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The Significance of Syncytial Tissues for the Position of the Hexactinellida in the Metazoa[1]

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SYNOPSIS. Hexactinellid sponges are metazoans in which the major tissue component is a multinucleated syncytium. The preferred deepwater habitat of these sponges makes collection of hexactinellids in good condition difficult, and has hindered extensive examination of their body plan. Nonetheless, over the last three decades a number of studies have explored their ecology, histology and physiology. It has been shown that hexactinellids are extremely long-lived animals. Their cytoplasm consists of a giant, multinucleated tissue, the trabecular syncytium, which is connected via open and plugged cytoplasmic bridges to cells such as archaeocytes, choanoblasts, and cells with spherical inclusions. Because all of the sponge is cytoplasmically interconnected, electrical signals can propagate through the animal. The effector response is arrest of the feeding current. The perforate plugged junction apparently allows tissues to specialize in different ways while maintaining limited cytoplasmic continuity. Larvae of hexactinellid sponges are already largely syncytial. Although it is not known when the first syncytial tissues are formed or when perforate plugged junctions first appear during embryogenesis, evidence that embryos are cellular until gastrulation suggests that hexactinellid sponges may have evolved from cellular sponges and that syncytial tissues are not a primitive trait of the Metazoa.

INTRODUCTION

Hexactinellids (''glass sponges'') are the least studied of sponges because most of them inhabit deep waters. Their siliceous skeletons have left a fossil record as far back as the Cambrian/Pre-Cambrian, making them possibly the earliest living metazoans on earth. During the Jurassic, siliceous sponges formed vast reefs, the largest of which occupied margins of the Tethys Sea and now forms rocky outcrops through much of Europe (Ghiold, 1991). Sponge reefs were thought to have become extinct in the Triassic, but the recent discovery of 4 such reefs in Hecate Strait, north of Vancouver Island, British Columbia is testimony to the continuing success of these animals (Conway *et al.*, 1991). Today hexactinellids inhabit all the oceans of the world, but are accessible only by dredge or submersible except at a few sites, where the upper edge of the population range just reaches depths accessible by SCUBA.

Hexactinellid sponges are highly unusual in that approximately 75 percent of the tissue mass is multinucleated; the remaining tissue consists of uninucleated ''cells'' that are connected to the multinucleated tissue by open or plugged cytoplasmic bridges. Given our growing knowledge of the role of highly conserved genes for transcription factors in the establishment of the metazoan body plan (Slack *et al.*, 1993; Carroll *et al.*, 2001; Davidson, 2001), it becomes increasingly important to understand how the organization of hexactinellid sponges compares to that of cellular sponges, let alone other metazoans.

The tissue organization of several hexactinellids has been described in detail (see Mackie and Singla, 1983; Leys, 1995, 1999; and references therein). The goal of the present paper is to review knowledge of the body plan of a generalized hexactinellid sponge, in order to emphasize the key structural features that make this animal syncytial rather than cellular, and to assess the implications of this organization for the status of the Hexactinellida within the Metazoa.

HISTORICAL BACKGROUND

Since the earliest descriptions of sponges from this group in the late 19th Century, hexactinellid tissues have been thought to be largely syncytial (Schulze, 1880, 1887, 1899; Ijima, 1901, 1904). Both Schulze and Ijima described the major tissue component as a system of fine branching, multinucleate trabeculae, which held the flagellated chambers in place. The thinness of the trabeculae was attributed to the minimal development or complete lack of a collagenous mesohyl. It was agreed that although there were several cells that stained distinctly, the majority of the tissue constituted a giant multinucleated syncytium.

Bidder (1929) recognized the histological distinctness of hexactinellids and separated them from the Calcarea and Demospongiae by creating two phyla, the Nuda (Hexactinellida) and the Gelatinosa (Calcarea and Demospongiae), names that reflected the lack of a well-developed mesohyl in hexactinellids compared with other sponges. By this classification scheme, Bidder also implied that this characteristic was evidence that sponges evolved from different choanoflagellate ancestors, justifying the separation at the phylum level. More recently Reid (1963) suggested a separation at the subphylum level to reflect the probable common

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origin of sponges based on similarities in descriptions of the larvae (Ijima, 1904; Okada, 1928).

These proposals were largely ignored until the histology of hexactinellids could be verified with modern techniques. In the 1970s SCUBA was used to collect specimens found in relatively shallow waters off the coast of British Columbia and initial attempts were made to fix the tissue for electron microscopy. Studying *Aphrocallistes vastus* and *Heterochone calyx*, Reiswig (1979) discovered that normal fixation protocols for electron microsocopy (protocols which work well for fixing demosponges) do not work well with hexactinellid tissue. Mackie and Singla (1983) had better success, after much trial and error, by using a one-step cocktail fixative. Both investigations concluded that the major part of the sponge consists of a multinucleated tissue that they termed the trabecular syncytium.

The further discovery that hexactinellids can propagate signals that cause the arrest of their feeding current (Mackie, 1979; Lawn *et al.*, 1981) was fully consistent with the histological evidence that their tissues were syncytial as the absence of membrane barriers would allow impulses to spread. A new proposal put forward by Reiswig and Mackie (1983) placed hexactinellids in the subphylum Symplasma, and all other sponges in the subphylum Cellularia to reflect this profound structural difference.

THE NATURE OF SYNCYTIAL ORGANISMS

Because no other metazoan possesses such extensive syncytial tissues, or such an unusual manner of maintaining cytoplasmic continuity between uninucleate and syncytial components, it is worthwhile briefly discussing what is generally meant by the terms syncytial and cellular in other organisms.

Before tissue could be viewed by electron microscopy, the boundaries between cellular and syncytial tissue were less clear. Although most tissues were thought to be cellular, in some instances cells appeared to be tenuously connected by protoplasmic bridges, and in other cases, such as in *Drosophila* embryos, cells were clearly multinucleate by way of incomplete cytokinesis. In addition, botanists had found many algae, such as *Caulerpa*, which clearly possessed multinucleate cells. Several attempts were made to standardize terminology. Sachs (1892) felt that a nucleus presiding over an area of cytoplasm, whether in a uninucleate or multinucleate cell, was the functional unit and described these regions as ''energids.'' Rubaschkin and Besuglaja (1932) suggested the term 'protoplasts' for individual cells or unions of cells, and ''symplasts'' for multinucleate tissues in which none of the nuclei has individual territory. In 1934, Studnička (1934) proposed terminology that attempted to combine these two viewpoints, using ''symplasma'' for cells which had cytoplasmic continuity as a result of incomplete cytokinesis but which otherwise remained independent, ''syncytia'' for multinucleated structures whose cytoplasm was not organized around centrioles (such as muscle cells), and ''plasmodia'' for multinucleated tissue which had been formed by fusion of separate cells or by division of nuclei in a growing cell.

With the advent of modern microscopy techniques the structures of tissues have become less elusive, and the question of what defines a syncytium is no longer given much consideration. Currently, the term syncytium is used to describe any tissues that lack membrane barriers between adjacent nuclei. For example, a large number of algae in the Charophyta, Rhodophyta, and Chrysophyta are coenocytic (=syncytial) by incomplete cytokinesis; the slime mold *Physarum* is a multinucleate plasmodium; the epithelia of some cnidarians and skeletogenic tissues of echinoids are syncytial by incomplete cytokinesis; the giant neurons in squid and vertebrate striated muscle cells are multinucleate by fusion of separate cells; some embryonic tissues (*e.g.*, *Drosophila*) are multinucleate by incomplete cytokinesis; and developing sperm cells in many organisms are syncytial because of their connection by cytoplasmic bridges (see Mackie and Singla, 1983 for thorough review). Syncytial tissues have come about in many different organisms, and though the structure of each multinucleate tissue depends on how it was formed, the general function of syncytia is to enhance intercellular transport by way of cytoplasmic continuity. Interestingly, although plant cells connected by plasmodesmata could also be considered syncytial because they are connected cytoplasmically, they nonetheless function independently and are therefore considered by plant biologists to be cells. The same argument is applied to the terminology used for hexactinellid tissue. The uninucleate components of the tissue function independently and as such are termed cells; yet because all components are connected cytoplasmically these animals are considered to be syncytial.

DESCRIPTION OF WHOLE SPONGES

Although some hexactinellids are bilaterally symmetrical (see Tabachnick, 1991), the majority are radially symmetrical, and vase- or tube-shaped. From the work of Reiswig (1979), Mackie and Singla (1983), and Reiswig and Mehl (1991) we know that the main component of the soft tissues is a single continuous multinucleated tissue called the trabecular syncytium that stretches in a cobweb-like manner between the dermal and atrial sides of the sponge (Fig. 1A). The trabecular syncytium is apparently continuous throughout the sponge. The center of the body wall is the choanosome, which consists of oval flagellated chambers that house the collar-flagellar units that generate the water current through the sponge (Fig. 1B, C). Other components of the sponge are uninucleate, but all appear to be connected to other uninucleate components and to the multinucleated trabecular syncytium by cytoplasmic bridges. The membrane is continuous from one component through the cytoplasmic bridges to the other components. The cellular com-

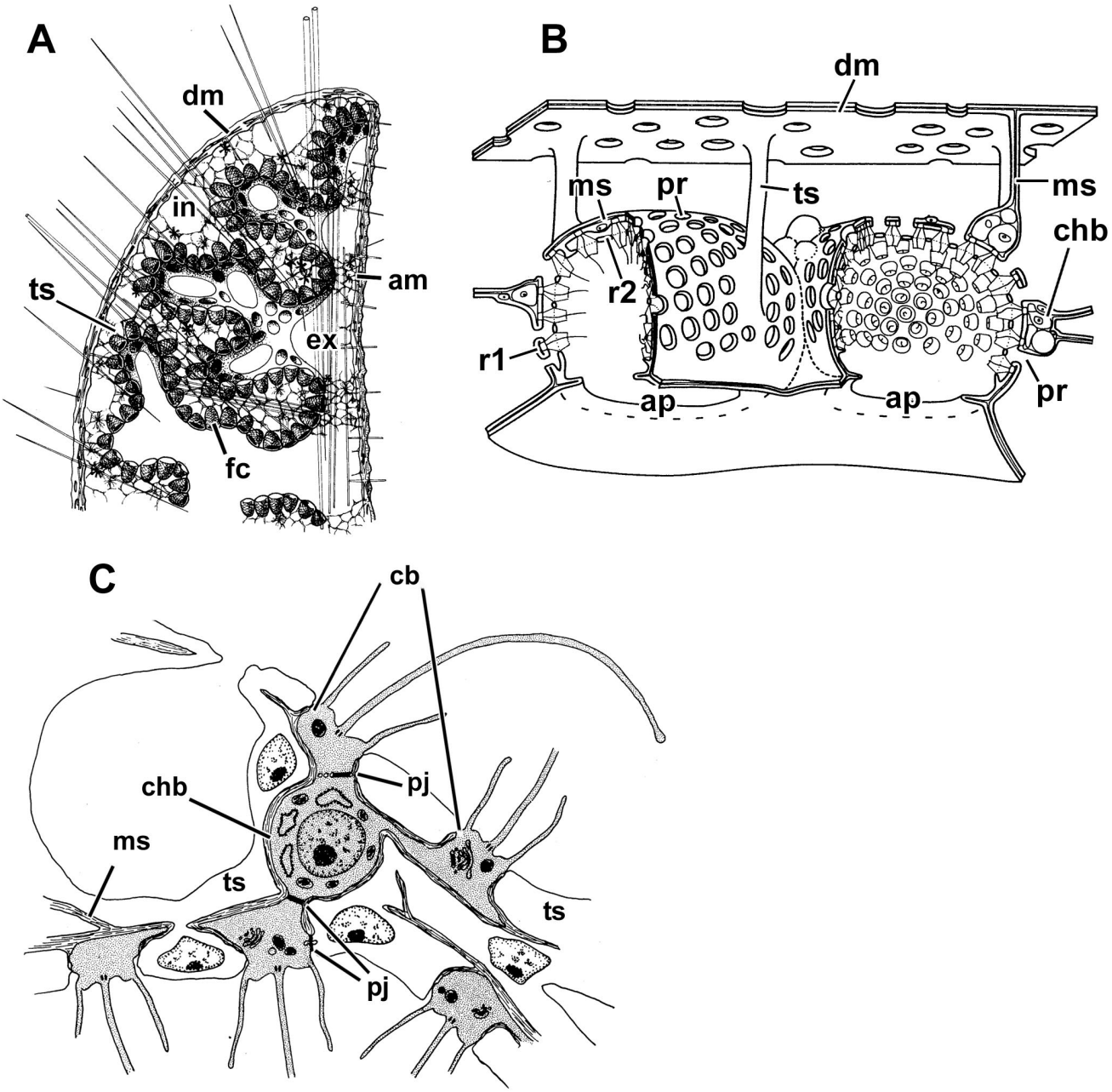

FIG. 1. Anatomy of hexasterophoran hexactinellid sponges. A Body wall of *Acanthascus grossularia*, redrawn from Schulze (1887). B Choanosome of *Farrea occa*, redrawn from Reiswig and Mehl (1991). C Drawing of a section of the choanosome tissue, modified from Mackie and Singla (1983). Abbreviations: am, atrial membrane; ap, apopyle; cb, collar body; chb, choanoblast; dm, dermal membrane; ex, excurrent channel; fc, flagellated chamber; in, incurrent channel; ms, mesohyl space; pj, plugged junction; pr, prosopyle; r1, primary reticulum; r2, secondary reticulum; ts, trabecular syncytium.

ponents include: archaeocytes, a pluripotent cell type common to all sponges; choanoblasts, the nucleated base from which eunucleate collar bodies bud; thesocytes or cystencytes, a cell that contains large spherical inclusions and some paracrystalline rods; and spherulous cells, that contain small spherical inclusions. In all adult hexactinellid tissue that has been sectioned to date, sclerocytes—the spicule-forming tissues—are known to be multinucleate, but no bridges have been found connecting this tissue to the multinucleated tissue. Nonetheless, sections of hexactinellid embryos show that in the developing larva sclerocytes are connected to other tissues by cytoplasmic bridges (Leys, 2003), thus their absence in adult sponges may simply reflect the difficulty of finding such a small feature by thin section electron microscopy.

The cytoplasmic bridges connecting uninucleate and multinucleate components are occasionally open, but more often they are plugged by an intrasyncytial proteinaceous junction (except possibly in *Dactylocalyx* [Reiswig, 1991]), that is unique in the Metazoa. It is because the plugged junction allows the uninucleate components to be independent that it is conventional to refer to these components as cells. Consequently, knowledge of the structure, formation, and placement of the plugged junction is critical to understanding hexactinellid syncytia.

PERFORATE PLUGGED JUNCTIONS

The structure of the plugged junction has been described in some detail by Mackie (1981), Mackie and Singla (1983), and Jan Köster (communication to G. Mackie). The plug is not a membrane barrier, and contains no lipid bilayer. It is a flattened disc consisting of three layers and containing cylindrical pore particles (Fig. 2A–D). The mature plug has a ring of rods around the periphery, and throughout the disk there are large pore particles, which have a 5 nm channel. Some plugs have membranous saccules as if in transit (Fig. 2E). Because the cytoplasm is often different on the two sides of a plugged junction (Fig. 2B, F), it is presumed that the plug limits the transport of certain materials while allowing others to pass. As it is not a membranous structure, it is permeable to the flow of ions and electric currents. In embryos, archaeocyte-like cells are connected by particularly large plugged junctions (Fig. 2G), which may suggest that a large amount of material is transferred between cells.

Thin sections of adult tissue and of aggregates formed from dissociated tissue of *Rhabdocalyptus dawsoni* indicate that the plug arises as a mass of ''amorphous, electron-dense material'' lying between the nucleus and the Golgi apparatus (Pavans de Ceccatty and Mackie, 1982). The early plug consists of small vesicles that lie among and around the electron-dense material (Fig. 3A; Pavans de Ceccatty and Mackie, 1982). When it is fully formed, the plug lies within a ring- or tube-shaped vesicle that fuses with the ''cell'' membrane and becomes lodged within a cytoplasmic bridge (Fig. 3B, C; Mackie and Singla, 1983). Hence the plug is a specialized cytoplasmic structure, unlike desmosomes, gap junctions, or septate junctions, which are formed from membrane appositions.

CYTOSKELETAL ARCHITECTURE AND INTRASYNCYTIAL TRANSPORT

One of the difficulties in interpreting hexactinellid tissue from sections is its extreme thinness. The cytoplasm is so sparse that multitudes of sections must be made to observe features such as cytoplasmic bridges and the formation of plugged junctions. However, because of the great regenerative capacity of sponge tissue, dissociated pieces of the adult sponge will reaggregate to form a single large piece of tissue. Pavans de Ceccatty and Mackie (1982) first used this technique to study plugged junction formation, and found that during aggregation the syncytial ''giant cell'' becomes compartmentalized by these junctions. In later work using similar preparations, S.A. Arkett (unpublished observation) noted that occasionally such aggregates adhered to the substrate. In thin areas of adherent aggregates, the cytoplasm could be seen to be in constant motion.

Using an acellular tissue extract made from the adult sponge it was possible to generate adherent aggregate preparations with which to study the cytoskeletal organization of the hexactinellid tissue using immunofluorescence, and the phenomenon of cytoplasmic streaming using video microscopy (Leys, 1997). Like Pavans de Ceccatty and Mackie (1982), Leys (1995) found that dissociated pieces of tissue from the sponge are either multinucleated pieces of the trabecular syncytium, or cellular components, still connected to each other by perforate plugged junctions. However, instead of compartmentalizing, the adherent pieces contact other pieces and fuse to form a single larger piece (Leys, 1995). Over the course of several hours the tissue can cover a 15 cm-diameter petri dish with a single continuous syncytial sheet.

The cytoskeleton of adherent preparations examined by immunocytochemistry shows tracts of microtubule bundles that stretch for hundreds of micrometers; nuclei are dispersed around the microtubules (Fig. 4A). Rather than forming a cell soma from which the microtubules radiate, the nuclei are in fact transported along the tracts of microtubules (Fig. 4B). Microtubule organizing centers were only found in the ''cellular'' archaeocytes (Leys, 1996); it remains unclear where the microtubules of the multinucleated tissues originate. Actin microfilaments are equally extensive; they traverse hundreds of micrometers around and across the adherent preparations, and form giant rod-like extensions from the edges of the adherent aggregates (Leys, 1995). In summary, the cytoskeleton of adherent tissue of hexactinellid sponges is that of a multinucleated giant cell.

Movement of individual organelles is evident within young adherent aggregates, and with time, the cytoplasm within the giant syncytial sheets becomes organized into 20 μm-wide rivers. Streams of cytoplasm contain nuclei, mitochondria, spicule pieces, and tubulovesicular organelles (Leys, 1995, 1998). Organelles move continuously in streams, at an average rate of $2.15\ \mu m \cdot s^{-1} \pm 0.33\ \mu m \cdot s^{-1}$, and by saltation in thin lamellipodia, at 0.32 to $3.6\ \mu m \cdot s^{-1}$ (Leys, 1996). Thin section and negative stain electron microscopy provide evidence for viscous coupling of organelles in bulk cytoplasm to microtubule bundles via a tubulovesicular network (Leys, 1996) as in the 500 μm wide swaths of streaming cytoplasm in the coenocytic characean alga *Chara* (Kachar and Reese, 1988).

Cytoplasmic dynein is implicated in organelle transport based on preliminary experiments showing immunoreactivity by whole cell lysate to antibodies to cytoplasmic dynein, and reversible inhibition of trans-

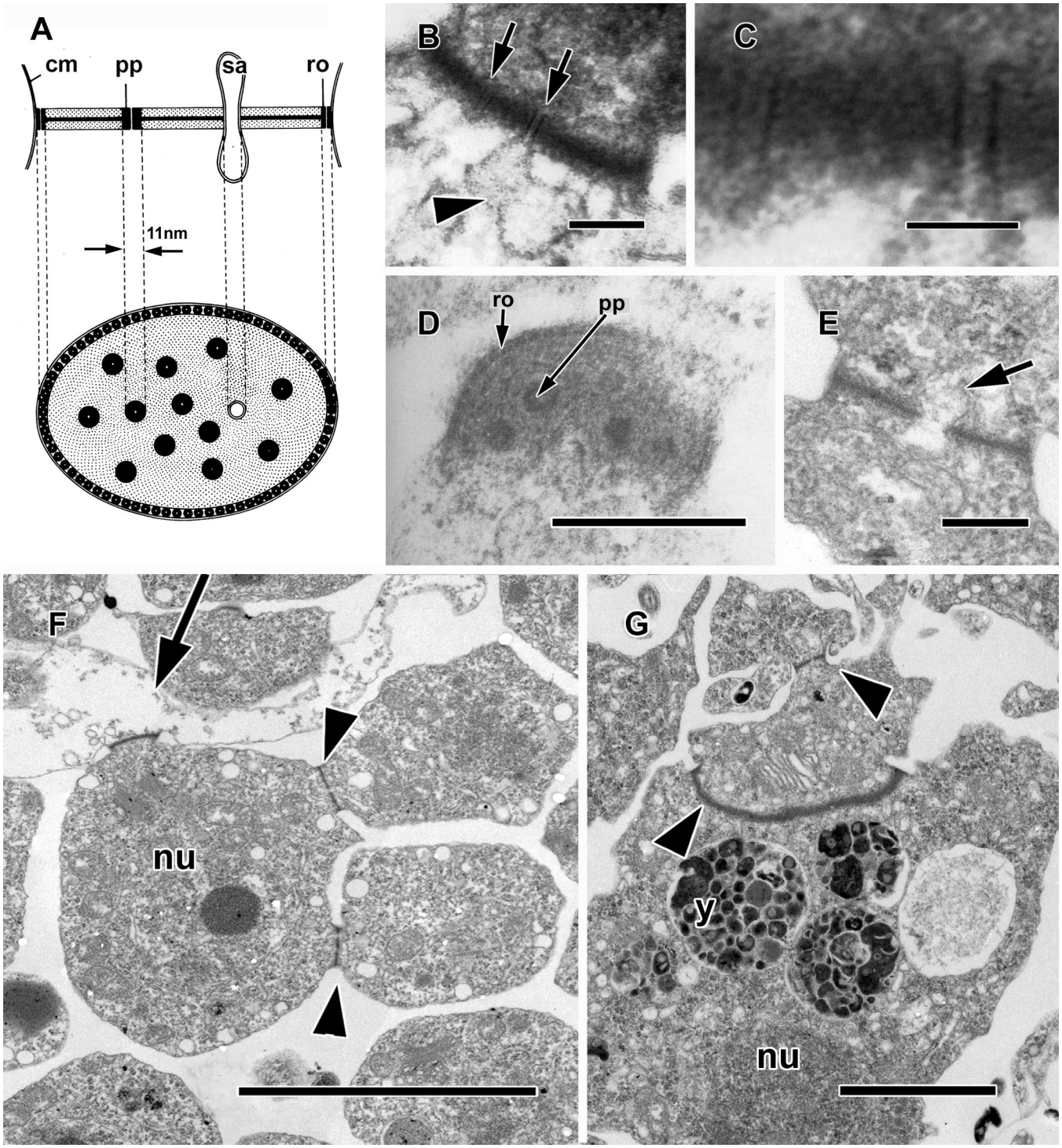

FIG. 2. Structure of the perforate plugged junction. A Diagram of a 'plug' in cross and transverse section, redrawn from Mackie and Singla (1983). Abbreviations: cm, cell membrane; pp, pore particle; ro, rod; sa, saccule. B–G Transmission electron microscopy (TEM). B Cross section of a plug showing pore particles (arrows), and electron dense material passing through the pore (arrowhead). C Magnification of the pore particles in B. D Transverse section through a plug showing pore particles (pp) and rodlets (ro), from Mackie and Singla (1983). E Cross section of a plug traversed by a membranous saccule (arrow). F Plugged junctions between archaeocytes (arrowheads) and between an archaeocyte and the trabecular syncytium (arrow) showing the distinct cytoplasm on either side of the junction. nu, nucleus. G Two plugged junctions (arrowheads) in a blastomere of an embryo. nu, nucleus; Y, yolk inclusion. Scale bars: B, 25 nm; C, 10 nm; D, 0.2 μm; E, 0.1 μm; F,G, 1 μm.

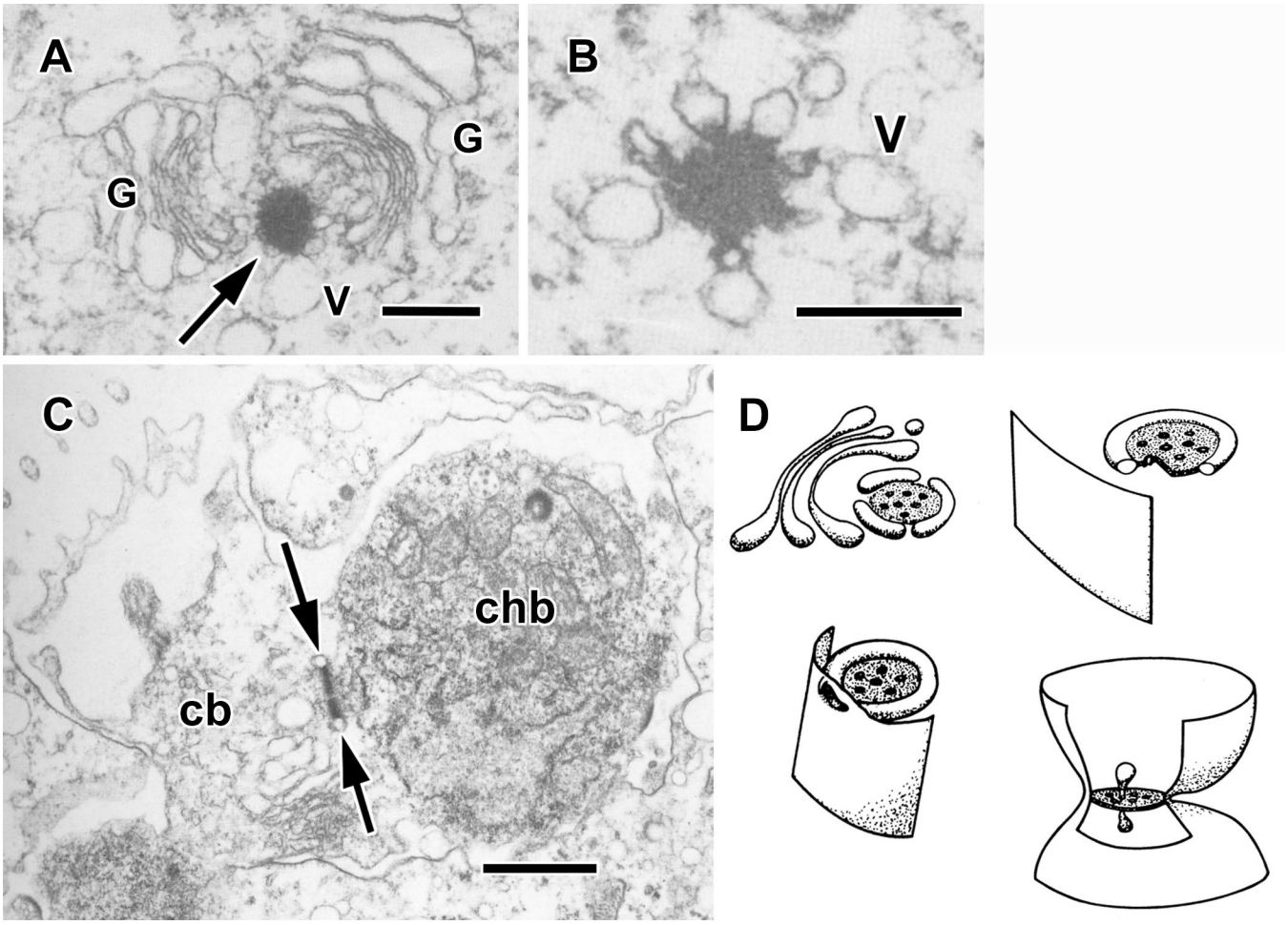

FIG. 3. Formation of the perforate plugged junction. A An early plug consisting of amorphous osmiophilic material (arrow) and small vesicles (v) forming beside the Golgi apparatus (G). B Membranous vesicles (v) surround early plugs (A and B from Pavans de Ceccatty and Mackie, 1982). C Rings of membrane (arrows) on either side of a plug that is fully formed, but not yet inserted into a cytoplasmic bridge (modified from Mackie and Singla, 1983). D Schematic of plug formation and insertion into a cytoplasmic bridge, as proposed by Mackie and Singla (1983). Scale bars: A,B, 0.5 μm; C, 1 μm.

port by n-ethyl maleimide (Leys, 1996). However, as numerous attempts to generate reactivated lysed preparations have been unsuccessful (for a summary of experiments see Leys, 1996), the role of kinesin in organelle transport cannot be ruled out. Furthermore, both the rates and mode of saltatory transport of organelles in giant lamellipodia resemble myosin-driven transport along actin in extruded squid axoplasm (Bearer *et al.*, 1993).

HEXACTINELLID PHYSIOLOGY

The syncytial condition of hexactinellid sponges has far reaching implications for feeding and behavioural physiology in these animals.

In the absence of a well-developed collagenous mesohyl, nutrient transport is symplastic or intrasyncytial in streams of cytoplasm, rather than apoplastic, as in cellular sponges (Leys and Reiswig, 1998) and other metazoans. Feeding experiments have shown that latex beads are taken up by the trabecular syncytium at the choanosome (Wyeth, 1999); it is presumed they are then transported into streams. Latex beads pipetted onto the surface of adherent aggregates attach to the membrane and are internalized (Leys, 1996). Uptake occurs irrespective of proximity to streaming cytoplasm, yet after uptake, beads are caught up and transported in nearby streams.

The syncytial tissues and perforate plugged junctions also allow the propagation of electrical impulses through the sponge. Despite many histological investigations there is no evidence for the existence of nerves in any member of the Porifera. Transmission through gap junctions can also be ruled out. Loewenstein (1967) reported the only experiment demonstrating electrical coupling between two dissociated sponge cells, but this experiment has never been replicated, and no evidence for gap junctions or any similar connecting junction has been found in calcareous sponges or demosponges (Garrone *et al.*, 1980). The contractions seen in some demosponges in response to tidal changes (Reiswig, 1971), or sediment in the water (Gerrodette and Flechsig, 1979), at 10–20 $\mu m \cdot s^{-1}$—

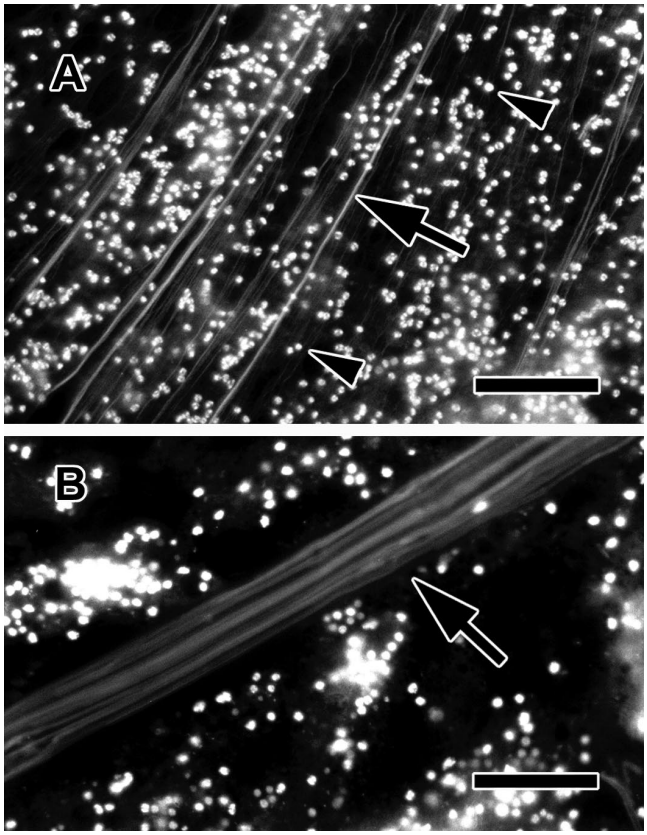

FIG. 4. The microtubule cytoskeleton and nuclei in adherent tissue from *Rhabdocalyptus dawsoni*. A Fluorescence microscopy of the microtubule cytoskeleton (arrows) of a 24-hour adherent tissue preparation labeled with anti-tubulin, and nuclei (arrowheads) labeled with Hoechst. B Fluorescence microscopy of nuclei labeled with Hoechst in a live adherent tissue preparation. Nuclei that were moving in a stream of cytoplasm (arrow) leave a trail of light marking their paths. Scale bars: A,B, 10 μm.

approximately the rate of a calcium wave in astrocytes (Nedergaard, 1994)—are far slower than any known rates of nervous or non-nervous impulse conduction.

Hexactinellid sponges are capable of much faster responses. *Rhabdocalyptus dawsoni* arrests its feeding current upon mechanical or electrical stimulation, both in the field and in laboratory experiments (Mackie, 1979; Lawn *et al.*, 1981). The speed of the response to stimuli, 0.26 $cm \cdot s^{-1}$, indicated the event is electrical, rather than mechanical or chemical—although both of those possibilities have been examined and eliminated (Mackie *et al.*, 1983). However, the thinness and fragility of the tissue defied attempts to record from the sponge until a new preparation was conceived.

The first recording of a propagated action potential (AP) in a sponge was achieved using a preparation that consists of aggregates grafted onto the atrial side of the host sponge (Leys and Mackie, 1997). The rate of propagation of the AP (0.29 $cm \cdot s^{-1}$), though slower than AP propagation in most invertebrate epithelia and nerve nets, agrees well with the rate calculated for propagated arrests of the feeding current (Mackie *et al.*, 1983). The impulse has the characteristics of a calcium-based event (Leys *et al.*, 1999). Though there is no published work on ion channels in any member of the Porifera (Hille, 1992), calcium and potassium channels are found in protists, whereas sodium channels first appear in the Cnidaria. Consequently, the demonstration that hexactinellid sponges are capable of propagated electrical events that may be calcium-based has implications for the evolution of excitability within the Metazoa.

CONCLUSIONS: STATUS OF HEXACTINELLIDS AS METAZOANS

Given their highly divergent body plans, can hexactinellid sponges be considered to be metazoans? Accumulated molecular evidence, including sequences from small and large subunit rRNA and genes coding for proteins that characterize the Metazoa, indicates that hexactinellids and other animals are monophyletic (Garrone, 1995; Müller, 1995; Cavalier-Smith *et al.*, 1996; Collins, 1998; Schütze *et al.*, 1999; Borchiellini *et al.*, 2001). At the same time, analysis of slowly evolving gene sequences, such as PKC (Kruse *et al.*, 1998), and of large subunit rRNA (Medina *et al.*, 2001) suggests that the Porifera is paraphyletic; hexactinellids and demosponges form one clade, while calcareous sponges are more closely related to other metazoans.

Although this molecular data lends support to Reid's (1957) proposal to group sponges that have a siliceous skeleton together in the Silicea (a term recently reintroduced by Medina *et al.* [2001]), assumptions that demosponge and hexactinellid larvae are similar (Reid, 1963) are now known to be incorrect. While we know very little about the details of embryogenesis in hexactinellids, it is clear that the larva is already largely syncytial and does not resemble either the amphiblastula of the Calcarea and some demosponges, or the parenchymella larva of most demosponges (Boury-Esnault *et al.*, 1999).

Our knowledge of development in hexactinellids comes from only two studies (Okada, 1928; Boury-Esnault *et al.*, 1999). Okada's descriptions were from sections of paraffin-embedded material that had been fixed in Flemming's solution or a saturated solution of mercuric chloride in seawater. Using light microscopy, Okada concluded that the embryo underwent gastrulation and that the gastrula consisted of three ''cell elements'' on the outside, and two on the inside. However, as one of the outer elements is described as consisting of ''small cytoplasmic masses with yolk bodies but without the nuclei,'' it is unclear whether syncytial tissues already exist in the gastrula. According to the light microscope observations of Boury-Esnault and colleagues (1999) the larva is cellular until gastrulation, which occurs by cellular delamination to produce a population of micromeres surrounding a population of macromeres. The former differentiates into a smooth syncytial epithelium and a layer of multiciliated cells. The macromeres also differentiate into two distinct tissues, one syncytial and the other cellular,

which occupy the anterior-central and posterior-central portions of the larva. All multinucleate and uninucleate components of the larva are connected by perforate plugged junctions.

From the above descriptions it appears that the embryos are initially cellular like conventional sponges, and that synctyial tissues develop from cellular components prior to or at gastrulation. However, as thin section TEM has not been carried out on the earliest stages of embryogenesis, it is unclear when the perforate plugged junction first forms. Though it is possible that the early blastomeres are completely independent of one-another, it seems likely that in the early stages of development blastomeres may be connected by cytoplasmic bridges, as is seen in the development of squid embryos (*e.g.,* Arnold, 1974), and that subsequently a proteinaceous barrier, the perforate plug, may be inserted between the two daughter cells allowing separation of function. In this scenario, at a later stage, if no barriers are inserted following nuclear division, a multinucleate tissue would form (the trabecular reticulum), which allows intrasyncytial nutrient transport, electrical conduction, and coordinated responses by the whole organism. If this embryological sequence recapitulates phylogeny, hexactinellid sponges may be seen to have evolved from conventional sponges, and syncytial tissues are not a primitive trait in the Metazoa. Current work is focused on understanding the earliest stages of syncytia formation during embryogenesis of hexactinellid sponges.

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