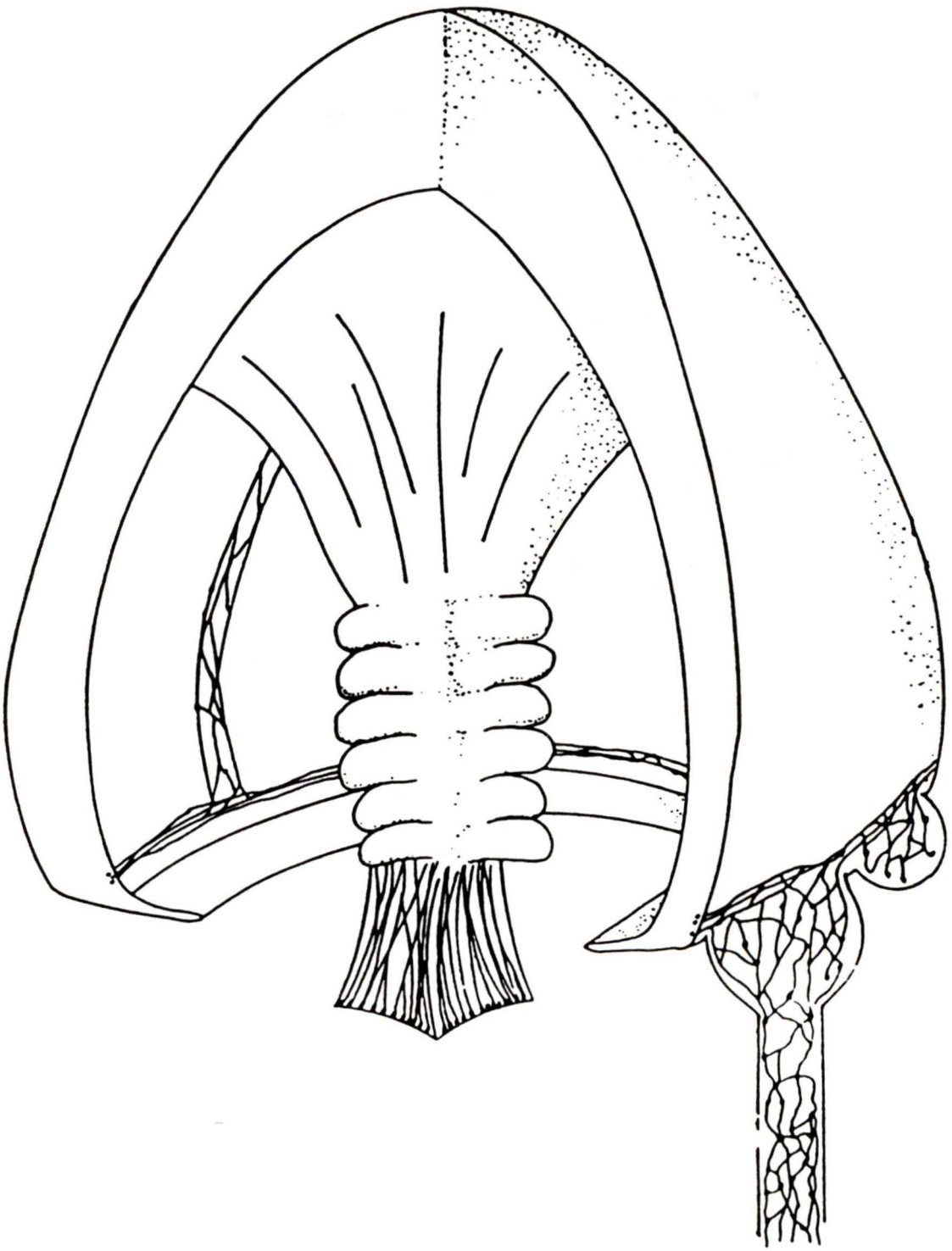

Figure 14: Fa-IR in *Halitholus* sp.

a) Tentacle nerve plexus (scale: 50 µm).

b) Neurites within the inner nerve ring. Elements run in two bands separated by the ring canal (scale: 100 µm).

c) Individual neurites overlying radial canal (scale: 50 µm).

d) Processes extending from radial plexus out onto subumbrella end blindly (arrow, scale: 100 µm).

e) Plexus overlying radial canal (scale: 20 µm).

f) Manubrial plexus showing large multipolar cell bodies (scale:20 µm).

g) Manubrial nerve plexus and sensory cells around lips (scale:50 µm).

h) Whole manubrium showing extensive plexus and immunoreactivity around margin (scale: 0.1mm).

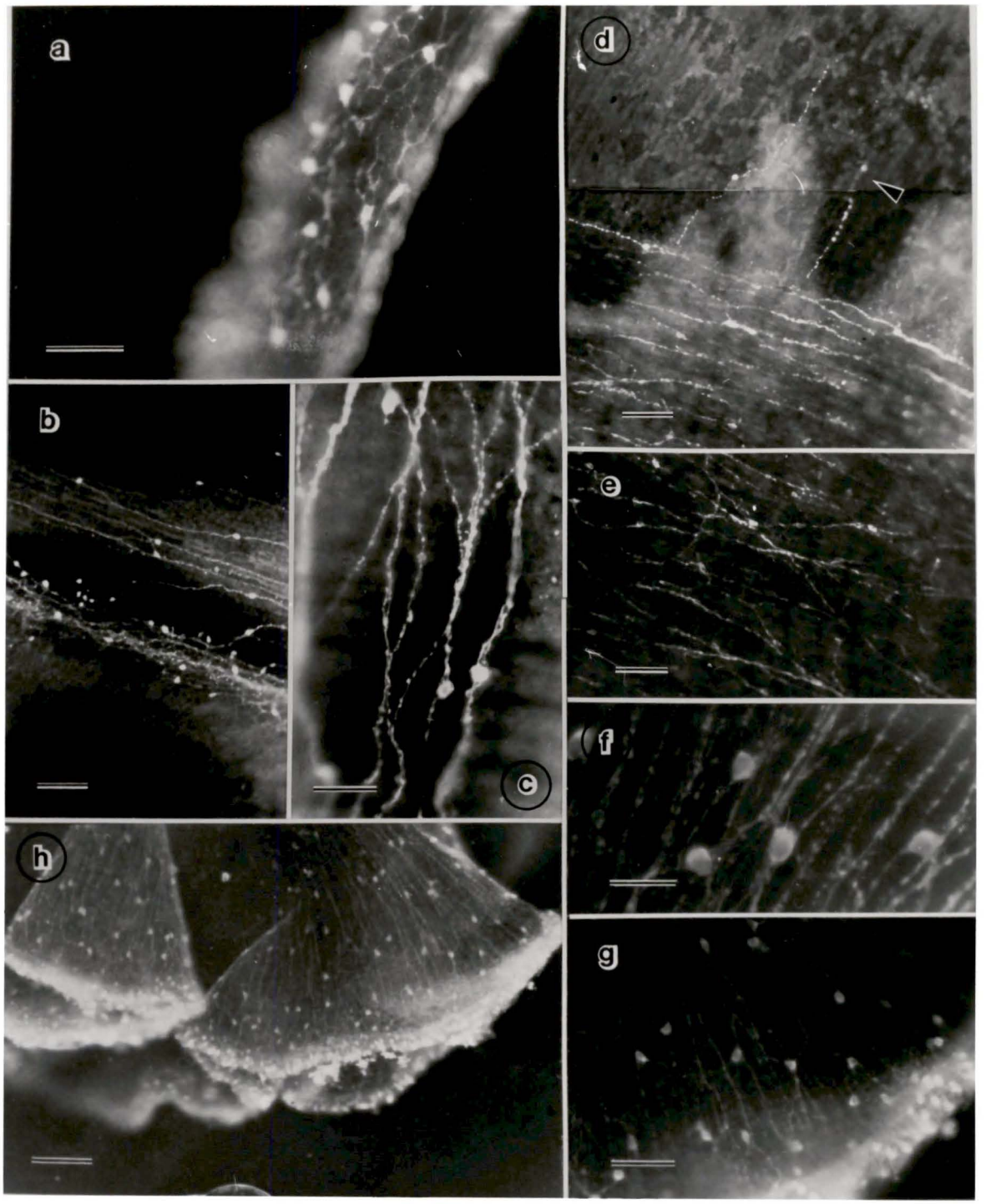
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Figure 15: Drawing showing the distribution of Fa-IR in *Halitholus* sp.

Fa-IR is present in elements of the inner nerve ring but is not present in the outer nerve ring. The immunoreactive elements of the inner nerve ring are separated into two bands which run on either side of the ring canal. An immunoreactive plexus extends up each radial canal from the inner nerve ring. A few elements extend out from this onto the interradial subumbrella muscle sheet. Pigmented tissue obscures staining (if present) in the stomach but a plexus and sensory cells showing Fa-IR are present in the manubrium. The tentacle plexus exhibits Fa-IR but this disappears as the plexus enters the tentacle bulb.

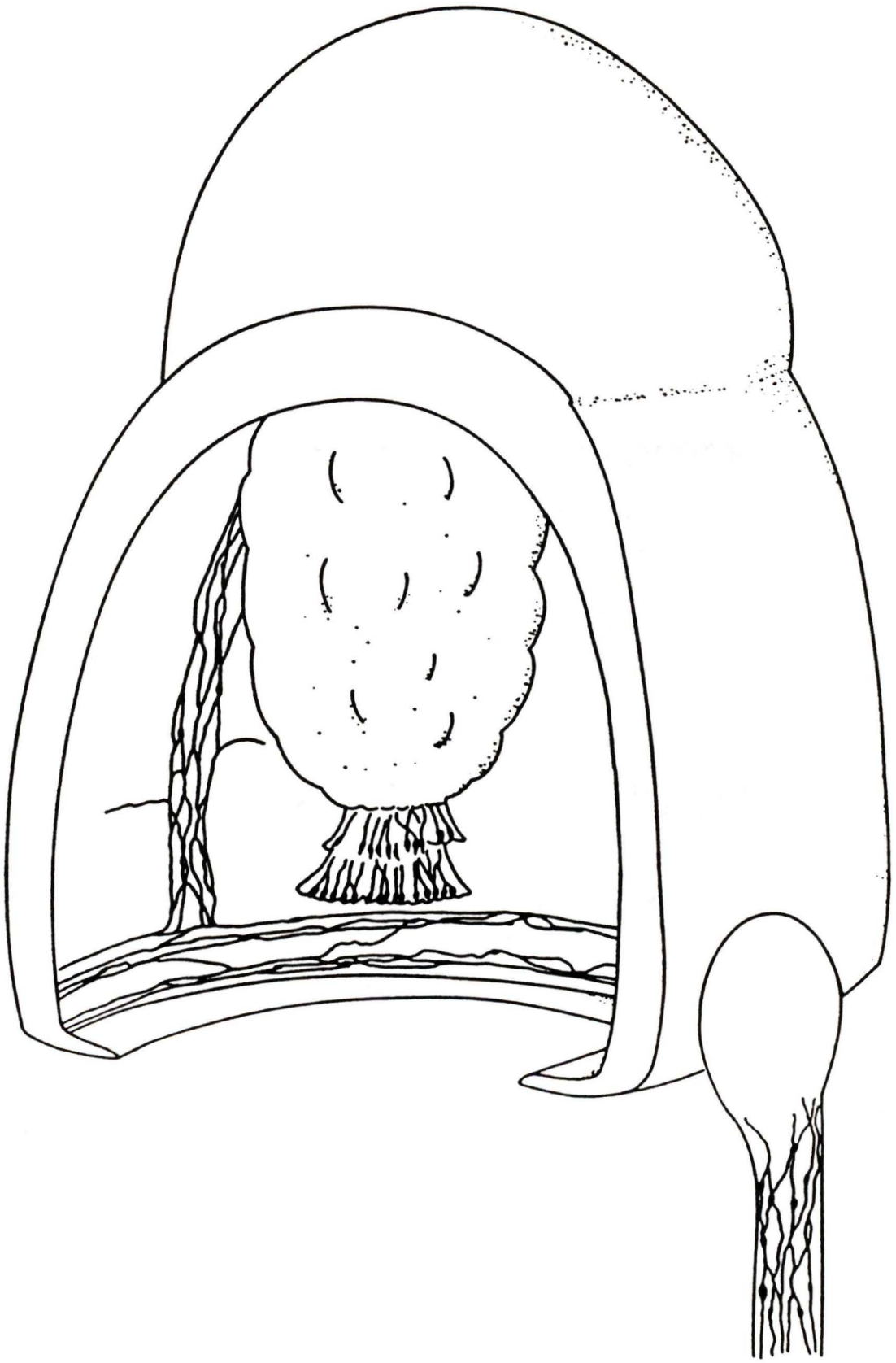

Figure 16: Fa-IR in *Gonionemus vertens*

a) Neurites within the inner nerve ring (scale: 100 µm).

b) Plexus extending from inner nerve ring and overlying radial canal (scale: 100 µm).

c) Higher magnification of radial nerve plexus (scale: 50 µm).

d) Manubrial nerve plexus and sensory cells (scale: 100 µm).

e) Cell body and processes within subumbrella (scale: 50 µm).

f) Circularly orientated nerve plexus within subumbrella (scale: 100 µm).

g) Tentacle plexus and immunoreactive sensory cells concentrated at nematocyst ring (scale: 100 µm).

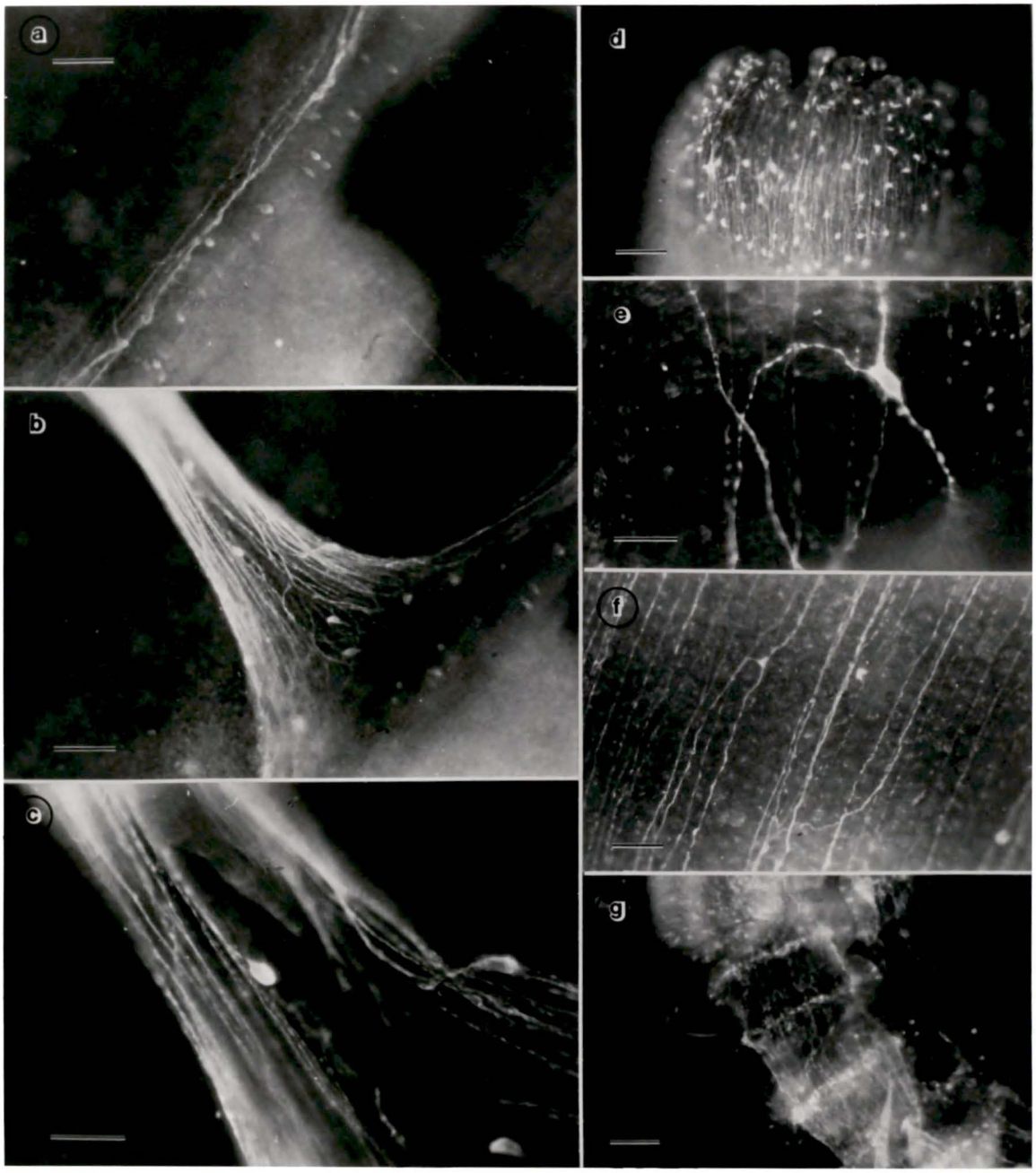
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Figure 17: Drawing showing the distribution Fa-IR in *Gonionemus vertens*

Fa-IR is present in a few elements of the inner nerve ring but pigmentation obscures detail of staining (if present) in the outer nerve ring. Immunoreactive sensory cells are present around the margin of the bell and appear to send processes into the inner nerve ring. Fa-IR is present in the nerve plexus which extends up each radial canal. Processes run out from this plexus onto the interradial muscle sheet and these become more numerous as the canal nears the stomach. Detail of staining in the stomach is obscured by pigmentation but Fa-IR is present in a plexus and many sensory cells of the more distal regions of the manubrium. Fa-IR is present in a tentacle plexus and immunoreactive sensory cells are obvious in the nematocyst rings (ner).

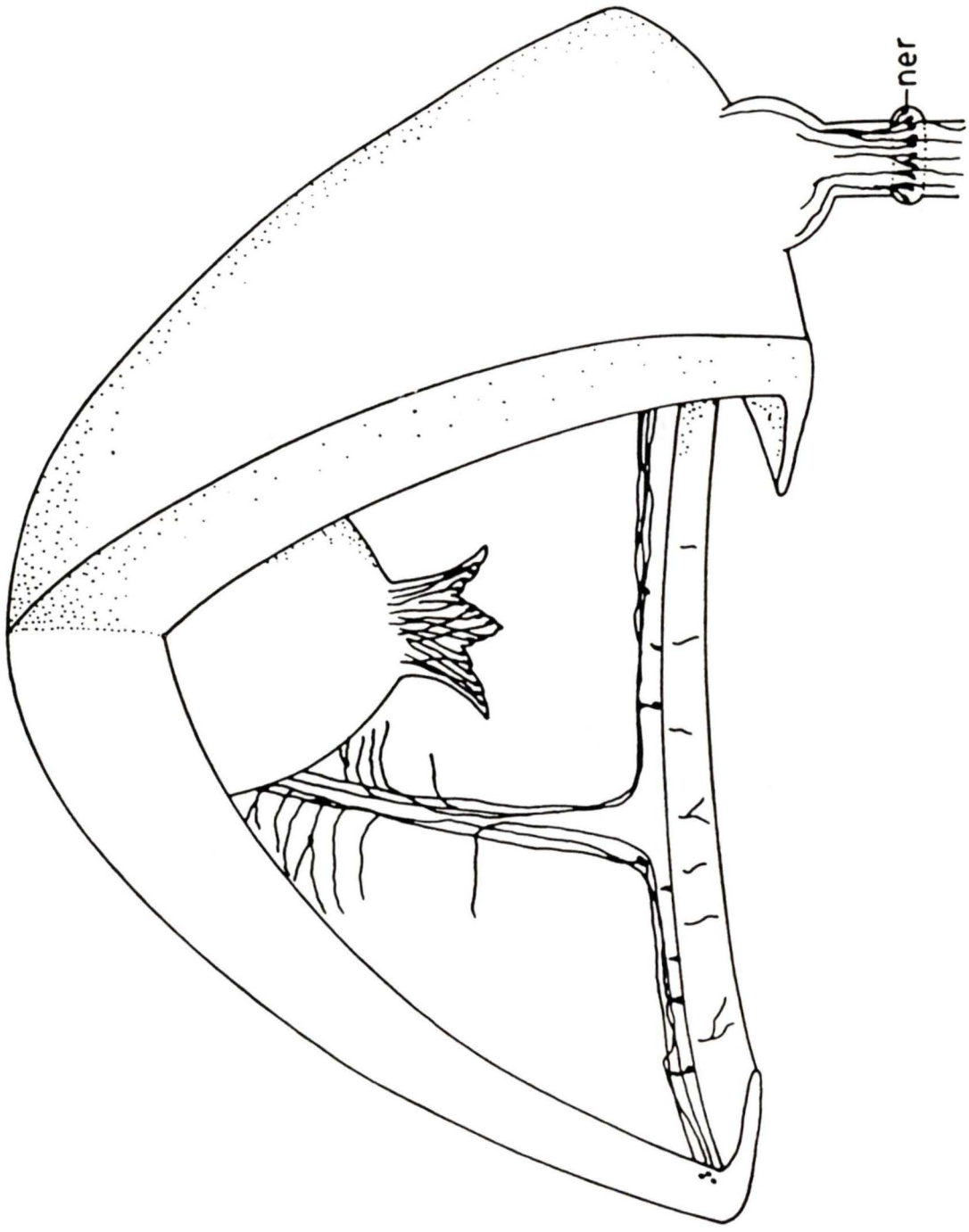

ner

Figure 18: Fa-IR in *Proboscidactyla flavicirrata*

a) Manubrium showing autofluoresence and immunoreactive sensory cells around lips (scale: 100 µm).

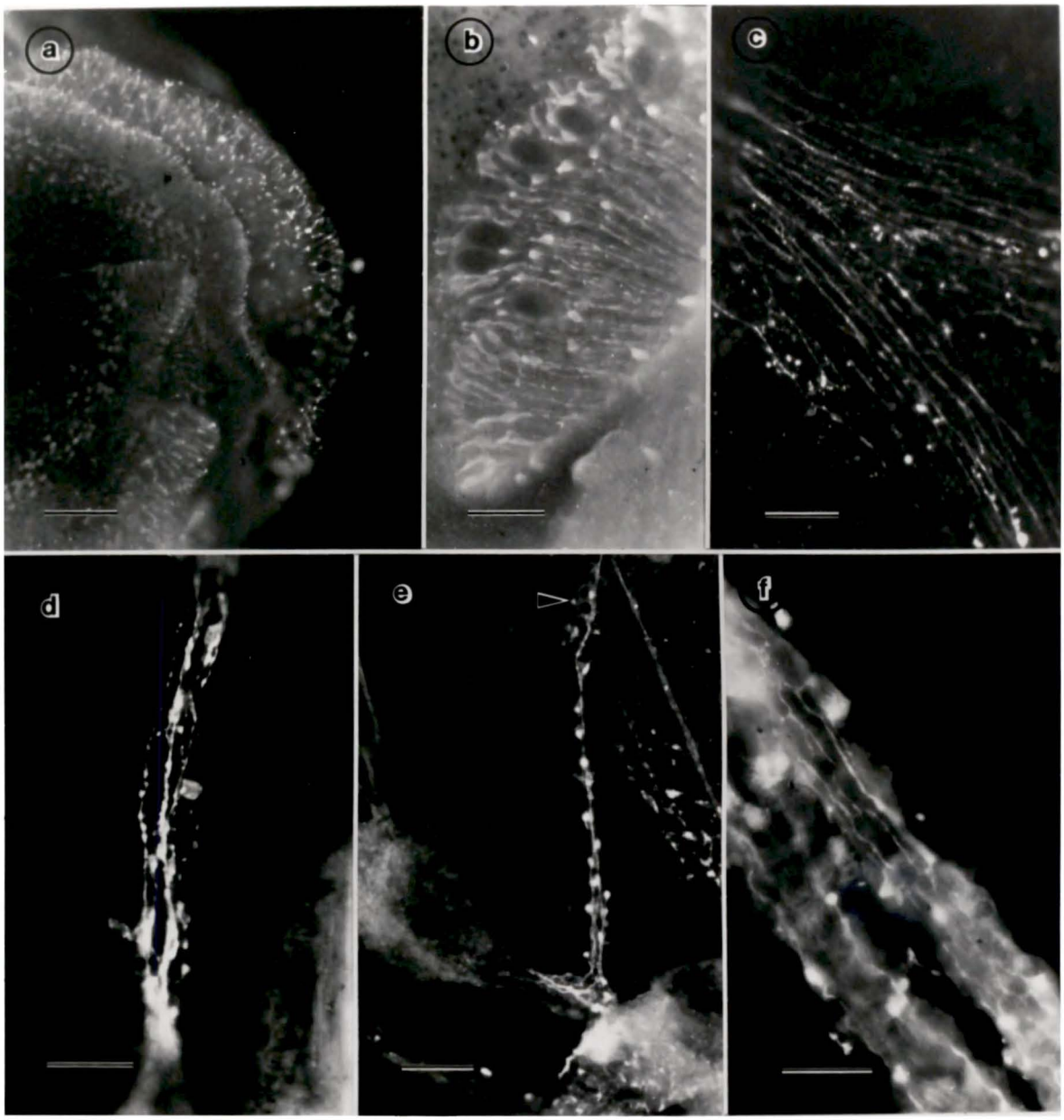
b) Manubrial plexus and sensory cells (scale: 50 µm).

c) Nerve plexus overlying radial canal (scale: 100 µm).

d) Central portion of cnidothylax tract showing several separate immunoreactive neurites (scale: 50 µm).

e) Cnidothylax showing nematocyst cluster (arrow) and neurites extending into outer nerve ring (scale: 100 µm).

f) Tentacle nerve plexus (scale: 50 µm).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e
f

Figure 19: Drawing showing the distribution Fa-IR in *P. flavicirrata*

Fa-IR is present in elements of both the outer and inner nerve rings. From the inner nerve ring an immunoreactive plexus extends up each radial canal and merges with a manubrial plexus. Fa-IR is present in a few sensory cells at the margin of the manubrium. From the outer nerve ring immunoreactive elements extend out onto the velum and a plexus extends down each tentacle. Fa-IR is also present in up to six neurites in each cnidothylax tract (cnid).

nec=nematocyst cluster

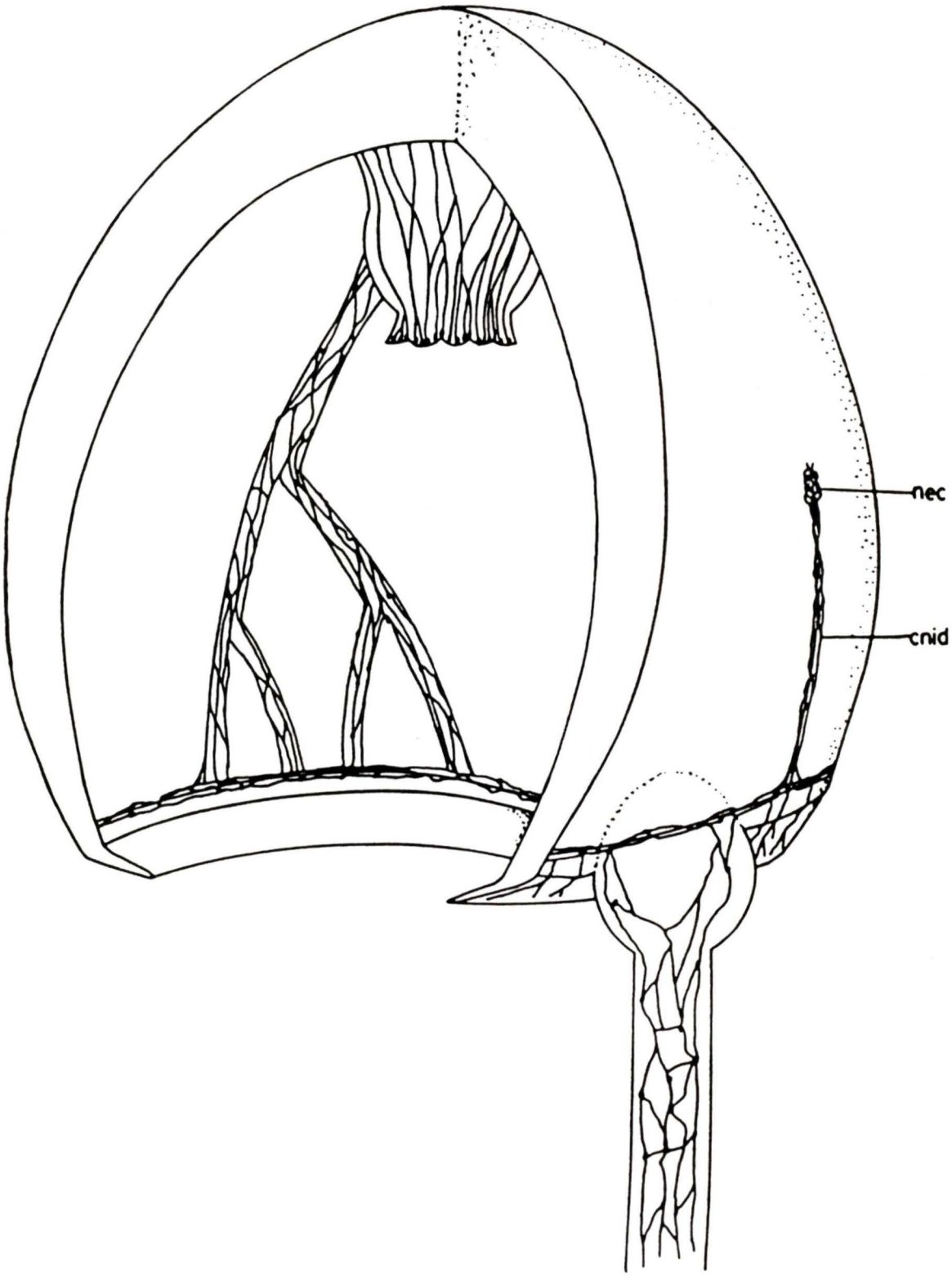

nec
cnid

Figure 20: Drawing showing Fa-IR in *Aegina citrea*

Fa-IR is present in a tenatacle plexus and in elements which extend down either side of each peronium. Some elements in the velum show Fa-IR but this cannot be traced to either nerve ring (neither of which exhibit Fa-IR). An immunoreactive plexus extends over the stomach wall above the stomach pouches but there are no immunoreactive sensory cells around the mouth.

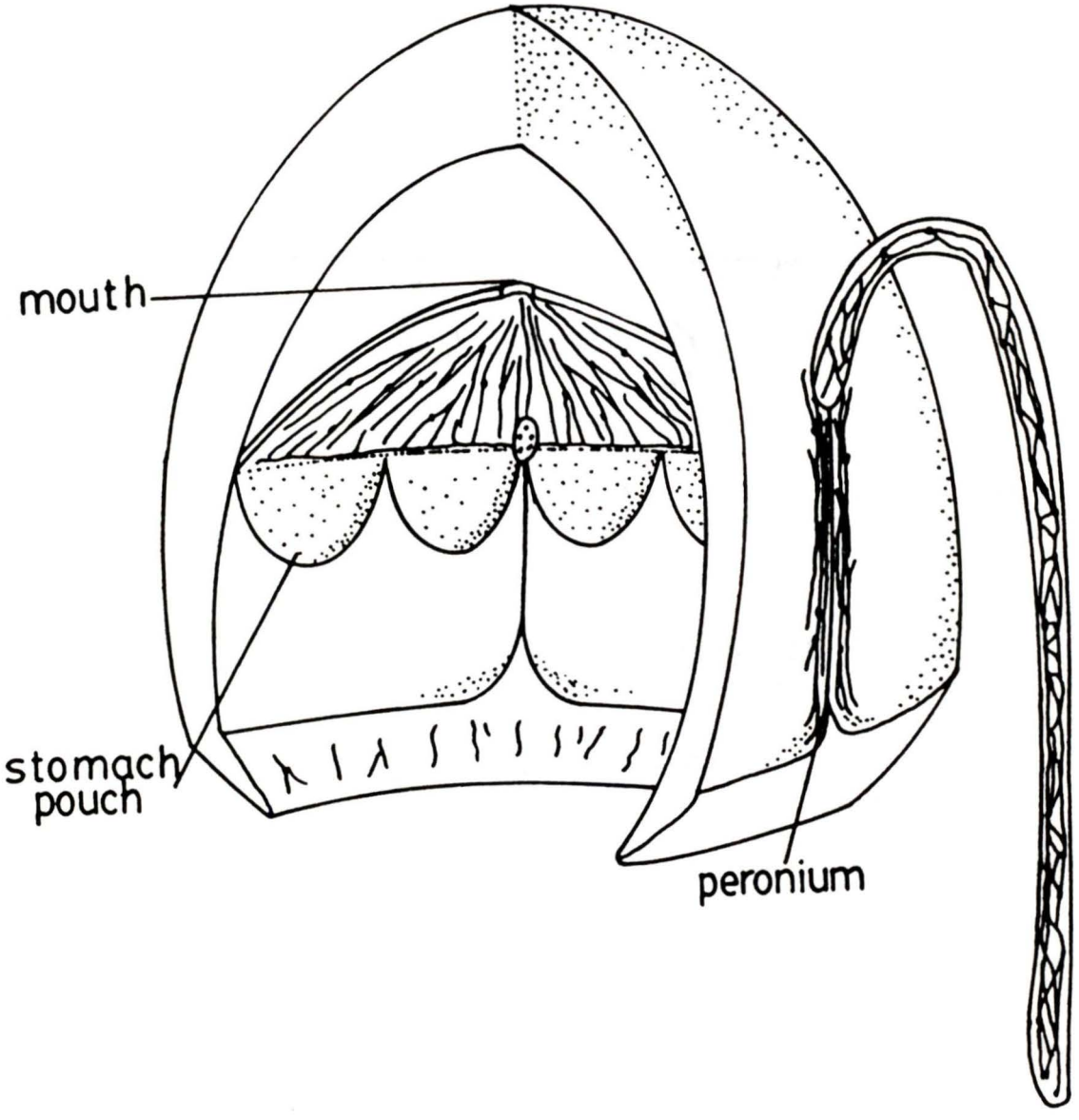

mouth
stomach pouch
peronium

Figure 21: Controls showing absence of Fa-IR in *Gonionemus vertens*

Primary antibody has been omitted and there are no visible immunoreactive elements.

a) Part of the manubrium viewed using phase contrast microscopy.

b) Same view as in a) but viewed using fluorescence microscopy.

c) Part of a tentacle viewed using phase contrast microscopy (ner= nematocyst ring).

d) Same view as in c) but viewed using fluoresence microscopy.

e) Radial canal (rc) viewed using fluoresence microscopy.

f) Radial canal viewed with phase contrast microscopy.

g) Same view as in f) but viewed using fluoresence microscopy.

(scale (a)-(g): 100 µm).

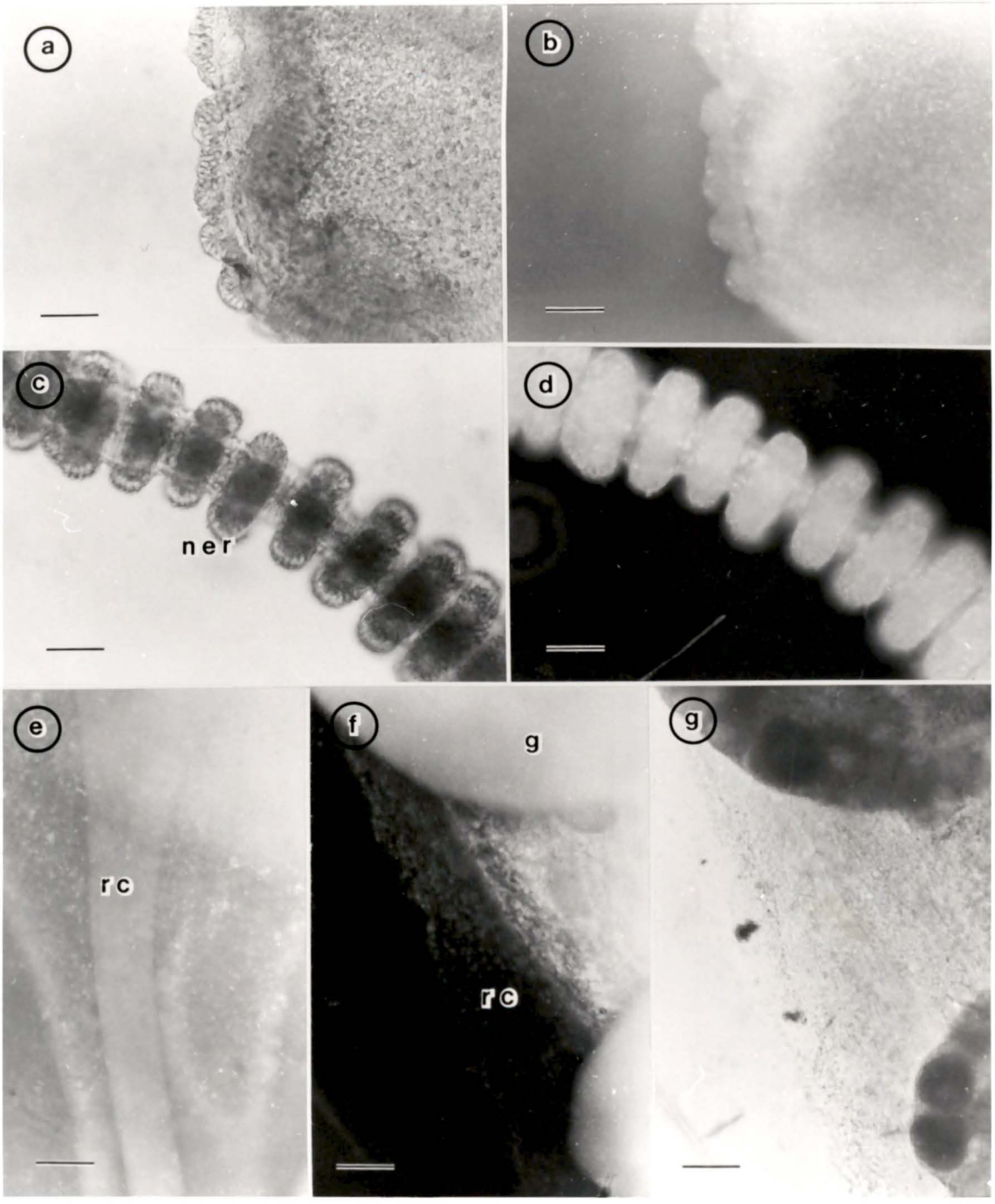

a
b
c
ner
d
e
rc
f
g
rc
g

Figure 22: Tri-radius region of margin in *Phialidium* sp.

Inner nerve ring (INR), outer nerve ring (ONR) and part of the ring canal in the endoderm (en) can be seen. Neurites extend from across the mesogloea (m) from one nerve ring to the other (scale: 1 μm).

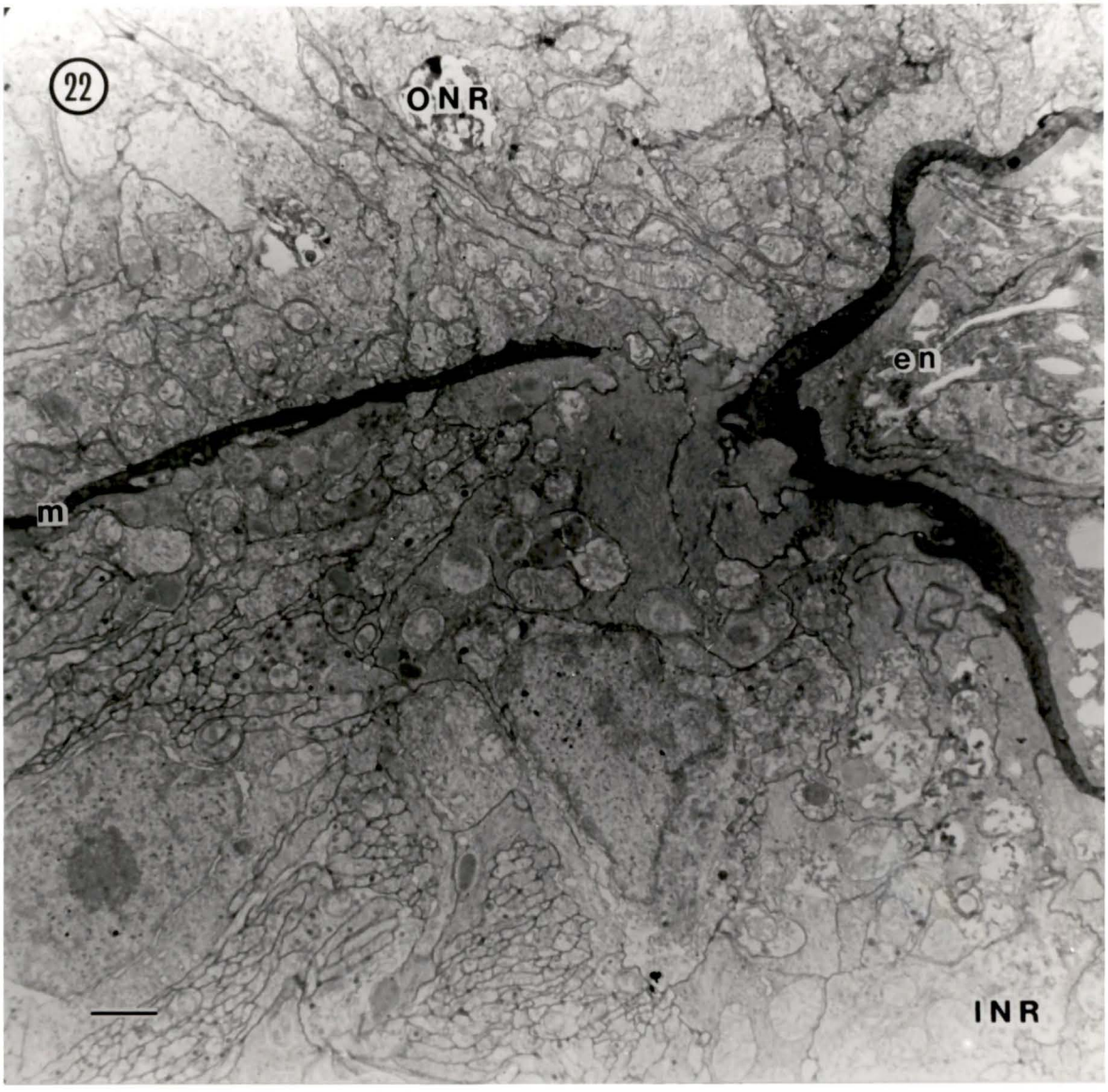

22
ONR
en
m
INR

Figure 23: Axon profiles in the INR and ONR of *Phialidium* sp.

A). Various sizes of neurite profile can been seen including one slightly larger cell (asterisk). (m:mesogloea; scale: 0.5 µm).

B). Numerous small neurite profiles are present in the outer nerve ring. A cross-section of one neurite showing neurotubules is arrowed (scale: 0.5 µm).

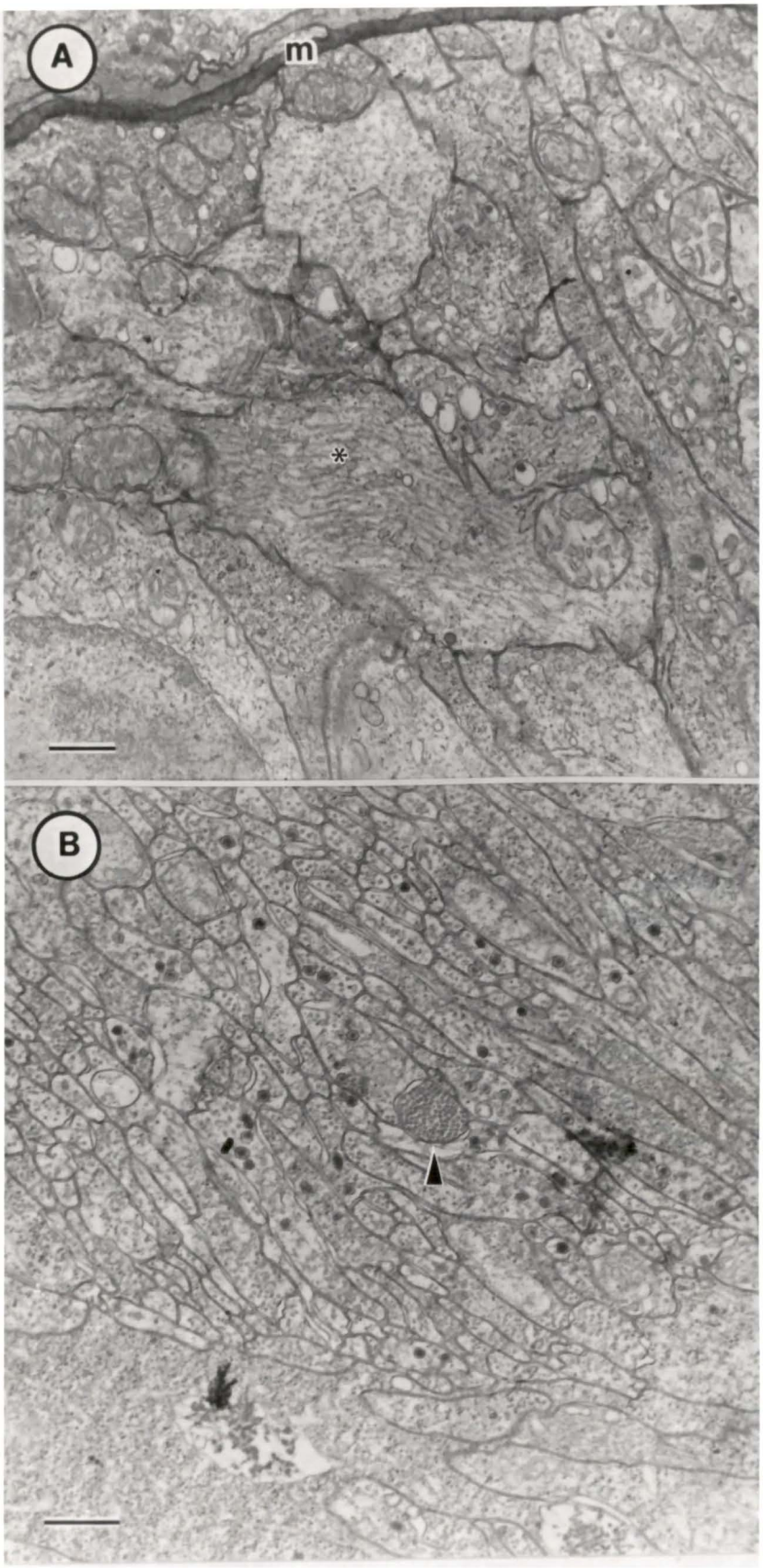

A
m
*
B

Figure 24: The inner nerve ring of *Phialidium* sp.

A). Neuro-neuronal synapses (arrows) are common within the nerve ring (scale: 0.2 µm).

B). An accumulation of dense-core vesicles within one neurite (scale: 0.25 µm).

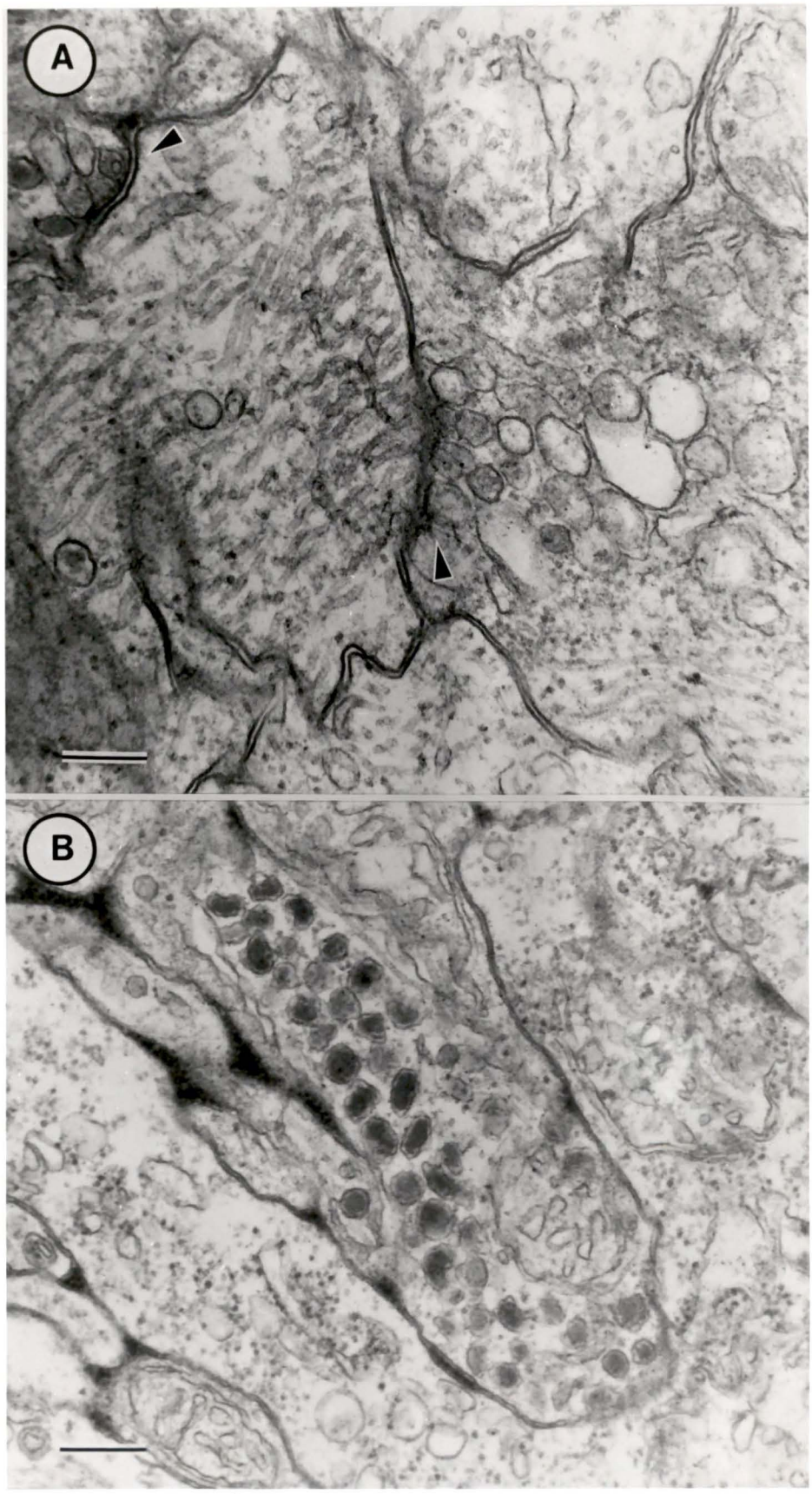

A
B

Figure 25: The outer nerve ring of *Phialidium* sp.

A). The ONR forms a ridge which runs around the outer edge of the bell (scale: 5 µm).

B). Dense-core vesicles in the neurites in the ONR (scale: 0.25 µm). The inset shows a neuro-neuronal synapse (scale: 0.1 µm).

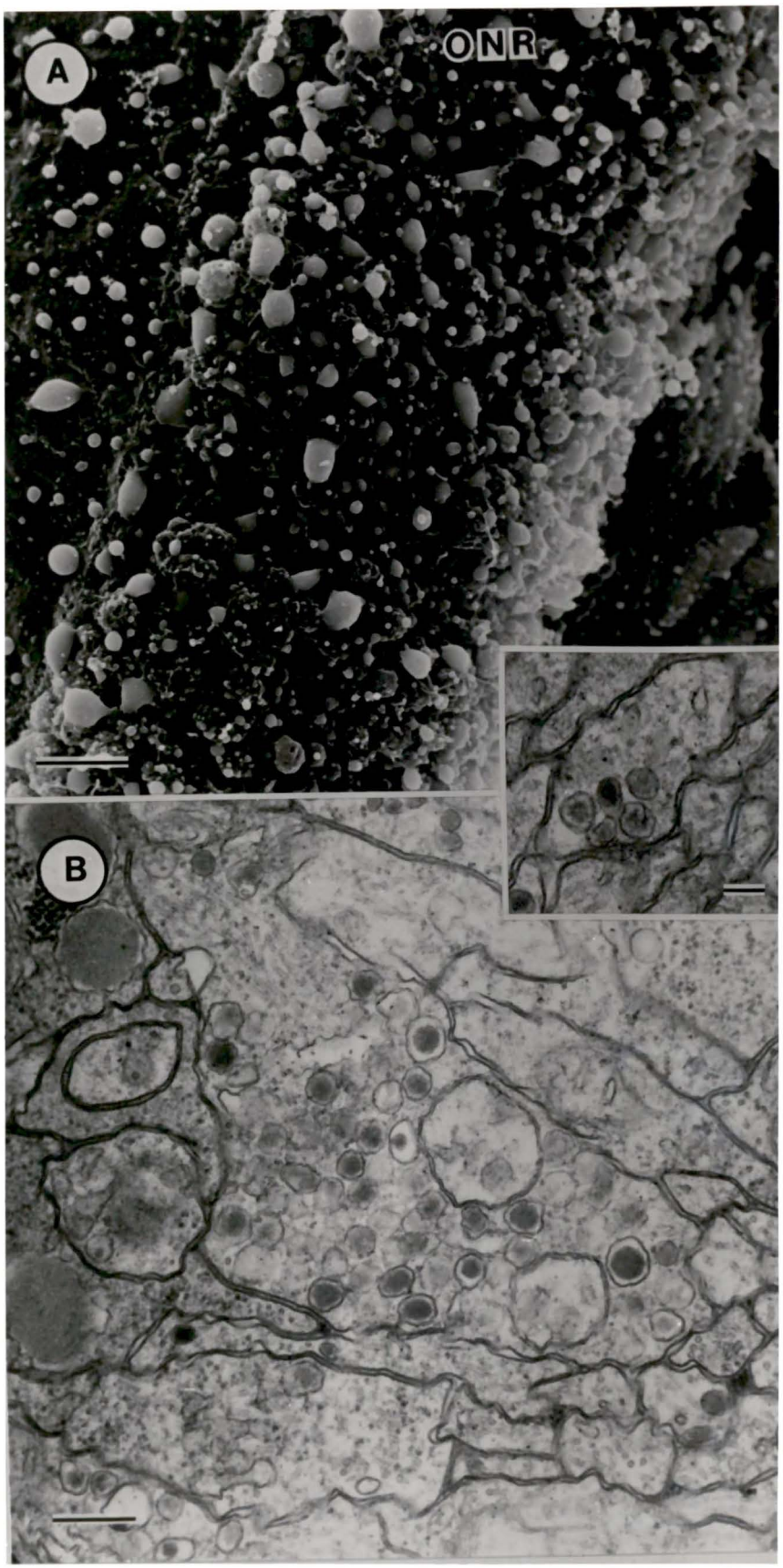

A
ONR
B

Figure 26: Frequency distributions of dense-core vesicles in INR and ONR

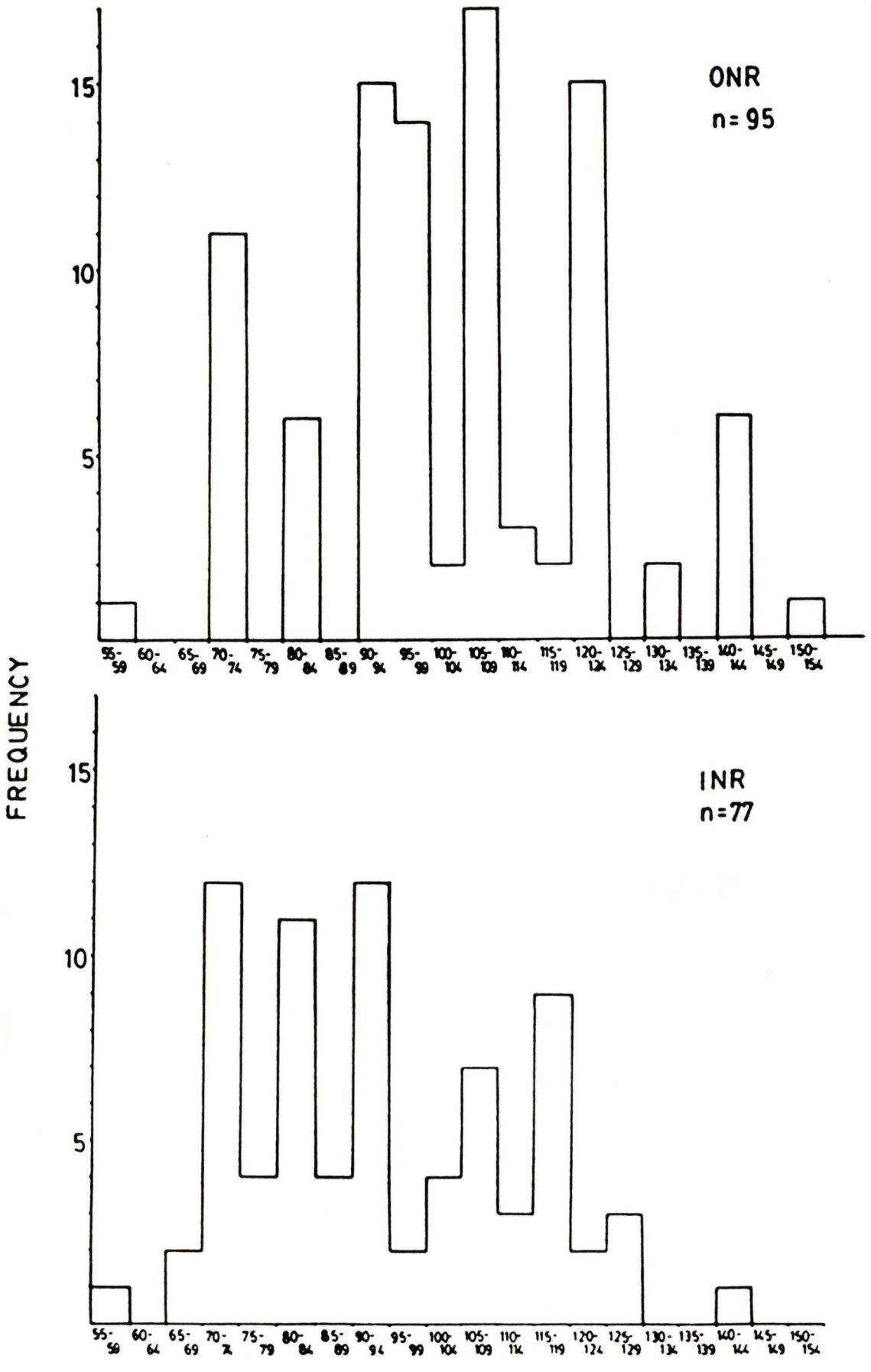

ONR
n=95
INR
n=77
FREQUENCY
VESICLE DIAMETER (nm)
15
10
5
55-59
60-64
65-69
70-74
75-79
80-84
85-89
90-94
95-99
100-104
105-109
110-114
115-119
120-124
125-129
130-134
135-139
140-144
145-149
150-154

Figure 27: Cross-sections through the subumbrella of *Phialidium* sp.

A). There is a close association between epithelial cells containing blocks of muscle (mu) and nerve cells (arrow) in the ectoderm (ec), (en:endoderm; scale: 1 µm).

B). A magnified view of the cell arrowed in A). In it dense-core vesicles and neurotubules can be seen (scale: 0.5 µm).

C). Nerve cells (arrows) closely associated with musculoepithelial cells (scale: 0.5 µm).

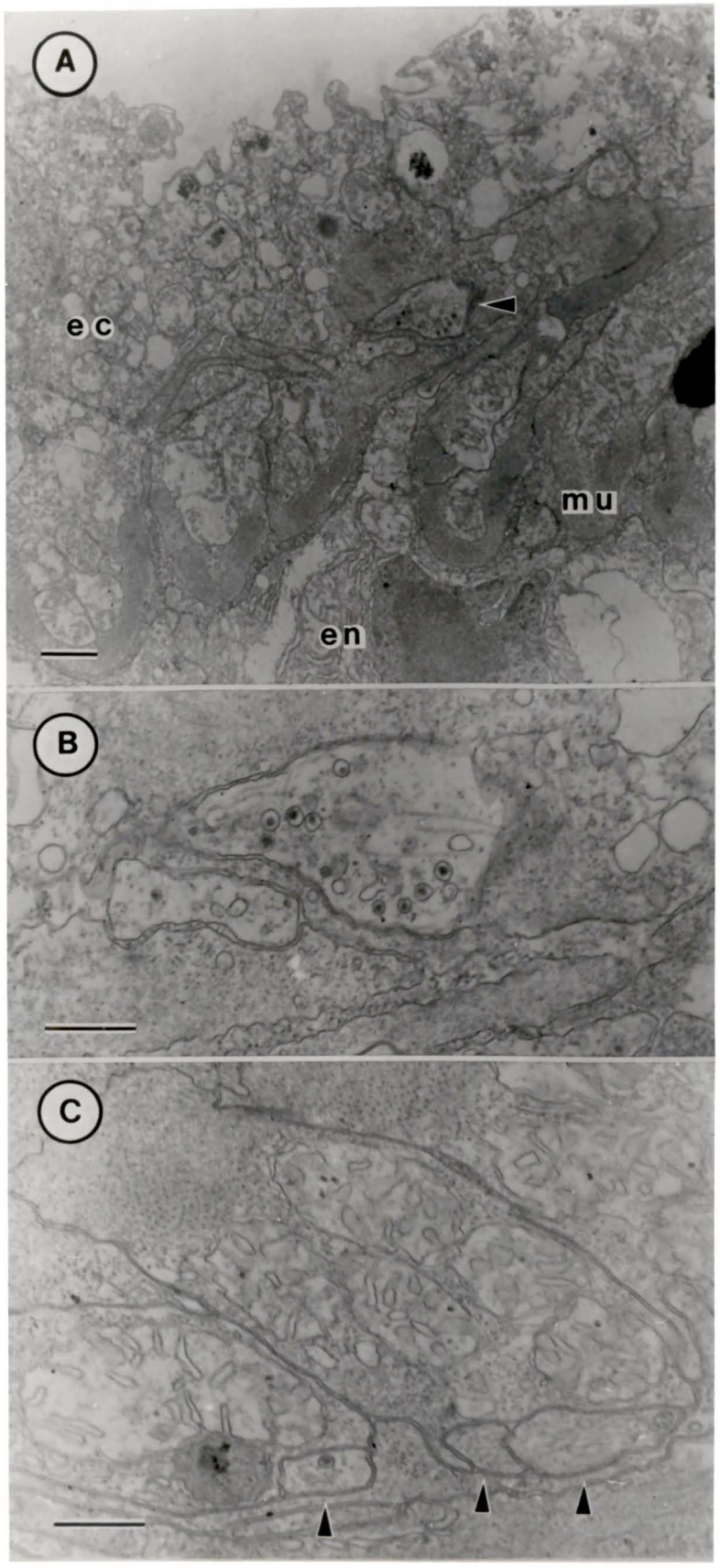

A
ec
mu
en
B
C

Figure 28: Sensory structures in the tentacle of *Phialidium* sp.

A). The tentacle bulb (tb) and tentacle (scale: 25 µm).

Inset; A cnidocil (arrow) consisting of one long flagellum and a surrounding cone of stereocilia (scale: 0.5 µm).

B). A sensory cell (sc) in tentacle ectoderm. The sensory cilium (arrow) extends beyond the surface 0.5-1 µm (ne:nematocyst; scale: 1 µm).

Inset; Cross-section through a cnidocil. (scale; 0.2 µm).

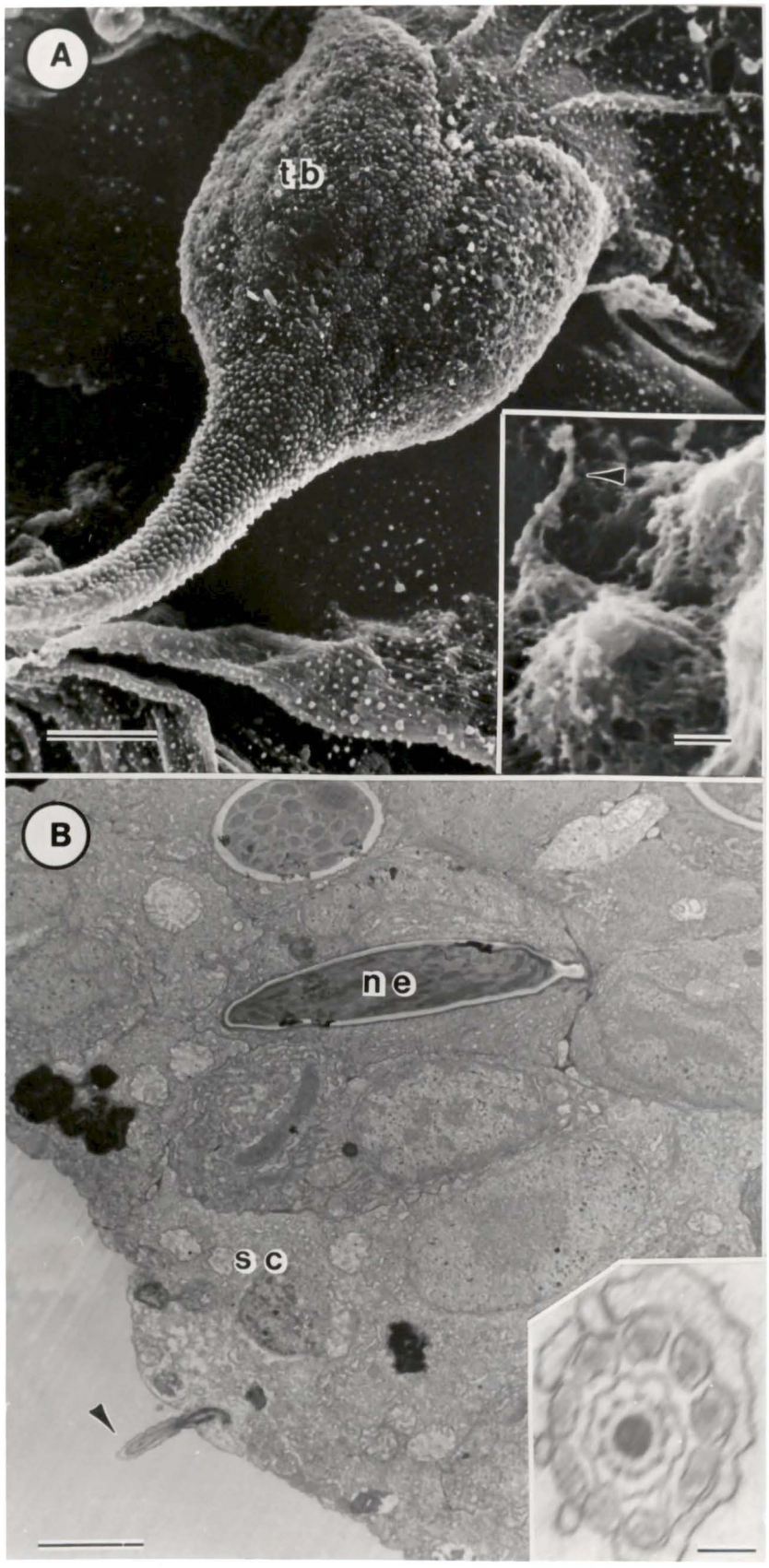

A
t-b
B
ne
sc

Figure 29: Cross-sections through tentacle of *Phialidium* sp.

A). Nerve bundles (arrows) are scattered throughout the ectoderm (ec). Blocks of muscle (mu) lie next to the mesogloea (m), (en:endoderm, ne:nematocyst; scale: 2 µm).

B). Nerve bundles are often lie next to the mesogloea (scale: 0.25 µm).

C) and D). Single nerve profiles in the tentacle ectoderm. Dense-core vesicles are present in some of the profiles (scale: 0.5 µm).

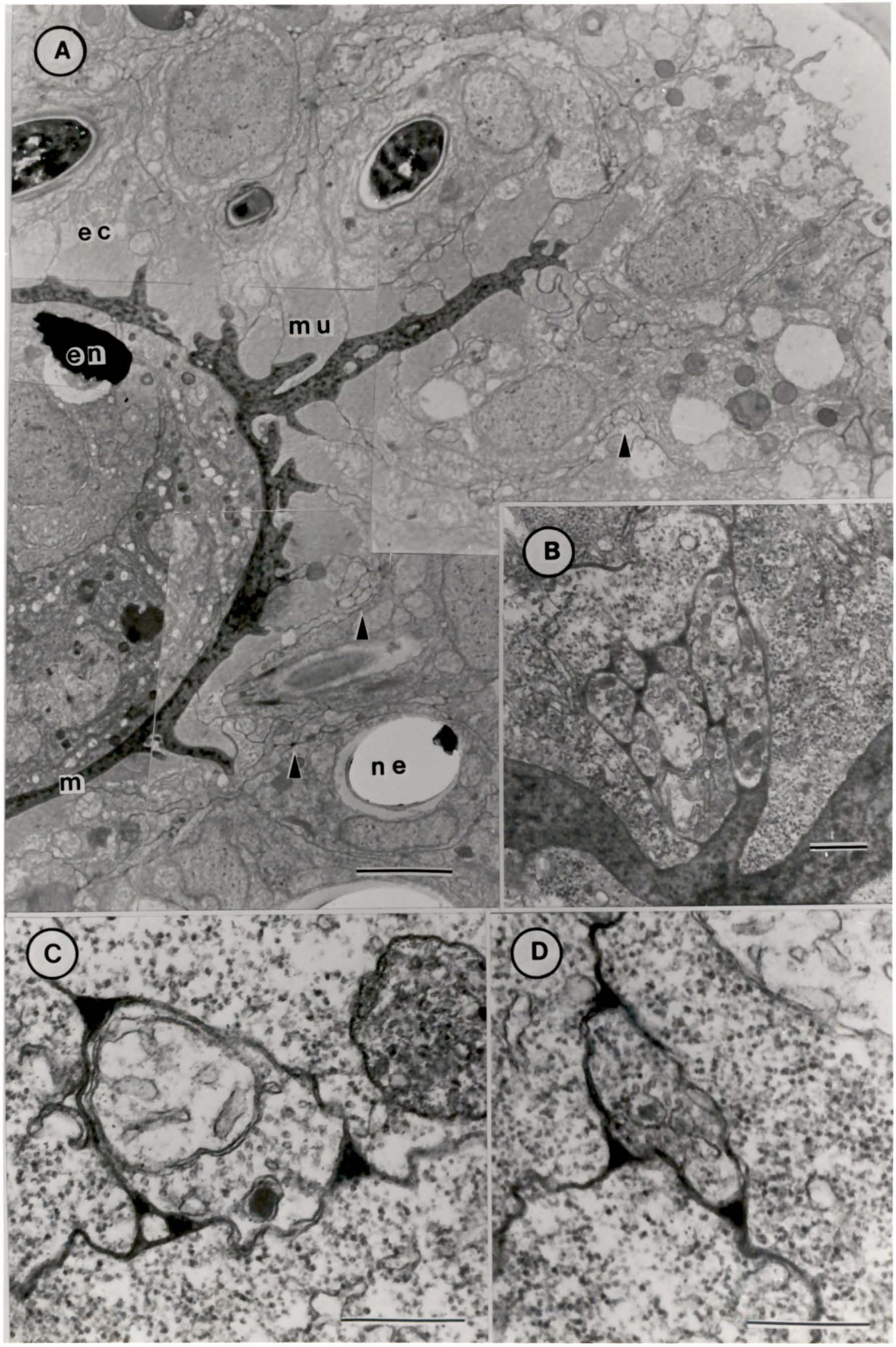

A
ec
mu
en
ne
m
B
C
D

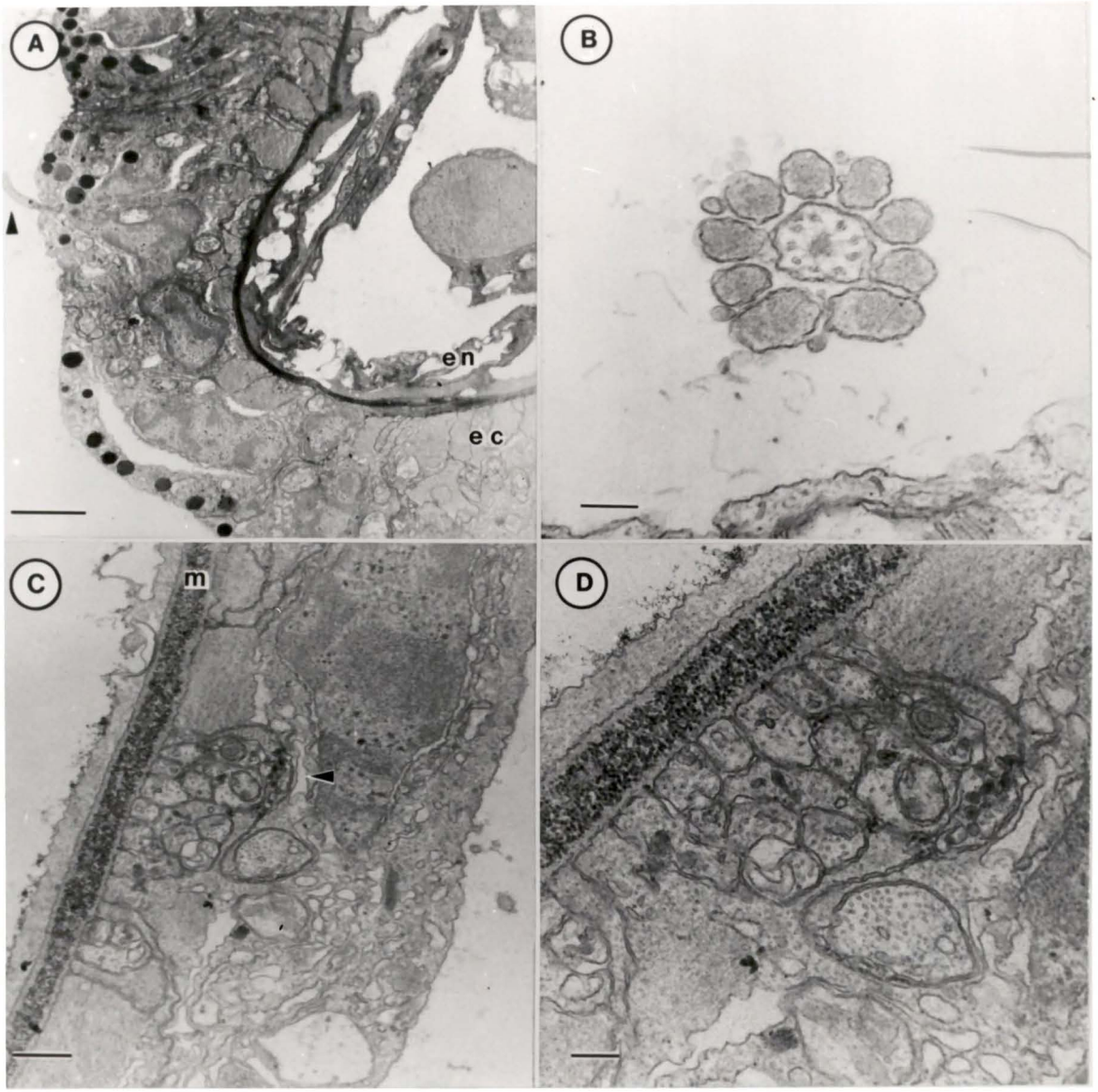
Figure 30: Cross-sections of the manubrium of *Bougainvillia* sp.

A). Sensory cells (arrow) are present in the ectoderm (ec) (en:endoderm; scale: 2 µm).

B). Nematocytes with cnidocils are also present (scale: 0.2 µm).

C). Bundles of nerves (arrow) are visible in association with the musculoepithelial cells of the ectoderm (m:mesogloea; scale: 0.5 µm).

D). Dense-core vesicles are present in some axon profiles (scale: 0.2 µm).


A
en
ec
B
C
m
D

DISCUSSION

Immunocytochemistry

The presence of Fa-IR in a portion of the nervous system of representatives of five orders of hydromedusae (including work by Mackie *et al.* 1985) suggests that a FMRFamide-like compound is a ubiquitous putative neurotransmitter or neuromodulator in Hydrozoa and possibly all coelenterates i.e. cnidaria and ctenophora (Grimmelikhuijzen 1983). That Fa-IR is restricted to a distinct subset of nerves in hydromedusae accords with previous findings (Grimmelikhuijzen and Spencer 1984; Mackie *et al.* 1985) and supports the concept that cnidaria (and possibly ctenophores) possess both physiologically and pharmacologically distinct pathways in their nervous system (see Spencer and Arkett 1984). The FMRFamide-immunoreactive subset of nerves seems to be associated with the same elements in all orders, namely smooth muscle and especially certain (but not all) sensory structures. This suggests that the FMRFamide-like compound may be acting in two different systems. FMRFamide itself is known to modulate tension in smooth muscle in molluscs (Price and Greenberg 1977; Greenberg and Price 1979; Painter 1982). A FMRFamide-like peptide may be functioning in a similar way in cnidaria, acting over a relatively long time course to vary, for instance, tentacle length in *Stomotoca atra*. Rapid responses such as swimming contractions in large medusae or escape behaviour in *Aglantha digitale* utilize large or giant motor neurons (e.g. Satterlie and Spencer 1983; Roberts and Mackie

1980) and these consistently do not show Fa-IR. Certainly chemical synapses have been identified on purely morphological grounds between radial nerves and smooth muscle in *Stomotoca atra* (Mackie and Singla 1975) and *Polyorchis penicillatus* (King 1979); also between the ONR and both velar radial muscle and tentacle longitudinal muscle in *Gonionemus vertens* (Westfall 1970a), *S. atra*, (Mackie and Singla 1975) and *P. penicillatus* (Spencer 1979). The physiological aspects of these presumed chemical junctions remain unknown since no one to one correlation of structure to function is normally possible. It is worth noting, however, that the vesicles present at these synapses vary in size from 80-120nm which is within the range for neuropeptide-containing vesicles in other groups (Hökfelt *et al.* 1980; Pelletier *et al.* 1981; van den Pol 1986).

Fa-IR is also strongly associated with nerves in the tentacles and manubrium which are both regions of high nematocyte and sensory cell density. Therefore a FMRFamide-like molecule could be acting within a system that modulates the sensitivity or stimulus threshold of nematocytes. Nematocyte discharge does not seem to be under direct nervous control but synapses are known to be present between nematocytes and neurons in hydromedusae (Westfall 1970b; Mackie and Singla unpubl. observations). The dense-core vesicles present at these synapses are of a size range that is consistent with them containing neuropeptides.

Fa-IR is not observed in the nerves coming from statocysts or ocelli in this study although it has been localized in the ocellar nerves of the anthomedusan *Polyorchis penicillatus* (Grimmelikhuijzen and Spencer 1984). Why there should be this difference is not clear, although it may be due to visibility of the ocellar nerves of *P. penicillatus* which are more distinct than those of *Sarsia* sp. or

Bougainvillia sp. making Fa-IR in this species easier to see. Further work using a well-characterised antiserum is needed before any conclusions can be made concerning Fa-IR in ocelli or ocellar nerves in the cnidaria. Equally difficult to explain is the lack of Fa-IR in the subumbrella nerve net in only *Sarsia* sp. and *P. penicillatus* (Grimmelikhuijzen and Spencer 1984). If the FMRFamide-like molecule is functioning within a system acting on smooth muscle such as the manubrial pointing response then it would be expected to be found in the nerves innervating the radial muscle bands. Since it is, not this may be an indication that in these species the putative neurotransmitter is more involved in sensory systems than in neuromuscular ones (or again it may be a problem with visualisation, see Mackie *et al.* 1985).

Fa-IR in *Eirene viridula*, as in many strains of *Hydra*, is strongly associated with the hypostome and tentacles. In *Hydra oligactis*, immunoreactive elements are concentrated into a ring around the hypostome (Grimmelikhuijzen *et al.* 1982). Although single nerve cells can act as motor, sensory and interneurons in hydroids (Westfall 1973; Westfall and Kinnamon 1978) some morphological centralization has been shown to exist in the basal region of the hydroid *H. littoralis* (Kinnamon and Westfall 1981). This weak centralization of neural elements is mirrored in the distribution of Fa-IR in the nervous sytem of both *H. oligactis* and *E. viridula*. In the latter, the ganglion-like fan cells appear to be in a position to control or influence information flow between the hypostome, tentacles and stalk. Whether or not they do so is unknown but the assumption that hydroid polyps possess a nervous system without any morphological centralization (Tardent and Weber 1976) can no longer be sustained. The fan cells themselves have not been

previously identified although numerous neurosensory cells, each with its own cilium, are known to be present in the region of the tentacle bases (Hündgen 1978). The fan cells however do not possess cilia and since there is no Fa-IR between them it would seem that the neurosensory cells here do not contain the FMRFamide-like compound. Therefore, in addition to possessing some morphological centralization, the hydroid would also seem to have pharmacological subsets of nerves. In *Eirene viridula* this subset consists of nerve cells that may perform several functions so the FMRFamide-like molecule may be acting in either or both of the systems suggested for hydromedusae. It will be recalled that *Hydra* appears to have about 6 pharmacologically distinct nerve sets (Grimmelikhuijzen *et al.* 1980; Grimmelikhuijzen *et al.* 1981a; Grimmelikhuijzen *et al.* 1981b; Grimmelikhuijzen *et al.* 1981c; Grimmelikhuijzen *et al.* 1982). Fa-IR has been found in smooth muscle bands in *Metridium senile*, an anthozoan, suggesting that a FMRFamide-like molecule may play a role in muscle contraction (Grimmelikhuijzen 1983) but in hydrozoan hydroids it is also strongly associated with sensory cells around the hypostome and in the tentacles so it may also be involved in a nematocyte modulatory system or in some other sensory function. Bundles of neurites can be seen in close association with nematocytes in cross-sections of tentacles of *E. viridula* although synapses have not been reported (Hündgen 1978).

The presence of Fa-IR in a specific subset of nerves in the nervous system of representatives of the Hydrozoa as well as in other cnidaria (Grimmelikhuijzen 1983) and the lack of evidence for the presence of classical transmitters may indicate that primitive neurotransmitters were peptides and that the classical

transmitters found in the higher invertebrates and vertebrates evolved later. It is however rash to assume that present members of an ancient group such as the coelenterates are identical or even similar to their ancestors in terms of their neuropharmacology. This may not be the case and although cnidarian nervous systems may be simple they are not necessarily primitive.

Peptide transmission may provide the nervous system with a more generalised, less precise, simpler method of signalling since peptides may be exocytosed at any part of the plasma membrane and often have different effects depending on the target organ. This more general nature of neuropeptides might be especially appropriate for a nervous system which lacks a high degree of specialisation or differentiation. Classical transmission (involving, for instance, acetylcholine) could be a specialised development to produce a faster but more inflexible means of information transfer. There is no reason to suppose that classical transmitters have not evolved in cnidaria. Indeed, a synaptic delay of 0.7-0.8 ms in *Aglantha digitale* suggests something quicker than a peptide (Kerfoot *et al.* 1985). The classical transmitters already known from vertebrate systems may not be present in identical forms in cnidaria and would therefore be difficult to detect or identify using current techniques. Neuropeptides tend to be larger compounds (though not exclusively) and although this allows more potential for variation, polyclonal antisera can often recognise several members which may differ only in one or a few amino acids. Thus neuropeptides may be easier to detect especially in the lower invertebrates and there should be a greater emphasis on this sort of work on cnidaria. Hydromedusae are particularly good for standard fluorescent immunocytochemistry since many individuals are small and thin enough to be used

as whole mounts. Previous work on cnidaria (Grimmelikhuijzen 1983; Grimmelikhuijzen and Spencer 1984) has used paraffin-embedded thin sections which sometimes limits the amount of information that can be obtained regarding the extent and nature of Fa-IR. This is due in part to poor tissue preservation and in part to the problems of reassembling a three-dimensional animal from many thin sections.

It is clear from the results of this study that, despite similaritites in distribution of Fa-IR, there are variations in the 'layout' of the nervous system between different orders of hydromedusae especially in the subumbrella region. Differences in the way the subumbrella swimming muscle sheets are innervated have already been noted (Satterlie and Spencer 1983). The neuroanatomy of the narcomedusae in particular has been little studied since the work of the Hertwig brothers (1878). It is now evident that this order possesses a subumbrella nerve net (similar to the Leptomedusae) which covers the main stomach wall and runs up to a small opening or 'mouth'. Whether it extends to cover the stomach pouches and lower subumbrella remains to be seen. In contrast with other orders the 'mouth ' is not surrounded by immunoreactive sensory cells and seems to lack any specialisations for feeding.

In the Anthomedusae it seems that all subumbrella nerves are restricted to the muscle bands overlying the radial canals, either as a random plexus (e.g. *Euphysa* sp.) or as a relatively organised structure containing regularly arranged cell bodies (e.g. *Bougainvillia* sp.). These kinds of subtle differences have not been noted before. Within the order it does not seem uncommon for processes to extend beyond the radial tracts onto the subumbrella muscle sheet. Thus, there is

potential for neural imput into an otherwise myoid conducting system. It would now appear that a subumbrella nerve net is present in most, if not all, Leptomedusae but in the Limnomedusae there are obvious differences and it is not clear whether there is a 'standard' neural layout for the subumbrella in this order. With these kinds of variations both within and between the orders, attempts at selecting a 'typical' hydromedusan to study become more difficult and results obtained with one species may not be directly applicable to another.

Electron Microscopy

The genus *Phialidium* sp. has been used to study various aspects of cnidarian biology such as hydrozoan development (Bonner 1955; Roosen-Runge 1962, 1970; Worthman 1974) and ultrastructure of various non-nervous tissues (Roosen-Runge and Szollosi 1965; Leik and Kelly 1970; Singla 1975). The morphology and ultrastructure of the nervous system, however, have been investigated on only a few previous occasions (Hertwig and Hertwig 1878; Horridge and Mackay 1962; Satterlie and Spencer 1983). The medusa is small, flimsy and easily damaged and is therefore not ideal for standard electron microscopical techniques compared to other medusae such as *Polyorchis penicillatus* but since it is widely available and exhibits strong Fa-IR within its nervous system it is a good candidate for such a study in this case.

The basic layout of the nervous system of the genus is similar to that in other hydromedusae (Jha and Mackie 1967; Mackie and Singla 1975; Singla 1978; Spencer

1979). The nerve tracts are not as large or distinct as in species such as *Polyorchis penicillatus* and therefore *Phialidium* sp. is not as attractive for physiological work. The total diameter of the inner and outer nerve rings in *Phialidium* sp. is smaller than in many better known medusae but the rings do contain a comparable number of axon profiles to other genera. As a consequence of the small overall size, the motor giants are difficult to distinguish especially in small or immature specimens as is sometimes the problem with other medusae (Satterlie and Spencer 1983). Horridge and Mackay (1962) could not identify axons by the presence of neurotubules within the nerves of *Phialidium* sp. and suggested that they were not present. Aldehyde fixation now preserves neurotubules and they are consistent in size with those found in other phyla. These workers also divided neuronal vesicles into two categories, type A and type B. The former correspond to what are now known as dense-core vesicles often associated with synapses and typical of putative peptidergic neurons in many phyla (Hökfelt *et al.* 1980). The larger clear type B vesicles are not synaptic and are associated with the Golgi body. Small clear vesicles (20-40nm) of the type associated with cholinergic junctions have not been reported.

The axo-axonal chemical synapses and dense-core vesicles found in this study are similar to those found in the only other well-studied Leptomedusan *Aequorea victoria* although the dense-core vesicles in the latter are slightly smaller in average size (Satterlie 1985). In other genera neuro-epithelial synapses have been identified but are associated with clear vesicles 90-130nm in diameter (Satterlie and Spencer 1983).

The subumbrella nerve net consists of single neurites running up the subumbrella surface between the radial and circular muscle from inner nerve ring to manubrium. As in *Aequorea victoria* (Kerfoot 1980) neurites appear to innervate both muscle layers equally. Small loose bundles of neurites also lie between the muscle layers so it is not possible to determine whether the single immunoreactive elements seen at the light microscope level correspond to individual neurites or to several neurites in close proximity to one another. The subumbrella nerve net described in *Phialidium* sp. seems to be common to the Leptomedusae and was originally identified morphologically by the Hertwigs (1878) and later ultrastructurally by others (Kerfoot 1980; Satterlie and Spencer 1983). Although neuromuscular synapses were not found in either muscle layer in the subumbrella in this study, they have been identified in this region in other medusae (Satterlie and Spencer 1983). In *Aequorea victoria* small neurons were identified on the subumbrella side of the velum (Kerfoot 1980) in contrast to *Phialidium* sp. Very small profiles may have been missed in this species although an extensive survey was made of velar tissue.

In this study dense-core vesicles have been found in all the regions that have exhibited Fa-IR at the light microscope level but without further work at characterising the contents of these vesicles it is impossible to say for certain whether these are the ultrastructural sites of the FMRFamide-like molecule. Indeed in recent years, the vesicular hypothesis of storage and release has been questioned (*e.g.* Tauc and Baux 1980) and it is evident that one transmitter, acetylcholine, is present in both the cytoplasm and vesicles of the synaptic region. Neuropeptides however have been localised to dense-core vesicles on more than

one occasion (Cuello *et al.* 1977; Pelletier *et al.* 1981; Priestly 1984; van den Pol 1986). It therefore seems safe to assume that at least some of a FMRFamide-like compound is stored in dense-core vesicles in coelenterates. If this is the case, then according to this study 30-50% of the neurons in the nerve rings are FMRFamide immunoreactive. It is more difficult to determine the proportion of FMRFamide immunoreactive elements in the tentacles, subumbrella and manubrium since nerves are less concentrated, but an estimate would be from 10-50%. It seems unlikely that the contents of all the dense-core vesicles would be the same and thus the true proportions are probably smaller. It remains clear however that a distinct pharmacological subset of nerves exists in the nervous system of the Hydrozoa.

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