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CICLO XXV

Evolution of the secondary sensory cells in tunicates and their development in *Ciona intestinalis*

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ABSTRACT

This thesis attempts to clarify the evolution of secondary sensory cells in Tunicata (Chordata), the Vertebrata sister group, to study these cells in species belonging to different classes using a morphological approach, and to gain insights into their development using a molecular approach based on important developmental genes.

In **chapter 1,** we analysed the mouth of three species of Thaliacea, representative of the three orders, to verify the presence of secondary mechanoreceptors comparable with the ones in the coronal organ described in the Ascidiacea class. We showed that oral secondary sensory cells are present with the exception of one species. We hypothesised that the presence of these cells in the oral region is related to the animal feeding mechanism. In **chapter 2,** we extended the morphological study to the class Appendicularia. These species are known to possess an oral sensory organ, the circumoral ring, composed of secondary sensory cells. We described these cells in detail, thus completing the data collection of the main tunicate groups. Moreover, based on these results and data from the literature, we performed a cladistics analysis using the cephalochordate amphioxus and vertebrates as outgroups. Our research showed that all tunicate secondary sensory cells may have evolved from a monociliated prototype, which successively differentiated into the present variety of secondary sensory cells.

Chapter 3 describes the development of the oral secondary sensory structures in the model *Ciona intestinalis* (Ascidiacea). In this animal, we found that secondary sensory cells after the metamorphosis could be identified as cells with short cilia and microvilli, which later become pluriciliated. Moreover, we have provided evidence that secondary sensory cells can proliferate during development. Finally, **chapter 4** shows that some genes involved in neural and sensory cell differentiation in vertebrates (*Notch, Delta, Hairy/Hes, Atoh, Musashi*) and other molecules implicated in neurotransmission (TRP channels and glutamate) are expressed in the secondary sensory cells of *C. intestinalis*.

Taken together, the results presented in this thesis confirm that secondary sensory cells are a common feature of tunicates and have an important role as mechanoreceptors, likely playing a role in the feeding process. Moreover, our data suggest that important genes involved in the differentiation and function of these cells might have been present in the common ancestor of tunicates and vertebrates. Thus, these cells represent a plesiomorphic feature in these two groups.

RIASSUNTO IN ITALIANO

Il lavoro presentato in questa tesi è volto a chiarire l'evoluzione delle cellule sensoriali secondarie nel subphylum Tunicata (Chordata), ritenuto attualmente il sister group dei Vertebrata. Sono stati utilizzati due approcci, uno morfologico che prende in considerazione numerose specie appartenenti a classi diverse, e un approccio molecolare che intende chiarire quali geni siano coinvolti nel differenziamento di questo tipo cellulare.

Nel **capitolo 1** viene descritta l'analisi delle strutture sensoriali orali di tre specie rappresentative dei tre ordini costituenti i Thaliacea. In solo due delle specie analizzate è stato riconosciuto un organo, paragonabile all' organo coronale descritto per la classe degli Ascidiacea, costituito da cellule sensoriali secondarie; l'assenza dello stesso nella terza specie è stata da noi interpretata come un adattamento alla diversa modalità di nutrizione che caratterizza questi animali. Nel **capitolo 2** viene estesa l'analisi morfologica a due specie appartenenti alla classe Larvacea, nelle quali è stata osservata una struttura sensoriale orale, chiamata anello circumorale, simile all'organo coronale di Thaliacea e Ascidiacea. I nuovi dati, a completamento di quelli già presenti in letteratura, sono stati utilizzati per effettuare un'analisi cladistica delle cellule sensoriali secondarie dei Tunicata, utilizzando specie appartenenti ai Cephalochordata e Vertebrata come outgroups. I risultati mostrano che il prototipo cellulare ancestrale era rappresentato da una cellula dotata di un apparato apicale con un solo ciglio, circondato da corti microvilli, dal quale poi si sarebbero differenziati gli altri tipi cellulari osservati.

Il **capitolo 3** presenta la descrizione dello sviluppo dell'organo coronale nell'ascidia solitaria *Ciona intestinalis*, dove le cellule sensoriali dell'organo coronale sono state osservate fin dalla metamorfosi, con un apparato apicale dotato di corte ciglia e microvilli, queste successivamente acquisiscono l'aspetto pluricigliato tipico dell'adulto. È' stata inoltre analizzata la proliferazione delle cellule dell'organo coronale durante lo sviluppo. Infine, nel **capitolo 4** sono stati considerati alcuni importanti geni coinvolti nel differenziamento cellulare in senso neurale e sensoriale (*Notch, Delta, Hairy/Hes, Atoh, Musashi*) e altre molecole

coinvolte nella neurotrasmissione (canali TRP e il neurotrasmettitore glutamate), dimostrandone la loro espressione nell'organo coronale di *Ciona intestinalis*.

Considerati nel loro insieme questi risultati confermano che le cellule sensoriali secondarie osservate nei tunicati rappresentano un carattere comune di questo subphylum, probabilmente con un ruolo principale nel meccanismo di alimentazione. Inoltre il fatto che condividano con le cellule sensoriali secondari dei vertebrati l'espressione di alcuni importanti geni coinvolti nello sviluppo e nel funzionamento, ci porta a pensare che questa tipologia di cellule sensoriali rappresentino un carattere plesiomorfico di questi due gruppi.

INTRODUCTION

THE SENSORY SYSTEMS

The term “sensory systems” indicates structures that are functionally linked to the nervous system and receive and transmit sensory information from external or internal environments to the brain. Sensory perception is mediated by highly differentiated and specialised receptors, which are categorised according to the type of stimulus they receive: photo-, chemo-, mechano-, thermo-, and noci-receptors. These cells convert information into an electrical signal that is transmitted directly, or by means of neurons, to specific regions of the brain. In the brain, the information is processed and a response is evoked from the organism through the nerve efferent way. Signal transmission involves ion channels that may alter the membrane ionic permeability, thus generating an action potential, or may induce the release of molecules (i.e., neurotransmitters) at the synaptic level.

EVOLUTION OF MECHANORECEPTORS

The term “mechanosensation” specifically refers to the response to mechanical stimuli, and it is responsible for the perception of touch, pain, hearing and balance. These perceptions are all mediated by mechanoreceptors, which are morphologically very heterogeneous. Mechanosensory cells can be divided into two principal groups on the basis of their neuronal connections. Primary neurons possess a basal axonal prolongation that allows for the direct transmission of signals to the brain. On the other hand, secondary receptors lack these axonal prolongations. Thus, they transmit signals via synaptic connections between their own basal plasmalemma and neurons. Mechanoreceptors possess an apical apparatus for signal transduction that has a shared generalised arrangement formed by a single cilium surrounded by microvilli. This organisation has been retained or changed during evolution. In vertebrates, for example, the mechanoreceptors responsible for hearing and balance typically have stereocilia and lack the cilium. The basic organisation of ciliated mechanoreceptors is comparable to that of choanoflagellates, and it has been proposed that this

unicellular organism might resemble the ancestral prototype for all of the mechanoreceptors that evolved later, both as unicellular sensory units and multicellular organs (Fritzsch et al., 2007; Burighel et al., 2011). Mechanoreceptor cells in the three main lineages, lophotrochozoans, ecdysozoans and chordates, are characterised by an important variation with respect to the choanoflagellate prototype. In lophotrochozoans, the best studied groups are molluscs, in particular cephalopods, which have both primary and secondary mechanoreceptors, are able to respond to mechanical stress, and have a few short microvilli and many cilia, which in some cases are variable in length. In the ecdysozoan lineage, only primary receptors have been observed, including a single kinocilium (a specialised immobile cilium), while microvilli are absent.

MECHANOSENSATION IN THE VERTEBRATE ACUSTICO-LATERALIS SYSTEM

In vertebrates, one of the best studied mechanoreceptor classes is the inner ear hair cells, which are responsible for hearing and balance. Hair cells are also present in fish and the amphibian lateral line in which they detect hydrodynamic movements and vibrations during swimming and predatory behaviours. In general, the structure responsible for mechanotransduction in hair cells is characterised by a sensory hair bundle bearing a kinocilium with a 9+2 microtubule arrangement. However, in some vertebrates, such as mammals and birds, the kinocilium is lost during differentiation, while the basal body is retained. The kinocilium is surrounded by stereovilli, which are rigid, actin-filled, structures of graded lengths that are connected to each other with thin tip-links and are fundamental for signal transduction (Manley et al., 2004). The hair cells in the lateral line differ from inner ear hair cells mostly in the length of the cilia and stereovilli. Furthermore, the lateral line hair cells are embedded in a cupula filled with a gelatinous matrix and are arranged in several groups called neuromasts, which are extended from head to tail. Together, the inner ear and lateral line constitute the acustico-lateralis system, which is also found in agnatans, such as lampreys and myxines. These organisms have hair cells with characteristics that

are comparable to those of other vertebrates, suggesting a common evolutionary origin of the hair cells in agnatans and gnatostomes.

The lateral line and inner ear are derived from the embryonic lateral line and otic placodes. Placodes are thickenings of the cranial ectoderm that are able to give rise to sensory organs through invagination movements and the expression of a specific set of genes. Recently, a paratympanic placode has been shown to be able to generate hair cells in the vertebrate middle ear (O'Neill et al., 2012).

According to Schlosser's (2010) hypothesis, the process of placode determination requires several steps. Initially, many diffusible factors (e.g., Wnts, Fgfs, Bmps, Shh) are involved in the patterning of the "panplacodal region", a horseshoe-shaped territory along the anterior and lateral edge of the neural plate. Subsequently, other factors (e.g., Eya, Six, Pax) stabilise the two main pattern forming areas, one anterior and one lateral. Finally, each single placode is differentiated (i.e., adenohypophyseal, olfactory, lens, trigeminal, profundal, lateral line, otic, paratympanic and epibranchial), primarily prompted by the expression of bHLH transcription factors, which are characterised by the typical basic helix-loop-helix domain.

In the lateral line and otic placodes, three important proneural bHLH transcription factors are involved in differentiation: Atoh1, Neurod1 and Neurog1 (Fritzsch et al., 2010). Different experiments using mutant mice have determined, at least in part, the role of each factor and the complex network (Jahan et al., 2010; Jahan et al., 2012a; Pan et al., 2012a). In the mammalian ear, *Neurog1* is upregulated in neurons, while *Atoh1* is subsequently upregulated in sensory hair cells. *Neurod1* is expressed in both cell types. Later in development, *Atoh* expression becomes restricted to the hair cells. Functional studies have shown that the absence of this gene results in a lack of hair cells, whereas its overexpression leads to the production of extra hair cells. Notch and its ligand, Delta, are implicated in hair cell differentiation. *Notch* expression is restricted to supporting cells, while *Delta* is found in nascent hair cells. Their pathway has been particularly well studied during the lateral inhibition process, which, on one side, promotes hair cell differentiation and on the other, inhibits neighbouring cells from taking the same differentiation pathway. *Hes* is a gene that acts downstream of *Notch*. *Hes* belongs to the bHLH family, acts as an antagonist of *Atoh,* and is expressed in cells that do not become hair cells. Another gene expressed

downstream of the Notch pathway is *Musashi*, which regulates cell fate and maintains the undifferentiated status of the cell (Muller and Littlewood-Evans, 2001).

Neural placodes were traditionally considered to be unique to vertebrates, based on the “new head hypothesis”, and their appearance was thought to be related to the transition from a filter-feeding system to an active predatory lifestyle that led to the formation of a compartmentalised brain and associated complex sensory structures (Northcutt, 2005).

Vertebrates, together with tunicates and cephalochordates, constitute the chordate phylum. They share some common features of embryonic development, in particular the presence of a notochord, the ability to induce neural tube differentiation, and a similarly patterned nervous system.

In fact, embryonic structures resembling placodes have been observed in tunicates. These embryonic structures show the expression of the orthologs of the *Six, Eya,* and *Pax* genes, the ability to invaginate and the ability to give rise to body structures bearing sensory cells and neurons. Thus, it is possible that sensory placodes did not arise *de novo* in vertebrates but evolved from an ectodermal area in the common chordate ancestor (Mazet et al., 2005; Mazet and Shimeld, 2005; Baker et al., 2008; Graham and Shimeld, 2012).

TUNICATES

Tunicates are divided into three classes: appendicularians and thaliaceans, which are the planktonic groups, and ascidians, which are the benthic group. Whereas appendicularians are exclusively solitary and thaliaceans are exclusively colonial, the ascidian class includes both solitary and colonial organisms. The ascidian life cycle is characterised by the development of a free swimming tadpole-larva, with the typical chordate organisation (a rod-like notochord and a neural tube extending anteriorly in a sensory vesicle and regionalised into three parts). The mature larva tastes the substrate by means of three rostral papillae, and once an appropriate substrate has been selected, it settles and undergoes a deep metamorphosis, consisting of tail absorption and the rearrangement of visceral organs and the nervous system. The larval brain is partially retained and partially formed *ex novo* (Horie et al., 2011). In the adult,

the brain is located between the two siphons and is associated with a neural gland, forming the neural complex.

After metamorphosis, the organism opens its siphons for the circulation of seawater and becomes a filter. The oral siphon is a wide, fissured branchial basket with many beating ciliated cells that provoke an incoming current of water into the oral siphon. Once water is passed through the branchial stigmata, it passes into the atrial chamber and then exits through the atrial siphon. Food particles contained in seawater are captured and directed to the gut after being trapped by a mucous net secreted by the endostyle. The atrial siphon is also used for gamete release.

SENSORY SYSTEMS IN TUNICATES AND EVOLUTIONARY CONSIDERATIONS

Adult ascidians possess two different types of mechanosensory systems based on primary or secondary ciliated sensory cells. The cupular organ, the cupular strand and the capsular organ are all based on primary mechanoreceptors. They are placed in the atrial chamber and atrial siphon, where they have a hydrodynamic function in monitoring water movements and are responsible for vibration perception. All of them exhibit ciliated sensory cells embedded in or covered by a tunic layer and are arranged as an array (such as in the cupular strand), in groups (such as in the cupular organ), or in a patch (such as in the capsular organ) (Mackie and Burighel, 2005). Mechanosensory organs based on primary receptors have also been observed in pelagic tunicates, both in appendicularians and thaliaceans, especially close to the mouth region (e.g., cupular-like organ, triads of sensory cells, and ventral sense organ) (Bone and Ryan, 1978).

In ascidians, the only organ based on secondary sensory cells is the coronal organ, which is arranged in a continuous row on the tentacle surface of the oral siphon facing the incurrent water flow (Burighel et al., 2003). As primary sensory cells, the coronal organ sensory cells are ciliated and are accompanied by supporting cells, but they do not possess any axonal prolongation or an apical bundle covered by a tunic layer. The coronal organ plays a role in detecting particles entering the oral siphon, possibly evoking the squirting response typical

of these organisms whenever unsuitable substances are introduced (Mackie et al., 2006). In the last ten years, several species of ascidians were analysed, and all of them were shown to have a coronal organ, although variations in the apical specialisations of the sensory cells (presence of one or more cilia, sometimes surrounded by microvilli or stereovilli) or in the accompanying cells (supporting and secreting cells) have been observed (Manni et al., 2006; Caicci et al., 2010a). To understand the evolution of secondary sensory cells, it is important to know if the presence of the coronal organ is common to all tunicate groups and, if so, if the features of the coronal sensory cells in thaliaceans and appendicularians are shared with ascidians.

Prior to the discovery of the coronal organ, ascidian cupular organs were hypothesised to be equivalent to vertebrate neuromasts based on their aspect and function. Moreover, developmental studies suggested that cupular organs have a placodal origin, sharing both the embryonic position along the neural plate and gene expression patterns with vertebrate atrial placodes.

What renders this hypothesis controversial is that the sensory cells constituting the cupular organ are primary in ascidians, whereas they are secondary in vertebrate neuromasts. This observation suggests that the secondary sensory cells of the coronal organ might be homologous to vertebrate hair cells. On the one hand, several morphological data support the latter hypothesis; on the other hand, some aspects of coronal organ development are controversial. The coronal organ develops from a placode, the stomodeal placode (as well as the cupular organs), expressing the gene pattern typically found in all placodes; however, the stomodeal placode is located in an anterior position with respect to the neural plate and expresses genes (i.e., *Pitx*) (Mazet et al., 2005; Tiozzo et al., 2005) that render it more similar to the vertebrate olfactory/adenohypophyseal placode (which does not give rise to secondary sensory cells) instead of to the acustico-lateralis placodes.

AIMS OF THE PROJECT

To gain insights into the homology between tunicate and vertebrate secondary sensory cells, the following aims are proposed:

1. to analyse tunicate secondary sensory cells, extending the study to thaliaceans and appendicularians. Moreover, to performe a cladistic analysis on the evolution of secondary sensory cells in chordates using original data and data from the literature;

2. to morphologically study the development of the coronal organ in the ascidian *Ciona intestinalis* across different developmental stages;

3. to identify and determine if some of the molecular markers that characterise vertebrate hair cells are expressed in the coronal organ and to analyse them during the organ development in *C. intestinalis*.

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Chapter 1

The oral sensory structures of Thaliacea (Tunicata) and consideration of the evolution of hair cells in Chordata.

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ABSTRACT

We analyzed the mouth of three species, representative of the three orders of the class Thaliacea (Tunicata) - *Pyrosoma atlanticum* (Pyrosomatida), *Doliolum nationalis* (Doliolida) and *Thalia democratica* (Salpida) – to verify the presence of mechanoreceptors, particularly hair cells. In vertebrates, hair cells are well known mechanoreceptors of the inner ear and lateral line, typically exhibiting an apical hair bundle composed of a cilium and stereovilli but lacking an axon. For a long time, hair cells were thought to be exclusive to vertebrates. However, evidence of a mechanosensory organ (the coronal organ) employing hair cells in the mouth of tunicates, considered the sister group of vertebrates, suggests that tunicate and vertebrate hair cells may share a common origin. This study on thaliaceans, a tunicate group not yet investigated, shows that both *P. atlanticum* and *D. nationalis* possess a coronal organ, in addition to sensory structures containing peripheral neurons (*i.e.*, cupular organs and triads of sensory cells). In contrast, in *T. democratica*, we did not recognize any oral multicellular sensory organ. We hypothesize that in *T. democratica,* hair cells were secondarily lost, concomitantly with the loss of branchial fissures, the acquisition of a feeding mechanism based on muscle activity and a mechanosensory apparatus based on excitable epithelia. Our data are consistent with the hypothesis that hair cells were present in the common ancestor of tunicates and vertebrates, from which hair cells progressively evolved.

INTRODUCTION

Hair cells are highly specialized mechanoreceptors of the inner ear and the lateral line organ of vertebrates, which detect movements of fluids and allow animals to perceive sounds, vibrations, touches, gravity and acceleration. Hair cells differentiate during embryogenesis from placodes, thickenings of the cranial ectoderm, which represents the source of hair cells, supporting cells and associated neurons (Northcutt, 2005; Schlosser, 2010; O'Neill et al., 2012). In their mature form, hair cells typically possess an apical hair bundle for the detection of stimuli, which displays variability both among and within species. At their base, hair cells are contacted by neurites from the central nervous system, establishing both afferent and efferent synapses with the plasmalemma. Afferent synapses are involved in the transmission of mechanical information to the brain, while efferent synapses are involved in the control of hair cell activity by the central nervous system. The absence of an axon and the presence of synapses at the base of hair cells classifies them as secondary sensory cells. These features render hair cells unique mechanoreceptors. For a long time, hair cells were considered exclusive to vertebrates, without any homologous counterpart outside of the vertebrate lineage. However, in recent years, alternative evolutionary scenarios have been proposed on the basis of both comparative molecular studies of metazoans and morphological analysis of tunicates (Fritzsch et al., 2002; Bouchard et al., 2010; Pan et al., 2012b).

The coronal organ is a mechanoreceptor organ based on hair cells that has been described in tunicate ascidians (Burighel et al.,2003; Burighel et al., 2011 for review). Tunicates belong to the phylum of chordates together with cephalochordates and vertebrates (Delsuc et al., 2008). Traditionally, the subphylum Tunicata is divided into three classes, the sessile ascidians and the planktonic thaliaceans and appendicularians (Berrill, 1950). In ascidians, the coronal organ is located in the mouth, *i.e.,* the oral siphon, where it forms a continuous sensory row bordering the crown of oral tentacles and the velum. It controls the flow of water entering the mouth and evaluates the size and number of particles transported in the incoming seawater. The coronal organ prevents entry of large particles that could damage the delicate branchial basket by evoking a squirting response, the typical protective response used to expel

seawater and particles from the basket (Mackie et al., 2006). Coronal hair cells are secondary sensory cells, contacted at their base by afferent and efferent synapses, and contain an apical hair bundle that shows variability among species (Manni et al., 2006; Caicci et al., 2010a). During embryogenesis, the ascidian coronal organ develops from the oral siphon rudiment, the stomodeum, which exhibits properties of neural placodes, deriving from an ectodermal thickening and expressing a set of pan-placodal genes present in vertebrate placodes (Bolles, 1891a; Manni et al., 2004b; Mazet and Shimeld, 2005). However, the stomodeum also expresses a molecular marker (the *Ptx* gene) typical of anterior vertebrate placodes, whereas hair cells are characteristically derived from vertebrate posterior placodes. Secondary sensory cells with a function similar to that of coronal organ are also found in the mouth of appendicularians and also derive from an embryonic placodal area (Bone, 1998; Bassham and Postlethwait, 2005). For these reasons, it has been proposed that hair cells could be a plesiomorphic feature of tunicates and that vertebrate and tunicate hair cells share a common origin (Manni et al., 2006).

In addition to scattered sensory neurons, ascidians possess other multicellular mechanoreceptors concentrated in the oral siphon or the atrial chamber, including the atrial cupular organs of *Ciona intestinalis* and *Corella eumniota* (Deibel et al., 1988; Mackie and Singla, 2004), the atrial capsular organs of *Cheliosoma productum* (Mackie and Singla, 2003), the atrial cupular strand of *Corella inflata* (Mackie and Singla, 2004), and the oral and atrial tentacular sensory cells of *Polyandrocarpa misakiensis* (Koyama, 2008). All of these organs derive from embryonic regions with placodal features (namely, the stomodeum and the atrial rudiments) (Bolles, 1891a). From a cytological point of view, these organs are based on primary sensory cells and have their own axon. In metazoans, gene expression data on primary and secondary sensory cells suggest that an ancestral sensory cell with its own axon evolved into two distinct cell types: a hair cell that specialized in mechanotransduction and a sensory neuron that connects the hair cell to the central nervous system (Pierce et al., 2008; Bouchard et al., 2010; Pan et al., 2012b). The presence of the coronal organ in tunicates could suggest that a primary sensory cell in the chordate ancestor represented the evolutionary precursor of both secondary and primary sensory cells in tunicates and vertebrates (Burighel et al., 2011).

To verify if secondary sensory cells are a plesiomorphic or apomorphic feature of tunicates, we analyzed the mouth of three species of thaliaceans, pelagic tunicates in which hair cells have not yet been characterized (Fig. 1 A-D). In agreement with classical taxonomic views, all recent phylogenetic reports based on the analysis of nuclear and mitochondrial genes have consistently identified the monophyletic nature of thaliaceans, although their position with respect to the other tunicates is still under discussion (Wada et al., 1998; Swalla et al., 2000; Stach and Turbeville, 2002a; Yokobori et al., 2005; Tsagkogeorga et al., 2009b). Within the thaliaceans, a commonly accepted view places pyrosomes in a basal position, with doliolids and salps being sister groups, following a trend towards branchial sac simplification and muscle-band development (see Govindarajan et al., 2011).

Thaliaceans are transparent suspension feeders with a complex life cycle comprising an obligatory alternation of sexual (blastozooid or aggregate) and asexual (oozooid or solitary) life-history stages or generations. Thaliaceans possess different mechanisms of feeding: Pyrosomatida and Doliolida closely resemble ascidians because a stream of water enters the oral opening and is pumped through the stigmatal openings in the wall of the branchial sac by the activity of ciliated stigmatal cells. Nevertheless, in Doliolida the circumferential muscles can contract when need to effect an escape response; in *D. nationalis*, this reaction is extremely rapid because the gill cilia cease beating less than 100 ms after a large or noxious particle touches the mouth (Deibel et al., 1988). Salpida exhibit an extreme variation of the ascidian type of feeding and achieve the highest rates, per individual, of water clearance and transport. The branchial basket in salps is reduced to a single gill bar, and they use their circumferential muscle bands, instead of cilia, to pump large volumes of water through the pharynx and atrium. Thaliaceans, in contrast to ascidians, also use the flow of water through the branchial cavity for locomotion by continuous or intermittent jet propulsion. In pyrosomes, the cilia produce the water flow, whereas in salps and doliolids, locomotion is the result of the contraction of striated muscle fibers (Bone, 1998).

The species considered in this study are *Pyrosoma atlanticum* (Pyrosomatida), *Doliolum nationalis* (Doliolida) and *Thalia democratica* (Salpida) (Fig. 1). These species represent the three orders of thaliaceans and are the

objects of numerous ecological studies due to their important role in oceans. Unfortunately, the histology and the fine anatomy of the nervous systems and sensory organs of these species is based on very few, dated papers. However, more recent studies on their physiology have provided additional information.

In *Pyrosoma atlanticum*, the mouth senses mechanical stimulation when the oral opening is delicately touched or stimulated by the collision of large particles or their entry into the gill. The animal responds by arresting ciliary beating and contracting the oral siphon, which corresponds to the squirting of ascidians (Deibel et al., 1988). Such stimuli could act upon tentacular receptors located around the oral opening (Neumann, 1935). However, mechanosensory cells are also distributed on the siphon rims and on the oral flaps (or lobes) bordering the mouth (Brien, 1948).

Doliolum nationalis is barrel shaped and surrounded by eight muscle bands, the first and last of which control the oral and atrial apertures by regulating flaps or valves. The interior of the body is largely hollow, with a transverse gill septum perforated by several slits; the gill cilia pump water without assistance from the body muscles. This is fundamentally different from the feeding mechanism of salps, which swim continuously to feed using rhythmic contractions of their body muscles. In doliolids, the flaps of the anterior and posterior siphons bear ciliated sensory cells that react to touch by evoking the escape reaction. In the oral siphons, triads of mechanosensory cells without accompanying supporting cells lie at the base of each scalloped extension of the flaps (Bone, 1959).

The oral siphon of *Thalia democratica* exhibits two flexible lips (or valves); a dorsal (posterior or rear) lip and a ventral (anterior or front) lip extending as a mobile velum. Both lips are provided with a series of small muscle bands regulating their closure during movements. An elongated structure called the languet is located on the dorsal side of the pharynx anterior to the peripharyngeal band and the neural gland aperture and has been compared to a single tentacle with possible tactile functions (Delage and Hérouard, 1898; Neumann, 1935). Salps possess numerous sensory receptors on the lip border, on the outer body surface and inside the pharynx (Fedele, 1933; Bone, 1959). Observations on their feeding behavior suggest that food is not selected, and the only apparent constraint limiting food particle size is the diameter of the oesophageal opening

(Fedele, 1933; Madin, 1974). As a striking feature, Salps possess excitable epithelia that propagate action potentials when mechanically stimulated and control animal behavior. These epithelia are linked to the central nervous system to form the most complicated suite of excitable epithelia-nervous system interactions known in any animal (Bone, 1998). Skin impulses of the inner oral epithelium inhibit feeding and are presumably evoked by large particles inhaled into the front of the pharynx, similar to the squirting of a sessile ascidian (Mackie and Bone, 1977). Fedele (1933) suggested that impulses reach the nervous system via axons of the sensory cells in the inner epithelium of the ventral lip, although Mackie and Bone (1977) were unable to find these cells.

Our results show that, differently from Salpida, Pyrosomatida and Doliolida evolved different mechanoreceptors in the oral siphon. We discuss the variety of adaptive solutions for mechanoreception in tunicates and hypothesize a secondary loss of hair cells in salps, where a novel feeding mechanism evolved, coupled to a highly specialized mechanosensitive system for the propagation of stimuli through excitable epithelia. Our data give further support to the idea that hair cells are a plesiomorphic feature of tunicates and that they share a common origin with vertebrate hair cells.

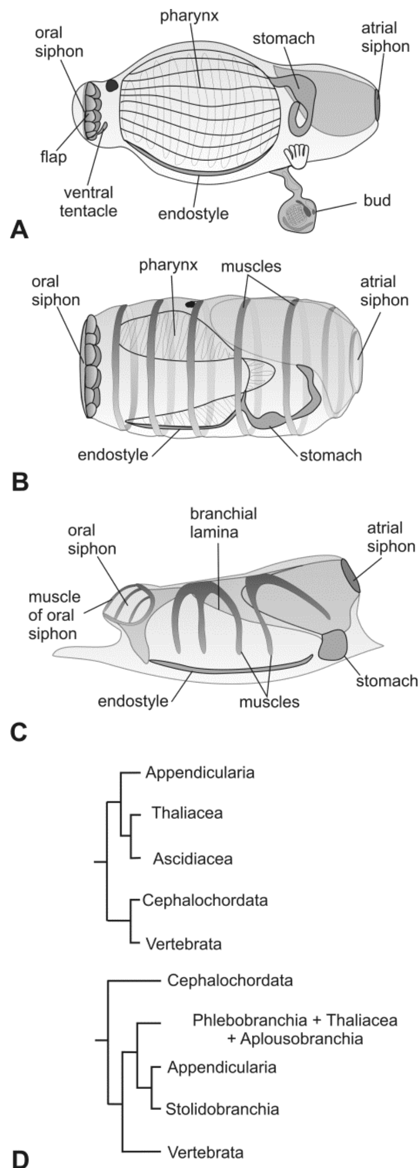

Fig. 1. Schematic drawing of **A**. *Pyrosoma atlanticum*; **B**. *Doliolum nationalis*; **C**. *Thalia democratica*. **D**. Phylogeny of Chordata (modified from Govindarajan et al., 2011).

MATERIALS AND METHODS

Specimens of *Pyrosoma atlanticum* (order Pyrosomatida) are blastozooids that were collected at the Station Zoologique in Villefranche-sur-Mer (France) and in Maeda-point, Okinawajime-Island (Japan); specimens of *Doliolum nationalis* (order Doliolida) are blastozooids collected at the Station Zoologique in Villefranche-sur-Mer (France) and in the Adriatic Sea in front of Trieste (Northern Italy); specimens of *Thalia democratica* (order Salpida) are oozooids and

blastozooids that were collected in front of the Marechiaro site in the Gulf of Naples (Southern Italy).

Transmission electron microscopy

Specimens were anesthetized with 0.02 % MS222 at 4°C. After complete relaxation of the siphon, specimens were fixed in 1.7% glutaraldehyde buffered with 0.2 M sodium cacodylate plus 1.7% NaCl at pH 7.4,. After washing in buffer with 1.7% NaCl, specimens were dissected with surgical forceps to isolate the area of the oral siphon. After post-fixation in 1% OsO4 in 0.2 M cacodylate buffer, specimens were dehydrated and embedded in Epon Araldite 812. Thick sections (1 µm) were counterstained with toluidine blue; thin sections (80 nm) were stained with uranyl acetate and lead citrate. Micrographs were taken with a Hitachi H-600 (operating at 75kV) and an FEI Tecnai G^2 electron microscope (operating at 100 kV). All photos were collected and labeled using Corel Draw X3; no modifications were made to the images.

Scanning electron microscopy

Specimens were fixed in glutaraldehyde solution and dissected as described for transmission electron microscopy. After post-fixation and dehydration, specimens were critical-point dried, sputter-coated with gold, and observed under a Cambridge Stereoscan 260 electron microscope. Micrographs were collected and typeset labeled Corel Draw X3; no modifications were made to the images.

RESULTS

General organization of the thaliacean oral siphon

The histological organization of the oral siphon was analyzed in *Pyrosoma atlanticum* (Figs. 2-3), *Doliolum nationalis* (Figs. 4-5), and *Thalia democratica* (Fig. 6). These species do not have tentacles arranged in a ring at the base of the siphon, as typically occur in ascidians: *P. atlanticum* possesses a dozen rounded

flaps accompanied by a unique long, ventral tentacle (Fig. 2 A-B); *D. nationalis* is characterized by a dozen flaps, forming scalloped extensions of the lips, without tentacles (Figs. 4 A-B, 5 A-B); *T. democratica* has never tentacles nor flaps, but the ventral lip is furnished with a mobile velum (Fig. 6 A). The epithelium lining the internal wall of the siphon is continuous with an external epidermis, and it is covered by a layer of tunic, which terminates as a thin sheet at the base of the flaps in *D. nationalis* (Fig. 4 C-E) and in *P. atlanticum* (Fig. 2 B), where it covers part of the tentacle. In *T. democratica*, the tunic covers the internal wall of the lips (Fig. 6 G). The flaps and the tentacle can be relaxed, hanging down toward the branchial cavity, or held up to project across and reduce the opening of the siphon. In *T. democratica*, valves can be closed or held open by muscles.

Our morphological analysis of the oral siphon of the three species allowed us to recognize different kinds of sensory organs that we identified as follows: cupular organs (Fig. 2) and the coronal organ (Fig. 3) in *P. atlanticum*, and triads of sensory cells (Fig. 4) and the coronal organ (Fig. 5) in *D. nationalis*. On the contrary, we did not recognize any multicellular sensory organs in the oral siphon of *T. democratica* (Fig. 6).

Pyrosoma atlanticum

Cupular organ. In *P. atlanticum*, we found sensory organs composed of sensory cells with an apical bundle covered by an amorphous cupula; we called them "cupular organs" (Fig. 2). These organs are scattered on the flaps, on the side opposite to the siphon aperture. In transverse sections, they consist of a sensory cell flanked by supporting cells (Fig. 2 C, G). Latero-apical tight junctions seal the cells together. The sensory cell has a pyriform shape with a narrow apex protruding above the surface of the contiguous cells, a large basal nucleus, and rough endoplasmic reticulum cisterns and Golgi fields in the supranuclear region (Fig. 2 C). The apical sensory apparatus exhibits a long cilium, with a typical 9+2 microtubular configuration, surrounded by 50-60 long microvilli. The bundle is embedded in a tunic-like cupula secreted by the adjacent supporting cells. The proximal part of the cilium is located in a deep and narrow extracellular pocket surrounded by a cytoplasm rich in microtubules. At the cilium base, the pocket enlarges to form a sort of extracellular ampullar ring (Fig. 2 C, D). Transverse sections of the cell apex reveal that a cylindrical flange of cytoplasm surrounds

the cilium (Fig. 2 E). Microvilli accompanying this flange are well developed and contain microfilaments connected to the apical cell cytoskeleton.

Both supporting and sensory cells rest on a basal lamina, which is crossed by a number of neurites. In some cases, a neurite appears to emerge directly from a sensory cupular cell (Fig. 2 F). Synapses between these neurites and sensory cells were not observed.

Coronal organ. The coronal organ consists of a continuous row of ciliated sensory cells running along the outer border of the flaps and along the lateral and apical borders of the ventral tentacle (Fig. 3 A-C). In transverse sections, the sensory row appears to consist of two of cells lying side by side (Fig. 3 C).

C-shaped, non-ciliated supporting cells flank the sensory cells on both sides (Fig. 3 C, F). Supporting cells are similar to adjacent epithelial cells in cytoplasmic characteristics, but they are taller and curved so as to partially enclose the sensory cells. Supporting cells possess a dense apical glycocalyx, which is also present on contiguous epithelial cells but not on sensory cells. A few electron-dense glycogen granules are scattered through the cytoplasm of supporting cells. All of the cells of the flaps and tentacle, both sensory and non-sensory, are joined apico-laterally to each other by dense tight junctions (Fig. 3 F).

The coronal sensory cells are cylindrical or flask-shaped and possess a large central nucleus, scattered mitochondria, glycogen granules, and rough endoplasmic reticulum cisternae (Fig. 3 D, F). The Golgi complex lies above the nucleus and is composed of a few stacks of cisternae and associated vesicles. The cells bear an apical structure composed of one long cilium surrounded by short microvilli, all of the same length, and do not show any particular arrangement with respect to the cilium. Each cilium has a conventional 9+2 microtubular arrangement and a dense, short basal body with poorly developed ciliary rootlets. A secondary basal body is sometimes recognizable, perpendicular to the first one. Loose, fibrillar material extends between the cilium and microvilli (Fig. 3 E).

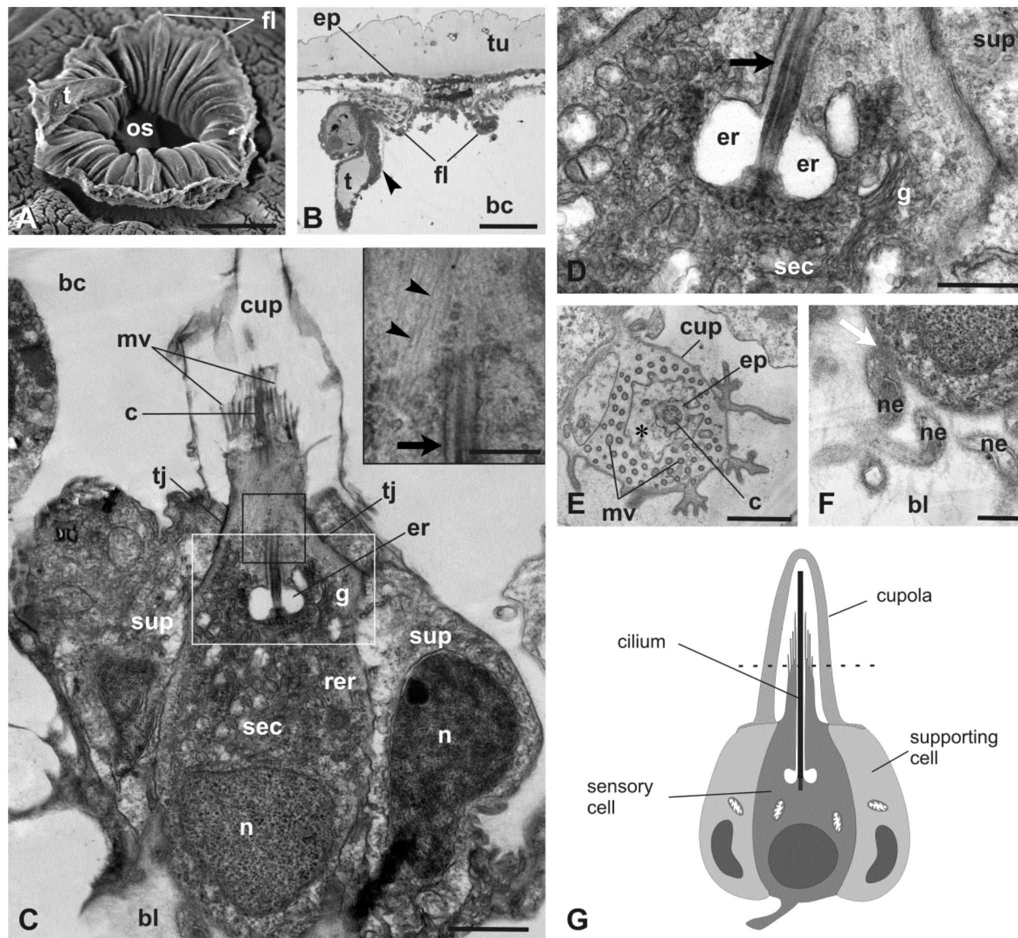

Fig. 2. Cupular organ of *Pyrosoma atlanticum*. **A**. Inner view of the oral siphon (os) by scanning electron microscopy to show the ring of flaps (fl) and the single ventral tentacle (t). **B**. Histological sagittal section of the oral siphon. Note the extension of the tentacle (t) with respect to the flaps (fl). The siphon is contracted, and its aperture is not recognizable. Arrow: inner limit of tunic on tentacle. bc: branchial chamber; ep: epidermis; tu, tunic. Toluidine blue staining. **C-F**. Electron microscopy. C. The cupular organ has sensory cells (sec) possessing a cilium (c) surrounded by short microvilli (mv) embedded by a cupula (cup). The white square indicates the area shown in D; the black square indicates the area enlarged in the inset showing the extracellular pocket (arrow in inset and in D) enlarged basally in an extracellular ring (er) containing the cilium. Note numerous microtubules (arrowheads in inset) surrounding the pocket. E is a transverse section of the cilium at the level of microvilli emergence, showing the cylindrical flange of cytoplasm (black asterisk) surrounding the cilium to delimit the extracellular pocket (ep) filled with electron-dense material. In sensory cells, the nucleus (n) is basal; in the cytoplasm, cisterns of rough endoplasmic reticulum (rer) and Golgi apparatus (g) are recognizable. Supporting cells (sup) accompany sensory cells. Both the cell types lie on a basal lamina (bl), where numerous neurites (ne) are present. The white arrow in F points to a neurite emerging from a sensory cell. bc, branchial chamber; bl, basal lamina; tj: tight junctions. **G**. Schematic drawing of the cupular organ. The dotted line marks the level of the section shown in E. Scale bar: 100 μm in A; 50 μm in B; 2.5 μm in C, 600 nm in inset; 1 μm in D; 800 nm in E; 1 μm and F.

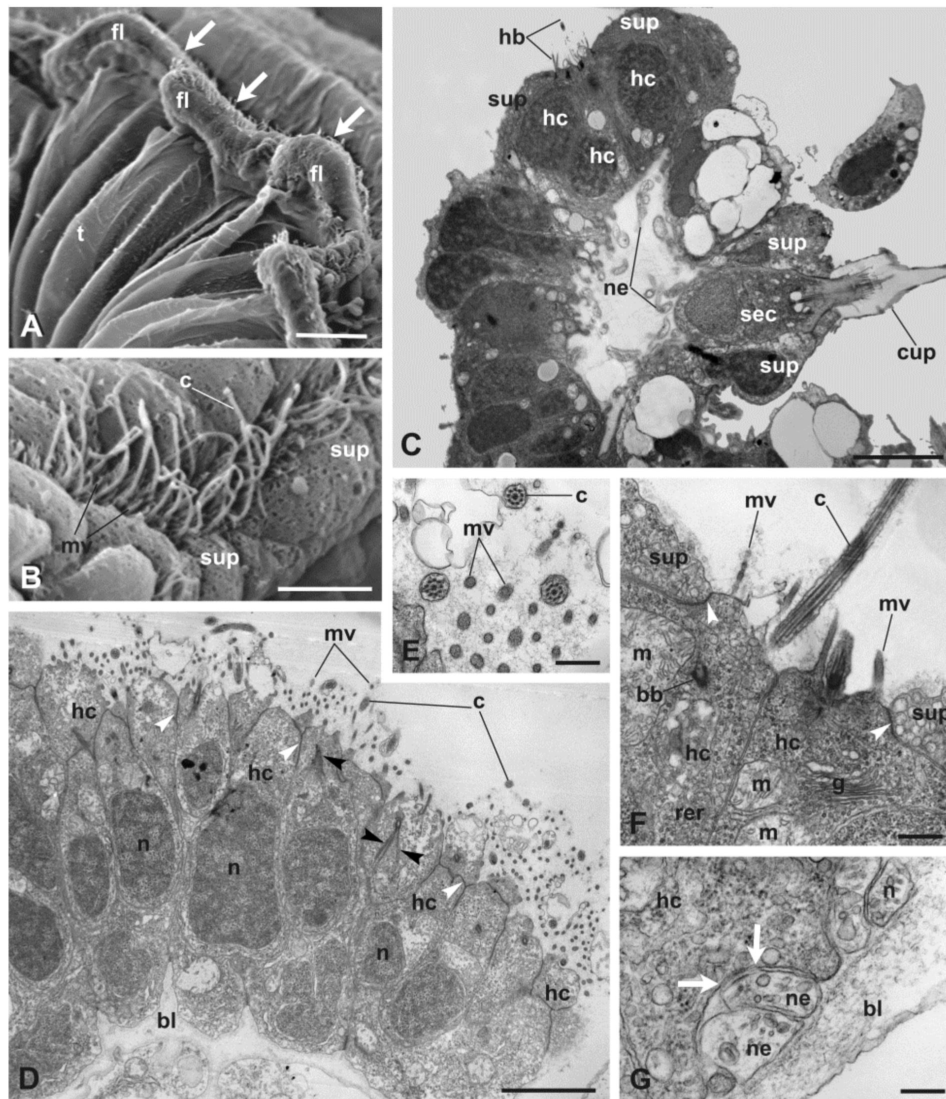

Fig. 3 Coronal organ of *Pyrosoma atlanticum.* **A-B**. Scanning electron microscopy. A. the coronal organ (arrows) is visible on the flap (fl) borders; the inner epithelium of the oral siphon is covered by the tunic (t). B. Note the hair bundles of the row of sensory cells, furnished with a cilium (c) and short microvilli (mv) that emerge from the groove formed by the flanking supporting cells. **C-G**. Transmission electron microscopy of coronal organ. C. Section of a flap (close to the one shown in figure 2 C) to show the relationship between the coronal organ, recognizable due to a hair bundle (hb) of hair cells (hc), and a cupular organ, marked by a cupular sensory cell (sec) and related supporting cells (sup) surmounted by cupula (cup). Note numerous neurites (ne), associated with cupular sensory cells and hair cells in the blood space. In D, the coronal organ is longitudinally cut so that a continuous row of hair cells (hc) is recognizable. Black arrowheads: ciliary rootlets; white arrowheads: tight junctions between hair cells. Cilia have a typical 9+2 configuration, as visible in E. Note the loose, fibrillar material extending between the cilia and microvilli. F. Detail of apical cytoplasm of hair cells (hc): scattered mitochondria (m), rough endoplasmic reticulum cisternae (rer) and the Golgi complex (g) can be recognized. White arrowheads: tight junction between hair cells and supporting cells (sup). The basal plasmalemma of hair cells forms a groove (G) where numerous neurites (ne) are located. White arrows delimit a synaptic area (enlarged in inset). Inset in G: the black arrowhead indicates a synaptic vesicle contacting the hair cell presynaptic membrane. bb: basal body; bl: basal lamina; c: cilium; mv: microvilli; n: nucleus. Scale bar: 15μm in A; 2.5μm in B, D; 5μm in C; 400nm in E, F; 400nm in G, 200 nm in inset.

The basal plasmalemma of each sensory cell lies on the basal lamina, which forms a continuous fibrous layer supporting both sensory and other epithelial cells (Fig. 3 D, G). Each cell forms a groove, where the typical extracellular matrix of the basal lamina is absent (Fig. 3 G). The grooves contain neurites in close proximity to the sensory cell membrane. Synapses, identifiable by paired, thickened, electron-dense plasma membranes and small vesicles attached to the membrane on one side of the cleft, were occasionally observed (Fig. 3 G). Most were afferent synapses, clearly identifiable by the presence of synaptic vesicles in the sensory cell cytoplasm adjacent to the junction. In contrast, efferent synapses were less clearly defined but were recognizable by the presence of synaptic vesicles on the neurite side of
the junction, making the sensory cell postsynaptic to the neurite in question. Axons were never seen directly emerging from coronal cells, and their neuronal somata were not observed anywhere in the vicinity of the coronal organ.

Doliolum nationalis

Triads of sensory cells. On the side exposed to the water flow, the flaps of *D. nationalis* bear a dozen triads of sensory cells regularly arranged around the mouth aperture (Fig. 4 A-E). Triads are at the base of each scalloped extension of the flaps, covered by the tunic, so that they are not visible under a scanning electron microscope. Each triad is formed by three sensory cells connected to adjacent thin epithelial cells by tight junctions; supporting cells are not present (Fig. 4 F). Sensory cells possess a long cilium projecting into the tunic, the microtubule configuration of which changes along its axis. In its proximal portion, located in a deep extracellular pocket that enlarges at the base in a ring, the cilium lacks dynein arms (Fig. 4 F-G); moreover, each doublet of microtubules is linked to the plasmalemma, which appears scalloped. The plasmalemma delimiting the pocket shows regular densities alternating with each doublet of microtubules in such a way that radial fibrils link the densities with the scalloped ciliary membrane. The cytoplasm surrounding the pocket housing the cilium is devoid of organelles but rich in microtubules. The apical portion of the sensory cell protrudes into the tunic where the cilium, inside the extracellular pocket and enveloped by a cylindrical flange of cytoplasm, contains two central microtubules.

Typical bridges between the two central microtubules and the nine lateral doublets are absent (Fig. 4 H).

The nucleus of sensory cells is large and exhibits a nucleolus. Mitochondria are abundant, and both the Golgi apparatus and rough endoplasmic reticulum are recognizable. At its base, each sensory cell extends an axon to the brain. Figure 4 F shows a sensory cell with an axon (enlarged in 4 I) followed in adjacent ultrathin sections, demonstrating its continuity with the cytoplasm of the cell (showed in Fig. 4 J). Synapses were never observed at the base of cells.

Coronal organ. The margin of the flaps is bordered by the coronal organ that, in transverse sections, is composed of two ciliated sensory cells flanked on both sides by supporting cells characterized by a thick apical glycocalyx (Fig. 5 A-F).

The coronal sensory cells are cuboidal and possess one rigid cilium, approximately 5 μm long, with a conventional configuration surrounded by short laminar microvillar protrusions (Fig. 5 C, D, F). The apical surface of the sensory cells is laterally raised so as to form an elongated ridge defining the hair bundle (Fig. 5 D-F).

The cytoplasm of coronal sensory cells is characterized by scattered mitochondria, a supranuclear Golgi complex composed of stacks of numerous cisternae, and some perinuclear rough endoplasmic reticulum cisterns. The basal plasmalemma of the ciliated cells is folded to delimit an extracellular groove containing neurites (Fig.5 F, G). As in *P. atlanticum*, the basal lamina, which supports the epithelial cells of the flaps, borders the groove. The neurites contain many microtubules and mitochondria; synapses between neurites and sensory cells were also occasionally observed (Fig 5 G).

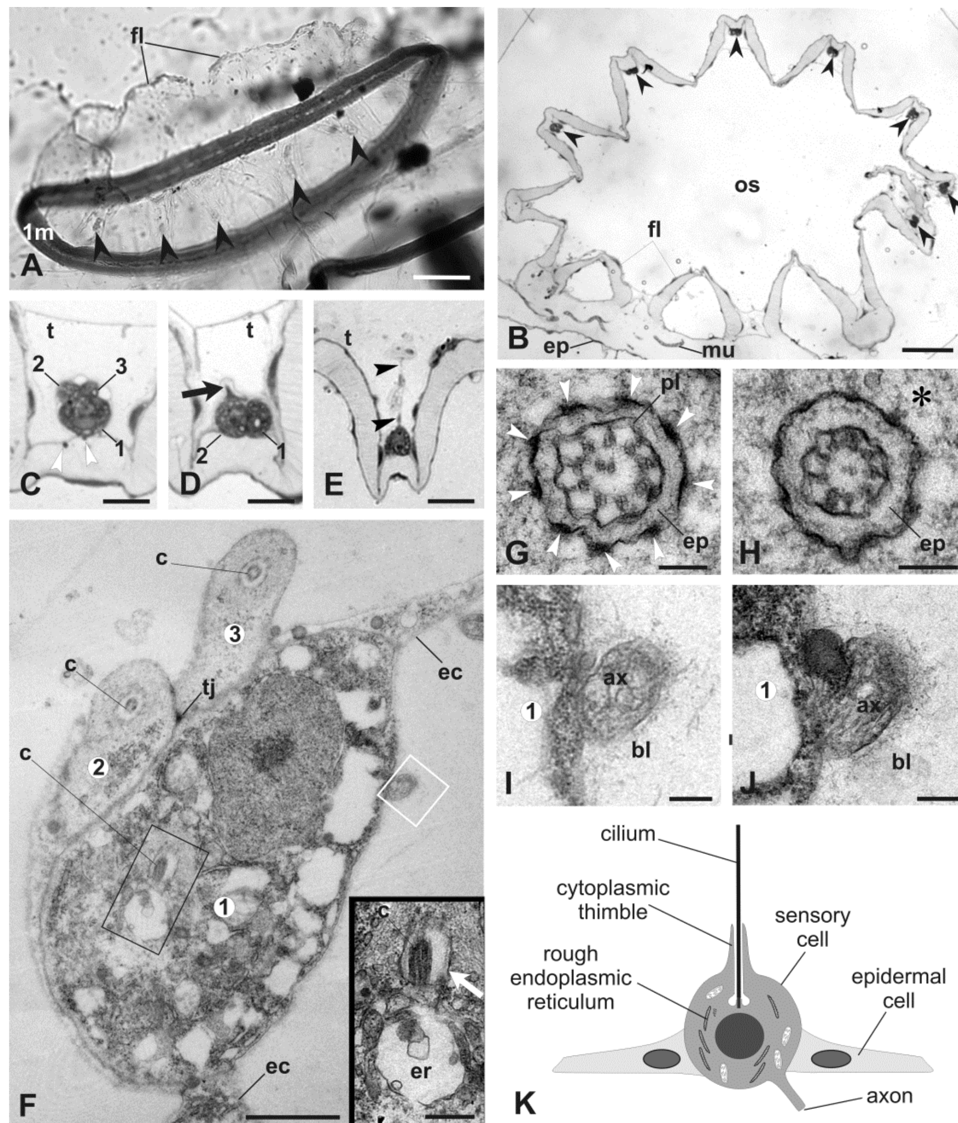

Fig. 4. Triads of sensory cells in *Doliolum nationalis*. **A**. Detail of the mouth of a whole mount blastozooid embedded in Epon Araldite resin. Flaps are held up and bear triads of sensory cells (arrowheads) regularly arranged around the mouth aperture. 1m: first muscle ring. **B-E**. Cross (B-D) and longitudinal (E) histological sections of the oral siphon (os), showing regularly arranged triads (black arrowheads in B) on flaps (fl). In C, three cells (indicated by numbers 1-3) are recognizable under the tunic (t) contiguous with thin epithelial cells; neurites (white arrowheads) are close to the basal plasmalemma of sensory cells. In D, two cells (indicated by numbers 1-2) are visible; the cell indicated by the number 2 shows a cylindrical flange of cytoplasm (black arrow) containing the cilium (not recognizable in this figure). Note, in E, the cilium extension (arrowheads) into the tunic. ep: epidermis; mu: musculature. Toluidine blue staining. **F-J** Electron microscopy of sensory cells. In the triad, sensory cells (indicated by numbers 1-3) are joined to each other and with adjacent epithelial cells (ec) by means of tight junctions (tj) and possess a cilium (c). The black squared area in F is enlarged in the inset to show the proximal portion of the cilium located in a deep extracellular pocket (white arrow) enlarged at the base in an extracellular ring (er). G shows a transverse section of a cilium in its proximal portion; note that it is in the extracellular pocket (ep), it lacks dynein arms, and each doublet is linked to the plasmalemma (pl) that appears scalloped. The cell plasmalemma delimiting the extracellular pocket shows regular densities alternate to each couple of microtubules (white arrowheads). Fibrils link these densities to the scalloped ciliary membrane. In H, the apical portion of the cilium in the extracellular pocket (ep), surrounded by a cylindrical flange of cytoplasm (asterisk), is visible. I-J are two very close ultrathin sections presenting the same axon (ax) to show its relationship with cell indicated by the number 1 in F. I is an enlargement of area delimited by the white square in F. In J, the axon is emerging from the sensory cell. bl: basal lamina. **K**. Sketch of a sensory cell belonging to a triad. Scale bar: 50 μm in A; 75 μm in B; 7 μm in C, D, E; 1.4 μm in F, 300 nm in inset; 150 nm in G; 300 nm in H; 200 nm in I, J.

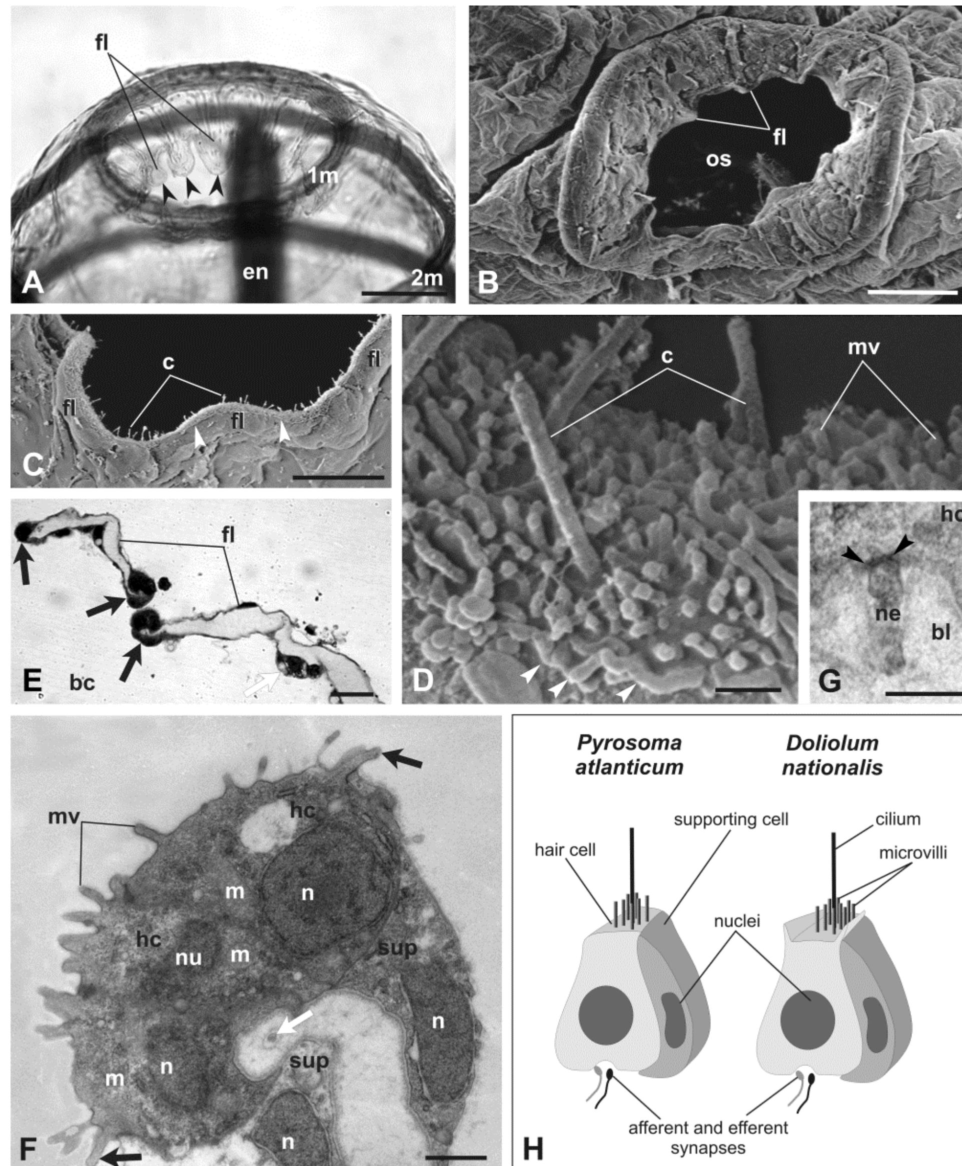

Fig. 5. Coronal organ of *Doliolum nationalis.* **A**. Detail of the oral siphon of a whole mount blastozooid embedded in Epon Araldite resin that shows the flap (fl) border where the coronal organ is located (arrowheads). 1m and 2m: first and second muscle ring, respectively; en: endostyle. **B-D**. Scanning electron microscopy of the oral siphon (os) viewed from the outer side. The coronal organ is recognizable thanks to cilia (c) and microvilli (mv) bordering the flaps (fl). Note the ridge (arrowheads) formed by the apical plasmalemma of sensory cells raised to delimit their sensory apical bundles. **E**. Histological section of flaps (fl), showing a position of the coronal organ (black arrows); a triad of sensory cell is also present (white arrow). bc: branchial chamber. Toluidine blue staining. **F-G**. Transmission electron microscopy of the coronal organ. Transverse section (F) of two hair cells (hc) flanked on both sides by supporting cells (sup). In the cytoplasm, scattered mitochondria (m) are visible. The basal plasmalemma of hair cells is folded to form a groove containing a neurite (white arrow). In G, a neurite (ne) establishes a synapse (arrowheads) with the basal plasmalemma of a hair cell (hc). Black arrows in F: ridge formed by apical plasmalemma of a sensory cell; bl: basal lamina; n: nucleus. **H**. Scheme of coronal cells in *Pyrosoma atlanticum* and *Doliolum nationalis*. Scale bar: 150 μm in A; 100 μm in B; 20 μm in C; 2 μm in D; 10 μm in E; 1 μm in F; 250 nm in G.

Thalia democratica

In *T. democratica*, we carefully analyzed the rim of the mouth and the velum extending from the ventral lip using light, scanning and transmission electron microscopy for sensory cells (Fig. 6 A, B). To search for the coronal organ, we concentrated our attention on the inner epithelium of the siphon, where the tunic stops, because the coronal organ is located in this area in other tunicates; however, no line of ciliated sensory cells was found. Moreover, we analyzed the dorsal languet, which has been compared to a sensory tentacle (Delage and Hérouard, 1898). Contrary to a previous report, we found that the languet is posterior (not anterior) to the neural gland aperture. We observed that the languet exhibits a smooth epithelium and lacks ciliated cells (Fig. 6 C-E).
Scanning electron microscopy revealed that the rim of the lips, which is covered by the tunic, is irregular and marked by short crests. These crests are also visible in histological sections and are formed by epidermal cells covered by the tunic (Fig. 6 G-I). Epidermal cells are flat and laterally joined to each other by a deeply folded plasmalemma. Cells protrude apically under the tunic forming the crests visible under a scanning electron microscope. These cell protrusions contain the nucleus, long and parallel cisterns of rough endoplasmic reticulum and Golgi fields. Cell apexes possess free ribosomes and rough endoplasmic vesicles. At the apex, the plasmalemma is thickened and contacts the cuticle, the superficial thin and dense layer of tunic. Occasionally, cells projecting a cilium into the tunic, possibly representing primary sensory cells, are scattered among epidermal cells (Fig. 6 G). The basal plasmalemma of epidermal cells is smooth

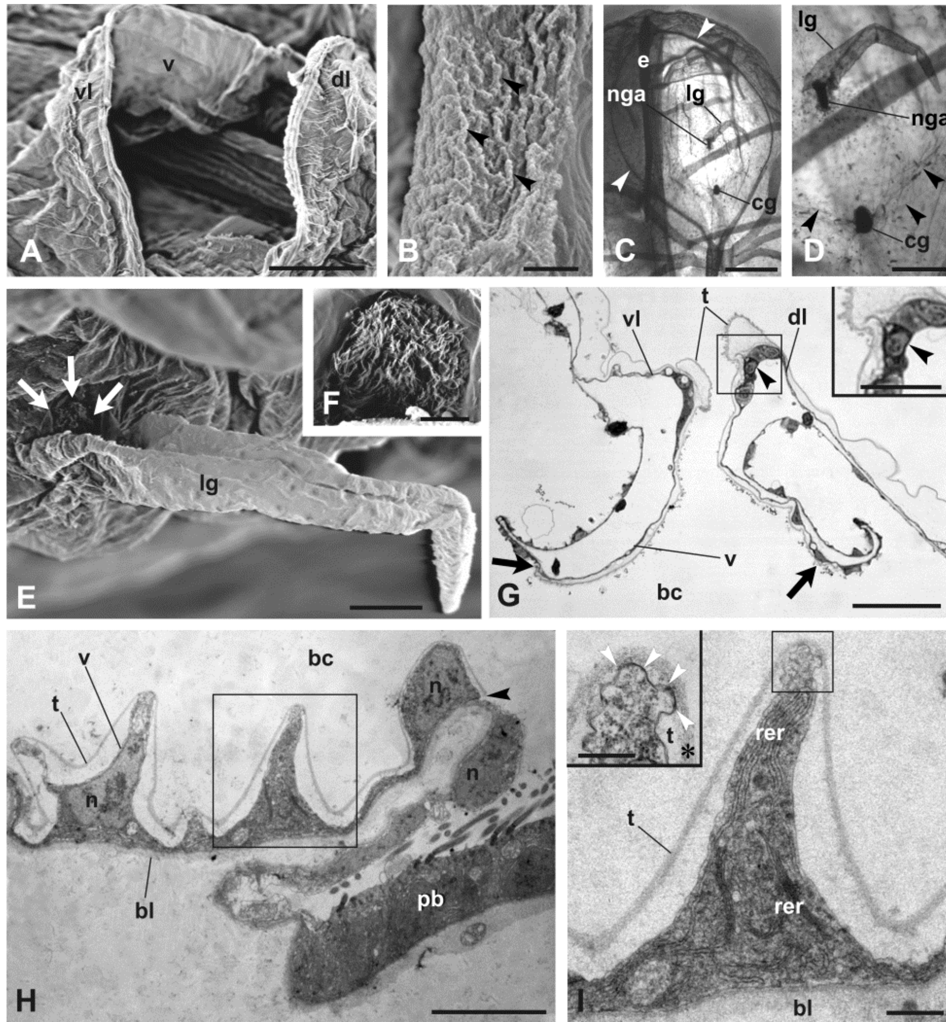

Fig. 6. *Thalia democratica*. **A-B**. Scanning electron microscopy of the oral siphon, delimited by a dorsal (dl) and a ventral (vl) lip, the latter bearing the velum (v). The lip rim (B) is characterized by short, irregular crests (arrowheads). **C-F.** Dorsal languet. In a whole mounted oozooid embedded in Epon Araldite resin (C-D), the languet (lg) seems to be anterior to the neural gland aperture (nga), but scanning electron microscopy (E) reveals that it is posterior to the aperture (white arrows in E). The languet surface is smooth, and ciliated cells are absent. F shows a detail of the neural gland aperture, characterized by numerous cilia. The languet is at the bottom. White arrowheads in C: peripharyngeal band; black arrowheads in D: nerves emerging from the cerebral ganglion (cg); e: endostyle. **G**. Histological longitudinal section of the mouth showing the tunic (t) covering the epidermis of the dorsal (dl) and ventral (vl) lips and the velum (v). Note the cell (black arrowheads in G and inset) of the dorsal lip projecting a cilium into the tunic. The tunic limit inside the mouth is marked by black arrows. bc: branchial chamber. Toluidine blue staining. **H-I**. Transmission electron microscopy of the velum (v) apex and inner epithelium extending to the peripharyngeal band (pb). Note the tunic covering the velum, and its inner limit (black arrowhead). The inner epithelium extending from the tunic limit to the peripharyngeal band does not contain a coronal organ or other sensory elements. The boxed area in H is enlarged in I to show a velum cell, exhibiting an irregular profile and long and parallel cisterns of rough endoplasmic reticulum (rer). The inset in I shows the cell apex with free ribosomes; note the plasmalemma thickened at points contacting (white arrowheads) the cuticle (asterisk) covering the tunic. bc: branchial basket; bl: basal lamina; n: nucleus. Scale bar: 100 μm in A; 2.5 μm in B; 300 μm in C; 100 μm in D; 50 μm in E; 10 μm in F; 20μm in G, 10μm in inset; 5μm in H; 1μm in I, 400nm in inset.

and lies on a thick basal lamina. Few neurites are recognizable in the space underlying the epidermis, sometimes in close association with muscles.

DISCUSSION

Despite their considerable impact on marine ecosystems for their role in the downward flux of carbon and nitrogen from the surface to the benthos (Sutherland et al., 2010), thaliaceans are infrequently studied tunicates, due to the difficulty of obtaining and maintaining specimens in the laboratory. Therefore, although their biology has long been studied, very few morphology studies on them have been published in the past decades. Our study on the sensory organs in the mouth of species representative of the three thaliacean orders, *Pyrosoma atlanticum*, *Doliolum nationalis* and *Thalia democratica*, furnishes new data on morphology of mechanoreceptors, and updates data coming from literature from the late 19th and early 20th century.

The three species lack the typical crown of tentacles of sessile ascidians. *P. atlanticum* and *D. nationalis* exhibit scalloped flaps (associated, in the first species, with a single tentacle), while *T. democratica* shows a dorsal and a ventral lip, the latter prolonged in a mobile velum. Considering that pyrosomes are believed to be the order most closely related to ascidians, on the base of phylogenetic, physiological and anatomical data (Burighel et al., 1992; Wada, 1998; Swalla et al., 2000; Stach and Turbeville, 2002; Yokobori et al., 2005; Tsagkogeorga et al. 2009; Govindarajan et al., 2011), we hypothesize that the flaps result from a reduction of the original crown of tentacles, whereas the single long tentacle is reminiscent of the original condition in the taxon. Conversely, *T. democratica* shows the most dissimilar anatomy compared to ascidians, following the trend of specialization that has identified salps as the most derived group with respect to the thaliaceans (Berrill, 1950). However, some authors consider thaliaceans a polyphyletic group, *i.e.*, an artificial class resulting from convergence between different evolutionary lines, with each one derived from different sessile ascidians (Godeaux, 1998).

Our results show that thaliaceans adopted a variety of solutions for mechanoreception. Some solutions are unique within tunicates, while others are shared; some are based on primary sensory cells, while others are based on

secondary sensory cells. This allows for several interesting evolutionary considerations, discussed below.

Oral sensory organs based on primary sensory cells: the cupular organ of *Pyrosoma atlanticum* and the triad of sensory cells of *Doliolum nationalis*

In pyrosomes, Brien (1948) reported the presence of flask-shaped mechanosensory cells with a cilium extended into the tunic, grouped together with supporting cells to form a tactile organ on the siphon rim and the oral flaps. Doliolids and salps are known to sense a variety of stimuli (mechanical, optical, chemical, thermal, hydrostatic, vibratory), which cause a number of reflexive responses (defensive, motor, protective, regulatory, expulsive, ciliary, visceral) (Fedele, 1923). Oral siphon responses have been attributed to a number of sensory structures. In doliolids, Bone (1959) reported the presence of triads of sensory cells without accompanying supporting cells at the base of the oral flaps.

We did not observe the flask-shaped ciliated cells reported by Brien (1948) on the oral siphon of *P. atlanticum.* However, we observed organs that we call cupular organs, characterized by pyriform primary sensory cells with a cilium and a corolla of microvilli embedded in a cupula. Based on their location and morphology, we suggest that the cupular organs are a new finding in *P. atlanticum*. There are several examples of mechanoreceptors containing a cupula in tunicates. Cupular organs of *P. atlanticum* are comparable to the cupular organs of the atrial chamber of *Ciona intestinalis* (Deibel et al., 1988) (Fedele, 1923; Deibel et al., 1988), which consist of a pad of supporting and sensory cells whose cilium is basally located in a pocket and projects, together with a corolla of microvilli, into a cupula composed of tunic-like material. Approximately 75–100 of these organs are found in *Ciona*, each containing 15–20 sensory cells. Cupular organs like those of *Ciona*, with a similar location, have also been reported in *Corella eumyota* (Mackie and Singla, 2004). These organs are termed simple cupular organs to distinguish them from the cupular strand, which is a much larger, elongated organ found in *Corella inflata* (Mackie and Singla, 2004). Cupular organs were also described in blastozooids of *T. democratica* (but not in oozooids of *T. democratica*, nor in other salps) (Bolles, 1891b). It is assumed that cupular organs detect vibrations and local water movements that displace the

cupula and the cilium within it, resulting in electrical responses in the sensory cells (Deibel et al., 1988). By analogy with ascidian cupular organs, *P. atlanticum* cupular organs could have a similar function because the flaps bend as the swimming muscles of the body contract. Deformations of sensory cells, caused by displacement of the cupula, could be involved in the reflex patterns involved in swimming control.

In the flaps of *D. nationalis*, we confirm the presence of the triads of sensory cells observed by Bone (1959) in whole-mount preparations. In the present paper, we document their fine-structural features for the first time. Triads of sensory cells are not accompanied by supporting cells. The sensory cells are large and rich in mitochondria, with an immobile cilium (as indicated by its unconventional configuration of microtubules) located in a deep pocket that projects into the tunic. They are clearly primary sensory cells. Based on their location and morphology, triads are an original innovation of doliolids. The structure of the triads suggests a possible role in mechanoreception. According to Bone (1959), these cells may be stimulated by the deformation of their processes when water flows through the mouth as the animal swims.

A comparison of tunicate multicellular mechanoreceptors based on primary sensory cells (for which we have cytological information available) reveals that the corolla-like configuration of the sensory bundle represents a general feature (Table 1). All of the organs are positioned close to the sphincters (oral or atrial), where they can evidently better monitor the water flowing into the animals' cavities. The matrix embedding the sensory apparatus varies in different species: it is represented by the tunic in the triads of sensory cells of *D. nationalis* (present data) and the sensory cells at the base of the oral tentacles of *Polyandrocarpa misakiensis* (Koyama, 2008) or by a cupula (composed of a tunic-like material) in the cupular organs of *P. atlanticum* (present data), *Ciona intestinalis* (Deibel et al., 1988) , *Corella eumnyota* (Mackie and Singla, 2004) and the cupular strand of *Corella inflata* (Mackie and Singla, 2004). A fluid-filled capsule protects sensory cells in the capsular organs of *Cheliosoma productum* (Mackie and Singla, 2003). All of these organs resemble each other due to the convergent evolution of a system for amplification of forces acting on the cilium.

In some species (like some of genus *Corella*), information detected by multicellular organs must be integrated with information coming from thousands

of primary sensory cells scattered in the epidermis immediately below the tunic (Mackie and Wyeth, 2000; Mackie et al., 2006). Animals probably use these sensors to pick up mechanical stimuli transmitted through their soft and pliable body walls.

The variety of receptors probably indicates the importance and complexity of tunicate mechanoreception and its behavioral integration. In agreement with Mackie and Singla (2003), we posit that ascidian mechanosensory systems evolved from clusters of simple ciliated mechanoreceptors, and we attribute the similarity of multicellular organs in different species to evolutionary convergence.

Oral sensory organs based on secondary sensory cells: the coronal organ in *Pyrosoma atlanticum* and *Doliolum nationalis*

Our results show that *P. atlanticum* and *D. nationalis* possess a coronal organ. In pyrosomes, previous reports indicated the presence of tentacular receptors located around the oral opening (Neumann, 1935). *P. atlanticum* responds to mechanical stimulation of the oral siphon (touch or collision of large particles) by ascidian-like squirting (Bone and Best, 1978b) , which is elicited by the coronal organ (Mackie et al., 2006). In doliolids, ciliated sensory cells in the oral flaps can evoke a similar defensive response causing escape reactions when the animal is touched or stimulated by water movements (Fedele, 1923). Unfortunately, papers produced in the first decades of the 1900s lack adequate illustrations, and thus, we are not able to say if the sensory structures reported by Neumann and Fedele correspond to the coronal organ. More importantly, the authors did not specify the nature of the sensory cells (primary or secondary), probably because it was impossible to define on the basis of whole mount preparations. Here, we report that thaliaceans possess mechanoreceptor organs based on secondary sensory cells.

A comparison between the coronal organs of *P. atlanticum* and *D. nationalis* shows that they share general features both in gross anatomy and cytology. In both the species, the coronal organ lies on the flap border where it is always exposed to the inhalant water flow. The organ is formed of sensory cells aligned side by side in a linear array, with a corolla-like configuration. The secondary sensory status of these cells is indicated by the absence of axons and

their ability to form synapses with neurites running below the basal plasmalemma. Supporting cells in the two species do not show any special features; they resemble flap epithelial cells and adhere to adjacent sensory cells with their lateral plasmalemma.

We observed some difference in the details of the basic architecture of the coronal organ and sensory cells between the two species, probably representing adaptations in the ability to respond to different stimuli, such as particles with diverse dimension ranges and/or water flow variations. For example, in *D. nationalis*, we found sensory cells with short, laminar microvilli, whereas *P. atlanticum* possesses short microvilli of typical design. Moreover, in the sensory cells of *D. nationalis*, the lateral region of the apical plasmalemma cells has laminar protrusions delimiting a groove containing the sensory hair bundle apparatus. A similar groove-like configuration is typical of ascidian coronal organs, but is formed by laminar protrusions of supporting cells. These laminar extensions are not present in *P. atlanticum*.

The thaliaceans' monociliated hair cells, with a corolla-like configuration, are comparable to those of stolidobranch ascidians, whereas all described aplousobranchs and phlebobranchs possess pluriciliated cells, sometimes accompanied by short microvilli (Burighel et al., 2011). However, actin-filled microvilli (stereovilli), surrounding or grouped to one side of a single cilium (or a couple of cilia), were reported in some stolidobranchs, but were never found in aplousobranchs, phlebobranchs or in the two thaliaceans described here. In some phlebobranchs, the coronal organ is compound, *i.e.*, it is accompanied by secretory cells. Although nothing is known about the functional significance of the morphological differences found in the coronal organs of various tunicates, it is conceivable that these differences reflect the ability of organs to respond to different types of signals and to produce different physiological responses, as has been demonstrated in various octavo-lateralis organs in vertebrates.

The original solutions to mechanoreception adopted by *Thalia democratica*

Our observation of the mouth of *Thalia democratica* failed to find multicellular sensory organs, although we carefully checked the lips to verify the

presence of sensory cells described by previous authors (Fedele, 1933; Bone, 1959).

According to Fedele (1933), the mouth of salps is richly innervated, and sensory neurons are particularly abundant on the rim of the lips. The ventral lip border, where the velum originates, is defined as “a rich sensitive edge” where sensory structures are distributed almost continuously. Moreover, the velum projecting from the ventral lip possesses other sensory structures, in the form of bulbs, which, in *T. democratica,* have an apical extension running along the velum until its border. These sensory structures could be chemoreceptors. Such a detailed description is also accompanied by sketches of Fedele’s (1933) observations. The same sensory elements were also seen by Bone (1959), who distinguished between two types of sensory cells. The first type lies at the anterior edge of the lips and bears a long process extending forward in the plane of the lip; their deformations are probably involved in reflex patterns that control normal forward swimming. The second type are numerous, irregularly arranged cells all around the lips, lying between the edges of the lips and the first muscle band; these cells do not bear long processes and probably correspond to the chemoreceptors described by Fedele.

Fedele, as was the custom of his time, would observe animals with a phase-contrast microscope, taking advantage of the natural transparency and thinness of tunicates. Smaller forms were examined as a whole, whereas the larger specimens were dissected and small pieces were examined in the same way. In addition, specimens were fixed and stained in various ways to visualize sensory elements. Even today, knowledge of the nervous systems of various tunicates relies heavily on his accurate work, published in a series of papers describing both the structure and the function of the nervous systems in different groups of tunicates. Based on the work of Fedele and Bone, we carefully analyzed the mouth of *T. democratica*, making transverse and longitudinal serial section reconstructions using a transmission electron microscope. We were able to find only rare (2-4 per animal) scattered ciliated cells, probably primary sensory cells. For a better definition of the sensory net in these animals, other methods that have been successfully used to visualize the peripheral nervous system of ascidians could be useful (Takamura, 1998; Imai and Meinertzhagen, 2007). Recently, for example, utilizing immunofluorescence, numerous peripheral

neurons possessing long cilia have been visualized in the epidermis of *T. democratica* (Pennati et al., 2012).

The absence of a coronal organ was surprising because all tunicates analyzed thus far contain this organ. Its absence means that, at the moment, *T. democratica* is the only tunicate species known to lack secondary sensory cells. We carefully observed the inner epithelium of the mouth where the tunic stops because this is the typical position of the coronal organ in ascidians, in appendicularians (Burighel et al., 2011), in *P. atlanticum* and *D. nationalis* (present data). Failing to find it, we observed also the "languet", the dorsal epithelial protrusion, which, according to Delage and Hérouard (1898) and Neumann (1935), is anterior to the pericoronal band and the neural gland aperture and may represent a reduction of the tentacle crown to a single tentacle with possible tactile function. However, we found that the languet is smooth and does not exhibit any sensory structures. Moreover, the languet is posterior (not anterior) to the neural gland aperture, and therefore, it cannot be equivalent to a tentacle.

On the basis of anatomical and behavioral specializations that evolved in salps, we hypothesize that the absence of the coronal organ in *T. democratica* represents a secondary loss. Salps are the only tunicates that feed based on the activity of muscles instead of cilia. Salps lack both tentacles and flaps, but exhibit lips composed of muscles. Salps have the highest *per* individual filtration rates of all marine zooplankton filter feeders (Alldredge and Madin, 1982). Because of their unique feeding mechanism, salps are able to filter the largest volume of water among tunicates. Water passes through a branchial basket reduced to a single bar. There is no grading of particles entering the oral aperture (a role ascribed to the coronal organ in ascidians, (see Mackie et al., 2006), but everything small enough to enter the mouth is accepted. The diameter of the oesophageal aperture is the limit for particle admission (Fedele, 1933; Madin, 1974). Water flow is also responsible for swimming and rapid jet reactions. Moreover, salps evolved an exceptional system for mechanoreception based on excitable epithelia. Skin impulses of the inner epithelial layer in the anterior region of the lips inhibit the regular rhythmic bursts of forward swimming and are presumably evoked by large particle objects inhaled into the front of the pharynx (Bone, 1998). Although Fedele (1933) stated that sensory cells are present in the

inner epithelium lining the pharynx, Mackie and Bone (1977) did not observed nerve endings in the gill or pharyngeal epithelia.

Currently, we cannot generalize our observations on *T. democratica* to all species of the order. Further comparative studies are necessary to understand how oral mechanoreception is organized and functions in salps. However, the functional considerations discussed above, coupled with the information from classical and modern phylogenies of thaliaceans, suggest that secondary sensory cells of the coronal organ were originally present in the common ancestor to thaliaceans and were secondarily lost in salps. We therefore maintain the hypothesis that hair cells are a plesiomorphic feature of tunicates, and they share a common origin with vertebrate hair cells.

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Chapter 2

Evolutionary diversification of secondary mechanoreceptor cells in Tunicata

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ABSTRACT

Hair cells are vertebrate secondary sensory cells located in the ear and in the lateral line organ. Until recently, these cells were considered mechanoreceptors exclusively found in vertebrates and evolved within this group. However, evidence of secondary mechanoreceptors in some tunicates, the proposed sister group of vertebrates, led to the hypothesis that vertebrate and tunicate secondary sensory cells share a common origin. Secondary sensory cells were described in ascidians an thaliaceans, where their role in feeding system were hypothesized. However, it is unknown whether such cells are present in appendicularians; therefore it remains unclear whether these cells were present in the ground plan of tunicates. In order to investigate the evolution of secondary sensory cells in chordates, we studied the detailed cellular and subcellular morphology of oral mechanoreceptors of appendicularians, the third remaining taxon of tunicates. Using light- and electron microscopy, we showed that two appendicularian species, *Oikopleura dioica* and *Oikopleura albicans*, possess a mechanosensory organ, called circumoral ring, constituted of secondary sensory cells. We described the ultrastructure of this organ and thus taxonomically completed the data collection of tunicate secondary sensory cells. Basing on our morphological results on appendicularians combined with other data, we constructed a matrix consisting 19 characters derived from detailed ultrastructural studies in 20 different tunicate genera, using the cephalochordate amphioxus and vertebrate species as outgroups. Our study clearly showed that the circumoral ring is the appendicularian counterpart of the coronal organ in other tunicate taxa. The cladistic analysis of the matrix enabled us to reconstruct the ground pattern of tunicates with a simple monociliated prototype cell, which successively differentiated to the current variety of oral mechanoreceptors in the different tunicate lineages. Moreover, we demonstrated that the inferred evolutionary changes coincide with major transitions in the feeding strategies in the respective lineages.

INTRODUCTION

Tunicates include the sessile ascidians and the planktonic appendicularians and thaliaceans, and are considered a key group to investigate the evolution of the more complex vertebrates; indeed, recent chordate phylogenies, based on molecular data, place tunicates as sister group of vertebrates, with the cephalochordates at the most basal position (Delsuc et al., 2006; Delsuc et al., 2008). During the last years, several studies on ascidians and thaliaceans have been focused on identification and description of tunicate sensory systems, in order to understand the evolution of vertebrate sensory organs. The latter are particularly elaborated and the comprehension of their structural origin and evolution will further understanding of their organization and function.

Secondary sensory cells lack their own axon but form synapses with other neurons at the level of their basal plasmalemma. In ascidians, they have been hypothesized as possible homologues of vertebrate secondary sensory cells, i.e. hair cells of ear and lateral line. In all the ascidians analysed so far, sensory cells are located in the oral aperture and are arranged to form an organ, called coronal organ, which is constituted of a row of cells bordering the oral tentacle surface. Its role is to monitor the inflowing water during the feeding process. Coronal sensory cells show considerable variability in different species (Manni et al., 2006; Caicci et al., 2007; Caicci et al., 2010a), especially regarding the apical bundle, which can exhibit one or more cilia, surrounded by microvilli or stereovilli, of the same or different length. In thaliaceans (Caicci et al., 2013: Chapter 1, present thesis), the coronal organ was described in *Doliolum nationalis* and *Pyrosoma atlanticum*, representative of the orders Doliolida and Pyrosomida, respectively. The organ is absent in the salp *Thalia democratica* and it has been hypothesized that this condition is due to the different feeding system adopted by this group of animals. Contrary to ascidians, doliolids, and pyrosomes , salps actively pump a large amount of water into their mouth utilizing muscular contractions, instead of pharyngeal ciliary beating.

Secondary sensory cells have been identified also in the mouth of appendicularians. In *Oikopleura dioica*, they are pharyngeal ciliated cells (Olsson et al., 1990; Bone, 1998), which constitute a ring of ciliated mechanoreceptor

cells. The ring is oblique with respect to the transversal plane of the animal: caudally it is located in the roof of the pharynx, ventrally it reaches the tip of the lower lip, so that it divides the rostral pharynx into an upper and a lower portion. Unfortunately, at present there is no information on the detailed cytoarchitecture of these cells, in particular there is poor information about the organization of their apical sensory structure (e.g., we do not know if they are monociliated, multiciliated, with microvilli or stereovilli). Circumoral ring cells are connected to the brain through the ventral branch of the second nerve (Olsson et al., 1990), and it is known that the cells located on the lower lip have the ability to detect mechanical stimuli. Their stimulation provokes a response of ciliary reversal in the spiracles, the rings of ciliated cells responsible for water pumping (Fol, 1872). Similarly to the coronal organ on the oral tentacles of other tunicates, the lip receptors in appendicularians prevent larger particles from entering the mouth via incurrent seawater flow. More recent authors have confirmed that these receptors respond to tactile stimuli (Lohmann, 1933; Galt and Mackie, 1971). In contrast to the remaining tunicates, appendicularians possess other secondary mechanoreceptors, the so-called Langerhans cells, in the posterior of trunk. When stimulated, Langerhans cells trigger the escape response of the animal (Bone and Ryan, 1979).

In order to improve our understanding of the evolution of oral secondary sensory cells in chordates, we (i) investigated the structural details of the circumoral ring cells in two species of appendicularians, *Oikopleura dioica* and *Oikopleura albicans*, by means of electron microscopy in order to obtain the cytological data necessary to compare them to the sensory cells in the coronal organs of ascidians and thaliaceans, and complete the picture of the oral secondary sensory cells in tunicates. (ii) Second, we traced the evolution of secondary sensory cells within tunicates, by conceptualizing a data matrix based on the morphological characters derived from our studies of secondary sensory cells of the tunicate species, using as outgroup representative species of both cephalochordates and vertebrates.

Within chordates, cephalochordates possess several types of secondary sensory cells, spread in different head regions (Bone and Best, 1978b). In particular, the oral spines are sensory structures placed around the mouth; if stimulated, they are able to provoke a rejection response, with expulsion of water

(Stokes and Holland, 1995; Lacalli and Hou, 1999; Lacalli, 2004). On the basis of their position and morphology, oral spines were proposed to be homologues to vertebrate taste buds; moreover, because the nerve plexus contacting these cells shares common features with adoral nerves and ganglia of echinoderms (Lacalli et al., 1999), a possible homology between these two structures has also been hypothesized. It is noteworthy, that echinoderm and hemichordate mouths do not display any secondary sensory cell (Harrison and Ruppert, 1997; Lacalli et al., 1999). Thus, Lacalli and coauthors proposed that amphioxus has retained some features typical of hemichordates and echinoderms (e.g. regarding the nerve network), and furthermore had acquired new structures (e.g. secondary sensory cells of oral spines), as occurred during evolution in other chordates.

Vertebrate hair cells of ear and lateral line were believed to be exclusive for vertebrates and to have evolved within this group because of their typical morphology and development (Northcutt and Gans, 1983). They are secondary sensory cells exhibiting and apical sensory apparatus usually constituted of a no-motile cilium (kinocilium) and graded in length, actin-filled microvilli (stereovilli). Vertebrate hair cells develop from a number of embryonic placodes (Schlosser, 2010; O'Neill et al., 2012); these are ectodermal thickenings characterized by the expression of some common placodal genes (e.g. *Eya, Pax, Six*), and others more specifically related to the single type of placode (Schlosser, 2010; O'Neill et al., 2012). In tunicates, embryonic territories marked by the same set of genes and able to give rise to sensory organs were recognized both in ascidians (Mazet and Shimeld, 2005; Kourakis et al., 2010) and appendicularians (Bassham and Postlethwait, 2005). Among ascidian placodes, the stomodeal one is placed anterior to the neural plate border; the oral siphon and coronal organ develop from it (Manni et al., 2005; Veeman et al., 2010). In addition to general placodal genes, it expresses also the *Pitx* gene (Mazet et al., 2005), a gene characterizing the vertebrate adenohypophyseal and olfactory placodes (Schlosser, 2010), which in vertebrates do not give rise to secondary sensory cells. In the same way, in *O. dioica* the mouth derives from the stomodeal placode, expressing genes such as *Eya*, *Six 3/6, Pax 2/5/8* and *Pitx*. However, the orthologue of *Six 1/2*, which in vertebrates is expressed specifically in the otic placode, has been detected also in appendicularian stomodeum, with a persistent expression (Mazet et al., 2003; Bassham and Postlethwait, 2005; Canestro et al., 2008).

Differently from tunicates, in cephalochordates true placodes have not been identified, although several studies evidenced that some broad ectodermal regions are characterized by expression of typical placodal genes and are able to differentiate both primary neurons and secondary sensory cells. It has been suggested that the ability to differentiate neurons from the neural ectoderm may have been present in all chordates, initially in broad regions, as seen in cephalochordates, and subsequently in restricted, specialized regions, differentiating placodes, as found in tunicates and vertebrates (Kozmik et al., 2007; Graham and Shimeld, 2012).

Our data evidence that the circumoral ring of cells in appendicularians can be considered homologues to the coronal organs of ascidians and thaliaceans, being located in a correspondent position and constituted of secondary sensory cells having the same mechanoreceptor function. Moreover, our phylogenetic analysis shows that the chordate oral secondary sensory cells may be derived from a simple monociliated prototype cell, from which the actual diversity of sensory cells progressively evolved.

MATERIALS AND METHODS

Specimens of *Oikopleura dioica and Oikopleura albicans* were collected in front of the Zoological Station in Villefranche-sur-Mer (France). In addition, developmental stages of *Oikopleura dioica* were obtained in the SARS High Technology Center in Bergen, Norway. Precisely timed stages were obtained by mixing ripe eggs and sperm and pipetting the animals directly into the primary fixative on ice.

Transmission electron microscopy

Specimens were anesthetised with 0.02 % MS222 at 4°C. After complete relaxation, specimens were fixed in 1.7% glutaraldehyde buffered in cacodylate buffer (0.2M sodium cacodylate, pH 7.4, plus 1.7% NaCl), or in 1% glutaraldehyde buffered in phosphate buffer (1.28 mM NaH_2PO_4 plus 5.38 mM Na_2HPO_4, pH = 7.4). After post-fixation in 1% OsO4 in 0.2M cacodylate buffer, specimens were dehydrated and embedded in Epon Araldite 812. Thick sections (1 μm) were counterstained with toluidine blue; thin sections (80nm) were given

contrast by staining with uranyl acetate and lead citrate. Micrographs were taken with a Hitachi H-600 (operating at 75kV) and FEI Tecnai G^2 electron microscope (operating at 100kV). All photos were collected and labeled in Corel Draw X3.

Scanning electron microscopy

Specimens were fixed in glutaraldehyde solution as described for transmission electron microscopy. After post-fixation and dehydration, they were critical-point dried, sputter-coated with gold, and observed under a Cambridge Stereoscan 260 and under a Fei Quantum 200 scanning electron microscopes. Micrographs were collected and then labeled in Corel Draw X3.

Phylogenetic analysis

Construction of morphological character matrix

We constructed a matrix based on 19 characters derived from detailed ultrastructural studies of oral secondary sensory cells using Mac Clade 4.08 (Maddison and Maddison, 2000). Phylogenetic analysis allows to detect phylogenetic information present in the examined structures and to complement other matrices, morphological or molecular. Character definition and description of character states is detailed in the Results-section. Character coding was strictly binary in order to maximize information content (Swofford, 2003).

Analyses

All phylogenetic analyses were performed using PAUP* (version 4.0b10) (van Name, 1945; Monniot, 1965; Monniot and Monniot, 1978; Kott, 1985; Monniot and Monniot, 1988; Kott, 1990; Monniot and Monniot, 1990; Kott, 1992; Monniot et al., 1992; Rodrigues et al., 1998; Kott, 2001). Parsimony analyses were conducted using the branch and bound option. A strict consensus tree and a 50% Majority Rule tree were calculated. Jackknife values were calculated for 1000 replicates using an heuristic search strategy with n = 10 random addition sequence replicates, TBR branch swapping, retaining all optimal trees, and 30% random character deletion. We tracked transformations of character states in the

resulting trees using Mac Clade 4.08 with standard settings, i.e. acctran-optimization (Maddison and Maddison, 2000).

RESULTS

Secondary sensory cells in the mouth of *Oikopleura dioica* and *Oikopleura albicans*

In both *O. dioica* and *O. albicans,* the mouth is delimited by two lips, a dorsal and a ventral one, the latter protruding anteriorly. Sensory cells are arranged to form the circumoral ring, that is continuous in *O. dioica*, while it is interrupted at its lateral edges in *O. albicans* (Figs. 1-2). Scanning electron microscopy analysis reveals that in both species sensory cells possess apical cilia arranged in multiple rows. The cilia have different lengths, with the longest in the central position ehereas gradually they gets shorten, conferring to the circumoral ring a waved aspect (Fig. 1D-F). In *O. albicans*, cilia of ventral lip receptors are accompanied by short microvilli (Fig. 2 B).

Transmission electron microscopic observations were performed in *O. dioica*. Sagittal sections of the mouth region reveal that the circumoral ring consists of a single row of sensory cells (Fig. 1 E-H). The sensory cells are flange by non-ciliated supporting cells that appear C-shaped in cross sections. These supporting cells form a continuous groove that houses the apical cilia of the

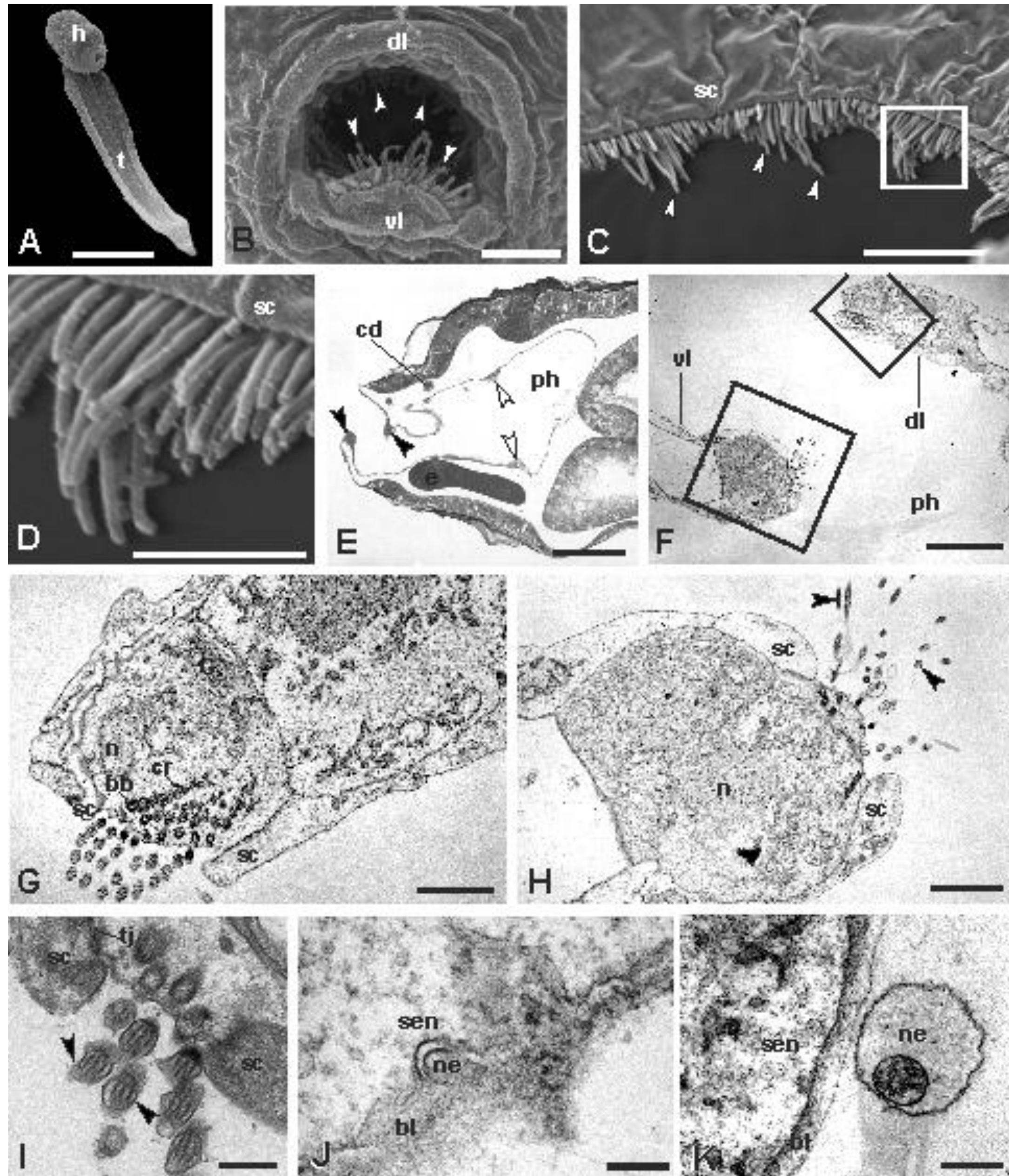

Fig. 1. Secondary sensory cells in *Oikopleura dioica*. h: head; t: tail. **A-D** Scanning electron micrographs A-B. Juvenile 26 hours old (A) and detail of the mouth (B) to show cilia of sensory cells belonging to the circumoral ring. Arrow: mouth; arrowheads: cilia belonging to sensory cells; dl: dorsal lip; t: tail; tr: trunk; vl: ventral lip. **C-D**. Mouth of an adult animal showing cilia (white arrowheads) of sensory cells located on the dorsal lip (dl). Cilia are of different lengths; this confers a wavy arrangement to the circumoral ring. Note that the apical membrane of supporting cells (sc) forms a crest delimiting the sensory bundle. Squared area in C is enlarged in D to show that each sensory cell possesses a number of ciliary rows forming the sensorial apparatus. vl: ventral lip. **E**. Sagittal section of the head showing the ventral (vl) and dorsal (dl) lips and the circumoral ring (black arrowheads); white arrowheads: perypharyngeal band; cd, ciliated duct of the neural gland; e, endostyle; ph, pharynx. **F-K**. Transmission electron microscopy of circumoral organ. The organ is formed by a single cell row dorsally located on the roof of the pharynx (ph), ventrally on the tip of the ventral lip (vl). Squared areas in F are enlarged in G and H to show ventral and dorsal receptor cells, respectively. The hair bundle is multmulticiliated and delimited by apical extensions of supporting cells (sc). Cilia (arrowheads) show a conventional 9+2 microtubular arrangement and a dense, short basal body (bb) with developed ciliary rootlets (cr). bl, basal lamina; dl, dorsal lip; G, Golgi complex; n, nucleus; tj, tight junction. Note in J and K neuritis (ne) very close to the sensory cell membrane (sen). Scale bar: 100 µm in A; 5 µm in B and C; 2 µm in D; 40 µm in E; 10 µm in F; 2 µm in G; 2,5 µm in H; 500 nm in I; 160 nm in J; 800 nm in H.

sensory cells throughout the circumoral ring (Fig. 1C-D, 2 B). All of the cells of the lips, both sensory and non-sensory, are joined apico-laterally to each other by tight junctions. The sensory cells ring are flask-shaped and possess an oval central nucleus, scattered mitochondria, and few cisternae of rough endoplasmic reticulum (Fig. 1 G-H). The Golgi complex is composed of few stacks of cisternae and associated vesicles. The cells apically bear many cilia, with a conventional 9+2 microtubular arrangement and a dense, short basal body with developed ciliary rootlets (Fig. 1 I). The basal plasmalemma of each sensory cell rests on the basal lamina, which forms a continuous fibrous layer supporting both sensory and other epithelial cells. Neurites were often seen in close proximity to the sensory cell membrane (Fig. 1 J-K). We never recognized axonal extensions of the basal plasmalemma of the sensory cells. Instead, the basal plasmalemma was always flat and approached by nerve fibres projecting from the brain. Occassionally, synapses could be identified.

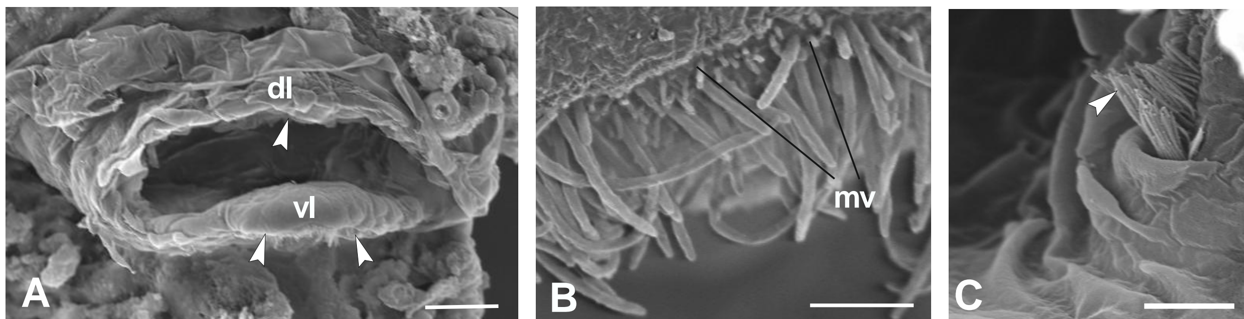

Fig. 2. Secondary sensory cells in *Oikopleura albicans*. Scanning electron microscopy. The mouth (A) is furnished of a dorsal (dl) and a ventral (vl) lip, bearing the circumoral ring (arrowheads pointing to cilia). Cilia are of different lengths and, in the ventral lip (B), are accompanied by short microvilli (mv). In this species, the circumoral ring is not continuous, but interrupted at the lateral edges; in C, left limit of the upper row of sensory cells (arrowhead). Scale bar: 25 µm in C; 2.5 µm in B; 5 µm in C.

Phylogenetic analysis of coronal organs

Taxon sampling

In order to analyze the phylogenetic information potentially present in the diversity pattern of secondary sensory cells around the mouth of chordates, we compiled a data matrix covering 20 different taxa of chordates. The cephalochordate *Branchiostoma floridae* (amphioxus) and the vertebrates

Lampetra fluviatilis, *Eptatretus stouti,* and *Danio* rerio were selected as outgroups.

In amphioxus, two types of secondary sensory cells were described. Type II sensory cells are monociliated secondary sensory cells, usually solitary or arranged in small clusters, and most of them may be mechanoreceptors (Lacalli and Hou, 1999; Holland and Yu, 2002). Since these cells are scattered throughout the epidermis, we did not select them for our analysis. Differently, we considered the sensory cells forming oral spines in larvae (Lacalli et al., 1999; Lacalli, 2004): these sensory cells are monociliated and clustered so that 8-10 cilia together form a spine; many spines are arranged in a discontinuous row on the outer margin of the mouth. Spines are able to respond to contact with debris by initiating a cough response: the pharyngeal slits close as the pharynx contracts in order to expel water out of the mouth and dislodge the debris.

Among vertebrates, we considered hair cells of the lateral line, because the organ extends also roughly around the mouth region. We selected the Pacific hagfish *Eptatretus stoutii*, the lamprey species *Lampetra japonica*, and the zebrafish, *Danio rerio* as Osteichthyan, mainly because they have been extensively studied (Braun and Northcutt, 1997; Yamada, 1972 Metcalfe et al., 1985; Williams and Holder, 2000).

In the context of tunicates, we limited our analysis to genera, since the number of species (listed in Table 1) was too high with respect to the limited amount of characters and species belonging to the same genus possess the same kind of secondary sensory cells (except for *Oikopleura dioica* and *Oikopleura albicans*). We considered six genera of stolidobranch ascidians (*Botryllus*, *Botrylloides*, *Styela*, *Polyandrocarpa*, *Molgula*, and *Pyura*), two genera of aplousobranch ascidians (*Clavelina* and *Diplosoma*), and five genera of phlebobranch ascidians (*Ciona*, *Ascidiella*, *Phallusia*, *Chelyosoma,* and *Corella*). *Oikopleura dioica* and *Oikopleura albicans* were chosen as representative of the class Appendicularia and sensory cells of the circumoral ring were considered in the phylogenetic analysis. We did not consider the Langherans cells, because they are not located around or inside the mouth, and they innervate their axon via electrical synapses (gap junctions) instead of classical chemical synapses (Bone and Ryan, 1979). Within the class of thaliaceans, the genera *Pyrosoma* and *Doliolum* as representatives of the two orders Pyrosomatida and Doliolida, were

selected; no species of the order Salpida was chosen, because the only salp analyzed, *Thalia democratica*, does not possess secondary sensory cells in the mouth (see chapter 1 present thesis). Including *T. democratica* in the data matrix would result in an almost completely empty row of characters.

Traditionally recognized higher taxonomic groupings				**Species**	**References**
Tunicata	Ascidiacea	Pleurogona	Stolidobranchiata	*Botryllus schlosseri*	Burighel et al., 2003
				Botrylloides leachi, B. violaceus	Burighel et al., 2003; 2008
				Styela plicata, S. montereyensis, S. gibsii	Manni et al., 2004; Caicci et al., 2010
				Polyandrocarpa zorritensis	Caicci et al., 2010
				Molgula socialis	Caicci et al., 2007
				Pyura stolonifera, P. haustor	Caicci et al., 2010
		Enterogona	Aplousobranchiata	*Clavelina lepadiformis*	Manni et al., 2006
				Diplosoma listerianum	Manni et al., 2006
			Phlebobranchiata	*Ciona intestinalis*	Manni et al., 2006: Mackie et al., 2006
				Ascidiella aspersa	
				Phallusia mammillata	
				Chelyosoma productum	
				Corella inflata; C. willmeriana	
	Appendicularia			*Oikopleura dioica; O. albicans*	Bone 1998; present thesis
	Thaliacea	Pyrosomatida		*Pyrosoma atlanticum*	Present thesis
		Doliolida		*Doliolum nationalis*	Present thesis

Table1. List of tunicate species considered in the cladistic analysis.

Character coding

The formal coding of the characters is given in Tables 2 and 3; for all the characters, 1 denotes presence and 0 absence. The anatomical references used are listed in Table 1 for tunicates; for other species, references are cited in the previous paragraph (Taxon sampling). Figure 3 presents schematic drawings summarizing the main features of considered secondary sensory cells.

	Character definition
1	Single type of secondary sensory cells (present = 1, absent = 0)
2	Secondary sensory cells with a single cilium (monociliary) (present = 1, absent = 0)
3	Secondary sensory cells with two cilia (biciliary) (present = 1, absent = 0)
4	Secondary sensory cells with more than two cilia (pluriciliary) (present = 1, absent = 0)
5	Cilia in multiciliary sensory cells of same length (0) / different lengths (1)
6	Microvilli or stereovilli on sensory cells (present = 1, absent = 0)
7	Microvilli on monociliary sensory cells (present = 1, absent = 0)
8	Stereovilli on monociliary sensory cells (present = 1, absent = 0)
9	Cilium of monociliary sensory cell surrounded by a ring of microvilli (0) or cilium eccentric to microvilli (1)
10	Microvilli on pluriciliary sensory cells (present = 1, absent = 0)
11	Cilia in pluriciliary sensory cells in a single line (0) or in multiple lines (1)
12	Accessory secretory cells in coronal organ (present = 1, absent = 0)
13	Supporting cells form a wall or crest alongside the coronal organ (present = 1, absent = 0)
14	Electron dense granules in sensory cells (present = 1, absent = 0)
15	Width of coronal organ uniform along oral rim (0) or wider at certain areas (1)
16	Accessory centriole in sensory cells (present = 1, absent = 0)
17	Tentacles or flaps present (present = 1, absent = 0)
18	Tentacles simple (0) / branched (1)
19	Secondary sensory cells in continuous row (present = 1, absent = 0)

Table 2. Definition of characters used for the construction of the morphological character matrix. See text for details.

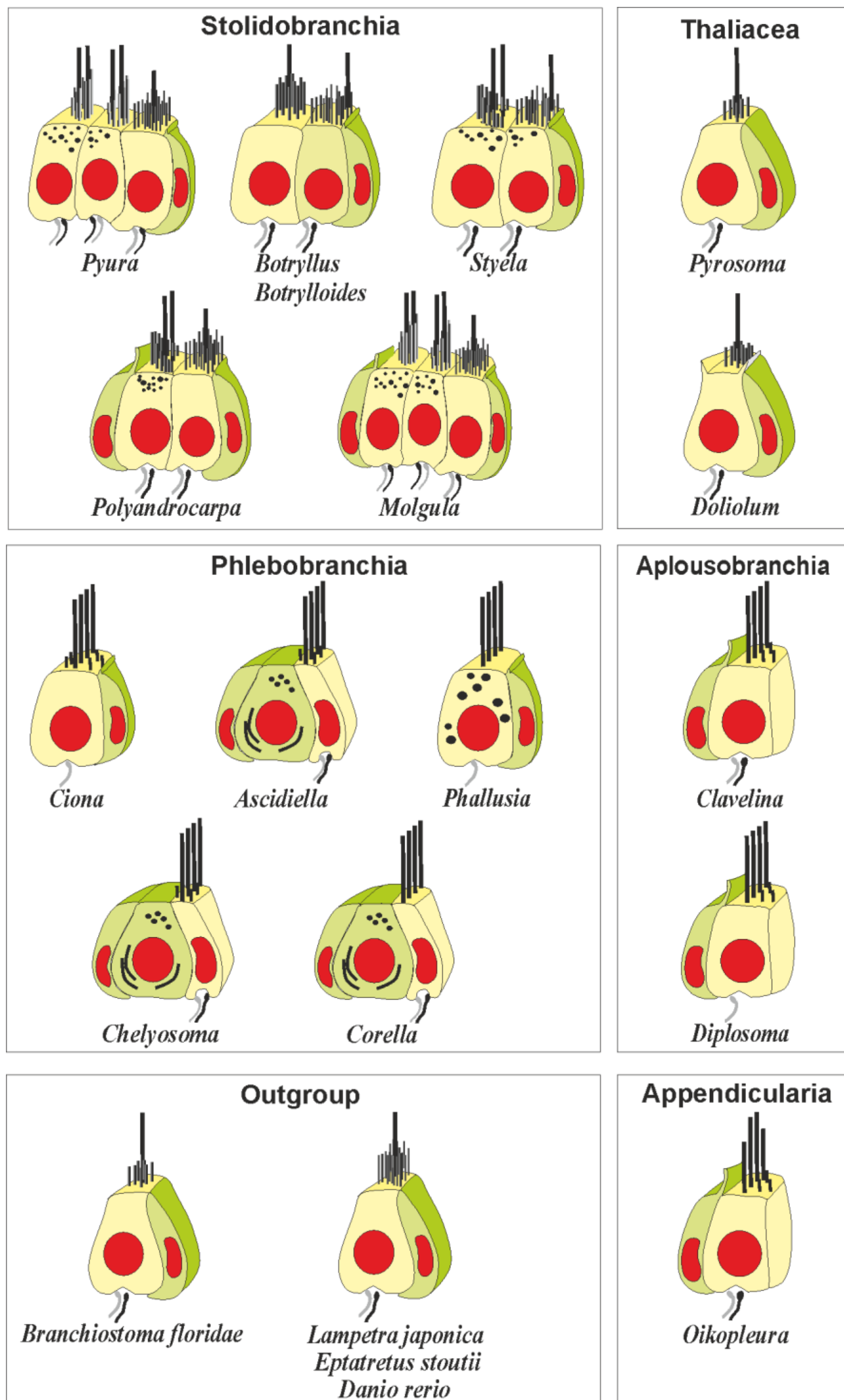

Fig. 3. Schematic depictions of secondary sensory cells of representatives of all tunicate suborders (Stolidobranchiata, Phlebobranchiata, Thaliacea, Aplousobranchiata, and Appendicularia,) and of outgroups (*Branchiostoma floridae, Lampetra japonica, Eptatretus stoutii, Danio rerio*). Microvilli are drawn as shorter sticks, compared to stereovilli, and cilia are represented by the longest sticks. Light green: supporting and secretory cells; light yellow: secondary sensory cells.

1. Single type of secondary sensory cells (present = 1, absent = 0)

The tunicate coronal organ is generally constituted of a single type of secondary sensory cells; however, stolidobranch ascidians may possess two or three different types of mechanoreceptors. For example, *Molgula socialis* exhibits a very complex condition. In this species, three different types of ciliated sensory cells have been identified: cells with a single cilium central to a group of short microvilli (type 1), and two kinds of cells bearing a more complex apical structure, composed of two long cilia accompanied by a group of stereovilli graded in length; stereovilli may form a crescent (type 2) or a complete ring around the two cilia (type 3). Cells follow a characteristic arrangement: types 2 and 3 are located towards the proximal side of tentacles, mostly exposed to inflowing water; type 1 is located more peripherally.

Amphioxus oral spines, and the lateral line organ of the three vertebrate species show a single type of sensory cells.

2. Secondary sensory cells with a single cilium (monociliary) (present = 1, absent = 0)

All considered chordates, except enterogon ascidians and the two *Oikopleurid* species, possess sensory cells with a single cilium. It is noteworthy, however, that in *Ciona intestinalis* (an enterogon species) differentiating coronal sensory cells reach the multiciliated state starting from a monociliated immature cell (Chap. 3 present thesis). This ontogenetic aspect of the transitory monociliated condition has not been considered in this phylogenetic analysis.

3. Secondary sensory cells with two cilia (biciliary) (present = 1, absent = 0)

Stolidobranch species, except for *Botryllus schlosseri* and *Botrylloides leachi* (belonging to the same subfamily Botryllinae), possess biciliated sensory cells. In these genera, the couple of cilia is surrounded by a crescent or a ring of graded in length stereovilli. The row of cilia is parallel to the coronal organ axes.

4. Secondary sensory cells with more than two cilia (pluriciliary) (present = 1, absent = 0)

Enterogon ascidians and the two *Oikopleurid* species present numerous cilia per cell. Cilia are not randomly distributed to form a bundle, but constitute oriented rows, parallel to coronal organ/circumoral ring arrangement.

5. Cilia in pluriciliary sensory cells of same length (0) / different lengths (1)
Among species with multiciliated secondary sensory cells, *Oikopleura dioica* and *Oikopleura albicans* display the unique feature of cilia of different length, orderly arranged: cilia are shorter toward the cell edges, whereas they are longer in the center. This organization confers a wavy aspect to the circumoral ring.

6. Microvilli or stereovilli on sensory cells (present = 1, absent = 0)
With few exceptions (genera *Phallusia* and *Corella*), the apical surfaces of secondary sensory cells possess microvilli or stereovilli; these two apical specializations are never contemporary present in a same sensory cell.
In some vertebrate species, the role of stereovilli in stimuli transduction has been clarified (Gillespie, 1995; Thurm et al., 1998): stereovilli are linked each other and with the cilium (where present) by fibrillar links representing the structural device for transduction of the stimulatory force acting on the cilium to the site of mechanosensitive ion channels. In stolidobranch ascidians, extracellular radial filaments connecting the cilium/a to the surrounding stereovilli have been described (Burighel et al., 2003; Caicci et al., 2010), although the mechanism of signal transduction is not known. It is noteworthy that a loose fibrillar matrix is generally present also among microvilli and cilia.

7. Microvilli on monociliary sensory cells (present = 1, absent = 0)
Generally, monociliated secondary sensory cells show short, unbranched microvilli. Lamprey and zebrafish differ in possessing stereovilli.

8. Stereovilli on monociliary sensory cells (present = 1, absent = 0)
Stereovilli associated to a single cilium have been described in hair cells of the lateral line organ in lamprey and zebrafish; this condition was not found in hagfish, amphioxus and tunicates. Stolidobranch ascidians are characterized by stereovilli in their coronal apical bundle, but they are associated to a couple of cilia.

9. *Cilium of monociliary sensory cell surrounded by a ring of microvilli (0) or cilium eccentric to microvilli (1)*

Usually microvilli are short, of equal length, and form a corolla surrounding a central cilium. In *Botryllus schlosseri*, some coronal sensory cells possess an eccentric cilium, placing the cilium lateral with respect to the group of microvilli.

10. *Microvilli on multiciliary sensory cells (present = 1, absent = 0)*

The presence of microvilli is not constant in multiciliary sensory cells. Some enterogon ascidians (*Clavelina*, *Ciona*, *Ascidiella*, *Chelyosoma*) show short, not branched microvilli, accompanying a single row of cilia; differently, in *Diplosoma*, *Phallusia* and *Corella*, microvilli are absent. Interestingly, in *Ciona intestinalis* development, microvilli undergo a dynamic process of maturation: in early development they are relatively long with respect to the cilium, then they become branched, and finally they lose their ramification again while the cilium elongates (see chapter 3 in present thesis).

In *Oikopleura albicans*, a number of short microvilli accompany the cilia of sensory cells located on the ventral lip, whereas those on the dorsal lip lack this specialization.

11. *Cilia in multiciliary sensory cells in a single line (0) or in multiple lines (1)*

In coronal sensory cells of enterogon ascidians, cilia of equal lengths are aligned to form a single line; differently, *Oikopleura* presents a number of ciliary lines per cell, cilia have different lengths and result in the wavy arrangement of the sensory organ.

12. *Accessory secretory cells in coronal organ (present = 1, absent = 0)*

Three enterogon ascidian genera, *Ascidiella*, *Chelyosoma,* and *Corella*, possess a more complex coronal organ that might be defined as “compound”. In these taxa the coronal organs consist of ciliated sensory cells flanked by secretory cells. The latter face the middle of the tentacles; they do not form synapses with the nerve fibres that contact the ciliated sensory cells; they appear to be involved in protein synthesis, as evidenced by the presence of numerous, large cisternae of rough endoplasmic reticulum and Golgi complexes. The strict association of

secretory cells with ciliated sensory cells suggests that the secretion mechanism might be activated by sensory cell stimulation; however, the exact role of these cells has not been clarified by means of physiological studies.

13. Supporting cells form a wall or crest alongside the sensory organ (present = 1, absent = 0)

In some ascidians and *Oikopleura*, supporting cells possess an expanded, apical lamina delimiting a sort of canal, in which the sensory cilia and stereovilli are found. In *Molgula*, supporting cells are also polarised with respect to organ orientation: the inner supporting cells (facing the longitudinal axis of the tentacle, hence mostly exposed to inflowing water), possess an expanded, apical lamina limiting the apical sensory structures, whereas the outer supporting cells have a shorter, irregular apical lamina. It has been hypothesised that the cytoplasmic laminae of supporting cells are a functional device to maximize the water flow against the hair bundles, facilitating the maintenance of laminar flows on them for optimal perception of particles entering the branchial basket.

14. Electron dense granules in sensory cells (present = 1, absent = 0)

Some ascidians species exhibit sensory cells with cytoplasmic granules of different type (glycogen-like granules, multi-vesicular bodies and secretory granules). Their role is not known.

15. Width of sensory organ uniform along oral rim (0) or wider at certain areas (1)

Molgula and *Pyura* have a particularly complex coronal organ, because it extends on branched tentacles and it is constituted by a variable number of rows of sensory cells: generally, in the basal part of a tentacle branch the coronal organ exhibits few rows of sensory cells; the number of rows increases toward the tentacle branch apex.

16. Accessory centriole in sensory cells (present = 1, absent = 0)

In many of the considered species, sensory cilia possess a basal body in which two centrioles are recognizable.

17. Tentacles or flaps (present = 1, absent = 0)

All ascidians possess a crown of tentacles at the base of the oral siphon. In thaliaceans, *Pyrosomas* has a dozen of flaps and a single ventral tentacle, whereas *Doliolum* possesses only flaps. Amphioxus has oral tentacles. *Oikopleura* does not present tentacles or flaps, but the mouth is delimited by two lips. Vertebrates do not exhibit tentacles or flaps.

18. Tentacles simple (0) / branched (1)

Only the genera *Molgula* and *Pyura* possess branched tentacles. In other tunicates and in amphioxus, if present, tentacles are not branched and usually cylindrical in cross section.

19*. Secondary sensory cells in continuous row (present = 1, absent = 0)*

The tunicates coronal organ is constituted of sensory cells, which are adjacent to each other along the entire coronal organ; differently, oral spines in amphioxus and neuromasts in vertebrates are formed by focalized groups of sensory cells.

Phylogenetic Analysis

We coded the 19 morphological characters listed above (Table 2) for the 16 tunicate genera (Table 1) and completed the matrix with scorings for the four outgroup species, the cephalochordate *Branchiostoma floridae* and the vertebrates *Lampetra fluviatilis*, *Eptatretus stouti*, and *Brachydanio rerio* (Table 3). 17 characters were parsimony informative and two characters were autapomorphic for *Oikopleura dioica* after we pruned a second species of the genus *Oikopleura* (*O. albicans*) from the taxa list, because all character states except character 6 (*Microvilli or stereovilli on sensory cells*, see there) were identical in the two *Oikopleura* species and we aimed for a more balanced ratio between taxa versus characters. The branch and bound analysis in PAUP* (4.0 b10) (van Name, 1945; Monniot, 1965; Monniot and Monniot, 1978; Kott, 1985; Monniot and Monniot, 1988; Kott, 1990; Monniot and Monniot, 1990; Kott, 1992; Monniot et al., 1992; Rodrigues et al., 1998; Kott, 2001; Stach, 2007; Nakashima et al., 2011) resulted in 158 equally parsimonious trees with a tree length of

TL=26, a consistency index of CI=0.71, and a rescaled consistency index of rCI=0.64. As a summary of the most parsimonious trees, the 50%-majority-rule-consensus-tree in Fig. 4 shows a strongly supported monophyletic Stolidobranchia, a strongly supported monophyletic Molgulidae plus Pyuridae, a strongly supported Vertebrata, a less strongly supported group consisting of Enterogona plus *Oikopleura*, and a weakly supported monophyletic Tunicata. Thus, despite the limited nature of the phylogenetic analysis, being restricted to characters derived exclusively from the oral secondary sensory cells and their supposed homologues, our phylogenetic analysis recovers major clades traditionally recognized within Tunicata and Chordata.

Taxa	**Character state**																		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Botryllus	0	1	0	0	-	1	1	0	1	-	-	0	1	0	0	1	1	0	1
Botrylloides	0	1	0	0	-	1	1	0	1	-	-	0	1	0	0	1	1	0	1
Styela	0	1	1	0	0	1	1	0	0	-	-	0	1	1	0	1	1	0	1
Polyandrocarpa	0	1	1	0	0	1	1	0	0	-	-	0	1	1	0	1	1	0	1
Molgula	0	1	1	0	0	1	1	0	0	-	-	0	1	1	1	1	1	1	1
Pyura	0	1	1	0	0	1	1	0	0	-	-	0	1	1	1	1	1	1	1
Clavelina	1	0	0	1	0	1	-	-	-	1	0	0	1	0	0	0	1	0	1
Diplosoma	1	0	0	1	0	1	-	-	-	0	0	0	1	0	0	0	1	0	1
Ciona	1	0	0	1	0	1	-	-	-	1	0	0	0	0	0	1	1	0	1
Ascidiella	1	0	0	1	0	1	-	-	-	1	0	1	0	0	0	0	1	0	1
Phallusia	1	0	0	1	0	0	-	-	-	0	0	0	0	1	0	0	1	0	1
Chelyosoma	1	0	0	1	0	1	-	-	-	1	0	1	0	0	0	0	1	0	1
Corella	1	0	0	1	0	0	-	-	-	0	0	1	0	0	0	0	1	0	1
Oikopleura	1	0	0	1	1	0/1	-	-	-	1	1	0	1	0	0	0	0	-	
Pyrosoma	1	1	0	0	-	1	1	0	0	-	-	0	0	0	?	0	1	0	1
Doliolum	1	1	0	0	-	1	1	0	0	-	-	0	0	0	0	0	1	0	1
Branchiostoma floridae	1	1	0	0	-	1	0	0	0	-	-	0	0	0	-	1	1	0	0
Lampetra japonica	1	1	0	0	-	1	0	1	-	-	-	0	0	0	-	0	0	-	0
Eptatretus stoutii	1	1	0	0	-	1	1	0	-	-	-	0	0	0	-	?	0	-	0
Dario rerio	1	1	0	0	-	1	0	1	-	-	-	0	0	0	-	?	0	-	0

Table 3. Morphological character matrix used for cladistic analysis.

This result demonstrates that the morphological variation seen in sensory organs based on secondary sensory cells contains phylogenetic information.

Several uncontroverted synapomorphies are reconstructed for the lineages of the monophyletic clades recovered within Tunicata (Fig. 4). In the stem lineage of Enterogona plus *Oikopleura,* secondary mechanoreceptor cells became multiciliated (character # 4, Fig. 4), whereas in the stem lineage of Stolidobranchia secondary mechanoreceptors cells diversified and more than a single type of such cells is present in the coronal organ (character # 1, Fig. 4). This diversification of cell types is then accentuated again in the stem lineage of botryllid species, where the position of the cilium in relation to the microvilli diversified (character # 9, Fig. 4). In the stem lineage of the solitary styelids, molgulids, and pyurids, secondary mechanoreceptor cells became biciliated (character # 3, Fig. 4). Thus we infer a diversification of secondary mechanoreceptor cells in stolidobranch ascidians.

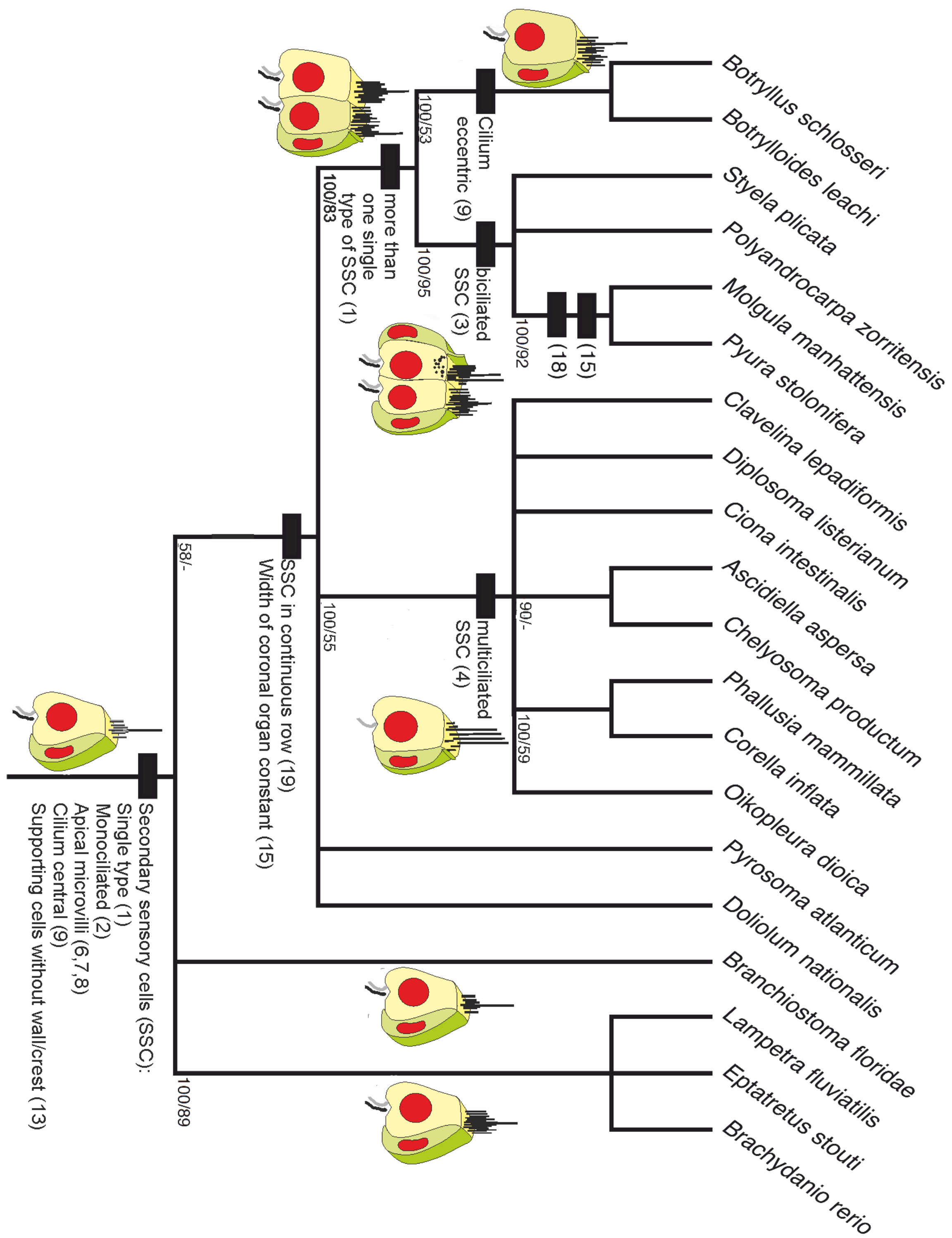

Fig. 4. 50%-majority-rule-consensus-tree obtained from the PAUP* version 4.0b10. Major apomorphic changes are indicated along the branches by black rectangles, short descriptions of the character with corresponding characters number in bracket). Characteristic schematics correspond to those in Fig. 3. Numbers indicate percentage of occurrences in the 50%-majority-rule-consensus-trees/jackknife percentages from 100 replicates with 30% character deletion.

DISCUSSION

The circumoral secondary sensory cells of appendicularians

Our morphological study suggests that appendicularian secondary sensory cells constitute the homologue of ascidian coronal organ, on the base of their corresponding position, morphology, and function. In the two analyzed oikopleurid species, the circumoral ring is located around the oral aperture, in the ectodermal tissue anterior to the pharynx; in ascidians, the coronal organ has a similar position, being located around the mouth (the oral siphon) aperture, in the ectoderm anterior to the pharynx (Manni et al., 2004; Burighel et al., 2011). Moreover, the embryonic primordia of both coronal organ and circumoral ring express orthologs of vertebrate placode genes (Basham et al., 2005; Mazet et al., 2005).

The circumoral ring cells are directly exposed to the inhalant water flow because they are not covered by the matrix constituting the external "house", the sheath similar to tunic in composition (Bone, 1998; Fenaux, 1998), in which they live and which serves as a filtration apparatus (Burighel et al., 2003; Manni et al., 2006). Water is pumped through the house by undulatory movements of the tail and at the same time a ciliary current draws food particles into the mouth. The food is trapped in mucus and ingested while the water passes out through two ventrolateral stigmata. This additional characteristic renders the circumoral cells comparable in position to that of coronal organ: indeed, in ascidians and thaliaceans, the tunic layer envelopes the entire external animal surface, including part or the inner wall of oral aperture, but the tunic ends just anterior of the position of the coronal organ, so that coronal sensory cells are directly exposed to the water current (Burighel et al., 2003; Mackie and Burighel, 2005; Manni et al., 2006). This is different from what generally occurs in mechanoreceptor organs based on primary sensory cells, in which sensory elements are covered by the outer tunic, or by cupulae (Caicci et al., 2010; Mackie and Burighel, 2005).

Both circumoral cells and coronal cells are secondary receptors; synapses were clearly identified at the base of coronal sensory cells (Burighel et al., 2011), though the evidence is less obvious in the case of the base of the circumoral ring

cells (see Figure 1 K). Nevertheless, axons extending directly from the base of circumoral ring cells were never found (Bone, 1998; Olsson et al., 1990). Finally, the circumoral ring and the coronal organ share similar mechanoreceptor function linked to the alimentary activity: they are responsible for the expulsion of unwanted particles entered the pharynx with the water flow. In ascidians this response is the typical squirting, with muscles involvement. In appendicularians, no muscles reaction or squirting behaviour occur, but the reflex consists in ciliary reversals that cause beating arrest, followed by changing in ciliary beating with water flow inversion and expulsion of particles (Bone, 1998; Galt and Mackie, 1971). A peculiar feature of circumoral ring cells is the arrangement of their apical cilia. The multiple cilia are of different lengths in a cell, the shorter toward the cell border, the longer in the center. This organization confers a wavy aspect to the circumoral ring and has never been observed before in tunicates, representing a novel form of organization among the wide variability of apical structures in secondary sensory cells.

We propose that the appendicularian oral sensory system is homologous to ascidian and thaliacean coronal organ.

Phylogenetic information inferred by tunicate secondary sensory cells

While traditional taxonomy of tunicate taxa is highly refined and the respective literature is substantial (e.g., (Stach and Turbeville, 2002) phylogenetic analyses of morphological characters of tunicate taxa are rare and problematic. For example, Moreno and Rocha (2008), presented a cladistic analysis of tunicate taxa on the level of traditionally recognized families. With the difficulties associated with coding for higher taxa and the principal reliance on characters traditionally used in this study, the resulting phylogeny was poorly resolved, with merely Stolidobranchia and Aplousobranchia, of the traditionally recognized higher tunicate taxa, being recovered monophyletic. Another attempt in cladistically analyzing morphological characters was published by Moreno and Rocha (2008), but these authors predominantly focused on the taxon Aplousobranchia. Our present phylogenetic analysis is not intended as a comprehensive cladistic analysis of tunicate taxa, but as a preliminary test for

phylogenetic information content in a recently discovered sensory system (Stach and Turbeville, 2002). The formal cladistic analysis of our data matrix is congruent with traditional taxonomy and other morphological analysis in supporting the monophyly of Stolidobranchia (Delsuc et al., 2008; Govindarajan et al., 2011; Stach and Turbeville, 2002; Tsagkogeorga et al., 2009; Turon and Lopez-Legentil, 2004; Zeng and Swalla, 2005). This indicates that the characters coded do indeed contain phylogenetic signal and could therefore be useful in future attempts to cladistically resolve tunicate phylogeny based on a broader character sampling. Comparison to some recent molecular phylogenies shows that the monophyly of Stolidobranchia is about the only corner stone recovered in most molecular systematic studies, whereas the position of Appendicularia is extremely uncertain and neither the monophyly of Phlebobranchia, Aplousobranchia, Thaliacea, or Enterogona are unambiguously supported (Fiala-Médioni, 1978; Petersen, 2007; Petersen and Svane, 2002). Thus, interestingly, our cladistic analysis, albeit limited to a single organ system, mirrors the general picture seen in many recent molecular studies, strongly indicating the presence of phylogenetic information in our data.

Evolutionary implications

The phylogenetic hypothesis derived from the analysis of morphological characters pertaining to oral secondary sensory cells implies some interesting character transformations. Most notably there is a diversification of sensory cell types in the stem lineage of Stolidobranchia from a single cell type as the plesiomorphic condition in the ground pattern of Tunicata (see Fig. 3). This diversification coincides with a considerable elaboration of the branchial basket in the form of internal longitudinal blood vessels and folds that improve the filtration efficiency by increasing the surface area of the branchial basket (Burighel and Cloney, 1997; Godeaux et al., 1998). Within Thaliacea, salps have probably secondarily lost the coronal organ and, contrary to other thaliaceans, no secondary sensory cells are found around the mouth opening. It is interesting that here again a drastic change of feeding mode coincides with this loss of the coronal organ: contrarily to all remaining thaliaceans, salps feed by using their

substantial body muscles and not cilia to create the feeding current (Godeaux et al., 1998). Coincident with this new mode of feeding, is also a reduction of the branchial basket to a single pair of large gill openings, a dorsal gill bar, and a sturdy mucus net (Bone et al., 1991; Delsuc et al., 2008; Fenaux, 1986; 1998; Flood, 1991; 2003; Flood and Deibel, 1998; Govindarajan et al., 2011; Tsagkogeorga et al., 2009). In contrast, the evolutionary origin of multiciliarity in the secondary sensory cells in Enterogona and Appendicularia cannot be easily related to feeding biology, because this group contains the Aplousobranchiata with a simple branchial basket as well as Phlebobranchiata with more complicated forms. Interestingly Appendicularia, which show a unique morphology of their secondary sensory cells in possessing cilia of different length alongside apical microvilli, have also a unique mode of feeding by using an external house to sort and concentrate particles before they enter their mouths (Delsuc et al., 2008; Govindarajan et al., 2011; Tsagkogeorga et al., 2009). If the distribution of character states revealed in our studies is mapped on recent molecular phylogenies, the inferred character transformations would be essentially the same as sketched here, with the exception that multiciliarity in appendicularians and Enterogona would be interpreted as convergent. Thus, in conclusion, it seems clear that the evolution of sensory cells in the coronal organs of tunicates is intimately correlated with the evolution of the respective feeding system although the precise nature of this correlation remains to be investigated in greater depth.

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Chapter 3

Mechanoreceptor differentiation in a tunicate: insight into the evolution and proliferation of vertebrate hair cells

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Submitted

ABSTRACT

Tunicates are privileged animals in the study of the origin and evolution of vertebrates because they are considered vertebrates' closest living relatives and possess structures, such as neural placodes, classically thought to be exclusive to vertebrates. Neural placodes are transient thickenings of the cranial ectoderm, giving rise to various types of sensory cells, such as hair cells, axonless secondary receptors of the inner ear and the lateral line, which have an apical apparatus typically bearing cilia and stereovilli. To contribute to understanding the evolution of these cells, we analysed their development in *Ciona intestinalis*. In this ascidian, the stomodeum develops into the oral siphon, with its mechanoreceptor coronal organ, which extends at the base of the siphon all along the border of the velum and tentacles. The coronal organ represents the best candidate homolog for the vertebrate octavo-lateralis system. We found that coronal sensory cells can be identified from the moment of metamorphosis, before tentacles form, as cells with short cilia and microvilli. As they develop, they acquire mature features and become pluriciliated, while the associated supporting cells differentiate. The coronal organ grows throughout the animal's lifespan, accompanying growth of the tentacle crown. Anti-phospho Histone H3 immunostaining indicates that both hair cells and supporting cells can proliferate. This finding suggests that both ancestral cell types were able to proliferate and that this property was restricted to the supporting cells of vertebrates and definitively lost in mammals.

INTRODUCTION

Tunicates are invertebrate chordates, considered to be the sister group of vertebrates (Delsuc et al., 2006). They comprise the most frequently studied ascidians which reproduce sexually, producing swimming chordate-like larvae which metamorphose in sessile filter-feeding adults. Thanks to their strategic phylogenetic position, study of tunicates can provide information on how vertebrates, mobile predators with sophisticated brains and sensory systems, evolved from ancestors which were probably filter-feeding animals, such as the present-day non-vertebrate chordates, tunicates and cephalochordates.

Adult tunicates possess a mechanoreceptor, the coronal organ, forming a continuous row running all along the borders of the tentacles and the velum, at the base of the oral siphon (Fig. 1 A-B). It is composed of sensory cells (hair cells) provided with specialised apical bundles for detecting particles entering the siphon with the water flow (Mackie et al., 2006; Burighel et al., 2011). The coronal organ recalls the vertebrate octavo-lateralis system, which includes the lateral line and inner ear sensory organs, characterised by hair cells, *i.e.*, secondary sensory cells that lack their own axons. Vertebrate hair cells are mechanoreceptors that are always accompanied by supporting cells and show a variety of apical sensory apparatuses, which are considered specialisations that better detect various types of mechanical stimuli (*e.g.*, touch, hearing, gravity, acceleration) (Fritzsch et al., 2002).

Secondary mechanoreceptors are not only present in vertebrates but also appear in several groups of invertebrates, and present-day hair cells may be derived from primary ciliated cells (with their own axons) similar to those of choanoflagellates and coelenterates, bearing a single cilium, surrounded by microvilli and expressing transcription factors homologous to those of vertebrates during sensory cell differentiation (Fritzsch et al., 2002; Bouchard et al., 2010; Pan et al., 2012b). From that ancestor cell, three distinct lines of ciliated cells most likely evolved (Burighel et al., 2011): the lophotrochozoan lineage, best represented by mollusc sensory cells, in which secondary sensory cells have many kinocilia and no stereovilli; the ecdysozoan lineage, in which only primary sensory cells with a single cilium are recognisable; and the chordate lineage, in which the secondary sensory cells in all three subphyla (Vertebrata, Tunicata and

Cephalochordata) show a variety of apical specialisations. Although comparison of lophotrochozoan and vertebrate secondary sensory cells generally indicates convergent adaptations in response to similar selective pressures, within the chordate group, debate arises as to whether these sensory cells have a common evolutionary origin and, in particular, whether the coronal hair cells of tunicates and the hair cells in vertebrates derived from a common cell precursor (Manley and Ladher, 2008). However, an evolutive scenario of metazoan sensory cells can be better defined by examining developmental molecular mechanisms because gene expression data on primary and secondary sensory cells suggest that an ancestral sensory cell with its own axon evolved into two distinct cell types: a hair cell that specialised in mechanotransduction and a sensory neuron that connects the hair cell to the central nervous system (Pierce et al., 2008; Bouchard et al., 2010; Pan et al., 2012b).

In vertebrate embryos, the ear components and lateral line derive from a number of placodes, which are thickenings of the cranial ectoderm able to produce several types of cells, such as sensory neurons, secondary sensory cells and supporting cells (Northcutt, 2005; Schlosser, 2010; O'Neill et al., 2012). In non-mammalian vertebrates, these organs not only grow continuously, with turnover of cellular components, but can also regenerate new hair cells after pharmacological or mechanical damage. The sources of new sensory cells are the supporting cells, able to transdifferentiate and divide, as shown in zebrafish, amphibians and chicken (Stone and Cotanche, 2007; Jahan et al., 2010; Warchol, 2011). Conversely, in the inner ear of adult mammals, hair cells cannot regenerate and their loss or damage results in irreversible deafness or hearing impairment (Burns et al., 2012).

In tunicates, the coronal organ is the only structure characterised by secondary sensory cells and, in the embryo, it derives from a placodal-like territory, the stomodeum, involved in the formation of the oral region and expressing the homologs of typical vertebrate placodal genes (*Eya*, *Six*, *Pax, Ptx*) and differentiating sensory cells (coronal hair cells) (Manni et al., 2005; Mazet et al., 2005; Mazet and Shimeld, 2005). Molecular and morphological data are not easy to interpret taken together because the stomodeum seems comparable to the anterior vertebrate placodes (for the expression of *Ptx*), whereas coronal hair cells are similar to vertebrate hair cells, which derive from posterior placodes.

However, coronal hair cells are considered the best candidate for explaining vertebrate hair cell evolution (Manni et al., 2004a; Caicci et al., 2007; Burighel et al., 2011). Furthermore, the cephalochordate amphioxus shows sensory cells that could represent the equivalents of vertebrate hair cells (Candiani et al., 2011): due to the expression of miR-183, a microRNA expressed also by hair cells in vertebrate neuromasts (Pierce et al., 2008), patches of sensory cells (possibly the corpuscles of de Quatrefages) have been individuated in amphioxus rostrum.

To gain more insight into the evolution of the sensory system in chordates, we studied the differentiation of the coronal organ in the solitary ascidian *Ciona intestinalis*, the "mouth" of which is represented by the oral siphon, with a short velum and tentacles of several orders of length, according to the animal's size (Fig. 1 A-B). We followed morphogenetic events, including changes in hair cells, supporting cells and innervation patterns, from the swimming larval stage until the second juvenile stage (Chiba et al., 2004). Because this ascidian grows continuously throughout its lifespan, with a corresponding increase in the size of each structure, we analysed which cell components contribute to coronal organ growth. Our data showed that, unlike vertebrates, both hair cells and supporting cells can divide mitotically and participate in coronal organ growth.

MATERIALS AND METHODS

Animals

Specimens of *Ciona intestinalis* (family Cionidae, order Enterogona) were collected from the Lagoon of Venice and kept in aquaria at 18-20°C; isolated oral siphons from fixed juveniles and adults were used for analysis of cell proliferation and innervation patterns. Animals were selected and fixed from the swimming larval stage to the second ascidian stage, following the method of Chiba et al. (2004) (Fig. 1 C). Briefly, the metamorphosis stage is reached in animals when the body axis can rotate and the rudiments of one oral and two atrial siphons are beginning to invaginate; the following stages (I, IV protostigmata; II, III protostigmata; V protostigmata; VI protostigmata) are based on the appearance of the protostigmata (in *C. intestinalis,* the six protostigmata subdivide to form the many elliptical branchial stigmata of the adult). The second ascidian stage starts

when juveniles bear the rudiments of two atrial siphons fused with each other to form the cloacal siphon, and this stage lasts until the adult stage, in which the gonads are mature. In our laboratory conditions, some aspects of developmental timing differed from those reported by Chiba et al. (2004): in particular, the swimming larva stage was reached one day post-fertilisation (dpf); metamorphosis stage, 3 dpf; I, IV protostigmata stage, 4 dpf; II, III protostigmata stage, 6 dpf; V protostigmata stage, 7-8 dpf; VI protostigmata stage, 10-14 dpf; and second ascidian stage, 15-30 dpf.

Scanning electron microscopy

Juveniles were anaesthetised in MS222 (Sigma-Aldrich, cat. n. A5040) (0.01%) and selected under the stereomicroscope. Specimens were fixed in glutaraldehyde (1.7%, 0.2 M, pH 7.4) and NaCl (1.7%), washed, and post-fixed in OsO_4 (1%) in 0.2 M cacodylate buffer. After dehydration through increasing concentrations of ethanol, they were critical-point dried, sputter-coated with gold, and observed under the scanning electron microscope (Cambridge Stereoscan 260).

Transmission electron microscopy

Specimens of each stage were fixed as described above, washed through propylene oxide and embedded in Epon resin at 60°C. They were then serially sectioned with longitudinal and transverse orientation (L.K.B. and Pabisch ultramicrometers). One-micrometre sections were labelled with toluidine blue, and pictures were taken with a Leica 5000B light microscope with an accessory Leica DFC 480 digital photo camera; ultrathin sections of 80-120 nm were treated with uranyl acetate and lead citrate, and pictures were taken with a FEI Tecnai G^2 transmission electron microscope operating at 100 kV.

Immunohistochemistry

Adults were anaesthetised in MS222 (0.01%) and fixed overnight in freshly prepared MOPS buffer with 4% paraformaldehyde. The oral siphons were then isolated, and the fixative was removed by washing in phosphate buffer solution

(PBS). For visualisation of proliferation sites, specimens were then incubated in a 10% H_2O_2 solution in methanol to eliminate endogenous peroxidase, rehydrated through increasing concentrations of PBS in ethanol, permeabilised with Triton X-100 and digested with Trypsin. Nonspecific binding sites were saturated with BSA and sheep serum in PBS. Samples were incubated overnight at 4°C in the same blocking solution with a mix of two antibodies: polyclonal rabbit anti-phospho-Histone H3 and monoclonal mouse anti-α-tubulin (Table 1). Samples were then washed and incubated with a mix of secondary antibody in the blocking solution: fluorescein-conjugated goat anti–mouse IgG (1:100 dilution) (Calbiochem, 401234) and horseradish peroxidase-linked donkey anti-rabbit IgG (1:1000 dilution) (GE Healthcare Life Sciences, NA934). The TSA Cy5 Plus Kit (PerkinElmer, NEL745E001KT) was used, following data sheet instructions, to detect peroxidase-linked secondary antibody. Lastly, each sample was incubated with DAPI to visualise nuclei. Specimens were mounted in 75% glycerol on slides, observed under a Leica 5000 B fluorescence microscope, and pictures were taken with a Leica DFC 480 digital camera.

Antibody Characterisation

Table 1 lists the primary antibodies used in this study. The polyclonal rabbit anti-phospho-Histone H3 (anti-PH3), which binds cells in active division, recognises Human Histone H3 when phosphorylated at Ser10. On a Western blot of Colcemid treated HeLa acid extract, the antibody specifically detected Histone H3, and a distinct band at approximately 17 kDa was observed (manufacturer’s technical information). The antibody stained cell nuclei following previously reported patterns in the same and other species (Liu et al., 2006; Auger et al., 2010). Monoclonal anti-α-tubulin, used to visualise cilia and nerves, recognises an epitope located in the C-terminal end of the α-tubulin isoform in a variety of organisms (e.g., human, sea urchin, tunicate, *Chlamydomonas*); on a Western blot of Human foreskin fibroblast whole cell extracts, a distinct band at 50 kDa was observed (manufacturer’s technical information). The antibody labelled microtubules according to a pattern identical with previous reports (Piperno et al., 1987; Gasparini et al., 2008).

RESULTS

Anatomy of tentacles and coronal organ

In adults of *Ciona intestinalis* (Fig. 1 A-B), the coronal organ is composed of pluriciliated sensory cells bordering the velum and tentacles, at the base of the oral siphon. The cross-section of each tentacle shows three crests, one median and two lateral; the coronal organ is located on the two lateral crests (Fig. 1 D) (Manni et al., 2006). The epithelium lining the wall of the siphon is continuous with the external epidermis and is covered by the tunic, which terminates as a thin sheet at the base of the velum and tentacles, close to but not covering the coronal organ. The tentacles may be relaxed, hanging down towards the branchial cavity, or held up in such a way that they project across the lumen of the siphon, forming a type of filter. Our results on the gross anatomy of larvae and juveniles confirm previously published data (Chiba et al., 2004; Manni et al., 2005).

Coronal organ development

At the swimming larva stage, the stomodeal placode, the ectodermal thickening representing the rudiment of the oral siphon, is deeply invaginated: at its base, it touches the roof of the pharynx endoderm; posteriorly, it connects to the sensory vesicle through the neurohypophyseal duct (Fig. 1 E-J), from which the adult central nervous system differentiates. The opening of the stomodeum is covered by a thick tunic layer penetrated by a few small apical protrusions from the underlying ectodermal cells. At this stage, the coronal organ cannot be identified. When the larva settles on the substrate, rapid metamorphosis begins (Manni et al., 2005).

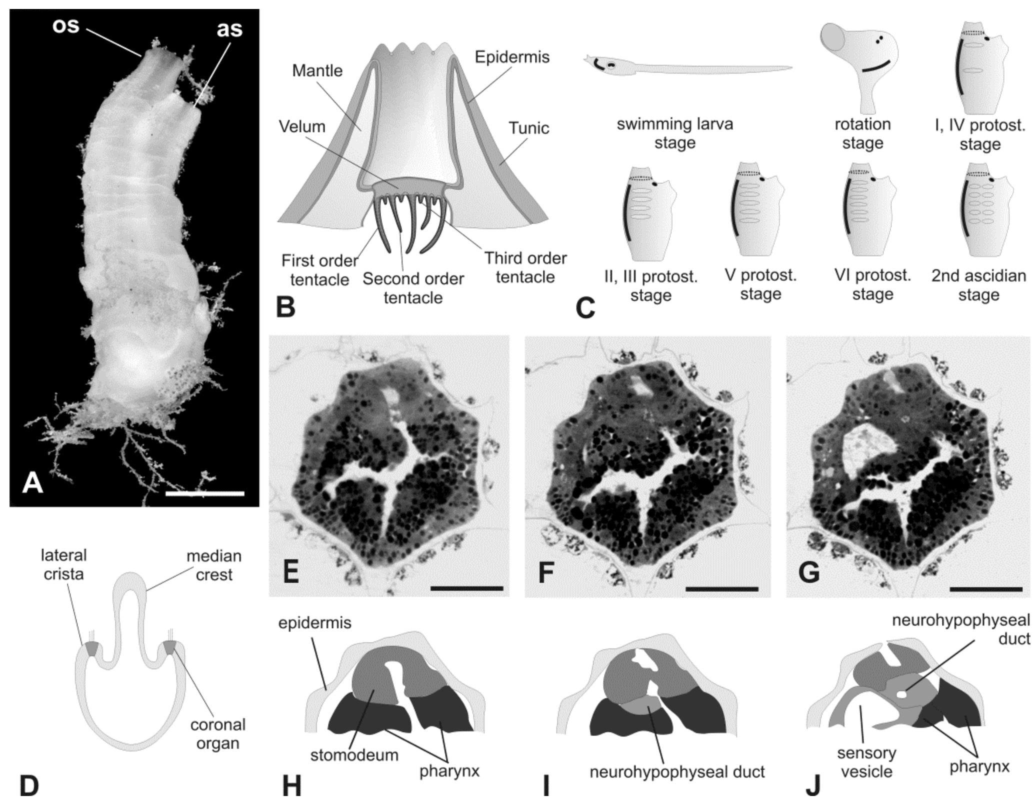

Fig. 1. (**A**) Adult of *Ciona intestinalis*. as: atrial siphon; os: oral siphon. Scale bar: 1200 μm. (**B**) Sketch of the oral siphon of *Ciona intestinalis*. Coronal organ lies on the tentacles and velum. (**C**) Sketch of animals at the stages used in this work. (**D**) Scheme of the cross-section of an adult tentacle, showing the position of the coronal organ in the lateral crests. (**E**-**G**) Three serial cross-sections (1 μm thick) collected every 3 μm of a swimming larval cephalenteron, showing the relationships between the stomodeum, pharynx and neurohypophyseal duct. Pharynx lumen communicates with the stomodeum lumen (E), but the latter is filled with tunic. In F, note the point of aperture of the neurohypophyseal duct at base of the stomodeum. Toluidine blue. Scale bar: 50 μm. (**H-J**) Sketch of figures E-G, in which the areas of interest are drawn for clarity; the tunic is omitted for clarity.

During metamorphosis, the body axes rotate, and all the organs rearrange themselves to reach the definitive adult position: the larval tail is gradually absorbed; the oral siphon displays its main components (musculature, inner and outer walls), but is still completely filled with tunic; the larval nervous system is degenerating and being substituted by the juvenile nervous system (Fig. 2 A-B). The tunic layer stops at the base of the siphon and leaves the ectodermal tissue

exposed to seawater. This represents the presumptive territory of the velum and tentacles (Fig. 2 C) at the boundary with the underlying prebranchial zone. The prebranchial wall is distinguishable in section by its thickness (Fig. 2 A-B); its internal border is marked by the endodermal peripharyngeal band, already easily identified by its long cilia. At this stage, the tentacles and velum are not present as projections of the inner siphon wall (towards the siphon lumen); however, early coronal sensory cells are now identifiable (Fig. 2 C-D), being columnar, with large nuclei and apical surfaces with cilia and occasional microvilli.

At the next stage (I, IV protostigmata), when the juvenile possesses two rows of small stigmata, the body axis acquires its definitive position, with the oral siphon with its oral lobes anteriorly oriented and the endostyle marking the ventral side (Fig. 2 E); the oral siphon is open, and the animal begins to filter through the stigmata cilia; the heart begins beating. At this stage, the velum and four to six short oral tentacles (approximately 20 μm in length) become recognisable. The tentacles are oval-shaped in transverse section, and their walls are formed of a single layer of cubical cells. The sensory cells of the coronal organ, including the characteristic hair cells, can now be recognised on the anterior side of the tentacles (facing the siphon aperture), on their lateral borders and on the velum. These cells have large nuclei and bear a single apical cilium, which is now accompanied by long, branching microvilli (Fig. 2 F). The basal plasmalemma of each sensory cell lies on the basal lamina, which forms a continuous fibrous layer, supporting both sensory and other epithelial cells; neurites run very close to the basal plasmalemma and form the coronal nerve parallel to the sensory cell line (Fig. 2 G). Supporting cells can be identified, adjacent to sensory cells and embracing them, exhibiting a C-shaped profile in section; basally, the supporting cells almost reach the synaptic areas of the sensory cells. Sensory cells and supporting cells extend along the borders of the velum and tentacles to form a continuous row.

At the II, III protostigmata stage, the animal possesses an oral siphon bordered by 6-8 oral lobes with distinct oral pigmented spots. The whole oral area (lobes, siphon and prebranchial region) is more elongated with respect to the previous stage. Six symmetrically arranged tentacles approximately 30 μm long are clearly recognisable, projecting towards the central area of the siphon. The coronal sensory cells have a few cisterns of rough endoplasmic reticulum (Fig. 2 H) and

many free ribosomes; mitochondria are rare. The basal plasmalemma of sensory cells folds slightly to define a type of groove, where the typical extracellular matrix of the basal lamina is absent. Here, neurites are in a close relationship with the sensory cell membrane (Figs. 2 H inset; 2 I). Synapses, identifiable by paired, thickened, electron-dense plasma membranes and small vesicles attached to them on one side of the cleft, are occasionally observed. Most are afferent synapses, clearly identifiable as such by the presence of synaptic vesicles in the sensory cell cytoplasm adjacent to the junction. Efferent synapses, by contrast, are less clearly defined but are still recognisable due to the synaptic vesicles on the neurite side of the junction, making the sensory cells post-synaptic to the neurite in question. The supporting cells now exhibit their definitive aspect (Fig. 2 H): they are curved, to partially enclose the sensory cells; their cytoplasm is rich in ribosomes and mitochondria. Tight junctions between supporting and sensory cells are also recognisable. Both supporting cells and the adjacent epithelial cells bear short apical cilia, together with a thin glycocalyx layer.

At the V protostigmata stage (Fig. 2 J), scanning electron micrographs show that two orders of tentacles are now present, due to the development of new series of tentacles alternating with the first ones: the first-order longer tentacles (the old series) are 70-80 μm long and dorso-ventrally flattened (approximately 10 μm thick); the second-order tentacles (the new series) are approximately 10 μm long (Fig. 2 K-M). The coronal organ extends as a continuous line of sensory cells bordering each tentacle and the velum. Apically, these cells are very rich in branched and unbranched microvilli and a central cilium (Fig. 2 M), as also demonstrated in thin section; synaptic areas become more clearly defined.

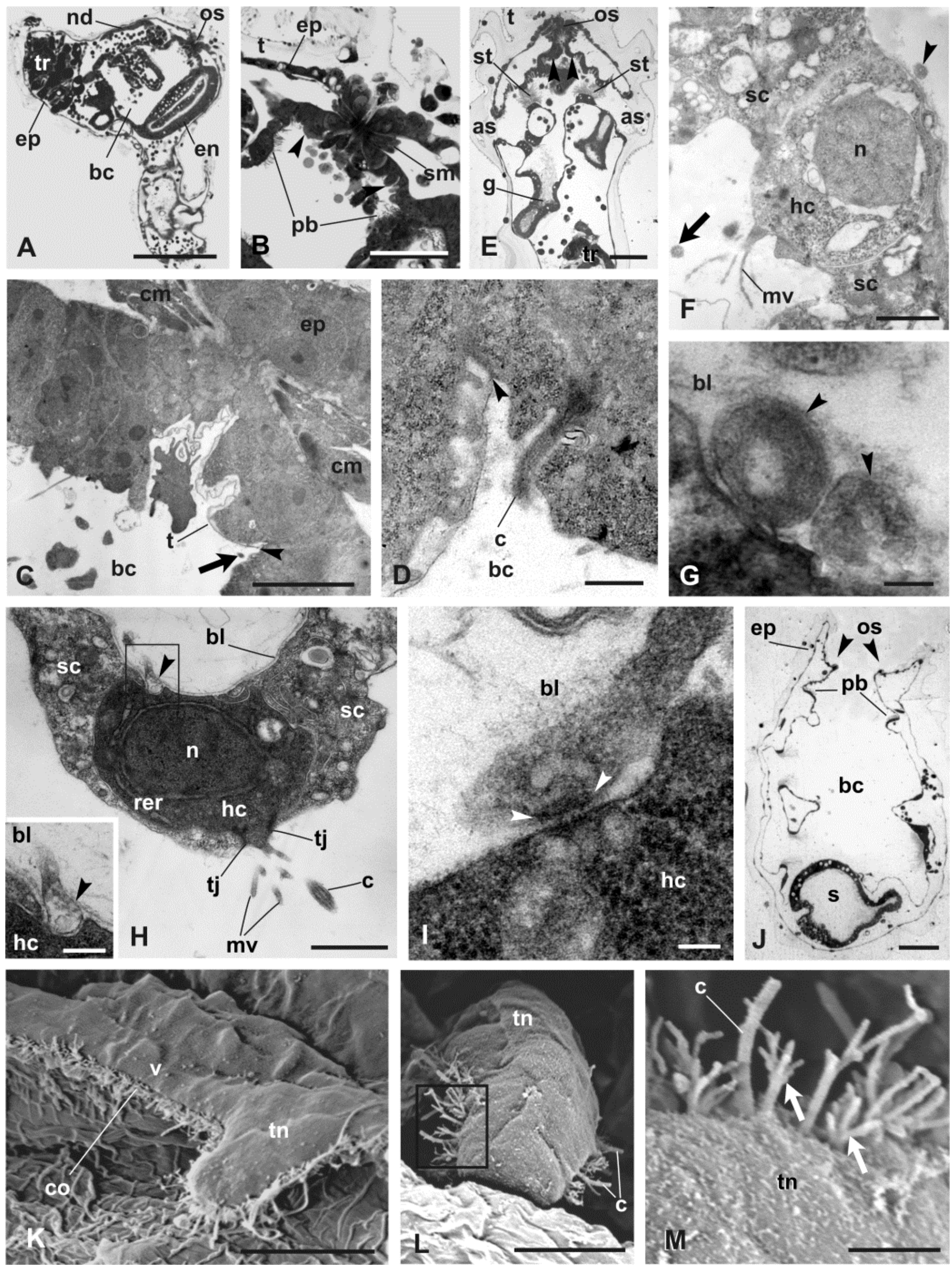
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Fig. 2. Coronal organ development during metamorphosis (3 days post-fertilisation) (**A-D**), and then at 4 days (**E-H**), 6 days (**I-J**) and 8 days post-fertilisation (**K-M**). (A) Sagittal medial section of a metamorphosing animal to show the relationship between the oral siphon (os), neurohypophyseal duct (nd), branchial chamber (bc) and endostyle (en). ep: epidermis; tr: tail remnants. Toluidine blue. Scale bars: 130 μm. (B) Detail of the oral siphon shown in A, not yet open. Arrowheads: prebranchial zone, where the rudiment of the coronal organ is located. ep: epidermis; pb: peripharyngeal band; sm: sphincter siphon muscles; t: tunic. Toluidine blue. Scale bar: 32 μm. (C-D) Electron micrographs of the oral siphon region, showing the prebranchial zone. Internal boundary of the tunic (t) marked by arrowheads. Figure D taken from an ultrathin section very close to that of figure C; arrow in C indicates the section of the cilium belonging to a coronal sensory cell, the same (c) is shown in D. bc: branchial chamber. Scale bar: 5 μm in C; 500 nm in D. (E) Transverse section of a juvenile showing the velum (arrowheads) at base of the oral siphon (os). at: atrial siphon; bc: branchial chamber; ep: epidermis; g: terminal gut; st : stigma; t: tunic; tr: tail remnants. Toluidine blue. Scale bar: 40 μm in E. (F-G) Electron micrographs of a coronal hair cell (hc). Apically, it presents a cilium (arrow) and branched microvillus (mv); note large nucleus (n); a. A neurite (arrowhead) is close to basal plasmalemma. bl: basal lamina; sc: supporting cell. Scale bar: 14 μm in F; 200 nm in G. (H-I) Ultrastructure of a coronal organ in transverse section. Coronal hair cell (hc) with cisterns of rough endoplasmic reticulum (rer). Squared area in I is enlarged in inset to show the groove formed by the basal plasmalemma of a sensory cell; note the presence of a neurite (black arrowhead). Figure I shows a synapse (white arrowhead) between the neurite and basal plasmalemma of hair cell. bl: basal lamina; c: cilium of hair cell; mv: microvilli; n: nucleus; sc: supporting cell; tj: tight junction between the hair cell and supporting cell. Scale bar: 14 μm in H; 350 nm in inset in H; 200 nm in I. (J) Longitudinal section of a juvenile 8 days post-fertilisation passing through the oral siphon (os). Arrowhead: coronal organ on velum; bc: branchial chamber; ep: epidermis; pb: peripharyngeal band; s: stomach. Toluidine blue. Scale bar: 50 μm. (K-M) Scanning electron microscopy of an oral siphon. Coronal organ (co) is recognisable due to a sensory hair bundle. Note cilia (c) surrounded by branched microvilli (arrows). tn: tentacle; v: velum. Scale bar: 10 μm in K; 3 μm in L; 1 μm in M.

At the VI protostigmata stage (Fig. 3 A-E), the sensory cells are characterised by appreciable shortening of the microvilli, which lack branches; in addition, the cilia belonging to adjacent sensory cells are now aligned along a single row. The hair bundle is located in a shallow area defined by supporting cells. Scattered cilia are also present on the apical surface of each tentacle (Fig. 3 B, D). Some lipid droplets occur in the sensory cell cytoplasm; synapses are better defined than in previous stages (Fig- 3 E).

At the second ascidian stage, the juveniles have almost reached their final morphology; typical branchial stigmata, oval in shape and derived from the protostigmata, are present, and the two atrial siphon primordia are completely fused along the midline in a single dorsal opening. SEM images show that third-order tentacles are now identifiable, in the form of button-shaped primordia on the velum (Fig. 3 F-G). At this stage, first- and second-order tentacles also have one median and two lateral crests, as typically found in the tentacles of adults (Fig. 3F). Now, some

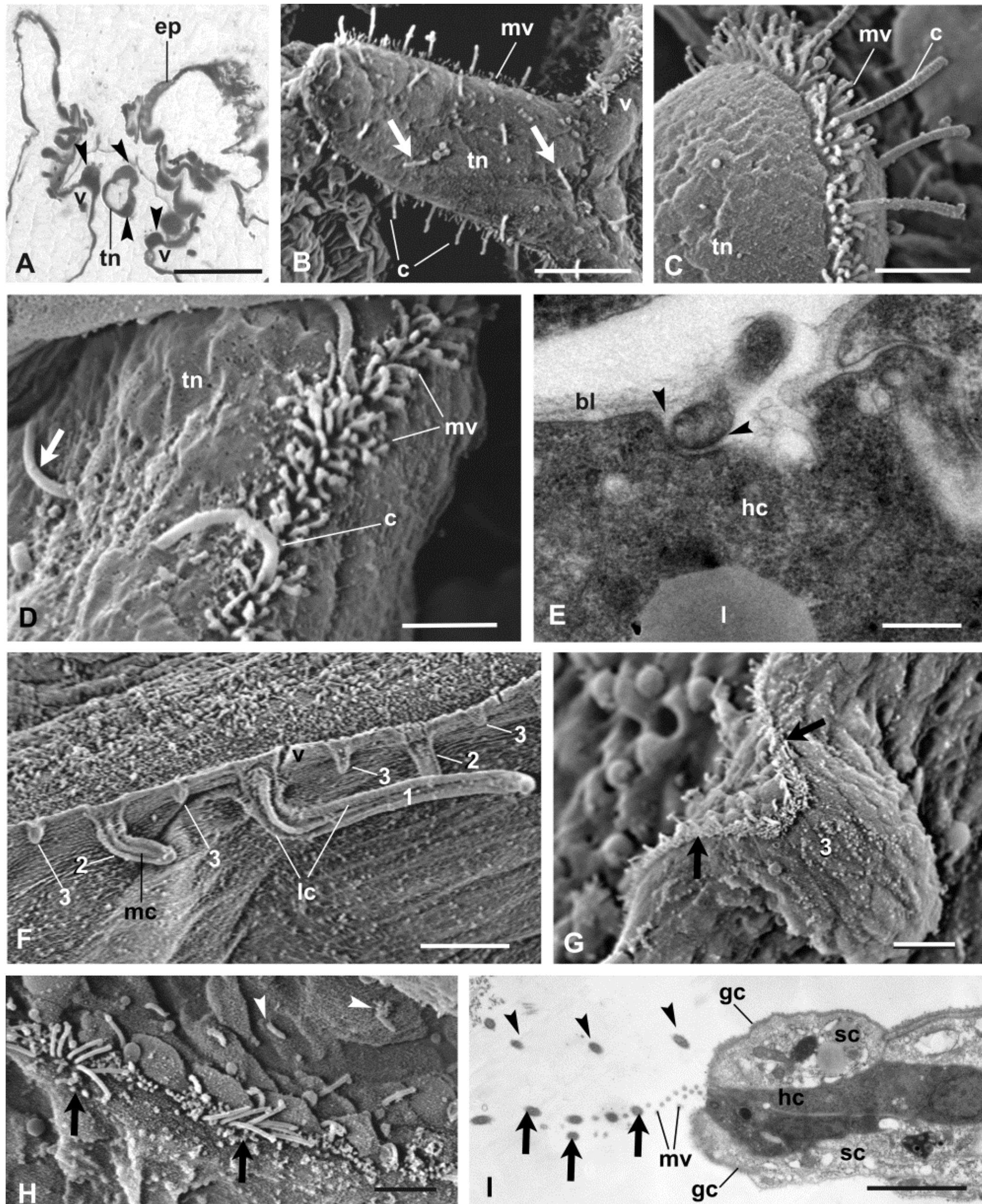

Fig. 3. Coronal organ development during metamorphosis 10-14 days post-fertilisation (**A-E**) and second ascidian stage (**H-I**). (A) Detail of an oral siphon, with the coronal organ (arrowheads) on a velum (v) and a tentacle (tn). ep: epidermis. Toluidine blue. Scale bar: 25 µm in A. (B-D) Scanning electron microscopy of a coronal organ to show short, unbranched microvilli (mv) and coronal cilia (c) arranged in rows. Arrows: cilia belonging to tentacle epithelial cells. tn: tentacle; v: velum. Scale bar: 5 µm in B; 2 µm in C; 1.5 µm in D. (E) Micrograph of basal area of sensory cell (hc) showing lipid droplet (l) and synapse (arrowheads). bl: basal lamina. Scale bar: 250 nm. (F-H) Scanning electron microscope images showing tentacles of different orders: first- (1) and second- (2) order tentacles characterised by two lateral crests (lc) and one median crest (mc). Third-order tentacles (3), enlarged in G. Coronal organ (arrows) on lateral crest of tentacle, due to continuous line of cilia of sensory cells. Scattered cilia (arrowheads) arise from epithelial cells of upper wall of a tentacle. v, velum. Scanning electron microscopy. Scale bar: 100 µm in F; 10 µm in G; 2.5 µm in H. (I) Detail of hair bundle, showing many cilia (arrows) aligned according to coronal organ axes and accompanying hair cells (hc). Arrowheads: cilia belonging to supporting cells (sc). gc: glycocalyx; mv: microvilli. Scale bars: 3 µm.

coronal hair cells are pluriciliated, as demonstrated by scanning images and thin sections, and the cilia are aligned along the axis of the coronal organ (Fig. 3 H-I). The microvilli are very short and similar in length but do not possess any particular orientation with respect to the cilia. Both cilia and microvilli show a thin fibrillar sheath, but links between the cilia and the nearest microvilli, or among adjacent microvilli, such as those typically observed in the hair cell tufts of vertebrates and other tunicates (Burighel et al., 2003), were not identified. The supporting cells possess a thick glycocalyx; the two rows of supporting cells flanking the sensory cells can be distinguished from each other by the single short cilium present on the cells in the row located towards the middle of the tentacle (Fig. 3 I).

On the lateral crests, the coronal organ soon reaches its mature configuration, with all hair cells displaying a single row of 5-10 long cilia, accompanied by a very few short microvilli, all the same length, and showing no particular arrangement with respect to the cilia (Manni et al., 2006). The phases of hair bundle formation are shown in Figure 4.

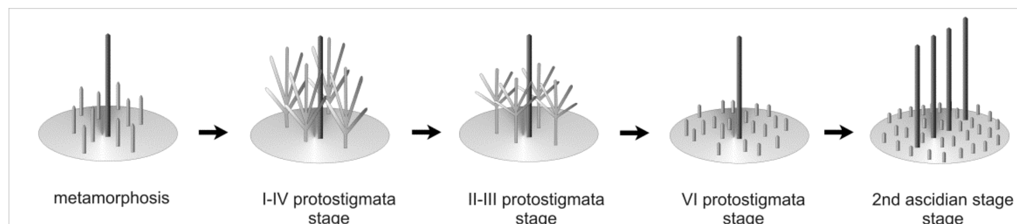

Fig. 4. Sketch showing the main steps in hair bundle formation.

Coronal cell proliferation and innervation pattern

Immunohistochemical techniques were used to verify the presence of any proliferation sites along the tentacles and velum of the oral siphon in juveniles (Fig. 5 A-L). In all samples, we found that a few scattered cells, including both epithelial cells of the tentacle wall and non-epithelial cells of the coronal organ, were positive for the anti-PH3 mitosis marker. This result was verified by visualisation of the same specimens by a triple staining method, *i.e.*, also applying DAPI and anti-α-tubulin, which revealed the nuclei and also gave good views of nerves and cilia.

Comparison between signals showed that, in some cases, two close but not completely divided nuclei were marked by anti-PH3, indicating late karyokinesis (Fig. 5 C-D). Frequently, no apical cilia in dividing cells could be recognised by anti-α-tubulin as, conversely, was always the case for sensory cells, in which the hair bundle was clearly identifiable by the strong signal. However, the anti-PH3 signals were adjacent to those of sensory cells, indicating that mitotic cells were of the supporting type. Two types of mitotic division were observed: i) the mitotic plane was perpendicular to the row of supporting cells, so that the daughter cells were in the same row (Fig. 5 A-D); ii) the mitotic plane was parallel with the row of supporting cells, so that one daughter cell remained among the supporting cells, and the other was directed towards the sensory cell row (Fig. 5 E-H).

In very occasional cases, and only when the coronal organ was observed frontally, mitotic cells appeared among sensory cells. In addition, the overlap of all three signals revealed that dividing nuclei belonged to cells that apically bore the hair bundle and basally were approached by a fibre of the coronal nerve (Fig. 5I-L). Staining with anti-α-tubulin identified nerves in the tentacles and velum (Fig. 6 A-C).

One pair of anterior nerves emerged from the ganglion and entered the pericoronal blood sinus, at the base of the oral siphon, constituting the pericoronal nerves encircling the siphon. A rich nerve plexus is present at the base of the velum, in association with the velar sphincter muscle (Mackie et al., 2006). Bundles of neurites run from it into the tentacle lumen, one under each side of the coronal organ. Although the sensory components of this nerve plexus are not distinguishable from motor ones, in the absence of muscles inside the tentacle, the neurites forming these bundles are presumably dendrites and axons belonging to cell bodies lying in the cerebral ganglion, which are responsible for afferent and efferent synapses, respectively, with coronal sensory cells. Transverse connections between the two bundles are frequently observed.

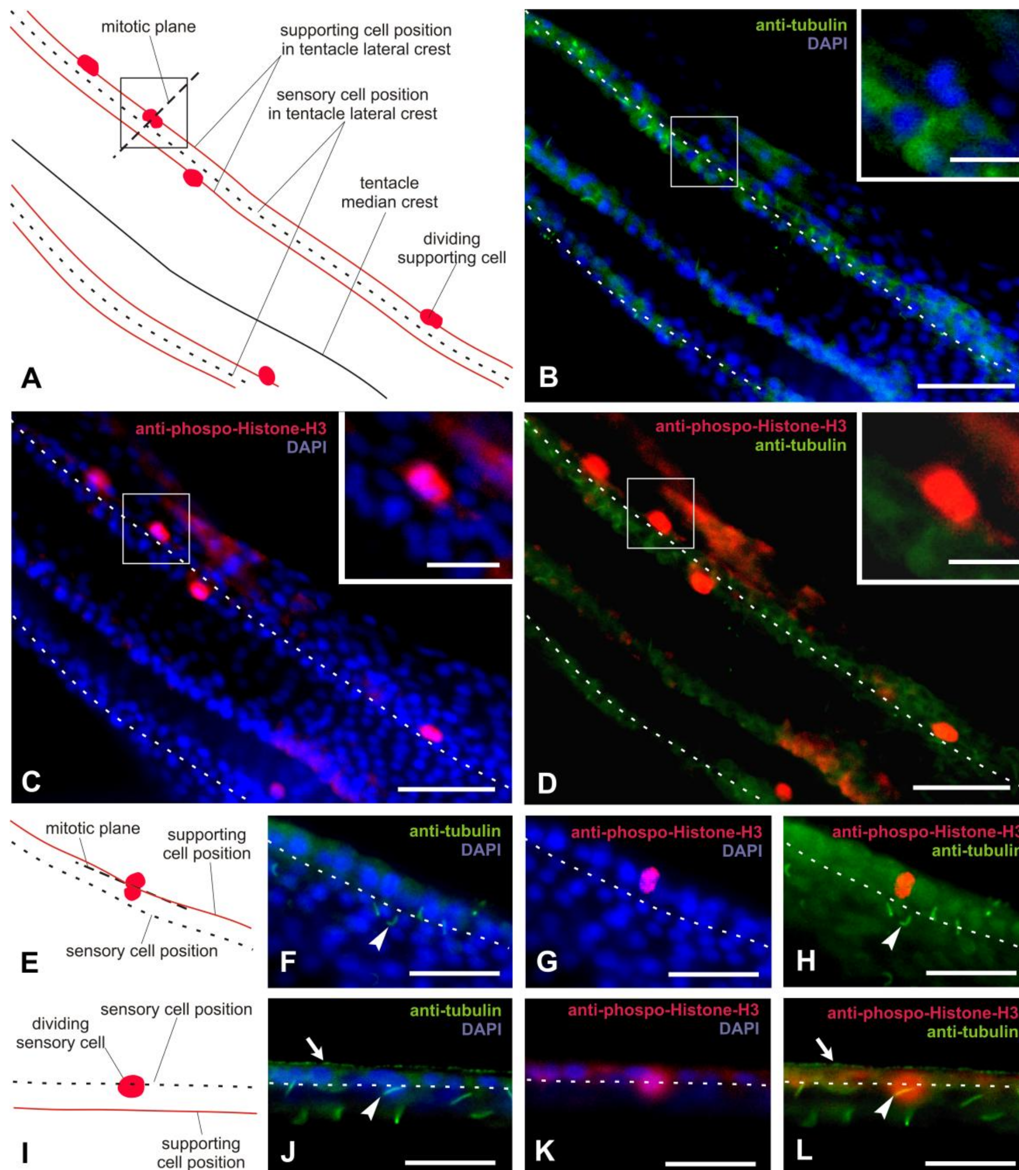

Fig. 5. Immunofluorescence analysis of a coronal organ in flat-mount preparations of an oral siphon, in which proliferating cells were detected with monoclonal phospho-Histone H3 antibody (red), nuclei visualised by DAPI staining (blue) and tubulin in cilia and nerves marked by anti-tubulin antibody (green). Dotted lines in the photographs mark the sensory cell position. (**A-D**) Tentacle area (sketched in A) showing proliferation in the supporting cells. Squared areas in B-D are enlarged in insets and identify one supporting cell dividing along a mitotic plane perpendicular to that of the row of supporting cells. Scale bars: 50 µm in B-D; 10 µm in insets of B-D. (**E-H**) Supporting cell in mitosis (sketched in E), with the division plane parallel to the row of supporting cells, such that one daughter cell becomes a supporting cell and the other becomes a hair cell. Scale bar: 25 µm in F-H. (**I-L**) dividing hair cells (sketched in I). Sub-coronal nerve (arrows) discriminates the sensory cell row from a nearby row of monociliated supporting cells. Arrowheads: cilia belonging to the dividing coronal sensory cell; lc: tentacle lateral crest; mc: tentacle median crest. Scale bar: 25 µm in J-L. B, F and J: merging between anti-tubulin antibody and DAPI signals; C, G and K between anti-phospho-Histone H3 and DAPI; D, H and L between anti-phospho-Histone H3 and anti-tubulin signals.

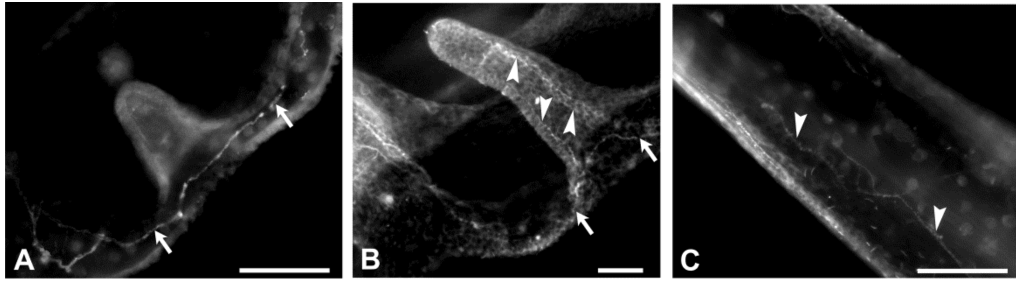

Fig. 6. (**A-C**) Nerves labelled with anti-tubulin antibody. A nerve runs along the base of velum (arrows); its branches enter each tentacle as coronal nerves, running along each side of the tentacle under the coronal organ (arrowheads). Scale bars: 50 µm A-C.

DISCUSSION

How vertebrates evolved from their ancestors and which key innovations led to their ecological success remains an interesting and debated aspect of evolutionary biology. This was especially so after the classical view of chordate phylogeny - which placed cephalochordates close to vertebrates and tunicates at the base of the chordate tree - was reversed (Delsuc et al., 2006), and the hypothesis was proposed that structures considered exclusive to vertebrates, such as neural crests and neural placodes, have a lengthier evolutionary history than previously supposed (Holland and Holland, 2001; Streit, 2001; Manni et al., 2004a; Manni et al., 2004b; Bassham and Postlethwait, 2005; Schlosser, 2005; Donoghue et al., 2008; Jeffery et al., 2008; Graham and Shimeld, 2012). In this respect, comparative and developmental studies on sensory systems in chordates are still contributing to the definition of a complex picture, according to which the derivatives of neural placodes were assembled over a protracted period of time, rather than arising collectively with the vertebrates. The hypothesis that vertebrate hair cells share a common origin with tunicate hair cells is discussed within this scenario, which is strongly supported by molecular data (Pierce et al., 2008; Bouchard et al., 2010; Burighel et al., 2011; Pan et al., 2012b).

Data exploring the hypothesis of the possible common evolutionary origin of vertebrate and tunicate hair cells revealed that, during *Ciona intestinalis* embryogenesis, the oral siphon primordium (i.e., the stomodeum) expresses

genes homologous to those expressed by vertebrate placodes (see Graham and Shimeld, 2012). Stomodeal cell derivatives include both primary and secondary sensory cells: the adult oral siphon is extremely rich in scattered epidermal neurons functioning as mechanoreceptors for perception of near-field vibrations, and it also possesses coronal hair cells, which are dedicated epidermal cells that are sensitive to touch (Mackie et al., 2006; Manni et al., 2006). Although detailed studies of cell lineage are not available for these various types of sensory cells, it is still possible that they first originated in the stomodeal ectoderm, as occurs in vertebrate placode sensory cells, and do not reach their location by migrating from elsewhere.

Comparative analysis of ascidian hair cells (for a review, see Burighel et al., 2011) revealed that the coronal organ may be considered a plesiomorphic feature of this group, being found in all species analysed thus far; moreover, secondary sensory cells with similar functions and locations have also been described in the putatively most basal-living group of tunicate appendicularians (Bone, 1998), suggesting that tunicate hair cells are as ancient as the subphylum.

Hair cell differentiation in tunicates

The first evidence of coronal hair cells occurs at the base of the rudiment of the oral siphon three dpf, before the formation of the tentacles and velum. Hair cells are distinguishable from adjacent cells due to their columnar shape and the presence of a hair bundle, composed of one cilium and microvilli. The region where they are located, the prebranchial zone, is ectodermic in origin, being anterior to the neural gland aperture, which is also ectodermic (Manni et al., 2005; Horie et al., 2011). Early coronal hair cells recall precocious auditory receptors described in birds and several mammal species, which derive from a population of columnar cells of ectodermic origin with an apical apparatus composed of a single central cilium (kinocilium) and numerous microvilli (Tilney and Tilney, 1986; Kaltenbach et al., 1994; Zine and Romand, 1996; Frolenkov et al., 2004). Therefore, the corolla-like configuration of the hair bundle (cilium central to a corona of microvilli), in both tunicates and vertebrates, is the cell prototype from which the various hair cells differentiate during embryogenesis.

However, it also appears to be the ancestral prototype from which distinct lines of ciliated cells may have evolved (Burighel et al. 2011).

The further development of the apical pole of coronal hair cells is characterised by branching of microvilli (4 days post-fertilisation) and their progressive shortening with respect to the cilium (up to 10-14 days post-fertilisation); the length of the microvilli then stabilises. This phase is followed by the cilia arranged in a line, surrounded by short, scattered microvilli (second ascidian stage). The ability to gradually modify the apical sensory tuft displayed by coronal hair cells recalls hair cell differentiation in chicks and mammals, in which many gradual, profound transformations have been documented, mainly in reference to the number, structure and position of microvilli (Frolenkov et al., 2004).

In *C. intestinalis*, hair cell differentiation parallels changes in the entire oral area. In particular, tentacles of different orders are progressively added, according to a stereotyped pattern, which provides for the appearance of new tentacles, which are always intercalated with those already present (Millar, 1953), that acquire the features typical of tentacles in adults, with a central crest flanked by two shorter side-crests, bearing the coronal organ (Manni et al., 2006).

The innervation pattern of the coronal organ has been studied by immunocytochemistry in some ascidian species, with both simple and branched tentacles (Burighel et al., 2001; Mackie et al., 2006; Caicci et al., 2010b). Our results in *C. intestinalis* are in agreement with previous reports, revealing that a nerve (the subcoronal nerve) runs at the base of each row of coronal cells. Thin nerve fibres ultimately reach the coronal sensory cells and form chemical synapses with them. Although synapses have rarely been observed during differentiation, neurites have been shown at the base of hair cells at a very precocious phase (during metamorphosis, three days post fertilisation), before the aperture of the oral siphon and the beginning of filtering activity – at a stage when the juvenile nervous system is still being defined. This early relationship between differentiating sensory cells and nervous system parallels that described in the ascidian *Botryllus schlosseri*, in which a close temporal relationship linking the development of a local nervous network and its target organ was shown (Zaniolo et al., 2002). Our observations in *C. intestinalis* suggest that the nervous

system takes part in the specification and differentiation of sensory cells, as occurs in vertebrate development (Ramekers et al., 2012).

Supporting cells are a common feature of the coronal organ and present various aspects in different ascidians (Burighel et al., 2011). Our data on *C. intestinalis* show that supporting cells in their mature form are polarised with respect to the water flow because supporting cells towards the middle of the tentacles are ciliated differently from the others; their simple organisation reflects the organization commonly found in species of the same order (Manni et al., 2006). During development, supporting cells are recognisable four days post-fertilisation, due to their C-profile embracing the sensory cells, and, in a few days, they achieve their definitive aspect, also showing a thin glycocalyx layer.

Regenerative ability is most likely the 'default condition' of chordate hair cells

C. intestinalis is characterised by the continuous increase in number and length of tentacles during its lifespan. Thus, during tentacle elongation, hair cells must multiply to allow for the increase in length of the coronal organ.

We observed that both epithelial and coronal organ cells proliferate and that both sensory and supporting cells were observed in mitosis in the coronal organ. In this respect, in the ascidian *Pyura haustor*, unequivocal sensory coronal cells with typical features of mitosis were found (Caicci et al., 2010a). In our work, some observations allowed us to define the orientation of the mitotic plane, due to the position of the dividing nuclei. In some cases, the dividing cell was of the supporting type; this finding suggested that daughter cells could give rise to both supporting and hair cells.

The evidence that both hair cells and supporting cells in ascidians can divide allows interesting considerations about the evolution of this feature in chordates. Whereas in birds and mammals the full complement of hair cells is produced during embryogenesis, in fish and amphibians, hair cells are added throughout life. In addition, all non-mammalian vertebrates appear to be capable of regenerating hair cells after injury; when this occurs, regeneration is mediated by the proliferation or transdifferentiation of supporting cells or, frequently, a combination of both mechanisms (Warchol, 2011). The ability to regenerate hair

cells was lost during the evolution of the mammalian ear, for reasons which are only now beginning to be explored (Collado et al., 2011; Sinkkonen et al., 2011; Burns et al., 2012). In view of the phylogeny of the vertebrate ear, it is probable that regenerative ability was the norm for most of the ear's evolutionary history. It has been speculated that the cellular dynamics of more 'primitive' hair cell epithelia resembled those in the sensory organs of the vertebrate olfactory and gustatory systems, in which sensory receptors are constantly being generated, maturing, and dying (Warchol, 2011). Although we cannot exclude the possibility that the mitogenic ability of sensory coronal cells was acquired independently in tunicates, our data are in agreement with the hypothesis that hair cells proliferate in ascidians. This finding prompted us to propose that both hair and supporting cells had this ability in the common ancestor of tunicates and vertebrates and that this ability has been maintained in tunicates but restricted to supporting cells in non-mammalian vertebrates as well as definitely lost in mammals.

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Chapter 4

Inside the *Ciona intestinalis* secondary sensory cells: a case of convergence or homology with the vertebrate hair cells?

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ABSTRACT

Vertebrate hair cell mechanoreceptors have been intensively studied because of their role in hearing, balance and perception, however their evolution within chordates remains to be elucidated. Among the different invertebrate mechanoreceptors invoked as possible homologs of vertebrate hair cells, the tunicate secondary sensory cells seem to be at moment the best candidates. They belong to the coronal organ, a sensory apparatus formed by a continuous row of ciliated cells located at the base of the mouth, which detects particles entering with the inhalant sea water flow. Here we analysed the coronal organ of the ascidian *Ciona intestinalis* both from a genetic and functional perspective, considering genes involved in neural and sensory cell differentiation (Notch, Delta, Hairy/Hes, Atoh, Musashi), and other molecules implicated in neurotransmission (TRP channels and glutamate). We found that the tunicate secondary sensory cells share with the vertebrate hair cells the expression of some genes important for their development and function. Our data suggest that these genes might have been already expressed in such cells in the common ancestor of tunicates and vertebrates, and thus their secondary sensory cells would represent a plesiomorphic feature in the two subphyla.

INTRODUCTION

All living organisms use their sensory systems to communicate with the external environment, receiving stimuli by means of highly specialized sensory receptors. The study of sensory cell evolution is intriguing and debated because it tries to explain how complex sensory systems, such as those of vertebrate, could have originated.

Mechanoreceptors are a subclass of sensory cell, with a polarized collar structure in which they exhibit an apical surface characterized by a ciliated apparatus sensitive to physical stimuli (i.e. vibration, hydrodynamic movements, touch, sound, acceleration and gravity) (Gillespie and Walker, 2001). Mechanosensory cells can be classified in two main groups: primary and secondary receptors. Primary receptors are neurons possessing at the basal membrane an axonal prolongation that allows the direct transmission of the signal to the brain. Secondary receptors do not possess axons, but at the base they are in contact with neuronal axons by means of synapses. It has been hypothesized that secondary sensory cells might derive from primary cells (Fritzsch et al., 2007; Fritzsch et al., 2010; Burighel et al., 2011). Secondary receptors were observed in several groups of organisms: in mollusks for example, but also in the three chordate subphyla: vertebrates, tunicates and cephalochordates. Vertebrate secondary receptors are commonly indicated with the term “hair cell” and are found in the acoustico-lateralis system, which includes the lateral line and inner ear.

In vertebrates these structures arise from the lateral line and otic placodes, which are specialized cranial ectodermal patches of the embryo characterized by the expression of particular genes (e.g. *Eya, Six, Pax*) (Butler, 2000; Streit, 2001; Schlosser, 2006; Schlosser, 2010). Placodes were considered a novelty of vertebrates and according to the “new head hypothesis” their appearance is related to the transition from a filter-feeding to an active predatory lifestyle, with the formation of a compartmentalized brain and the associated complex sensory structures (Northcutt, 2005; Butler, 2006). Embryonic structures resembling placodes were recognized also in tunicates (Bassham and Postlethwait, 2005; Mazet and Shimeld, 2005; Kourakis et al., 2010), among them the stomodeal and

atrial placodes which possess the ability to invaginate and give origin to organs provided with sensory cells and neurons, through the expression of the *Six, Eya, Pax* orthologs genes (Manni et al., 2005; Mazet et al., 2005; Mazet and Shimeld, 2005; Kourakis and Smith, 2007).

In tunicates, the stomodeal placode is the embryonic structure from which the coronal organ develops. This sensory system is unique in tunicates as it is based on secondary sensory cells (Manni et al., 2005). The coronal organ was described for the first time in detail in the colonial ascidian *Botryllus schlosseri* (Burighel et al., 2003), and subsequently its presence was evidenced in all the numerous species of ascidians analyzed (Manni et al., 2006; Caicci et al., 2007; Burighel et al., 2011) and also in thaliaceans and appendicularians (Chaps. 1 and 2 present thesis; Bone, 1998). The coronal sensory cells are always accompanied by supporting cells, and are placed all along the edge of the oral rim (on tentacles, flaps or lobes, or on the lips, depending on the species). Their apical apparatus is turned toward the incurrent water flow (Manni et al., 2006; Caicci et al., 2007), mirroring their function in detection of the particles entering the mouth (Mackie et al., 2006).

Recently an analysis of coronal sensory cell structure and development has been carried out in the ascidian *Ciona intestinalis* (present thesis), evidencing both similarities and differences with vertebrate hair cells, especially from a morphological point of view. *C. intestinalis* adults bear a coronal organ characterized by pluriciliated sensory cells with many short microvilli (Manni et al., 2006) forming a continuous row, located on the tentacles and velum, at the base of the oral siphon. As the animal grows, the number of tentacles increases, and in parallel the coronal organ grows. Mature tentacles contain a blood sinus and are furnished with two lateral and one median crest: the coronal organ is located on the lateral crests, with sensory cells facing the water flow entering the oral siphon.

In consideration of the most recent chordate phylogenies that place tunicates as sister group of vertebrates, (Delsuc et al., 2008; Tsagkogeorga et al., 2009b), discovery of the coronal organ lead us to the hypothesis that tunicate and vertebrate secondary sensory cells might have the same evolutionary origin, and hence represent a plesiomorphic feature of these two groups. In the present study we aim to verify if genes characterizing vertebrate neural and hair cell

differentiation, such as *Notch, Delta, Hairy, Atoh, Musashi*, are present and expressed in *C. intestinalis* coronal sensory cells.

Notch and *Delta* take part in one of the best studied pathways in neural differentiation, being responsible for the lateral inhibition mechanism that produces, for example, the mosaic of hair and supporting cells in the inner ear and lateral line (Haddon et al., 1998; Haddon et al., 1999; Riley et al., 1999; Eddison et al., 2000; Akanuma et al., 2002; Reed, 2004; Daudet and Lewis, 2005).

Hairy, the ortholog of *Hes* in vertebrates, is a basic helix-loop-helix transcription factor acting as a repressor in the *Notch/Delta* pathway, with an important role in the maintenance of the neural stem cells status (Fekete et al., 1998; Kageyama and Ohtsuka, 1999; Kageyama et al., 2008; Tateya et al., 2011); *Ciona intestinalis* possesses three *hairy* genes: *hairy a*, *hairy b,* and *hairy c* (Satou and Satoh, 2005).

Atoh, that acts in the same pathway as an antagonist of *Hairy*, and possesses a role as an activator in hair cell differentiation (Cafaro et al., 2007; Millimaki et al., 2007; Fritzsch et al., 2010; Jahan et al., 2012b; Pan et al., 2012a).

Musashi (*Msi*) is also involved in the same genetic pathway: it is a RNA-binding protein responsible for the post-transcriptional regulation of neural differentiation.

We also considered other markers characterizing mechanotransduction in vertebrate hair cells: the *TRP* channel family has an important role in mechanotransduction, and among these channels the two implicated in hair cell signal transduction are *TRPA* and *TRPN*, whose important role has been widely reported (Corey, 2003; Sidi et al., 2003; Christensen and Corey, 2007; Brierley et al., 2009; Arnadottir and Chalfie, 2010). We further considered the most important excitatory neurotransmitter in mechanotransduction, glutamate, which is the major afferent neurotransmitter of hair cells (Ottersen et al., 1998; Fuchs et al., 2003; Glowatzki et al., 2008; Obholzer et al., 2008).

Our data evidence that secondary sensory cells in vertebrates and ascidians share important neural differentiation markers and some functional features.

MATERIAL AND METHODS

C. intestinalis specimens were collected in the Lagoon of Venice (Italy) and Plymouth sea (U.K.), the adults were kept in aquaria at 18-20°C. They were dissected in order to obtain gametes for the *in vitro* cross fertilization; larvae and juveniles of the first and second stages were fixed for the immunohystochemical and *in situ* hybridization experiments. For RT-PCR, isolated tentacles were collected from adults, rinsed in filtered sea water in order to eliminate possible blood cells, and the RNA was extracted.

RT-PCR.

Tentacles were isolated from about 20 *C. intestinalis* adult oral siphons and the RNA was extracted using RNeasy mini kit (Qiagen). cDNA was synthesised using Superscript III Reverse Transcriptase RNaseH (Invitrogen). RT-PCR was performed using Taq DNA polymerase (Invitrogen), and specific forward and reverse primers, reported in Table 1.

In situ hybridization.

Larvae and juveniles were anesthetized in MS222 (0.01%) and fixed overnight in freshly prepared MOPS buffered (0.1 M MOPS (Fluka, 69947), 1 mM MgSO4 (Sigma-Aldrich, M2773), 2 mM EGTA (Fluka, 03780), 0.5 M NaCl) 4% paraformaldehyde (PFA, Electron Microscopy Sciences, 19208). Samples were washed in PBT (Phosphate Buffered Saline solution and 0.1% Tween-20), treated with Proteinase K 0.4 µg/ml for 1 hour at 37°C (to allow the tunic removal), post-fixed in 4% PFA in PBT for 1 hour and left in hybridization buffer (50% formamide, 2X SSC pH 7, 100 µg/ml yeast RNA, 50 µg/ml Heparin, 0.1 % CHAPS, 0.1% Tween-20) at 60°C over night. Riboprobes were obtained from *Ciona* Gene Collection release 1 (Satou et al., 2002) (BlueScript Vector) and synthesised using T7 and T3 polymerase (for the antisense and sense probes, respectively) with Dig conjugated rUTP (Dig-rUTP label mix - Roche). To reveal the transcripts, we used the antibody AP-anti DIG (Roche) diluted 1:3000 and the NBT/BCIP (8 µg/ml - Roche) system. Specimens were mounted in glycerol 75%

on slides, observed under the microscope Leica 5000 B and images were taken using a Leica DFC 480 digital camera.

Gene		Primer
Notch	For	5'- GAGCAACAACATAACAGGGTGGATAGCG -3'
	Rev	5'- GTGGGTAAACTCGCATTCTCTGGTG -3'
Hairy a	For	5'- GATTGTTCTCACCGCCGTCGC -3'
	Rev	5'- GAATCGCTACTACTGGTGTGGGGGG -3'
Hairy b	For	5'- AGCCTGTCCATCAACTTCCACCAC -3'
	Rev	5'- CCGATGGCGAGATGGAACGAAC -3'
Hairy c	For	5'- TGCGAAGGAGTTACTGTTGATGTGCGA -3'
	Rev	5'- GTGGATTACGGCAGGCAGCAGC -3'
Delta	For	5'- GTCTGTATGCCTGGGTGGATGGATAC -3'
	Rev	5'- CGGTAACTCCACCCTTGCCTCTG -3'
Musashi	For	5'- GGCGAGAGGAACAGCAAGGAGGA -3'
	Rev	5'- GCACCAGGAGAGAGGGCGGAAGGATG -3'
Delta-like	For	5'- ACCGACACAACGACCTGCCCATC -3'
	Rev	5'- GCGACCCGTAGTAACCTTCCGAGC -3'
TrpN	For	5'- CACTCTGCTGCGAAAAACAACCACG -3'
	Rev	5'- CTTGCGAATAACAGCGGAGACCAACC -3'
TrpA	For	5'- GCGGCGGCGTTTGGTCAC -3'
	Rev	5'- GCCAATGCCGCCGTATGTAGAGG -3'
Atoh	For	5'- GCTGTTCCTCCGCTGTCTTCTGTCAT -3'
	Rev	5'- CCTTCCATTCTTCGCCGTTCCC -3'

Table 1. List of primers

Immunohistochemical staining.

Second stage juveniles were used for immunohistochemistry procedures. Tissues were permeablized in Triton X-100 (Sigma-Aldrich, T8787). Aspecific binding sites were saturated using BSA (1%) (Sigma-Aldrich, A9647) and sheep serum (Sigma-Aldrich, S3772) (2%) in PBS; to detect the presence of VGlut neurotransmitter, a polyclonal primary antibody IgG anti Ci-VGlut produced in mouse was used (following (Horie et al., 2008); samples were incubated at 4°C over night. After washes in PBT, samples were incubated for 2 hours with a secondary antibody (fluorescein-coniugated anti-mouse) (1:500 dilution). Cell nuclei were stained with DAPI in Milli-Q water (5 µg/ml, 5’) (Sigma-Aldrich, D9542). Specimens were mounted in 75% glycerol on slides, observed under a

fluorescence microscope Leica 5000 B and images were taken using a Leica DFC 480 digital camera.

RESULTS

Neural differentiation markers.

Atoh, Notch, Delta, Hairy and *Musashi*, the genes considered here, have a conserved involvement in neural differentiation. As mentioned before, *C. intestinalis* has one ortholog for *Atoh*, *Notch*, and *Msi*, while it has at least two orthologs for *Delta* (coding for Delta-like protein and Delta protein), and three orthologs for *Hairy* (*Hairy a, b* and *c*).

Reverse transcription PCR using gene-specific primers (Tab. 1) and cDNA obtained from adult oral siphon tentacles gave bands with the expected molecular weights, for Notch, Musashi, Delta-like, Atoh and Hairy b (Fig.1). For Delta, Hairy a and Hairy c, no bands were observed. Considering that primers were designed on different exons, we can conclude that the bands were amplified only from the transcripts, hence we exclude genomic DNA contamination.

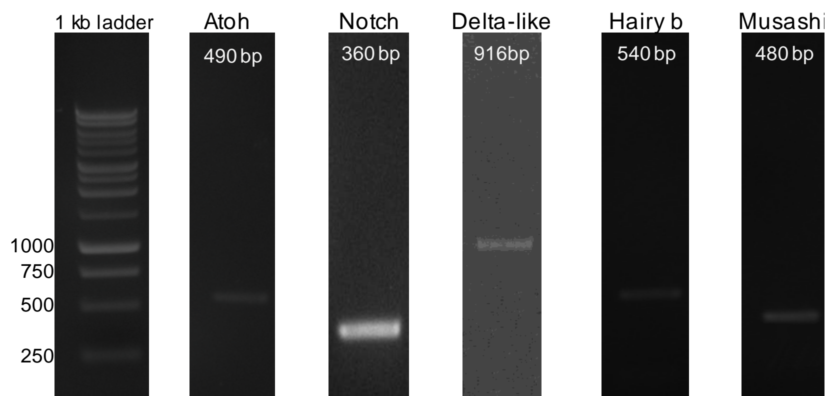
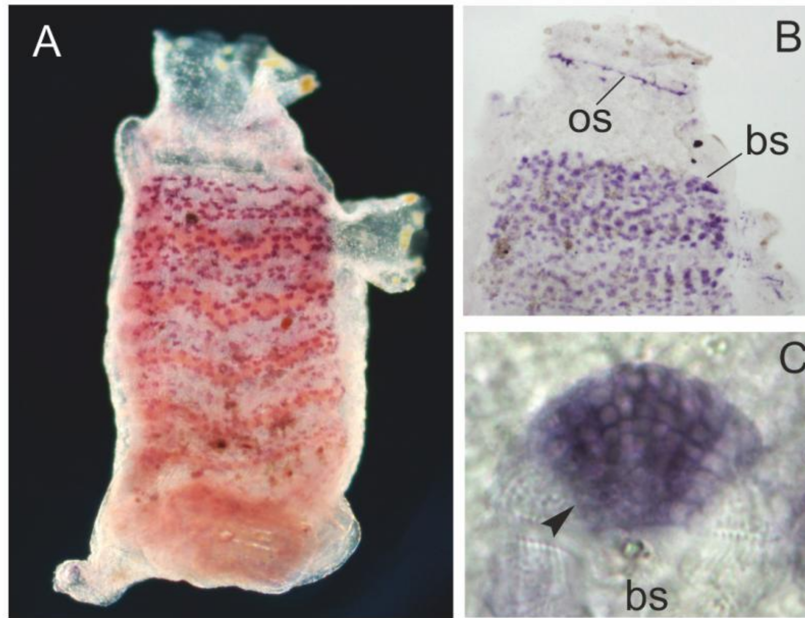


Fig. 1. RT-PCR on isolated oral tentacles of adult *C. intestinalis*, for the transcripts Atoh, Notch, Delta-like, Hairy b and Musashi, with the correspondent molecular weights.

Since the tentacles contain blood sinuses, we considered whether contaminating blood cells might be responsible for the observed gene expression. To test this we also checked for the expression of two transcripts (CRB and CC6) that are

exclusively expressed by blood cells. Neither showed expression and hence we conclude that our sample was not contaminated by blood cells.

We then performed whole mount *in situ* hybridizations on the oral siphon of adults and the whole body of second stage juveniles in order to localize the transcripts on the coronal organ (Fig.2). Only *Hairy b* and *Msi* gave a clear result (Fig. 2 A-E). Notch, Delta, and Atoh transcripts were not detected. In both of the stages, we observed *Hairy b* expression at the level of the pharyngeal gills, where stained cells were located at the two extremities of the elliptical stigmata (Fig. 2 A-C). At the same developmental stages, *Msi* expression was found in the ciliated cells of the coronal organ (Fig. 2 E, F), and was also detected in the endostyle, in the peripharyngeal bands, at the base of the oral siphon, and in the ciliated funnel (Fig. 2 D).



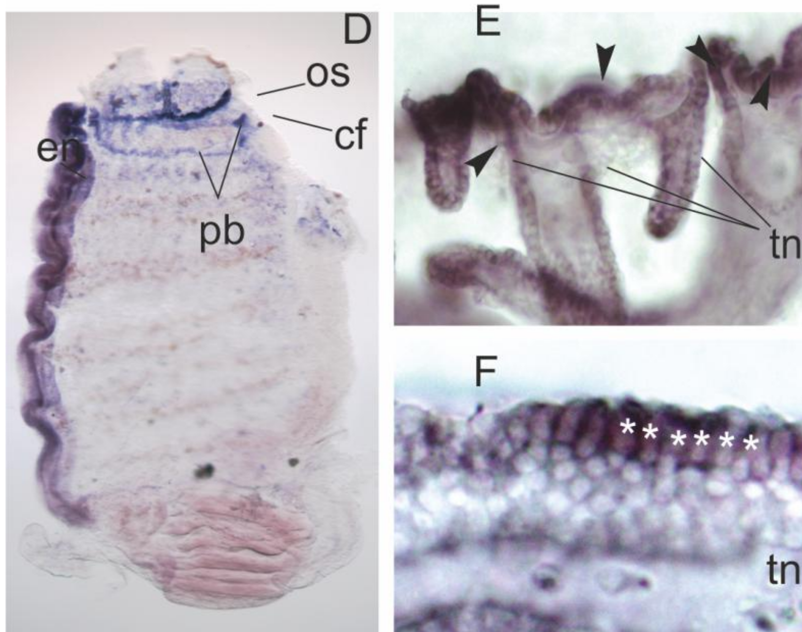


Fig. 2. *In situ* hybridization on *C. intestinalis* young adult. **A-C**. *Hairy b* is expressed in the branchial stigmata (bs). Positive signal is also present in the oral siphon (os), at the base of the tentacles (tn, B). In the stigmata, the stained cells belong to an undifferentiated population, that gives rise to the ciliated cells (C). **D-F.** *Musashi* is expressed in many ciliated tissues: endostyle (en), peripharyngeal bands (pb), oral siphon (os), ciliated funnel (cf). In the oral siphon, tentacles show a weak signal, especially concentrated at the base (black arrowheads in E), but labelled cells are present also along the tentacles crests (asterisks in F).

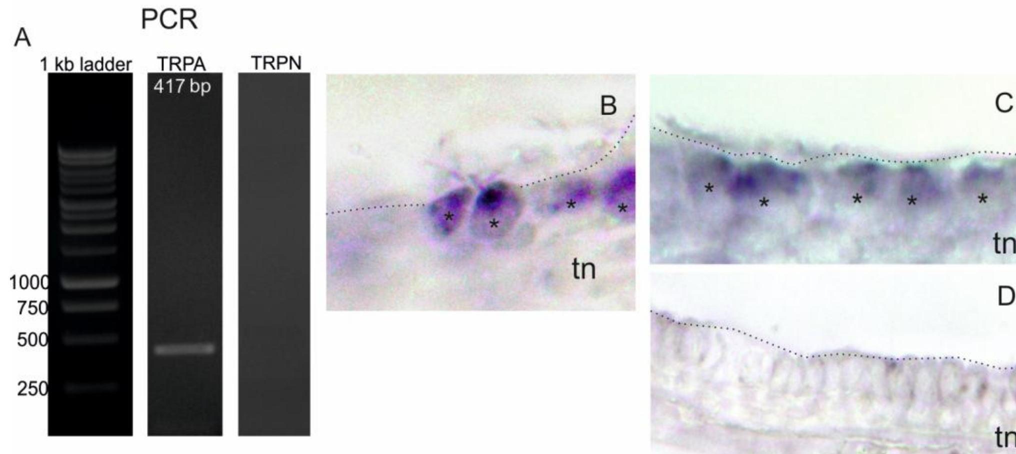


Fig. 3. RT-PCR on adult *C. intestinalis* tentacles (**A**) and in situ hybridization on adult (**B-D**) for *TRP* channels. *TRPA* is expressed in the tentacles (tn) and in particular in the sensory cells of the coronal organ (asterisks in B,C). Expression of *TRPN* has not been detected (D). Dotted lines evidences the coronal organ.

TRP.

The presence of *TRP* channel expression was investigated by means of RT-PCR. We detected only one TRPA channel transcript at tentacle level, TRPA1 (417 bp), and TRPN was not expressed (Fig. 3 A). Whole mount *in situ* hybridization on the adult oral siphon and second stage juveniles confirmed the results obtained with RT-PCR. Only *TRPA1* was expressed in the coronal organ, and it was localized in the sensory cells (Fig. 3 B-D).

Ci-VGlut.

We performed immunostaining on *C. intestinalis* adults using a Ci-VGlut antiserum (Horie et al., 2008), in order to trace the nerve network, with special attention to the nerves connecting the cerebral ganglion to the coronal organ. Ci-VGlut immunostaining evidenced two nerves running under the coronal organ (Fig. 4 A,B), placed in correspondence of the two lateral crests of each tentacle, and with several bridges crossing the tentacle and connecting the two nerves. At the base of each tentacle, the nerves joined one another to form a bigger nerve (the pericoronal nerve), running at the base of the oral siphon, in correspondence

of the velum (Fig. 4A) This nerve converged toward the cerebral ganglion, into the main anterior nerves (Fig.4 C).

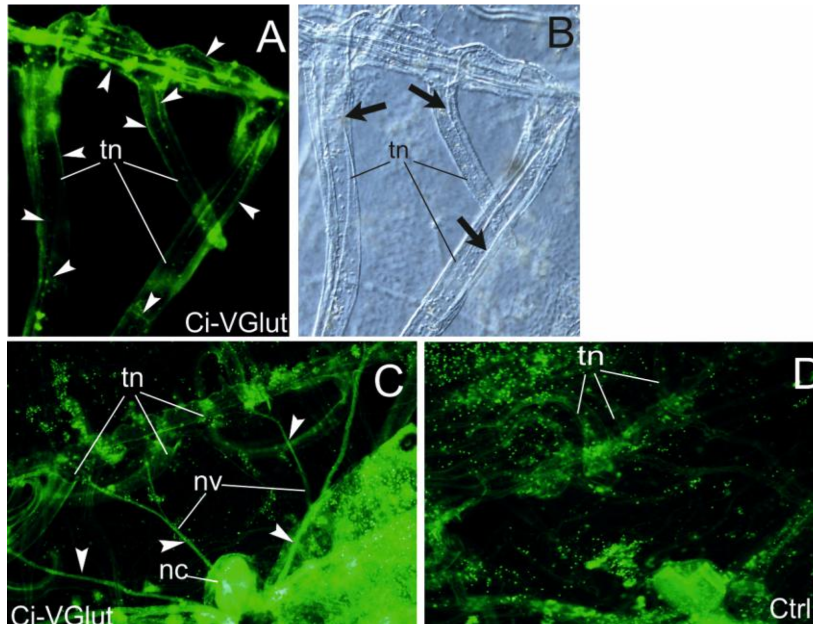


Fig. 4. Immunohistochemistry for Ci-VGlut. Green fluorescent signal indicates the nerves labelled with Ci-VGlut (**A-C**). High magnification on the tentacle region (tn) evidences the nerves (white arrowheads) running under the coronal organ (A). B, bright field of tentacles with the coronal organ (black arrows). Neural complex (nc) with the labelled anterior nerves (nv) directed toward the tentacles (C); negative control do not show any staining (**D**).

DISCUSSION

Neural differentiation of hair cells.

Among the many genes involved in neural and hair cells differentiation, we considered *Notch, Delta, Hes (Hairy), Atoh,* and *Msi* (Fig. 5).

Notch codes for a transmembrane receptor that becomes active after ligand binding, and one of its classical ligands is Delta. The *Delta/Notch* pathway is a conserved mechanism by which the regulation of hair and supporting cells production is regulated in the inner ear and lateral line (Haddon et al., 1998; Haddon et al., 1999; Riley et al., 1999; Eddison et al., 2000; Akanuma et al., 2002; Reed, 2004; Daudet and Lewis, 2005). Our findings by the RT-PCR showed the presence of Notch and Delta-like transcripts in *C. intestinalis* tentacles, however their localization was not detected by *in situ* hybridization,

probably because of a low level of gene expression combined with high background signal. These results suggest that *Notch/Delta* pathway might also play a role in coronal organ development of *C. intestinalis* juveniles. *Delta-like* is expressed at pharyngeal gill level, in a cell population constituting the precursor of both the stigmata neural and non-neural ciliated cells (Arkett et al., 1989; Shimazaki et al., 2006). Considering that the coronal organ is formed by neural ciliated cells, it is possible that *Delta-like* maintains a similar role in this region.

In situ hybridization also revealed that *Hairy b* shows an expression pattern comparable to that of *Delta-like* (Shimazaki et al., 2006), that is it is also expressed by the ciliated cell precursors at stigmata level. Although *Hairy b* did not give a clear signal in the coronal organ, the presence of its transcript detected by RT-PCR, suggests that tentacles possess an undifferentiated cell population able to produce the ciliated sensory cells of the coronal organ.

Atoh1 has been demonstrated to be crucial for hair cell differentiation (Cafaro et al., 2007; Millimaki et al., 2007; Fritzsch et al., 2010; Matsuda and Chitnis, 2010; Pan et al., 2012a): *Atonal* is expressed in *Drosophila*, where it is involved in the differentiation of the mechanosensory cells in the auditory Johnston's organ (Eberl et al., 2000; Boekhoff-Falk, 2005); in vertebrates there are two orthologs, *Atoh1* and *Atoh7* derived through multiple duplication steps from the ancestral *atonal*; *Atoh1* is expressed in inner ear and lateral line and is responsible of hair cell differentiation (Cafaro et al., 2007; Pan et al., 2012a). All these genes share the highly conserved bHLH domain (Fritzsch et al., 2007; Pan et al., 2012a). In *C. intestinalis* there is only one ortholog and very little is known about its expression and function; but in our experiments the expression of *Atoh* has been detected in the tentacles suggesting that it is involved in coronal sensory cell differentiation, as these are the only sensory cells at that level (Burighel et al., 2003; Mackie et al., 2006). Its detection in the adult organ might indicate a role in differentiation of new sensory cells during development, as it keeps growing its entire life. In addition we found, both by RT-PCR and *in* situ hybridization, the expression of the RNA binding protein Msi, which plays an important role in neural differentiation, maintaining stem cell status. *Msi* is conserved both in invertebrates and vertebrates: it was identified for the first time in *Drosophila* where it functions as regulator in the asymmetric cell division in sensory organ precursors by indirect activation of *Notch* (Nakamura et al., 1994; Okabe et al.,

2001). In mouse, there are two orthologs of *Msi*: *Msi1* and *Msi2*. For the latter the role is still unclear, while *Msi1* is known to be expressed in neural stem/precursor cells of the central nervous system, where it controls stem cell maintenance (Kaneko et al., 2000; Imai et al., 2001; Siddall et al., 2006). Moreover, *Msi1* has an important role in hair cell differentiation in the mouse inner ear, showing an expression pattern similar to that of *Notch*. During sensory organ maturation *Msi1* expression is lost in differentiated hair cells but persists in supporting cells, where it regulates in particular their differentiation (Sakaguchi et al., 2004; Savary et al., 2007). *C. intestinalis* has only one ortholog for *Msi*- *Ci-Msi* - which has a role very similar to the one observed in vertebrates in regulation of neural cell differentiation (Kawashima et al., 2000). In adulthood *Ci-Msi* is expressed in many ciliated epithelia: endostyle, intestine, branchial fissures, peripharingeal bands and oral tentacles. In agreement with previous studies our result supports the hypothesized role of *Msi* as regulator in ciliated cell differentiation. (Kawashima et al., 2000; Awazu et al., 2004).

All together our data evidence that the molecular mechanisms leading to mechanosensory differentiation of vertebrate hair cells are also involved in the formation of coronal organ sensory cells, although further studies are needed, both to refine the exact expression pattern of these genes and to characterize the gene network by means of functional analysis. We hypothesize that the presence of common transcription factors and RNA binding proteins in the secondary sensory cells of tunicates and vertebrates is due to the derivation from a common ancestor rather than an independent evolution of these cell types in the two groups.

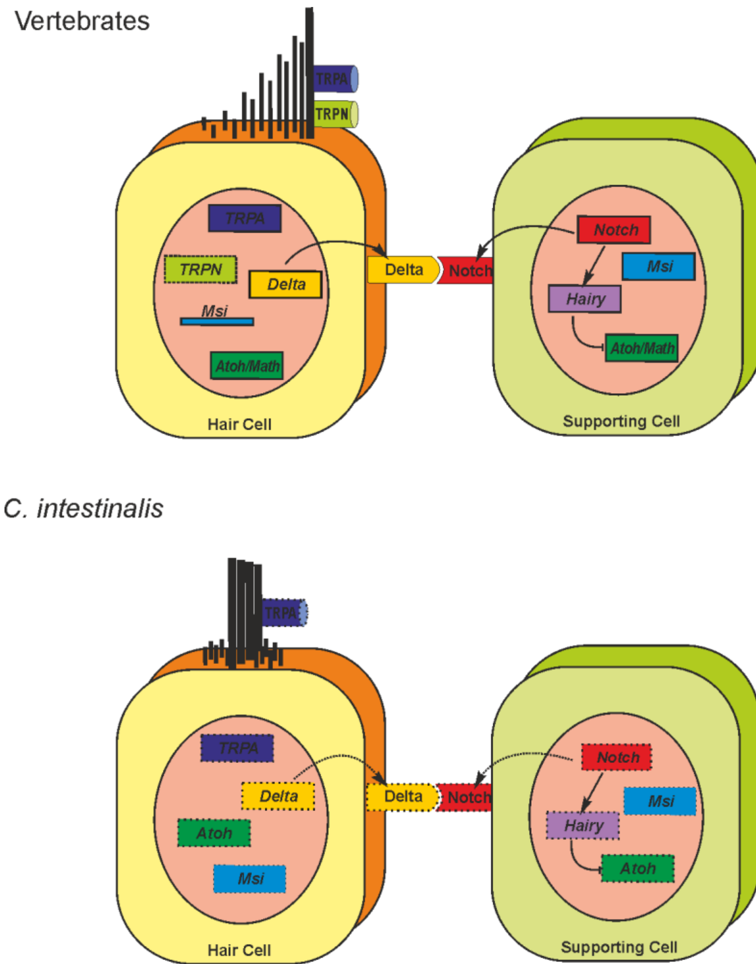


Fig. 5. Schematic draw summarizing the genetic pathway acting during vertebrate hair cell differentiation (on the top) and the genetic pathway hypothesis (indicated by the interrupted border lines) in *C. intestinalis* (on the bottom). Interrupted border line of *TRPN* in the vertebrate hair cell nucleus indicates that the gene is not ubiquitously expressed (e.g. absent in mammals).

Hair cell neurotransmission.

The two aspects related to neurotransmission here surveyed concerned the *TRP* genes, codingfor different cation membrane channels, and the neurotransmitter glutamate.

The TRP (Transient Potential Receptor) superfamily is a group of cation channel genes that play a critical role in sensory physiology; it includes several members encoding proteins sharing a common basic structure consisting in six transmembrane domains that render them non selective cation channels and Ca^{2+} permeable. TRP proteins can be divided in seven categories: TRPC, TRPV,

TRPN, TRPM, TRPA, TRPP, TRPML. We consider two groups in particular: TRPA and TRPN, both implicated in hair cell signal transduction (Corey, 2003; Sidi et al., 2003; Christensen and Corey, 2007; Brierley et al., 2009; Arnadottir and Chalfie, 2010). TRPA channel is activated in vertebrate hair cells by mechanical stimuli (i.e. sound, vibration, pain and touch). Studies on its gene expression revealed that the mRNA is present in the sensory epithelium and the protein product is located in the sterocilia. Despite controversial results on knockdown mice, TRPA still seems to be the best candidate as the primary protein implicated in auditory transduction in hair cells (Corey et al., 2004). *C. intestinalis* possesses four genes encoding TRPA proteins and we focused in particular on *TRPA1* because is the most closely related to ANKTN1, the unique mammal orthologue. Its expression was detected in the coronal organ both by RT-PCR and *in situ* hybridization, with specific signal present in the coronal sensory cells (Fig. 3 B,C). Expression of *TRPA1* involves only some cells in the coronal organ of adults and this may be explained by the continuous process of mechanosensory differentiation so that the mRNA could be present just in those cells not yet active, according to Corey (2004). Thus, we infer *TRPA1* participates in coronal organ sensory cell signal transduction even though the exact role of this channel remains to be elucidated. However its identification is particularly important in shedding light on the evolutionary relationships between the coronal sensory cells and vertebrate hair cells.

In spite of the importance of TRPN channel in vertebrate hair cell mechanosensation (Sidi et al., 2003), in our analysis we find absence of the TRPN transcript in the coronal organ; we can hypothesize that this channel is not essential for mechanotransduction, similar to what occurs in mammals where no *TRPN* gene has been recognized so far (Arnadottir and Chalfie, 2010), and it is supposed that its role might be replaced by other proteins.

Glutamate is the main vertebrate neurotransmitter in the excitatory afferent synapses implicated in photo-, chemo- and mechano- sensation (Jahr and Lester, 1992). Currently, it is proposed to be the most important neurotransmitter in vertebrate hair cell function (Ottersen et al., 1998). Previous studies on *C. intestinalis* larvae evidenced the glutamatergic network showing some parallels with the glutamatergic network in vertebrates (Horie et al., 2008). Here we focused on the coronal organ and the nerves connecting it with the cerebral

ganglion. We found labeled axons at the base of the coronal organ, indicating that the coronal organ sensory cells use this molecule to transmit the signal to the CNS. The nerve network evidenced here corresponds to the one described in a previous study (Chap. 3, present thesis). Currently it is unknown whether other neurotransmitters may be involved in this secondary sensory cell function, and further analyses are necessary. In the ascidian *Botryllus schlosseri* the acetyocholine esterase enzyme was evidenced in tentacle nerves (Burighel et al., 2001) and acetylcholine is the main vertebrate inhibitory neurotransmitter acting in hair cell efferent synapses (Simmons, 2002). Since efferent synapses are present we may expect to find it also in *C. intestinalis* (Manni et al., 2006).

CONCLUSION

On the base of their morphological features, their wide distribution in tunicates and their embryonic origin form a placodal-like territory, coronal sensory cells have been hypothesized to be homologous to vertebrate hair cells (see Burighel et al., 2011 for review and present thesis), Here we evidence that the secondary sensory cells of the coronal organ are characterized by genes commonly expressed in mechanoreceptors, mostly reflecting the high conservation of the molecular machinery controlling their differentiation across metazoans. Furthermore, some important functional aspects of their activity, i.e., an ionic membrane channel and a neurotransmitter, are in common with vertebrate hair cells. Therefore these data reinforce our hypothesis of homology between the secondary receptors in tunicates and vertebrates. Considering the phylogenetic relation of these two groups it may be reasonable to think about a common ancestor of the two cell types already provided with these molecular tools, instead of independent evolution, although the latter still represents a possible explanation. Future functional studies on ascidians could help to clarify the gene network behind coronal sensory cell differentiation, allowing a more detailed comparison between this sensory organ and the vertebrate counterpart.

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CONCLUDING REMARKS

This doctoral research proposed to investigate tunicate secondary sensory cells using an Evo-Devo approach, which aimed to combine the comparative study of their morphology in different tunicates, the cladistics analysis to infer their evolution, and the molecular study of their development in the ascidian model *Ciona intestinalis*.

In the present thesis, I present, for the first time, a detailed morphological description of the coronal organ in thaliaceans and appendicularians, two classes of tunicates that have only been partially considered in previous studies, thus completing the picture of the secondary sensory cells in tunicates. The widespread distribution of the oral secondary sensory cells within tunicates, with the exception of salps (thaliaceans), confirms that these sensory cells are a common feature of this class and play an important role as mechanoreceptors in the feeding process.

The common and different morphological features of these sensory cells were considered in detail and were combined with the current knowledge of chordate phylogeny, including both the data presented here and the data present in the literature, to propose an evolutionary hypothesis for tunicate secondary sensory cells. The first important conclusion based on these results is that the tunicate ancestor might have possessed secondary sensory cells with a single cilium surrounded by short microvilli that is, nevertheless, the basic arrangement observed in several ciliated mechanoreceptors across metazoans.

The analysis of the ascidian *C. intestinalis* described the morphological changes that occur during the development of the coronal organ and the expression of genes constituting the genetic machinery that underlies mechanosensation and, in particular, the differentiation of vertebrate hair cells. Thus, another important consideration is that tunicate sensory cells and vertebrate hair cells also share molecular features, which are, in part, very well conserved across all metazoans, suggesting that the common ancestor of these two groups might have already possessed the genetic tools necessary for the development of secondary sensory cells.

In light of previous work and the current data, the results presented in this thesis reinforce the hypothesis that tunicate secondary sensory cells and vertebrate hair cells might be a plesiomorphic feature of these two groups, rather than the effect of evolutionary convergence with the subsequent acquisition of different specialisations according to the environmental pressures and needs of the organism.

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PARTICIPATION MEETINGS

Rigon F., Shimeld S.M., Degasperi V., Gasparini F., Burighel P., Manni L. “Placodal genes during asexual development of a colonial chordate” BSDB meeting on “Development and sensory systems” 7-9 Sept 2010, Oxford (UK).

Rigon F., Shimeld S. M., Stach T., Gasparini F., Caicci F., Burighel P., Zainolo G., Manni L. “Morphological and molecular development of coronal hair cells in *Ciona intestinalis*: an evo-devo approach to investigate possible homology to vertebrate hair cells” PhD Day, 31 May-1 June 2011 Università di Padova (Italy).

Rigon F., Shimeld S.M. Gasparini F., Caicci F., Burighel P., Zaniolo G., Manni L. “Sviluppo delle cellule capellute in *Ciona intestinalis.*”. GEI meeting, Monteortone. 5th-8 June, 2011 (PD, Italy).

Caicci F., Stach T., Burighel P., **Rigon F**., Gasparini F., Zaniolo G., Manni L. “Strutture sensoriali orali nei Thaliacea (Tunicata) ed evoluzione delle cellule capellute nei Chordata” 72° Congesso dell’Unione Zoologica Italiana (UZI). 5th-8th September 2011, Bologna (Italy).

Stach T., Caicci F., Burighel P., **Rigon F**., Gasparini F., Zaniolo G., Manni L. “Hair cells in Tunicata: when did the lateral line evolve?” Deep Metazoan Phylogeny 2011, new data, new challenges. 11- 14 October 2011, Munich.

Rigon F., Caicci F., Gasparini F., Zaniolo G., Burighel P., Manni L. “Sviluppo dell’organo coronale: alla ricerca dell’antenato del sistema acustico-laterale nell’ascidia *Ciona intestinalis*” 58° Convegno GEI. 13-15 Giugno 2012, Torino.

Rigon F., Caicci F., Gasparini F., Burighel P., Manni. L. “Coronal organ development: in search of the hair cell ancestor in the ascidian *Ciona intestinalis* (Tunicata, Chordata).” EVO-DEVO meeting. 10-13 July 2012, Lisbon.

Rigon F., Shimeld S.M., Stach T., Caicci F., Gasparini F., Burighel P., Zaniolo G.,
Manni. L. “Coronal organ evolution and development: in search of the hair cell ancestor in tunicates”. PhD Day. 25-26 June 2012, Università di Padova (Italy)

PRACTICAL COURSES

EMBO practical course "Marine animal models in evolution and development". 12-23 July 2010. Fiskebackskil, Sweden.

Field course on "Animal biodiversity". 30 August-3 September, 2011.Nettlecombe Court, The Leonard Wills Field Centre, Williton, Taunton, Somerset (UK).

UZI Spring School. "Development of the nervous system: a comparative and behavioural approach". 11-13 May 2012. Venice (Italy).