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Antisera to the sequence Arg-Phe-amide visualize neuronal centralization in hydroid polyps

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Summary. Antisera to the sequence Arg-Phe-amide (RFamide) have a high affinity to the nervous system of fixed hydroid polyps. Whole-mount incubations of several *Hydra* species with RFamide antisera visualize the three-dimensional structure of an ectodermal nervous system in the hypostome, tentacles, gastric region and peduncle. In the hypostome of *Hydra attenuata* a ganglion-like structure occurs, consisting of numerous sensory cells located in a region around the mouth opening and a dense plexus of processes which project mostly radially towards the bases of the tentacles. In *Hydra oligactis* an ectodermal nerve ring was observed lying at the border of hypostome and tentacle bases. This nerve ring consists of a few large ganglion cells with thick processes forming a circle around the hypostome. This is the first direct demonstration of a nerve ring in a hydroid polyp.

Incubation of *Hydractinia echinata* gastrozooids with RFamide antisera visualizes an extremly dense plexus of neuronal processes in body and head regions. A ring of sensory cells around the mouth opening is the first group of neurons to show RFamide immunoreactivity during the development of a primary polyp. In gonozooids the oocytes and spermatophores are covered with strongly immunoreactive neurons.

All examples of whole-mount incubations with RFamide antisera clearly show that hydroid polyps have by no means a "diffuse nerve net", as is often believed, and that neuronal centralization and plexus formation are common in these animals. The examples also show that treatment of intact fixed animals with RFamide antisera is a useful technique to study the anatomy or development of a principal portion of the hydroid nervous system.

Key words: RFamide – Neuropeptides – Nervous system – Reproduction – Hydrozoa

The primitive nervous systems of coelenterates produce peptides, some of which are related to bombesin/gastrin-releasing peptide, oxytocin/vasopressin, and the molluscan neuropeptide Phe-Met-Arg-Phe-amide (cf. Grimmelikhuijzen 1984, for a recent review). Especially Phe-Met-Arg-Phe-amide (FMRFamide)-related peptides are abundant in the nervous systems of coelenterates (Grimmelikhuijzen et al. 1982a; Grimmelikhuijzen 1983a; Grimmelikhuijzen and Spencer 1984). Whole-mount staining of *Hydra* with methylene blue during the last 90 years has often led to the assumption that hydroid polyps have "an elementary network of loosely interconnected neurons" with no "morphologically definable center of coordination" (Tardent and Weber 1976), despite the fact that in the original papers a concentration of neurons in hypostome and foot was often recognized (Schneider 1890; Hadži 1909; McConnell 1932; Spangenberg and Ham 1960; Burnett and Diehl 1964). More recent work on light- and electron-microscopic sections has further shown that "some" centralization occurs in the hypostome of *Hydra* (Davis et al. 1968; Kinnamon and Westfall 1981), but the misconception of a decentralized nervous system in hydroid polyps is still presented in most of the introductory textbooks of zoology. In the present paper I will clearly demonstrate, using whole-mount incubations with antisera to the sequence Arg-Phe-amide (RFamide), that hydroid polyps have by no means a diffuse, loosely interconnected nerve net, and that neuronal centralization and plexus formation are common.

Materials and methods

Animals

Hydra attenuata, *Hydra circumcincta*, *Hydra oligactis*, *Hydra viridis* and *Hydractinia echinata* were cultured in the laboratory (Grimmelikhuijzen et al. 1982a, b; Müller 1984). *H. circumcincta*, *H. oligactis* and *H. viridis* were a kind gift from Dr. T. Holtstein (Munich). A different batch of *H. oligactis* was kindly provided by Dr. H. Bode (Irvine). Larvae of *Hydractinia echinata* were brought to metamorphosis (and form primary polyps) through a treatment with Cs^+ ions (Müller 1984). Sexuality in *H. circumcincta* was induced by irregular feeding and washing. Sexuality in *H. oligactis* was induced by keeping an asexual male and female stock at 4° C for 10 days and returning them afterwards to normal (20° C) culture conditions.

Antisera

Antisera to the sequence RFamide were obtained by immunizing four rabbits with synthetic RFamide (Bachem), which was coupled via carbodiimide to bovine thyroglobu-

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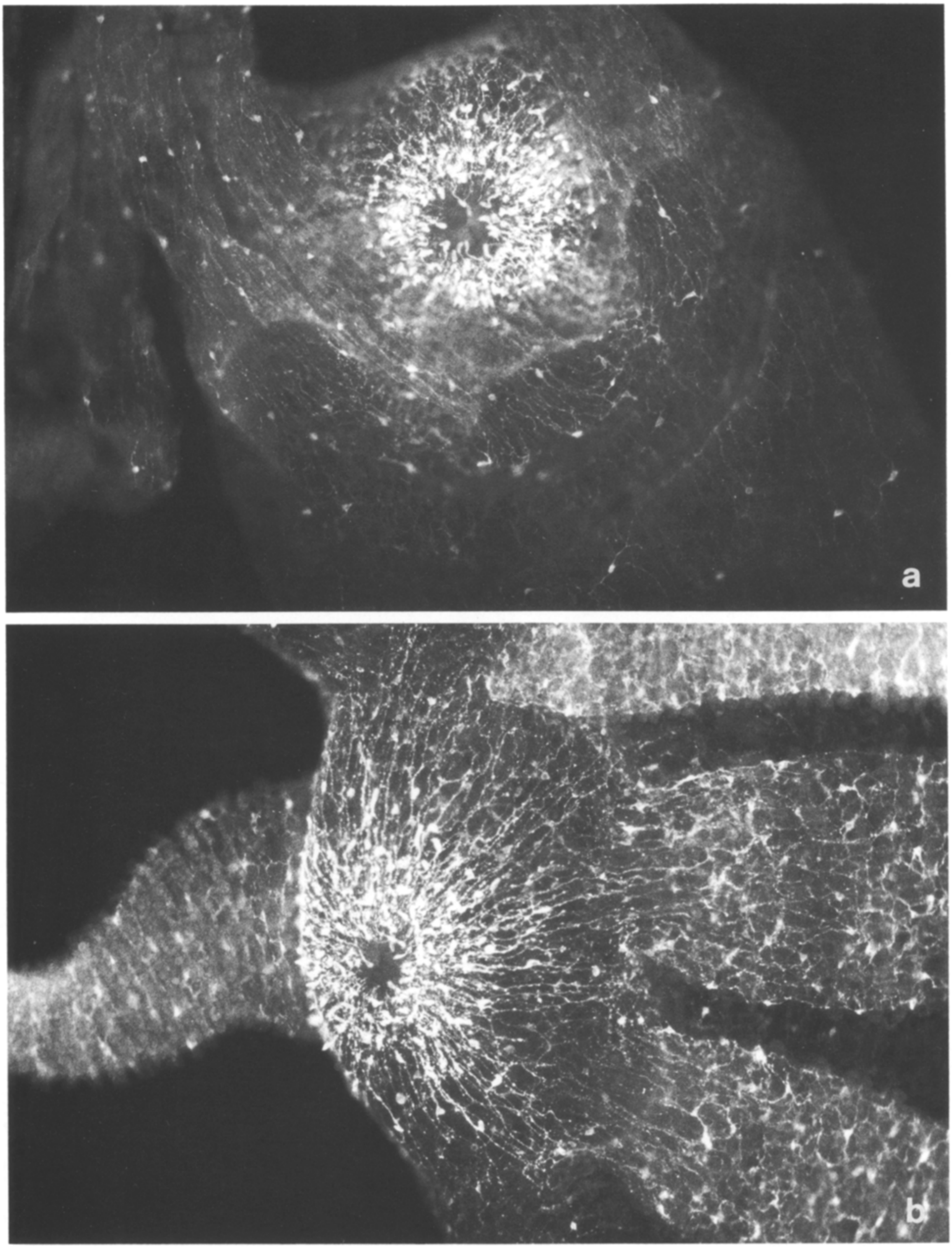

Fig. 1a, b. Whole-mount staining of *Hydra attenuata* by RFamide antiserum 146II. **a** Low power micrograph showing a strong agglomeration of sensory cells around the mouth opening and a weaker innervation of tentacles and upper gastric region. × 160. **b** Side view showing numerous sensory cells and a dense plexus of radially orientated processes in the apical half of the hypostome. × 160

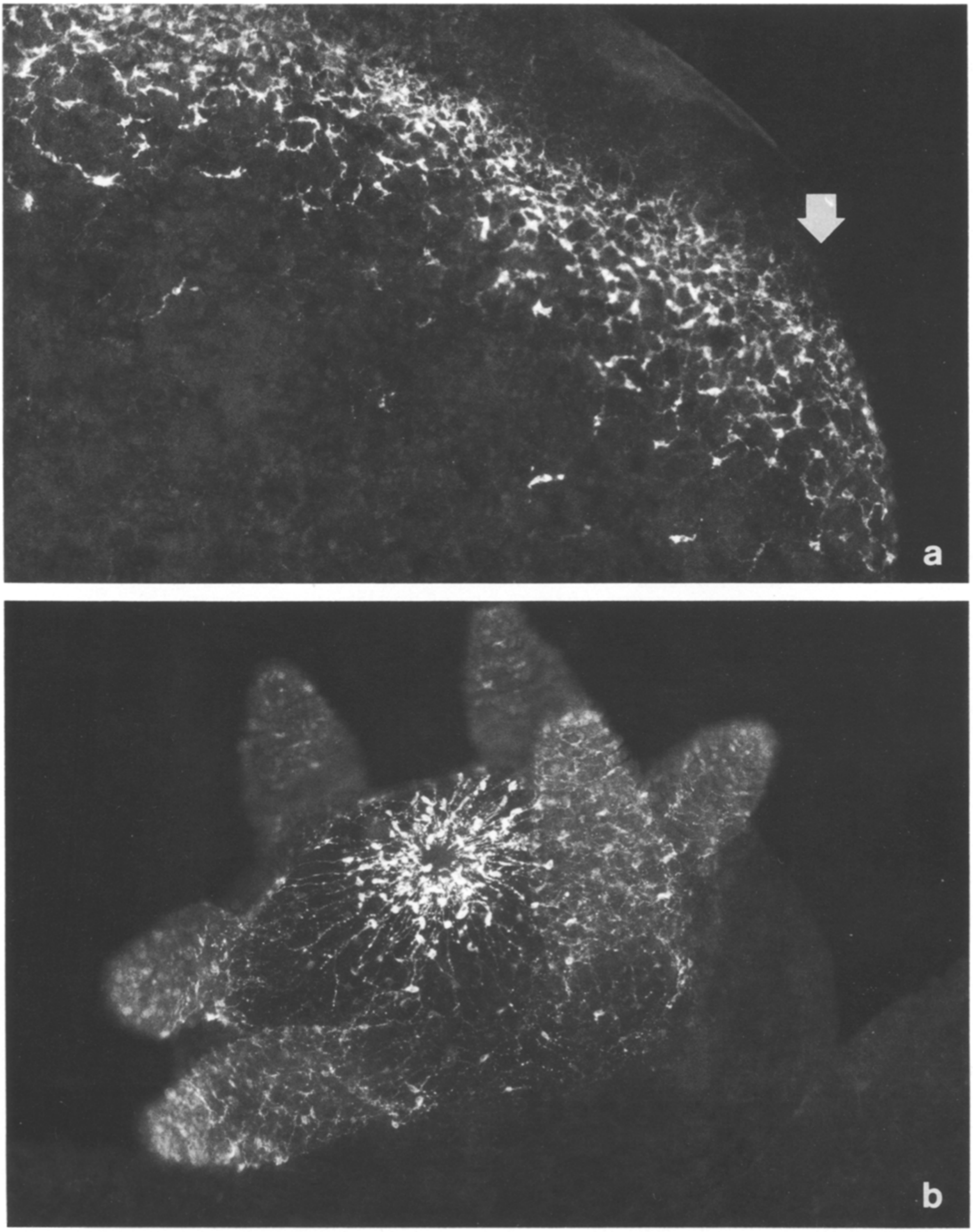

Fig. 2a, b. Whole-mount staining of *Hydra attenuata* by RFamide antiserum 146II. **a** A collar of densely packed multipolar ganglion cells in the ectoderm of the lower peduncle. The *arrow* points to the basal disk. ×160. **b** Bud, half-way development. Note that the agglomeration of sensory cells around the mouth is already well developed. ×160

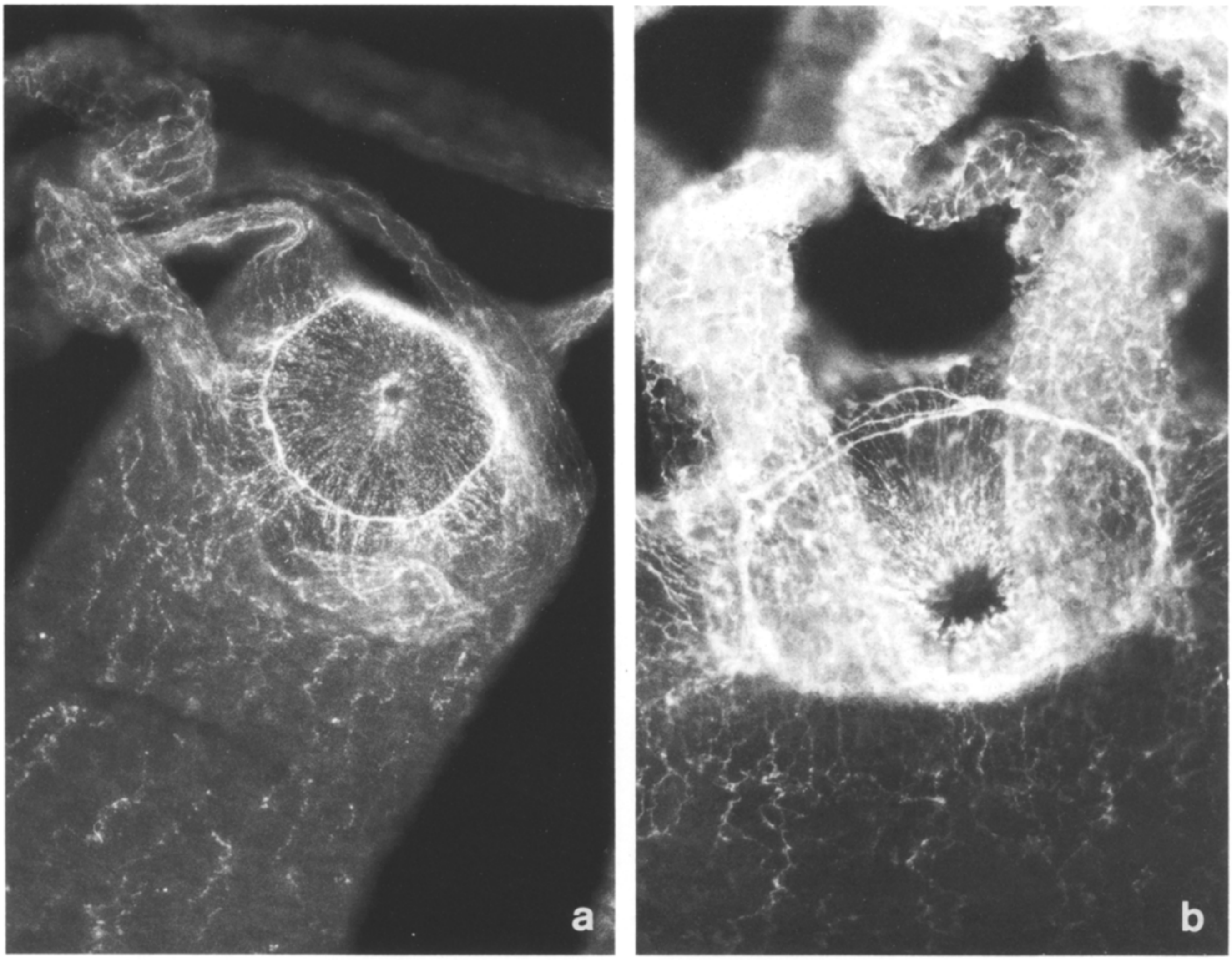

Fig. 3a, b. Whole-mount staining of *Hydra oligactis* by RFamide antiserum 146II. **a** Low power micrograph showing the strong radial bias of the dense plexus of fibers in the hypostome and the nerve ring running between hypostome and tentacle bases. As the sensory cells of the hypostome are only weakly immunoreactive, they are not visible here. Note that the upper gastric region is rather well innervated. × 110. **b** Closer view of the hypostomal nerve ring to show that the ring consists of thick, interlaced processes of ganglion cells. × 180

lin (Sigma). The antisera were treated overnight at 0° C with 2 mg/ml thyroglobulin, followed by centrifugation to remove antibody populations specific for the carrier protein. All four rabbits produced antisera with excellent staining properties (Codes 143, 144, 145, 146). In the present study only one antiserum (146, bleeding II; 146II) has been used.

Immunocytochemistry

Polyps were fixed in a fresh solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0, for 24 h at 0° C. Polyps of *Hydractinia* were anesthetized before fixation by incubation in sea water, mixed 1:1 with isotonic (0.4 M) $MgCl_2$. Such a procedure, however, could not be developed for *Hydra*. After fixation the specimens were washed, at room temperature, with 0.1 M phosphate buffer, pH 7.0 (2 h), with 0.4 M glycin, pH 7.0 (4 h), and finally with PBS-Triton (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.0, 0.25% Triton) for 2 h. The polyps were then transferred in wells of ELISA plates and incubated overnight at 0° C in a humid chamber with antiserum 146II diluted 1:1000 with PBS-Triton-HSA (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.0, 0.25% Triton, 0.25% human serum albumin). The following day the polyps were rinsed several times with PBS-Triton at room temperature (1 h) and subsequently incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Miles Laboratories), diluted 1:80 with PBS-Triton-HSA (3 h). The animals were then shortly rinsed in PBS-Triton (10 min), incubated in a 1% solution of Evans Blue (Merck) in PBS (1 min) and frequently washed for 1–2 h in PBS-Triton until only little colour could be washed from the animals. The specimens were then mounted in buffered glycerol and examined with a Zeiss IM35 microscope equipped with BP450–490, FT510 and LP520 filters for fluorescein-isothiocyanate visualization (Evans Blue gives a red counterstaining at these wavelengths).

Specificity of the RFamide antisera

Solid-phase absorption of the RFamide antisera (diluted 1:1000) with 100 µg/ml RFamide-Sepharose did abolish all staining in hydroid polyps. This suggests (but does not prove) that the hydroid antigen contains the sequence RF-amide. All RFamide antisera stained endocrine cells in chicken and rat pancreas, which shows that the RFamide antisera have the same shortcoming as most of the FMRF-amide antisera, namely a cross-reaction with peptides containing the sequence RYamide such as avian and bovine pancreatic polypeptide (cf. Triepel and Grimmelikhuijzen 1984a). However, several guinea-pig FMRFamide antisera have been raised by the author that do not cross-react with peptides of the pancreatic polypeptide family (Triepel and Grimmelikhuijzen 1984b; Grimmelikhuijzen 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 of the other known brain peptides (Triepel and Grimmelikhuijzen 1984b). By using a double-labeling technique (Grimmelikhuijzen et al. 1982b; Grimmelikhuijzen 1983b) with guinea-pig antiserum 550 and rabbit antiserum 146II, I found that in hydroid polyps both antisera stained exactly the same structures. Thus, the hydroid antigen is not one of the commonly occurring brain peptides, but might be related to any neuropeptide bearing the RFamide moiety (such as FMRFamide).

Results

Whole-mount staining of different Hydra species

Polyclonal antisera to the sequence RFamide have excellent staining properties for the *Hydra* nervous system. These types of antisera are, in *Hydra*, even superior to the best antisera raised against the sequence FMRFamide, such as antisera 114I and 117I (Grimmelikhuijzen 1983a). This quality of the RFamide antisera, together with the relatively high transparency of hydroid polyps, makes it possible to stain the nervous system of whole, fixed animals.

Such a whole-mount staining of *Hydra attenuata* shows an extremly dense nervous structure in the ectoderm of the hypostome. This structure consists of a cluster of closely packed "sensory cells" (unipolar neurons containing a cilium) which lie around the mouth opening and in the apical half of the hypostome (Fig. 1). In adult animals this cluster consists of 150–300 cells. Every sensory cell produces one or two processes at its basal part, which aborize and project mainly radially towards the bases of the tentacles (Fig. 1b). Weakly immunoreactive "ganglion cells" (multipolar neurons) occur in the basal half of the hypostome. The processes of these ganglion cells have a radial or a more random orientation and are intermingled with the processes of the sensory cells. The hypostomal plexus forms a unity with the nervous net of the tentacles. This net consists of strongly immunoreactive multipolar ganglion cells, which project in all directions and occasionally produce a small process that reaches the surface of the tentacle ectoderm. Immunoreactive sensory cells are also present in the tentacles, but to a much lesser extent (mainly in the tip).

Low amounts of immunoreactive neurons occur in the subhypostomal and upper gastric region of *Hydra attenuata* (Fig. 1a). The numbers of these neurons decrease strongly in the midgastric region. Immunoreactive perikarya are absent in the lower gastric area, where only few, very weakly immunoreactive processes can be found. In the lower part of the peduncle, just above the basal disk, a collar of densely packed, strongly immunoreactive neurons occurs (Fig. 2a). These neurons have many short processes, which project in all directions, also towards the ectodermal surface. A few strongly immunoreactive neurons lie between the peduncle region and the presumptive budding area. These "solitary" neurons are connected via very fine, weakly immunoreactive processes to the band of neurons in the peduncle. A schematic representation of the RFamide-positive nervous system in *Hydra attenuata* is given in Fig. 9a.

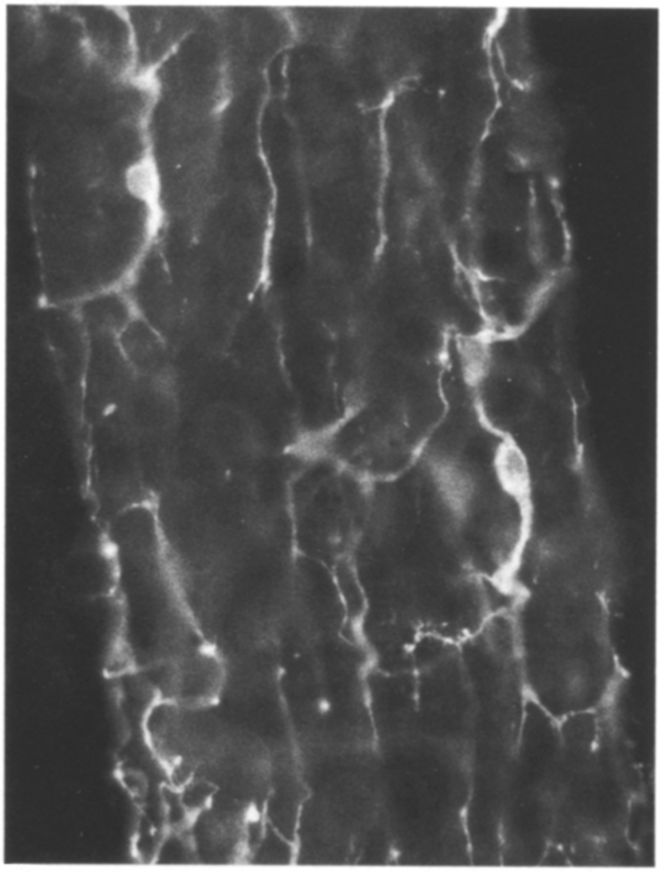

Fig. 4. Basal part of a tentacle of *Hydra oligactis* stained by RF-amide antiserum 146II. Note the strongly immunoreactive bipolar ganglion cells. ×600

Whole-mount staining of *Hydra oligactis* shows an immunoreactive nervous system, which is different from that of *Hydra attenuata*. The sensory cells in the hypostome are somewhat fewer in number, distributed over a larger surface (the apical 3/4 of the hypostome), and are far less immunoreactive than in *Hydra attenuata*. The processes of the sensory cells, on the other hand, are numerous, very immunoreactive and have a prominent radial orientation (Fig. 3a). In the basal 1/4 of the hypostome few ganglion cells occur, which have the same radial projections as the sensory cells. Between the hypostome and the tentacle bases a distinct nerve ring exists (Fig. 3). This nerve ring is made up of one or a few thick, interlaced processes originating from fused ganglion cells. The number of ganglion cells constituting the nerve ring is difficult to estimate, as the ring (4–12 µm) and the perikarya of the ganglion cells

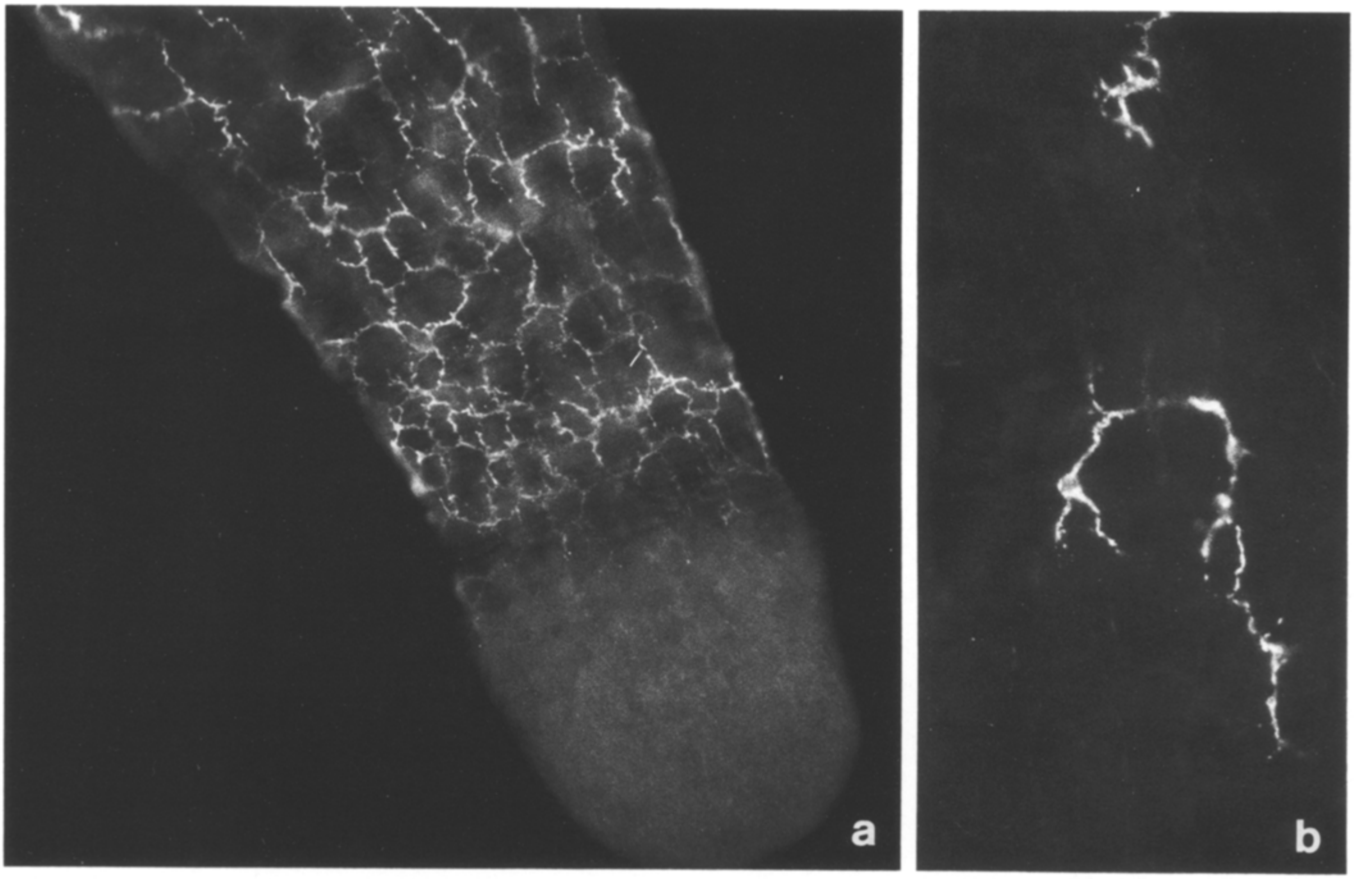

Fig. 5a, b. Whole-mount staining of the foot of *Hydra oligactis* by RFamide antiserum 146II. **a** Lowest part of the peduncle, where a collar of immunoreactive ganglion cells occurs (the small perikarya are only weakly immunoreactive). Note the absence of immunoreactive neurons in the basal disk. ×200. **b** Scattered strongly immunoreactive neurons in the upper part of the peduncle. ×400

(8–16 μm) have a similar diameter. Other ganglion cells outside the ring project processes to the ring, and also the ring itself forms branches, which integrate with the tentacle-nerve net. Most radial processes from the hypostomal plexus appear not to synapse onto the ring, but project "over" the ring to the tentacle bases, where they integrate with the tentacle-nerve net and the branches from the nerve ring.

The tentacles of *Hydra oligactis* are more richly innervated than those of *Hydra attenuata*. Especially striking are the strongly immunoreactive ganglion cells in the basal part of the tentacles, of which many are bipolar (Fig. 4). Many small sensory cells occur, especially in the apical parts of the tentacles, where also numerous processes project to the surface of the ectoderm.

The gastric region of *Hydra oligactis* is rather well innervated by RFamide-positive neurons (Fig. 3a). This innervation is relatively strong in the subhypostomal and midgastric region, but nearly absent in the lowest gastric area. In the lower peduncle region there is, again, a collar of strongly immunoreactive neurons, but this plexus is less dense than that of *Hydra attenuata* (Fig. 5a). Strongly immunoreactive ganglion cells are scattered between lower peduncle and budding area. These neurons are connected via fine, weakly immunoreactive processes to the peduncular collar of neurons (Fig. 5b). Fig. 9b gives a drawing of the RFamide-positive nervous system in *Hydra oligactis*.

The RFamide-positive nervous systems of *Hydra viridis* and *Hydra circumcincta* are not much different from that of *Hydra attenuata*. In both species, however, the ganglion cells at the bases of the hypostome have a more circular orientation. Especially in *Hydra circumcincta* the ganglion cells and their fine processes are arranged in such a way that the impression is gained of a ring running around the hypostome. This ring, however, is essentially different from the one in *Hydra oligactis*.

In all four *Hydra species*, buds develop an immunoreactive nervous system after the appearance of the tentacles (Fig. 2b). In *Hydra oligactis* the hypostomal plexus emerges first and the hypostomal nerve ring is formed afterwards by a circular arrangement and fusion of ganglion cells.

Whole-mount staining of Hydractinia echinata

Intact gastrozooids of *Hydractinia echinata* show a dense plexus of immunoreactive processes in the ectoderm of the whole body column (Fig. 6). These processes have a strong longitudinal bias (Fig. 6b). Many strongly immunoreactive perikarya in the body column are of sensory-cell type, although also numerous weakly immunoreactive bi- and multipolar ganglion cells occur. The sensory cells are especially abundant in the tip of the proboscis, where they project not only cilia, but also processes to the oral cavity (Fig. 6). The tentacles are only moderately innervated by regularly spaced sensory and bipolar neurons and longitudinally orientated processes (Fig. 6a). No nerve ring occurs, and also

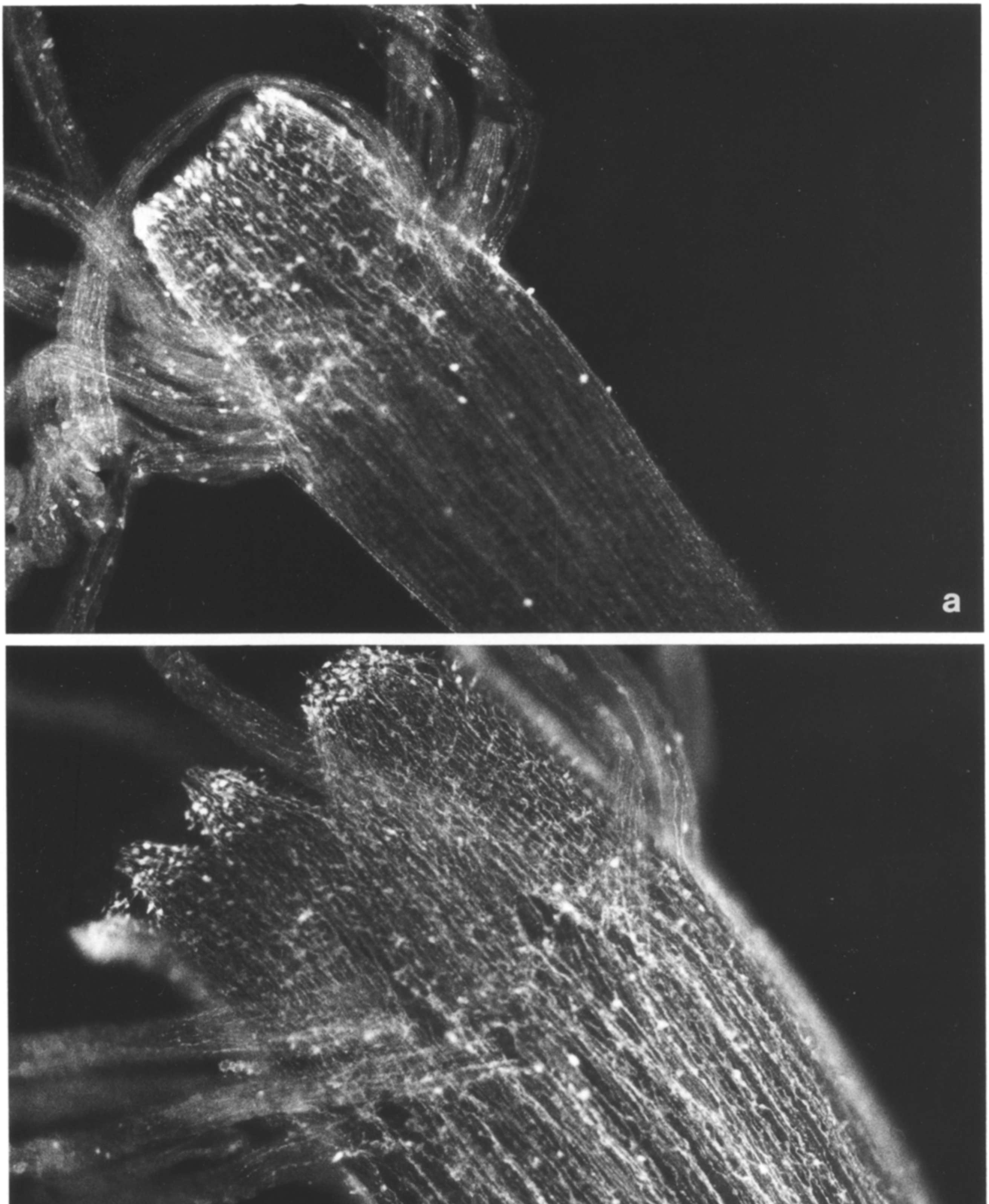

Fig. 6a, b. Whole-mount staining of gastrozooids of *Hydractinia echinata* by RFamide antiserum 146II. **a** Micrograph showing the innervation of body column, proboscis and tentacles. Note that many perikarya of the body column contact the surface of the ectoderm, and that sensory cells around the mouth project cilia into the oral cavity. × 180. **b** View of an animal pressed between the coverslips to show more clearly the extremely dense nervous plexus. × 180

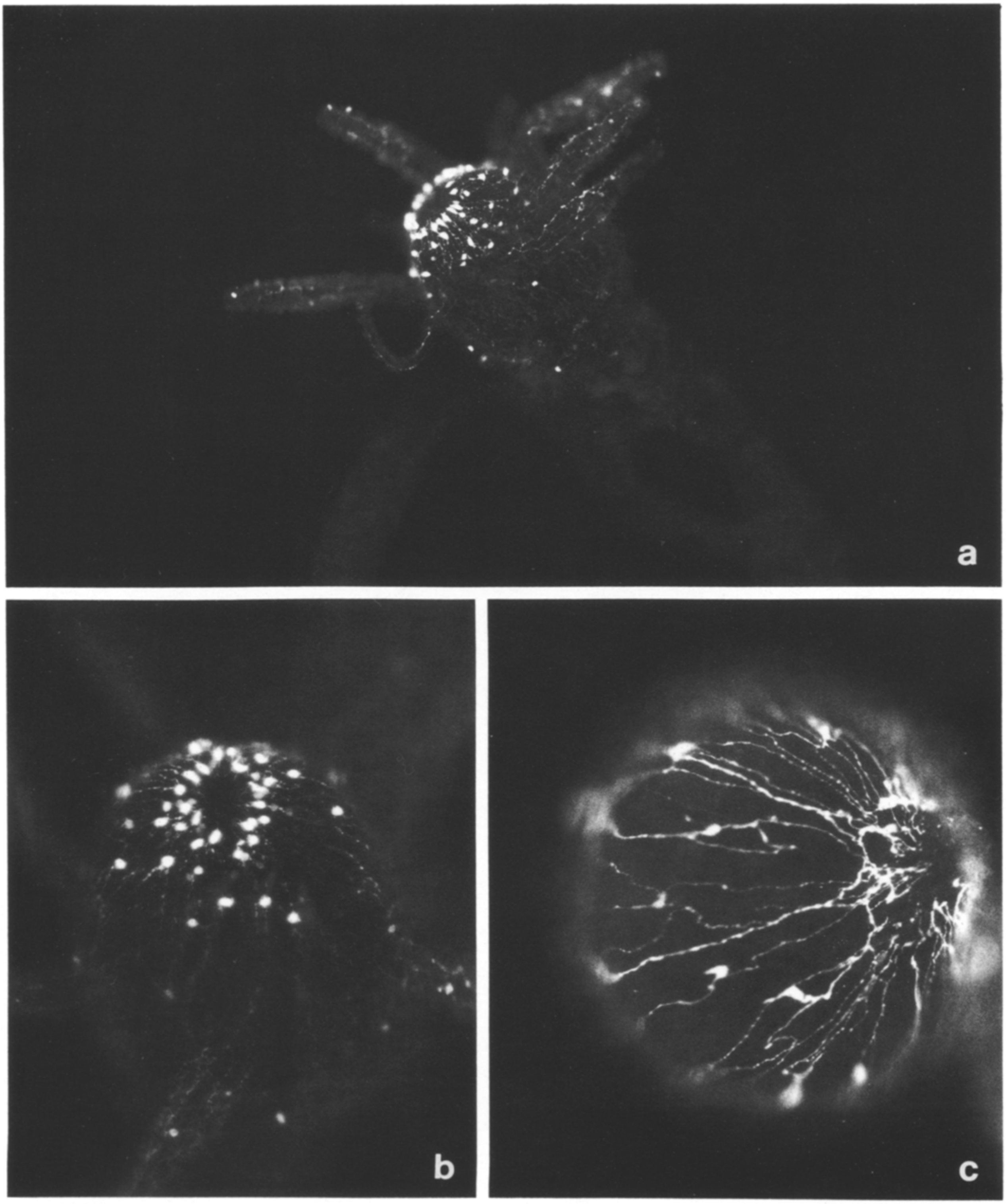

Fig. 7a–c. Whole-mount staining of *Hydractinia echinata* by RFamide antiserum 146II. **a** Micrograph of a primary polyp 120 h after the onset of metamorphosis. Note the row of round perikarya around the mouth opening. ×150. **b** Closer view of a primary polyp of the same age to show that all perikarya around the mouth project cilia into the oral cavity. ×240. **c** Isolated spermatophore viewed from its base. Note the characteristic strongly immunoreactive neurons covering its surface. ×280

no neurons have been observed that project circularly around the basis of the proboscis.

The RFamide-positive plexus of the body column stops in an area just above the stolon. This lowest part of the column has an increased number of sensory cells. In the stolons only very few, weakly immunoreactive fibres and perikarya were found.

In the developing primary polyp, immunoreactive neurons appear 30–40 h after the onset of metamorphosis in a planula larva. These first neurons are arranged in a ring

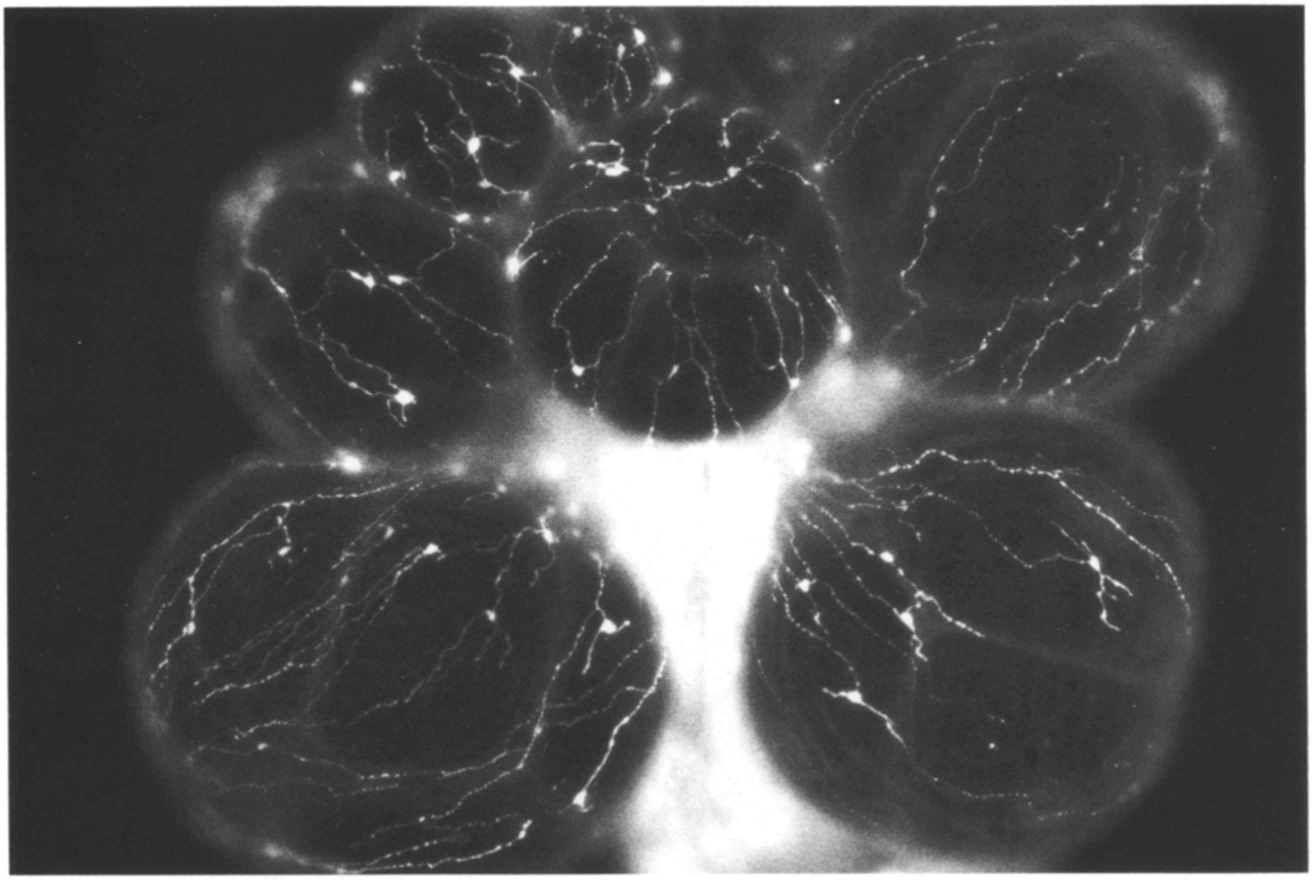

Fig. 8. Whole mount of a female gonozooid of *Hydractinia echinata* stained by RFamide antiserum 146II. Note that the gonophores are covered with highly immunoreactive ganglion cells. These neurons have long beaded processes, which often end "blindly" with a thick final varicosity. ×200

around the mouth opening, and form short longitudinally orientated processes. The perikarya are round and have cilia projecting to the center of the oral cavity and are, therefore, sensory in nature. Later, more and more rows of immunoreactive sensory cells emerge in the proboscis, and also the body column and tentacles start to develop their nervous system (Fig. 7a, b). When the first neurons appear in the primary polyp, the tentacles have already been formed, and the situation is thus quite similar to that of the developing buds in *Hydra*.

Whole-mount staining of sexual hydroid polyps

Female gonozooids of *Hydractinia echinata* have an immunoreactive neuronal plexus similar to that of the gastrozooids. The head contains a moderate number of sensory neurons just below the bases of the tentaculoids, and also the tentaculoids themselves have a few sensory cells. The lower part of the body column, however, and the area near the bases of the gonophores, are more richly innervated than the upper half of the animal. A striking neuronal structure occurs at the surface (ectoderm) of the gonophores. This structure consists of strongly immunoreactive perikarya with long, beaded, processes (Fig. 8). There appears to be little synaptic contact among the neurons of the gonophores and most processes end blindly with a thick "final" varicosity. The nervous system of the gonophores is connected via the gonophore stem to the plexus of the body column.

Male gonozooids of *Hydractinia echinata* have an immunoreactive nervous system which is quite similar to that of the female polyps. Also here, the spermatophores are covered by strongly immunoreactive multipolar neurons (Fig. 7c).

The hermaphroditic form of *Hydra circumcincta* and the female and male polyps of *Hydra oligactis* have been inspected. In these species both oocytes and testes are devoid of RFamide-positive neurons.

Discussion

Staining of intact, fixed hydroid polyps with antisera to the sequence RFamide is an effective technique to demonstrate the three-dimensional structure of a major portion of the animal's nervous system. In addition, the method gives an important clue to the transmitter substance that is being used. Both aspects are equally important.

The value of the immunocytochemical method is illustrated in the present paper with several examples. Thus, whole-mount staining of different *Hydra* species shows that two neuronal centralizations occur, namely in the ectoderm of hypostome and lower peduncle. The centralization in the hypostome of *Hydra attenuata* consists of an agglomeration of sensory cells and neuropile around the mouth and

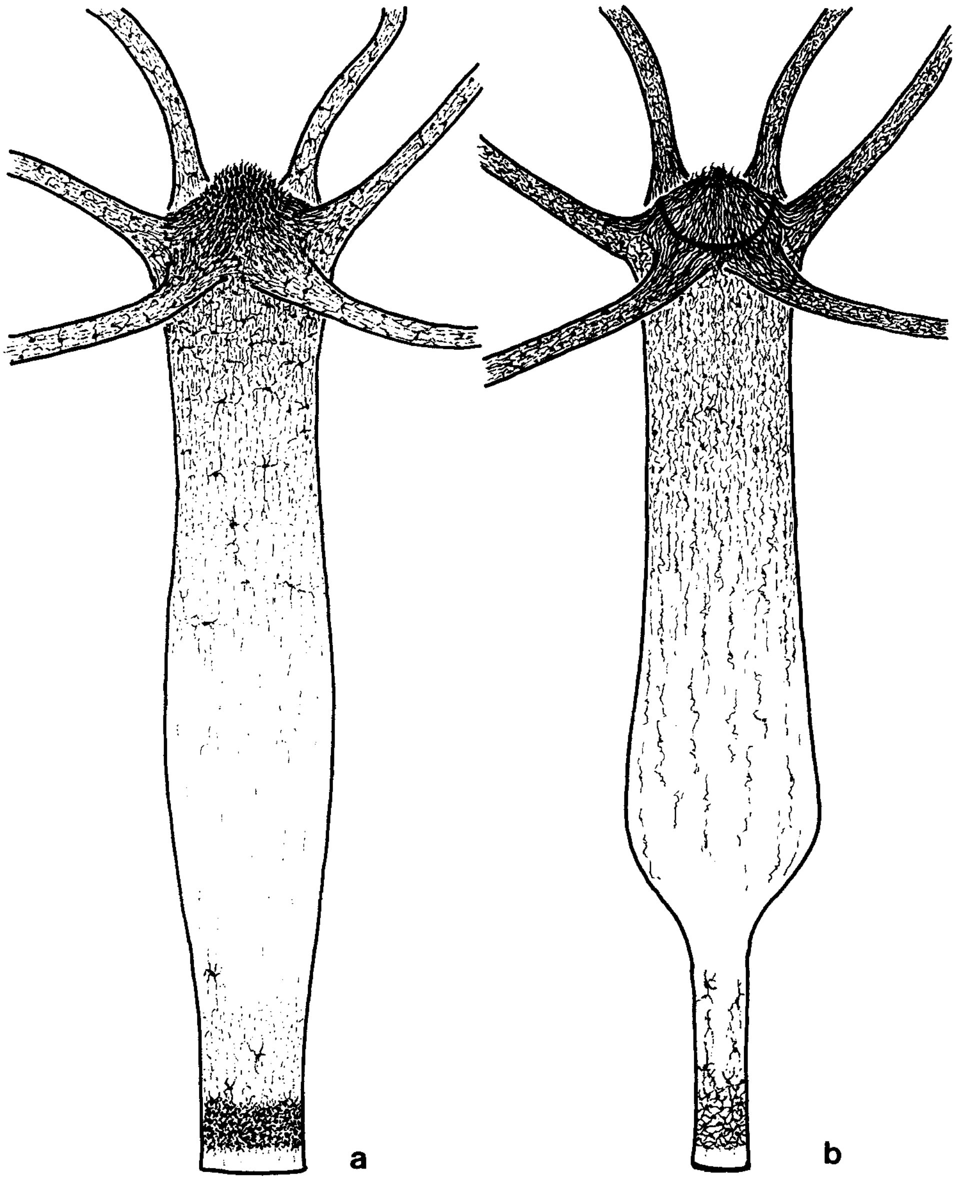

Fig. 9a, b. Drawings showing the RFamide-positive nervous system in two *Hydra* species.
a *Hydra attenuata*, where two centralizations occur: The ganglion-like agglomeration of sensory cells and processes around the mouth, and the collar of densely packed neurons in the peduncle.
b *Hydra oligactis*, which has a dense plexus of radially orientated processes in the hypostome and a nerve ring between hypostome and tentacle bases. The collar of neurons in the peduncle is less well developed

in the apical half of the hypostome (Figs. 1, 9a). Such a strong condensation of neurons resembles closely a ganglion. The ganglion-like structure must be even denser and more complex than shown in this contribution, since it is known that also vasopressin-positive neurons, which are different from the RFamide-positive neurons, occur in the hypostomal area (Grimmelikhuijzen et al. 1982b; Grimmelikhuijzen 1983b). The centralization in the hypostome of *Hydra oligactis* is especially evident from its nerve ring (Figs. 3, 9b). The reason that the neuronal centralization in the hydroid hypostome has not previously been demonstrated in its full extent is two-fold. Firstly, methylene-blue staining of the early days (Schneider 1890; Hadži 1909; McConnell 1932; Spangenberg and Ham 1960; Burnett and Diehl 1964) is inferior to antiserum staining in whole mounts. Secondly, a three-dimensional reconstruction of electron micrographs of serial sections through the hypostome, as carried out by Kinnamon and Westfall (1981) can only, for obvious reasons, take into account the positions of the different perikarya, and therefore omits the most important part of the hypostomal plexus, namely, the dense wiring of the neuronal processes.

Davis and coworkers (1968) have found a small "neural ring" in *Hydra oligactis*, using toluidine-blue stained light-microscopic sections of the hypostome, but not much attention has been paid by later workers to this interesting finding. The present work shows the hypostomal nerve ring in its entirety. A well-developed nerve ring is unusual in hydrozoan polyps. In fact, only polyps of a totally different class, those belonging to the Cubozoa, are recognized as having such rings (Werner et al. 1976; Spencer and Schwab 1982).

Nerve rings are common in hydrozoan medusae, where they consist of electrically coupled neurons (Anderson and Mackie 1977; Spencer 1978, 1981; Spencer and Schwab 1982; Anderson and Schwab 1982; Spencer and Arkett 1984). The outer nerve ring and the outer part of the inner nerve ring in the hydrozoan medusa *Polyorchis penicillatus*, are RFamide-positive (Grimmelikhuijzen and Spencer 1984), and it could well be that the hypostomal nerve ring of *Hydra oligactis* has a similar function as its counterparts in medusae, i.e., it is either involved in some synchrone motor action, or it functions as an integration and pacemaker center (Spencer 1982; Spencer and Arkett 1984). By virtue of its position near the tentacle bases, its connections to the tentacle-nerve net, and its unusual diameter (4–12 μm), it seems likely that the nerve ring is involved in synchronizing and initiating tentacle movements. However, concerted tentacle movements can also be initiated without a neuronal ring (e.g., in *Hydra attenuata*), and it would be interesting to find a clear behavioural correlate for the nerve ring in *Hydra oligactis*.

Passano and McCullough (1964, 1965) have postulated two pacemaker centers in the head region of *Hydra*, one producing periodic contraction bursts, the other causing contraction bursts associated with locomotion. The ganglion-like neuronal condensation in the hypostome of *Hydra* (and possibly also the nerve ring in *Hydra oligactis*) could certainly account for these activities. At the other hand, it has to be realized that most neurons in the hypostomal agglomeration are sensory, so that at least a part of its activity must be involved in sensory input and integration (feeding).

The existence of a well-developed nerve ring in *Hydra oligactis* clearly places this animal apart from the other *Hydra* species. This shows that whole-mount staining of the nervous system of hydroids with RFamide antisera may also contribute to the taxonomy of these animals.

Gastrozooids of *Hydractinia echinata* do not show such a clear centralization as polyps of the different *Hydra* species. Their nervous system, on the other hand, can certainly not be called a loosely interconnected net, since the plexus of immunoreactive fibres in the body column and proboscis is extremly dense. Such an extremely dense plexus in the ectoderm of the whole polyp has not been recognized before by electron-microscopic studies (Stokes 1974). As in *Hydra*, the cluster of immunoreactive sensory cells around the mouth indicates that at least a part of the RFamide-positive nervous system must be involved in feeding.

The existence, in *Hydractinia*, of strongly immunoreactive neurons covering the gonophores of male and female gonozooids could, in principle, have two different meanings. Either they are just a remainder of the nervous plexus of the body column, or they are especially produced for the ovaries and testes and are involved in gametogenesis or spawning. Examination of the morphology of the neurons does not at all give the impression that these neurons are without any function; they are highly immunoreactive and have long, vital, beaded processes. This favours the second possibility. RFamide-positive neurons were not found to be associated with oocytes and testes of *Hydra*. This shows that RFamide-like peptides are not generally involved in gametogenesis of hydroids. Sexual polyps of *Hydractinia*, in contrast to those of *Hydra*, are known to have a light-sensitive spawning (Ballard 1942; Yoshida 1959; Müller 1961). This difference between *Hydractinia* and *Hydra* could mean that the RFamide-positive neurons on the gonophores of *Hydractinia* are involved in the light-sensitive or circadian release of oocytes and sperm. Spawning was found to be Ca^{2+}-dependent (Ballard 1942; Yoshida 1959). This would fit very well with a transmitter-mediated process.

Neurons covering the gonophores have not been recognized in a recent electron-microscopic study on the development of these organs in *Hydractinia* (Hertwig and Hündgen 1984). This example shows that whole-mount incubation of hydroid polyps with RFamide antisera should be of interest for anyone dealing with hydrozoan physiology.

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