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Ultrastructural study of the proboscis endothelium of *Riseriellus occultus* (Nemertea, Heteronemertea)

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Abstract

We have examined with transmission electron microscopy the epithelial layer exposed to the rhynchocoel fluid of the proboscis in the heteronemertine *Riseriellus occultus*. This epithelium is organized as a monociliated, pseudostratified myoepithelium consisting of two cell types: apically situated monociliated supportive cells and subapical myocytes lacking cilia. The low supportive cells form a continuous adluminal sheet and reach with numerous cytoplasmic processes into the extracellular matrix; these cells are characterized by numerous, irregularly shaped, apical folds projecting into the rhynchocoel fluid, delimiting broad extracellular spaces. The authors suppose that both apical and basal folds could accommodate stretching of the endothelium when the proboscis is everted. The apical folds of the supportive cells increase the interface of these with the rhynchocoel fluid; this feature, together with the presence of pinocytotic vesicles in such cells, suggest that they could be involved in the exchange of substances between the rhynchocoel fluid and the proboscis. The myocytes are scattered singly within the monociliated pseudostratified myoepithelium. They are situated between the supportive cells and the subjacent extracellular matrix. Basement membrane separating both cells types is lacking. Myofibrillar parts protrude basally from the myocyte somata. The myofibrillar parts lie in direct apposition to the extracellular matrix, and are oriented circular to the longitudinal axis of the proboscis. We consider the myocytes to be intra-epithelial, myoepithelial cells.

Introduction

The principal diagnostic feature of the phylum Nemertea is a cylindrical, eversible proboscis that is enclosed in the rhynchocoel. This organ consists of concentric tissue layers (for review see Gibson, 1972, 1985; Norenburg, 1993): (1) a glandular epithelium exposed to the external environment when everted, (2) muscle layers with arrangements that vary with higher taxa; and (3) a squamous epithelium or endothelium (terminology after Gibson, loc. cit., and Norenburg, loc. cit.), facing the rhynchocoel fluid. Among these layers, extracellular matrix and nervous tissue are variably disposed.

Although the nemertean proboscis has been the subject of numerous histological studies, little is known about its ultrastructure. The only studies of this organ using electron microscopy are those of Ling (1971), Anadón (1976), Stricker & Cloney (1981, 1983), Stricker (1985), Turbeville & Ruppert (1985), Turbeville (1991) and Montalvo et al. (1996). However, proboscis endothelium has not been given all the attention it deserves. Because the proboscis itself is never vascularized (Gibson, 1972), the endothelial cells can be expected to be involved in transport of nutrient from the rhynchocoel to the proboscis.

The present study forms part of a series of works (Montalvo et al., 1996) the main purpose of which is to extend the knowledge of the fine structure of the nemertean proboscis. This paper focuses on the ultrastructure of the proboscis endothelium of the heteronemertine *Riseriellus occultus* Rogers, Junoy, Gibson &

Thorpe, 1993, and describes a systematic examination of serial sections of several regions of the proboscis. The putative function of the endothelium is discussed in relation to its fine structure. It was also of interest to find and examine the musculature associated with endothelium described in other species (e.g. Ling, 1971; Turbeville, 1991), but not detected in *R. occultus* by light microscopy (Rogers et al., 1993).

Materials and methods

Specimens of the heteronemertine *Riseriellus occultus*, were collected by hand at low tide from the Foz Estuary, northwestern Spain. The individuals were found on the upper shore in consolidated mud among roots of *Spartina* sp and in muddy sands with *Zostera noltii* Hornemann, 1832. The worms were kept with muddy sand in an aquarium supplied with running seawater at about 16 °C. Small segments of the anterior, middle and posterior regions of both everted and uneverted proboscides were obtained, fixed and prepared for the transmission electron microscopy (TEM) according to the methods described in a previous report (Montalvo et al., 1996). For scanning electron microscopy, small segments representing different regions of the proboscis were fixed by the same methods used for TEM. After dehydration through a graded series of ethanol, they were transferred into acetone and critical point dried with CO_2. Dried samples were mounted on aluminum stubs and coated with gold-palladium. They were examined with a Zeiss DSM-950 scanning electron microscope at an accelerating voltage of 20 kV.

Results

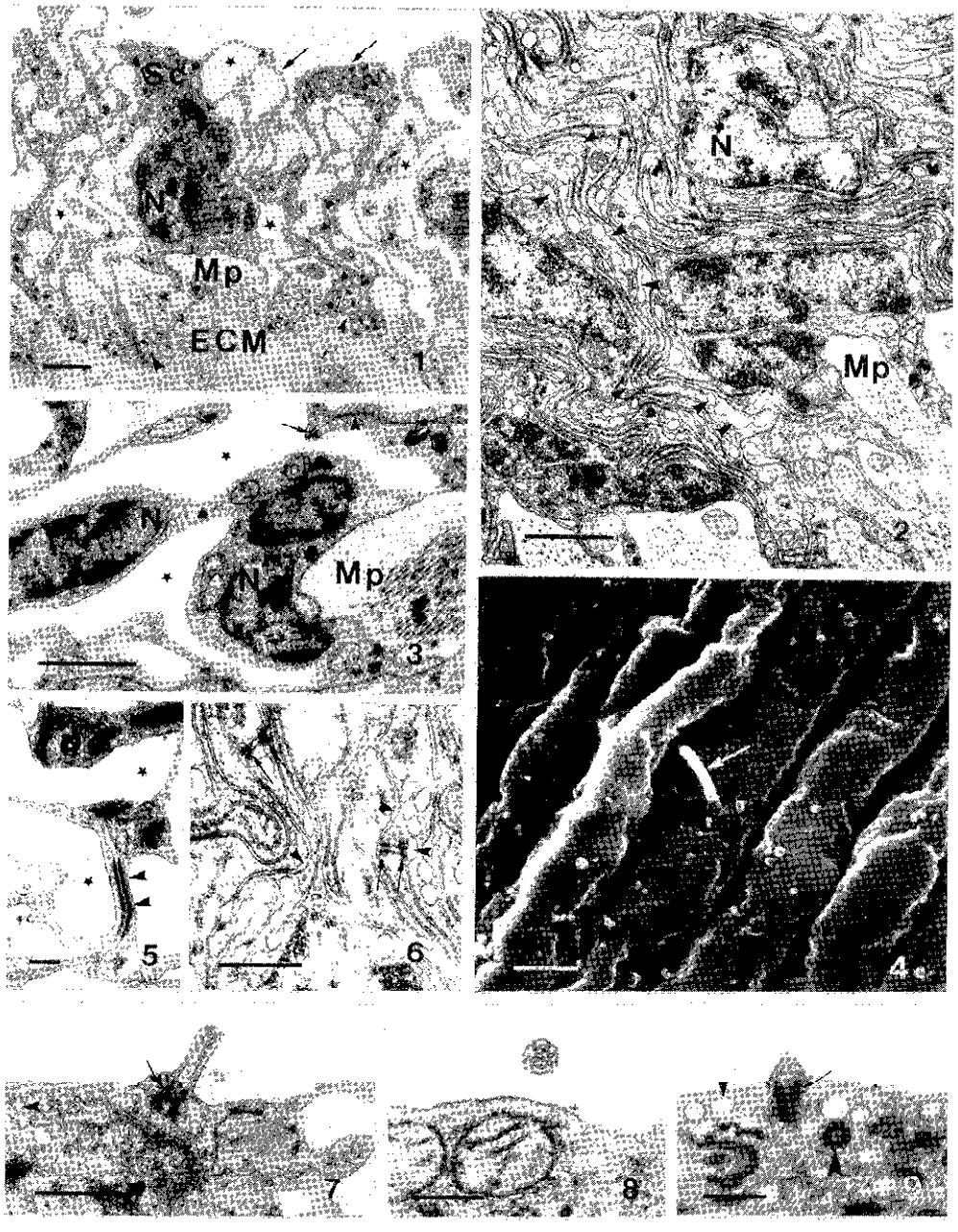
The general organization of the proboscis of *Riseriellus occultus* closely agrees with generalized heteronemertine form described by Ling (1971). The epithelial layer exposed to the rhynchocoel fluid exhibits an organization like that of a monociliated pseudostratified myoepithelium (MPM) according to the terminology of Rieger & Lombardi (1987). The MPM consist of two cell types resting on a basal extracellular matrix (ECM): apically situated monociliated supportive cells, and subapical myocytes lacking cilia (Figure 1).

The shape of the low supportive cells varies with the stretching of the proboscis and they form a continuous adluminal sheet (Figures 1, 2). These cells are characterized by reduced perikaryon and greatly elongated cytoplasmic processes. The perikaryon contains a nucleus of an irregular shape; heterochromatin clumps are distributed along the nuclear envelope and in the nucleoplasm (Figures 1, 2).

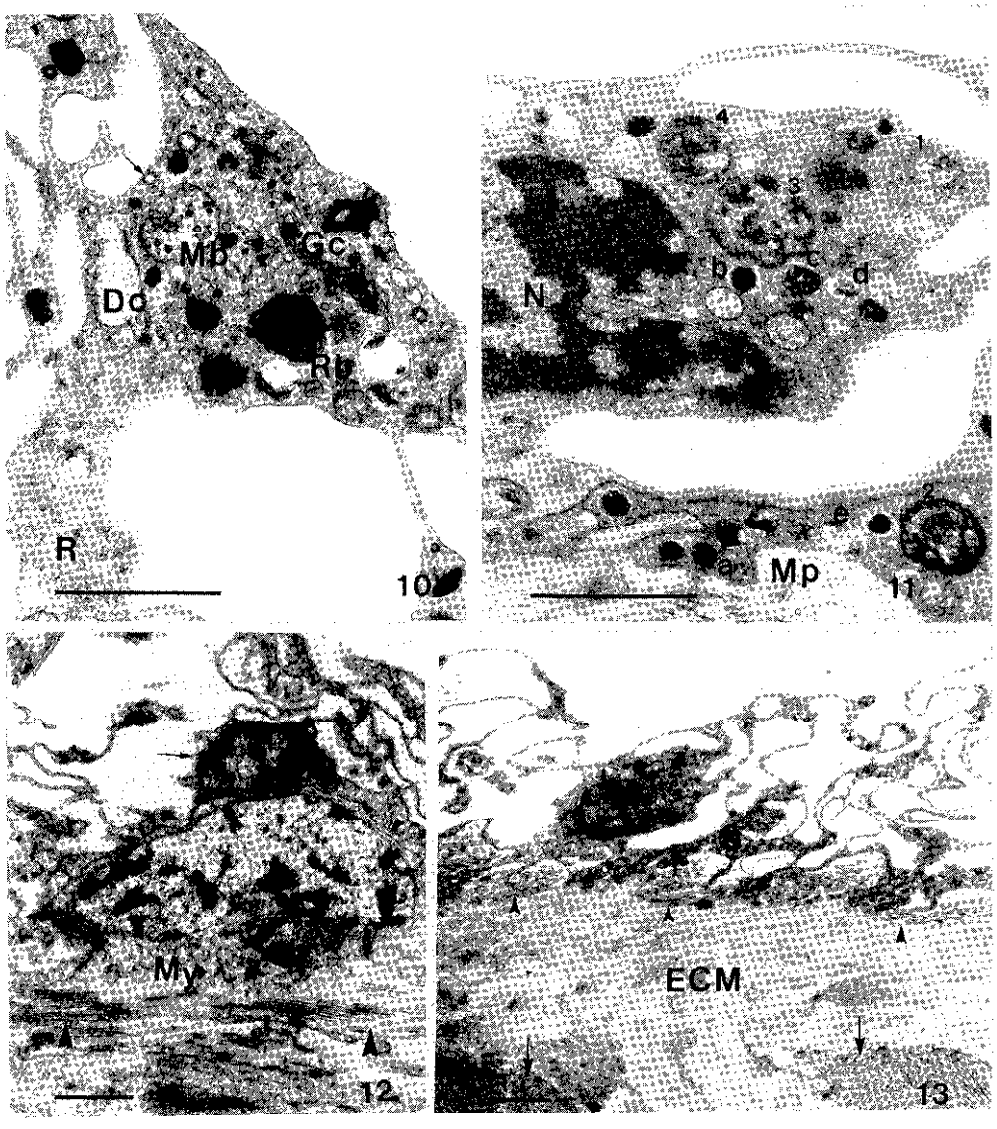
The thin cytoplasmic portion investing the nucleus of each supportive cell attenuates basally into a thin cytoplasmic sheet that spreads over the myocytes and is anchored to the ECM by folds interposed between the myofilament-containing processes of the myocytes (Figure 1). Adjacent supportive cells are joined by an apicolateral, tiny junctional structure that resembles a zonula adherens (Figure 3). The apical plasma membrane of these cells shows numerous, slender folds in irregular and tortuous directions projecting into the rhynchocoel fluid (Figure 1). These irregularly shaped folds branch, and thicker parts and thinner parts are readily distinguished in them. The thicker folds are mostly oriented circular to the longitudinal axis of the proboscis (Figure 4). Frequently, small surface areas of the folds are apposed side by side leaving an intercellular space of approximately 22.5 nm in width attached by adherens-type junctions, which are recognized as such by the increased density on the cytoplasmic surface in the junctional areas (Figure 5). Moreover, the tips of these adluminal folds are joined to those of adjacent supportive cells by similar junctional structures that resemble zonulae adherentes (Figure 6). The apical borders of the supportive cells appear alveolate due to spatial arrangement of these folds (Figure 1). The alveoli actually are extracellular spaces showing large dilatations of irregular shapes in random places; they form a extracellular space system that is, as a whole, continuous. These extracellular spaces appear obliterated in the everted proboscis (Figure 2), due to the narrowing and the consequent lengthening of the supportive cells.

Scanning electron micrographs and observations of serial sections of several regions of the proboscis have allowed us to ascertain that each supportive cell bears a single rudimentary cilium (Figure 4). This extremely short cilium (0.36 μm long and about 0.29 μm in diameter) projects freely into the rhynchocoel fluid (Figure 7). The cilium arises from a typical basal body; its axoneme consists of usually ten, but sometimes nine or eleven, microtubules without an easily defined arrangement (Figure 8). An accessory centriole oriented perpendicular to the basal body is consistently observed near this (Figure 9).

The supportive cells usually contain a variety of cytoplasmic organelles. Short, flattened cisternae of



Figures 1–9. Supportive cells of the monociliated pseudostratified myoepithelium (MPM) of *Riseriellus occultus* proboscis. *Figure 1.* General view of the MPM in a longitudinal section of an uneverted proboscis showing supportive cells and myofibrillar parts of myocytes. Note the apical (arrow) and basal (arrowhead) folds, and the broad extracellular spaces (asterisks). *Figure 2.* General appearance of the MPM in a longitudinal section of an everted proboscis. Arrowheads indicate collapsed extracellular spaces. *Figure 3.* Perikaryon of a supportive cell containing a nucleus deeply indented. Arrowhead indicate the lateral cell membrane presenting an adherens-type junction (arrow) near the apical cell border. Asterisks indicate extracellular spaces. *Figure 4.* Scanning electron micrograph of the apical surface of the MPM. Annularly oriented folds and the single rudimentary cilium (arrow) of a supportive cell are observed. *Figure 5.* Detail of folds laterally joined by adherents-type junctions (arrowheads). Asterisks indicate extracellular spaces. *Figure 6.* High magnification of the MPM in an everted proboscis showing adherens-type junctions (paired arrows) between tips of folds delimiting collapsed extracellular spaces (arrowheads). *Figure 7.* Apical cytoplasmic region showing the rudimentary cilium and its basal body (arrow). Arrowhead indicates endocytotic vesicles. *Figure 8.* Cross section of a rudimentary cilium showing the axonemal microtubules without an apparent arrangement. *Figure 9.* Detail of the apical cytoplasm region showing ciliary basal body (arrow) and accessory centriole (large arrowhead). Small arrowhead indicate endocytotic vesicles. Abbreviations: *ECM* – extracellular matrix; *Mp* = Myofibrillar parts of myocytes; *N* = nucleus of the supportive cell; *Sc* = Supportive cells. Scale bars – 1 μm (Figures 1–4, 7), 0.5 μm (Figures 6, 9) and 0.2 μm (Figures 5, 8).



Figures 10–13. MPM of the *Riseriellus occultus* proboscis. *Figure 10.* Thicker part of a supportive cell fold containing a multivesicular body whose small vesicles are similar to those (arrowheads) associated to the Golgi complex. Arrow indicated coated endocytotic pit. *Figure 11.* Perikaryon of a supportive cell showing autophagosome (*1*), autophagic vacuole (*2*) and residual bodies (*3-4*). Secretion granules at different stages of maturation (*a-e*) are also present. *Figure 12.* Myoepithelial cell of the MPM. Note its myofibrillar parts (large arrowheads). Arrow indicates supportive cell covering the myoepithelial cell. Note the absence of a basement membrane (small arrowhead) between supportive cells and myoepithelial cells. *Figure 13.* Survey electron micrograph of the MPM. Note the highly developed ECM separating the myocytes (arrowheads) of the MPM from the innermost longitudinal muscles (arrows) of the proboscis. Abbreviations: *Dc* = dilated rough endoplasmic reticulum cisterna; *ECM* = extracellular matrix; *Gc* = Golgi complex; *Mb* = Multivesicular body; *Mp* = Myofibrillar parts of myocytes; *R* = Free ribosomes; *Rb* = Osmiophilic residual body; *Sc* = Supportive cells. Scale bars = 1 μm (Figures 10–13).

rough endoplasmic reticulum, numerous free ribosomes, and abundant mitochondria are distributed throughout the cytoplasm. Numerous clear pinocytotic vesicles (120 nm in diameter) occur mostly in the perikarya and in the thicker folding cytoplasm, often showing a close spatial relationship with the plasmalemma (Figure 10). A Golgi complex develops generally in the paranuclear region, near the apical plasma membrane, but it also occurs in the cytoplasm of the thicker folds. It consists of few flattened cisternae and many, small vesicles (30 nm in overall diameter) containing an amorphous material that

exhibits varied electron density (Figure 10). Multivesicular bodies are common close to the Golgi complex (Figure 10). In these supportive cells, there are also numerous autophagosomes and residual bodies exhibiting different contents (Figure 11). Characteristic granules of variable size (ranging from 0.2 μm to 0.7 μm in diameter) and appearance occur simultaneously in small clumps in these cells (Figure 11). They apparently come from the Golgi complex, subsequently becoming countless and dispersed throughout the cytoplasm. Their different morphology may represent stages of the maturation process. It has not been possible to ascertain the final fate of these granules.

A variable number of myocytes contributes to the MPM. They are covered by the cytoplasmic sheets of the supportive cells and do not reach the rhynchocoel lumen because they are situated between these cells and the subjacent ECM (Figure 12); i.e., the myocytes are intra-epithelial (myoepithelial cells). They are scattered singly (Figures 1, 13), but all over the MPM, throughout the length of the proboscis. A basement membrane separating supportive cells and myoepithelial cells is lacking. Apposed cell membranes of both cell types are separated from one another by an intercellular space of about 20 nm. The oval nucleus of the myocytes usually bulges out from the peripheral sarcoplasm opposite the ECM (Figure 12), and the cytoplasm contains relatively few mitochondria, granular and agranular endoplasmic reticulum, free ribosomes, and vacuoles. Myofilament-containing cytoplasmic processes protrude basally from the myoepithelial cell somata (Figure 12). These delicate myofibrillar parts, containing both thick and thin myofilaments, lie in direct apposition to the ECM and are oriented circularly to the longitudinal axis of the proboscis (Figure 1). They have an epithelial arrangement, no bundles could be delineated, and the ECM separates them from the innermost longitudinal muscle layer of the proboscis (Figure 13).

The ECM underlies the basal surface of the MPM. It consists of a ground substance of moderate electron-density in which abundant collagen fibrils occur (Figures 1, 13).

The ultrastructure of the proboscis endothelium of *R. occultus* is presented diagrammatically in Figure 14.

Discussion

In *Riseriellus occultus* the epithelium of the proboscis exposed to the rhynchocoel fluid is a monociliated pseudostratified myoepithelium (MPM). This layer has been called outer epithelium (e.g. Bürger, 1895), endothelium (e.g. Thompson, 1901; Gibson, 1972) or peritoneum (Turbeville & Ruppert, 1985; Turbeville, 1991). This epithelium, as seen by light microscopy has been described as one thin layer of only one type of lining cells that can show some size differences along the proboscis (e.g. Thompson, 1901), associated or not with an underlying circular musculature (Norenburg, 1986). The few ultrastructural studies of the proboscis agree in principle with these observations (Ling, 1971; Stricker & Cloney, 1981, 1983; Turbeville & Ruppert, 1985).

The myoepithelium of *R. occultus* is pseudostratified because the supportive cells and the myocytes are situated at different levels. Both types of cells are completely underlain by the extracellular matrix (ECM). This pseudostratified myoepithelial organization may be the result of the apical displacement of non-contractile cells (supportive cells) and basal displacement of contractile cells (myocytes) as has been suggested by Rieger & Lombardi (1987) in the evolution of the coelomic linings in the Bilateria.

The supportive cells present numerous greatly elongated folds that branch and are united with each other by adherens-type junctions. Wide extracellular spaces, crossed by these folds, have been observed in the uneverted proboscis. In this position, the extracellular spaces could be confused with large vacuoles if no junctional specializations between such folds were present. The spaces are obliterated in the everted proboscis, due to narrowing and consequent lengthening of the supportive cells. Probably, these narrow spaces were mistaken for so-called pinocytotic channels by Ling (1971). We suppose that both apical and basal folds of the supportive cells could accommodate stretching of the endothelium when the proboscis is everted.

Gibson (1972) and Turbeville (1991) suggested that in nemertines the proboscis, which possesses no blood supply of its own, is supplied with nutrients by way of the rhynchocoel, the rhynchocoel fluid serving as the medium whereby soluble substances were passed from the blood system to the proboscis. Circumstantial evidence in favor of this hypothesis comes from the occurrence in the proboscis endothelium of alkaline phosphatases in the archinemertine *Cephalothrix* (Jennings & Gibson, 1969), and arylamidases in the hoplonemertine *Gononemertes* (Gibson & Egan, 1976). Our ultrastructural observations support this view. Thus, the heavy folding of the supportive cells increases the

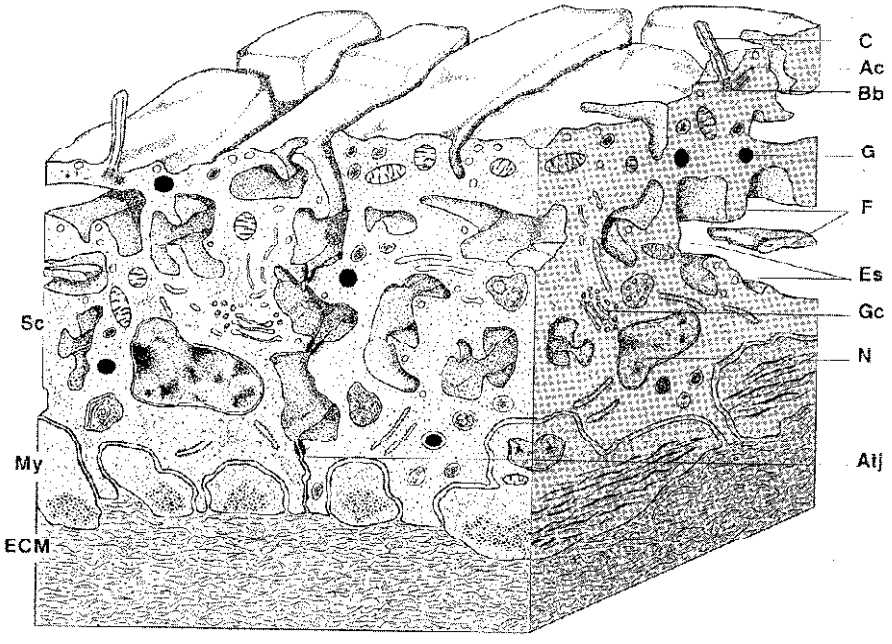


Figure 14. Diagrammatic representation of the MPM of the *R. occultus* proboscis, showing its ultrastructural organization. Abbreviations: *Ac* = Accessory centriole; *Atj* = Adherens-type junction; *Bb* = Basal body; *C* = Cilium; *G* = Granules; *Gc* = Golgi complex; *ECM* = Extracellular matrix; *Es* = Extracellular spaces; *F* = Folds; *My* = Myocyte; *N* = Nucleus; *Sc* = Supportive cell.

cell surface exposed to the rhynchocoel fluid in the uneverted proboscis. This architecture, together with the presence of pinocytotic vesicles, would be expected in an epithelium involved in the uptake or secretion of substances, as Ling (1971) pointed out for the proboscis endothelium of the heteronemertine *Lineus ruber* (Müller, 1774).

In *R. occultus* we have observed rudimentary cilia in the supportive cells. The only known cilium in the proboscis endothelium of a nemertine was shown by Ling (1971) in *L. ruber*. This cilium has peripheral but with no central microtubules, whereas those of *R. occultus* have a modified axoneme with 9–11 unordered microtubules and, apparently, are not functional. Rudimentary cilia have been reported from other body cavities lined with mesothelium, as in the rhynchocoel and lateral vessels of the paleonemertines *Carinoma tremaphoros* Thompson, 1900 and *Tubulanus rhabdotus* Corrêa, 1954 by Turbeville & Ruppert (1985) and Turbeville (1991).

The layer of circular musculature associated with the nemertine proboscis endothelium has been called endothelial (Thompson, 1901), subepithelial (Thompson, 1901; Turbeville, 1991), subendothelial (Ling, 1971; Anadón, 1976; Norenburg, 1993), inner circular (e.g. Sánchez & Moreto, 1984; Gibson, 1985; Norenburg, 1993), subperitoneal (Turbeville & Ruppert, 1985), and basiperitoneal (Turbeville & Ruppert, 1985). Although the majority of these terms refer to the topological position of this muscle layer under the endothelium, they do not indicate clearly the relative position of this muscle layer with respect to the ECM. Therefore, it is difficult to state if all the observed muscle layers associated with the endothelium are formed by myoepithelial cells. In *R. occultus* and other nemertine proboscides studied by TEM (Ling, 1971; Turbeville & Ruppert, 1985; Turbeville, 1991) this muscle layer is situated adjacent to and below the supportive cells, over the ECM. A possibly different position for this muscle layer, under the endothelial ECM, is hinted at in two ultrastructural studies. Anadón (1976) stated that all the proboscis musculature in the heteronemertine *Cerebratulus* sp. is situated 'bajo la membrana basal', including the inner circular musculature ('externa' in her work) but also indicated that some of the fibers of the outer circular musculature ('interna' in her work) 'se separen, y cruzando la capa de musculatura longitudinal, se sitúen bajo el endotelio'.

Stricker & Cloney (1983) indicated the presence of a layer of 'connective tissue' in the proboscis of the hoplonemertine *Paranemertes peregrina* Coe, 1901,

between the circular muscle layer and the endothelium (Stricker & Cloney, loc. cit.: 95); but in their diagrams (Stricker & Cloney, loc. cit.: Figure 2B, C), the circular muscle layer is drawn between the connective tissue and the endothelium.

According to the original description of *R. occultus* (Rogers et al., 1993), the proboscis of this species possesses an outer circular and inner longitudinal muscle layers. Myocytes were not detectable by light microscopy even in holotype sections (J. Junoy, personal observations) nor in sections of specimens collected in the type locality. The presence of this layer detected by TEM make the proboscis organization similar to that of *Zygeupolia* in the middle region (Thompson, 1901; see also Norenburg, 1993). Although many nemertine proboscides observed by light microscopy are described without musculature associated with the endothelium (see Gibson, 1985; Norenburg, 1993), it would become apparent with future ultrastructural studies.

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References

Anadón, N., 1976. Aportaciones a la estructura y ultraestructura de los heteronemertinos (Segunda parte). Bol. r. Soc. esp. Hist. nat. Secc. Biol. 74: 83–114.

Bürger, O., 1895. Die Nemertinen des Golfes von Neapel und der angrenzenden Meeres-Abschnitte. Fauna Flora Golf. Neapel 22: 1–743.

Gibson, R., 1972. Nemerteans. Hutchinson, London, 224 pp.

Gibson, R., 1985. The need for a standard approach to taxonomic descriptions of nemerteans. Am. Zool. 25: 5–14.

Gibson, R. & E. Egan, 1976. Some histochemical observations on digestive and other enzymes of the entozoic hoplonemertean *Gononemertes australensis* Gibson, with comments on its possible feeding behaviour. J. exp. mar. Biol. Ecol. 24: 285–296.

Jennings, J. B. & R. Gibson, 1969. Observations on the nutrition of seven species of rhynchocoelan worms. Biol. Bull. 136: 405–433.

Ling, E. A., 1971. The proboscis apparatus of the nemertine *Lineus ruber*. Philos. Trans. r. Soc., Lond. Biol. 262: 1–22.

Montalvo, S., J. Junoy, C. Roldán & P. García-Corrales, 1996. Ultrastructural study of sensory cells of the proboscidial glandular epithelium of *Riseriellus occultus* (Nemertea, Heteronemertea). J. Morph. 229: 83–96.

Norenburg, J., 1986. Redescription of a brooding nemertine, *Cyanophthalma obscura* (Schultze) gen. et com. n., with observations on its biology and discussion of the species of *Prostomatella* and related taxa. Zool. Scr. 15: 275–293.

Norenburg, J., 1993. *Riserius pugetensis* gen. n., sp. n. (Nemertina: Anopla), a new mesopsammic species, and comments on phylogenetics of some anoplan characters. Hydrobiologia 266: 203–205.

Rieger, R. M. & J. Lombardi, 1987. Ultrastructure of coelomic lining in echinoderm podia: significance for concepts in the evolution of muscle and peritoneal cells. Zoomorphology 107: 191–208.

Rogers A. D., J. Junoy, R. Gibson & J. P. Thorpe, 1993. Enzyme electrophoresis, genetic identity and description of a new genus and species of heteronemertean (Nemertea, Anopla) from northwestern Spain and North Wales. Hydrobiologia 266: 219–238.

Sánchez, M. & H. J. Moreto. 1984. Redescription of the heteronemertean *Lineus atrocaeruleus* (Schmarda, 1859). Zool. Scr. 13: 183–188.

Stricker, S. A., 1985. The stylet apparatus of monostiliferous hoplonemerteans. Am. Zool. 25: 87–97.

Stricker, S. A. & R. A. Cloney, 1981. The stylet apparatus of the nemertean *Paranemertes peregrina*: its ultrastructure and role in prey capture. Zoomorphology 97: 205–223.

Stricker, S. A. & R. A. Cloney, 1983. The ultrastructure of venom-producing cells in *Paranemertes peregrina* (Nemertea, Hoplonemertea). J. Morph. 177: 89–107.

Thompson, C. B., 1901. *Zygeupolia litoralis*, a new heteronemertean. Proc. Acad. nat. Sci. Phila. 53: 657–739.

Turbeville, J. M., 1991. Nemertina. In F. W. Harrison & E. Ruppert (eds), Microscopic Anatomy of Invertebrates 3, Platyhelminthes and Nemertina. Wiley-Liss, New York: 285–328.

Turbeville, J. M. & E. E. Ruppert, 1985. Comparative ultrastructure and the evolution of nemertines. Am. Zool. 25: 53–71.