Integrative and Comparative Biology, volume 55, number 6, pp. 1018–1027
doi:10.1093/icb/icv014
Society for Integrative and Comparative Biology

SYMPOSIUM

Sensory Flask Cells in Sponge Larvae Regulate Metamorphosis via Calcium Signaling

Nagayasu Nakanishi, Daniel Stoupin, Sandie M. Degnan and Bernard M. Degnan[1]

School of Biological Sciences, University of Queensland, Brisbane QLD 4072, Australia

From the symposium "Origins of Neurons and Parallel Evolution of Nervous Systems: The Dawn of Neuronal Organization" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2015 at West Palm Beach, Florida.

[1]E-mail: b.degnan@uq.edu.au

Synopsis The Porifera (sponges) is one of the earliest phyletic lineages to branch off the metazoan tree. Although the body-plan of sponges is among the simplest in the animal kingdom and sponges lack nervous systems that communicate environmental signals to other cells, their larvae have sensory systems that generate coordinated responses to environmental cues. In eumetazoans (Cnidaria and Bilateria), the nervous systems of larvae often regulate metamorphosis through Ca^{2+}-dependent signal transduction. In sponges, neither the identity of the receptor system that detects an inductive environmental cue (hereafter "metamorphic cues") nor the signaling system that mediates settlement and metamorphosis are known. Using a combination of behavioral assays and surgical manipulations, we show here that specialized epithelial cells—referred to as flask cells—enriched in the anterior third of the *Amphimedon queenslandica* larva are most likely to be the sensory cells that detect the metamorphic cues. Surgical removal of the region enriched in flask cells in a larva inhibits the initiation of metamorphosis. The flask cell has an apical sensory apparatus with a cilium surrounded by an apical F-actin-rich protrusion, and numerous vesicles, hallmarks of eumetazoan sensory-neurosecretory cells. We demonstrate that these flask cells respond to metamorphic cues by elevating intracellular Ca^{2+} levels, and that this elevation is necessary for the initiation of metamorphosis. Taken together, these analyses suggest that sponge larvae have sensory-secretory epithelial cells capable of converting exogenous cues into internal signals via Ca^{2+}-mediated signaling, which is necessary for the initiation of metamorphosis. Similarities in the morphology, physiology, and function of the sensory flask cells in sponge larvae with the sensory/neurosecretory cells in eumetazoan larvae suggest this sensory system predates the divergence of Porifera and Eumetazoa.

Introduction

Sponges (phylum Porifera) are sessile filter-feeding animals and one of the earliest, if not the earliest, phyletic lineages to branch off the metazoan tree (Medina et al. 2001; Philippe et al. 2009; Ryan et al. 2013; Moroz et al. 2014). As animals, they show a variety of coordinated responses to sensory stimuli, including contracting the body and/or arresting the internal flow of water upon mechanical stimulation (Summarized by Elliot and Leys [2007] and Mackie [1979]), and changing the direction of swimming in larvae upon photic stimulation (Leys and Degnan 2001; Elliott et al. 2004). However, conclusive evidence is lacking that sponges have nervous systems (Jones 1962; de Ceccatty 1974; Mackie 1979). Thus, coordinated behaviors of sponges generally are explained in non-neural terms (summarized by Leys and Meech [2006] and Mackie [1979]), with an assumption that the sensory cells directly respond to the external environmental stimuli and thus are also the effector cells (Parker 1919; Leys and Degnan 2001; Maldonado et al. 2003). The lack of nervous systems in sponges has traditionally been considered primitive in Metazoa, although the possibility that this system may have been secondarily lost in sponges has been raised recently (Ryan et al. 2013). Regardless, understanding of sponges' sensory biology in a comparative framework is critical for reconstructing the deep history of the evolution of sensory-neural systems in Metazoa. In this article,

Advanced Access publication April 21, 2015

we investigate the sensory biology of larval settlement and metamorphosis in the demosponge *Amphimedon queenslandica*.

Sponges typically have a biphasic (pelagobenthic) life cycle in which embryogenesis results in a free-swimming lecithotrophic larva, which then settles and metamorphoses into a sessile, filter-feeding juvenile that grows into a mature adult (reviewed by Ereskovsky [2010]). Sponge larvae were previously thought to lack the sensory capacity to select specific substrates for settlement (Simpson 1984), although more recent studies show that demosponge larvae settle preferentially on a range of substrata including biofilms, coral rubble, and coralline algae (Jackson et al. 2002; Whalan et al. 2008, 2012; Woollacott and Hadfield 1996). These observations suggest demosponges have receptor and signaling systems that trigger coordinated metamorphic responses to environmental cues. However, the identity of specific receptors and sensory cells is still unknown in sponges.

The nervous systems of eumetazoan larvae are thought to function as systems receiving metamorphic cues (Morse 1990; Leitz and Lay 1995; Conzelmann et al. 2013). The sensory receptor cell typically has a single apical sensory cilium, numerous small vesicles in the cytoplasm, and basal neurites that form a network with other neurons (Piraino et al. 2011; Conzelmann et al. 2013). In initiating metamorphosis, sensory cells perceive natural metamorphic cues that lead to depolarization of these cells through the modulation of potassium channels. Depolarization of the membrane presumably activates voltage-gated Ca^{2+} channels, thereby increasing cytosolic Ca^{2+} levels (Freeman and Ridgway 1990; Freeman 1993; Clare 1996; Biggers and Laufer 1999). The increase in intracellular Ca^{2+} triggers exocytosis and the release of neurotransmitters/neurohormones, propagating the metamorphic signal through the nervous system to the effector cells. Alternatively, metamorphic signals may spread through epithelial conduction of Ca^{2+} via gap junctions, as proposed for the hydrozoan cnidarian *Mitrocomella polydiademata* (Freeman and Ridgway 1990).

In addition to natural cues, elevation of K^+ and Cs^+ levels in seawater triggers metamorphosis in many eumetazoan larvae (summarized by Degnan et al. [1997], Herrmann [1995], and Woollacott and Hadfield [1996]). Increased levels of K^+ and Cs^+ are thought to depolarize the larval cells that receive the metamorphic cue, including neurons, by diffusion of K^+ into the cytoplasm or by Cs^+ blocking potassium channels (Hadfield et al. 2000). Partial depolarization of membrane potential can result in the opening of voltage-gated Ca^{2+} channels, thereby increasing the intracellular levels of Ca^{2+} and propagating the metamorphic signals through the nervous system, or through epithelial conduction, as described above.

Interestingly, increased levels of K^+ and Cs^+ synergize with biofilmed substrates to induce metamorphosis in a demosponge *Aplysilla* sp. (Woollacott and Hadfield 1996). This raises the possibility that the mechanism of transduction of metamorphic signals might be shared across sponges and eumetazoans. However, since the pathway(s) of such transduction in sponge larvae remain unexplored and clearly does not include neurons, the nature of these mechanisms is currently unclear, specifically whether intracellular Ca^{2+} signaling is required.

In this article, we investigate the identity, morphology, and physiology of a sensory receptor cell in the marine demosponge *A. queenslandica* (Hooper and Van Soest 2006). An adult *A. queenslandica* has brood chambers within which embryos develop into parenchymella larvae. Larvae emerge daily from the parent sponge throughout the year, either naturally or by heat-treatment (Leys et al. 2008; Maritz et al. 2010) and are competent to settle on specific species of inductive coralline algae within as little as 4 h post-emergence (Jackson et al. 2002; Degnan and Degnan 2010). Their responsiveness to specific inductive substrata is consistent with a capacity to sense metamorphic cues. Using a combination of larval-settlement experiments, morphological analyses of cells, and calcium assays, we show here that the sensory receptor system of *A. queenslandica* larvae consists of an anteriorly enriched set of sensory-secretory epithelial cells called flask cells and that these cells respond to metamorphic cues through Ca^{2+}-dependent signaling system.

Methods

Biological material

Adult *A. queenslandica* were collected from Heron Island Reef, transported to the University of Queensland, and maintained at 25°C in recirculating aquaria. Larvae were collected upon release from adults after 2 h of mild heating (approximately 2°C above ambient temperature) as previously described (Leys et al. 2008). Larvae were kept in 0.2 µm filtered seawater (FSW) at 25°C. Articulated coralline algae (*Amphiroa fragilissima*) were also collected from Heron Island Reef and maintained in aquaria at the University of Queensland.

Larval settlement assays

For settlement experiments, 10–20 larvae or larval fragments per replicate were incubated for 1 h in a dish containing 5 mL FSW with *A. fragilissima* (covering approximately 10% of the dish's surface area). For experiments involving bleached algae, *A. fragilissima* were incubated in 5 mL household bleach (Clorox) for 1 h until the algae completely turned from their original red-pink to white. The bleached algae were rinsed in fresh water prior to their use as substrates. After 1 h of exposure to *A. fragilissima* the rate of settlement was scored as the proportion of larvae or larval fragments that physically attached to the substrate and did not swim away upon mild degrees of physical disturbance (i.e., movement of surrounding water). Larvae were deemed to have initiated metamorphosis when epithelial infolding had begun.

Fluorescent dye labeling and confocal microscopy

Fixation, followed by labeling with fluorescent dyes, were conducted as previously described (Larroux et al. 2006; Nakanishi et al. 2014). Filamentous actin was labeled using AlexaFluor 488-conjugated phallacidin (1:25, Molecular Probes). The lipophilic tracer CM-DiI (Molecular Probes, C7000) was used to label flask cells of larvae prior to fixation (Nakanishi et al. 2014). Nuclei were labeled with the fluorescent dye DAPI (1:1000, Molecular Probes). ProlongGold antifade reagent (Molecular Probes) was used to mount the specimens. Images were recorded using a Zeiss LSM 510 META Confocal Microscope and confocal stacks were viewed using ImageJ.

Transmission electron microscopy

A free-swimming larva was processed for transmission electron microscopy (TEM) by using the high-pressure freezing (HPF) technique, and ultrathin sections were prepared and analyzed as previously described (Nakanishi et al. 2014).

Calcium imaging

A 1 mM Fluo-4-AM (Molecular Probes, F-14201) stock solution was prepared in DMSO. Larvae were incubated in FSW containing 1 μM Fluo-4-AM for 30 min, and then washed in FSW and incubated without the dye for another 30 min to allow complete de-esterification of intracellular AM esters. These larvae were exposed to fragments of *A. fragilissima* for 30 min. Using a Zeiss LSM 510 META Confocal Microscope for high-resolution imaging, and a Nikon Eclipse Ti inverted fluorescence microscope for time-lapse imaging, intracellular free Ca^{2+} was imaged in larvae that were firmly attached to *A. fragilissima* but which had not yet initiated epithelial infolding.

Treatment with calcium chelator BAPTA-AM

A 50 mM BAPTA-AM (Molecular Probes, B-1205) stock solution was prepared in DMSO. Larvae were incubated in 100 μM BAPTA-AM in FSW for 1–2 h prior to the induction of settlement with fragments of *A. fragilissima* and the rate of settlement was scored as described above. The rate of larval settlement was also scored for larvae pre-incubated in 100 μM EDTA in FSW; this was used to control for the effect of chelating free extracellular calcium ions. Larvae were also subjected to 0.01% DMSO in FSW and induced to settle as described above.

Results

Amphimedon queenslandica larvae have a sensory system that detects metamorphic cues

Within 4 h post-emergence from the adult, *A. queenslandica* larvae are competent to settle and initiate metamorphosis upon contact with an inductive environmental cue (Jackson et al. 2002; Degnan and Degnan 2010). The larvae typically settle by firmly attaching their anterior end or anterior-lateral region to the substrate. The coralline alga *A. fragilissima* induces settlement of *A. queenslandica* larvae at high rates (Fig. 1; Degnan S.M. and Bayes J. unpublished data). About 70% of day-old larvae settle within 1 h when exposed to live *A. fragilissima*, while settlement and metamorphosis rarely occur when *A. fragilissima* is either absent or bleached (Fig. 1; live *A. fragilissima* $n = 5$ replicates, bleached *A. fragilissima* $n = 3$ replicates; two-tailed *t*-test, $P < 0.001$). These data are consistent with *A. queenslandica* larvae possessing a system to sense metamorphic cues associated with live *A. fragilissima*.

Flask cells are necessary for the induction of settlement and metamorphosis in *A. queenslandica*

Next we sought to determine the identity of the larval territory and cells that receive and respond to the inductive metamorphic cues associated with *A. fragilissima*. *Amphimedon queenslandica* larvae possess several morphologically and spatially distinct epithelial cell types (Fig. 2A) (Leys and Degnan 2001, 2002). In particular, cuboidal cells at the anterior pole, flask cells enriched in the anterior region, and globular cells intercalated throughout the larval epithelium all have been implicated as having a potential sensory

function based on their morphology, spatial distribution, and/or expression of genes related to bilaterian neural markers (Leys and Degnan 2001; Sakarya et al. 2007; Richards et al. 2008). The posterior region of the larva houses pigment cells both with and without long cilia, as well as long-ciliated cells adjacent to pigment cells that are presumed to be photosensory-effector cells that function in steering the larvae away from light (Leys and Degnan 2001; Rivera et al. 2012).

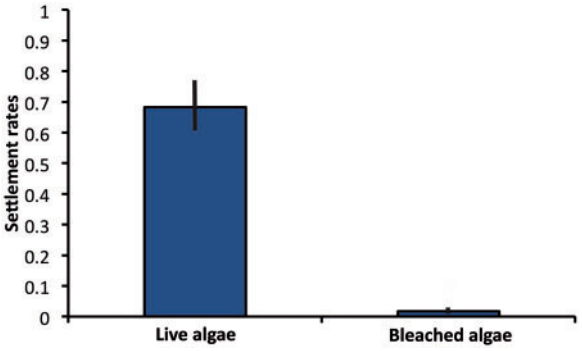

Fig. 1 Settlement of *Amphimedon queenslandica* larvae onto live, but not bleached, articulated coralline alga *A. fragilissima*. A histogram showing the settlement rates for day-old larvae in the presence of live and bleached *A. fragilissima*. Normal rates of settlement on the live alga is significantly higher that the non-bleached algae (two-tailed *t*-tests, $P<0.001$). Standard error bars are shown. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology* online.)

Taking advantage of the distinct spatial distribution of different kinds of candidate sensory epithelial cells, we transversely cut day-old larvae to generate anterior, middle, and posterior fragments, each of which contained distinct populations or enrichments in putative sensory cells. The anterior fragment uniquely has cuboidal cells and flask cells, the middle fragment has a small number of flask cells but lacks cuboidal cells, and the posterior fragment has pigmented cells (Fig. 2A, i–iii); all fragments contain globular and columnar epithelial cells. Surgical manipulations did not diminish the ability of the larvae to swim, or alter the polarity of their swimming. None of the larval fragments regenerated the removed parts (within 24 h).

These larval fragments were exposed to *A. fragilissima* fragments and their rates of settlement were determined relative to untreated control larvae. We found that the settlement rates of the anterior fragment was not significantly different from normal larvae, while those of the middle or posterior fragment were significantly lower (Fig. 2B; trisected larvae $n=4$ replicates, uncut control $n=5$ replicates; two-tailed *t*-test, anterior-control $P=0.354$, middle-control $P<0.01$, posterior-control $P<0.001$). This result is consistent with the anterior larval fragments containing cells that can detect the *A. fragilissima* inductive cue and that can induce metamorphosis.

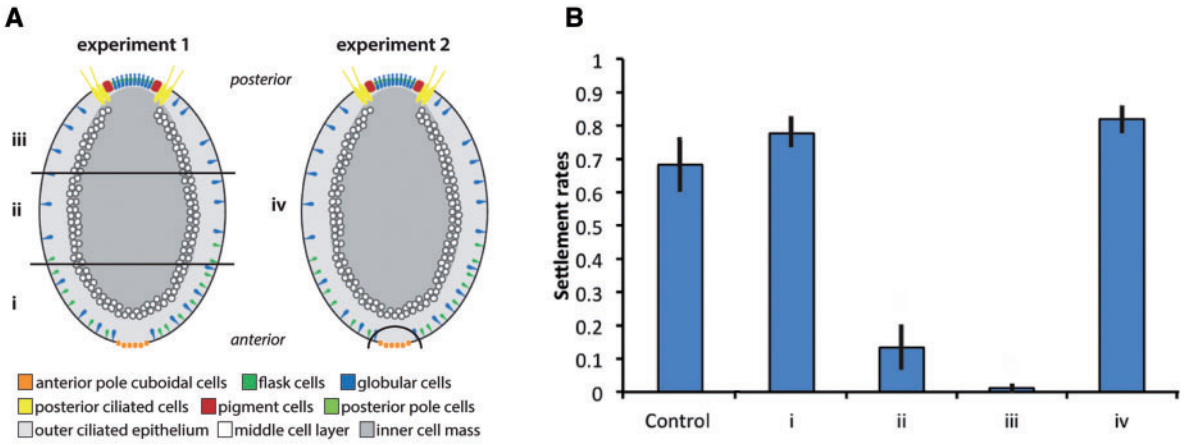

Fig. 2 Only larval fragments enriched with flask cells can be induced to undergo metamorphosis. (**A**) Summary illustration of the ablation experiments. Day-old larvae were transversely trisected (larval fragments i–iii, experiment 1), or their anterior-most region containing cuboidal cells was surgically removed (larval fragment iv, experiment 2). Note that each of the surgically manipulated larvae contains distinct types of epithelial cells. (**B**) A histogram showing settlement rates for surgically manipulated larvae (i–iv) and uncut normal control larvae ("Control") in the presence of the coralline alga *A. fragilissima*. The settlement rates of anterior larval fragments and larvae free of cuboidal cells (iv) did not differ significantly from the normal control larvae, while larval middle fragments (ii) and posterior fragments (iii) were significantly lower (two-tailed *t*-test, i-control $P=0.354$, ii-control $P<0.01$, iii-control $P<0.001$, iv-control $P=0.150$). Flask cells are the only type of epithelial cell uniquely shared between surgically manipulated larvae that showed normal rates of settlement (i.e., i and iv), consistent with it functioning as a sensory receptor cell that can detect and respond to metamorphic cues associated with *A. fragilissima*. Standard error bars are shown. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology* online.)

As mentioned above, the anterior fragment is enriched with cuboidal cells and flask cells, either or both of which could be sensory receptor cells of metamorphic cues. To distinguish between these alternatives, we generated larvae without cuboidal cells by surgically ablating the anterior-most region (Fig. 2A, iv). These larvae retained the normal pattern of swimming, and did not regenerate cuboidal cells (within 24 h). We found no difference in settlement rates between larvae lacking cuboidal cells and normal larvae (Fig. 2B; $n = 5$ replicates, two-tailed *t*-test, $P = 0.150$), indicating that cuboidal cells are not necessary for the induction of settlement and metamorphosis. Together, these experiments suggest that flask cells are instrumental in the control of settlement and metamorphosis in *A. queenslandica*.

Flask cells have morphological hallmarks of sensory-neurosecretory cells

We then examined the morphology of flask cells using confocal and TEM. In accordance with a previous morphological study (Leys and Degnan 2001), we found that flask cells have a single apical cilium emanating from a deep indentation of the apical cell membrane and possess numerous vesicles of various sizes (200–400 nm in diameter) in the apical cytoplasm (Fig. 3A, B). The nucleus is round (about 2 μm in diameter) and mediobasal in position; it is enriched with diffuse chromatin, and most of the condensed chromatin occurs near the nuclear envelope. Large vacuoles (600–700 nm in diameter) occupy the region basal to the nucleus. Mitochondria appear less numerous than in adjacent columnar epithelial cells. No chemical synapses were found, although a putative intercellular junction between a flask cell and a columnar epithelial cell was detected (Fig. 3C). In addition, we identified an apical F-actin-rich protrusion that surrounds the base of the cilium, and enrichment of F-actin in the apical region of the flask cell by electron and confocal microscopy (Fig. 3A, D). By differentially staining flask cells with the lipophilic dye DiI, which labels the plasma membrane (Nakanishi et al. 2014), we were able to visualize the morphology of the entire cell. This revealed basal neurite-like processes that were variable in length and orientation and had varicosities (Fig. 3E; Supplementary Movie S1); these cellular projections were confirmed by electron microscopy (Supplementary Fig. S1). These basal processes were not found to form a network, but would presumably increase the number of cells with which a flask cell could communicate (e.g., Supplementary Fig. S1). The presence of an apical sensory apparatus consisting of a cilium surrounded by an F-actin-rich protrusion, numerous vesicles, and basal neurite-like processes are consistent with flask cell having both sensory and secretory capabilities, as observed in larval sensory neurons in bilaterian and cnidarian larvae (Piraino et al. 2011; Conzelmann et al. 2013).

Initiation of metamorphosis requires the elevation of intracellular calcium levels in flask cells

As Ca^{2+} signaling is instrumental in the induction of metamorphosis in a range of eumetazoans (Freeman and Ridgway 1990; Freeman 1993; Clare 1996; Biggers and Laufer 1999), we sought to determine whether it is also required for metamorphosis in *Amphimedon*.Using the cell-permanent Ca^{2+} indicator Fluo-4-AM, we detected high levels of intracellular Ca^{2+} specifically in flask cells in larvae less than 30 min after settling on the coralline alga *A. fragilissima* (Fig. 4A; Supplementary Movie S2). Intracellular Ca^{2+} was not detected above background levels in flask cells of free-swimming larvae of the same age. Time-lapse imaging of a settled larva undergoing metamorphosis on *A. fragilissima* shows that cells with high intracellular Ca^{2+} initially are clustered in the anterior region. More posteriorly-localized cells exhibited an increase intracellular Ca^{2+} levels later in metamorphosis (Supplementary Movie S2). Fluo-4-AM signals were always confined to scattered individual cells, and were not seen to spread to neighboring cells; we found no evidence for epithelial conduction of Ca^{2+} in the form of a calcium wave. These data are consistent with calcium signaling occurring in flask cells shortly after coming in contact with the inductive cue associated with *A. fragilissima* and suggests that this signaling event is mediating metamorphic signal transduction to initiate metamorphosis in *A. queenslandica*.

Treatment of *A. queenslandica* larvae with 100 μM membrane-permeable, Ca^{2+}-chelating agent BAPTA-AM, which reduces the availability of free cytosolic Ca^{2+}, prior to contact with the coralline alga *A. fragilissima* settlement significantly lowered rates of settlement and metamorphosis compared with DMSO controls (Fig. 4B; BAPTA-AM $n = 4$ replicates, DMSO $n = 4$ replicates; two-tailed *t*-test $P < 0.001$). Larvae subjected to 100 μM BAPTA-AM swam normally and the morphology of the flask cells appeared normal. We considered the possibility that the charged form of BAPTA-AM in the cytosol might leak out of the cell and chelate extracellular free

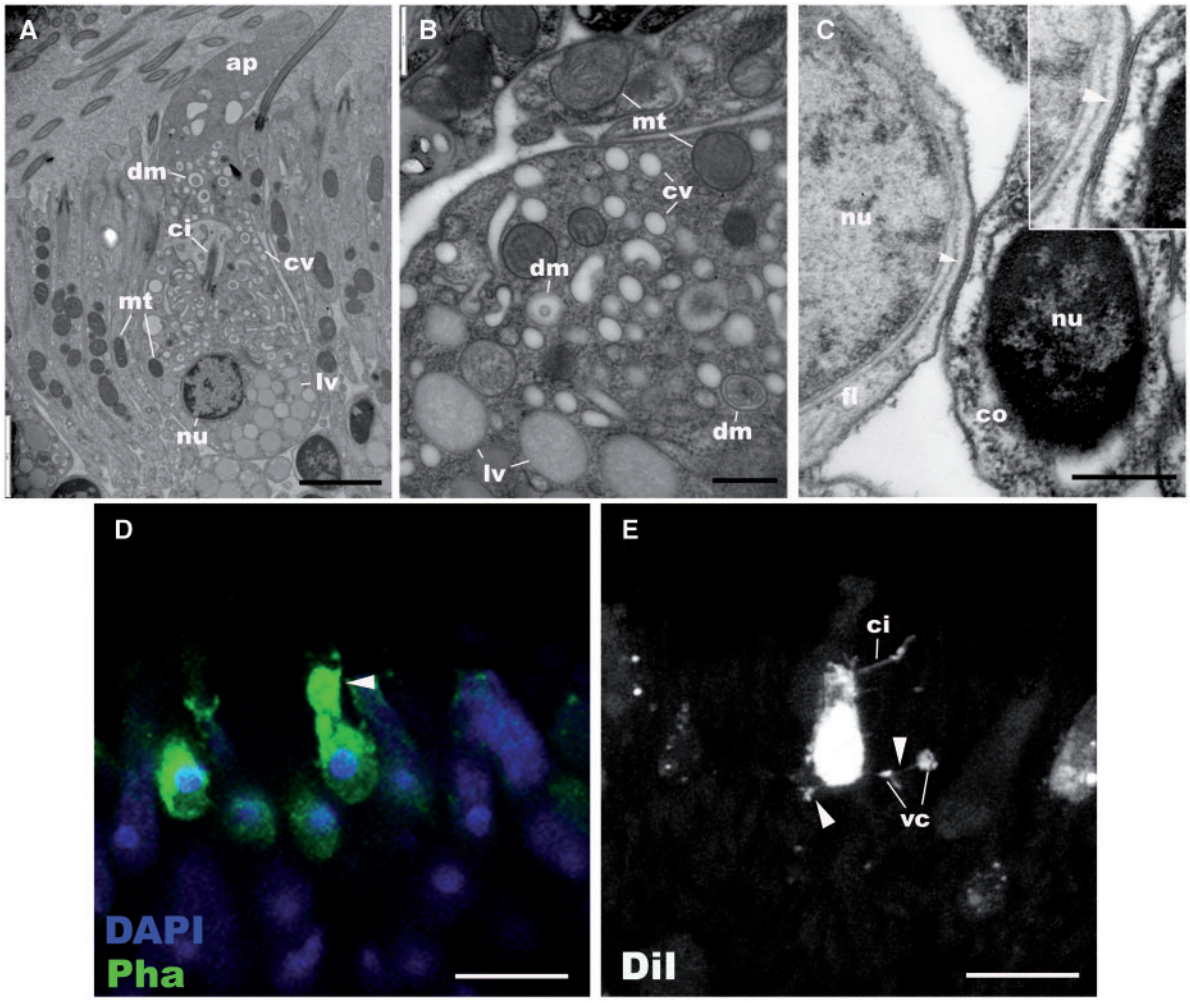

Fig. 3 Flask cells show the morphological hallmarks of sensory-neurosecretory cells. (**A–C**) Transmission electron micrographs of flask cells. The apical side is up. Note in A the flask-shaped morphology with a prominent apical protrusion (ap), a single apical cilium emanating from a deep pit (ci), and a mediobasal nucleus (nu) with condensed chromatin enriched at the peripheral region. Numerous clear (cv) or double-membraned (dm) vesicles occur apical to the nucleus; clear vesicles are 200–230 nm in diameter, and double-membraned vesicles are 270–380 nm in diameter (A, B). Large vacuoles (lv; 600–700 nm in diameter) cluster in the region basal to the nucleus (A, B). (C) A putative mediolateral intercellular junction (arrowhead) between a flask cell (fl) and a columnar epithelial cell (co); the inset shows a magnified view of the junction. D, E: Confocal sections of flask cells labeled with phalloidin (pha) (**D**) or CM-DiI (DiI) (**E**). Apical side is up. Phalloidin is used to label F-actin, and CM-DiI is used to label the plasma membrane. In D, nuclei are labeled by DAPI. Note enrichment of F-actin in the apical region of the flask cell (arrowhead in D). (E) Basal neurite-like processes (arrowheads) with varicosities (vc). Note: mt, mitochondrium. Scale bar: 2 μm (A); 500 nm (B, C); 10 μm (D, E).

Ca^{2+}, and reduce settlement rates without affecting intracellular Ca^{2+} signaling. To address this, we used the unmodified divalent-cation chelating agent EDTA as a control. The settlement rates for larvae incubated with 100 μM EDTA were not significantly different from those of the DMSO controls, and were significantly higher than the rates for larvae incubated with BAPTA-AM (Fig. 4B; $n = 3$ replicates; two-tailed t-test, EDTA-DMSO $P = 0.766$, EDTA-BAPTA-AM $P < 0.001$), indicating that the reduced rates of settlement of larvae exposed to BAPTA-AM resulted from the chelating of intracellular Ca^{2+}.

Discussion

These results are consistent with *A. queenslandica* larvae having a receptor and signaling system that recognizes and responds to exogenous environmental cues that induce settlement and metamorphosis. We find evidence that the sensory system consists of an anteriorly enriched sensory-secretory type of epithelial cell, the flask cell, which converts exogenous cues into an internal developmental signal that triggers metamorphosis. Promulgation of this signal requires an internal calcium signaling event within the flask cells.

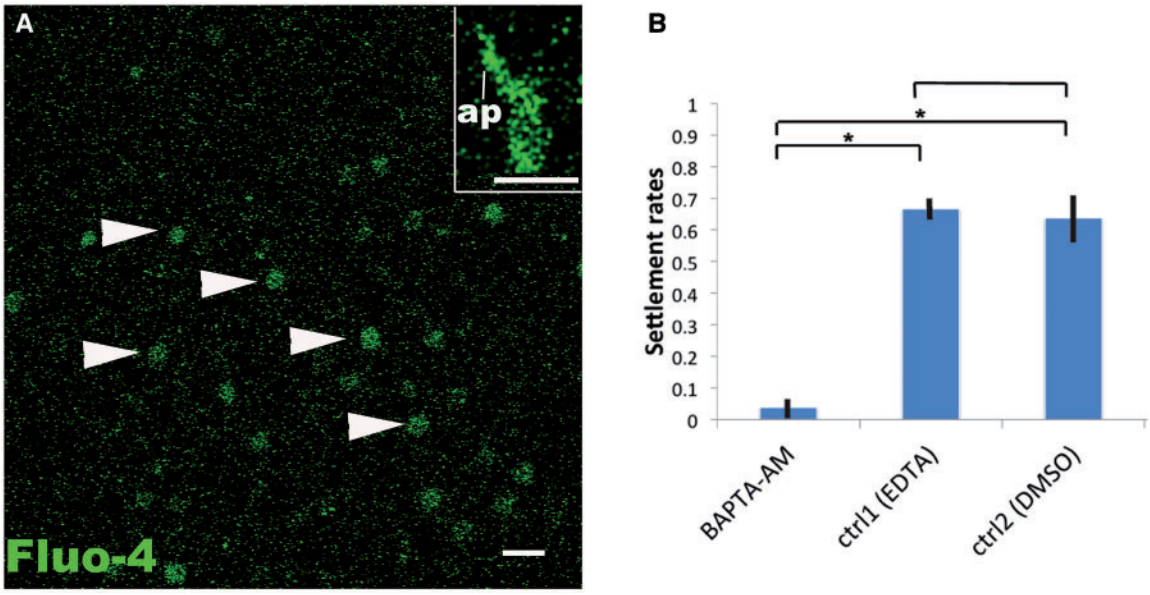

Fig. 4 Cytosolic free Ca^{2+} increases in flask cells at settlement, and is required for the initiation of metamorphosis. (**A**) A confocal tangential section of the anterior-lateral epithelium of an early settled larva (less than 30 min post-settlement), labeled with the calcium indicator Fluo-4-AM. The section is at the superficial level of the epithelium. Strong labeling occurs specifically in flask cells (arrowheads). The inset shows the lateral view of one of the labeled flask cells; apical surface is to the top. Note the extension of the apical process (ap) characteristic of flask cells undergoing changes in form at settlement (unpublished). (**B**) A histogram showing settlement rates for day-old larvae exposed to 100 μM BAPTA-AM, 100 μM EDTA, and 0.01% DMSO in FSW in the presence of the coralline algae *A. fragilissima*. Rates of settlement of larvae incubated in BAPTA-AM were significantly lower than the rates for those incubated with EDTA or DMSO (two-tailed *t*-test, BAPTA-AM-EDTA $P<0.001$, BAPTA-AM-DMSO $P<0.001$). The rates of settlement of larvae incubated in EDTA and DMSO did not significantly differ from each other (two-tailed *t*-test, $P=0.766$). Standard error bars are shown, and asterisk (*) denotes a statistically significant difference ($\alpha=0.05$) based on two-tailed *t*-tests. Scale bar: 10 μm. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology* online.)

Metamorphosis in *A. queenslandica* entails drastic reorganization of the entire larval body (Leys and Degnan 2002; Nakanishi et al. 2014). It is initiated when the anterior region of the newly settled larva, where the flask cells are enriched, contacts an appropriate substrate. The settled larva then undergoes a series of epithelial infolding that occurs sequentially from the base (i.e., the former anterior region) to the apex (i.e., the former posterior region), giving a wrinkled appearance. The epithelial integrity begins to break down, and extensive cell migration and apoptosis of the former larval epithelial cells are initiated (Nakanishi et al. 2014). The results presented here are consistent with flask cells being instrumental in the initial detection of the exogenous signal, and the transmission of an endogenous signal to other larval cells. This signal triggers a cascade of changes in cellular behavior and/or state that results in larval settlement and metamorphosis. At settlement, flask cells undergo epithelial-to-mesenchymal transition to transform into the archeocytes, a migratory stem cell that later gives rise to cell types such as the choanocyte in the juvenile (Nakanishi et al. 2014). Hence, the flask cells contribute to metamorphosis in three ways: (i) acting as a sensory receptor cell that detects environmental metamorphic cues; (ii) providing an intercellular signal to induce metamorphosis; and (iii) transdifferentiating into postlarval stem cells and subsequently other types of cells that contribute to the juvenile body plan.

Although the mechanisms of metamorphic signal transmission from flask cells to other cells remain unresolved, the presence of a diversity of secretory vesicles in these cells suggests that they may release signaling ligands via Ca^{2+}-dependent exocytosis. Alternatively, epithelial conduction of Ca^{2+} could potentially propagate the metamorphic signal. In this case, Ca^{2+} may directly signal to neighboring cells through diffusion via intercellular junctions, and function as a second messenger by associating with calcium-binding proteins. However, our Ca^{2+} time-lapse imaging shows no evidence of diffusion of Ca^{2+} (i.e., calcium "wave"), lending little support to the epithelial-conduction model of metamorphic signal propagation. This is consistent with previous studies that failed to find evidence of gap junctions in sponge larvae or adults (Bergquist and Green 1977; Green and Bergquist 1979; Garrone et al. 1980; Lethias et al. 1983; Garrone and Lethias 1990). Nonetheless, it is worth noting that our

HPF preparations show evidence of intercellular junctions between flask cells and neighboring ciliated epithelial cells in *A. queenslandica*. While these may act as communicating junctions, the *A. queenslandica* genome lacks genes encoding gap-junction proteins (innexin or connexin) (Srivastava et al. 2010).

In eumetazoans, a neurosecretory mode of signal transmission at metamorphosis appears to be largely conserved. In annelids, the anterior apical organ of the trochophore larvae houses sensory-neurosecretory cells with an apical sensory apparatus consisting of a single cilium surrounded by a collar of microvilli and numerous dense-cored vesicles, which is structurally very similar to the sponge flask cell. A subset of these sensory cells express myoinhibitory peptides (MIPs) that can trigger settlement and that are thought to be released upon the larva sensing metamorphic cues (Conzelmann et al. 2013). In hydrozoan cnidarians, a subset of sensory-neurosecretory cells with an apical sensory cilium and secretory vesicles in the anterior region of planula larvae express GLWamides (Leitz and Lay 1995; Piraino et al. 2011), which belong to the Wamide neuropeptide family together with MIPs (Liu et al. 2008). GLWamides trigger metamorphosis across Cnidaria (Leitz et al. 1994; Schmich et al. 1998; Iwao et al. 2002; Erwin and Szmant 2010), and GLWamide-positive sensory cells have been identified in larvae of the anthozoan *Nematostella vectensis* (Nakanishi et al. 2012) and the scyphozoan *Aurelia* sp. (N. Nakanishi and D. K. Jacobs, unpublished data).

Interestingly, GLWamides have been reported to induce metamorphosis in two coral-reef demosponges (*Coscinoderma matthewsi* and *Rhopaloeides odorabile*; Whalan et al. 2012), thereby raising the possibility that GLWamide-related peptides might have a conserved role as morphogens that induce metamorphosis in sponges and eumetazoans. However, genes encoding GLWamides or related peptides have not been found in sponges, including the *A. queenslandica* genome (Srivastava et al. 2010).

Members of all four classes of Porifera—Demospongiae, Calcarea, Homoscleromorpha, and Hexactinellida (Erpenbeck and Worheide 2007; Gazave et al. 2012)—have a pelagobenthic life cycle that includes the settlement of a planktonic larva, which metamorphoses into a juvenile that subsequently grows and matures (Ereskovsky 2010). Comparison of disparate sponge larvae reveals that epithelial secretory cell types that resemble flask cells in structure are found throughout the phylum, although they have been given a range of names. For instance, the "globular flagellate cell" in *Haliclona tubifera* larvae has a single apical cilium emanating within a pit, numerous small vesicles in the apical cytoplasm, and a mediobasal nucleus with chromatin being largely diffuse, and is localized predominantly to the anterior region of the larva (Woollacott 1993). Also, the cruciform cell of calcaronean amphiblastula larvae (Amano and Hori 1992), the bottle cell of calcinean calciblastula larvae (Amano and Hori 2001), and the stout cell of homoscleromorphan cinctoblastula larvae (de Caralt et al. 2007) are similar to flask cells in having secretory characteristics with numerous vesicles apical to the nucleus, large vacuoles basal to the nucleus, and a nucleus with diffuse chromatin. These cells differ from flask cells in lacking cilia; nevertheless, the surface of the apical cell is exposed to the outer environment, and thus the possibility of sensory function in these cells cannot yet be excluded. Whether these cells regulate metamorphosis in a manner similar to *A. queenslandica* flask cells has yet to be determined.

The presence of sensory flask-like cells in a range of sponge and eumetazoan larvae, and their widespread role in regulating settlement and metamorphosis, suggests there exists a deeply conserved larval sensory system that was present in the last common ancestor to sponges and eumetazoans. This system is required to translate environmental information into internal developmental signals that coordinate larval settlement and metamorphosis, probably via intracellular calcium signaling. Importantly these observations support the proposition that the biphasic or pelagobenthic life cycle is a metazoan synapomorphy that antedates the cladogenesis of contemporary animal lineages (Degnan and Degnan 2010). Studies into the sensory and neural systems in other early-diverging animal taxa will be critical to address this possibility.

Acknowledgments

The authors thank the staff at the Heron Island Research Station for their assistance, Dr Kathryn Green at the Centre for Microscopy and Microanalysis at the University of Queensland for preparing EM sections, Dr Gemma Richards for drawing the schematic of larvae used in Fig. 2A, and members of the Degnan laboratory for constructive discussions.

Funding

This work was supported by Australian Research Council grants [to S.M.D and B.M.D.] and an Australian Research Council Laureate Fellowship grant [to B.M.D.].

Supplementary data

Supplementary Data available at *ICB* online.

References

Amano S, Hori I. 1992. Metamorphosis of calcareous sponges .1. Ultrastructure of free-swimming larvae. Invert Reprod Dev 21:81–90.

Amano S, Hori I. 2001. Metamorphosis of coeloblastula performed by multipotential larval flagellated cells in the calcareous sponge *Leucosolenia laxa*. Biol Bull 200:20–32.

Bergquist PR, Green CR. 1977. Ultrastructural-study of settlement and metamorphosis in sponge larvae. Cahiers Biol Mar 18:289–302.

Biggers WJ, Laufer H. 1999. Settlement and metamorphosis of Capitella larvae induced by juvenile hormone-active compounds is mediated by protein kinase C and ion channels. Biol Bull 196:187–98.

Clare AS. 1996. Signal transduction in barnacle settlement: calcium re-visited. Biofouling 10:141–59.

Conzelmann M, Williams EA, Tunaru S, Randel N, Shahidi R, Asadulina A, Berger J, Offermanns S, Jekely G. 2013. Conserved MIP receptor-ligand pair regulates Platynereis larval settlement. Proc Natl Acad Sci USA 110:8224–9.

de Caralt S, Uriz MJ, Ereskovsky AV, Wijffels RH. 2007. Embryo development of Corticium candelabrum (Demospongiae:Homosclerophorida). Invert Biol 126:211–9.

de Ceccatty MP. 1974. Coordination in sponges—foundations of integration. Am Zool 14:895–903.

Degnan BM, Souter D, Degnan SM, Long SC. 1997. Induction of metamorphosis with potassium ions requires development of competence and an anterior signalling centre in the ascidian Herdmania momus. Dev Genes Evol 206:370–6.

Degnan SM, Degnan BM. 2010. The initiation of metamorphosis as an ancient polyphenic trait and its role in metazoan life-cycle evolution. Philos Trans R Soc Lond B Biol Sci 365:641–51.

Elliott GRD, Leys SP. 2007. Coordinated contractions effectively expel water from the aquiferous system of a freshwater sponge. J Exp Biol 210:3736–48.

Elliott GRD, MacDonald TA, Leys S. 2004. Sponge larval phototaxis: a comparative study. Boll Mus Ist Biol Univ Genova 68:291–300.

Ereskovsky AV. 2010. The comparative embryology of sponges [Internet]. New York: Springer. (http://dx.doi.org/10.1007/978-90-481-8575-7).

Erpenbeck D, Worheide G. 2007. On the molecular phylogeny of sponges (Porifera). Zootaxa 1668:107–26.

Erwin PM, Szmant AM. 2010. Settlement induction of Acropora palmata planulae by a GLW-amide neuropeptide. Coral Reefs 29:929–39.

Freeman G. 1993. Metamorphosis in the brachiopod terebratalia—evidence for a role of calcium-channel function and the dissociation of shell formation from settlement. Biol Bull 184:15–24.

Freeman G, Ridgway EB. 1990. Cellular and intracellular pathways mediating the metamorphic stimulus in hydrozoan planulae. Rouxs Arch Dev Biol 199:63–79.

Garrone R, Lethias C. 1990. Freeze-fracture study of sponge cells. In: Rutzler K, editor. New perspectives in sponge biology. Washington, DC: Smithonian Institution Press. p. 121–8.

Garrone R, Lethias C, Escaig J. 1980. Freeze-fracture study of sponge cell membranes and extracellular matrix preliminary results. Biol Cell 38:71–4.

Gazave E, Lapebie P, Ereskovsky AV, Vacelet J, Renard E, Cardenas P, Borchiellini C. 2012. No longer Demospongiae: Homoscleromorpha formal nomination as a fourth class of Porifera. Hydrobiologia 687:3–10.

Green CR, Bergquist PR. 1979. Cell membrane specialisations in the Porifera. In: Levi C, Boury-Esnault N, editors. Biologie des spongiaires sponge biology. Paris: CNRS. p. 153–8.

Hadfield MG, Meleshkevitch EA, Boudko DY. 2000. The apical sensory organ of a gastropod veliger is a receptor for settlement cues. Biol Bull 198:67–76.

Herrmann K. 1995. Induction and regulation of metamorphosis in planktonic larvae—Phoronis Mulleri (Tentaculata) as Archetype. Helgolander Meeresuntersuchungen 49:255–81.

Hooper JNA, Van Soest RWM. 2006. A new species of Amphimedon (Porifera, Demospongiae, Haplosclerida, Niphatidae) from the Capricorn-Bunker Group of Islands, Great Barrier Reef, Australia: target species for the 'sponge genome project'. Zootaxa 1314:31–9.

Iwao K, Fujisawa T, Hatta M. 2002. A cnidarian neuropeptide of the GLWamide family induces metamorphosis of reef-building corals in the genus Acropora. Coral Reefs 21:127–9.

Jackson D, Leys SP, Hinman VF, Woods R, Lavin MF, Degnan BM. 2002. Ecological regulation of development: induction of marine invertebrate metamorphosis. Int J Dev Biol 46:679–86.

Jones WC. 1962. Is there a nervous system in sponges. Biol Rev Camb Philos Soc 37:1–50.

Larroux C, Fahey B, Liubicich D, Hinman VF, Gauthier M, Gongora M, Green K, Worheide G, Leys SP, Degnan BM. 2006. Developmental expression of transcription factor genes in a demosponge: insights into the origin of metazoan multicellularity. Evol Dev 8:150–73.

Leitz T, Lay M. 1995. Metamorphosin-A is a neuropeptide. Rouxs Arch Dev Biol 204:276–9.

Leitz T, Morand K, Mann M. 1994. Metamorphosin A: a novel peptide controlling development of the lower metazoan Hydractinia echinata (Coelenterata, Hydrozoa). Dev Biol 163:440–6.

Lethias C, Garrone R, Mazzorana M. 1983. Fine-structure of sponge cell membranes—comparative study with freeze fracture and conventional thin section methods. Tissue Cell 15:523–35.

Leys SP, Degnan BM. 2001. Cytological basis of photoresponsive behavior in a sponge larva. Biol Bull 201:323–38.

Leys SP, Degnan BM. 2002. Embryogenesis and metamorphosis in a haplosclerid demosponge: gastrulation and transdifferentiation of larval ciliated cells to choanocytes. Invert Biol 121:171–89.

Leys SP, Larroux C, Gauthier M, Adamska M, Fahey B, Richards GS, Degnan SM, Degnan BM. 2008. Isolation of amphimedon developmental material. CSH Protoc 2008:pdb.prot5095.

Leys SP, Meech RW. 2006. Physiology of coordination in sponges. Can J Zool 84:288–306.
Liu F, Baggerman G, Schoofs L, Wets G. 2008. The construction of a bioactive peptide database in Metazoa. J Proteome Res 7:4119–31.
Mackie GO. 1979. Is there a conduction system in sponges? In: Levi C, Boury-Esnault N, editors. Colloques internationaux du centre national de la recherche scientifique. Paris: CNRS. p. 145–51.
Maldonado M, Durfort M, McCarthy DA, Young CM. 2003. The cellular basis of photobehavior in the tufted parenchymella larva of demosponges. Mar Biol 143:427–41.
Maritz K, Calcino A, Fahey B, Degnan BM, Degnan SM. 2010. Remarkable consistency of larval release in the spermcast-mating demosponge *Amphimedon queenslandica* (Hooper and van Soest). Open Mar Biol J 4:57–64.
Medina M, Collins AG, Silberman JD, Sogin ML. 2001. Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. Proc Natl Acad Sci USA 98:9707–12.
Moroz LL, Kocot KM, Citarella MR, Dosung S, Norekian TP, Povolotskaya IS, Grigorenko AP, Dailey C, Berezikov E, Buckley KM, et al. 2014. The ctenophore genome and the evolutionary origins of neural systems. Nature 510:109–14.
Morse DE. 1990. Recent progress in larval settlement and metamorphosis—closing the gaps between molecular-biology and ecology. Bull Mar Sci 46:465–83.
Nakanishi N, Renfer E, Technau U, Rentzsch F. 2012. Nervous systems of the sea anemone *Nematostella vectensis* are generated by ectoderm and endoderm and shaped by distinct mechanisms. Development 139:347–57.
Nakanishi N, Sogabe S, Degnan BM. 2014. Evolutionary origin of gastrulation: insights from sponge development. BMC Biol 12:26.
Parker GH. 1919. The elementary nervous system. Philadelphia: Lippincott.
Philippe H, Derelle R, Lopez P, Pick K, Borchiellini C, Boury-Esnault N, Vacelet J, Renard E, Houliston E, Queinnec E, et al. 2009. Phylogenomics revives traditional views on deep animal relationships. Curr Biol 19:706–12.
Piraino S, Zega G, Di Benedetto C, Leone A, Dell'Anna A, Pennati R, Carnevali DC, Schmid V, Reichert H. 2011. Complex neural architecture in the diploblastic larva of clava multicornis (hydrozoa, cnidaria). J Comp Neurol 519:1931–51.
Richards GS, Simionato E, Perron M, Adamska M, Vervoort M, Degnan BM. 2008. Sponge genes provide new insight into the evolutionary origin of the neurogenic circuit. Curr Biol 18:1156–61.
Rivera AS, Ozturk N, Fahey B, Plachetzki DC, Degnan BM, Sancar A, Oakley TH. 2012. Blue-light-receptive cryptochrome is expressed in a sponge eye lacking neurons and opsin. J Exp Biol 215:1278–86.
Ryan JF, Pang K, Schnitzler CE, Nguyen AD, Moreland RT, Simmons DK, Koch BJ, Francis WR, Havlak P, Program NCS, et al. 2013. The genome of the ctenophore *Mnemiopsis leidyi* and its implications for cell type evolution. Science 342:1242592.
Sakarya O, Armstrong KA, Adamska M, Adamski M, Wang I-F, Tidor B, Degnan BM, Oakley TH, Kosik KS. 2007. A post-synaptic scaffold at the origin of the animal kingdom. PLoS One 2:e506.
Schmich J, Trepel S, Leitz T. 1998. The role of GLWamides in metamorphosis of *Hydractinia echinata*. Dev Genes Evol 208:267–73.
Simpson TL. 1984. The cell biology of sponges. New York: Springer-Verlag.
Srivastava M, Simakov O, Chapman J, Fahey B, Gauthier MEA, Mitros T, Richards GS, Conaco C, Dacre M, Hellsten U, et al. 2010. The *Amphimedon queenslandica* genome and the evolution of animal complexity. Nature 466:720–U3.
Whalan S, Ettinger-Epstein P, Battershill C, de Nys R. 2008. Larval vertical migration and hierarchical selectivity of settlement in a brooding marine sponge. Mar Ecol Prog Ser 368:145–54.
Whalan S, Webster NS, Negri AP. 2012. Crustose coralline algae and a cnidarian neuropeptide trigger larval settlement in two coral reef sponges. PLoS One 7:e30386.
Woollacott RM. 1993. Structure and swimming behavior of the larva of haliclona-tubifera (porifera, demospongiae). J Morphol 218:301–21.
Woollacott RM, Hadfield MG. 1996. Induction of metamorphosis in larvae of a sponge. Invert Biol 115:257–62.