Nervous system characterization during the development of a basal echinoderm, the feather star Antedon mediterranea

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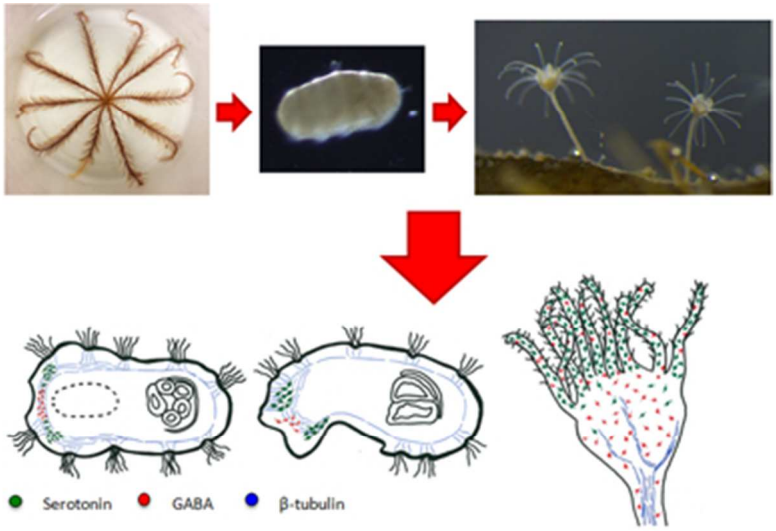

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Graphical Abstract text

Crinoids are basal echinoderms, developing through a swimming doliolaria larva and a post-metamorphic pentacrinoid. We characterized the nervous system of both these stages. Our results revealed the presence of a highly composite nervous system, suggesting that different larval/juvenile activities are under the control of different neural populations.

Nervous system characterization during the development of a basal echinoderm, the feather star *Antedon mediterranea*

Running Title: Neural development of *A. mediterranea*

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Abstract

Neural development of crinoids, and more in general, of echinoderms have always been elusive, as larval neurons degenerate and a tripartite nervous system differentiates in the adult. Despite their key phylogenetic position as basal echinoderms, crinoids has been scarcely exploited in developmental research. However, they are the only extant echinoderms retaining the ancestral body plan of the group and studies on these models are precious to clarify neural evolution in deuterostomes. *Antedon mediterranea* is a feather star, endemic to the Mediterranean Sea. Its development includes a swimming lecithotrophic larva, the doliolaria, with basiepithelial nerve plexus, and a sessile filter-feeding juvenile, the pentacrinoid, whose nervous system has never been described in detail. Thus, we characterized the nervous system of both these developmental stages by means of immunolocalization and, for the first time, *in situ* hybridization techniques. The results confirmed previous descriptions of doliolaria morphology and revealed that the larval apical organ contains two bilateral clusters of serotonergic cells while GABAergic neurons are localized under the adhesive pit. This suggested that different larval activities (e.g. attachment and metamorphosis) are under the control of different neural populations. In the pentacrinoid, analyses showed the presence of a cholinergic entoneural system while the ectoneural plexus appeared

more composite, displaying different neural populations. The expression of three neural-related microRNAs was described for the first time, suggesting that these are evolutionary conserved also in basal echinoderms. Overall, our results set the stages for future investigations that will allow to complete the intriguing puzzle of echinoderm evo-devo neurobiology.

Keywords: crinoids, nervous system, serotonin, GABA, microRNA

1. Introduction

Crinoids are invertebrate deuterostomes and the only living representatives of Pelmatozoa. They are recognized as the sister group of the Eleutherozoa, a taxon including the other four traditional echinoderm classes (echinoids, holothuroids, asteroids and brittle stars) (Reich, Dunn, Akasaka, & Wessel, 2015; Telford et al., 2014). They are usually divided into the free living feather stars (or comatulids) and the fixed sea lilies, a distinction that, based on recent molecular analyses, corresponds to two different ways of life rather than a true taxonomic relationship (Hemery, Michel, Améziane, & Eléaume, 2013; Rouse et al., 2013).

Crinoids and echinoderms, in general, display a number of peculiar anatomical and physiological features, among which the nervous system is one of the most enigmatic. In the adult, this has been traditionally described as a tripartite system whose components differ for anatomical localization and function: a basi-epithelial and sensory ectoneural system, a sub-epithelial and motor hyponeural system and an inner entoneural system (Cobb, 1987). In Eleutherozoa, the ecto and hyponeural components are dominant whereas in crinoids the entoneural system is the main part of the adult nervous system, forming well-developed brachial nerves (Candia Carnevali, Galassi, Bonasoro, Patruno, & Thorndyke, 2001; Candia Carnevali & Bonasoro, 2001; Heinzeller & Welsch, 1994). The developmental origin of these sub-systems has been under major debate, particularly that related to the hyponeural components (Heinzeller & Welsch, 1994). Larval nervous system generally consists of a main anterior structure (apical organ) and ciliary band-associated neurons and it has little if any relationship with adult nervous system as it is completely lost at metamorphosis. Adult neural structures are therefore developed *ex-novo* from rudiment tissues (Burke, 2011).

Our present understanding of echinoderm neural development is mainly derived from studies on the Eleutherozoa (Byrne, Nakajima, Chee, & Burke, 2007; Cary & Hinman, 2017; Hirokawa, Komatsu, & Nakajima, 2008; Katow et al., 2010; Nakano, Murabe, Amemiya, & Nakajima, 2006) and less information is available on crinoid neural development. Indeed, despite their key phylogenetic position, crinoids have been scarcely exploited in both developmental and evolutionary studies and only recently interest in crinoid developmental processes has aroused (Amemiya et al., 2015; Comeau, Bishop, & Cameron, 2017; Nakano, Nakajima, & Amemiya, 2009; Omori, Akasaka, Kurokawa, & Amemiya, 2011).

Most crinoids develop through two main post-embryonic forms: a swimming doliolaria larva and a stalked pentacrinoid juvenile. Doliolaria is a lecithotrophic larva found in holothurians and ophiuroids as well (McEdward & Miner, 2001; Nakano et al., 2006). Through metamorphosis doliolaria becomes a sessile filter-feeding pentacrinoid, which grows into the adult form in sea

lilies, whereas in feather stars the juvenile detaches from the stalk after several months, becoming a free-living adult (Barbaglio et al., 2012; Mladenov & Chia, 1983). Nervous system anatomy of doliolaria larvae has been reported in few species (Barbaglio et al., 2012; Nakano et al., 2009) whereas pentacrinoid neural structures have never been described in detail. As nervous system organization and development are fundamental aspects of animal body plan (Byrne et al., 2007; Nakajima, Kaneko, Murray, & Burke, 2004), the scant data available in crinoids strongly reduce our possibilities to understand the phylogenetic origin of echinoderm nervous system. Difficulties in larva collection and the lack of effective protocols for molecular investigations have strongly limited the potentiality of this model. Crinoids, however, are the only extant echinoderms retaining the ancestral body plan of the group (Guensburg & Sprinkle, 2009; Paul & Smith, 1984).

Thus, in the present study, we characterised the nervous system of doliolaria and pentacrinoid stages of the feather star *Antedon mediterranea*, a species endemic to the Mediterranean Sea. This comatulid has been highly exploited in regeneration studies (Candia Carnevali et al., 2001; Candia Carnevali & Bonasoro, 2001; Di Benedetto et al., 2014; Sugni et al., 2010) whereas its development has received less attention. After spawning, *A. mediterranea* females keep their embryos attached to their genital pinnules until, 3-4 days post fertilization, the swimming doliolariae hatch. Doliolaria larva displays a distinctive barrel-like shape with two ciliated grooves: the anterior adhesive pit and the ventrally located vestibulum. Moreover, its body is characterised by five transverse ciliary bands and, anteriorly, by a long tuft that emerges from an apical pit. After a few days, doliolaria settles on a proper substrate and undergoes metamorphosis. Through a transient cystidean stage, the larva reaches the pentacrinoid stage. This juvenile consists of a cup-like calyx attached to the substrate by a long stalk. Tube feet progressively emerge from the calyx opening, surrounding the mouth and already bearing several thin processes called papillae (Barbaglio et al., 2012).

Considering the emerging role of crinoids in evolutionary and developmental studies, we investigated neural organization of both doliolariae and pentacrinoids by mean of microscopy, immunohistochemistry and, for the first time, *in situ* hybridization technique. The type and distribution of neural populations were described and their molecular markers explored through the expression of three nervous system-enriched microRNAs (miRNAs; miR-7, miR-124 and miR-184), a class of small non-coding RNAs highly conserved among metazoans. Overall, our results provide precious data about crinoid neuroanatomy and set the stage for further investigations that will help to complete the intriguing puzzle of echinoderm neurobiology.

2. Materials and Methods

2.1. Animal maintenance and sample collection

Adults of *A. mediterranea* (Fig. 1 A) were collected in winter 2017 at Le Grazie (La Spezia Gulf, Ligurian Sea, Italy). Animals were immediately transferred to the laboratory, where they were maintained in aquaria filled with artificial sea water (ASW) and provided with close multi-circulation system as well as mechanical, chemical and biological filters. ASW was prepared by mixing commercial aquarium salt (Instant Ocean®, Aquarium System) with partially deionized water in order to reach the Mediterranean Sea salinity (37-38‰). Temperature was set at 17±1°C and photoperiod was fixed at 16h:8h (dark:light), mimicking natural conditions. Aquaria physical (temperature, salinity, pH) and chemical (nitrite and nitrate levels) parameters were regularly checked and promptly adjusted if necessary. Five days per week each adult individual was fed with artificial feed for seawater filter-feeding animals (Clam and Filter Feeder, H_2Ocean). Since *A. mediterranea* females keep their embryos attached to their genital pinnules (Fig 1 B and C) until the swimming doliolariae (Fig. 1 D) hatch, the latter were collected directly from the pinnular surfaces by gently pipetting water on them to help larvae release. Pentacrinoids (Fig. 1 E) were left to develop in aquaria: to facilitate doliolaria settlement, plastic grids were put inside the aquaria. Collection of both dololariae and pentacrinoids was performed under a stereomicroscope. Before fixation, pentacrinoids were relaxed by a 10-minute incubation in a solution of 3.5% $MgCl_2$ in ASW.

2.2. Microscopy

Standard methods of light microscopy were employed (Mercurio & Sugni, 2016). Briefly, samples were pre-fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer and 1.4% NaCl for 2 h. After overnight washing in the same buffer, samples were post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer. After standard dehydration in ethanol series (25%, 70%, 90% and 100%), samples were washed in propylene oxide and embedded in Epon-Araldite 812 resin. Semi-thin sections (about 1 µm) were cut with a Reichert-Jung ULTRACUT E using glass knives, stained with crystal violet and basic fuchsin and then mounted with Eukitt (Bio Optica). Samples were observed under a Leica light microscope and photographed using a Leica DFC-320 camera and LAS (Leica Application Suite, Leica) software.

2.3. Immunolocalization

Doliolaria samples were processed for immunofluorescence staining on paraffin sections, as published whole mount protocols (Barbaglio et al., 2012; Nakano et al., 2009) failed in providing staining in the innermost structures. Samples were fixed in a solution of 4% paraformaldehyde, 0.5 M NaCl, 0.1 M morpholinopropanesulfonic acid (MOPS fixative; pH 7.5) for 90 minutes,

dehydrated in ethanol series (30%, 50%, 70%, 90%, 95% and 100%), cleared in xylene and left overnight in a solution of xylene and paraffin (1:1). Doliolariae were then rinsed in paraffin wax and embedded. Longitudinal sections (7 µm) were cut with Reichert OmE sledge microtome and placed in gelatin-coated slides. Then, paraffin was removed through a 20-minutes incubation in xylene and doliolaria sections progressively rehydrated through ethanol series. After washes in Phosphate Buffer Saline (PBS) and a pre-incubation in 50% Normal Goat serum (NGS; Sigma-Aldrich) for 1 hour, samples were incubated overnight in a solution of 10% NGS in PBS with the primary antibody (1:250) at 4°C in a wet chamber. Samples were washed in PBS, treated with 1% Bovine Serum Albumin (BSA; Sigma-Aldrich) solution for 1 hour and incubated with the secondary antibody (1:400) for 1 hour at 4°C. Finally, sections were washed with PBS, counter-stained with DAPI to mark the nuclear DNA (Sigma-Aldrich: D9542) mounted with 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma Italy) plus MOWIOL (Sigma, Italy) and observed using a Leica SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany), equipped with an argon/krypton laser. Negative controls omitting primary or secondary antibodies were always performed.

Pentacrinoid samples were processed for whole mount immunofluorescence staining. Samples were fixed in MOPS fixative for 90 minutes and then washed with 0.1% Tween-20 in Phosphate Buffer Saline (PBT). Decalcification was achieved by a 2-day incubation in 5% Ethylenediaminetetraacetic acid (EDTA) at room temperature. Pentacrinoids were permeabilised by washing them with a solution of 0.25% Triton-X in PBT for 30 minutes and pre-incubated in 50% NGS for 2 hours. Then, samples were incubated overnight at 4°C in a solution of 10% NGS in PBT with primary antibody diluted 1:250. After several washings in PBT, pentacrinoids were pre-incubated in 1% BSA in PBT and incubated overnight at 4°C in PBT with the corresponding secondary antibody diluted 1:400. After PBT washings, samples were mounted with DABCO on microscope slides and observed using a Leica SP2 confocal laser scanning microscope. Negative controls were performed omitting the primary or the secondary antibodies.

2.4. Antibody characterization

The following primary antibodies were used: monoclonal anti-ß-tubulin antibody, produced in mouse (clone 2-28-33; Sigma-Aldrich); anti-serotonin polyclonal antibody, developed in rabbit (Sigma-Aldrich: S5545); anti-dopamine antibody, produced in rabbit (Sigma-Aldrich: AB122S); rabbit anti-GABA polyclonal antibody (Sigma-Aldrich: A2052); anti-choline acetyltransferase (ChAT) polyclonal antibody developed in rabbit (Sigma-Aldrich: AB143).

Fluorescent secondary antibodies: Alexa Fluor 488 Goat Anti-Mouse IgG Antibody (ThermoFisher: A-11001), Alexa Fluor 568 Goat Anti-Mouse IgG Antibody (ThermoFisher: A-11004); Alexa Fluor 488 Chicken Anti-Rabbit IgG Antibody (ThermoFisher: A-11008).

2.5. Whole mount in situ hybridization

Expression patterns of three neural-related miRNAs, miR-7, miR-124 and miR-184 plus the muscle specific miR-133 were investigated by whole mount *in situ* hybridization in both doliolariae and pentacrinoids. All steps were performed in RNase free conditions at room temperature unless otherwise specified. After sample fixation in MOPS fixative, doliolariae were permeabilised with 2 µg/ml Proteinase K in PBT at 37°C for 5 minutes, while pentacrinoid were decalcified for 2 days with a solution of 5% EDTA. Then both kinds of samples were post-fixed in a solution of 4% paraformaldehyde in PBT for 1 hour, incubated for 2 hours in the hybridization solution (50% formamide, 5x SSC, 100 µg/ml yeast RNA, 50 µg/ml, 0.1% Tween-20) at hybridization temperature. Hybridization was carried out with Dig-labelled LNA probes (Locked Nucleic Acid; Exiqon, Norway) for 5 days at the hybridization temperature (according to the manufacturer instructions). Probes were designed based on published sequences (Peterson Kevin, Su, Arnone Maria, Swalla, & King Benjamin, 2013). After several washes in 50% formamide, 5x SSC, 0.1% Tween-20 in DEPC-treated water at hybridization temperature and subsequently in PBT, samples were incubated for 2 hours in blocking buffer solution (1:4 deactivated Sheep Serum in PBT). Specimens were incubated overnight in a dilution 1:2000 of alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics, Germany) in blocking buffer. The following day, samples were washed in PBT and incubated in filtered APT buffer (5 M NaCl, 1 M $MgCl_2$, 0.2 M Tris pH 9.5, 0.1% Tween-20) for 15 minutes. Signal detection was obtained in APT buffer containing NBT (nitrobluetetrazolium salt; Roche Diagnostics) and BCIP (5-bromo-4-chloro-3-indolylphosphate; Roche Diagnostics) substrates. When colour reaction developed, samples were fixed for 1 hour in 4% paraformaldehyde, mounted on glass slides with 80% glycerol and photographed under a Leica microscope equipped with Leica DFC-320 Camera.

Finally, doliolariae and pentacrinoids were embedded in Epon 812-Araldite resin and 5 µm thick sections were cut and stained with diluted basic fuchsin.

3. Results

3.1. Morphological analysis of doliolaria

The overall anatomy of *A. mediterranea* doliolaria was described in Barbaglio et al., 2007, here we recall and detail the main aspects related to the nervous system, to facilitate the comprehension of immunohistochemical and *in situ* hybridization results. Histological sections revealed that the

doliolaria body was covered by a thick epidermis characterized by a conspicuous basiepithelial nerve plexus which ran along the entire length of the larva. The plexus presented a dense network of basally located fiber bundles. These bundles were remarkably thicker in the anterior portion of the larva, particularly under the apical pit and along two lateral longitudinal areas (Fig. 2A and B); however, it was scarcely developed at the level of the vestibulum roof (Fig. 2 A). In the posterior region, the nerve plexus became thinner and nervous fibers were hardly detectable in the epithelial tissues. Larval posterior region was almost completely occupied by the enterohydrocoel cavities, that will ultimately differentiate into the hydrocoel and enteric sac. From semi-thin sections, it was not possible to understand whether neural elements of the future visceral and enteric nervous system were already differentiated at this stage (Fig. 2 A). The rest of the body was composed of loosely distributed mesenchymal cells filling the spaces between the cavities and the epidermis (Fig. 2 A and B).

3.2. Immunolocalization of doliolaria nervous system

To better describe the nervous system of doliolaria larvae, nervous fibers were immunolabeled using a monoclonal antibody against β-tubulin. In whole mount preparations antibodies failed to penetrate, thus immunolocalization was performed on sections. Labelling was observed in nerve fibers concentrated in the neural plexus and in neural processes directing toward the body surface. Fibers were particularly concentrated towards the dorsal apical pit (Fig. 2 C and D). The antibody also labelled the ciliated cells of the adhesive pit and of the five transverse bands (Fig. 2 C and D).
Neural organization was then explored employing antibodies specific to three neurotransmitters: serotonin, GABA and dopamine. Serotonin-positive cells were observed at the level of the apical neural plexus, concentrated in two clusters at each side of the plexus (Fig. 2 E). In sagittal sections, serotonergic cells localized right under the apical pit and in a thin area between the adhesive pit and the vestibulum (Fig. 2 F). GABAergic neurons were concentrated in a restricted area of the apical neural plexus, located in its anterior-most part. These neurons apparently underlay the adhesive pit between the two clusters of serotonergic cells (Fig. 2 G and H). No dopamine-positive cell was observed in doliolaria sections.

3.3. Morphological analysis of pentacrinoid

Histological analysis revealed a prominent skeleton underlying the epidermis, both in the calyx and along the stalk (Fig. 3 A and B). In the oral region, tube feet were covered by thin epidermis and characterised by hollow central cavities (Fig. 3 C). Papillae were regularly arranged along tube feet (Fig. 3 B) and cells located at their base sent projections to their tip (Fig. 3 C). In the calyx, the hydrocoel could be easily recognised as a conspicuous cavity right under the tube feet, bordered by a thin epithelium (Fig. 3 B). Instead, a thick epithelium of ciliated cells was observed at the level of

the mouth and continued to the intestine, where cells were filled with heterogeneous vesicles. All the structures described above were surrounded by the somatocoelic cavities delimited by a thin monostratified epithelium (Fig. 3 B). At the base of the calyx, a large cluster of neurons was visible, forming an aboral neural centre, surrounded by thick skeletal plates (Fig. 3 B). This cluster sent processes towards the oral end and inside the stalk, where a central bundle of nerves could be recognized, reaching the attachment disk (Fig. 3 D).

3.4. Immunolocalization of pentacrinoid nervous system

Anti-β-tubulin antibody was used to clarify nervous system architecture of pentacrinoids. Neural processes were detected at the centre of the stalk, running from the base of the calyx to the attachment disk (Fig. 3 E and F). Inside the calyx, five main nerve branches radiated from the aboral neural centre; these fibers were interconnected by transversal commissures and directed to the oral surface. These neural structures form the developing entoneural system. Along the entire surface of the pentacrinoid, short fibers were also detected as part of the other two components of the nervous system, the iponeural and ectoneural systems (Fig. 3 C). A strong signal was observed in each papilla, labelling the central microtubule-rich cell previously described in Heinzeller and Welsch (1994) (Fig. 3 E-G). Neuronal populations were explored using antibodies specific to serotonin, GABA, dopamine and choline acetyltransferase. Serotonin-positive cells were observed lying in a sub-epidermal position (Fig. 3 H). In tube feet, numerous serotonergic neurons created a dense neural net that did not project into the papillae (Fig. 3 K). Other positive cells were detected in the sub-epidermal neural plexus of the calyx, concentrated in the oral region, whereas in the aboral part of the calyx and the stalk fluorescence was completely absent (Fig. 3 H). GABAergic neurons were widely distributed in the whole animal (Fig. 3 I). GABA-positive cells were also present in the sub-epidermal plexus of the stalk (Fig. 3 F). Along the tube feet GABAergic neurons were detected: they appeared as flat cells connected by GABA positive fibres less numerous than serotonergic ones (Fig. 3 L). Cholinergic neurons were found in pentacrinoid entoneural system: fluorescence was observed in the central nerve cord running along the stalk and inside the calyx in the developing aboral nerve centre (Fig. 3 J). ChAT-positive cells were also detected along the tube feet and, particularly, at the level of the papillae (Fig. 3 M). No dopamine-positive cell was detected in pentacrinoid samples.

3.5. miRNAs expression during A. mediterranea development

A novel protocol for *in situ* hybridization in different crinoid post-embryonic stages was successfully developed. This was employed to characterize the expression pattern of three neural-related miRNAs (miR-124, miR-7 and miR-184) in both doliolariae and pentacrinoids. Indeed, most miRNAs are evolutionary conserved and some are specifically expressed in specific neural

territories of both vertebrate and invertebrate species. Their expression can therefore be used as molecular marker to identified neural populations.

miR-124 was abundantly expressed in the apical neural plexus of doliolaria larvae, forming a densely-stained arch characterised by two lateral spots of stronger signal (Fig. 4 A and C). miR-124 signal was observed especially in the neural region under the apical pit and in the neural plexus extending between the adhesive pit and the vestibulum (Fig. 4 B). In pentacrinoids, miR-124 mature transcript was present in neurons located along the tube feet, and in the neural plexus associated with the digestive system (Fig. 4 D-F). In tube feet, stained neurons were regularly interspersed along their entire length, although it was not possible to clearly define if those neurons were associated with the papillae.

In doliolaria, miR-7 expression was detected in the apical neural plexus, in a sub-set of neurons drawing an arch around the anterior part the vestibulum (Fig. 4 G). Transcript localization was confirmed by sagittal sections in which stained cells were observed in the ventral region between the adhesive pit and the vestibulum (Fig. 4 I). Mature transcripts were also found right under the apical pit (Fig.4 G), as confirmed in lateral view (Fig. 4 H). In the pentacrinoid stage, miR-7 was expressed in the proximal part of tube feet, whereas signal could not be detected in their tip. Furthermore, signal was detected around the mouth opening. miR-7 expression was found neither at the base of the calyx nor in the stalk (Fig. 4 J, K and L).

miR-184 mature transcripts were found in different tissues of doliolaria body. Expression was detected in the apical portion, where cell bodies of the neural plexus are concentrated, as well as in endodermal and mesodermal derivatives (Fig. 4 M and N). In fact, in post-*in situ* histological sections, miR-184 expression appeared widely distributed in the evaginates of the enterohydrocoel and in the surrounding mesenchymal cells (Fig. 4 O). In the apical neural plexus, signal was concentrated in cells located just under the apical pit, as observable in both whole mount and sectioned samples (Fig. 4 N and O). Also in pentacrinoids, miR-184 was expressed in both neural and non-neural tissues (Fig. 4 P). Several cells along the entire length of the tube feet were clearly marked. Moreover, miR-184 transcripts were found in the epithelium of the digestive tract, from the mouth to the intestine; the wall of the intestine was particularly stained in the aboral tract, as it was clearly visible both in whole mount samples (Fig. 4 Q) and in sections (Fig. 4 R).

miR-133, a muscle specific miRNA, was used as a negative control since it has been demonstrated that *A. mediterranea* doliolariae and pentacrinoids lack muscles (Barbaglio et al. 2012; Chia et al., 1986). Indeed, both the developmental stages processed with miR-133 probe never showed positive signal (data not showed).

4. Discussion

As basal echinoderms, crinoids are promising model systems in developmental and evolutionary biology. An increasing number of studies have been performed on the developmental stages of these organisms, mostly concerning skeletogenesis (Amemiya et al., 2015; Comeau et al., 2017) and molecular phylogeny (Hemery et al., 2013; Reich et al., 2015; Rouse et al., 2013; Telford et al., 2014), whereas the development of the nervous system has been only partially explored (Barbaglio et al., 2012; Nakano et al., 2009). This strongly hampers the overall understanding of echinoderm nervous system evolution. Thus, in this work we investigated the neuroanatomy of the two main developmental stages of the feather star *A. mediterranea*.

The histological analysis of doliolaria larva confirmed its neuroanatomy, previously described in Barbaglio et al. (2012), consisting mainly in a basiepithelial neural plexus integrated in larval epidermis and particularly developed in the apical part of the doliolaria. The lateral plexus thickenings, visible on both side of the body, reflect the overall bilateral symmetry of the larva. In addition, our immunohistochemistry analysis revealed neural fibers directing from the plexus towards the body surface. These connections were particularly appreciable under the apical pit from which the sensory apical tuft emerges, suggesting that the apical neural plexus may function as an integrating centre for sensory inputs (Burke, 2011). The integrative activity of the apical neural plexus was further supported by the presence of different neural populations (Fig. 5). Serotonin localization was already detected in the anterior region of neural plexus of the crinoid *Oxycomanthus japonicus* larvae but a detailed description of its organization was missing (Nakano et al., 2009). In all other Ambulacraria (Echinodermata + Hemichodrata) larvae, an apical organ containing serotonergic neurons has been extensively described. Comparative studies reported that the ancestral apical organ was organized into a bilateral serotonergic nerve plexus associated with ciliary cells in the anterior-most end of the larva. From this simple organization, the apical organ has been differentially modified in each larval form (Byrne et al., 2007; Nielsen, 2005). In asteroid bipinnaria and holothuroid auricularia larvae, the apical organ consists of serotonergic cells along the anterior ciliary band sectors. In particular, in asteroids, the apical organ is more complex, being organised in two ganglia associated with a plexus. Similarly, ophioplutei have a pair of serotonergic ganglia located close to the ciliary bands (Hirokawa et al., 2008). The simple apical organ of auricularia is instead similar to that of hemichordate tornaria larva (Byrne et al., 2007; Chee & Byrne, 1999; Nakano et al., 2006; Nezlin & Yushin, 2004) whereas in echinopluteus this structure is outside the ciliary epithelium, forming two cluster of serotonergic cells joined by a neuropil (Bishop & Burke, 2007; Byrne et al., 2007). In this context, our description of doliolaria

serotoninergic neurons complete the framework of larval apical organ in Ambulacraria supporting its ancestral bilateral organisation.

GABAergic neurons in echinoderms have received much less attention. In the doliolaria of *A. mediterranea* we detected GABA only in a restricted area of the neural plexus, right under the adhesive pit. This may indicate a role during settlement either by controlling the release of sticky substance by the adhesive pit, or by integrating information from anterior sensory cells. Indeed, as observed in the ascidian *Ciona intestinalis* (Takamura, Minamida, & Okabe, 2010), in many mollusc species (García-Lavandeira et al., 2005; Laimek et al., 2008; Rumrill & Cameron, 1983) and in sea urchin GABA induces settlement (Rahman & Ueharai, 2001) through the regulation of swimming activity (Bisgrove & Burke, 1987). In the pluteus stage of the echinoid *Hemicentrotus pulcherrimus*, GABA immunoreactive cells were detected in the ciliary band of the arms and functional analyses demonstrated the contribution of GABAergic system in the regulation of larval movements (Katow, Abe, Katow, Zamani, & Abe, 2013). We never observed GABA immunoreactivity in proximity of ciliated bands suggesting that in *A. mediterranea* doliolaria, this neurotransmitter could be involved in settlement through a different mechanism. The localization allows excluding any involvements of this neurotransmitter in the regulation of ciliary beating. The differences in larval GABAergic systems observed between *A. mediterranea* and *H. pulcherrimus* could also be due to the highly-derived morphology displayed by the echinopluteus but further functional and comparative analyses, including different echinoderm larvae are needed to clarify this intriguing topic.

The organization of the nervous system in the transient cystidean phase has been partially described in *O. japonicus* (Nakano et al., 2009), but until now only scant and incomplete data were available about the neural organization of the subsequent pentacrinoid stage. In particular, in the pentacrinoid stage of *A. mediterranea*, the entoneural system has never been described in details and it was thought to appear only late in development (Hyman, 1955). In the present study, combining histological analysis and β-tubulin immunodetection, the first description of the developing aboral nerve centre is reported. Indeed, at the base of the calyx a conspicuous concentration of somata was evident, from which a thick nerve cord ran in the stalk reaching the attachment disk. Five β-tubulin-positive neurite bundles could be observed directing towards the oral surface, suggesting that primordia of the brachial nerves appear long before the development of the arms. Most of the epidermal plexus could not be detected by semi-thin sections but somata and cell processes associated with the papillae were clearly recognizable along tube feet and appeared β-tubulin immunoreactive, in agreement with previous description (Byrne & Fontaine, 1983).

Regarding neural type distribution, serotonergic neurons were numerous in tube feet, where they

formed a complex network of fibers under the thin epidermis, and in the oral region of the calyx, where they were arranged in a looser network and possessed short processes. These results differ from those obtained in the adult, where serotonin was detected in the brachial nerve and coelomic epithelium, and only weak signal was found in the ambulacral groove (Candia Carnevali et al., 1996). This may be due to differences in the techniques used or may indicate that during pentacrinoid growth neurogenesis proceeds, producing new cell types in internal tissues. The GABAergic system appeared more widely distributed than the serotonergic one: numerous sub-epithelial neurons are present in both the stalk and the calyx, in which the neural plexus reaches the digestive epithelium. GABAergic neurons were observed also in tube feet, although they were arranged differently from serotonergic cells: nerve fibers proceeded along each side of the tube feet, flanking the hydrocoelic canal. A similar arrangement was displayed by cholinergic neurons but ChAT-positive cells were also detected in the papillae. Cell bodies and fibers of the aboral nerve centre and stalk nerves, (entoneural system) were also found to be cholinergic. This is in line with the neurobiology of the adult, as this system constitutes the major part of the nervous system and has motor functions (Heinzeller & Welsch, 1994). In adult specimens of the asteroid *Asterias rubens*, GABAergic neurons were observed in radial nerve cords, tube feet and regions of the digestive system. Particularly, the distribution of this neurotransmitter in the nerve plexus of asteroids (Newman & Thorndyke, 1994) together with pharmacological investigations in both asteroid and echinoid species demonstrated that GABA had an excitatory effect on tube feet (Florey, Cahill, & Rathmayer, 1975; Protas & Muske, 1980). Acetylcholine also caused tube foot contraction. It was therefore proposed that GABA responses were mediated through cholinergic motor neurons even if the two neurotransmitters seemed to interact with different receptors (Newman & Thorndyke, 1994; Protas & Muske, 1980). The occurrence of cross-talk between different neuronal populations was also reported for other neurotransmitters systems: in sea urchin larvae, dopamine and serotonin regulated forward and backward swimming speed in a complementary manner (Yoshihiro, Keiko, Chieko, Akemi, & Baba, 1992). The similar organization displayed by cholinergic and GABAergic neurons in pentacrinoid tube feet, suggests a possible interaction between systems but only future functional analyses will clarify the involvement of each neurotransmitter in tube feet contraction.

Finally, a new protocol for *in situ* hybridization in crinoids was designed, taking into consideration the technical difficulties connected mainly with the yolk-rich body of the lecithotrophic doliolaria and the skeletal stereom of the pentacrinoid. This was exploited to study the expression of neural specific miRNAs, as they have been demonstrated to provide an effective tool to identify neural territories in different metazoans.

miR-124 is the major nervous system-specific miRNA and was found in all of the bilateral species studied to date. In particular, with very few exceptions, miR-124 is expressed in the central nervous system regardless of the neuroanatomy of the taxon (Chen & Zeller, 2011; Christodoulou et al., 2010; Sun & Lai, 2013). In the doliolaria of *A. mediterranea* miR-124 mature transcripts were restricted to the apical part of the neural plexus, in the area under the apical pit where a group of somata was found (Chia et al., 1986). This expression pattern seems to indicate that the apical organ and the rest of the apical neural plexus, where both serotonergic and GABAergic neurons were located, may be the coordinating area of the larval nervous system, a sort of central nervous system, as reported in other echinoderm larvae (Byrne et al., 2007). In the pentacrinoid, miR-124 expression was apparently limited to the basiepithelial (ectoneural) system, at the level of tube feet and the digestive tract. This was a surprising result given the predominance of the entoneural system in adult crinoids (Candia Carnevali & Bonasoro, 2001), which actually exerts "centralizing" functions, and suggested that in trying to make comparisons with other groups, the ectoneural system may be the best one to consider. To confirm these results, detection of miR-124 in other echinoderm larvae and adults is surely necessary and results will be precious to clarify echinoderm nervous system evolution.

miR-7 is another highly conserved, neuron-enriched miRNA, but during animal evolution its expression domains extended also to non-neural tissues, such as endocrine islets of mammal pancreas and pharyngeal endoderm of cephalochordates (Bravo-Egana et al., 2008; Candiani, Moronti, De Pietri Tonelli, Garbarino, & Pestarino, 2011). In the nervous system of both protostomes and deuterostomes, miR-7 appears to be restricted to specific areas: in insects and cephalochordates, miR-7 was detected in photoreceptors (Candiani et al., 2011; Li & Carthew, 2005). In vertebrates and annelids, it is predominantly expressed in the neurosecretory areas of their nervous systems, the hypothalamus and the neurosecretory centre respectively (Christodoulou et al., 2010). It has been proposed that in ancestral bilaterians miR-7 first appeared in dual sensory-neurosecretory neurons, that eventually led to the evolution of optimized sensory neurons and neurosecretory centres in modern species (Tessmar-Raible et al., 2007). In *A. mediterranea* doliolaria, miR-7 was expressed al low level in the ventral region of the apical neural plexus and in cells underlying the apical pit. In the pentacrinoid, miR-7 was restricted to the mouth region and the proximal part of tube feet. Although a more detailed analysis of cell types in crinoid larvae and juveniles are necessary to make relevant evolutionary considerations, the results appeared encouraging, particularly considering that the apical organs of many other larvae contain neurosecretory neurons (Marlow et al., 2014; Tessmar-Raible et al., 2007).

miR-184 is extremely conserved in Nephrozoa, a taxon encompassing protostomes and

deuterostomes, but its expression and function in the majority of animals still remain obscure. In vertebrates, miR-184 research concentrates on its involvement in tumours and corneal diseases (Emdad et al., 2015; Shalom-Feurstein et al., 2012; Wong, Chan & Wei, 2009), but a complete expression profile during development has never been performed. In amphioxus, miR-184 transcripts were found in the developing neural tube and somites, and later in the cerebral vesicle, the pharynx, the endostyle and the first two gill slits (Candiani et al., 2011). In *Drosophila*, miR-184 expression is highly dynamic during development: in embryogenesis, it is prevalently expressed in the ventral nerve cord and in restricted endodermal and mesodermal areas, while larvae also express this miRNA in several imaginary discs (Li et al., 2011). In line with these findings, in *A. mediterranea*, miR-184 was expressed in ectodermal, mesodermal and endodermal derivatives in both doliolaria and pentacrinoid stages. In doliolaria, this miRNA was found in anterior neurons, the mesenchyme and the enterohydrocoel. In the latter, the expression was not uniform, but regularly arranged in spots, suggesting the presence of different cell types. Similarly, in the pentacrinoid, miR-184 displayed both neuronal and endodermal expression. Our results show that there is conservation at least in the type of embryonic tissues that express this microRNA, but more detailed analysis of miRNA expression during nervous system development in crinoids and other echinoderms will be required to make appropriate comparisons.

In conclusion, our results confirmed and implemented previous descriptions of doliolaria nervous system, revealing that larval apical region includes both serotonergic and GABAergic neurons, which were differentially localized within the plexus. This suggests that different larval activities are under the control of different neural populations. In the pentacrinoid, analyses showed the presence of a cholinergic entoneural nervous system while the basiepithelial plexus appeared more composite, displaying different neural populations. The present work sets the stage for future investigations, providing a proper protocol to successfully perform *in situ* hybridization, allowing to answer many of the still highly-debated questions about echinoderm evolution.

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Figure legends

Figure 1. Overview of *Antedon mediterranea* life cycle. The adult stage (**A**) is free-living and feeds by capturing food particles with pinnules that grows on the ten arms. The proximal pinnules contain gonads, and during breeding season eggs released by the female are kept on pinnules thanks to gland secretions (**B**). After external fertilization, the eggs develop on the pinnules (**C**) until they hatch at the doliolaria stage (**D**). The doliolaria is a lecithotrophic larva, characterised by five transverse ciliary bands (*****) and two ciliated groves: the vestibulum (**ve**) and the adhesive pit (**ad**). Anteriorly, a long ciliary tuft emerges from the apical tip (**ap**). Scale bar = 50 µm. After 2-3 days, the larva settles to an appropriate substratum and undergoes metamorphosis, becoming a

pentacrinoid (**E**). The pentacrinoid grows attached to the substratum through a long stalk (**s**) and feeds by filtering water with tube feet (**tf**) that grows on the oral surface. Scale bar = 100 μm.

Figure 2. Histology (**A**, **B**) and immunolocalization (**C-H**) in doliolaria larva. Frontal section (**A**) showing a conspicuous anterior neural plexus at the base of the epidermis from which two main lateral bundles of neurites extend posteriorly. In sagittal section (**B**), the neural plexus can be identified in the anterior region, extending dorsally just under the apical pit. **C**, **D**) β-tubulin immunolocalization in green: frontal (**C**) and lateral section (**D**). β-tubulin-positive fibres localized in the neural plexus from which neural projections direct towards the larva body surface with particular intensity under the apical pit (*arrow*) are visible. Cilia of the five ciliary bands are also immunoreactive.**E**, **F**). Serotonin immunolocalization in green: frontal (**E**) and lateral section (**F**) Serotonergic neurons appear concentrated in the anterior region of the neural plexus arranged dorsally, right under the apical pit (*arrow*), in two groups at each side of the larva. **G**, **H**) GABA immunolocalization in red: frontal (**G**) and lateral section (**H**) GABA-positive cells appear located in the anterior part of neural plexus, in proximity of the adhesive pit (*arrowhead*). In immunolocalization images: nuclei are stained in blue with DAPI.

a = anterior side; **ap** = apical pit; **ad** = adhesive pit; **d** = dorsal side; **eh** = enterohydrocoelic evaginates; **np** = neural plexus; **p**= posterior side; **v** = ventral side; **ve**= vestibulum. **Scale bar** = 50 μm.

Figure 3. *Antedon mediterranea* pentacrinoid: histology (**A-D**) and immunolocalization (**E-M**). **A**) The body of the pentacrinoid is composed of a cup-like calyx (**c**) and a long stalk (**s**) that connects the body to the substrate through an attachment disk (**ad**). **B**) The calyx is mainly occupied by the digestive tract (**dt**) that opens in a centrally located mouth (**m**), surrounded by numerous tube feet (**tf**). Hydrocoelic (**hy**) and somarocoelic (**so**) cavities are recognisable. At the base of the calyx, a conspicuous aboral neural centre (**anc**) can be identified, from which a thick nerve bundle runs along the stalk (**sn**). **C**) Higher magnification of tube feet with papillae (**pa**): cells from the base of the papillae send processes to the tip. **D**) The thin stalk is made of skeletal ossicles that surround a central stalk nerve bundle (**sn**). **E-G**) β-tubulin immunolocalization in green. Anti β-tubulin antibody markes the nervous fibres of the pentacrinoid. Strong fluorescence is present in the stalk nerve (*arrowheads*) and in five symmetrically arranged nerve bundles that from the aboral nerve centre project towards the oral surface (*double arrowheads)*. β-tubulin-positive cells are also located in the papillae of the tube feet. Red: GABA. **H, I**) Serotonin immunolocalization in green. Serotonergic cells are located in basi-epidermal position in the oral region of the calyx and in the tube feet, where they form a dense net without projecting to the papillae. **F**, **I**, **L**) GABA immunolocalization in red. GABAergic neurons are mainly located in basi-epithelial plexus both in

the calyx and the stalk, particularly in neurons associated with the digestive tract. In tube feet, only few cells are stained. **J**, **M**) ChAT immunolocalization in green. Cholinergic neurons can be detected in the developing aboral nerve centre projecting into the stalk nerve (*arrowhead*), and also in the papillae of the tube feet. **Scale bars** = 100 µm (**A**, **E**, **H**, **I** and **J**); 50 µm (**B**, **D**, **F**, **G**, **K**, **L** and **M**); 10 µm (**C**).

Figure 4. microRNA expression in *A. mediterranea* developmental stages. **A-F**) miR-124 expression. **A**) miR-124 is present exclusively in the anterior part of the neural plexus forming an arch characterised by two lateral spots of stronger signal (d*ouble arrowhead*). **B**) Lateral view shows the signal both at the level of the apical pit (*arrowhead*) and of the adhesive pit. **C**) Frontal section of miR-124 doliolaria. **D**) In pentacrinoid, miR-124 mature transcripts ae present in neurons along the tube feet and in the neurons associated with the digestive system (*arrow)*. **E**) Magnification of the calyx of miR-124 sample, showing expression in cells regularly interspersed along tube feet. **F**) Longitudinal section of miR-124 pentacrinoid. **G-L**) miR-7 expression. **G**) In doliolaria, miR-7 expression is observable in a ventral subset of neurons of the neural plexus around the anterior part the vestibulum. **H**) Mature transcripts are also present right under the apical pit (*arrowhead*). **I**) Longitudinal section showing miR-7 signal in the ventral area of the neural plexus. **J**) In pentacrinoid, miR-7 localization is restricted in the proximal part of tube feet (*asterisks*) and around the mouth opening. **K**) Magnification of the oral region of the calyx. **L**) Longitudinal section of a miR-7 pentacrinoid. **M-R**) miR-184 expression. **M-N**) In doliolaria, miR-184 is expressed in the neural plexus (*double arrowhead*), reaching the area under the apical pit (*arrowhead*), but also in the mesenchyme and in the enterohydrocoel evaginates (*star*). **O**) Frontal section of miR-184 doliolaria showing strong signal in the enterohydrocoel evaginates. **P**) In pentacrinoid, miR-184 is widely expressed in the sub-epidermal plexus along the tube feet (*asterisks*) and in the digestive system (*arrows*). **Q**) Magnification of the calyx of miR-184 sample, showing expression in the digestive system. **R**) Sections of a miR-184 pentacrinoid confirme the ectodermal, mesodermal and endodermal expression of this miRNA. **Scale bars** = 10 µm (**C**); 50 µm (**A**, **B**, **D-R**). **a** = anterior side; **ab** = aboral side; **d** = dorsal side; **or** = oral side; **p** = posterior side; **v** = ventral side.

Figure 5. Schematic representation of the distribution of serotonergic (**green**), GABAergic (**red**) and cholinergic (**blue**) neurons in *A. mediterranea.* doliolaria **A**: Frontal section of doliolaria; **B**: Sagittal section of doliolaria. **C)** Pentacrinoid.

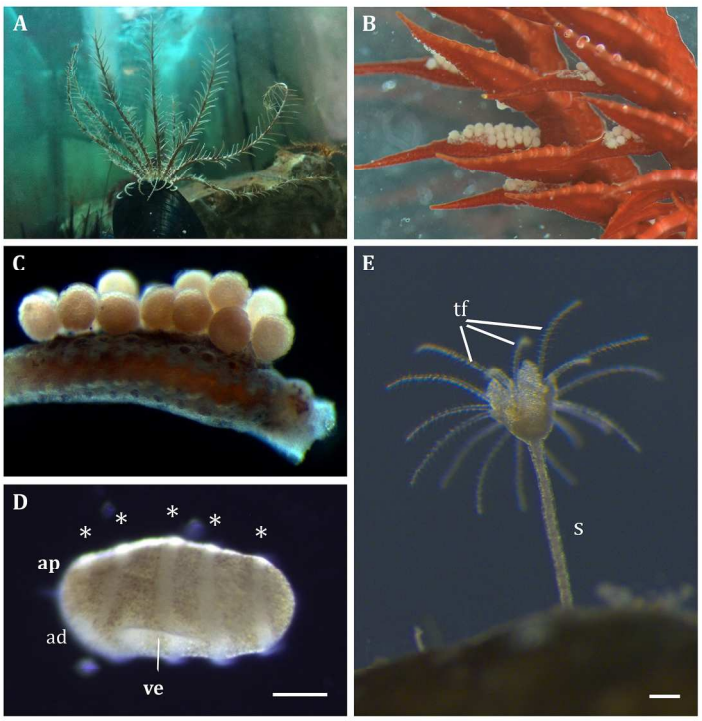


Figure 1. Overview of Antedon mediterranea life cycle. The adult stage (A) is free-living and feeds by capturing food particles with pinnules that grows on the ten arms. The proximal pinnules contain gonads, and during breeding season eggs released by the female are kept on pinnules thanks to gland secretions (B). After external fertilization, the eggs develop on the pinnules (C) until they hatch at the doliolaria stage (D). The doliolaria is a lecithotrophic larva, characterised by five transverse ciliary bands (*) and two ciliated groves: the vestibulum (ve) and the adhesive pit (ad). Anteriorly, a long ciliary tuft emerges from the apical tip (ap). Scale bar = 50 µm. After 2-3 days, the larva settles to an appropriate substratum and undergoes metamorphosis, becoming a pentacrinoid (E). The pentacrinoid grows attached to the substratum through a long stalk (s) and feeds by filtering water with tube feet (tf) that grows on the oral surface. Scale bar = 100 µm.

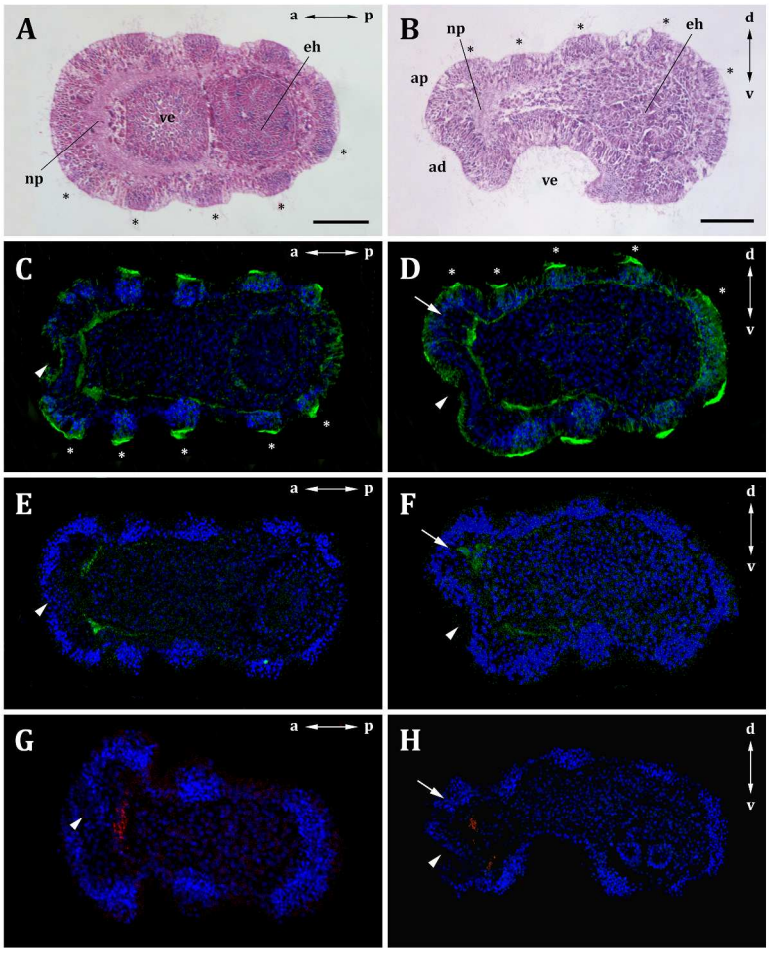


Figure 2. Histology (A, B) and immunolocalization (C-H) in doliolaria larva. Frontal section (A) showing a conspicuous anterior neural plexus at the base of the epidermis from which two main lateral bundles of neurites extend posteriorly. In sagittal section (B), the neural plexus can be identified in the anterior region, extending dorsally just under the apical pit. C, D) β-tubulin immunolocalization in green: frontal (C) and lateral section (D). β-tubulin-positive fibres localized in the neural plexus from which neural projections direct towards the larva body surface with particular intensity under the apical pit (arrow) are visible. Cilia of the five ciliary bands are also immunoreactive.E, F). Serotonin immunolocalization in green: frontal (E) and lateral section (F) Serotonergic neurons appear concentrated in the anterior region of the neural plexus arranged dorsally, right under the apical pit (arrow), in two groups at each side of the larva. G, H) GABA immunolocalization in red: frontal (G) and lateral section (H) GABA-positive cells appear located in the anterior part of neural plexus, in proximity of the adhesive pit (arrowhead). In immunolocalization images: nuclei are stained in blue with DAPI.
a = anterior side; ap = apical pit; ad = adhesive pit; d = dorsal side; eh = enterohydrocoelic evaginates; np

= neural plexus; p= posterior side; v = ventral side; ve= vestibulum. Scale bar = 50 μm.

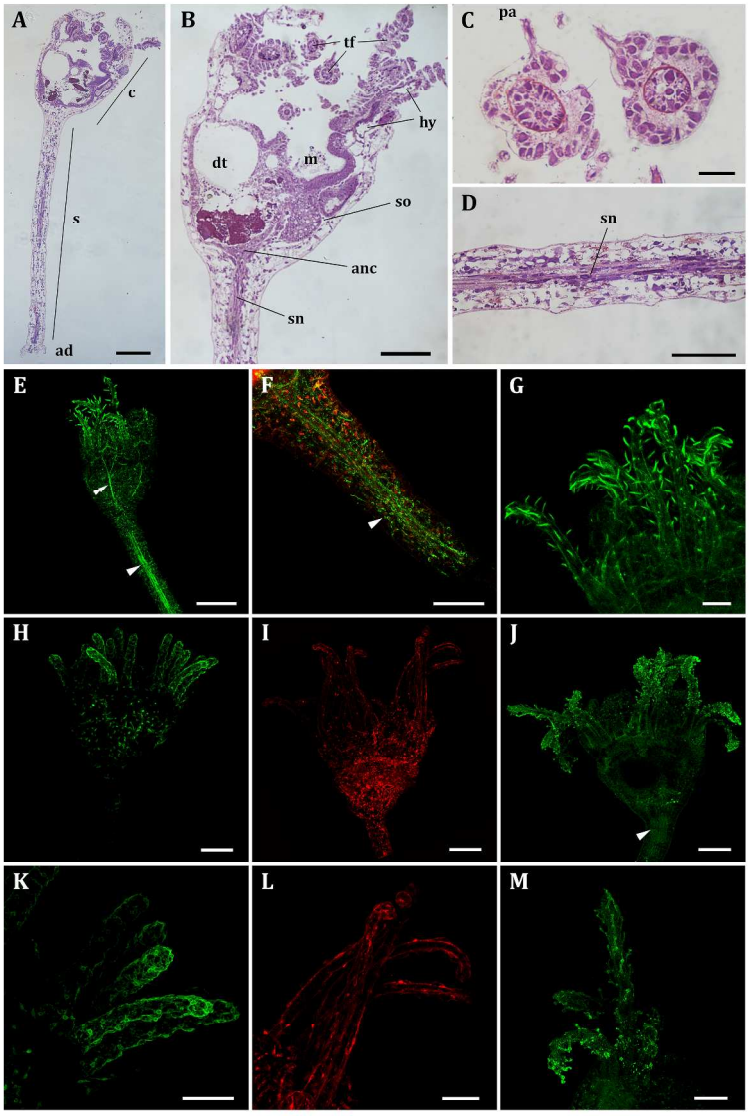


Figure 3. Antedon mediterranea pentacrinoid: histology (A-D) and immunolocalization (E-M). A) The body of the pentacrinoid is composed of a cup-like calyx (c) and a long stalk (s) that connects the body to the substrate through an attachment disk (ad). B) The calyx is mainly occupied by the digestive tract (dt) that opens in a centrally located mouth (m), surrounded by numerous tube feet (tf). Hydrocoelic (hy) and somarocoelic (so) cavities are recognisable. At the base of the calyx, a conspicuous aboral neural centre (anc) can be identified, from which a thick nerve bundle runs along the stalk (sn). C) Higher magnification of tube feet with papillae (pa): cells from the base of the papillae send processes to the tip. D) The thin stalk is made of skeletal ossicles that surround a central stalk nerve bundle (sn). E-G) β-tubulin immunolocalization in green. Anti β-tubulin antibody markes the nervous fibres of the pentacrinoid. Strong fluorescence is present in the stalk nerve (arrowheads) and in five symmetrically arranged nerve bundles that from the aboral nerve centre project towards the oral surface (double arrowheads). β-tubulin-positive cells are also located in the papillae of the tube feet. Red: GABA. H, I) Serotonin immunolocalization in green. Serotonergic cells are located in basi-epidermal position in the oral region of the calyx and in the tube feet,

where they form a dense net without projecting to the papillae. F, I, L) GABA immunolocalization in red. GABAergic neurons are mainly located in basi-epithelial plexus both in the calyx and the stalk, particularly in neurons associated with the digestive tract. In tube feet, only few cells are stained. J, M) ChAT immunolocalization in green. Cholinergic neurons can be detected in the developing aboral nerve centre projecting into the stalk nerve (arrowhead), and also in the papillae of the tube feet. Scale bars = 100 μm (A, E, H, I and J); 50 μm (B, D, F, G, K, L and M); 10 μm (C).

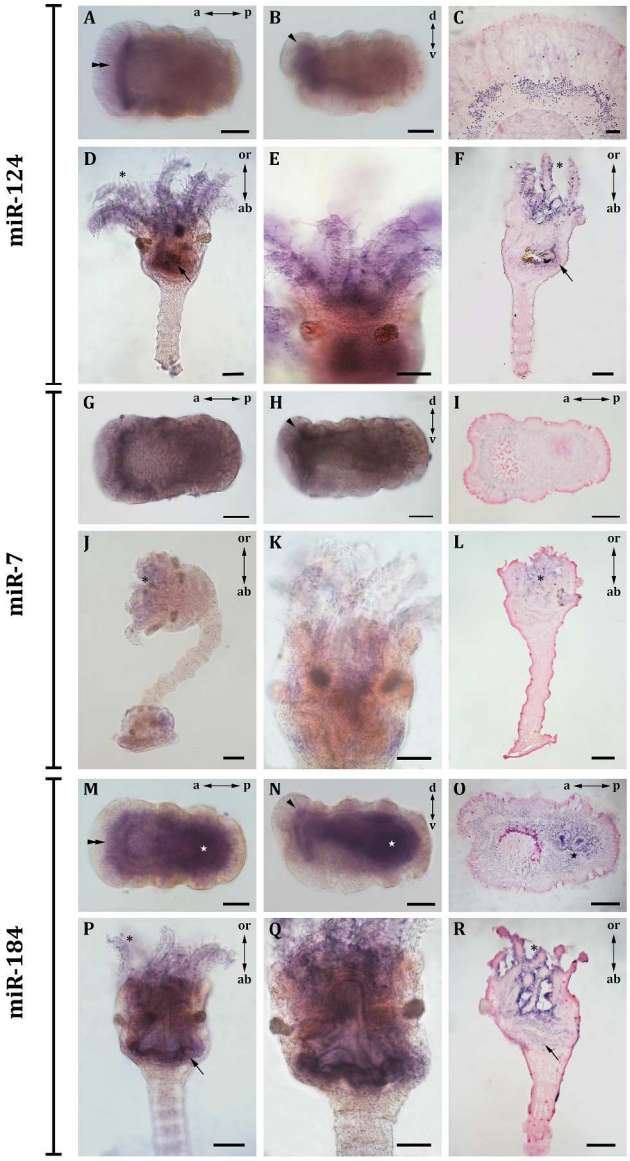
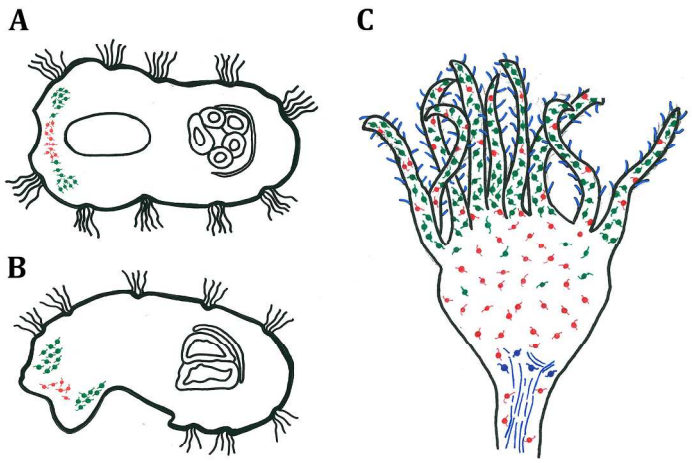


Figure 4. microRNA expression in A. mediterranea developmental stages. A-F) miR-124 expression. A) miR-124 is present exclusively in the anterior part of the neural plexus forming an arch characterised by two lateral spots of stronger signal (double arrowhead). B) Lateral view shows the signal both at the level of the apical pit (arrowhead) and of the adhesive pit. C) Frontal section of miR-124 doliolaria. D) In pentacrinoid, miR-124 mature transcripts ae present in neurons along the tube feet and in the neurons associated with the digestive system (arrow). E) Magnification of the calyx of miR-124 sample, showing expression in cells regularly interspersed along tube feet. F) Longitudinal section of miR-124 pentacrinoid. G-L) miR-7 expression. G) In doliolaria, miR-7 expression is observable in a ventral subset of neurons of the neural plexus around the anterior part the vestibulum. H) Mature transcripts are also present right under the apical pit (arrowhead). I) Longitudinal section showing miR-7 signal in the ventral area of the neural plexus. J) In pentacrinoid, miR-7 localization is restricted in the proximal part of tube feet (asterisks) and around the mouth opening. K) Magnification of the oral region of the calyx. L) Longitudinal section of a miR-7 pentacrinoid. M-R) miR-184 expression. M-N) In doliolaria, miR-184 is expressed in the neural plexus

(double arrowhead), reaching the area under the apical pit (arrowhead), but also in the mesenchyme and in the enterohydrocoel evaginates (star). O) Frontal section of miR-184 doliolaria showing strong signal in the enterohydrocoel evaginates. P) In pentacrinoid, miR-184 is widely expressed in the sub-epidermal plexus along the tube feet (asterisks) and in the digestive system (arrows). Q) Magnification of the calyx of miR-184 sample, showing expression in the digestive system. R) Sections of a miR-184 pentacrinoid confirme the ectodermal, mesodermal and endodermal expression of this miRNA. Scale bars = 10 µm (C); 50 µm (A, B, D-R). a = anterior side; ab = aboral side; d = dorsal side; or = oral side; p = posterior side; v = ventral side.



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