SEROTONIN IN PORIFERA? EVIDENCE FROM DEVELOPING *TEDANIA IGNIS,* THE CARIBBEAN FIRE SPONGE (DEMOSPONGIAE)

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Histochemical study of larvae and freshly settled juveniles of the Caribbean fire sponge *Tedania ignis* (Tedaniidae, Poecilosclerida) reveals evidence of serotonin-like immuno-reactivity, a possible indication for the presence of precursors of nerve cells in this species. Already in the earliest stages of its life, *T. ignis* is made up of two discernable cell types: monociliated cells arranged in quasi-epithelial fashion and covering the larva and the developing settled organism, and mesohylal cells (archeocytes). In the adult sponge, several mesohylal cell types can be distinguished which form a complex connective tissue. Serotonin-like immuno-reactivity demonstrated by us occurs only in two cell types: in some archeocytes of the parenchymella larvae, and in similar archeocytes and in a second, bipolar cell type of the settled, juvenile sponge. The discovery of a neuroactive substance in cells of developing sponges before and after metamorphosis provides new insights into the origin and evolution of nerve and muscle cells in the Eumetazoa. ❐ *Porifera, histochemistry, serotonin, Tedania ignis, larval development, evolution.*

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Although sponges, one of the oldest metazoan groups, possess the greatest diversity of biologically active compounds of any marine phylum, the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) has been reported only once, in myocyte-like cells of *Sycon ciliatum* (Sycettidae, Calcarea) (Lenz, 1966). Serotonin appeared early in the evolution of eucaryotes. For example, it is used in chemical signal chains in Protista where, in a species of the ciliate *Blepharisma,* a serotonin-like substance is known to function as a mating pheromone (Haldane, 1954; Miyake, 1984). It has also been shown that a number of lower organisms use serotonin as an internal messenger in their neurotransmitter-receptor systems (Carr et al., 1989) and that some of these characteristics of molecular structure that arose in unicellular organisms may have been inherited and modified by metazoans (Mackie, 1990; Van Houten, 1990).

It seems obvious that nerve cells developed gradually over a long period of time but the sequences of changes that must have occurred are difficult to establish. Being a primitive outgroup of the Eumetazoa, Porifera do not have neurons or myocytes that are present in organisms of higher levels of organisation. A common phylogenetic hypothesis such as the Planula or Phagocytella hypothesis (see Hyman, 1951; Ivanov, 1988; Rieger et al., 1991; Ax, 1995) encouraged the authors to search for precursors of nerve and muscle cells in sponge larvae in early developmental stages rather than in adults, a neglected area of research so far (Harrison & De Vos, 1991; Woollacott & Pinto, 1995). Such precursors of nerve cells and myocytes in sponges could represent the first stage in the evolution of integrative systems (e.g. Pavans de Ceccatty, 1974a, 1989; Mackie, 1990).

MATERIALS AND METHODS

Larvae of *Tedania ignis* (Durchassaing & Michelotti) (Tedaniidae, Poecilosclerida, Demospongiae) were sampled in the laboratory seawater system of the Smithsonian Coral Reef Field Station at Carrie Bow Cay, Belize, in March 1994 and November 1995. Larval release was induced in adult specimens collected in the nearby mangrove of Twin Cays (Rützler & Feller, 1996) and maintained in aerated seawater by exposing them to natural sunlight following a 12–24hr period of dark adaptation (Woollacott, 1993). The larvae were kept in seawater-rinsed glass dishes (10cm diameter) and fixed immediately after release and 80–100hrs after attachment to the substrate. To provide a

substrate suitable for fixation and removal for subsequent processing, the bottom of these dishes was coated with polymerised epoxy resin (Spurr).

Specimens were fixed in 4% paraform-aldehyde (PFA) in phosphate-buffered saline (PBS; 0.1M, pH 7.4) for 6–8hrs, rinsed in PBS, and treated with Triton X-100 (0.2%, 1hr) to permeabilise membranes. After labelling with the primary antibody (rabbit anti-serotonin, IMMUNOTECH 0601; 2.5%) overnight at 4°C, fluorescence-labelling was done for 1hr with a tetrarhodamine-isothiocyanate-(TRITC)-conjugated secondary antibody (swine anti-rabbit; DAKO, 1%) for 1hr. Specimens were then rinsed in PBS, whole-mounted (Gelmount) on slides, and examined under a REICHERT Polyvar epifluorescence microscope. Incubation in bovine-serum albumin-Triton (BSA-T) without primary antibody was used as the control for nonspecific binding of the secondary antibody. Three larvae and three settled juvenile sponges were sectioned (epoxy-embedded, 1μm thick, stained with toluidin blue) and investigated in detail. The immuno-staining of both larvae and freshly settled sponges was carried out by the labelled streptavidin-biotin method (LSAB kit; DAKO). Histochemical staining of peroxidase with amino-ethyl-carbazole (AEC, substrate buffer) was used to enhance visibility of the labelled cells.

RESULTS

Tedania ignis has a parenchymella larva composed of two types of cells (Bergquist et al., 1979). Peripherally, flagellated epithelium-like cells cover the organism. This "epithelium" is monociliated and 10-25μm high. The free-swimming larva exhibits coordinated ciliary action. A distinct basal lamina and typical eumetazoan apical junctional complexes are apparently lacking (but see below). Interior, apparently motile mesenchymal cells (mesohyl cells) are arranged beneath the epithelium-like sheath (Woollacott, 1990, 1993; Amano & Hori, 1994) (Fig. 1A). The live larvae are ovoid and have a size of 700–900μm long, 500–600μm wide, but the necessary Triton X-100 treatment weakens the cell membranes and larvae usually shrink and collapse (Fig. 1A, B). In the juvenile, settled sponge too– as in the adult–the exopinacoderm which covers the ectosome acts as the protective layer. Inside the sponge, choanocyte chambers connected by canals lined with endopinacocytes lie embedded among mesohyl cells (Fig. 2A). Using a whole-mount fluorescence technique, we found serotonin-like immuno-reactivity in special mesohyl cells of both larval and juvenile *T. ignis*. Spherical serotonergic cells appear to be randomly distributed and occur alone or in clusters (Figs 1B, 2B). In one larva, for example, 6 clusters of such serotonin positive cells were found, each composed of 2–4 single spherical cells with a diameter of 4–6μm. In some clusters as well as in several single cells the nuclei are clearly visible and appear as non-fluorescent regions (Fig. 1B).

In the juvenile, settled sponge, a few bipolar cells were found in addition to the spherical cells that superficially resemble bipolar neurons or 'myocyte-like' cells (actinocytes) reported by Bagby (1966) (Fig. 2B, C). These bipolar cells have a maximum length of 20-50μm. Both types of serotonin-positive cells (spherical and bipolar) appear to be located in the mesohyl as spicules can be seen on top of the labelled cells (Fig. 2C).

No information is yet available on whether interactions between these morphological types of serotonin-positive cells occur, nor do we know whether the bipolar cells differentiate from the spherical type. If these serotonergic cells are part of an integrative system, both cellular communication at a distance (spherical cells) or cell–cell contacts (bipolar cells) could be expected.

DISCUSSION

Our study is the first to report serotonergic cells in Demospongiae, a spherical type in both larva and post-metamorphosed sponge, and a second bipolar cell type that is exclusive to the post-larval developing organism . Up to now, serotonin was only demonstrated histochemically in myocyte-like cells of Calcarea (see below). Among the most primitive Eumetazoa, serotonin is well known to act as a neurotransmitter (e.g. in Anthozoa, Umbriaco et al., 1990). Actually, 5-HT has a wide range of functions in invertebrates, such as control of regeneration processes in Planaria (Kimmel & Carlyon, 1990) and of beat of cilia in echinoderm embryo (Mogami et al., 1992), and as inhibitors and activators of muscle of molluscs (Welsh, 1953; Twarog, 1988). As in Porifera, members of the phylum Placozoa do not differentiate nerve or muscle cells and are therefore counted among the most primitive eumetazoans (Grell, 1974; Ax, 1989; Grell & Ruthmann, 1991). Schuchert (1993) demonstrated in *Trichoplax adherens*

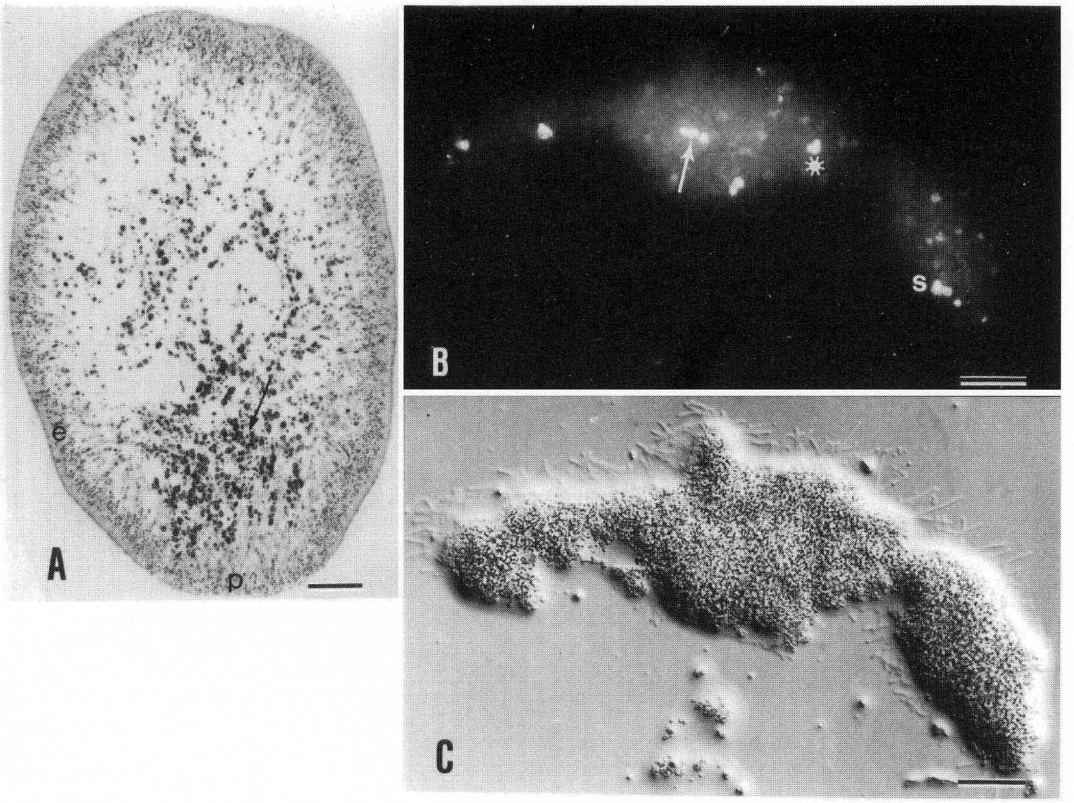

FIG. 1. *Tedania ignis,* histology of larva. A, Longitudinal section of an entire larva stained by toluidin blue showing epithelial-like cell layer (e) and dark-staining cells (archaeocytes, arrow) in clusters near the posterior pole (p) (scale bar=100μm). B, Serotonin-positive cells (s) are randomly distributed in the larval body; at least 6 clusters of 2-4 labelled cells are evident (one marked, asterisk). The nucleus in some of the cells is visible as a non-fluorescent region (arrow). As control for non specific binding of the secondary antibody specimen were incubated in BSA-T without primary antibodies. (Scale bar=100μm.) C, Nomarsky contrast view of the same specimen as in Fig. 1B. The collapsed and shrunk appearance is due to a necessary Triton X-100 treatment that weakens the cell membranes. (Scale bar=100mμ.)

(Placozoa) bottle-shaped cells (2.7–4μm) that stain specifically with the neuropeptide RF-amide. The author speculated about a possible sensory function of the bottle-shaped cells because the RF-amide positive cells were localised at the margin of the disc-like body of *T. adhaerens* and the neuropeptide RF-amide is regarded as functionally conservative in lower invertebrates.

Much effort has been made toward identifying attachment complexes between adjacent cells of the pinacoderm in adult sponges, as this layer controls the sponge's internal milieu which differs from the surrounding environment (Ledger, 1975; for review see Harrison & De Vos, 1991). This altered chemical composition in the tissue fluid of the sponge is regarded as a basic precondition for the evolution of nervous systems. Bandshaped, (epithelial-type) apical cell junctional complexes seem to be present in adult Porifera (e.g. apposed membrane junctions in Bagby, 1970; simple junctions in Ledger, 1975; parallel membrane junctions in Green & Bergquist, 1982; fig. 6 in Garonne & Lethias, 1990) in ultrastructural investigations of *Tedania ignis,* comparable structures seem to be evident (authors, unpublished). However, unequivocal clarification of the organisation of apical junctional complexes is still lacking in the Porifera. It has been stated repeatedly that permanent junctional complexes in epithelially organised cells, if present, are different in

& De Vos, 1991). The ability to contract or condense in response to endogenous events or external stimuli is a common feature in Calcarea and Demospongiae. It results in a decrease in body size and concurrent increase in number of cell contacts (review in Simpson, 1984; Weissenfels, 1989). One can speculate whether the increased number of cell contacts is only the result of a reduced body volume or possibly serves the intensification of 'signal transduction' in the network of actinocytes. Frequent cell–cell contacts between myocyte-like cells were also observed in *H. communis* (Pavans de Ceccatty et al., 1970). According to Pavans de Ceccatty (1974a), the microfilament-containing pinacocytes play an important role in this process, both for cell contraction and cell communication. Owing to the dynamic, 'loose' organisation of cellular features (Pavans de Ceccatty, 1974b; Bond, 1992) there are no nervous cells evident in sponges, but one can expect temporary, fixed pathways through connected mesohyl cells at the points of stable intercellular connections. Lentz (1966) reported acetylcholinesterase, monoamine oxidase, epinephrine, norepinephrin, and 5HT (serotonin) in 'myocyte-like' cells of *Sycon ciliatum*. These observations along with the association of cholinesterase and myofilaments in myocyte-like cells and the report of actin filaments in pinacocytes (Pavans de Ceccatty, 1989) may signify myoid and neuroid elements from a common integrative system that is coordinating 'tissue' contractions in sponges although electrophysiological evidence of a conducting mechanism is still lacking (Lawn, 1982).

In conclusion, we believe our findings of serotonergic cells in the Parazoa suggest an evolutionary specialisation of serotonin, separate from its function in Protists. We interpret our observations as supporting the recently emphasised sister-group relationship with the Eumetazoa (Morris, 1993; Müller, 1995; Ax, 1995) by exhibiting the very first steps in the evolutionary development of the integrative system of the Metazoa. Further research involving additional species and immuno-cytochemical, electrophysiological, and other approaches is clearly needed.

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BIOCALCIFICATION IN THE INDO-PACIFIC CORALLINE DEMOSPONGE *ASTROSCLERA WILLEYANA* LISTER - THE ROLE OF BASOPINACODERM. *Memoirs of the Queensland Museum 44: 666. 1999*:- The aragonitic calcareous basal skeleton of *Astrosclera* is composed of 20-60µm-sized aragonitic spherulites, produced by a combination of three processes. First, the spherulites are formed in large vesicle cells (LVC´s) inside large vesicles in the ectosome. In a second process, after release from LVC's, basopinacocytes transport the spherulites to the tips of the skeletal pillars, where they fuse together by epitaxial growth; and in a third process, during upward growth, the soft tissue is slowly rejected from the lowermost-parts of the skeletal cavities and the remaining spaces are subsequently filled by epitaxially-growing aragonite fibers. In the second and third process, basopinacocytes produce either the insoluble intracrystalline organic matrix, which does not consist of collagen, as well as the soluble intracrystalline matrix, which consists of highly acidic Ca^{2+}-binding mucus substances. Basopinacocytes control speed and direction of epitaxial growth in both of the latter two biocalcification processes. It is hypothesized that *Astrosclera* is able to control the rate of calcification by the regulation of its bacterial population. The mean growth rate of *Astrosclera* was measured at 230µm per year. A detailed description of soft tissue ultrastructure and its cellular composition has recently been published by Wörheide (1998). ❐ *Porifera, Astrosclera, skeletal development, calcification regulation, ultrastructure.*

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