Nerve net differentiation in medusa development of *Podocoryne carnea**

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SUMMARY: The phylum Cnidaria is the most primitive phylum with a well-developed nervous system. Planula larvae and polyps display a diffuse nerve net (plexus), which is densest in the polyp hypostome. In contrast, the nervous system of the medusa is more complexly structured and reflects the anatomical needs of a well differentiated non-sessile animal. We analyzed the nervous system of two life stages of the hydrozoan *Podocoryne carnea*. Nerve nets of both polyps and developing medusae were examined in whole mounts and gelatin sections by using antibodies and vital staining with reduced Methylene Blue. In the polyp, both RFamide-positive nerve cells and tyrosine-tubulin containing nerve cells form an ectodermal plexus. However, apical neuronal concentration is stressed by a particular nerve ring formed by tyrosine-tubulin positive nerve cells in the hypostome above the tentacle zone. This apical nerve ring is not detected with antisera against RFamide. In developing medusa buds, the earliest detected RFamide positive nerve cells occur at stage 4 at the location of the prospective ring canal. The nerve net of the developing medusa is fully differentiated at bud stage 8. Similar results were obtained with the anti tyrosine-tubulin antibody. Strikingly, two different nerve nets were discovered which connect the medusa bud with the plexus of the gonozoid, suggesting neuronal control by the polyp during medusa bud development. Vital staining with reduced Methylene Blue (Unna's) identified not only nerve cells at the ring canal but also bipolar cells within the radial canal. These cells may fulfill sensory functions.

Key words: differentiation, nerve cells, Hydrozoa.

INTRODUCTION

The phylum Cnidaria is subdivided into four classes, the Hydrozoa, Scyphozoa, Cubozoa and Anthozoa (Schuchert, 1993; Bridge *et al*., 1995). Aside from the planula larva, the polyp and medusa represent the main structural morphs. During the 17th and 18th Centuries, polyps and medusae were not recognized as animals but rather were referred to as plants. Pioneering anatomical analysis in the 19th Century revealed that these species belong to the animal kingdom and posses a well developed nervous system (Hertwig and Hertwig, 1879). For

example in 1850, Louis Agassiz first described marginal nerve rings in the hydromedusae *Sarsia* and *Bougainvillia* (reviewed in Mackie, 1989). Later, Hadži (1909) among others, carefully described the nervous system of *Hydra*. In the last nine decades, the *Hydra* nervous system has been intensively explored. In the ectoderm, 2 to 7 times more nerve cells are found compared to the endoderm and 11 different types of nerve cells have been morphologically distinguished (Epp and Tardent, 1978). In contrast to the uniformly distributed nerve cells in the endoderm, the ectodermal nerve cells are concentrated at the hypostome and in the foot of *Hydra* (Kinnamon and Westfall, 1981; Bode, 1996). Further evidence for a degree of adoral (i.e. uppermost

part of hypostome) nervous centralization was provided using an antiserum against the C-terminal fragment of neuropeptides (arginine-phenylalanine-amide, RFamide) which showed a nerve ring in *Hydra oligactis* but not in three other *Hydra* species (Grimmelikhuijzen, 1985). Moreover, RFamide positive nerve cells are not randomly distributed in *Hydra* but display a gradient like distribution in the body column with highest density in the hypostome (Grimmelikhuijzen, 1985; Bode *et al*., 1988). The nerve cells are synaptically and electrically connected and form a diffuse nerve net, which is called a plexus (Mackie, 1999). A plexus is also found in planula larvae (Tardent, 1978). The nerve cells derive from large interstitial cells (I-cells), which first divide into differentiation intermediates. When cell division is completed, they elaborate the morphological characteristics of either sensory cells or ganglion cells. This organized architecture rather than a random distribution is remarkable, since all cells continuously change their location within the animal and are lost by sloughing at the foot, head and tentacles (Campbell, 1967). Similar observations were obtained from vital staining experiments with polyps from *Podocoryne carnea* (Bravermann, 1969). Therefore, the constantly -moving cells abruptly switch their gene expression pattern and differentiation status in a region dependent manner.

Investigations of the medusa nervous system during the last 130 years have revealed a higher degree of complexity than that of polyps or planu-lae. Among others, *Polyorchis penicillatus* and *Aglantha digitale* are the best studied species (Spencer and Arkett, 1984; Arkett and Spencer 1986; Mackie and Meech, 1995a and b). In these species, the nervous system consists of a diffuse ectodermal plexus in the tentacles, manubrium and subumbrella, with concentrations below the radial canals and at the margin of the bell. But endodermal nerve rings were also reported close to the margin of the bell (Jha and Mackie, 1967; Mackie and Singla, 1975; Singla, 1974, 1975). Although ocelli have been described in the polyp of the scyphozoan *Stylocoronella riedli* (Blumer *et al*., 1995), well-developed sense organs only exceptionally occur in polyps. Nevertheless, many medusae have evolved sense organs such as ocelli and statocysts, which in hydromedusae are located on the exumbrellar surface of the tentacle bulb. The margin of the bell is the place where peripheral pathways converge and neurons receive input from sense organs and where activity patterns are generated (Mackie, 1990). Thus, the whole morphology of the nervous system of polyps and medusae differs considerably and reflects various behavior repertoires (Spencer, 1974, 1975), although most genes are expressed in both life stages (Bally and Schmid, 1988; Aerne *et al*., 1996). We analyzed the nervous system of the hydrozoan *Podocoryne carnea* which displays a full life cycle. Polyp colonies of *Podocoryne* produce medusae by asexual budding. The epimorphic medusa budding is a *de novo* differentiation and is
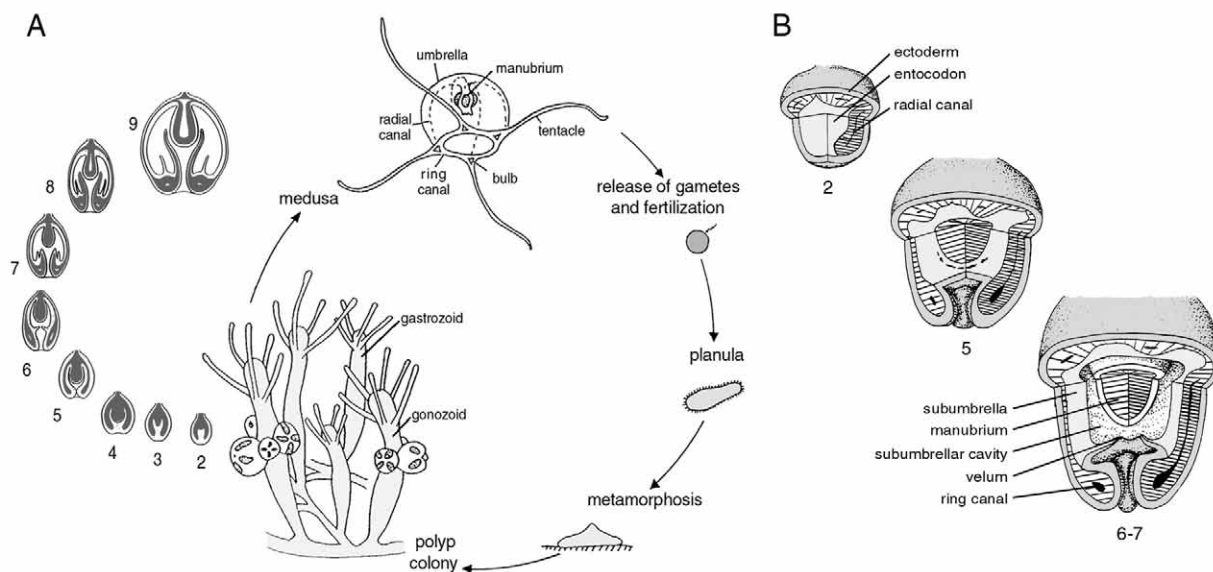

FIG. 1. – The life cycle of *Podocoryne carnea* and medusa bud development. (A) Schematic drawing of the life cycle. Gametes, planula and metamorphosing polyp are not proportionally drawn. On the left, the development of a medusa bud is schematically shown and the numbers indicate the bud stage according to Frey (1968). (B) Three dimensional sketch of bud stages after Boelsterli (1977). The numbers refer to the bud stage.

characteristic for the class Hydrozoa (Tardent, 1978). The bud of an Anthomedusa develops from a diploblastic evagination of the polyp (gonozoid) to the fully differentiated medusa within 7 days (Fig. 1). When *Podocoryne* medusae are released from gonozoids, they swim actively and shed gametes. Upon fertilization, planula larvae develop, settle on the appropriate substrate and metamorphose into primary polyps, the founders of new polyp colonies.

To approach the structure of the nervous system, we applied different methods. A vital staining procedure with reduced Methylene Blue by Unna's method (Pantin, 1948) can be used alternatively to silver staining experiments on whole mounts and reveals both the nerve net and sensory system (Batham *et al.,* 1960). The use of antisera against RFamides has demonstrated that these neuropeptides are widely found in the phylum Cnidaria (Grimmelikhuijzen and Westfall, 1995; Grimmelikhuijzen *et al.*, 1996). RFamides are produced by a plexus of neurons and thus, antisera against RFamide can be used in addition to silver or vital stainings. Since not all nerve cells produce RFamides and not all cells stain well with reduced Methylene Blue or with silver (Batham *et al.*, 1960), the use of additional markers is indispensable to uncover the nervous system in its entirety. We used a monoclonal antibody against tyrosine-tubulin, which is immunospecific for C-terminal tyrosine of mouse α-tubulin and crossreacts with many tyrosine-tubulin, including that of yeast (Kreis, 1987). The processes of neural elements (neurites) contain microtubules composed of tubulin (Thomas and Edwards, 1991), therefore the use of anti-tyrosine-tubulin antibody is suitable to visualize neurites.

Our data show the similarities between the *Podocoryne* and *Hydra* polyp nerve net structure. Moreover, we describe pattern formation and morphogenesis of the nervous system in the developing medusa bud which form the more complex and more highly- organized nervous system of the Anthomedusa *Podocoryne carnea*.

MATERIALS AND METHODS

Animals

Polyp colonies of *Podocoryne carnea* were cultured in artifical seawater as described by Schmid (1979). Medusae were obtained daily from polyp colonies by overnight-sampling. Medusa buds were staged according to Frey (1968) with bud stage 1 being the youngest and 10 being the oldest stage.

Immunocytochemistry on whole mount preparations

All steps were carried out at room temperature unless otherwise stated. Apart from planulae, the animals were relaxed (1 part seawater and 1 part 7% $MgCl_2*6H_2O$ in distilled water) for 5 minutes (medusae) or 15 minutes (polyps). Fixation was performed in phosphate-buffered saline (PBS) with 4% paraformaldehyde for 90 minutes, followed by three washes with PBS plus 0.1% Tween 20 (PBST), each for 30 minutes. Incubation with RFamide antiserum (rabbit antibody diluted 1:2000 in PBS; kindly provided by Dr. C.J.P. Grimmelikhuijzen) or with a monoclonal anti-tyrosine-tubulin antibody (a mouse antibody, clone TUB-1A2, Sigma; diluted 1:2500 in PBS containing 10% fetal calf serum) was accomplished at 4°C for 12 to 18 hours. Unbound antibodies were removed by three 30 minute washes in PBST. Subsequently, specimens were incubated at 4°C for 12 to 18 hours with either fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma; diluted 1:200 in PBST) or with FITC-conjugated goat anti-mouse IgG (Sigma; diluted 1:200 in PBST). For double staining, either tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Sigma; diluted 1:80 in PBST) or Alexa-conjugated anti-rabbit IgG (Alexa-568, Molecular Probes) was applied according the manufacturers' recommendations. Three washes in PBS for 30 minutes were followed by mounting the specimens in DABCO (2,5 g 1,4- diazabicyclo-[2.2.2.] octane triethylenediamine (Sigma) in 10 ml PBS and 90 ml glycerol) which retards photobleaching. Prior to DABCO mounting, nuclei were stained by incubation with DAPI (stock solution: 1 mg 4'6-diamidino-2-phenyl-indole ·2HCl (Serva) in 20 ml H_2O; working dilution: 30 μl stock solution in 4 ml seawater) for 30 seconds. The preparations were examined with either a Photomikroskop III (Zeiss) or with a laser confocal microscope (DMRXE, Leica). Confocal images were given false colours to match the fluorescent emission colours.

Immunocytochemistry on gelatin sections

Unless stated differently, all steps were performed at room temperature. Polyps and medusae were fixed upon relaxation as described above.

After three washes for 30 minutes in PBST, specimens were placed in a small trim block. Gelatin was dissolved to a final concentration of 8% by heating in water. This solution was cooled down to approximately 70°C and carefully pored in the trim block. The gelatin was deaerated by incubation in a vacuum oven (VD-23, Binder) at 65°C and 100 mbar for 40 minutes. Subsequently, gelatin was allowed to polymerize and specimens were oriented under the dissecting microscope. After 20 minutes at 4°C, the gelatin block was removed from the trim block and fixed in PBS with 4% paraformaldehyde at 4°C overnight. The gelatin block was trimmed and cut into sections of 50 μm thickness with a vibratome (Vibratome Series 1000, Technical Products International, Inc.). These free-floating sections were pooled in PBST, stained with antibodies, and processed as described above.

Vital staining with reduced Methylene Blue by Unna's method

Vital staining using reduced Methylene Blue was performed at room temperature as described by Batham *et al*. (1960). Briefly, animals were relaxed in narcotizing seawater (1 part seawater and 1 part 7% $MgCl_2*6H_2O$ in distilled water) for 5 minutes (medusae) or 15 minutes (polyps). About 20 specimens were kept in a volume of 4 ml and stained by addition of 8 drops of reduced Methylene Blue. Reduction of Methylene Blue was performed as follows: 50 mg Methylene Blue (Polychromes Methylenblau Unna, Ciba) were dissolved in 10 ml narcotizing seawater and filtered after the addition of 10 μl 24% HCl. 240 mg Rongalit (sodium formaldehyde sulfoxylate dihydrate, Fluka) were dissolved in 2 ml narcotizing seawater and added to the dissolved Methylene Blue with constant stirring and gentle warming. The mixture was set aside to cool with constant stirring until the color turned to pale yellow, and was finally filtered. Methylene Blue from various suppliers had to be tested for this protocol.

RESULTS

The nerve net of polyps (Fig. 2)

Since the processes of neural elements (i.e. neurites) contain microtubules, antibodies against this highly conserved structural component can be used to visualize the nervous system. Best results were obtained with a mouse anti-tyrosine-tubulin antibody. Whereas an antibody raised against mouse anti-α-tubulin also cross-reacted with flagellae, a mouse anti-β-tubulin antibody did not recognize any epitope in *Podocoryne carnea* (data not shown).

In *Podocoryne carnea*, the mouse anti-tyrosine-tubulin antibody binds to neurites and nematocysts. The neurites form a nerve net, which is regularly dispersed all over the ectoderm of the polyp column and forms a plexus in the tentacles as well. The neurites are mainly bipolar and run in a proximodistal direction in the body column and in the tentacles (Fig. 2A). Remarkably, just above the tentacle zone a few neurites run perpendicularly to the others and form a unique ring, which is interconnected with the proximodistal-oriented nerve net. Similarly, the RFamide-positive nerve cells form also a plexus along the body column but these neurites run exclusively in a base to apex orientation. Fewer RFamide-positive nerve cells are detected within the tentacles. RFamide expressing nerve cells occur predominantly in the hypostome and display a gradient-like distribution along the body column. No subhypostomal ring is formed by RFamide positive nerve cells (Fig. 2B). Double-staining confirmed differences in the distribution of nerve cells expressing RFamide and tyrosine-tubulin positive cells (Fig. 2C, D). Nevertheless, both nerve cell populations are localized in the ectoderm.

Two different types of neurons were detected with the anti-tyrosine-tubulin antibody. This is best demonstrated on gelatin sections of the body column. Large nerve cells which are oriented perpendicularly to the mesoglea and which project a cilium at the ectodermal surface are sensory cells. Smaller cells project their neurites parallel to the mesoglea and are termed ganglion cells. (Fig. 2E).

The nervous system in developing medusa buds (Fig. 3)

The developing bud is covered with a protective envelope, the perisarc. Since it interferes with penetration and diffusion, the technique of gelatin sections is an appropriate tool to investigate bud development.

RFamide-positive cells are first detected from stage 4 at the place of the prospective ring canal (Fig. 3A). RFamide expression in this region persists through all stages. In addition, RFamide-positive nerve cells are present in the developing
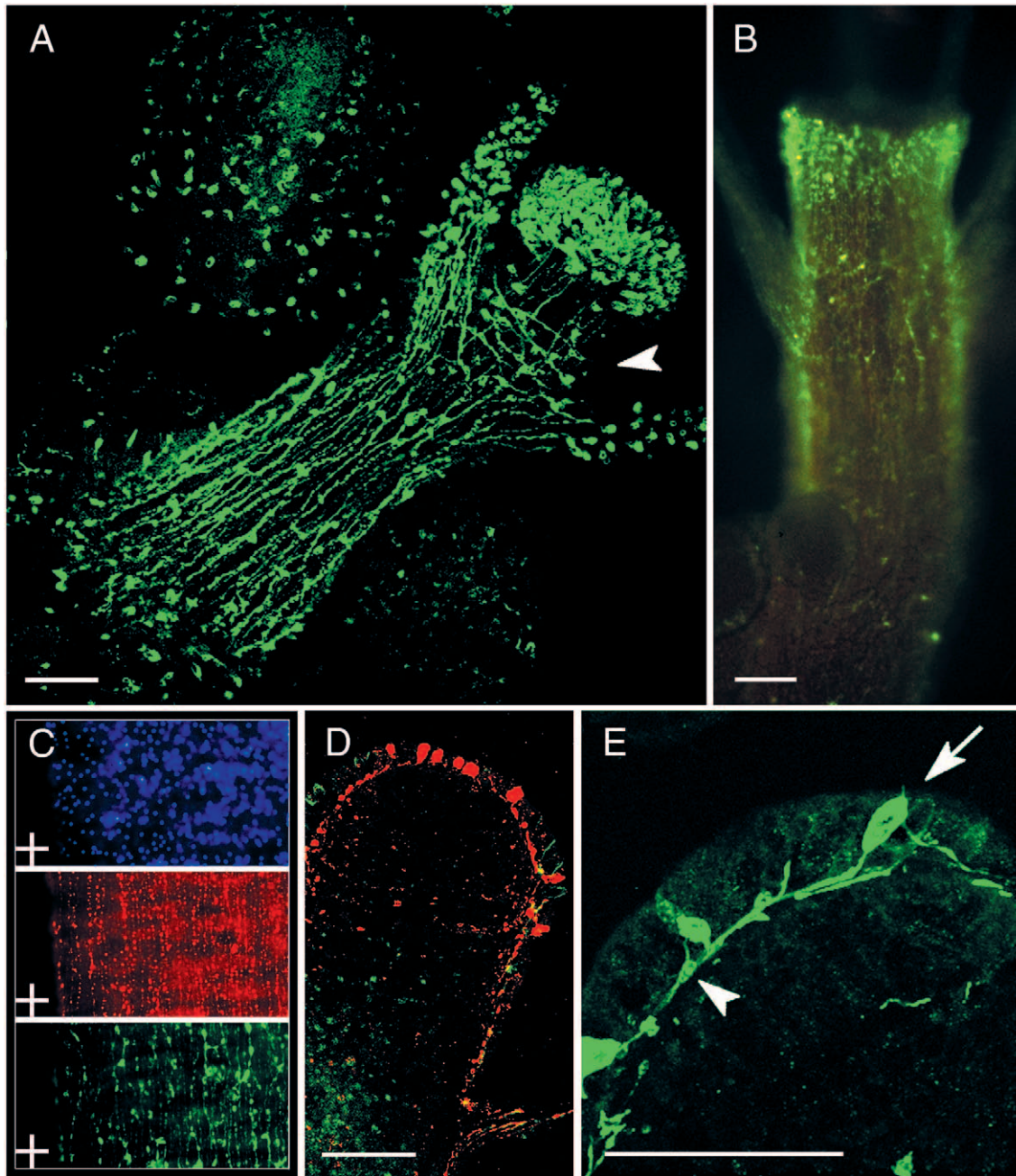
FIG. 2. – The nervous system of the polyp. Fluorescent signals were detected either with a laser confocal microscope (A, D and E) or with a conventional photomicroscope (B and C). (A) Nerve cells stained with anti-tyrosine-tubulin antibody in a gonozoid. The arrowhead points to the hypostomal nerve ring. (B) RFamide-positive nerve cells in a gonozoid display a gradient-like distribution, with the highest concentration in the hypostome. (C) Detail of DAPI-stained (visualization of all nuclei) and double-labeled body column. On top DAPI-labeled nucleotides in the body column are shown in blue, in the middle the tyrosine-tubulin positive net is shown in red, and at the bottom the RFamide positive plexus is shown in green. The cross on the left refers to the same position in all pictures. (D) Double-labeling of a hypostome in a gelatin section shows RFamide-positive nerve cells (red) and tyrosine-tubulin positive cells (green). (E) Cross section of body column. Arrow points to a sensory cell, arrowhead to a ganglion cell. Bars equal 100 μm.

manubrium from stage 5 to 6 on. Afterwards, nerve cells at the radial canal start to express RFamide (Fig. 3B). When tentacles develop (stage 7 to 8), the neurons at the radial canal connect the manubrial plexus to the nerve rings close to the ring canal (Fig. 3C). Interestingly, projecting neurites from the sub-

radial nerve cells immigrate into the developing tentacle bulbs (Fig. 3D). Thus, ectodermal neurites project into the bulb endoderm. Similarly, RFamide-positive neurons from the polyp ectoderm project from early bud stages on through the stalk, penetrate the mesoglea and project to the endodermal portion
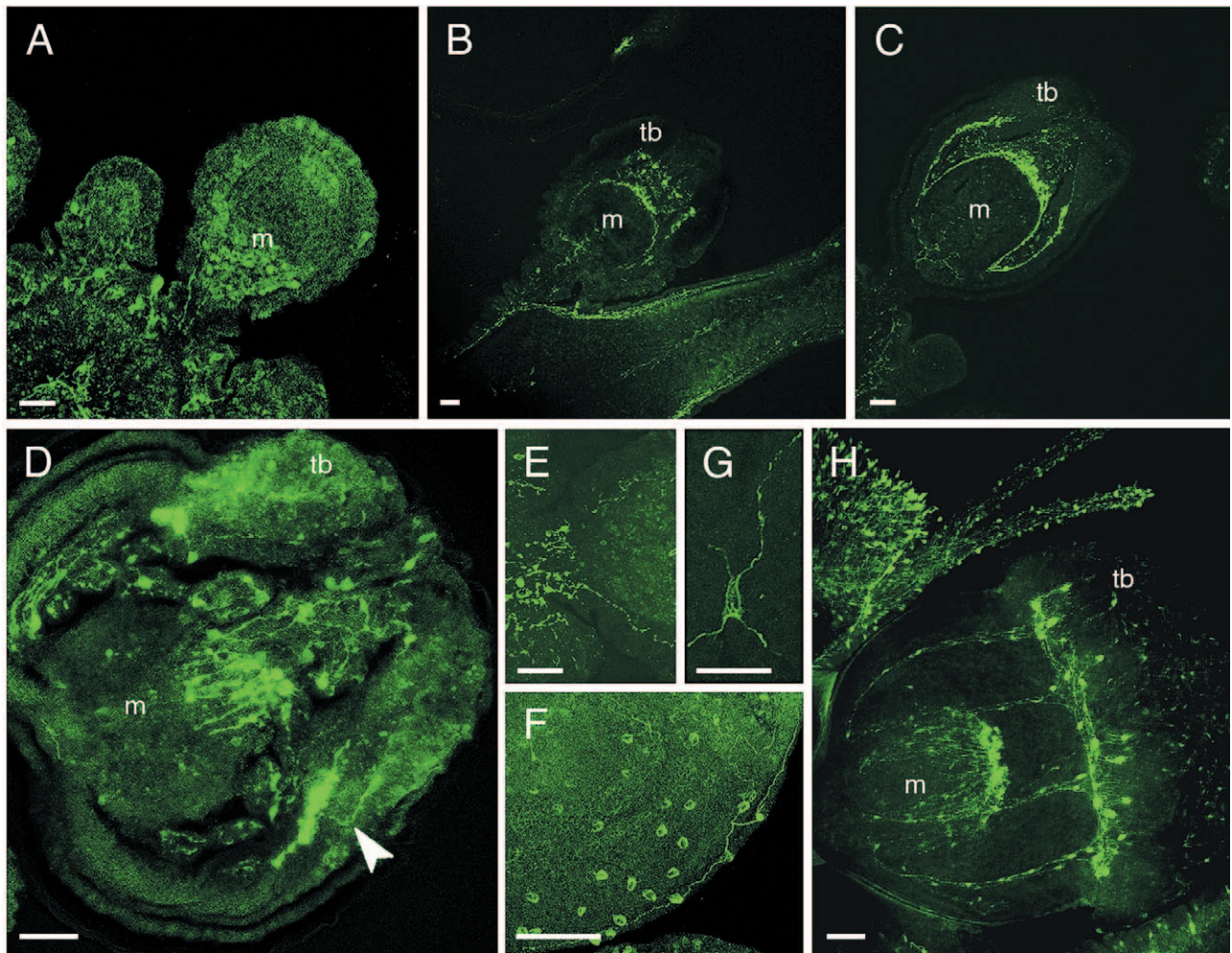
FIG. 3. – The developing nervous system in medusa buds. A-G are laser confocal images; H was done with a conventional photomicroscope. A-D and H are stained with antiserum against RFamide, the others with anti-tyrosine-tubulin. (A) Cross section of buds of stages 2 and 4 with RFamide expression in the apical part of the entocodon. (B) Section of a gonozoid with a bud of stage 5-6. RFamide -positive cells are detected at the developing ring canal, manubrium, and below the radial canals. Note the neurites which project through the stalk. (C) Cross section through bud stage 7-8. (D) Cross section of bud stage 8. The arrowhead points to the neurites, which project at right angles from the lower margin of the subumbrella into the tentacle bulb. (E) Projected neurites from the polyp (left) through the stalk into the bud (right). (F) Several, but not all nematocytes are connected to an ectodermal nerve net. (G) Multipolar nerve cell in the ectoderm of a medusa bud. (H) Whole mount of bud stage 8. Note that the developed nerve cells in the tentacles are not stained in this whole-mount preparation. m, manubrium; tb, tentacle bulb. Bars in A-F an H correspond to 100 μm, in G to 50 μm.

of the developing manubrium (Fig. 3A-C). When the tentacles are completed at stage 8, the nerve plexus is established (Fig. 3D).

The antibody against tyrosine-tubulin stained the developing nervous system in the bud similarly to the RFamide antiserum. Remarkably, tyrosine-tubulin positive neurites project through the stalk in the bud ectoderm corresponding to the prospective exumbrella (Fig. 3E,F). These neurites make contact with some, bud not all, nematocytes. Therefore, the bud communicates with the polyp by at least two different nerve nets. One is localized in the prospective exumbrella, connects the polyp through the stalk ectoderm and can be identified with the anti-

tyrosine-tubulin antibody. The other nerve net is RFamide positive and contains neurites, which are projected through the endoderm of the stalk. Fig. 3G depicts a multipolar nerve cell detected with the tyrosine-tubulin antibody. Not only the neurites but even the cell body is stained. The RFamide- positive nerve net is developed from bud stage 8 on, however, the RFamide-positive nerve cells of the tentacle are not labeled in whole mounts (Fig. 3H).

The nerve net of the mature medusa (Fig. 4)

The proportions of the fully developed nerve net are similar to those from bud stage 8 on. Four nerve
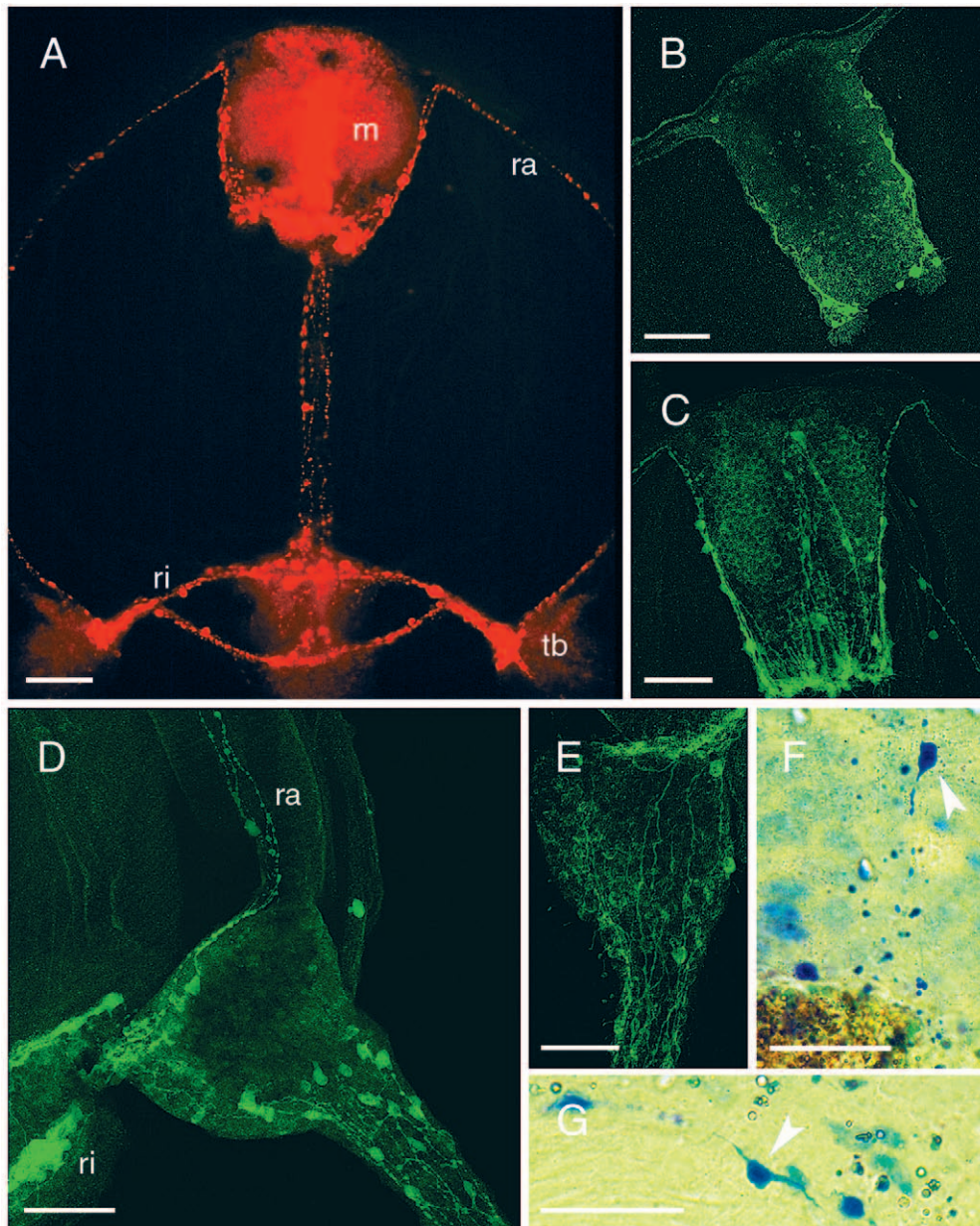
FIG. 4. – Nervous system of the mature *Podocoryne carnea* medusa. A to E are laser confocal images; F and G were done with a conventional lightmicroscope. A and B stained with antiserum against RFamide; C-E tyrosine-tubulin; F and G vital stainings with reduced Methylene Blue (Unna's). (A) Whole mount of a medusa. The picture is over exposed to visualize the outline of the medusa. (B) Cross section of a manubrium. (C) Whole mount shows projected neurites from the manubrial plexus to the subumbrellar fascicles. (D) Magnification of the bell margin with a tentacle bulb. (E) Tentacle bulb viewed from the inner side. Several neurites project side by side from the ring canal to the tentacles (F) Vital staining of a large cell with processes within the radial canal (arrowhead). (G) Vitally -stained bipolar cell at the ring canal (arrowhead). m, manubrium; ra, radial canal; ri, ring canal; tb, tentacle bulb. Scale bar = 100 μm.

fascicles, which lie in the subumbrellar ectoderm connect the nervous system of the manubrium with the nerve rings at the ring canal and the four nerve plexi of the bulbs and tentacles (Fig. 4A). RFamide-positive nerve cells of the manubrium surround the mouth opening and mingle with the four nerve fascicles in the subumbrellar ectoderm (Fig. 4B). This is also the case for tyrosine-tubulin positive nerve cells. Nevertheless, only the tyrosine-tubulin anti-

body stains nematocytes at the manubrial lips. Both nerve cell-types project their neurites additionally into the whole lower part of the manubrial ectoderm towards the base of the gonads (Fig. 4C). The neurites of the subumbrellar ectoderm connect to the ring canal nerves and to the plexus of bulb and tentacles (Fig. 4D). Most neurites enter the bulb at the inner ectoderm whereas the lateral and outer part seem not to be invaded (Fig. 4D,E). We detected no

immunoreactivity of nervous elements in the velum. Thus, the subradial neurites cross the mesoglea in the tentacle bulbs to connect to the nerve rings at the ring canal.

Interestingly, vital staining using reduced Methylene Blue revealed labeled cells within the radial canal (Fig. 4F). These large cells have processes similar to bipolar nerve cells at the ring canal that are detected with the same method (Fig. 4G). Strikingly, cells of the radial canal that are oriented to the periphery have been found to express a *Pax* gene (Gröger *et al.*, in preparation) and might fulfill a sensory function.

DISCUSSION

The structure of the nervous system of the hydrozoan *Podocoryne carnea* has been elucidated by means of vital staining and immunocytochemistry on both whole mounts and gelatin sections. Whereas both polyp and planula have a diffuse nerve net, the polyp shows a clear tendency for adoral concentration in the hypostome (Fig. 2A,B). The medusa nerve net, however, displays a clearly higher degree of organization typical for hydromedusae (Mackie *et al.*, 1985).

Polyps display a diffuse nerve net similar to planulae

Although nervous systems of both polyps and planulae are organized as a plexus, the polyp nervous system of *Podocoryne carnea* displays a tendency for concentration close to the mouth opening. Particularly, the tyrosine-tubulin positive nerve ring above the tentacles is exclusively found in the hypostome. Nowhere else in the body column can such ring-like connections be detected. If we superimpose the gradient-like distribution of RFamide -positive nerve cells with its highest concentration in the hypostome, a clear apical neural concentration becomes evident. Although no sense organs are found in *Podocoryne* polyps, recent data show that a few cells expressing a *Pax* gene are concentrated in the hypostome (Gröger *et al.*, in preparation). This *Pax* gene is probably involved in differentiation pathways of sensory cells and the apical localization of the *Pax* -expressing cells displays further evidence for a clearly apical sensory nerve cell concentration. As mentioned above, nerve cells, like epithelial cells, continuously move towards the end of the body column (Yaross *et*

al., 1986). Thus, the polyp nerve cells exhibit a fascinating position-dependent morphogenetic plasticity within the body column.

Pattern formation and morphogenesis in the developing medusa bud

The use of gelatin sections combined with immunocytochemistry improved the study of the developing nervous system of the medusa. At bud stage 8, nervous system formation is completed and coincides with the realization of the structural differentiation of the subumbrellar striated muscle cells (Boelsterli, 1977). It has been previously demonstrated that from bud stage 8 on, experimentally isolated buds develop into a medusa while younger isolated bud stages regenerate polyp structures (Schmid, 1972). These results indicate that accomplished morphogenesis at bud stage 8 is a prerequisite to maintain the medusa stage. The earliest RFamide positive nerve cells are found in bud stages 4 where the ring canal will develop. Boelsterli (1977) reported that bud stages 3-4 begin to harbor myofilaments in the entocodon. If we superimpose the detection of RFamide-positive nerve cells at stage 4 with the appearance of myofilaments it becomes apparent that nerve and muscle differentiation both begin synchronously around bud stage 3 to 4.

Striking observations include the invading neurites, which travel through the stalk of the bud. Spencer (1974) analyzed the responses of attached medusa buds to colonial excitation in *Proboscidactyla flavicirrata* and argued that ectodermal conduction causes crumpling while an endodermal conducted colonial pulse leads to swimming behavior. Our data demonstrate a neuronal interface bridging polyp and medusa in *Podocoryne*. Although, we found no evidence that these processes of the polyp nerve system connect to the medusan nervous system, these results indicate that the polyp - at least in part - may supervise medusa bud development by neuronal control. These findings may contribute to our understanding of why isolated buds can fulfill medusa development exclusively from stage 8 on. The ectodermal neurites, which project from the gonozoid into the prospective exumbrellar layer, make contact to many, but not all nematocytes. In the mature medusa, nematocytes are also found scattered all over the exumbrella, although we never observed neurites connecting them. Whether this network is temporally restricted to the developing medusa bud remains to be resolved. In *Hydra*,

synapses were reported between neurons, between neurons and epitheliomuscular cells and between neurons and nematocytes (Westfall, 1973; Kinnamon and Westfall 1984). But it seems unlikely that these synaptically-connected nematocytes in the medusa bud serve as mechano-receptors or may be discharged upon neuronal stimuli, as the perisarc firmly wraps the whole medusa bud.

The nerve system of the adult medusa displays higher organization

The organization of the *Podocoryne* medusa nervous system is in complete agreement with data from other hydromedusae (Mackie *et al.*, 1985). Cnidarians lack a predominant nervous center, a brain. However, above the inner side of the four tentacle bulbs, the main nervous junctions are made. At the tentacle bulbs, stimuli from tentacles and the manubrium (and sense organs, if present) are passed to the ring canal nerves, which represent the processing center. Spencer and Arkett (1984) argued that a radially symmetrical animal should have a ring-shaped central nervous system. Furthermore, the nerves at the ring canal are morphologically concentrated in different bundles. In general, an outer nerve ring, which is more marginally positioned, is distinguished from an inner nerve ring. Nerves within the nerve bundles are further differentiated, such as the giant axons for rapid conduction in *Aglantha digitale* (Mackie and Meech, 1995a,b). Moreover, an endodermal nerve ring has been reported in the hydromedusae *Sarsia*, *Euphysa* and *Stomotoca* (Jha and Mackie, 1967; Mackie and Singla, 1975). Our results show that the *Podocoryne* nervous system begins to develop at the prospective umbrella opening and only later develops in the manubrium, below the radial canals and finally in the tentacles. The high degree of morphological differentiation and the early onset of differentiation imply that the ring canal nerves represent the central nervous system of hydromedusae.

ACKNOWLEDGEMENTS

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