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Cornelis

Organization of the nervous system of physonectid siphonophores

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Summary. An antiserum to the sequence Arg-Phe-amide (RFamide) was used to stain the nervous systems of various physonectid siphonophores. In the stem of *Nanomia bijuga*, this antiserum stained an ectodermal nerve net, which was interrupted, at regular intervals, by transverse collars of neurons. Injection of Lucifer yellow into the "giant axon" of the stem showed that this axon was dye-coupled to an ectodermal nerve net that resembled the RFamide-positive network. Ectodermal nets of neurons were also found in the pneumatophore, gastrozooids, tentacles and tentilla. At the junctions of the pneumatophore, the gastrozooids, the dactylozooids and the gonozooids with the stem, and at the junctions of tentacles and tentilla, collars or rings of neurons occurred. The stem was connected to the phyllozooids and nectophores by muscular lamellae, which were bordered by chains of neurons. At the margin of the nectophores, an immunoreactive nerve ring was found. Connected to this ring and located in the "seitliche Zapfen" ("sidely-located patches"), were two agglomerations of nerve cells. On the upper side of the bell margin, positioned at 90° relative to the "seitliche Zapfen", a delta-shaped neuronal structure was found. This structure was connected to the nerve ring and was associated with a muscle, which ran a short distance along the exumbrellar surface.

The nervous systems of *Agalma elegans*, *Forskalia edwardsi*, *Forskalia leuckarti* and *Halistemma rubrum* resembled that of *Nanomia bijuga* in all major respects.

Key words: RFamide – Neuropeptide – Nervous system – Siphonophores – Coelenterates

The coelenterates were probably the first group of animals to have evolved a nervous system. Among the present-day coelenterates, the physonectid siphonophores represent an interesting group, as these hydrozoans form colonies consisting of many specialized individuals, which nevertheless are able to coordinate colonial behaviour such as fishing, swimming and escape (Mackie and Boag 1963; Mackie 1964). The activities of the individuals, which are all attached to a central stem (see Fig. 1 for a generalized illustration of physonectid siphonophores), are concerned with feeding (gastrozooids and tentacles), defence (dactylozooids

and tentacles), protection (phyllozooids), reproduction (gonozooids), buoyancy (pneumatophore) and swimming (nectophores). It is obvious that in physonectid siphonophores a rather specialized form of the ancestral coelenterate nerve net is required to allow for both colonial coordination and independent action of the individuals.

The anatomy of the nervous system of physonectid siphonophores has been described by Korotneff (1884), Schneider (1892), and Schaeppi (1898) using osmic-acetic acid maceration and methylene-blue staining, and more recently by Mackie (1964, 1973, 1978) and Jha and Mackie (1967) using light-microscopic and ultrastructural methods. Another method to stain components of the nervous system of coelenterates is to use antisera against certain vertebrate or invertebrate neuropeptides (see Grimmelikhuijen 1984 for a review). Antisera against the molluscan neuropeptide Phe-Met-Arg-Phe-amide (FMRFamide; Price and Greenberg 1977) and especially those against its carboxyterminal fragment Arg-Phe-amide (RFamide) have proven to be invaluable tools for determining the organization of the nervous system of coelenterates (Grimmelikhuijen et al. 1982a; Grimmelikhuijen 1983a, 1985; Grimmelikhuijen and Spencer 1984; Grimmelikhuijen and Graff 1985; Mackie et al. 1985). In the present study we have used staining with RFamide antisera in physonectid siphonophores and have attained a far clearer picture of the organization of the physonectid nervous system than has previously been possible.

Materials and methods

Animals

Animals were collected during May and June 1984 and April 1985 from the Mediterranean Sea near Villefranche-sur-Mer, France, either by making horizontal plankton hauls at 0–30 m, or by dipping them with a small net from the surface. Five species of physonectid siphonophores were used in this study: *Agalma elegans*, *Forskalia edwardsi*, *Forskalia leuckarti*, *Halistemma rubrum*, and *Nanomia bijuga*.

Immunocytochemistry of whole mounts

Specimens were anesthetized in a 1:1 mixture of sea water and 0.4 M MgCl₂, dissected if necessary, and fixed in a fresh solution of 4% paraformaldehyde in 0.1 M sodium-

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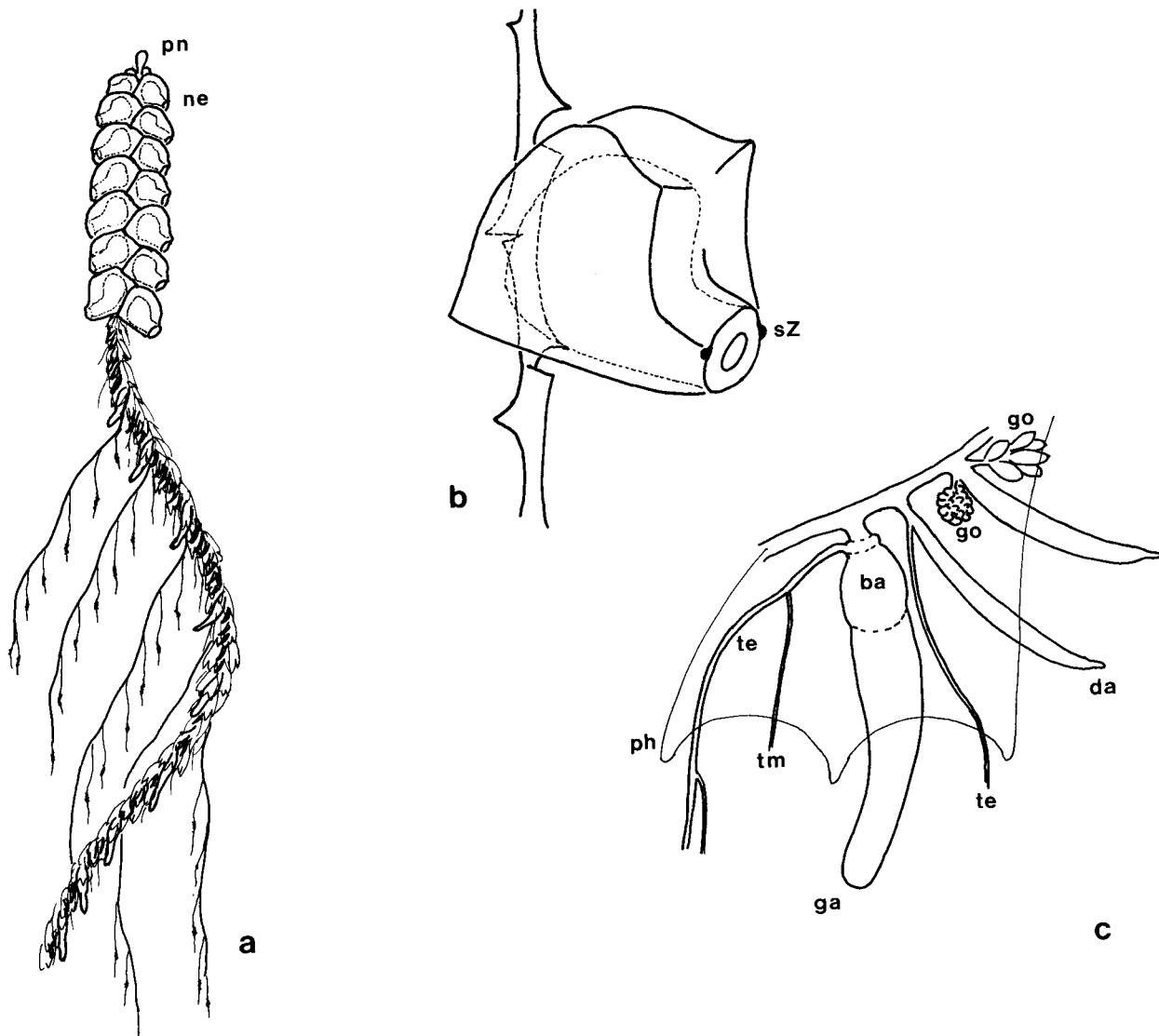


Fig. 1a–c. Schematic drawing of the physonectid siphonophore *Nanomia bijuga*: *ba* basigaster; *da* dactylozooid; *ga* gastrozooid; *go* gonodendron; *ne* nectophore; *ph* phyllozooid; *pn* pneumatophore; *sZ* “seitliche Zapfen”; *te* tentacle; *tm* tentillum. **a** Whole animal, consisting of the nectosome (upper region with nectophores and pneumatophore) and siphosome (lower region). **b** Part of the nectosome showing only one nectophore. **c** Part of the siphosome

phosphate buffer pH 7.0, at 0°C for 24 h. After fixing, the tissue was rinsed for 1 h in PBS (150 mM NaCl, 10 mM sodium-phosphate buffer, pH 7.0), for 4 h in 0.2 M glycine pH 7.0, to remove all traces of formaldehyde, and for 1 h in PBS containing 0.25% Triton X-100 (PBS-Triton). The specimens were then incubated overnight at 0°C in antiserum 146II diluted 1:1000 with PBS containing 0.25% Triton X-100 and 0.25% human serum albumin (PBS-Triton-HSA). The following day the specimens were rinsed several times for 1 h in PBS-Triton and subsequently incubated for 3 h in fluorescein isothiocyanate-labelled goat anti-rabbit IgG (Miles Laboratories) diluted 1:80 with PBS-Triton-HSA. The specimens were then rinsed (10 min) in PBS-Triton and counterstained in a 1% solution of Evans blue (Merck) in PBS (1 min). Excess stain was removed by rinsing for 1–2 h in PBS-Triton (the specimens should have a light-blue colour). The animals were mounted in buffered glycerol and examined with either a Leitz Orthoplan or

Zeiss IM35 microscope (BP 450–490, FT 510 and LP 520 filters).

Immunocytochemistry of sections

Animals were fixed as described above, rinsed for 10 min in PBS, dehydrated (70%, 96% 100% ethanol, 100% xylene; every step for 2 h) and embedded in paraffin. Sections, 10 µm thick, were mounted on gelatin-coated slides and quickly rehydrated (xylene 100%, 10 min; ethanol 100%, 96%, 70%, each 2 min; PBS-Triton, 10 min). The sections were subsequently incubated in antiserum 146II, diluted 1:1000 with PBS-Triton-HSA for 5 h in a humid chamber at room temperature. After rinsing for 20 min in PBS-Triton, the sections were incubated in fluorescein isothiocyanate-labelled goat anti-rabbit IgG for 1 h, rinsed for 10 min in PBS-Triton, counterstained for 1 min in 1% Evans blue in PBS and rinsed for 20 min in PBS-Triton until they had

a light-blue colour. The sections were then examined as described above.

Antisera

The RFamide antiserum 146II was raised in rabbit and characterized as described before (Grimmelikhuijzen 1985). This antiserum has a high affinity for RFamide, but also cross-reacts with peptides containing the sequence RY-amide, such as peptides of the pancreatic polypeptide family (cf. Triepel and Grimmelikhuijzen 1984a). However, several guinea-pig FMRFamide antisera have been raised that do not cross-react with members of the pancreatic polypeptide family (Triepel and Grimmelikhuijzen 1984b; Grimmelikhuijzen and Graff 1985). One of these antisera (code 550) has been extensively characterized on sections of guinea-pig brain, and was also found to lack cross-reaction with any known brain peptide (Triepel and Grimmelikhuijzen 1984b). By using a double labelling technique (Grimmelikhuijzen et al. 1982b; Grimmelikhuijzen 1983b) with guinea-pig antiserum 550 and rabbit antiserum 146II, we found that exactly the same structures were stained in siphonophores. Thus, the antigen in siphonophores is not one of the commonly occurring brain peptides, but might be related to any neuropeptide bearing the RFamide moiety.

Muscle staining

After staining with the primary antiserum and fluorescein isothiocyanate-labelled secondary antibody (see above), whole mounts were stained for actin with rhodamine-coupled phalloidin (1–10 µg/ml in PBS-Triton-HSA) for 2 h at room temperature. The preparation was then rinsed (10 min) and examined with BP 450–490, FT 510 and BP 520–560 filters for selective fluorescein-isothiocyanate visualization, and BP 546, FT 580 and LP 590 filters for selective rhodamine fluorescence. Rhodamin-coupled phalloidin was a kind gift of Dr. W. Jahn, Max-Planck-Institut für medizinische Forschung, Heidelberg.

Injection of Lucifer yellow

In intact, anesthetized animals, the superficial "giant axon" was penetrated with a micropipette, tip-filled with a 5% solution of Lucifer yellow CH and back-filled with 1 M LiCl. Lucifer yellow CH was iontophoresed for approximately 10 min with a continuous hyperpolarizing current of 5 nA.

Results

*The nervous system of *Nanomia bijuga**

Figure 2 summarizes our findings on the organization of the RFamide-immunoreactive nervous system of *Nanomia bijuga*. The reader is referred to this figure and to Fig. 1 for general features of physonectid morphology.

Whole-mount staining of the stem of *N. bijuga* with a RFamide antiserum, revealed an ectodermal network of strongly immunoreactive, multipolar neurons (Fig. 3). The neurons of this net appeared to be fused with each other, as the processes of one perikaryon were continuous with those of others (Fig. 3a). Only in a few cases was the "giant axon" of the stem found to be immunoreactive (Fig. 3c).

Iontophoresis of Lucifer yellow into the "giant axon" showed that the axon was "fused" with an ectodermal nerve net that resembled the RFamide-positive network of the stem (not shown, but see Fig. 9b).

At regular intervals, the network of the stem was interrupted by transverse bands of neurons (Fig. 3b). These bands consisted of rings of closely packed perikarya and dense neuropile. Double-labeling with a fluorescent stain for actin (rhodamine-coupled phalloidin) revealed that the longitudinal musculature in the stem was interrupted at the sites of the neuronal bands (not shown). In sections, the longitudinal muscle of the stem appeared to be innervated, superficially, as well as in the deeper layers, by processes of the nerve net.

The apex of the pneumatophore (float) contained a group of slender, unipolar neurons that projected cilia to the surface (Fig. 3d). This group of ectodermal "sensory" neurons was connected through a fine neuronal net to a collar of neurons at the junction of pneumatophore and stem (cf. Fig. 2b). The neurons of this collar were elongated "sensory" neurons with their processes connected to the stem network.

The nectophores (swimming bells) were connected to the stem by a muscular anchor (lamella) arising from the stem ectoderm. This structure had an elongated bowl-like shape and was supplied, around its edges, with a chain of spherical neurons (see Fig. 2c). This chain was connected by multipolar neurons to the nerve net of the stem. In the center of the anchor, a cluster of neurons occurred.

At the margin of the nectophores an immunoreactive nerve ring was observed (Figs. 4, 5). This ring consisted of aligned "sensory" neurons, the perikarya of which were lying at the exumbrellar side of the velar base (Fig. 4a, c). In two opposed patches of the margin ("seitliche Zapfen" of Claus 1878), clusters of "sensory" neurons were seen (Fig. 4b). Numerous processes connected these neurons to the nerve ring. At the upper edge of the "seitliche Zapfen" processes projected a short distance along the exumbrellar surface of the bell (see Fig. 2d). Staining for actin revealed that these projections were associated with muscle fibres (not shown). In the neighbourhood of the "seitliche Zapfen" several neuronal processes projected from the nerve ring, along the exumbrellar surface of the velum to its rim (Fig. 4d). In preparations that were stained for actin (Fig. 5d, e), these radial projections were found to lie adjacent, but outside the area where the "fibres of Claus" (Claus 1878; Mackie 1964) occurred. In contrast to a previous report (Mackie 1964), the staining for actin showed that the "fibres of Claus" were situated at the exumbrellar side of the velum (and not at the subumbrellar side). Two additional, large patches of muscle fibres were found at the exumbrellar side of the bell margin, close to the "fibres of Claus" (Fig. 5e, cf. Fig. 2d). None of these muscles were innervated by RFamide-positive neurons (Fig. 5d, e).

In between the "seitliche Zapfen", on the upper side of the bell margin, a delta-shaped structure of neuronal parikarya and processes projected from the nerve ring radially along the exumbrellar surface (Fig. 5a). This structure was not longer than 1/5 of the length of the nectophore. After actin staining, the delta-shaped structure was found to be associated with muscle fibres (Fig. 5b, c; cf. Fig. 2d). Sections confirmed that all RFamide-immunoreactive neurons in the nectophores were ectodermal. They also showed that most processes of the nerve ring were situated at the

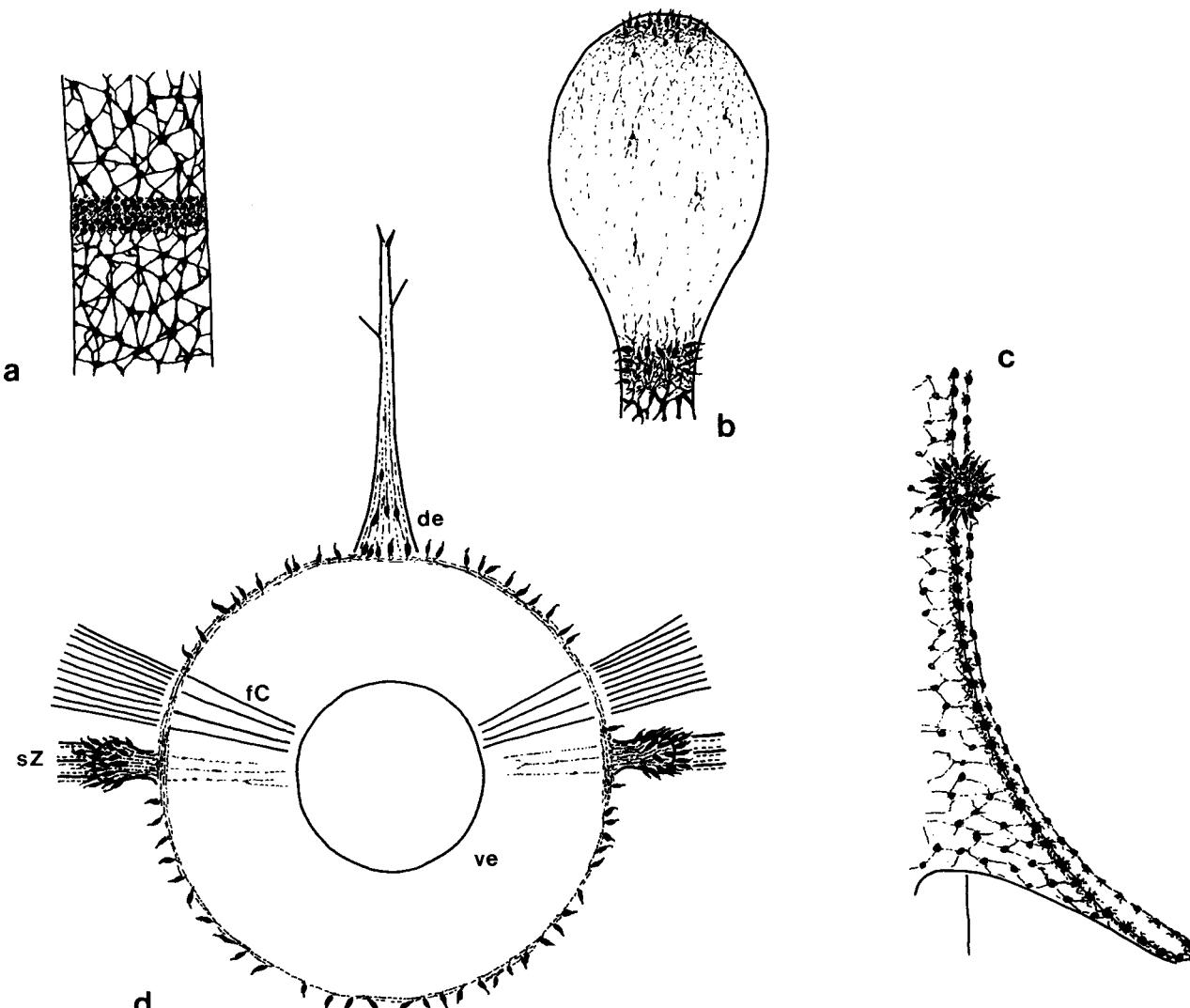


Fig. 2a–i. Schematic representation of the RFamide-positive nervous system in various parts of *Nanomia bijuga*: *de* delta-shaped structure; *fc* “fibres of Claus”; *sZ* “seitliche Zapfen”; *ve* velum. **a** Part of the stem with its nerve net and transverse neuronal collars. **b** The pneumatophore with a group of “sensory” neurons at the apex and a collar of neurons at the junction with the stem. **c** Part of the muscular lamella connecting the nectophore to the stem. The muscular lamella is bordered by a chain of spherical neurons. **d** View of the bell margin of the nectophore, showing the nerve ring, the agglomeration of neurons in the “seitliche Zapfen”, and the delta-shaped structure. Short muscle fibres (those at the “seitliche Zapfen”) and a long muscle – that of the delta-shaped structure – are innervated by RFamide-positive neurons. Two pairs of muscles (“fibres of Claus” and the patch of fibres in the ectoderm of the lower bell) lack such innervation. **e** Gastrozooid. Note the collar of neurons and dense neuropile at the junctions of basigaster, tentacle and peduncle. The collar interconnects the nerve nets of the gastrozooid, the tentacle and the stem. **f** Dactylozooid. This individual has only a few fibres in the apex and a small collar of neurons at the base. The collar interconnects the nerve nets of the tentacle, the gonodendron and the stem. **g** A lateral muscular lamella connecting the phyllozooid to the stem. The edges near the phyllozooid are bordered by a chain of spherical neurons. The neurons in the lateral positions bear clearly visible cilia. **h** Dorsal view of a gastrozooid tentacle. Two nerve tracts occur, which split up at the junction with a tentillum. **i** The peduncle of a gonodendron. Rings of round perikarya occur at the bases of the gonophores

exumbrellar side of the margin (“outer nerve ring”), although some profiles were also seen at the subumbrellar side (“inner nerve ring”).

Two lateral muscular lamellae attached each phyllozooid (bract) to the stem (Figs. 2g, 6f). An ectodermal nerve net, which was continuous with the nerve net of the stem, was associated with these lamellae (Figs. 6a, e). At the border of the lamellae and phyllozooids, a chain of spherical neuronal perikarya occurred. These perikarya, especially those in the lateral regions, carried clearly visible cilia projecting beyond the surface of the ectoderm (Fig. 6b). A double chain of neurons was also associated

with a medial, ectodermal, muscular lamella, which was lying close to the medial, endodermal bracteal canal (Figs. 6c, d). Sections confirmed that all immunoreactive structures in phyllozooids were ectodermal.

Whole mounts of gastrozooids showed a dense neuronal network in the ectoderm of the body column and mouth region (Fig. 7). This network consisted of perikarya projecting to the ectodermal surface (“sensory” neurons) and of associated processes running longitudinally between the underlying muscle fibres. The greatest density of perikarya was found around the mouth opening. Scattered neurons were found at the surface of the basigaster (Fig. 7b). In

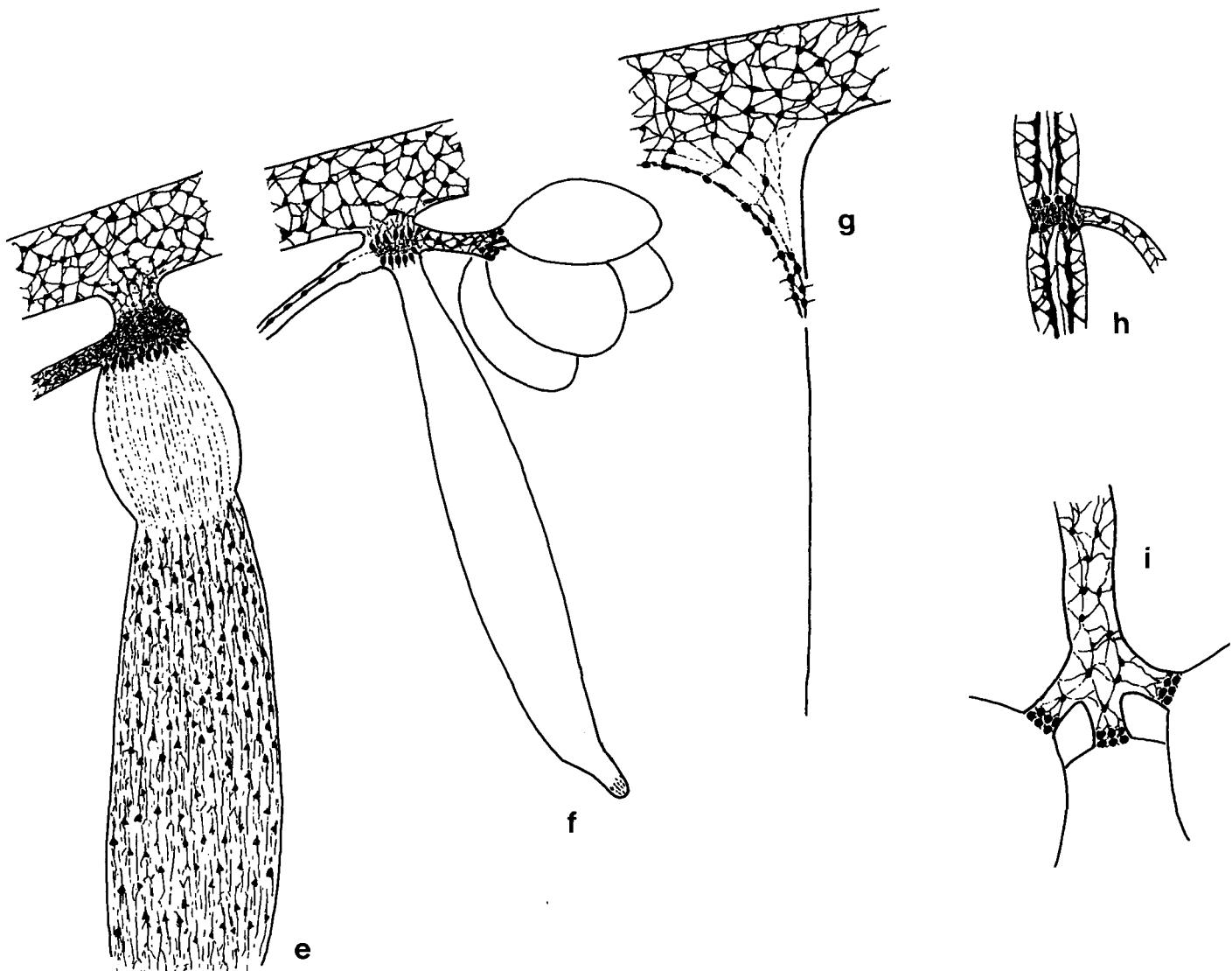


Fig. 2 (continued)

the deeper (muscular) layers of the basigaster, fine, longitudinally orientated processes could be seen. At the junction of the gastrozoid, tentacle and peduncle (cf. Fig. 1c), a collar of densely packed neurons occurred. This collar consisted of rows of "sensory" neurons and of an extremely dense network of fine processes. This neuropile-like network was connected to the fibres in the basogaster, to processes in the peduncle (which were connected to the nerve net of the stem) and extended a short distance into the tentacle. Because of the high density of immunoreactive (fluorescent) processes in the neuronal collar of the gastrozoid, we have been unable to obtain satisfactory photographs of this structure, but a drawing is given in Fig. 2e.

Sections of gastrozoids confirmed the ectodermal location of all immunoreactive structures (Fig. 8). Sections through the basigaster showed a dense plexus of fine processes, which was associated with longitudinal muscle fibres (Fig. 8d). The plexus of the basigaster connected the net of the more terminal portions of the gastrozoid with the neuronal collar at the base.

Whole mounts of dactylozooids (palpons) showed only a few fibres at the apex and no immunoreactive neuronal structures in more proximal regions. At the junction of dac-

tylozooid, peduncle and tentacle, a collar of ectodermal neurons occurred (see Fig. 2f). Sections confirmed the ectodermal location of this structure.

The tentacles of dactylozooids possessed one major tract of unipolar neurons, which at some locations diverged to form two or more chains. The tentacles of gastrozoids had a well-developed ectodermal nerve net and two closely aligned longitudinal tracts. These tracts were thicker than those of the dactylozooid tentacles and had apparently been formed by fusion of neurons of the ectodermal network (not shown, but compare Figs. 2h, 9c). At the junction with a tentillum, the tracts diverged to join two incomplete rings of round perikarya, lying each side of the junction (Fig. 2h). Neuropile connected these neuronal rings to each other and to the nerve net of the tentillum. The nerve nets of the tentilla were less well-developed than those of the tentacles (Fig. 2h). The cnidobands and their caps possessed a diffuse net containing "sensory" neurons. Sections showed that all immunoreactive neuronal structures in the tentacles and tentilla were ectodermal.

The peduncles of the gonodendra were covered by an ectodermal nerve net, which was continuous with the neuronal collar of a dactylozooid, or with the network of the

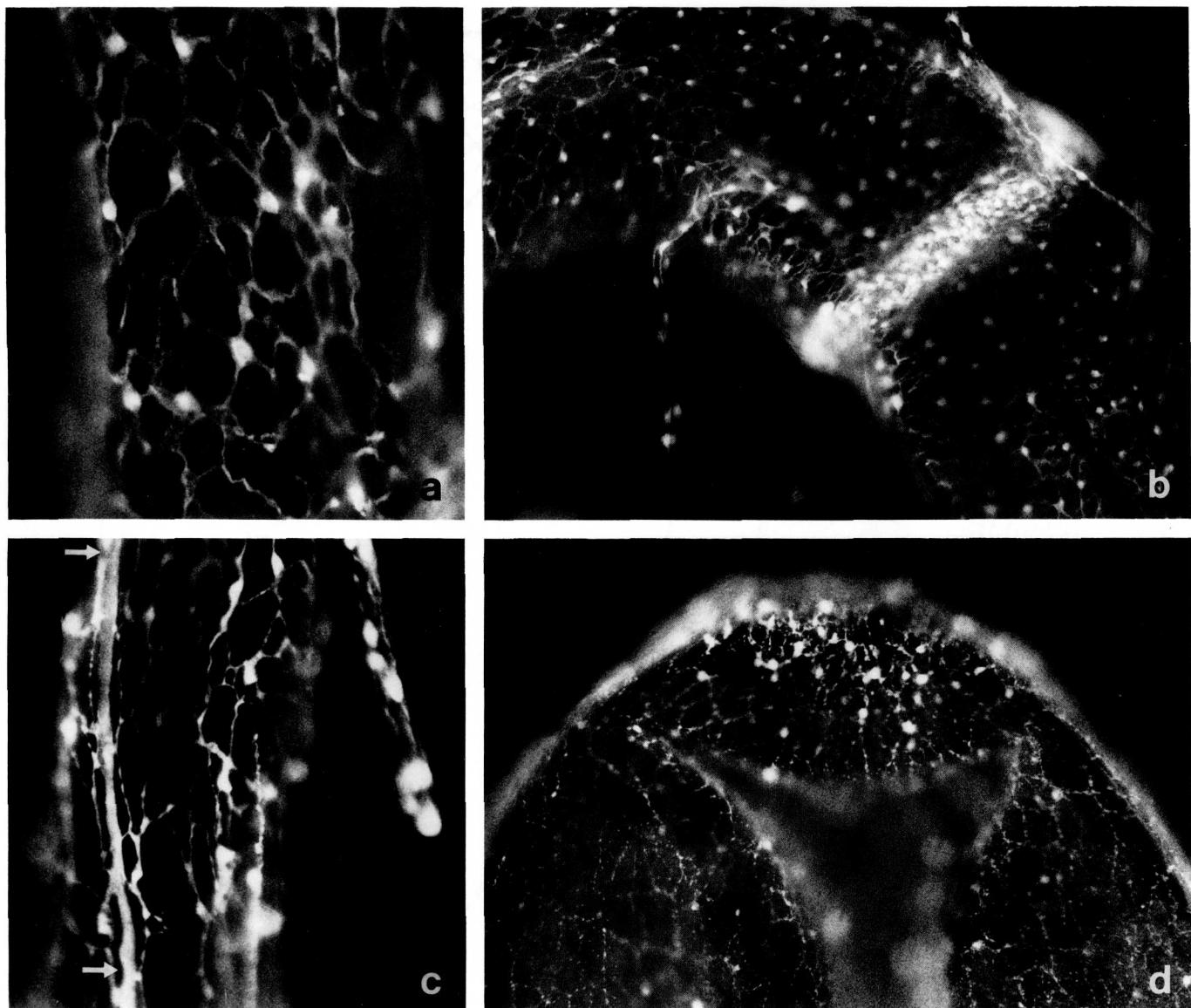


Fig. 3a-d. Whole mounts of *Nanomia bijuga*, stained by a RFamide antiserum. **a** The ectodermal nerve net of the stem. Note that the processes of this net are apparently fused with each other. $\times 440$. **b** A transverse neuronal collar in the ectoderm of the stem of the siphosome. $\times 160$. **c** "Giant axon" of the stem (arrows) and associated nerve net. $\times 280$. **d** Apex of the pneumatophore. $\times 180$.

stem (Fig. 2f). At the junction of the peduncle with the gonophore, rings of spherical perikarya were seen (not shown, but see Figs. 2i, 11b). In the gonophores themselves no immunoreactive structures occurred.

The nervous systems of *Agalma elegans* and *Halistemma rubrum*

Staining of *Agalma elegans* and *Halistemma rubrum* revealed neuronal systems, which were morphologically very similar to that of *Nanomia bijuga* (Fig. 9). In contrast to *N. bijuga*, however, the dactylozooids of *A. elegans* and *H. rubrum* had a well-developed nerve net in their main bodies (Fig. 10a, b).

The nervous systems of *Forskalia edwardsi* and *Forskalia leuckarti*

The immunoreactive nervous systems of *Forskalia edwardsi* and *Forskalia leuckarti* were, in a few respects, different

from that of *Nanomia bijuga*. The nerve net of the stem was only weakly stained, but also here transverse neuronal bands could be seen. In sections, the longitudinal muscle of the stem was found to be richly innervated (Fig. 10e).

Each nectophore was connected to the stem by a long muscular lamella, which was only sparsely innervated by scattered neurons and a few processes (see Fig. 12b). These processes were continuous with those of the stem ectoderm. At the junction of lamella and nectophore, a dense agglomeration of neurons occurred. This agglomeration consisted of rows of round perikarya, situated near the border of the lamella, and of numerous short processes (Fig. 11a, 12b). The neurons at the apex of young, developing lamellae were flask-shaped and carried clearly visible cilia ("sensory" neurons).

At the margin of the nectophores, a nerve ring was found consisting of regularly spaced "sensory" neurons (cf. Fig. 12a). The perikarya of these neurons were lying at the exumbrellar side of the bell margin and extended to

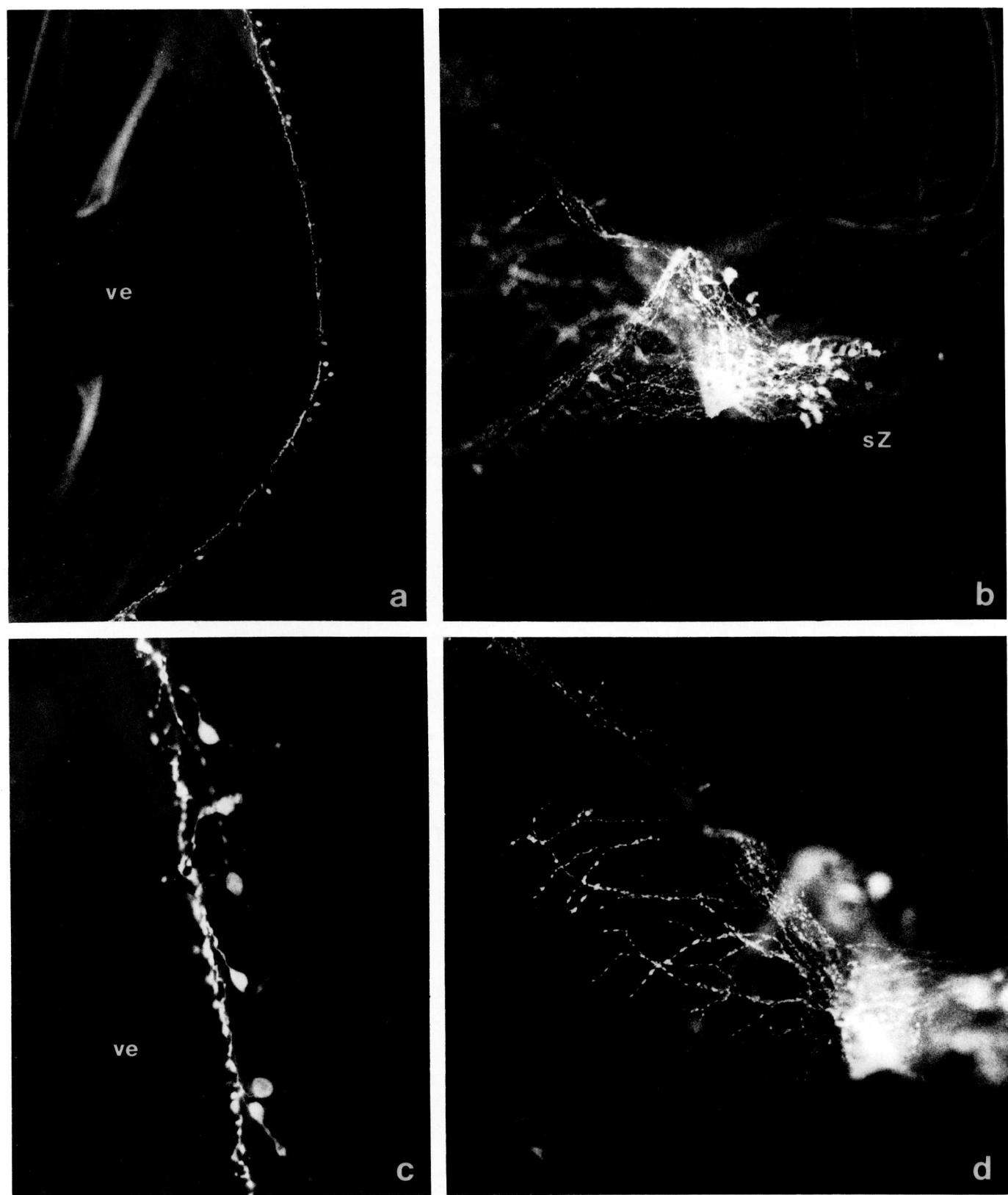


Fig. 4a-d. Whole mounts of the nectophores of *Nanomia bijuga*. sZ "seitlicher Zapfen"; ve velum. **a** General view of the lower side of the bell margin, showing the marginal nerve ring. $\times 110$. **b** "Seitlicher Zapfen". Note the presence of many sensory neurons and associated dense neuropile. $\times 160$. **c** Detailed view of the neurons of the nerve ring. $\times 440$. **d** Same "seitlicher Zapfen" as in **b**, which has now been focussed differently, to show the neuronal projections going from the nerve ring to the rim of the velum. $\times 220$

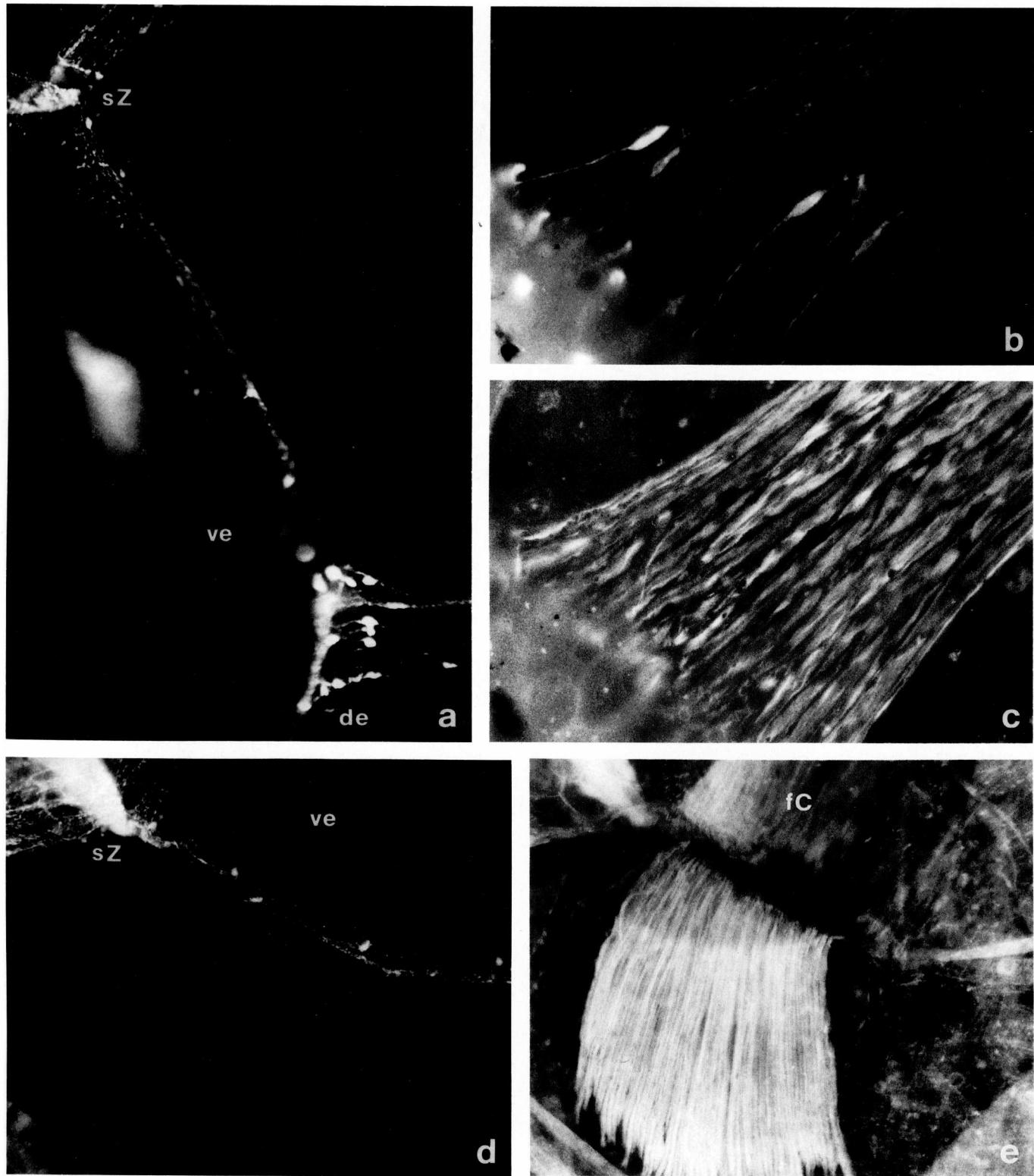


Fig. 5a–e. Whole mounts of the nectophores of *Nanomia bijuga*: *de* delta-shaped neuronal structure; *fC* “fibres of Claus”; *sz* “seitlicher Zapfen”; *ve* velum. **a** General view of the upper side of the bell margin to show the nerve ring, the “seitlicher Zapfen” and the delta-shaped neuronal structure. Note that from the “seitlicher Zapfen” and the delta-shaped structure neuronal fibres run upwards along the exumbrellar surface. $\times 180$. **b** RFamide-positive perikarya and processes of the delta-shaped structure, close to the nerve ring. $\times 420$. **c** Same view as in **b**, after switching filters specific for rhodamine fluorescence (see Materials and methods). Phalloidin-coupled rhodamine visualizes the presence of a muscle (actin). **d** Nerve ring and “seitlicher Zapfen” stained by the RFamide antiserum. $\times 180$. **e** Same view as in **d**, after changing filters for rhodamine fluorescence. Phalloidin-coupled rhodamine visualizes a large patch of muscle fibres at the exumbrellar surface of the lower bell. Opposite to this patch, at the exumbrellar surface of the velum, less well-developed fibres occur, known as the “fibres of Claus”. Note that the nerve ring is located in between these patches and that the muscles are not innervated by RFamide-positive processes.

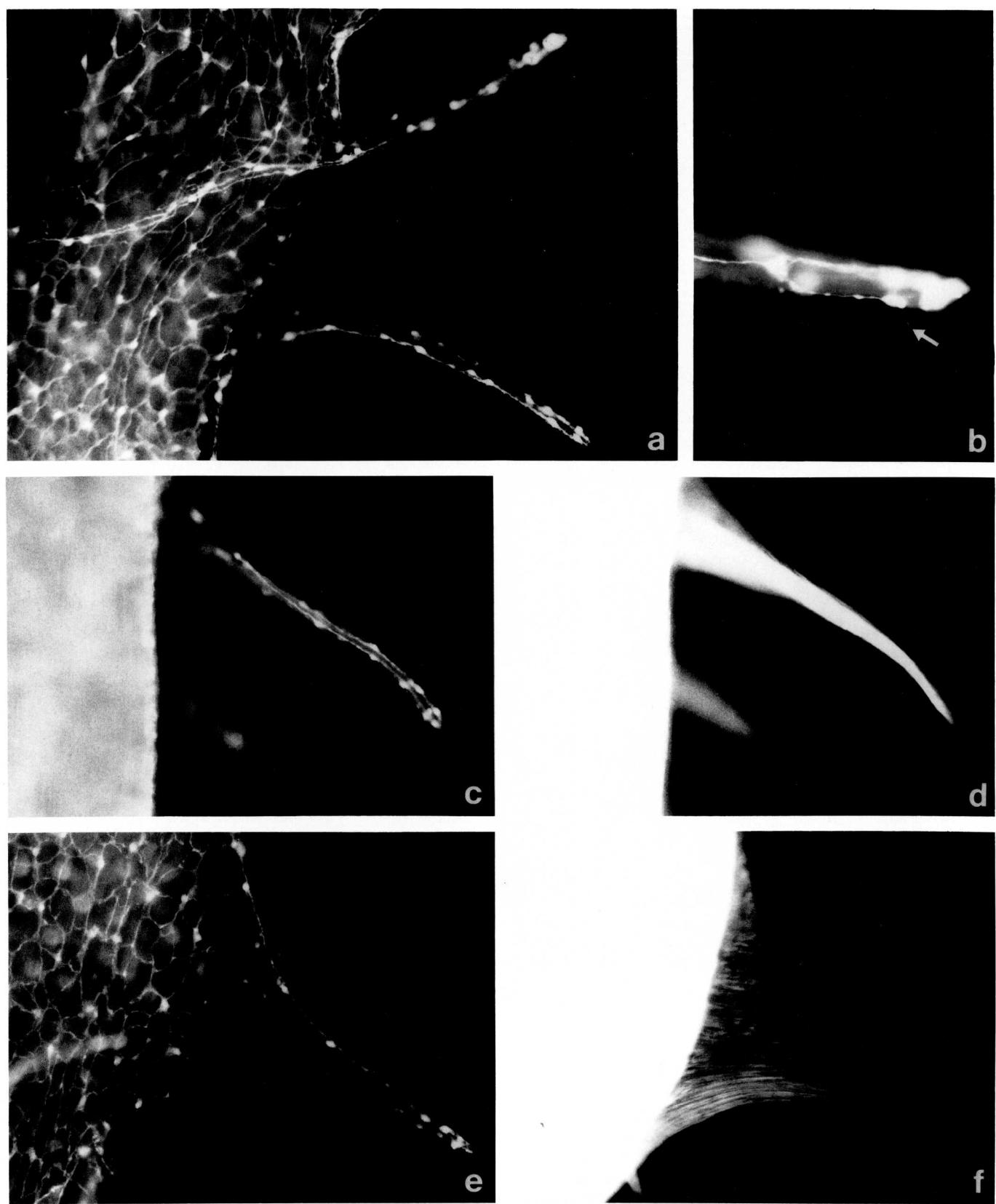


Fig. 6a–f. Muscular lamellae connecting the phyllozooids to the stem of *Nanomia bijuga*. **a** The neuronal connections of the lamellae with the stem. The upper row of neurons belongs to a medial, the lower row of neurons belongs to a lateral lamella. $\times 180$. **b** RFamide-positive neurons in the tip of a muscular lamella. Note that some neurons bear a clearly visible cilium (arrow). $\times 420$. **c** A double row of neurons, characteristic of a medial lamella. $\times 180$. **d** Same view as in **c**, after switching to filters specific for rhodamine fluorescence. Rhodamin-coupled phalloidine visualizes the medial muscular lamella. **e** The RFamide-positive nerve net of a lateral lamella. A single row of neurons occurs at the border of lamella and phyllozooid. $\times 180$. **f** Same view as in **e**, after changing to filters specific for rhodamine fluorescence. Rhodamine-coupled phalloidine visualizes the muscle fibres of the lateral lamella

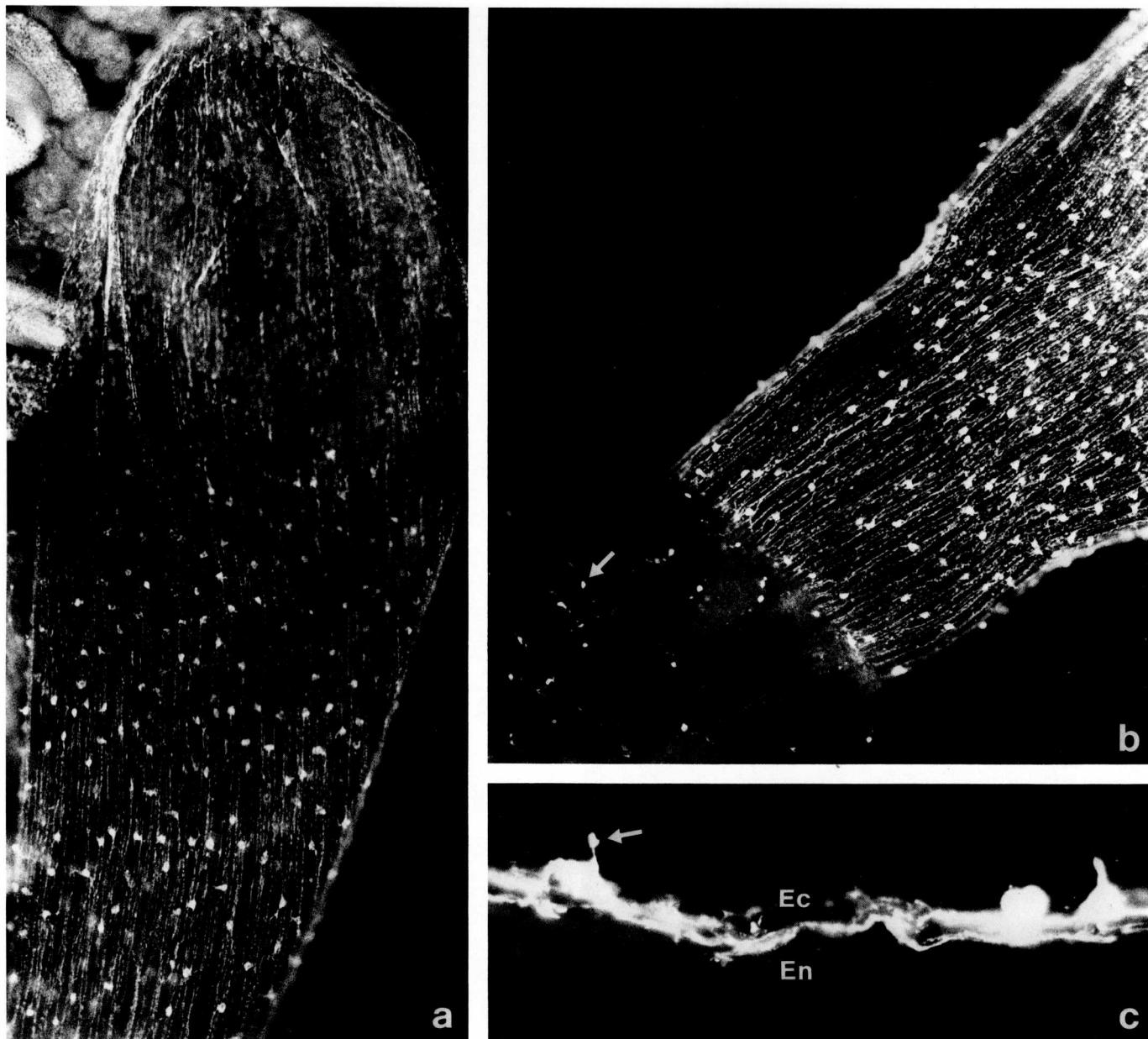


Fig. 7a–c. Gastrozoids of *Nanomia bijuga*. Ec ectoderm; En endoderm. **a** Whole mount showing a nerve net of pyramidal-shaped "sensory" neurons and longitudinally orientated processes in the upper part of the individual. $\times 160$. **b** Whole mount, showing the lower part of the gastrozoid. Note that the basigaster does not have a nerve net at its surface, but that it contains a few, scattered, superficially located neurons (arrow). $\times 160$. **c** Section through the body wall. The RFamide-positive neurons occur exclusively in the ectoderm. The perikarya have small projections reaching to the surface (arrow). $\times 500$

the surface of the ectoderm. They projected long, single processes towards the mesogleal "tri-radius", where they bifurcated at their junction with the nerve ring. At two opposite locations in the nerve ring, patches of neurons were found (Fig. 12a). These patches were located at 90° relative to the "lemon-yellow spot", which is present in swimming bells of *Forskalia* (Totton 1965). These neuronal structures were the counterparts of those found in the "seitliche Zapfen" of Claus in *Nanomia bijuga*. This became evident from two observations: (1) Neuronal projections were seen close to these patches passing radially from the nerve ring to the rim of the velum. (2) After staining for actin, pairs of muscles were observed ("fibres of Claus")

lying adjacent to these neuronal patches. No counterpart of the delta-shaped neuronal structure of *Nanomia bijuga* could be found.

Dactylozooids of *F. edwardsi* and *F. leuckarti* differed from those of *N. bijuga* by having a well-developed immunoreactive nerve net. Many fine, longitudinal fibres were seen close to the apex, and a network of multipolar neurons was found more proximal to this area (see Fig. 12c). The nerve net in the basal parts of the dactylozooids consisted of small, mainly bipolar perikarya, and of long processes which were often arranged in longitudinal tracts (Fig. 10c). As in *N. bijuga*, a neuronal collar occurred at the junction of dactylozooid, tentacle and stem (Fig. 10d).

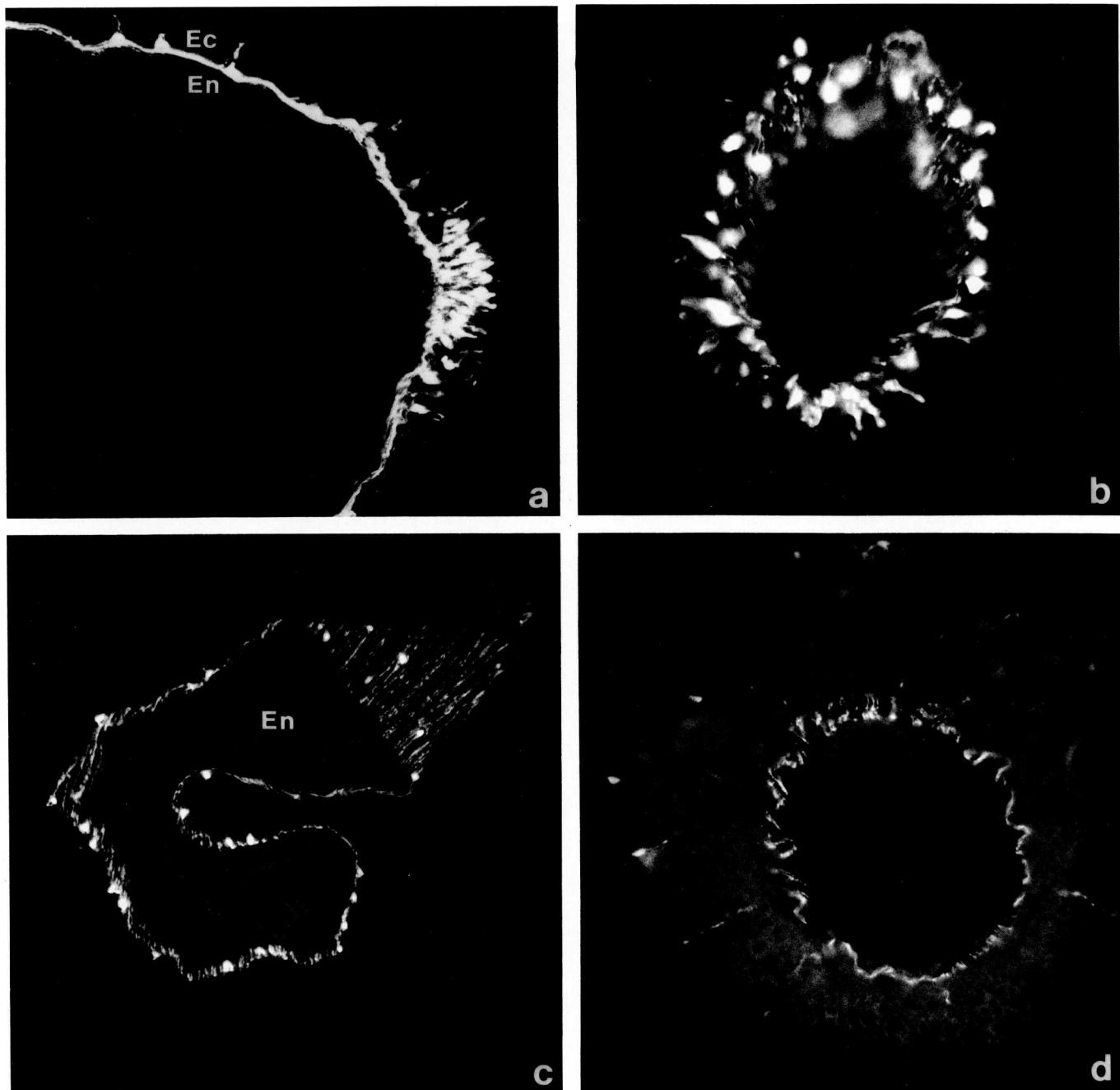


Fig. 8a-d. Sections through gastrozooids of *Nanomia bijuga*. *Ec* ectoderm; *En* endoderm. **a** Longitudinal section through the tip of a gastrozooid, to show a cluster of densely packed, slender "sensory" neurons near the mouth opening. $\times 200$. **b** Cross section through the mouth. $\times 480$. **c** Oblique section halfway up the gastrozooid. Note the longitudinal orientation of the fibres and the absence of immunoreactive neurons in the endoderm. $\times 200$. **d** Cross section through the basigaster, showing a plexus of fine, longitudinally orientated nerve fibres in the basal part of the ectoderm. The perikarya of some "sensory" neurons can be seen near the surface (cf. Fig. 7b). These neurons project to the basal plexus. $\times 280$

The nervous systems of gastrozooids of *F. edwardsi* and *F. leuckarti* were very similar to that of *N. bijuga*. The long muscular peduncles of the gastrozooids contained a diffuse network of fine nerve fibres, which connected the neuronal collars with the nerve net of the stem (Fig. 12d). The muscular lamellae, with which the peduncular bracts were connected to the peduncle (cf. Tutton 1965), were found to be bordered by chains of neurons (Fig. 12d).

Discussion

During the last hundred years several reports have appeared describing the neuroanatomy of physonectid siphonophores (Korotneff 1884; Schneider 1892; Schaeppi 1898; Mackie 1964, 1973, 1978; Jha and Mackie 1967). Owing to the techniques used, however, these reports have given a rather fragmented view of the physonectid nervous system. The

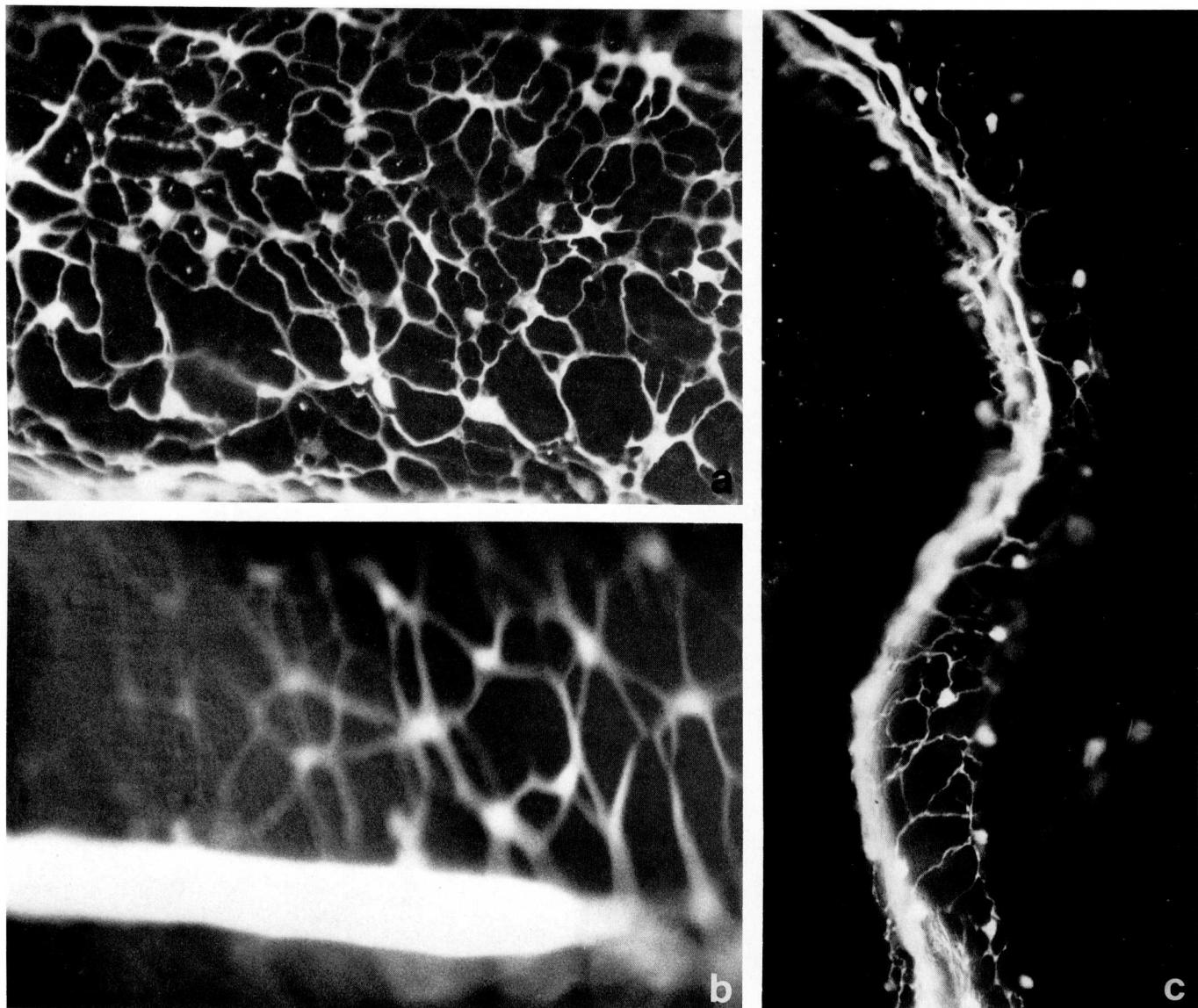


Fig. 9a–c. The nervous system in whole mounts of *Halistemma rubrum*. **a** Nerve net of the stem. Note that the processes of this net appear to be fused with each other. $\times 300$. **b** “Giant axon” of the stem after filling with Lucifer yellow. This “axon” is dye-coupled to an ectodermal nerve net that resembles the RFamide-positive network. $\times 500$. **c** Gastrozooid tentacle with two well-developed ectodermal nerve tracts. Note that the tracts appear to be formed of fused neurons. $\times 250$

present study has applied a new technique based on staining of whole mounts with an antiserum to the sequence RFamide (cf. Grimmelikhuijen and Spencer 1984; Grimmelikhuijen 1985). This method has, indeed, proven to be very useful, and we have been able to obtain a detailed, three-dimensional view of a major portion of the physonectid nervous system. In spite of the obvious advantages of this technique, however, it has to be realized that there may be elements of the nervous system that cannot be stained by the RFamide antiserum, so that our present picture of the physonectid nervous system might still be incomplete.

The ectodermal nerve net in the stem of physonectid siphonophores was formed from immunoreactive neurons, which seemed to be fused with one another (Figs. 3a, 9a). This observation is in good agreement with our finding that Lucifer yellow diffused rapidly from the “giant axon” into and through the neurons of the nerve net (Fig. 9b; Spencer and Mackie, unpublished; Mackie 1984). Coupling

or fusion of neurons appears to be quite common in hydrozoans (cf. Anderson and Mackie 1977; Spencer 1981; Spencer and Arkett 1984). In physonectid siphonophores it certainly favours rapid conduction of spike traffic through the ectoderm of the stem. A primary function of the stem nervous system is to act as a conducting substrate for escape and protective behaviours involving many individuals (Mackie 1964, 1978). The “giant axon” is the critical element for rapid longitudinal conduction, while the associated ectodermal nerve net obviously links the “giant axon” to the various individuals in the colony.

According to Mackie, there are two independent nerve nets in the ectoderm of the stem of physonectids, each associated with its own “giant axon” (Mackie 1973, 1976, 1978, 1984). Unfortunately, we have not been able to confirm this using our staining technique, as only one type of nerve net and one “giant axon” was ever seen. It is quite possible, of course, that a second nerve net and associated giant

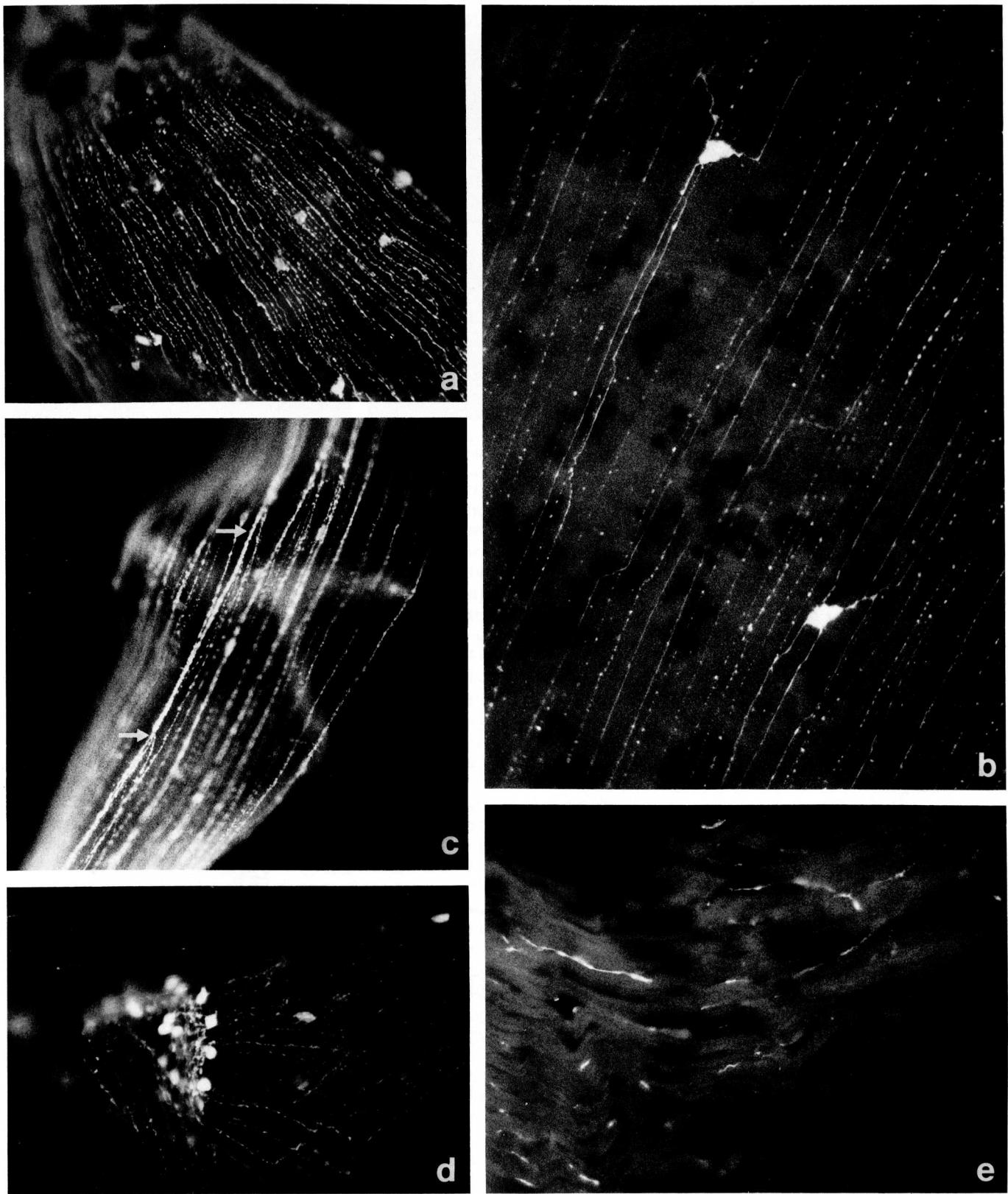


Fig. 10a–e. *Halistemma rubrum* and *Forskalia edwardsi*. **a** The apex of a dactylozooid of *Halistemma rubrum*. $\times 200$. **b** Middle region of a dactylozooid of *Halistemma rubrum*. Scattered multipolar neurons form a fine nerve net with long beaded processes. $\times 320$. **c** Basal region of a dactylozooid of *Forskalia edwardsi*. Longitudinally oriented processes are often arranged in tracts (arrows). $\times 200$. **d** A small neuronal collar at the border of dactylozooid and peduncle in *Forskalia edwardsi*. $\times 200$. **e** Section through the ectoderm of the stem of *Forskalia edwardsi*, showing longitudinal muscle fibres, associated with RFamide-positive neuronal processes. $\times 320$

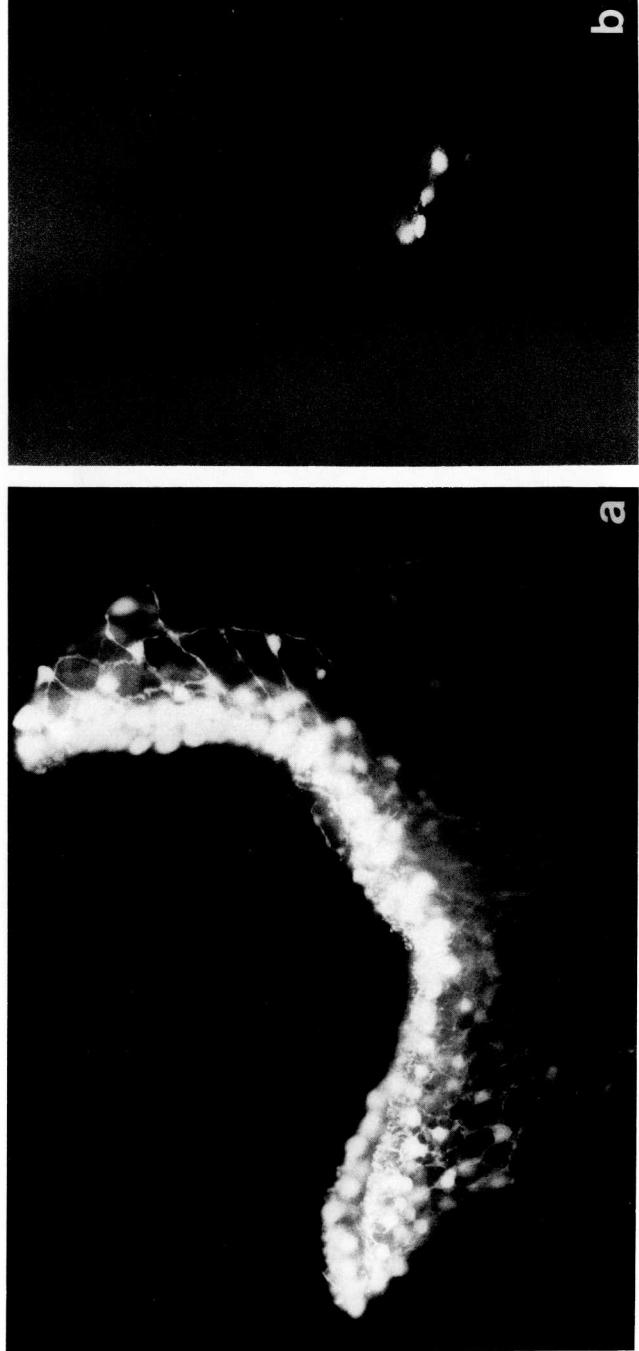


Fig. 11a, b. Whole mounts of *Forskalia edwardsi*. **a** Distal part of the long muscular lamella connecting the necrophore to the stem. Numerous spherical perikarya are seen bordering the junction of lamella and necrophore. $\times 200$. **b** Ring of round perikarya at the base of a gonophore. The peduncle of the gonodendron has no immunoreactive neurons. $\times 200$

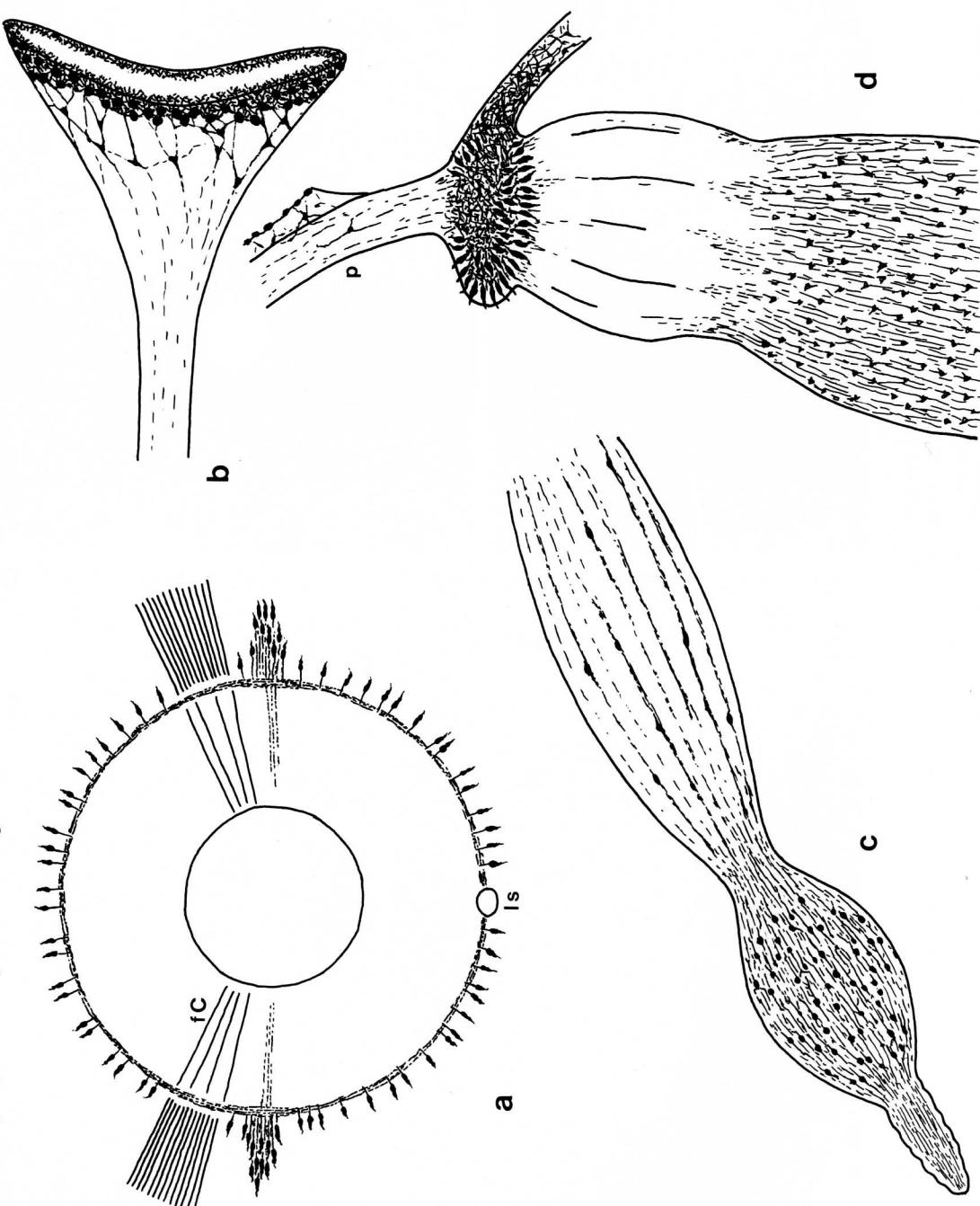


Fig. 12a-d. Schematic drawing of the RFamide-positive nervous system in some parts of *Forskalia edwardsi*. **a** "fibres of Claus"; *ls* lemon-yellow spot; *p* peduncle. **b** The long lamella connecting the stem with a necrophore. **c** The peduncle and base of a gastrozooid. **d** Peduncle and base of a zooid.

axon are present, but that it does not contain RFamide-immunoreactive material.

The function of the transverse neuronal bands, which appeared at regular intervals along the stem (Fig. 3b), is not clear at present. They could be sites where local (rather than colony-wide) contractions are initiated. In addition, they could be integration centres receiving inputs from the various adjacent individuals and deciding whether or not a colonial response should be elicited.

Korotneff (1884) and Schneider (1892) have described neurons in the pneumatophores of *Apolemia* and *Physophora*. The most interesting feature of the pneumatophore nerve net that we have visualized in *Nanomia bijuga* and other physonectid siphonophores, is the group of "sensory" neurons at its apex (Figs. 2b, 3d). It is possible that these neurons are mechanoreceptors responsible for initiating siphosomal contraction and reversed swimming when the colony receives anterior stimulation (Mackie 1964). This reversed swimming would presumably involve a neuronal-to-epithelial transfer of excitation (possibly at the junctions of muscular lamellae and nectophores; Figs. 2c, 11a, 12b), as the final pathway is supposed to be epithelial (Mackie 1964; Mackie, personal communication).

In nectophores, a marginal nerve ring has been previously described in several species, including *Nanomia* und *Forskalia* (Schaeppi 1898; Mackie 1964). In sectioned material Jha and Mackie (1967) were able to distinguish an inner (subumbrellar) and an outer (exumbrellar) nerve ring. The immunoreactive nerve ring seen in our present study (Figs. 4a, c; 5a, d), is mostly situated on the outer side, which is a situation quite similar to that found in the hydro-medusa *Polyorchis* (Grimmelikhuijen and Spencer 1984). As the nectophore and medusa are homologous individuals, it would be reasonable to presume that the RFamide-positive neurons of the outer nerve ring are involved in sensory integration and are presynaptic to the motor neurons controlling the subumbrellar swimming muscle (cf. Spencer and Arkett 1984).

In the "seitliche Zapfen" of Claus (Claus 1878), which are two areas of epithelial thickening at the bell margin of the nectophores (cf. Fig. 1), Mackie has reported finding "nervous tissue" (Mackie 1964). In our present study we have found a ganglion-like structure in these areas, consisting of sensory neurons and dense neuropile. Short processes connected these structures to the nerve ring. In the neighbourhood of the "seitliche Zapfen", long fibres projected radially from the nerve ring over the exumbrellar surface of the velum to the rim (Fig. 4). The function of these structures, which have not been described before, is unclear. They do not innervate the radial smooth muscle fibres ("fibres of Claus") which are adjacent to these regions (Fig. 5d,e), but might provide sensory input for the nerve ring. The processes which pass from the "seitliche Zapfen" upwards over the exumbrella are probably motor-neuronal, as they are associated with short muscle fibres.

At the upper and lower exumbrellar surface of the nectophore, Schaeppi (1898) has found tracts, which he regarded as being muscle fibres associated with nervous elements. Mackie (1964) rejected this view and concluded that these tracts were purely nervous. Thus, Mackie proposed a "lower nerve tract" running radially along the exumbrellar surface and connecting the nerve ring with the nervous system of the stem, and an "upper nerve tract" connected to the nerve ring and projecting only a short distance along

the exumbrellar surface. With respect to the upper tract, our present study confirms the original description by Schaeppi. Positioned at the upper exumbrellar surface and connected to the nerve ring, we found a delta-shaped neuronal structure, which was associated with a muscle running a short distance along the exumbrella (Figs. 2d, 5a-c). The delta-shaped plexus and the associated muscle resemble the neuronal projections and the associated short muscle fibres on top of the "seitliche Zapfen". RFamide-positive neurons, then, are associated with three exumbrellar muscles, all positioned in the upper half of the bell margin. These muscles, when contracted, might change the shape of the velum, thereby deflecting the water jet during swimming. This might cause the animal to move in a more sideward direction.

In addition to the muscles mentioned above, two pairs of radial muscles occurred near the bell margin (cf. Fig. 2d). Each pair consisted of a velar component and of a component located at the exumbrellar side of the bell. The velar component has been described by Claus (1878) and was later named "fibres of Claus" by Mackie (1964). With our staining technique for actin, we found that the "fibres of Claus" were located at the exumbrellar side of the velum, in contrast to Mackie's observation, who described them "in the superior-lateral corners of the velum on the subumbrellar side" (Mackie 1964; see, however, Jha and Mackie 1967). Our observations are in accordance with the original description of Claus, who when describing the velum, stated that "the muscle layer related to the epithelium of the inner side consists, exactly like that of the swim sack, exclusively of circular fibres ...", and "the outer epithelium produces, mainly on the borders of the velum, radial fibres of less uniformity ..." (p. 25/26, Claus 1878). The patches of muscle fibres at the exumbrellar surface of the lower bell (Fig. 5e) are far better developed than the "fibres of Claus". They have, to our knowledge, never been described in the literature. The two pairs of muscles were not found to be innervated by RFamide-positive neurons, and it may be that they are not neuronally controlled. This would be in accordance with Mackie, who proposed that the "fibres of Claus" are excited by an epithelial (neuroid) pathway involved in reversed swimming (Mackie 1964).

Our RFamide antiserum did not show a continuous neuronal pathway between the nerve ring, or the marginal centres of the nectophore, and the nervous system of the stem. From a series of experiments involving ablation of potential pathways along the bell, Mackie has concluded that there must be a neuronal pathway along the lower side of the bell involved in forward swimming (Mackie 1964). This "lower nerve tract" was later reported to consist of a "giant nerve" in *Nanomia cara* and of a bundle of fine fibres in *Forskalia edwardsi* (Mackie 1965, 1973). Due to the limitation of our staining method (not all neuronal structures in physonectids might be stained by our antiserum), we are unable to draw any conclusions about the existence of these continuous neuronal tracts.

Schaeppi has reported finding neurons ("Ganglienzenlen") in the lamellae attaching the phyllozooids to the stem (Schaeppi 1898). In our present study we found that the lamellae possessed chains of bipolar neurons and a nerve net, both of which were connected to the nerve net of the stem (Fig. 6). The presence of neurons in the muscular lamellae indicates that these lamellae can contract in a controlled way. Phyllozooids have a protective function in the

colony and are covered with numerous cnidocytes. In *Nanomia bijuga* they are not known to be mobile (Mackie 1964). Mobile phyllozooids, however, are found in other siphonophores, such as *Athorybia rosacea* (Kölliker 1853; Totton 1965). It is also possible that the neurons of the muscular lamellae, and especially those located at the borders and tip (see Fig. 6b) have a sensory function. These neurons could trigger contraction, and eventually autonomy, after a noxious stimulation.

The anatomy of the nervous system of gastrozooids has not been extensively described (cf. Mackie 1978). In the present study we have visualized a well-developed neuronal net with perikarya, which showed all the characteristics of sensory neurons (Figs. 7, 8). An increased density of these cells around the mouth (Fig. 8a, b) suggests that they are involved in feeding and are, therefore, likely to be chemosensory. Feeding movements such as mouth gaping and gastrozooid writhing are organized locally, since these activities are not transferred via the stem nervous system from one gastrozooid to another (Mackie and Boag 1963; Mackie 1978). Since the processes from the sensory neurons appear to innervate the gastrozooid longitudinal muscles (Figs. 7c, 8), it is easy to imagine how a local reflexive pathway could elicit gastrozooid feeding movements.

Protective retraction of gastrozooids, in contrast to feeding, can be synchronous and this is mediated by the nervous system of the stem (Mackie 1978). A likely site for interaction between the stem nervous system and the gastrozooid-nerve net, is the collar of densely packed neurons at the base of the polyps (Fig. 2e). This collar projected processes to the well-developed longitudinal muscles of the gastrozooid and was also connected to the nerve net of the stem. An important part of the neuronal collar (especially its neuropile) extended into the ectoderm of the tentacle. This is probably the structure controlling protective contraction of the tentacle and tentilla.

Pumping activity in gastrozooids involves peristaltic waves propagating through the endodermal muscles (Mackie 1978). The associated electrical events are presumably conducted in the epithelio-muscular cells and do not involve the nervous system. This matches our inability to demonstrate immunoreactive neurons in the endoderm.

There were only a few RFamide-positive processes in the tip of dactylozooids of *Nanomia*, while a very obvious ectodermal net could be seen in *Agalma*, *Forskalia*, and *Halistemma* (Fig. 10). This net resembled that of gastrozooids, except that the perikarya were less numerous and were not obviously sensory. In this context it may be relevant that the dactylozooids are far less muscular than the gastrozooids and are not involved in feeding. They do, however, have a defensive role, might be concerned with the reception of tactile stimuli, and do perform egestive movements and protective retraction (Mackie and Boag 1963; Mackie 1978). Again, the collar of neurons at the base could be a centre for integrating signals going to or coming from the stem.

Mackie found chains of bipolar neurons in the dactylozooid tentacles of *Nanomia cara* (Mackie 1973). With our immunocytochemical technique we could confirm this finding in *Nanomia bijuga* and the other physonectid siphonophores. In addition, we found that gastrozooid tentacles possessed a nerve net and two well-developed neuronal tracts, which had apparently been formed by fusion of neurons (Fig. 9c). The purpose of these tracts is obviously

to provide a rapid through-conducting system, although the meaning of the paired arrangement is not understood. At every junction with a tentillum, the two tracts diverged, joining two small neuronal collars each side of the junction. It is likely that these junctional collars have the same function as their counterparts at the bases of the gastrozooids and dactylozooids. In addition, they might have a pacemaker function initiating local contractions of tentacles and tentilla.

The overall picture of the neuronal organization of physonectid siphonophores, which emerges from our present immunocytochemical study, is the following: The stem nervous system is the central part of the colonial nervous system and mediates colonial behaviour. Every individual, however, has a local nerve net and a centralized nervous system, consisting of collars of neurons, neuronal rings or ganglion-like structures. These neuronal condensations probably initiate local activities and control the interaction with the nervous system of the stem.

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References

- Anderson PAV, Mackie GO (1977) Electrically coupled, photo-sensitive neurons control swimming in a jellyfish. *Science* 197:186–188
- Claus C (1878) Über *Halistemma tergestinum* n. sp. nebst Bemerkungen über den feinern Bau der Physophoriden. *Arb Zool Inst Univ Wien* 1:1–56
- Grimmelikhuijen CJP (1983a) FMRFamide immunoreactivity is generally occurring in the nervous systems of coelenterates. *Histochemistry* 78:361–381
- Grimmelikhuijen CJP (1983b) Coexistence of neuropeptides in hydra. *Neuroscience* 9:837–845
- Grimmelikhuijen CJP (1984) Peptides in the nervous system of coelenterates. In: Falkmer S, Häkanson R, Sundler F (eds) *Evolution and tumor pathology of the neuroendocrine system*. Elsevier, Amsterdam, pp 39–58
- Grimmelikhuijen CJP (1985) Antisera to the sequence Arg-Phe-amide visualize neuronal centralization in hydroid polyps. *Cell Tissue Res* 241:171–182
- Grimmelikhuijen CJP, Graff D (1985) Arg-Phe-amide-like peptides in the primitive nervous systems of coelenterates. *Peptides* 6 [Suppl 3]:477–483
- Grimmelikhuijen CJP, Spencer AN (1984) FMRFamide immunoreactivity in the nervous system of the medusa *Polyorchis penicillatus*. *J Comp Neurol* 230:361–371
- Grimmelikhuijen CJP, Dockray GJ, Schot LPC (1982a) FMRF-amide-like immunoreactivity in the nervous system of hydra. *Histochemistry* 73:499–508
- Grimmelikhuijen CJP, Dierickx K, Boer GJ (1982b) Oxytocin/vasopressin-like immunoreactivity is present in the nervous system of hydra. *Neuroscience* 7:3191–3199
- Jha RK, Mackie GO (1967) The recognition, distribution and ultrastructure of hydrozoan nerve elements. *J Morphol* 123:43–62
- Kölliker A (1853) Die Schwimmpolypen der Siphonophoren von Messina. Engelmann, Leipzig

- Korotneff A (1884) Zur Histologie der Siphonophoren. Mitt Zool Sta Neapel 5:229–288
- Mackie GO (1964) Analysis of locomotion in a siphonophore colony. Proc Roy Soc [Biol] 159:366–391
- Mackie GO (1965) Conduction in the nerve-free epithelial of siphonophores. Am Zoologist 5:439–453
- Mackie GO (1973) Report on giant nerve fibres in *Nanomia*. Publ Seto Mar Lab 20:745–756
- Mackie GO (1976) The control of fast and slow muscle contractions in the siphonophore stem. In: Mackie GO (ed) Coelenterate ecology and behavior. Plenum Press, New York, pp 647–659
- Mackie GO (1978) Coordination in physonectid siphonophores. Mar Behav Physiol 5:325–346
- Mackie GO (1984) Fast pathways and escape behavior in Cnidaria. In: Eaton RC (ed) Neural mechanisms of startle behavior. Plenum Press, New York, pp 15–42
- Mackie GO, Boag DA (1963) Fishing, feeding and digestion in siphonophores. Pubbl Staz Zool Napoli 33:178–196
- Mackie GO, Singla CL, Stell WK (1985) Distribution of nerve elements showing FMRFamide-like immunoreactivity in Hydrozoa. Acta Zool Stockh 66:199–210
- Price DA, Greenberg MJ (1977) Structure of a molluscan cardioexcitatory neuropeptide. Science 197:670–671
- Schaeppi T (1898) Untersuchungen über das Nervensystem der Siphonophoren. Jena Zeit Naturwiss 25:483–550
- Schneider KC (1892) Einige histologische Befunde an Coelenteraten. Jena Zeit Naturwiss 20:379–462
- Spencer AN (1981) The parameters and properties of a group of electrically coupled neurones in the central nervous system of a hydrozoan jellyfish. J Exp Biol 93:33–50
- Spencer AN, Arkett SA (1984) Radial symmetry and the organization of central neurones in a hydrozoan jellyfish J Exp Biol 110:69–90
- Totton AK (1965) A synopsis of the Siphonophora. Trustees of the British Museum (Natural History), London
- Triepel J, Grimmelikhuijen CJP (1984a) A critical examination of the occurrence of FMRFamide immunoreactivity in the brain of guinea pig and rat. Histochemistry 80:63–71
- Triepel J, Grimmelikhuijen CJP (1984b) Mapping of neurons in the central nervous system of the guinea pig by use of antisera specific to the molluscan neuropeptide FMRFamide. Cell Tissue Res 237:575–586

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