RESEARCH ARTICLE

New Insights on Ctenophore Neural Anatomy: Immunofluorescence Study in *Pleurobrachia pileus* (Müller, 1776)

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ABSTRACT

Ctenophores are non-bilaterian animals sharing with cnidarians and bilaterians the presence of sensory receptors, nerve cells, and synapses, absent in placozoans and sponges. Although recent immunofluorescence studies have renewed our knowledge of cnidarian neuro-anatomy, ctenophores have been much less investigated despite their importance to understanding the origin and early evolution of the nervous system. In this study, the neuro-anatomy of the ctenophore *Pleurobrachia pileus* (Müller, 1776) was explored by whole-mount fluorescent antibody staining using antibodies against tyrosylated α-tubulin, FMRFamide, and vasopressin. We describe the morphology of nerve nets and their local specializations, and the organization of the aboral neuro-sensory complex comprising the apical organ and polar fields. Two distinct nerve nets are distinguished: a mesogleal nerve net, loosely organized throughout body mesoglea, and a much more compact "nerve net" with polygonal meshes in the ectodermal epithelium. The latter is organized as a plexus of short nerve cords. This epithelial nervous system contains distinct sub-populations of dispersed FMRFamide and vasopressin immunoreactive nerve cells. In the aboral neuro-sensory complex, our most significant observations include specialized nerve nets underlying the apical organ and polar fields, a tangential bundle of actin-rich fibers (interpreted as a muscle) within the polar fields, and distinct groups of neurons labeled by anti-FMRFamide and anti-vasopressin antibodies, within the apical organ floor. These results are discussed in a comparative perspective. *J. Exp. Zool. (Mol. Dev. Evol.) 316:171–187, 2011.* © 2010 Wiley-Liss, Inc.

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The acquisition of the nervous system was a key event in animal evolution, providing the basis for sophisticated integration of environmental cues and active behaviors. Nervous systems reach their highest anatomical and functional complexity in the Bilateria, but typical nerve cells and both kinds of synapses (chemical and electrical) are also present in the two non-bilaterian phyla Ctenophora and Cnidaria. Cytological and physiological data suggest homology between these cnidarian/ctenophore nerve cells and their bilaterian counterparts (Bullock and Horridge, '65) and this hypothesis has received additional support from recent molecular studies showing the expression of

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several homologues of bilaterian neural genes in cnidarian neuro-sensory cells (reviews in Galliot et al., 2009; Watanabe et al., 2009).

Neural cells were probably acquired only once in animal evolution, in a common ancestor of ctenophores, cnidarians, and bilaterians (Eumetazoa), while sponges and placozoans are the only nerve-less metazoans. The branching order of the earliest metazoan lineages remains controversial, with some recent molecular phylogenies casting doubt on this scenario (see Dunn et al., 2008; Miller, 2009; Schierwater et al., 2009). However, in line with classical views, analyses of a phylogenomic data set optimized for species sampling of the non-bilaterian lineages (Philippe et al., 2009) lent support to eumetazoan monophyly and thereby to a single origin of the nervous system (as well as of muscle cells). Another significant outcome of the same study was a sister-group relationship of ctenophores and cnidarians rehabilitating the old ''coelenterate'' group (also obtained by Schierwater et al., 2009).

A good knowledge of the neural features of cnidarians and ctenophores is critical for reconstructing the earliest steps of nervous system evolution. Although recent investigations using modern tools, such as immunofluorescent antibody staining or gene *in situ* hybridization, have provided a renewed picture of cnidarian neuro-anatomy, much less is known about the ctenophore nervous system. Therefore, it remains unclear to what extent ctenophores share with cnidarians the same fundamental neuro-anatomical characteristics, a particularly important question in the context of a supposed sister-group relationship between these two groups (Philippe et al., 2009). In a previous work (Jager et al., 2008), we have started to characterize elements of the neuro-sensory system at the molecular level, in the adult ctenophore *Pleurobrachia pileus* (Fig. 1A) (the sea gooseberry, order Cydippida), using genes of the SOX family as markers. In this study, we make a contribution to improving our understanding of ctenophore neuro-anatomy by using immunofluorescence.

Previous studies using histological techniques (Chun, 1880; Eimer, 1880; Hertwig, 1880; Samassa, 1892; Bethe, 1895; Heider, '27) or electron microscopy (Horridge and Mackay, '62, '64; Horridge, '65; Hernandez-Nicaise, '73a,b,c, '74; Tamm and Tamm, '95, 2002) have described the ctenophore nervous system as consisting mainly of a polygonal plexus integrated in the ectodermal epithelium, usually called the ''subepithelial nerve net'' (e.g. Hernandez-Nicaise, '91) and here referred to as the ''polygonal nerve net'' (to avoid using the ambiguous term ''subepithelial'') (Fig. 1B). The existence of an additional nerve net located in the mesoglea is not firmly established. For example, Hernandez-Nicaise ('73b) could detect nerve fibers, but no nerve cell bodies, in the body mesoglea of *Beroe*. In other investigated ctenophores species (e.g. *Pleurobrachia rhodopis*, *Lampetia pancerina*, and *Callianira bialata*; Hernandez-Nicaise, '91), a specialized axial nerve cord has long been described in the tentacle mesoglea, but neural cells have never been observed in the body mesoglea.

At the cellular level, ctenophore neurons have been classified as bipolar (i.e. cell body bearing two outgrowths or neurites) and multipolar (with more than two neurites) (Hernandez-Nicaise, '73a). In addition, two types of sensory neurons distributed throughout the epidermis have been described (Fig. 1B) (Hernandez-Nicaise, '91). The first type, called ciliated sensory cells (or ''vibroreceptors''), is characterized by the presence of a single non-motile cilium with a rather complex root. Hoplocytes, the second type of sensory neurons, bear one to several actin-rich apical peg(s) occasionally accompanied by a cilium. At their basal extremity, both types of sensory cells have neurites that establish synapses with neurons of the polygonal nerve net (Hernandez-Nicaise, '74; Hernandez-Nicaise, '91).

Ctenophore synapses are uniquely characterized by their ''triadic'' pre-synaptic morphology, with tight association of a vesicule row, a specialized portion of endoplasmic reticulum, and one to several large mitochondria (Hernandez-Nicaise, '73c). The nature of molecules involved in neurotransmission remains unknown, but the presence of FMRFamide immunoreactivity in neurons has been previously reported (Grimmelikhuijzen, '83; Grimmelikhuijzen et al., '89b). Ctenophore neurons can establish synapses with various kinds of effector cells, mainly gland cells, ciliated epithelial cells, and muscle cells. The latter are true muscle fibers without a distinct epithelial cell body (an important difference with cnidarians, Hernandez-Nicaise, '91) and they occur in the basal region of the ectodermal epithelium (parietal muscle) as well as within the mesoglea (mesogleal muscle) (Hernandez-Nicaise, '91 and our unpublished TEM observations in *Pleurobrachia pileus*; Fig. 1B).

There are local differentiations of the nervous system, particularly in relation to specialized ciliated epidermal structures (Hernandez-Nicaise, '84) (Fig. 1A). Such specializations of the ciliated epithelia include the eight longitudinal rows of swimming paddles or combs used for locomotion, each comb comprising several hundreds of tightly linked cilia. Experimental evidence (reviewed in Tamm, '82) indicates that propagation of beating waves along the comb rows does not involve neural transmission, but the nervous system modulates their beating frequency upon environmental stimulation or during particular behaviors. Ctenophores detect gravity (and probably other kinds of environmental stimuli), thanks to a complex sensory organ (the apical organ), located at their aboral pole (Tamm, '82). The apical organ is essentially made of various types of ciliated cells, including four groups of multiciliated cells called the balancer cells, supporting a mineralized statolith. Each balancer functions as a pace maker controlling the beating frequency of a pair of comb rows through special strands of epidermal-ciliated cells (ciliated grooves) (Tamm, '82). Many synapses have been described in the apical organ epithelial floor (Hernandez-Nicaise, '73a, '74) but the organization of the associated nervous system is

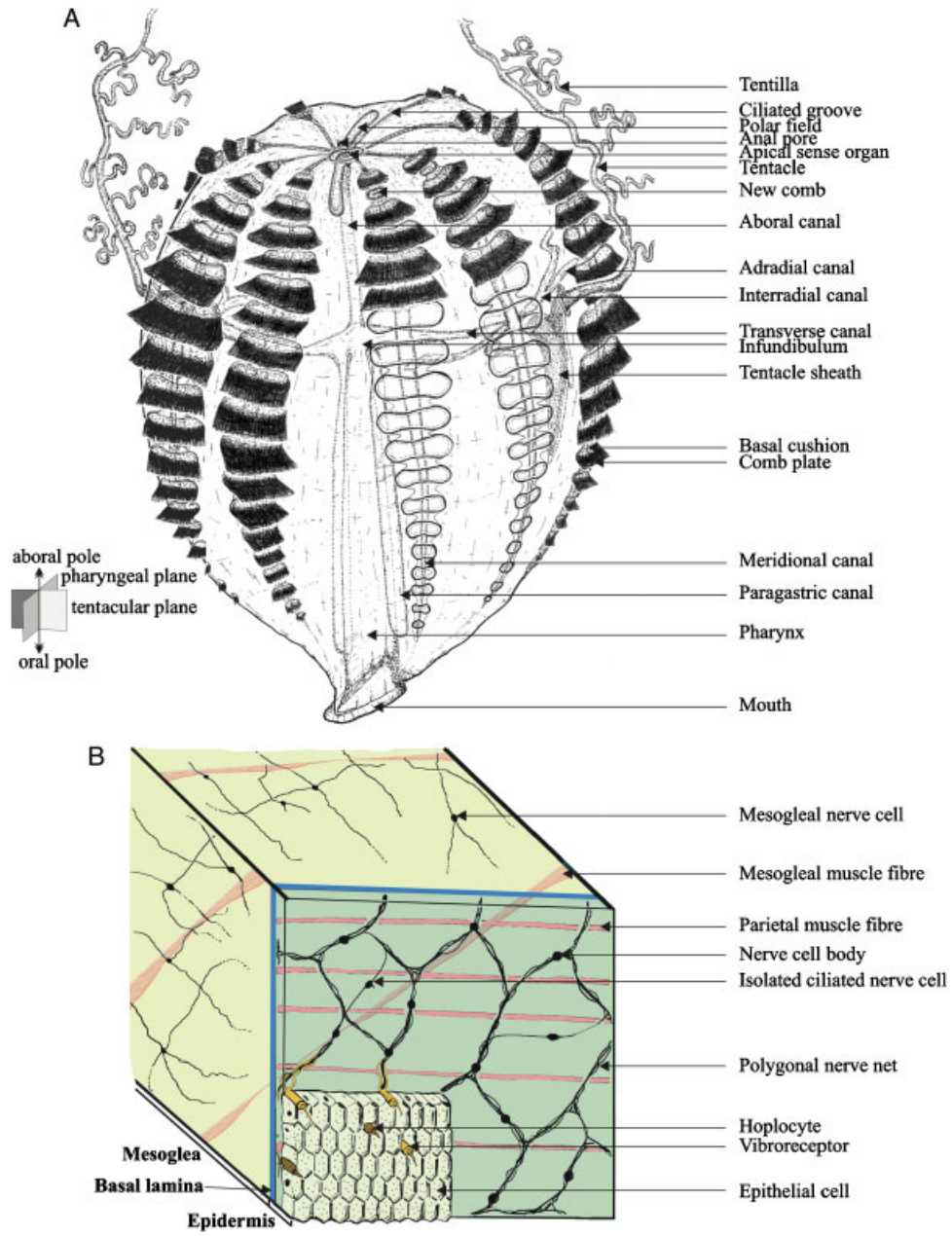

Figure 1. (A) General organization of *Pleurobrachia pileus* (from Jager et al., 2008). (B) Diagram summarizing the relative positions with respect to the basal lamina (in blue) of the polygonal (epithelial) nerve net and of the mesogleal nerve net, based on previous TEM investigations (references cited in the text) and on results from this study. Also included in the diagram are mesogleal and epithelial muscle cells and, at the bottom left corner, epithelial cells and both types of sensory cells (vibroreceptors and hoplocytes). Other cell types (e.g. gland cells) have been omitted for clarity. Note that the existence on the diagram of a space between the mesogleal nerve net and the basal lamina is intended to reflect uncertainty about a possible connection between the two nerve nets. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

not known. The apical organ is flanked by two tong-shape ciliated areas, the polar fields, extending in the pharyngeal plane. Their organization and functional significance remain unclear, but the expression of the *PpiSox3* gene revealed the presence of putative sensory organs ("Z bodies") aligned in the marginal zone of the polar fields (Jager et al., 2008). Another important feature of the ctenophore body plan is the presence of a pair of tentacles (absent in some groups, e.g. Beroida) used for catching preys and

covered with specialized sticking cells (colloblasts), thought by some to be highly modified ciliated cells (Hernandez-Nicaise, '91).

In this study, we re-explored the neuro-anatomy of the adult ctenophore *Pleurobrachia pileus* using antibodies directed against tyrosylated α-tubulin (a modified form of tubulin abundant in neurons; Hammond et al., 2008), FMRFamide, and vasopressin. The FMRFamide-like peptides or FLPs have widely been found as neurotransmitters or neuromodulators in all metazoan phyla with the exception of echinoderms (Grimmelikhuijzen et al., '96; Watanabe et al., 2009; Walker et al., 2010). The vasopressin-like neuropeptides (comprising oxytocins and vasopressins) are evolutionarily well conserved and have been identified in nervous system of various protostomes, cnidarians, and vertebrates (Grimmelikhuijzen et al., '82; Caldwell et al., 2008; Bardou et al., 2009). Compared with classical histology approaches, whole-mount immunodetection offers a more precise labeling of cell types (depending on the specificity of the antibody) and a better view of three-dimensional organization. This study is focused on neurons and neural structures and does not describe the types and distribution of epithelial sensory cells, which would deserve separate investigation. Furthermore, we did not investigate the nervous system associated with the endoderm. We provide the first evidence for the existence of a body mesogleal nerve net and for neuronal type specialization with respect to putative neurotransmitters in ctenophores. Furthermore, our observations highlight the structural complexity of the ctenophore body plan, with several structures being newly described, notably in the aboral sensory complex.

MATERIALS AND METHODS

Specimen Collection

Adult specimens of *Pleurobrachia pileus* were collected using plankton nets in Villefranche-sur-mer (France) during their reproductive season, between March and June. They were transferred into filtered natural seawater and kept at 16°C.

Immunofluorescence

Animals were immersed in a mix of 50% sea water and 50% of paraformaldehyde (8%) in phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.5). After fixation for 15 min, at room temperature, the samples were washed several times in PBS, dehydrated through a graded series of ethanol, and stored in methanol at −20°C. After stepwise re-hydration to PBS, samples were permeabilized with Triton-X100 (0.2% in PBS, then 0.01% in PBS, 10 min at room temperature). After blocking with 1% bovine serum albumin, samples were incubated with one or two of the following primary antibodies for at least 2 hrs at room temperature: rat monoclonal anti-tyrosylated α-tubulin or YL1/2 antibody (1:1,000 dilution, Serotec), rabbit polyclonal anti-FMRFamide (1:1,000 dilution, Abcam), and rabbit polyclonal anti-vasopressin (1:20 dilution, Abcam). The anti-FMRFamide and anti-vasopressin antibodies were pre-absorbed with *Pleurobrachia* tissue to reduce cross-reactivity. After washing with PBS Triton-X100 0.01% (PBST), samples were incubated overnight at 4°C with the appropriate secondary antibodies: Alexa Fluor® 568 goat anti-rat IgG and/or Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, Invitrogen, Cergy Pontoise, France). Dilutions of primary and secondary antibodies were made using PBST. Specimens for phalloidin staining were fixed but not dehydrated. They were incubated for 45 min in a dilute Phalloidin-TRITC (Sigma, Saint-Quentin, Fallovier, France) solution (10 µg/mL in PBST) and rinsed three times in PBST. All samples were finally stained with DAPI (1 µg/mL) for 15 min for DNA visualization, and then washed three times for 15 min in PBST.

Before mounting in Vectashield® solution, animals were micro-dissected. The epithelium was cut longitudinally between adjacent comb rows, and the specimens were spread out on a microscope slide, with the apical region in the centre. All fluorescence and DIC images were performed using a microscope (Olympus BX61, Olympus, France) with a Q-imaging Camera using Image Pro plus software® (Mediacybernetics, ScopPro, Itteville, France). To enhance fine details, Higauss filter was used on each picture (from Image Pro, ScopPro, Itteville, France). Confocal microscopy was performed using a Leica SP5 microscope.

RESULTS

Characterization of Polygonal (Epithelial) and Mesogleal Nerve Nets

The anti-tyrosylated-α-tubulin antibody (YL1/2) acts as an accurate marker of nerve cells, owing to the abundance of microtubules present in these cells (Hernandez-Nicaise, '74). It also stains several additional cell types (sensory cells, glandular cells, and muscle cells), generally with lower intensity. Neural cells are identified by their characteristic shape (with cell body and thin elongated neurites). Using the YL1/2 antibody, we could distinguish two different nerve nets in the adult ctenophore, as summarized in Figure 1B.

Throughout the epidermis, the YL1/2 antibody reveals a conspicuous epithelial nerve net (Fig. 2A and B). This nerve net has been called "subepithelial" in the literature (Hernandez-Nicaise, '91), and according to previous TEM investigations, it is located in the basal region of the epidermal cell layer, with its neural cell bodies and neurites being interspersed between other epidermal cell types (i.e. typical epithelial cells, glandular cells, sensory cells, parietal muscle cells…). In our YL1/2-stained preparations, its polygonal meshes are irregular in shape and size, their diameter ranging from about 25 to 130 µm (Fig. 2A and B), as previously described (Hernandez-Nicaise, '91). Most neurites are arranged in parallel bundles, so that the polygonal nerve net is not a network of single neurites, but of short "nerve cords" each comprising several (2–5) parallel neurites (Fig. 2A-A′-B). Nerve cell bodies (recognizable by their nuclei in Dapi

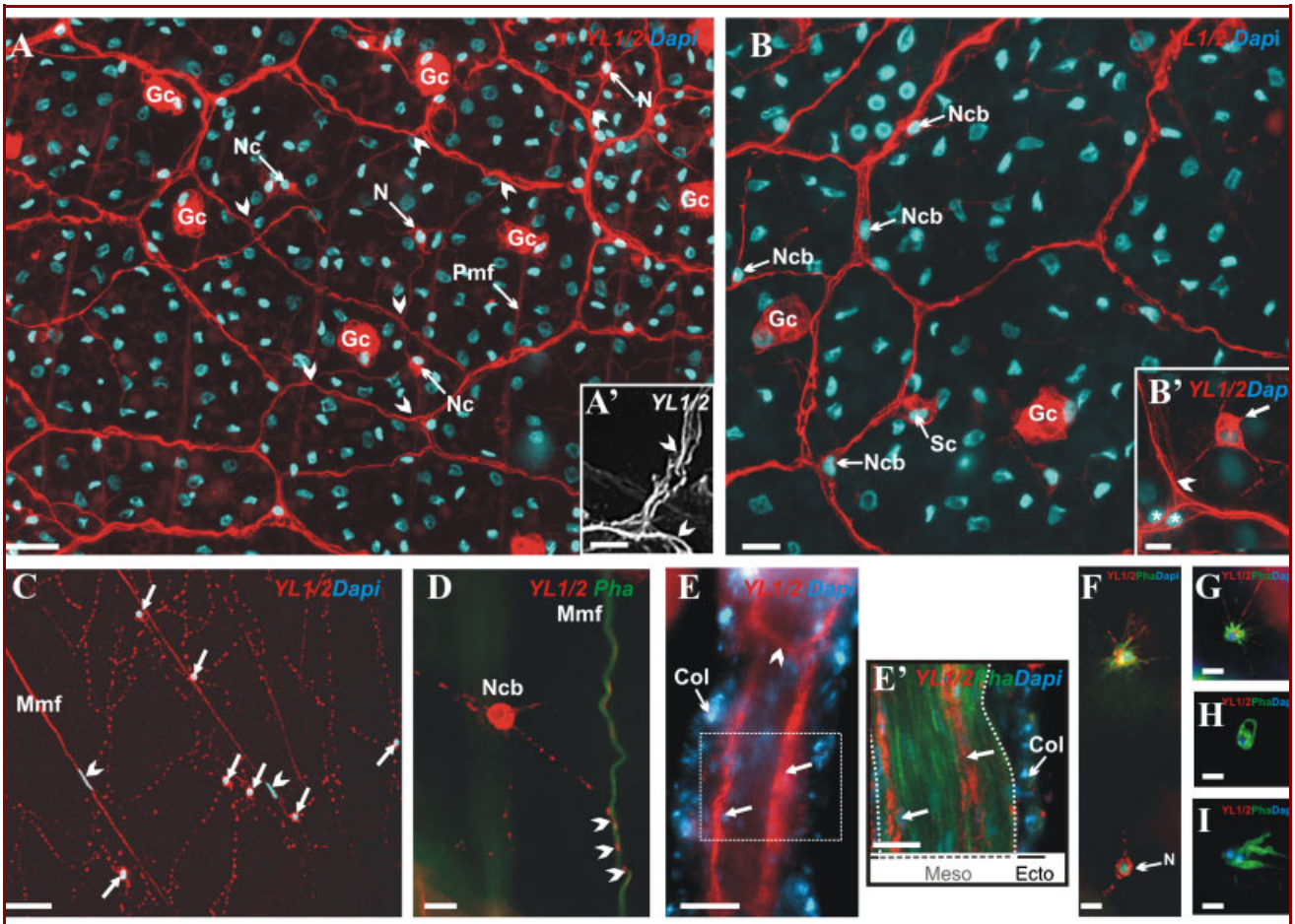

Figure 2. Characterization of *Pleurobrachia*'s nerve nets by anti-tyrosylated-α-tubulin (YL1/2) immunostaining (in red) and DAPI counterstaining (in blue): (**A**) General view of the polygonal nerve net showing its general organization. White arrowheads indicate where neurites of isolated neurons (N, Nc) join the main network. (**A′**) Higher magnification view showing that several parallel neurites are associated to form each segment of the network (white arrowheads). (**B**) Distribution of neural cell bodies (Ncb) in a closer view of the polygonal nerve net. (**B′**) Higher magnification view of an isolated ciliated neuron (white arrow: cilium) and its connection to the main network (white arrowhead; stars: nuclei of neural cells belonging to the polygonal nerve net). (**C**) A portion of the mesogleal nerve net (white arrows: nerve cell bodies; white arrowheads: nuclei of muscle cells). (**D**) Contact (white arrowheads) between a neurite of a mesogleal nerve cell and a mesogleal muscle fiber (Mmf). (**E**) The tentacle contains two parallel nerve cords stained with YL1/2 (white arrows) and linked by transverse connections (white arrowhead). (**E′**) Confocal longitudinal view of the tentacle counterstained with phalloidin (in green), showing that the two nerve cords (arrows) are embedded into the muscle layer and thereby belong to the mesoglea (Ecto: ectoderm; Meso: mesoglea). (**F**) A mesenchymal cell of the "vacuolated cell" type (stained by both YL1/2 and phalloidin) and a mesogleal nerve cell (N, only stained by YL1/2) in the same optical plan. (**G–I**) The three types of mesenchyme cells, (G) vacuolated cell, (H) clear cell, (I) filamentous cell. (C and E′) confocal microscopy (Z projection 14.7 μm in C, 20.44 μm in D); other pictures: classical microscopy. Col, colloblasts; Gc, glandular cell; Mmf, mesogleal muscle fiber; N, isolated nerve cell; Nc, isolated ciliated nerve cell; Ncb, nerve cell body; Pmf, parietal muscle fiber; Sc, sensory cell. Scale bars: A, B, E: 20 μm; C, E′: 10 μm; A′ B′,D, F–I: 5 μm.

counterstaining) are sometimes placed at nodes, but many nodes of the network lack a cell body, and many of the nerve cell bodies lie along the network interconnections (i.e. the segments linking two nodes) (Fig. 2B Ncb). Besides the main neural network, many isolated multipolar nerve cells are observed within the meshes (Fig. 2A and B′). Their neurites generally connect with the polygonal nerve net (Fig. 2A and B′, white arrowheads). Some of these isolated nerve cells bear a short cilium on their cell body (Fig. 2B′). These ciliated neurons, observed throughout the epidermis, are clearly distinct from the previously described

sensory cells (vibroreceptors and hoplocytes) by (i) the shortness of their cilium (much longer in vibroreceptors), (ii) the absence of an actin-rich peg (present in hoplocytes), (iii) the orientation of the cilium, not exposed to the external environment and projecting laterally or even in some cases toward the mesoglea (not shown).

A second nerve net was detected in the depth of the body mesoglea by YL1/2 staining (Fig. 2C and D and supp. Movie 1). Unlike the subepidermal nerve net, the mesogleal nerve net is a network of single neurites and thereby its organization is much more loose. The cell bodies are small (3–3.5 μm) and multipolar (Fig. 2C and D). In confocal (Fig. 2C) as well as conventional microscopy (Fig. 2D), staining of the neurites is discontinuous in the form of many aligned little puncta. This aspect could reflect either compositional difference between labile and stable microtubule domains along the neurites as documented for mammalian axons (Brown et al., '93) or artefactual modification of α-tubulin structure upon fixation. It is nevertheless clear that these mesogleal cells are organized in network (Fig. 2C) and that some of the neurites end in close contact with mesogleal muscle fibers (Fig. 2D). This mesogleal nerve net could be detected in all parts of the body, its density being higher in some particular regions (e.g. near the tentacle opening and close to the aboral sensory complex). We failed to detect clear connections between the epithelial and mesogleal nervous systems.

Since previous works on *Pleurobrachia* described mesogleal nervous components in the tentacles only, but failed to observe a nerve net throughout the body mesoglea (Hernandez-Nicaise, '91), we wanted to check whether the mesogleal nervous system of tentacles was also stained using the same anti-α-tubulin antibody. Two parallel nerve cords were stained with high intensity within the tentacle (Fig. 2E, E′). In confocal microscopy view (Fig. 2E′), these nerve cords clearly appeared embedded into the mesogleal tentacle musculature. We also observed thin transverse connections between the two nerve cords (Fig. 2E). These results confirm that YL1/2 immunofluorescence is a convenient tool for visualization of the ctenophore mesogleal nervous system.

In addition to nerve cells, the mesoglea also contains muscle fibers and various types of "mesenchyme cells" (Delage and Herouard, '01; Hyman, '40; Hernandez-Nicaise, '68a; Franc, '85) of unknown function. Because some of the mesenchyme cells bear thin processes, at first sight they could be confounded with neurons. We found that phalloidin staining offers a very efficient criterion for distinguishing between mesogleal neurons and mesenchyme cells, the latter being intensely stained with phalloidin while neurons are not (see YL1/2+phalloidin counter-staining in Fig. 2D and F). That mesenchyme cells have higher actin content is likely to reflect more intense cellular dynamics, compared with neurons, consistent with previous data suggesting that mesenchyme cells are highly mobile within the mesoglea (Franc, '85).

Among mesenchyme cells, we were able to recognize three main types (Fig. 2G–I). The first type is characterized by numerous long pseudopodial processes radiating from the cell body (Fig. 2G and F on the top). Although their morphology is somewhat neural-like, their cell bodies and processes are not only α-tubulin-rich (stained by YL1/2 antibody) but also actin-rich (stained by phalloidin). These cells are present at higher density in the most superficial region of the mesoglea between the epidermis and the mesogleal nerve net (supp. Movie 1) and they accumulate in huge numbers under cicatrizing portions of epithelium after healing (unshown observations). All these features unambiguously point to the "vacuolated cells" described by Franc ('85). The two remaining mesenchymal cell types are phalloidin-positive but YL1/2 negative. Some have a more or less ovoid shape and no process (Fig. 2H); they are not abundant and might correspond to the "clear cells" (Franc, '85). A last and much more abundant cell type is represented by elongated and sometimes ramified multinucleated cells (Fig. 2I). They probably correspond to the "filamentous cells" of Franc ('85), hypothesized by this author to be the precursors of mesogleal muscle fibers.

Local Specializations of the Polygonal Nerve Net

Local morphological differentiations (Fig. 3) of the polygonal nerve net can result from higher polygons density, thickening of polygon segments, preferential orientation of the polygons, or a combination of these three types of modification.

A first example of such condensations is the "tentacle nerve" (Hernandez-Nicaise, '73a, '91), a linear thickening integrated in the design of the polygonal nerve net, in the tentacular plane (Fig. 3A–D). In fact, it is a nerve cord, not a nerve, and to avoid confusion with the mesogleal nerve cords present in the tentacle itself (see above and Fig. 2E-E′), we propose to rename this structure "juxtatentacular nerve cord." It extends along the tentacular plane from the vicinity of the apical organ (Fig. 3A, white arrows) to the tentacle sheath (Fig. 3B) and then down to the oral hemisphere. The number of neurites comprised in the juxtatentacular nerve cord is difficult to estimate from whole-mount preparations but is clearly higher than five (see Fig. 3C). Interestingly, nerve cell bodies are not scattered along the juxtatentacular nerve cord but instead concentrated in small groups of 3–4 cells as revealed by DAPI staining (arrowheads in Fig. 3C).

We constantly observed that the juxtatentacular nerve cord becomes progressively thinner at its aboral extremity and finally vanishes within the polygonal nerve net, at some distance from the apical organ (Fig. 3A and D). Therefore, contrary to former suggestions (Hernandez-Nicaise, '74, '91), it does not reach the apical organ. This nevertheless does not imply the absence of a preferential communication pathway between the apical organ and the tentacle through the polygonal network, but this pathway if it exists is not morphologically identifiable in YL1/2-stained preparations.

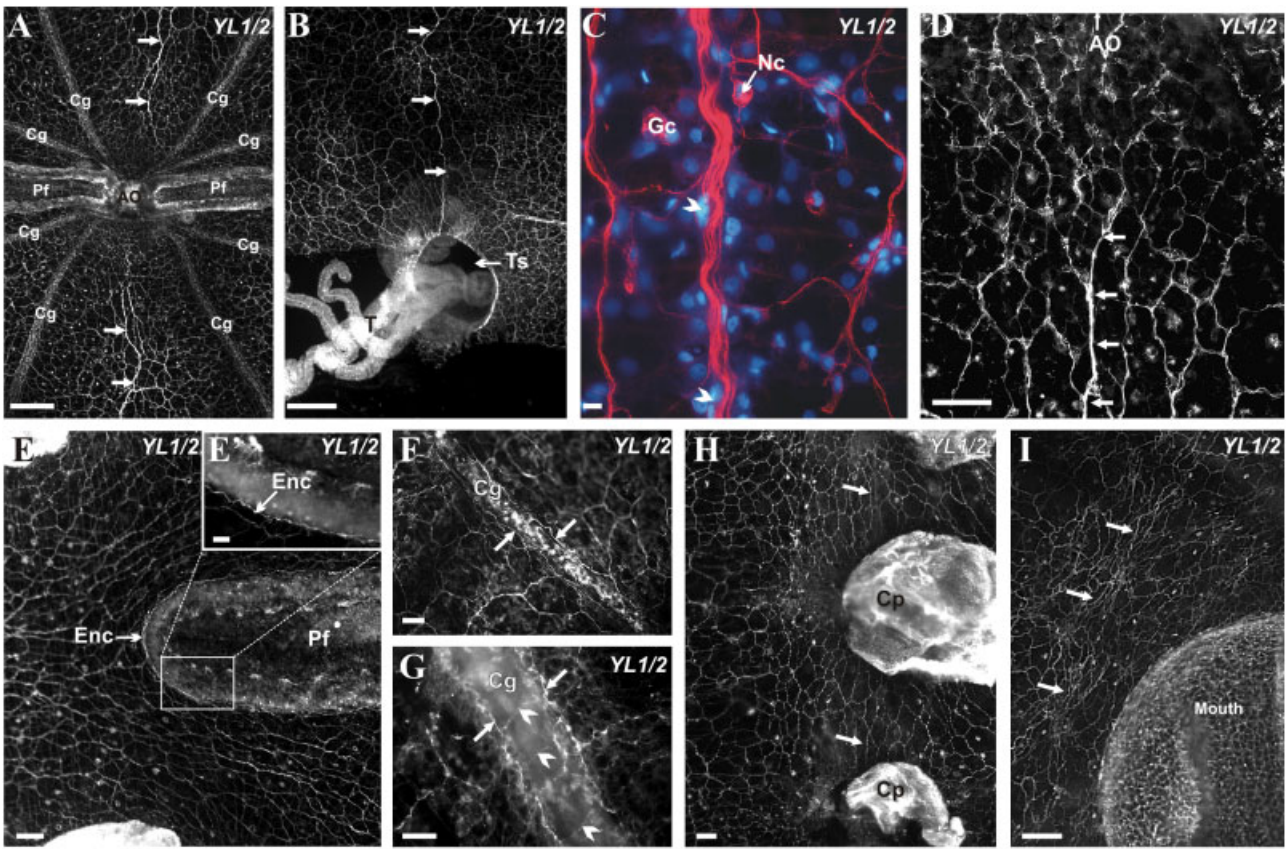

Figure 3. Modifications of the polygonal nerve net visualized by anti-tyrosylated-α-tubulin (YL1/2) immunostaining. (**A**) General view of the polygonal nerve net at the aboral pole showing the juxtatentacular nerve cords (white arrows) and a higher polygon density near the apical organ. (**B**) Juxtatentacular nerve cord (white arrows) entering into a tentacle sheath. (**C**) Closer view of the juxtatentacular nerve cord stained by YL1/2 (in red) and with Dapi counterstaining (in blue) showing its integration within the polygonal nerve net. White arrowheads indicate groups of nerve cell bodies (white arrows) of the juxtatentacular nerve cord. (**D**) The nerve net at the vicinity of the apical organ showing how the juxtatentacular nerve cord vanishes into the nerve net. (**E**) Extremity of a polar field and surrounding polygonal nerve net, showing the external circum-polar nerve cord (Enc) and the preferential orientation of the nerve net meshes around the polar field. (**E′**) Higher magnification view of the boxed area in E. (**F** and **G**) (higher magnification). Morphology of the nervous system along a ciliated groove (arrows: thin nerve cords running along the edges of the ciliated groove; arrowheads: transverse neurites joining both nerve cords). (**H**) Higher density and preferential orientation of the meshes (arrows) between three successive comb plates. (**I**) Higher density and preferential orientation of the meshes (arrows) around the mouth. AO, apical organ; Cg, ciliated groove; Cp, comb plate; Enc, external circum-polar nerve cord; Gc, glandular cell; Nc, isolated ciliated nerve cell; Pf, polar field; T, tentacle; Ts, tentacle sheath. Scales bars: A, B, E, H, I: 50 μm; D′, E, F, G: 20 μm; C: 5 μm. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

In the aboral region, several kinds of modifications are observable. In the tentacular plane, mesh density of the polygonal nerve net increases close to the apical organ, without particular change in the orientation or thickness of the connecting segments (Fig. 3A). Close to the polar fields, meshes are elongated and parallel to the polar field edges (Fig. 3E). Furthermore, the polygonal nerve net differentiates a continuous "external circum-polar nerve cord" (Jager et al., 2008) that encircles the polar fields (Fig. 3E and E′).

Along the ciliated grooves, we observed preferential orientation of the meshes, and a thin nerve cord-like structure running along each side of the ciliated groove (Fig. 3F and G, arrows). We could detect neurites crossing the ciliated groove and linking together both nerve cords (Fig. 3G, arrowheads), consistent with previous observations (Hernandez-Nicaise, '73a).

Similar longitudinal orientation of the meshes was also observed within the comb rows between two successive comb plates (Fig. 3H). But, there, interconnecting segments of the

network are not particularly thickened (Hernandez-Nicaise, '91). Finally, near the mouth, the polygonal nerve net is also modified with meshes being smaller and orientated parallel to the mouth margin (Fig. 3I).

Distinct Epithelial Nerve Cell Populations Revealed by Anti-Neuropeptide Antibodies

By using antibodies directed against neuropeptides, concomitantly with the structural marker YL1/2, we could identify various sub-populations of nerve cells within the epithelium (Fig. 4). With these anti-neuropeptide antibodies the staining appears discontinuous in the form of several puncta scattered within cell bodies and aligned along the neurites. We failed to detect positive mesogleal neurons with these anti-neuropeptides antibodies.

The anti-FMRFamide antibody revealed (i) a small subset of neurons integrated into the polygonal network itself, strongly counterstained by YL1/2 (yellow color on Fig. 4A, A′); and (ii) isolated nerve cells sparsely distributed throughout the epithelium, within the meshes of the polygonal nerve net, and only weakly stained by the YL1/2 antibody (green color on Fig. 4A, A″). These anti-FMRFamide-positive neurons have small cell bodies (between 4 and 5 μm). They are markedly more abundant near the ciliated grooves (Fig. 4A).

Throughout the epithelium, the anti-vasopressin antibody stains a much denser nerve cell sub-population than the anti-FMRFamide antibody (Fig. 4B). In addition, anti-vasopressin-positive cell bodies are larger (between 6 to 8 μm), and many of them bear a short and thin cilium (Fig. 4B′, Vc). These ciliated anti-vasopressin immunoreactive cells are dispersed throughout the epithelium and they closely look like the ciliated neurons described previously (Fig. 2B′). Again, these anti-vasopressin positive neurons are either integrated in the main polygonal network or isolated within the meshes (Fig. 4B–B′). In the former case, we never observed more than a single anti-vasopressin-positive neurite per network segment, implying that anti-vasopressin positive and negative neurons co-exist within a given segment.

Anti-Neuropeptide and Anti-Tyrosylated-α-Tubulin Antibodies Reveal Different Neural Structures in the Apical Organ Floor

In the epithelial floor (Hernandez-Nicaise, '91) of the apical organ (AO), our immunohistochemical investigation highlights a deep nerve net and several previously undescribed neural structures (Figs. 5 and 6). The precise localization of these structures can be described and compared, thanks to the positional markers provided by DIC views (Fig. 5A) and Dapi counterstaining (Fig. 5B–B′). Examples of such positional markers include the four balancers (B in Fig. 5A and B, B′), the lithocytes (Lt in Fig. 5B′), the four "refringent bodies" (Rb in Fig. 5A and B, B′), the two epithelial papillae (Ep in Fig. 5B′), and two regular rows of superficial nuclei, located in the AO floor between the refringent bodies, along the pharyngeal plane (Fig. 5B′) (see also Jager et al., 2008).

A close examination of the AO floor stained by YL1/2 reveals the presence of a deep nerve net (Fig. 5C). Since basal laminae are

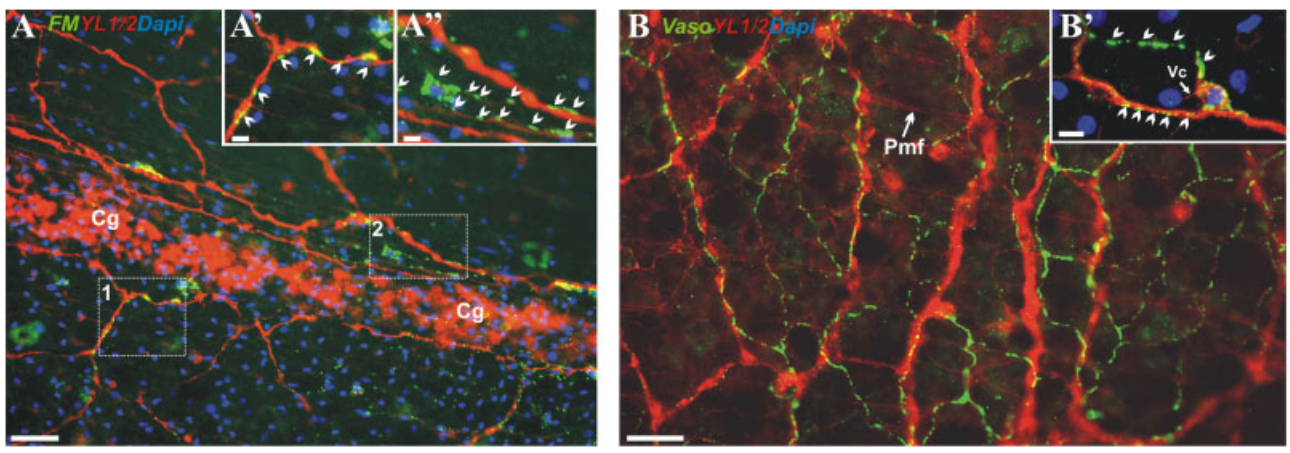

Figure 4. Distinct nerve cell sub-populations revealed in the epithelium by antibodies directed against neuropeptides (in green; with anti-tyrosylated-α-tubulin (YL1/2) counterstaining in red and Dapi in blue). (**A**) Anti-FMRFamide-(FM) positive cells near a ciliated groove. (**A′**) Higher magnification view corresponding to box 1 in A, showing a FMRFamide positive nerve cell integrated into the polygonal nerve net and counterstained by YL1/2 (yellow staining: white arrowheads). (**A″**) Higher magnification view corresponding to box 2 in A, showing an isolated FMRFamide-positive nerve cell not counterstained by YL1/2 (green staining: white arrowheads). (**B**) Distribution of anti-vasopressin immunoreactive neurons (green) throughout the polygonal nerve net. (**B′**) Higher magnification view of an anti-vasopressin positive cell with distribution of staining throughout neurites (white arrowheads) and cell body, the latter bearing a cilium (Vc). Cg, ciliated groove; Pmf, parietal muscle fiber; Vc, cilium. Scales bars: A, B: 20 μm; A′, A″, B′: 5 μm. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

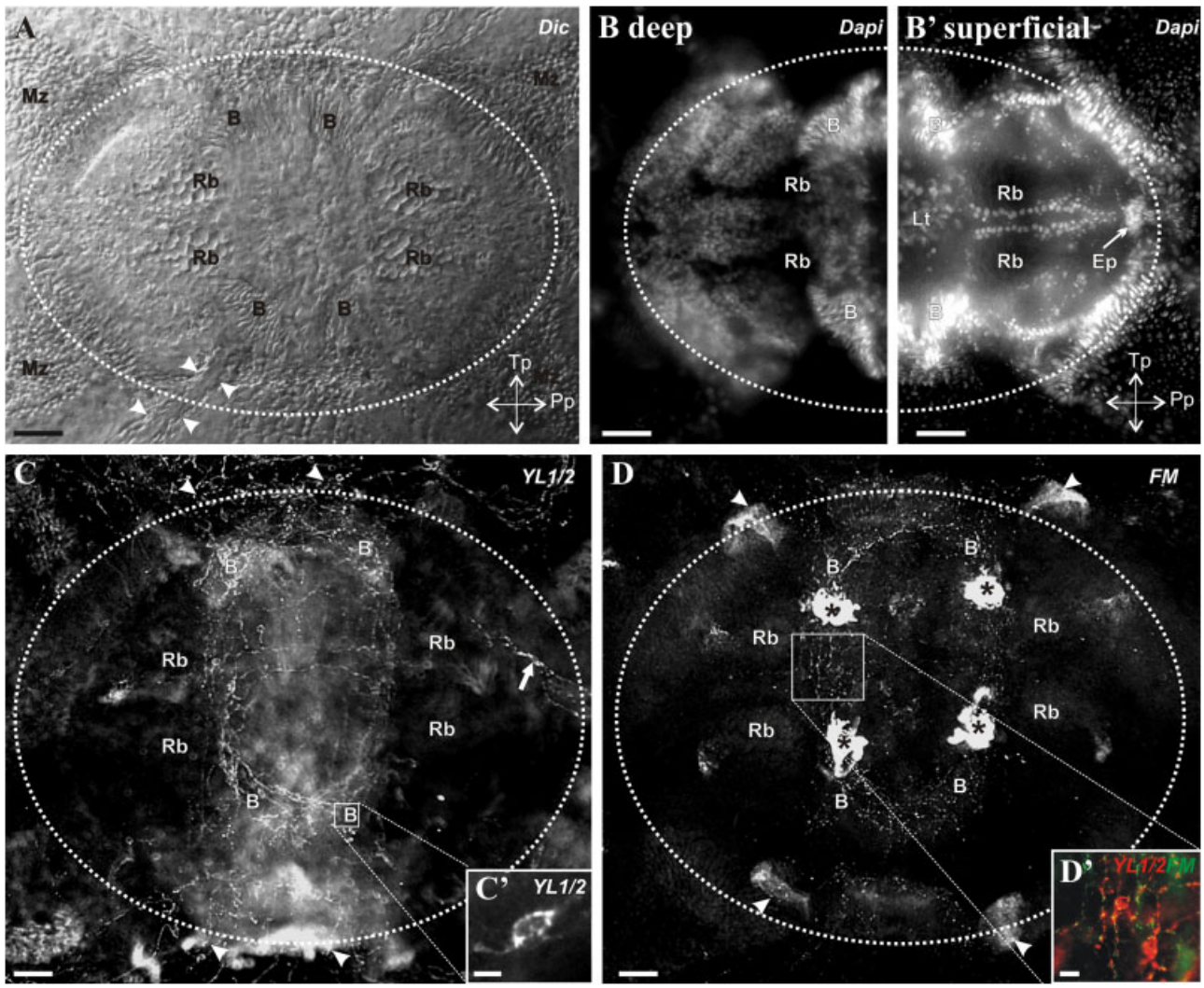

Figure 5. Organization of the apical organ and associated neural structures. All pictures are aboral views with the same orientation (Tp, tentacular plane; Pp, pharyngeal plane). The dotted circle delineates the border of the apical organ. (**A**) DIC view of the apical organ showing the four balancers (**B**) and four groups of large refringent round cells called "refringent bodies" (Rb). A pair of ciliated grooves near a balancer is indicated by arrowheads. (**B–B′**) DAPI staining views showing the distribution of nuclei in the epithelial floor of the apical organ in two different optical planes: basi-epithelial (deep) plane on the left (B) and superficial plane on the right (B′). (**C**) The deep nerve net of the apical organ floor revealed by YL1/2 staining. Note its continuity with the surrounding polygonal nerve net (arrowheads) and the polar field nervous system (arrow). (**C′**) Higher magnification view of the boxed area in C showing a nerve cell. (**D**) The deep nerve net of the apical organ floor is also positive with the anti-FMRFamide antibody. See Figure 6 for description of the four additional strongly stained structures (black stars). The four spots located on the border of the apical organ (white arrowheads) are not specific as they were also stained using the secondary antibody only. (**D′**) Composite view of YL1/2 (in red) and anti-FMRFamide (in green) staining in the area boxed in D. B, balancer; Ep, epithelial papillae; Lt, lithocytes; Mz, marginal zone of a polar field; Rb, refringent body; Tp, Tentacular plane; Pp, pharyngeal plane. Scales bars: A–C, D: 20 μm; C′: 5 μm; D′: 10 μm. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

not visible in our preparations, it is not possible to determine whether this nerve net is located in the basal portion of the AO epidermis or in the superficial region of the underlying mesoglea. This nerve net is mostly restricted to the region delimited by the four groups of balancer cells (Fig. 5C), but with lower density in the very central AO area. Within the AO nerve net, neurites are preferently orientated in two perpendicular directions corresponding to the pharyngeal and tentacular planes (Fig. 5C). The

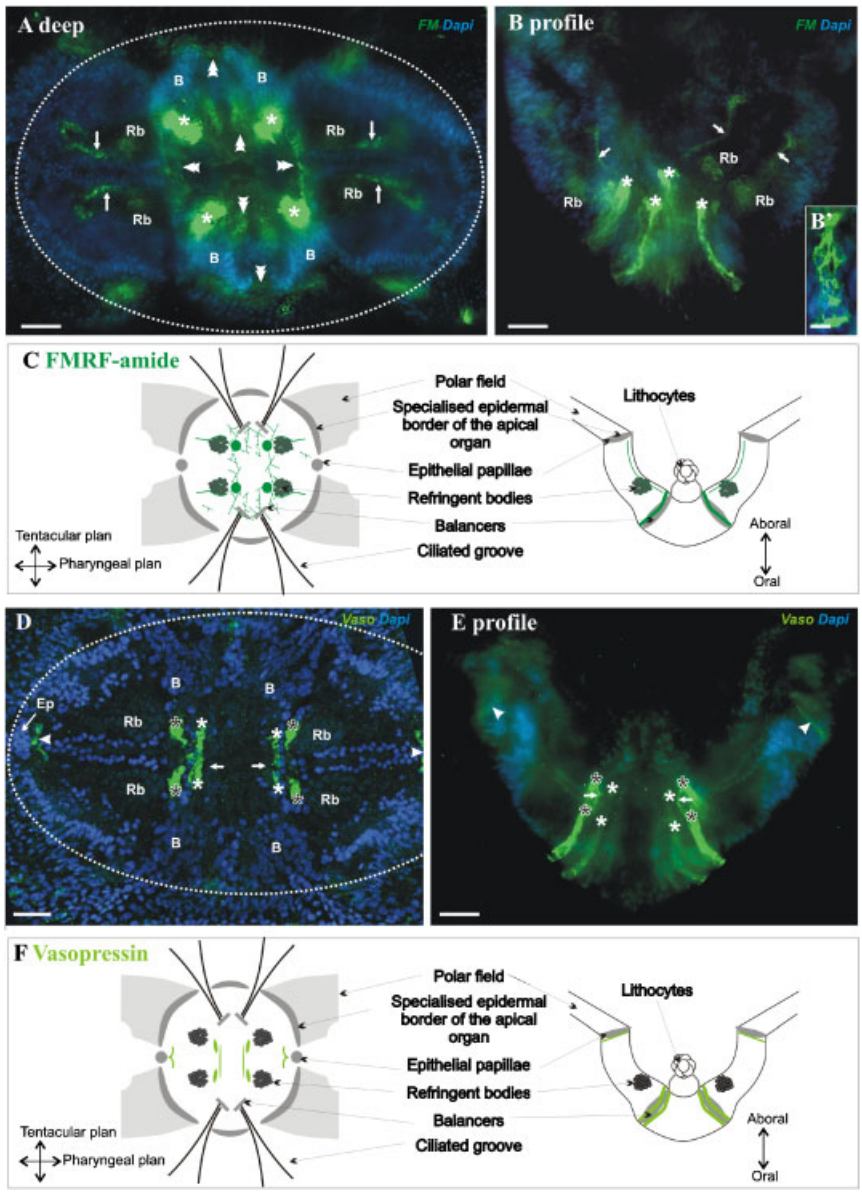

Figure 6. Anti-neuropeptide immunoreactive neural condensations in the apical organ. The dotted circle delineates the border of the apical organ. **(A–C)** Structures revealed by anti-FMRFamide staining (green) (DAPI counterstaining in blue). (A) Deep aboral view of the AO showing four intensely stained structures near the balancers (white stars) and four lines (white arrows) running from the refringent bodies (Rb) toward the periphery of the apical organ. The apical organ nerve net is also visible (double arrowheads). (B) Profile view of the apical organ (labels as in A). **(B′)** Higher magnification of one of the four strongly stained anti-FMRFamide positive structures. (C) Diagrams of the anti-FMRFamide staining (in green) in the apical organ. Note that the right diagram is not a section but a projection in profile view. **(D–F)** Structures revealed by anti-vasopressin staining (green) in the apical organ. (D) Aboral view showing central stained structures (white and black stars) near the balancers as well as peripheral V-shaped structures near the epithelial papillae (Ep) (arrowheads; note that epithelial papilla is not visible on the right side). (E) Profile view of the anti-vasopressin immunostaining in the apical organ (labels as in D). Note that this picture is from another specimen than the picture in D, and in this case a superficial line (white arrows) appears to join the two structures labeled with black stars. (F) Diagrams of the anti-vasopressin staining in the apical organ. Note that the right diagram is not a section but a projection in profile view. D: confocal microscopy (Z projection 5.54 μm); other pictures: classical microscopy. B, balancers; Ep, epithelial papillae, Rb, refringent bodies. Scale bars: A, B, D, E: 20 μm; B′: 10 μm. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

AO nerve net is markedly looser than the polygonal nerve net described above, since neurites are not associated in bundles. However, in the tentacular plane, the AO nerve net seems continuous with the polygonal nerve net surrounding the AO (Fig. 5C white arrowheads). A large proportion of the deep AO nerve net is stained by the anti-FMRFamide antibody (Fig. 5D), with clear co-localization of YL1/2+anti-FMRFamide in some neurites (Fig. 5D′).

In addition to these neurons integrated in the AO deep nerve net, anti-FMRFamide antibody staining revealed a complex pattern of condensed (putatively neuro-sensory) structures integrated in the thick epithelial floor of the apical organ (shown in Fig. 6A and B and schematized in Fig. 6C). First, we observed four symmetrical intensely stained structures, lying adjacent to the balancer cells (black stars in Fig. 5D and white stars in 6A and B). Profile views (Fig. 6B and B′) indicate that these structures span the entire thickness of the epithelium and are obliquely orientated, their basal extremity being more peripheral than their apical extremity. Both extremities are stained with particularly high intensity. Higher magnification views (Fig. 6B′) and confocal microscopy (Supp. Movie 2) reveal that these structures are made of numerous neurite-like processes weaved between cells nuclei. Additional structures stained by anti-FMRFamide antibody in the AO floor include the four refringent bodies (with low intensity), and four thin superficial lines (white arrows in Fig. 6A and B) running from the refringent bodies to the border of the apical organ, parallel to the pharyngeal plane. A movie displaying a three-dimensional view of the structures stained by anti-FMRFamide in the apical organ is provided in supplementary data (Supp. Movie 2).

Several anti-vasopressin immunoreactive structures were also observed in the AO floor (Fig. 6D–F): (i) close to each balancer, but not directly against it, four thick structures spanning the whole thickness of the apical organ floor (Black stars in Fig. 6D—apical view—and in Fig. 6E—profile view). Based on their position with respect to balancer cells, they are distinct from the FMRFamide immunoreactive structures indicated by white stars in Figure 6A; (ii) close to these structures, but more internal, two lines parallel to the tentacular plane and also spanning the whole epithelium thickness (white stars in Fig. 6D and E); and (iii) close to the AO peripheral border, in the pharyngeal plane, two symmetrical V-shape structures closely apposed to the epithelial papillae but clearly distinct from them (white arrowheads in Fig. 6D and E). A movie displaying three-dimensional view of the structures stained by anti-vasopressin antibody in the apical organ is provided in supplementary data (supp. Movie 3).

Complex Nervous System Associated With the Polar Fields

The organization of the nervous system associated with the polar fields was investigated with the same antibodies (Fig. 7). According to the few available histological studies, some hypothetical nervous elements may be present (Hertwig, 1880) but no sensory or nerve cells have been clearly identified until recently (Jager et al., 2008). The marginal zone of the polar field is characterized by cells bearing a single long cilium (Hertwig 1880) and forming a thickened rim around the central zone. In the central zone, epithelial cells bear several agglomerated cilia that create a continuous flow of seawater into the AO (Chun, 1880; Hertwig, 1880; Hernandez-Nicaise, '74, '84).

YL1/2 staining reveals conspicuous neuro-sensory structures distributed along the inner border of the marginal zone (Fig. 7A–C). These structures consist of several cells and have been previously described and named "Z bodies" (Fig. 7B and C, white stars; Jager et al., 2008). Each Z body sends many thin neurite-like processes toward the periphery of the polar field (Fig. 7B and C). Z bodies are more densely packed near the AO (Fig. 7A). They are connected with each others by neurites as previously described ("internal circum-polar nerve ring" in Jager et al., 2008; Fig. 7B and C arrowheads).

Along the polar field axis, YL1/2 also reveals a bundle of parallel fibers (Pfm in Fig. 7A–E), strongly stained by phalloidin (Fig. 7D) and thereby probably of muscular nature. They extend over the whole length of the polar fields, ending at some distance from their extremities (Fig. 7A and C, D). These fibers seem to lie on the same optical plane as the epithelial cells (unshown observations) and therefore are probably parietal muscle fibers.

Finally, we observed by YL1/2 antibody staining a superficial nerve net, in both the marginal and the central zones (Fig. 7E). This "polar field nerve net" is not polygonal and is markedly less condensed than the epithelial nerve plexus found in other parts of the body, but it nevertheless appears denser than the mesogleal nerve net. Some neurites seem to contact polar field muscle fibers (Fig. 7E white arrowhead). Other neurites extend toward the AO floor, in the vicinity of the dome orifice (Fig. 7F white arrowhead). Owing to technical limitations, we could not determine if this polar field nerve net is located above or below the basal lamina.

Some components of the polar field nervous system exhibit immunoreactivity with the anti-FMRFamide antibody (Fig. 7E′, G and H). In the marginal zone, anti-FMRFamide staining is closely associated with the Z bodies (Fig. 7G and H) but does not co-localize with YL1/2 staining. A movie displaying three-dimensional view of the FMRFamide immunoreactive structures in the polar field proximal region is provided in supplementary data (supp. Movie 4). In the most distal region of the polar fields, there is no FMRFamide signal associated with the Z bodies (not shown). In the central polar field zone, some isolated cells of the nerve net are also anti-FMRFamide reactive (Fig. 7E′). No signal could be detected in the polar fields with the anti-vasopressin antibody. Elements of the polar field nervous system and the polar field muscle are positioned on a diagram (Fig. 7I).

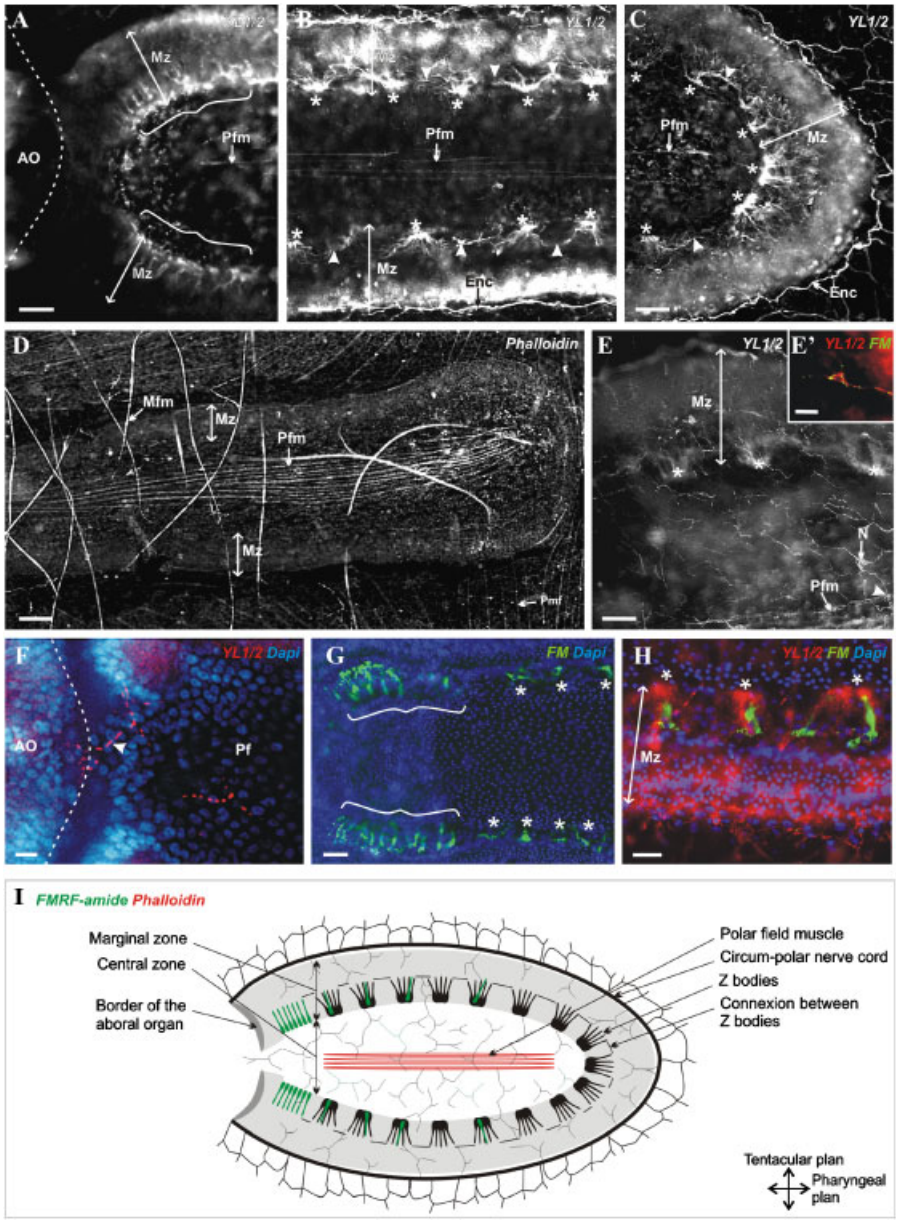

Figure 7. The nervous system associated with the polar fields. **(A–C)** Three distinct regions of a polar field stained by anti-tyrosylated-alpha-tubulin (YL1/2) and showing the iterated Z bodies (stars) along the inner margin of the marginal zone and parallel muscle fibers (Pfm) beneath the central ciliated epidermis. (A) Proximal polar field region showing densely packed Z bodies (brackets) and the extremity of the polar field muscular fibers. (B) Central part of a polar field showing regularly distributed Z bodies (stars) connected by thin processes (arrowheads). (C) Distal region of a polar field (labels as in B). **(D)** Phalloidin staining allows a better visualization of the polar field muscle (Pfm). **(E)** Nerve net underlying the polar field, with a contact between a muscular fiber and a neurite (arrowhead). The more superficial Z bodies are visible but out of focus (stars). **(E′)** Higher magnification of a nerve cell stained by both YL1/2 (in red) and anti-FMRFamide (in green). **(F)** YL1/2 immunostaining (in red) combined with DAPI counterstaining (in blue) reveals polar field neurites extending toward the apical organ (arrowhead). **(G)** FMRFamide immunostaining (in green) in the proximal region of a polar field (with Dapi counterstaining) showing immunopositive cells associated with the Z bodies (brackets and stars). **(H)** Detailed view of FMRFamide staining (in green) in Z-bodies combined with YL1/2 staining (in red) and DAPI counterstaining (in blue). **(I)** Diagram of the polar field summarizing all the described structures. AO, apical organ; Enc, external circum-polar nerve cord; Mfm, mesogleal muscular fiber; Mz, marginal zone; N, polar field nerve cell; Pf, polar field; Pfm, polar field muscular fiber. Scale bars: A–C, E, F, G: 20 μm, D: 50 μm, H: 10 μm; E′: 5 μm. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

DISCUSSION

The Characteristics of *Pleurobrachia* Nerve Nets in a Comparative Perspective

The non-specialized component of the *Pleurobrachia* nervous system comprises two nerve nets with sharply distinct localization and organization: the polygonal nerve net, integrated in the epidermal cell layer, and the mesogleal nerve net (Fig. 1B). Thanks to the immunofluorescence approach, we could for the first time establish the existence of a mesogleal nerve net extending throughout the body wall of a cyddipid ctenophore (Figs. 1B and 2C, D). By electron microscopy, Hernandez-Nicaise ('73b) could detect nerve fibers, but no nerve cell bodies, in the body mesoglea of the non-cyddipid *Beroe*. In the Cydippidae, mesogleal neural elements had been previously reported only in the tentacle (Hernandez-Nicaise, '73b, '91). The presence in ctenophores of an extensive nerve net running through the mesoglea was not unexpected given that these animals also possess truly mesogleal muscle fibers, of mesodermal embryonic origin (Martindale and Henry, '97, '99) and without any epithelial cell component. Moreover, we obtained evidence suggesting that these mesogleal muscles are innervated by some of the mesogleal neurons (Fig. 2D). The mesogleal nervous system is more condensed in the tentacle where it mainly consists of two parallel nerve cords (Fig. 2E, E′). However, the neural anatomy of tentacles and tentillae deserves further detailed investigations to reconcile our observations with Hernandez-Nicaise's ('73b, '91) description of a "thick ganglion-like strand of large nerve cells" in the tentacle axis, in addition to more peripheral neural components.

Contrasting with the situation observed in ctenophores, the nervous system of cnidarians is fundamentally epithelial (ectodermal and endodermal), as are also their muscle cells (Harrison and Westfall, '91). A strictly mesogleal nerve net is generally absent in cnidarian polyps or medusae, except in colonial octocorals (Pavans de Ceccatty and Buisson, '64; Titschack, '68, '70; Pernet et al., 2004), and the muscle cell bodies are likewise integrated within epithelia. Therefore, ctenophores and cnidarians differ fundamentally in terms of the development of the intermediate (mesogleal) compartment (Hernandez-Nicaise, '73b).

Although the *Pleurobrachia* mesogleal nerve net shows a loose arrangement of neural cells, reminiscent of the organization of cnidarian nerve nets, the polygonal nerve net has a sharply different structure. The interconnecting segments of the polygonal network do not consist of single neurites, but instead they appear as relatively thick strands containing several parallel neurites, so this "nerve net" is a plexus of anastomosed nerve cords and not of single neurons. Within this plexus, nerve cell bodies do not preferentially localize at nodes. Therefore, the intrinsic structure of the ctenophore "polygonal nerve net" reflects a degree of nervous tissue organization not far from certain bilaterian nervous systems (e.g. the Nemertodermatida, Raikova et al., 2000; the Acoela, Raikova et al., '98, Reuter et al., 2001). In comparison, cnidarian nerve nets outside from local specialization (e.g. oral region of polyps, peripheral region of hydromedusae) are plexus of single neurons, and thereby they have a lower degree of intrinsic condensation. It is unclear whether or not neural concentrations were present in the common ancestor of eumetazoans, but in any case the nervous system was probably affected by multiple independent events of condensation in cnidarians, ctenophores and bilaterians.

A last remarkable property revealed by our immunofluorescence analyses is the existence within the epithelial nerve net of several categories of neurons differing by their immunoreactivity. Thus, a minority of the anti-tyrosylated α-tubulin immunoreactive neurons that form the polygonal network were immunoreactive for vasopressin or FMRFamide as well (Fig. 4). Owing to technical limitations, we could not assess whether or not some of the nerve cells were immunoreactive for both anti-neuropeptide antibodies at the same time. On a global scale, however, current evidence suggests that vasopressin and FMRFamide immunoreactivities characterize distinct neural cell sub-populations within the network, the former being considerably more abundant and comprising monociliated neurons whereas FMRFamide immunoreactive cells are very few and lack a cilium. The existence among the nerve net of nerve cells with different biochemical properties is consistent with earlier suggestions based on cytological analyses of cytoplasmic and synaptic vesicles in ctenophores (Hernandez-Nicaise, '74). Similarly, immunodetection studies of neurotransmitter molecules (particularly neuropeptides) in cnidarians have revealed considerable diversity and specialization of neuronal types within the cnidarian nerve nets (Grimmelikhuijzen et al., '89a; Koizumi et al., '90, 2004; Grimmelikhuijzen et al., 2002; Kass-Simon and Pierobon, 2007).

These results furthermore suggest that the ctenophore nervous system may be partially peptidergic, which would represent a shared feature with cnidarians, but this will require confirmation by additional experimental evidence, since the antigens recognized by the anti-vasopressin and anti-FMRFamide antibodies used in this study have not been identified biochemically or genomically. Furthermore, anti-FMRFamide antibodies are capable of targeting a vast array of FMRFamide-like peptides (FLPs) in addition to FMRFamide itself. This explains in particular why a vast proportion of the cnidarian nervous system is FMRFamide immunoreactive (Grimmelikhuijzen, '83; our own observations in *Clytia hemisphaerica* with the commercial anti-FMRFamide antibody used in this study) while the neuropeptide FMRFamide itself has never been isolated from any cnidarian species and is known only from molluscs and annelids (Grimmelikhuijzen, 2002; Walker et al., 2010).

Unexpected Anatomical Complexity Revealed by Immunofluorescent Analyses of the Aboral Sensory Complex

Our immunohistochemical investigation of the apical organ (Figs. 5 and 6) and the polar fields (Fig. 7) highlights the

complexity of these structures and provides a renewed view of their organization.

Concerning the apical organ, anti-α-tubulin tyrosylated-tubulin (YL1/2) and anti-FMRFamide staining revealed a conspicuous nerve net lying in continuity with the polygonal nerve net of the surrounding epithelium (Fig. 5). This AO nerve net could not be observed previously by conventional techniques, although numerous efferent, afferent, and symmetrical synapses were known to occur at the base of balancer cells (Hernandez-Nicaise, '68b, '74). However, the exact position (basi-epithelial or mesogleal) of the AO nerve net could not be ascertained due to technical limitation.

Several intriguing neuro-sensory structures of the AO epithelial floor were revealed by their FMRFamide or vasopressin immunoreactivity (summarized in the drawings of Fig. 6C and F). Some of these structures are tetraradially arranged, whereas others have a biradial disposition. The puzzling fact is that none of them correspond to previously described structures of the apical organ anatomy. In the light of current evidence, we will refrain from naming these structures or proposing functional hypotheses in terms of connecting pathways or neuro-sensory integration. What is clear is that these FMRFamide and vasopressin immunoreactive structures add to an already impressive list of more or less enigmatic (putatively neuro-sensory) components described in the floor of the ctenophore apical organ (e.g. the epithelial papillae, the refringent bodies or lamellate bodies, the bridge, etc; Chun, 1880; Horridge, '64; Krisch, '73; Hernandez-Nicaise, '74; Tamm, '82, Tamm and Tamm, 2002).

The present work also provides the first detailed description of the neuro-sensory elements associated with the polar fields (Fig. 7). These ciliated areas located on each side of the AO have been poorly investigated in previous histological studies. Anti-tyrosylated-α-tubulin (YL1/2) immunofluorescence revealed a special loosely organized subepithelial nerve net (which includes some FMRFamide immunoreactive cells) extending throughout the polar fields and sending neurites toward the circum-polar nerve cord and the apical organ. We also showed the presence of FMRFamide immunoreactive cells associated with the previously described "Z bodies" (Jager et al., 2008), located at the boundary between the marginal and the central zones of the polar field.

The function of the polar fields remains unknown. Their proposed role as olfactory organs (Fol, 1869; Chun, 1880; Horridge, '74; Hernandez-Nicaise, '84) has not yet been confirmed by physiological studies. Polar fields have generally been considered as a simple continuation of the aboral organ floor, but this view is clearly contradicted by the presence of the Z bodies, unique (putatively sensory) structures without equivalent elsewhere in the body, notably in the AO. Given its highly organized neuro-anatomy, possible functions for the polar field might include the processing and conveying of sensory information from peripheral areas toward the apical organ; e.g. the circum-polar nerve cord is likely to be involved in communications between the aboral neuro-sensory complex and the rest of the body.

An additional new finding with potential implication regarding polar field function is the presence of a bundle of parallel fibers, extending all along the axis of the polar field (Fig. 7), revealed by YL1/2 and phalloidin staining. Their aspect in phalloidin preparations suggests they are muscle fibers, and they appear very similar to the parietal muscle fibers occurring in the integument throughout the rest of the body (Fig. 7D). The predicted effect of contraction of this tangential polar field muscles would be reduction of polar field length, generating a tension on the apical organ. It is well known that the AO is capable of withdrawal into a cavity delineated by the surrounding epithelium, following mechanical stimulation on the body surface (Horridge, '65; Hernandez-Nicaise, '74). This vertical movement is due to contraction of deep mesogleal muscle fibers. A possible function of the polar field epithelial muscles might be to antagonize this movement, i.e. to trigger the upwards migration of the apical organ.

Ctenophore Aboral Neuro-sensory Complex vs. Apical Organs and Brains of Other Animal Phyla

The body plan of most eumetazoan phyla comprises a more or less complex neuro-sensory structure localized at one pole of the organism. In the larvae of some cnidarians (Fu-Shiang and Koss, 2005; Rentsch et al., 2008) and in the planktonic ciliated larvae ("primary larvae") of lophotrochozoans and deuterostomes (Nielsen, 2005b), the neuro-sensory centre consists of a complex of ciliary tuft cells and neurons called the "apical organ." Most adult bilaterians possess a brain, here defined as a condensation of neural tissue at the anterior pole, integrating sensory inputs, and triggering various body responses mediated by motoneurons and neuro-endocrin cells. Whether or not the ctenophore aboral neuro-sensory complex is homologous (at least to some extent or at some level), with the apical organs and/or the brains observed in other animal phyla clearly represents a pivotal issue, notably if we wish to reconstruct the characteristics of the eumetazoan ancestor.

The ctenophore aboral sensory complex (apical organ+polar fields) features at least some characteristics of a "brain." It is a neuro-sensory condensation localized at one pole of the organism. It perceives (and probably integrates) various kinds of sensory stimuli and exerts a control on locomotion through regulation of the beating frequency of comb rows. Thus, there is undoubtedly a certain degree of anatomical and functional centralization of the neuro-sensory system in ctenophores. However, while a "true" brain (present in most Bilateria) is principally made of neurons, the dominant cell type in the ctenophore aboral sensory complex is represented by epithelial ciliated cells of various kinds (Hernandez-Nicaise, '91). This is

even true of the apical organ itself, since the truly neural structures revealed here by tyrosylated α-tubulin, anti-FMRFamide or anti-vasopressin immunoreactivity (Figs. 5 and 6) are sunk and integrated into the ciliated epithelial floor. Furthermore, physiological studies have demonstrated that the directional and motile responses to environmental stimuli triggered by the apical organ are conveyed by ciliated cells (of the balancers, ciliated grooves and polster cells), not by nerve cells (Tamm, '82; Hernandez-Nicaise, '91). Conversely, the main peripheral neural condensation, the juxtatentacular nerve cord, is not anatomically continuous with the aboral neuro-sensory condensations (this study). We therefore propose the qualificative of "ciliary brain" for the ctenophore aboral sensory complex (apical organ+polar fields). This term is intended to reflect a limited degree of analogy, but clear absence of primary homology, with the truly neural brains of bilaterian animals.

In bilaterians with planktonic ciliated larvae (e.g. trochophore larva), the adult brain in part derives from the larval apical organ (Nielsen, 2005a,b), and some morphologists have assumed homology between these larval "apical organs" of bilaterians and the homonymic structure of ctenophores (e.g. Nielsen 2001). Morphological observations and immunocytochemical experiments have indicated that bilaterian larval apical organs are mechano and chemosensory structures with neuroendocrine activities involved in different functions and notably in metamorphosis (Kempf et al., '97). At first sight, they could be considered similar to the ctenophore apical organ, based on sensory functions, cell condensation and apical position. More specifically, localized FMRFamide and vasopressin immunoreactivities reported here in the *Pleurobrachia* apical organ are reminiscent of the distinct RF-amide and vasotocin–neurophysin-expressing cells occurring in the apical organ of the annelid trochophore larva (Tessmar-Raible et al., 2007).

However, at the anatomical level, the resemblance between ctenophore and bilaterian "apical organs" is only superficial, and there are fundamental differences that strongly plead in favor of convergence. Whereas in ctenophores, the AO is a permanent structure found in cydippid larvae as well as in adults, the bilaterian apical organ is a transient structure, some populations of neurons disappearing during metamorphosis while others are incorporated into the adult nervous system (e.g. Dickinson and Croll, 2003; Byrne et al., 2007). Bilaterian larval apical organs contain only a few cells (most of ciliated protostomes larvae have an apical organ comprising 4–16 cells; Nielsen, 2004, 2005a), whereas the ctenophore AO comprises a much higher number of cells. The general organization of ctenophore vs. bilaterian "apical organs" fundamentally diverges in terms of their symmetry properties: bilaterality in the latter vs. a combination of tetraradial and biradial symmetry in the former (Figs. 5 and 6). It is also noteworthy that perception of gravity in bilaterian larvae is by sensory organs (statocysts) spatially separated from the apical organ (e.g. in gastropod trochophores; Kempf et al., '97), whereas in ctenophores, the apical organ by itself is a statocyst (notwithstanding its additional functions). Homology at the anatomical level is therefore unlikely. Similarly, the aboral condensation of monociliated sensory neurons sometimes called "apical organ" in some cnidarian planula larvae comprises few cell types, does not show any particular symmetry in its organization, and does not function as a statocyst, and therefore is certainly not homologous to the ctenophore apical organ (Chia and Koss, '79; Nakanishi et al., 2008; Marlow et al., 2009).

In spite of the absence of homology at the anatomical level, the shared existence of distinct RF-amide and vasopressin/vasotocin immunoreactive cells in ctenophore AO (this study) and in the apical organ of bilaterian larvae (Tessmar-Raible et al., 2007) might reflect homology at the level of particular types of sensory–neurosecretory cells, among distantly related eumetazoans, as previously suggested among distant bilaterians (Tessmar-Raible et al., 2007).

CONCLUSIONS

Coelenterate nervous systems share important common characteristics, such as a nerve net that extends over the whole organism and the presence of neuropeptides, but the ctenophore nervous system clearly departs from the cnidarian nervous system by several significant features including the condensed architecture of the polygonal nerve net (plexus of nerve cords), the presence of a truly mesogleal (i.e. mesodermal) nerve net, and a unique aboral neuro-sensory complex. These characters evoke body plan attributes that were classically considered typical for the Bilateria. Future comparative work on developmental genes might indicate whether these characters have evolved independently in the ctenophore lineage, or are ancestral features of the eumetazoan ancestor that have been lost in cnidarians. Further information is also expected to come from studies using a wider range of antibodies directed against putative neurotransmitters, as well as from investigation of other ctenophore species using the same approaches.

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LITERATURE CITED

Bardou I, Maubert E, Leprince J, Chichery R, Cocquerelle C, Launay S, Vivien D, Vaudry H, Agin V. 2009. Distributions of oxytocin-like and vasopressin-like immunoreactivities within the central nervous system of the cuttlefish *Sepia officinalis*. Cell Tissue Res 336:249–266.

Bethe A. 1895. Der subepitheliale Nervenplexus der Ctenophoren. Biol Zbl 15:140–145.

Brown A, Li Y, Slaughter T, Black M. 1993. Composite microtubules of the axon: quantitative analysis of tyrosinated and acetylated tubulin along individual axonal microtubules. J Cell Sci 104: 339–352.

Bullock TH, Horridge G. 1965. Structure and function in the nervous systems of invertebrates, Vol. I. San Francisco: W.H. Freeman.

Byrne M, Nakajima Y, Chee FC, Burke RD. 2007. Apical organs in echinoderm larvae: insights into larval evolution in the Ambulacraria. Evol Dev 9:432–445.

Caldwell HK, Lee H-J, Macbeth AH, Scott YoungIII W. 2008. Vasopressin: behavioral roles of an "original" neuropeptide. Prog Neurobiol 84:1–24.

Chia FS, Koss R. 1979. Fine-structural studies of the nervous system and the apical organ in the planula larva of the sea anemone *Anthopleura elegantissima*. J Morphol 160:275–298.

Chun C. 1880. Die Ctenophoren des Golfes von Neapel und der angrenzenden Meeres-Abschnitte: eine Monographie. Leipzig: Verlag von W. Engelmann.

Delage Y, Hérouard E. 1901. Traité de zoologie concrète. Paris: Librairie C. Reinwald.

Dickinson AJG, Croll RP. 2003. Development of the larval nervous system of the gastropod *Ilyanassa obsoleta*. J Comp Neurol 466:197–218.

Dunn CW, Hejnol A, Matus DQ, Pang K, Browne WE, Smith SA, Seaver E, Rouse GW, Obst M, Edgecombe GD, Sørensen MV, Haddock SH, Schmidt-Rhaesa A, Okusu A, Kristensen RM, Wheeler WC, Martindale MQ, Giribet G. 2008. Broad phylogenomic sampling improves resolution of the animal tree of life. Nature 452: 745–749.

Eimer T. 1880. Versuche über künstliche Teilbarkeit von Beroe ovata. Arch f mikr Anat 17:213.

Fol H. 1869. Ein Beitrag zur Anatomie und Entwickelungsgeschichte einiger Rippenquallen. Inaugural–Dissertation.

Franc JM. 1985. La mésoglée des cténaires: Approches ultrastructurale, biochimique et métabolique. Université Claude Bernard, Lyon I.

Fu-Shiang C, Koss R. 2005. Fine structural studies of the nervous system and the apical organ in the planula larva of the sea anemone *Anthopleura elegantissima*. J Morphol 160:275–297.

Galliot B, Quiquand M, Ghila L, de Rosa R, Miljkovic-Licina M, Chera S. 2009. Origins of neurogenesis, a cnidarian view. Dev Biol 332:2–24.

Grimmelikhuijzen CJP. 1983. FMRFamide immunoreactivity is generally occurring in the nervous systems of coelenterates. Histochemistry 78:361–381.

Grimmelikhuijzen CJP, Dierick K, Boer GJ. 1982. Oxytocin/vasopressin-like immunoreactivity is present in the nervous system of Hydra. Neuroscience 7:3191–3199.

Grimmelikhuijzen CJP, Graff D, McFarlane ID. 1989a. Neurones and neuropeptides in coelenterates. Arch Histol Cytol 52(Suppl): 265–278.

Grimmelikhuijzen CJP, Graff D, Spencer AN. 1989b. Structure, location and possible actions of Arg-Phe-amide peptides in coelenterates. In: Thorndyke MC, GoldWorthy GJ, editors. Neurohormones in invertebrates. Cambridge: Cambridge University Press. p 199–127.

Grimmelikhuijzen CJ, Leviev I, Carstensen K. 1996. Peptides in the nervous systems of cnidarians: structure, function, and biosynthesis. Int Rev Cytol 167:37–89.

Grimmelikhuijzen CJP, Williamson M, Hansen GN. 2002. Neuropeptides in cnidarians. Can J Zool 80:1690–1702.

Hammond JW, Cai D, Verhey KJ. 2008. Tubulin modifications and their cellular functions. Curr Opin Cell Biol 20:71–76.

Harrison FW, Westfall JA. 1991. Placozoa, Porifera, Cnidaria, and Ctenophora. New York: Wiley.

Heider K. 1927. Über das Nervensystem von Beroe ovata. Nachrichten der Gesellschaft der Wissenschaften zu Göttingen, Math.-phys. Kl. Göttingen: Vandenhoeck & Ruprecht.

Hernandez-Nicaise ML. 1968a. Specialized connexions between nerve cells and mesenchymal cells in ctenophores. Nature 217:1075–1076.

Hernandez-Nicaise ML. 1968b. Distribution and ultrastructure of symmetrical synapses in the Ctenaire nervous system. C R Acad Sci Hebd Seances Acad Sci D 267:1731–1734.

Hernandez-Nicaise ML. 1973a. The nervous system of ctenophores. I. Structure and ultrastructure of the epithelial nerve-nets. Z Zellforsch Mikrosk Anat 137:223–250.

Hernandez-Nicaise ML. 1973b. The nervous system of ctenophores. II. The nervous elements of the mesoglea of beroids and cydippids (author's transl). Z Zellforsch Mikrosk Anat 143:117–133.

Hernandez-Nicaise ML. 1973c. The nervous system of ctenophores. III. Ultrastructure of synapses. J Neurocytol 2:249–263.

Hernandez-Nicaise ML. 1974. Système nerveux et intégration chez. les cténaires; étude ultrastructurale et comportementale. Lyon, France: Univ. Claude Bernard.

Hernandez-Nicaise ML. 1984. Ctenophora. In: Bereiter-Hahn J, Matoltsy AG, Richards KS, editors. Dans: biology of the integument, Vol. 1: invertebrates. Berlin: Springer. p 96–111.

Hernandez-Nicaise ML. 1991. Ctenophora. In: Harrison FW, Westfall JA, editors. Dans: microscopic anatomy of the invertebrates, Vol. II: Placozoa, Porifera, Cnidaria, and Ctenophora. New York: Wiley. p 359–418.

Hertwig R. 1880. Ueber den Bau der Ctenophoren. Jena: G. Fischer.

Horridge GA. 1964. Presumed photoreceptive cilia in a Ctenophore. J Cell Sci 3:311.

Horridge GA. 1965. Relations between nerves and cilia in Ctenophores. Am Zool 5:357–375.

Horridge GA. 1974. Recent studies on the Ctenophora. In: Muscatine L, Lenhoff HM, editors. Dans: coelenterate biology: reviews and new perspectives. Academic Press. p 439–468.

Horridge GA, Mackay B. 1962. Naked axons and symmetrical synapses in coelenterates. J Cell Sci 3:531.

Horridge GA, Mackay B. 1964. Neurociliary synapses in Pleurobrachia (Ctenophora). J Cell Sci 3:163.

Hyman L. 1940. The invertebrates: protozoa through Ctenophora. New York: McGraw-Hill.

Jager M, Quéinnec E, Chiori R, Le Guyader H, Manuel M. 2008. Insights into the early evolution of SOX genes from expression analyses in a ctenophore. J Exp Zool (Mol Dev Evol) 310B: 650–667.

Kass-Simon G, Pierobon P. 2007. Cnidarian chemical neurotransmission, an updated overview. Comp Biochem Physiol A Mol Integr Physiol 146:9–25.

Kempf SC, Page LR, Pires A. 1997. Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch molluscs with emphasis on the structure and possible function of the apical sensory organ. J Comp Neurol 386:507–528.

Koizumi O, Mizumoto H, Sugiyama T, Bode HR. 1990. Nerve net formation in the primitive nervous system of Hydra—an overview. Neurosci Res (Suppl 13):S165–S170.

Koizumi O, Sato N, Goto C. 2004. Chemical anatomy of hydra nervous system using antibodies against hydra neuropeptides: a review. Hydrobiologia 530–531:1–3, 41–47.

Krisch B. 1973. Über das Apikalorgan (Statocyste) der Ctenophore Pleurobrachia pileus. Cell Tissue Res 142:241–262.

Marlow HQ, Srivastava M, Matus DQ, Rokhsar D, Martindale MQ. 2009. Anatomy and development of the nervous system of *Nematostella vectensis*, an anthozoan cnidarian. Dev Neurobiol 69: 235–254.

Martindale MQ, Henry JQ. 1997. Reassessing embryogenesis in the Ctenophora: the inductive role of e1 micromeres in organizing ctene row formation in the "mosaic" embryo, *Mnemiopsis leidyi*. Development 124:1999–2006.

Martindale MQ, Henry JQ. 1999. Intracellular fate mapping in a basal metazoan, the ctenophore *Mnemiopsis leidyi*, reveals the origins of mesoderm and the existence of indeterminate cell lineages. Dev Biol 214:243–257.

Miller G. 2009. Origins. On the origin of the nervous system. Science 325:24–26.

Nakanishi N, Yuan D, Jacobs DK, Hartenstein V. 2008. Early development, pattern, and reorganization of the planula nervous system in Aurelia (Cnidaria, Scyphozoa). Dev Genes Evol 218:511–524.

Nielsen C. 2001. Animal evolution: interrelationships of the living phyla, 2nd edition. Oxford: Oxford University Press.

Nielsen C. 2004. Trochophora larvae: cell-lineages, ciliary bands and body regions. 1. Annelida and Mollusca. J Exp Zool (Mol Dev Evol) 302B:35–68.

Nielsen C. 2005a. Trochophora larvae: cell-lineages, ciliary bands and body regions, 1. Annelida and Mollusca. J Exp Zool (Mol Dev Evol) 304B:401–447.

Nielsen C. 2005b. Larval and adult brains. Evol Dev 7:483–489.

Pavans de Ceccatty M, Buisson B. 1964. Le système nerveux intramésogleen dans les colonies de Veretillum cynomorium Pall. (Cnidaire, Pennatulidae). C R Acad Sci Paris 259:3611–3613.

Pernet V, Anctil M, Grimmelikhuijzen CJP. 2004. Antho-RFamide-containing neurons in the primitive nervous system of the anthozoan *Renilla koellikeri*. J Comp Neurol 472:208–220.

Philippe H, Derelle R, Lopez P, Pick K, Borchiellini C, Boury-Esnault N, Vacelet J, Renard E, Houliston E, Quéinnec E, Da Silva C, Wincker P, Le Guyader H, Leys S, Jackson DJ, Schreiber F, Erpenbeck D, Morgenstern B, Wörheide G, Manuel M. 2009. Phylogenomics revives traditional views on deep animal relationships. Curr Biol 10: 106–113.

Raikova OI, Reuter M, Kotikova EA, Gustafsson MKS. 1998. A commissural brain! The pattern of 5-HT immunoreactivity in Acoela (Plathelminthes). Zoomorphology 118:69–77.

Raikova OI, Reuter M, Jondelius U, Gustafsson MK. 2000. The brain of the Nemertodermatida (Platyhelminthes) as revealed by anti-5HT and anti-FMRFamide immunostainings. Tissue Cell 32: 358–365.

Rentsch F, Fritzenwanker JH, Scholz CB, Technau U. 2008. FGF signalling controls formation of the apical sensory organ in the cnidarian *Nematostella vectensis*. Development 135:1761–1769.

Reuter M, Raikova OI, Jondelius U, Gustafsson MK, Maule AG, Halton DW. 2001. Organisation of the nervous system in the Acoela: an immunocytochemical study. Tissue Cell 33:119–128.

Samassa P. 1892. Zur Histologie der Ctenophoren. Arch mikr Anat 40: 157–242.

Schierwater B, Eitel M, Jakob W, Osigus H, Hadrys H, Dellaporta SL, Kolokotronis S, Desalle R. 2009. Concatenated analysis sheds light on early metazoan evolution and fuels a modern "urmetazoon" hypothesis. PLoS Biol 7:e20.

Tamm SL. 1982. Ctenophora. In: Shelton GAB, editor. Dans: electrical conduction and behaviour in "simple" invertebrates. Oxford: Oxford University Press. p 266–358.

Tamm S, Tamm SL. 1995. A giant nerve net with multi-effector synapses underlying epithelial adhesive strips in the mouth of Beroë (Ctenophora). J Neurocytol 24:711–723.

Tamm SL, Tamm S. 2002. Novel bridge of axon-like processes of epithelial cells in the aboral sense organ of ctenophores. J Morphol 254:99–120.

Tessmar-Raible K, Raible F, Christodoulou F, Guy K, Rembold M, Hausen H, Arendt D. 2007. Conserved sensory-neurosecretory cell types in annelid and fish forebrain: insights into hypothalamus evolution. Cell 129:1389–1400.

Titschack H. 1968. Über das Nervensystem der Seefeder Veretillum cynomorium (Pallas). Cell Tissue Res 90:347–371.

Titschack H. 1970. Uber das mesogloeale Nervensystem der Oktocoral- len Alcyonium palmatum Pallas und Eunicella stricta (Bertoloni). Zool Anzeiger 185:68–75.

Walker RJ, Papaioannou S, Holden-Dye L. 2010. A review of FMRFamide- and Rfamide-like peptides in metazoa. Invert Neurosci, DOI 10.1007/s10158-1010-0097-7.

Watanabe H, Fujisawa T, Holstein TW. 2009. Cnidarians and the evolutionary origin of the nervous system. Dev Growth Differ 51: 167–183.