Early development and neurogenesis of Temnopleurus reevesii

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Early development and neurogenesis of *Temnopleurus reevesii*

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Abstract

Sea urchins are model non-chordate deuterostomes, and studying the nervous system of their embryos can aid in the understanding of the universal mechanisms of neurogenesis. However, despite the long history of sea urchin embryology research, the molecular mechanisms of their neurogenesis have not been well investigated, in part because neurons appear relatively late during embryogenesis. In this study, we used the species *Temnopleurus reevesii* as a new sea urchin model and investigated the detail of its development and neurogenesis during early embryogenesis. We found that the embryos of *T. reevesii* were tolerant of high temperatures and could be cultured successfully at 15 ˚C to 30 ˚C during early embryogenesis. At 30 ˚C, the embryos developed rapidly enough that the neurons appeared at just after 24 hours. This is faster than the development of other model urchins, such as *Hemicentrotus pulcherrimus* or *Strongylocentrotus purpuratus*. In addition, the body of the embryo was highly transparent, allowing the details of the neural network to be easily captured by ordinary epifluorescent and confocal microscopy without any additional treatments. Because of its rapid development and high transparency during embryogenesis, *T. reevesii* may be a suitable sea urchin model for studying neurogenesis. Moreover, the males and females are easily distinguishable, and the style of early cleavages is intriguingly unusual, suggesting that this sea urchin might be a good candidate for addressing not only neurology but also cell and developmental biology.

Keywords: serotonin, temperature tolerance

Introduction

As a model organism, the sea urchin has been used to address questions about the mysteries of life, especially in developmental and cell biology. This is because 1) the gametes are easily collected by simple injection of a small amount of KCl or acetylcholine into the body cavity, 2) the collected gametes from gonopores are not only mature and ready to be fertilized without any treatment but also can be stored for a few days or more (Kiyomoto et al. 2014), 3) multiple fertilized eggs develop synchronously, allowing the collection of millions of embryos at the same stage, and 4) molecular functions can be easily analyzed by simple microinjection of morpholino antisense oligos (MOs), DNA, and/or RNA (McClay 2011). These characteristics are also useful for teaching developmental biology in schools. Therefore, sea urchins have been important resources for both biology and education.

Phylogenetically, echinoderms, including sea urchins, form one clade with hemichordates and likely with Xenacoelomorpha, and this clade forms a sister group with chordates (Philippe et al. 2011). Thus, studying sea urchins from multiple scientific perspectives helps us to understand the conserved and diverged mechanisms during evolution. In fact, the gene regulatory network (GRN) established in many animals is a method for understanding the regulatory network of transcription factors and signaling molecules during embryogenesis, and sea urchin studies pose that these patterns are conserved across organisms (Davidson 2010).

Despite these valuable characteristics, sea urchins have not been used as a model organism in neuroscience. Although histochemical studies for the nervous system of sea urchin embryos/larvae were reported in the 1970s and 1980s (Ryberg 1977; Burke 1983; Bisgrove & Burke 1986), neither molecular mechanisms of their neural development nor physiological functions of the nervous system were investigated until the 21st century. One reason for this delay was a lack of genetic information related to neurogenesis compared with that in model organisms. After this limitation in sea urchins was overcome by the sequencing of the *Strongylocentrotus purpuratus* genome in 2006 (Sodergren et al. 2006), a small number of transcription factors and signaling molecules regulating neurogenesis of sea urchin embryos were identified, and their detailed functions were reported (Angerer et al. 2011). For example, the anterior neurogenic ectoderm is specified by two transcription factors, FoxQ2 and Six3 (Yaguchi et al. 2008; Wei et al. 2009), and TGF-β family members involved in body

axis formation (Duboc et al. 2004; Lapraz et al. 2009) pattern the specified region along the dorsal-ventral axis (Yaguchi et al. 2007). However, to understand the whole network controlling neurogenesis in the sea urchin embryo, a number of developmental events related to the differentiation of neurons must be elucidated at the level of molecular function.

Another reason for the slow progress in the study of the sea urchin nervous system is that neurogenesis in sea urchin occurs later in embryogenesis than other interesting phenomena, such as the specification of endomesoderm. In fact, in one of the model urchins, *Hemicentrotus pulcherrimus*, the migration of primary mesenchyme cells (PMCs) and the differentiation of serotonergic neurons become apparent within 21 hours and approximately 36 hours, respectively. In addition, the presence of PMCs is optically apparent from the outside of the embryo, whereas the neurons must be stained with specific antibodies, taking additional days for visualization. This difference has influenced scientists to study a more experimentally accessible target. In addition, later stage embryos have more cells (Mizoguchi 1999), and there are fewer neural cells than endomesodermal cells, making it difficult to collect material such as RNA or protein specifically from the neural cells. To overcome these disadvantages, although the ratio of neural cell number may be invariable within each species of sea urchin, we sought to establish a new model urchin with faster growth during embryogenesis. Here, we report that one sea urchin species, *Temnopleurus reevesii*, whose normal development has been reported in previous works (Kitazawa et al. 2014; Kitazawa et al. 2010), can be cultured at a wide range of water temperatures and grows faster in embryonic stages at high temperature than the common model species. Thus, this species can be a new model sea urchin, especially for studying neurogenesis.

Materials and methods

Animals and embryonic culture

Adults of *T. reevesii* were collected at the Shimoda Marine Research Center, University of Tsukuba and kept in an overflow tank using natural seawater. The gametes were collected by intracoelomic injection of 0.5 M KCl between November to January in this study, and the embryos were cultured in 3.5-cm plastic dishes or 200-ml glass beakers with filtered natural seawater (FSW). The embryos were cultured at 15-30 ˚C as described below. To grow them until later stages such as 6-arm pluteus larvae,

embryos were cultured in 3.5-cm plastic dishes with 3.0 ml FSW containing 100 μg/ml kanamycin. A diatom, *Chaetoseros calcitrans*, was used for larval food.

Whole-mount immunohistochemistry

Immunohistochemistry for detecting serotonin and synaptotagmin B (synB) (Nakajima et al. 2004; Burke et al. 2006) was performed as described previously (Yaguchi et al. 2006; Yaguchi et al. 2011). The primary antibodies (dilutions; anti-serotonin 1:2,000 and anti-synB 1:100) were detected with secondary antibodies conjugated with AlexaFluor-488 or AlexaFluor-568, respectively (Life Technologies, Carlsbad, CA, USA, 1:2,000 dilution). The specimens were observed using Olympus FV10i and IX70 microscopes, and optical sections were stacked and analyzed with ImageJ and Adobe Photoshop. FM1-43 (Life Technologies, 2.0 μg/ml in FSW) was used to stain cell membranes.

Results

Adults and early embryogenesis of *T. reevesii*

The adults of *T. reevesii* (Fig. 1A) that were collected at the Shimoda Marine Research Center had five gonopores around the anus, as do other typical sea urchins (Fig. 1B, asterisks). One gonopore was present on the outer edge of madreporite (Figure 1B, magenta asterisk). The males and females were easily distinguishable in this sea urchin under a dissecting microscope because the females had no obvious structure at the gonopores (Fig. 1C, arrow), whereas the genital papilla protruded from the gonopore in males (Fig. 1D). The shape of the genital papilla varied between individuals, but all of them contained a deferent canal and pigment cells within the soft tissue (Fig. 1D, E, arrowheads).

The diameter of unfertilized eggs was approximately 80 μm (Fig. 2A), and the transparency was so high that the female pronucleus was clearly observed (Fig. 2B, arrow). Fertilized eggs (Fig. 2C) underwent cleavages and reached the 4-cell stage within 2 hours of culture at 25°C (Fig. 2D). Compared with common model sea urchins such as *S. purpuratus* or *H. pulcherrimus*, the connection between the blastomeres during early cleavage stages was very loose in this species. Immediately after cleavage, a number of protrusions were present at the edge of daughter blastomeres in the cleavage furrow (Fig. 2E). The edges of the blastomeres at probable interphase

resembled filopodia, attaching to the outer extracellular matrix (ECM) and the hyaline layer (Fig. 2F). This was better observed with the combination of a dye staining the plasma membrane (FM1-43) and confocal microscopy (Fig. 2G, H). Figure 2I shows a single optical section of 4-cell stage of *T. reevesii* and indicated no clear connection between blastomeres, which were instead attached tightly to the hyaline layer. Because of this loose connection between blastomeres, micromeres at 16-cell stage appeared to be present independently of each other (Fig. 2J). The macromere-descendant tier at the 32-cell stage also had no strong interconnections (Fig. 2K), unlike those of the *H. pulcherrimus* 32-cell embryo (Fig. 2L). Time-lapse images of cleavages show that connections between the blastomeres formed after the 60-cell stage, during which strong FM1-43 signals indicating cell-cell contact appeared (Fig. 2M-O).

To establish a suitable temperature for embryonic culture and routine analysis of neurogenesis in *T. reevesii*, we grew embryos at 15˚C, 20˚C, 25˚C, 30˚C, and 37˚C. The temperature used for culturing mammalian cells or bacteria such as *E. coli*, i.e., 37˚C, was likely too high for this sea urchin species because none of the cleavage stages was observed (data not shown). However, 30˚C culture was successful until at least the 4-arm pluteus stage, and 15˚C culture was also sufficient for embryo growth (Fig. 3). As expected, the higher temperature promoted faster embryonic development, resulting in prism larvae by 24 hours in 30˚C culture, whereas embryos cultured at 15˚C required 72 hours to reach prism stage (Fig. 3). At 25 and 30˚C, embryos reached the 4-arm pluteus stage at approximately 48 hours, but they began to collapse after this stage unless they were fed. In summary, temperatures between 15˚C and 30˚C are suitable for culturing *T. reevesii* embryos, depending on the experimental purposes and schedules.

Neurogenesis in *T. reevesii* embryos

To investigate neurogenesis in *T. reevesii* embryos, we evaluated synaptotagmin B (synB) (Nakajima et al. 2004) and the neurotransmitter serotonin during embryogenesis at 4 different temperatures. SynB is a pan-neural marker in sea urchin embryos (Burke et al. 2006). As previously reported in other species (Bisgrove & Burke 1986; Yaguchi et al. 2000), *T. reevesii* embryos also had no neurons until the late gastrula stage. Thus, 15˚C and 20˚C embryos did not include any differentiated neurons at 24 hours, whereas 25˚C embryos had very weak serotonin signal at the anterior end (Fig. 4, white asterisk) and synB signal at the anterior and lateral regions

(Fig. 4, yellow asterisks). In 30˚C culture, the serotonin signal was stronger than in 25˚C embryos, and axon elongation was also more developed within the ciliary band ectoderm (Fig. 4). Because the development was slow in lower temperatures, the number of differentiated neurons in 15˚C embryos at 72 hours was similar to that in embryos cultured for 24 hours at 25˚C (*cf*. Fig. 5 and Fig. 4). The serotonin signal in 20˚C embryos was not yet observed in axons at 48 hours, although synB-positive axon-like structures had already formed in the anterior end region (Fig. 5). The axons elongated sufficiently to form a neural plexus after 72 hours at 20˚C. A developed neural network was observed in 48-hour embryos in culture at 25 or 30˚C (Fig. 5). Anterior views of 48-hour embryos from both temperatures clearly showed the presence of serotonergic neurons at the dorsal side of the anterior end region (Fig. 5).

To obtain additional information about neurogenesis in *T. reevesii* embryos, we used a confocal laser-scanning microscope to capture optical sections and construct z-stacks. For these experiments, we used embryos cultured at 25 and 30˚C for 24 and 48 hours. Although the serotonin signal observed with a standard epifluorescent microscope was very weak in 24-hour embryos cultured at 25˚C (Fig. 4), confocal imaging clearly showed the differentiation of serotonergic neurons at the anterior end of the embryos (Fig. 6). Within another 24 hours, each cell had extended an axon to form a plexus. The ciliary band neurons also had long axons beneath the ciliary band, and one pair of oral-ectoderm neurons connected their axons to the axon bundle located at the base of the post-oral arms (Fig. 6, white arrowheads). The ventral view of 30˚C embryos at 24 hours also showed that the oral ectoderm neurons extended their axons to the ciliary band. Both ventral and dorsal views at this stage showed that ciliary band neurons and anterior end neurons including serotonergic neurons extended their axons beneath the ciliary band toward each other (Fig. 6, blue arrowheads). Embryos cultured for 48 hours at 30˚C had well-developed lower lip ganglions (Fig. 6, asterisk) but showed no serotonin signal, unlike in *H. pulcherrimus* (Yaguchi & Katow 2003). Axons from those ganglion cells were connected to the axon bundle from the ciliary band neurons at the base of pre-oral arms.

After being fed a diatom, *Chaetoseros calcitrans*, for 1 week, embryos cultured at 20˚C grew to 6-arm larvae (Fig. 7A-C). Clusters of serotonergic neurons were located bilaterally at the left and right edges of the anterior/central part of the oral hood, and explicit axons extending from each cluster were present in between (Fig.

7D-F). Because of the absence of the high cell density at the anterior-central part of the oral hood, which characterizes the anterior neurogenic ectoderm, the absence of the ciliary band observed between gastrula and early pluteus stages (Fig. 7D) and no molecular markers available in this species yet, we cannot define the neurogenic ectoderm at this stage. However, the region in which serotonergic neurons clustered was within the high-cell-density lateral ciliary band region, and axons from serotonergic neurons were present at the dorsal side between pre-oral arms (Fig. 7G, arrow). By contrast, non-serotonergic neurons formed a network at the ventral side (Fig. 7H, I). SynB-positive ciliary band neurons extended their axons into the ciliary band region throughout the larva. In addition, synB signal was observed around the esophagus (Fig. 7B, arrowhead), indicating that the nervous system was well developed to control the muscle for swallowing food.

Discussion

Sea urchins have been used as one of the model organisms in studying developmental biology. The use of sea urchin is based in part on the ease of collecting large numbers of gametes and of the control of synchronous embryonic development. However, gender identification is sometimes stressful because the only prominent differences in the appearances of males and females are the genital papilla, but in model sea urchins such as *H. pulcherrimus*, which authors have used in previous experiments, the difference is so small (Tahara et al. 1960) that scientists have not used this characteristic to identify gender. By contrast, *T. reevesii* has a clear difference in the shape of the genital papilla between males and females (Fig. 1). Although the precise shape of the structure of the male is variable in individuals, it is still easily distinguishable from those of females because the female has no protrusion from the gonopores. Simple observation using dissecting microscopes allows the clear identification of *T. reevesii* gender. This will save time for scientists in research and for teachers/students in education to identify male or female with random injection of KCl into the body cavity and will save natural resources because it will allow the collection of fewer sea urchins.

One surprising finding regarding embryogenesis in this species is the style of early cleavages. In the embryos of established sea urchin models, blastomeres are tightly attached each other during non-mitotic phases (Fig. 2L). By contrast, *T. reevesii*

blastomeres are attached not to each other but to the ECM (Fig. 2G-L). Thus, several questions arose regarding the cleavage mechanisms, including the strength of the blastomeres' attachment to the ECM, the changes in the connection between blastomeres and ECM during mitosis, and how much the contractile ring in this species differs from that of other urchins. This type of loose connection between blastomeres has already been reported in one species of starfishes (Matsunaga et al. 2002), but the blastomeres attach to the fertilization envelope not to hyaline layer because of lacking the structure in the species. The present study did not include experiments investigating the cleavage system and thus addressing those questions, but the detailed mechanisms of cleavage and blastomere development in this species will be elucidated in future studies.

Because of the wide space between blastomeres during early cleavage stage, the process underlying cell-cell interactions, including signal transduction, remains unclear. For example, mRNA encoding Delta begins to be expressed in large micromeres as early as the 32-cell stage (Croce & McClay 2010), but at this stage in *T. reevesii*, the blastomeres are still not attached strongly to each other, which means that it is difficult for blastomeres to communicate through simple Delta-Notch complexes located on the cell membrane as in the model sea urchins. There is no gene expression or protein data so far for this species; thus, we could not assess when *delta* is expressed. However, because thin fibrous structures are present between blastomeres, visible in magnified images (Fig. 2H), signal molecules that function through the plasma membrane in normal urchins may utilize these fibers. In fact, receptors for pathways such as Hedgehog or Wnt are present on the primary cilia, which are protrusions from the cell, and receive their ligands there (He 2008; Goetz & Anderson 2010). High-resolution microscopy using protein-labeling techniques will improve our understanding of cell-cell communication in this species during early cleavage stages. After the 60-cell stage, the space between blastomeres becomes narrow, and compaction appears to occur comparably to that in mammalian embryos (Fig. 2M-O). Therefore, this species may be a good system for studying the molecular mechanisms of compaction during early cleavage.

The ability to culture embryos at a wide range of temperatures is useful for scientists because it allows easy control of developmental timing in the laboratory, depending on experimental contents and schedules. The temperature tolerance of other

urchin species, *H. pulcherrimus*, *Anthocidaris crassispina*, and *Pseudocentrotus depressus* (Fujisawa 1989), has previously been examined. Among them, embryos of *A. crassispina*, whose eggs and embryos have bad transparency, could grow between 16˚C and 29˚C. This is similar to the temperature range for *T. reevesii*, which is shown in this study. The factors underlying the temperature tolerance remain unclear, but one candidate is the temperature sensitivity of proteins involved in the cell cycle (Loeb 1969). Because it has been reported that the breeding season of *T. reevesii* in the Inland Sea of Japan is from July to January (Kitazawa et al. 2014), it is expected that sets of enzyme systems responsible for the life cycle of this sea urchin are adapted to higher temperatures than those of other model sea urchins such as *H. pulcherrimus* (15˚C culture in [Yaguchi et al. 2000]) and *S. purpuratus* (14˚C or 15˚C culture in [Yaguchi et al. 2006; Yaguchi et al. 2008]). It has been reported that the embryos of *Lytechinus variegatus*, another sea urchin model, can grow at high temperature close to 30˚C and its developmental speed is similar to that of *T. reevesii* (Roller & Stickle 1993; Cunningham & Watts 2010), being a good candidate in studying neurogenesis. However, the range of optimal temperature for embryonic culture is narrower than that of *T. reevesii* (Cunningham & Watts 2010).

Based on the previous reports, the serotonergic neurons are differentiated at the dorsal edge of the anterior neurogenic ectoderm in the other sea urchin embryos/larvae (Yaguchi et al. 2000; Yaguchi et al. 2006; Angerer et al. 2011). Embryos of *T. reevesii* also have serotonergic neurons at the dorsal side, judging from the anterior view of the stained sample (Fig. 5). Because molecular markers such as FoxQ2 or Six3, which label the anterior neurogenic ectoderm, are not yet available in this species, we cannot determine the exact positions of these neurons in the embryos/larvae. However, the presence of non-serotonergic neurons ventral to serotonergic neurons suggests that the dorsoventral pattern of anterior neurogenic region of this species is similar to that of other species. In addition, serotonergic neurons are the earliest neurons to develop in the anterior neurogenic ectoderm, and non-serotonergic neurons appear later (Fig. 5), indicating that some of the mechanisms of neurogenic ectoderm formation are well conserved with other model sea urchins (Yaguchi et al. 2006) .

In the prism and early pluteus stages, the distribution of serotonergic neurons in the anterior end of the larvae tends to be bilateral (Fig. 6), although some neurons are

observed around the central part of the neurogenic region (Fig. 5, 6). Still, larvae after 1 week show a completely bilateral pattern of serotonergic neurons (Fig. 7), unlike those of the modern sea urchin larvae (Nakajima et al. 1993). This is very similar to that in larvae of a primitive echinoid, *Eucidaris tribuloides*, in which the serotonergic neurons appear bilaterally in the late gastrula and form clusters on the left and right ciliary band at the anterior end (Bishop et al. 2013). However, *E. tribuloides* embryos have no thickened ectoderm and left-right transverse ciliary band at the anterior end, indicating that they do not have the anterior neurogenic ectoderm structure typically observed in model sea urchins. By contrast, *T. reevesii* embryos have typical animal plate characteristics during the early pluteus stage (Fig. 3), but the larvae at 7 days do not clearly show the high-cell-density region normally observed in the neurogenic ectoderm of model species (Fig. 7D-F). This suggests that *T. reevesii* larvae share features at the anterior end with other echinoderms in later stages. However, the rearrangement of the ciliary band and neurogenic ectoderm during the pluteus stage remains to be elucidated using tissue specific markers in *T. reevesii*.

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Figures

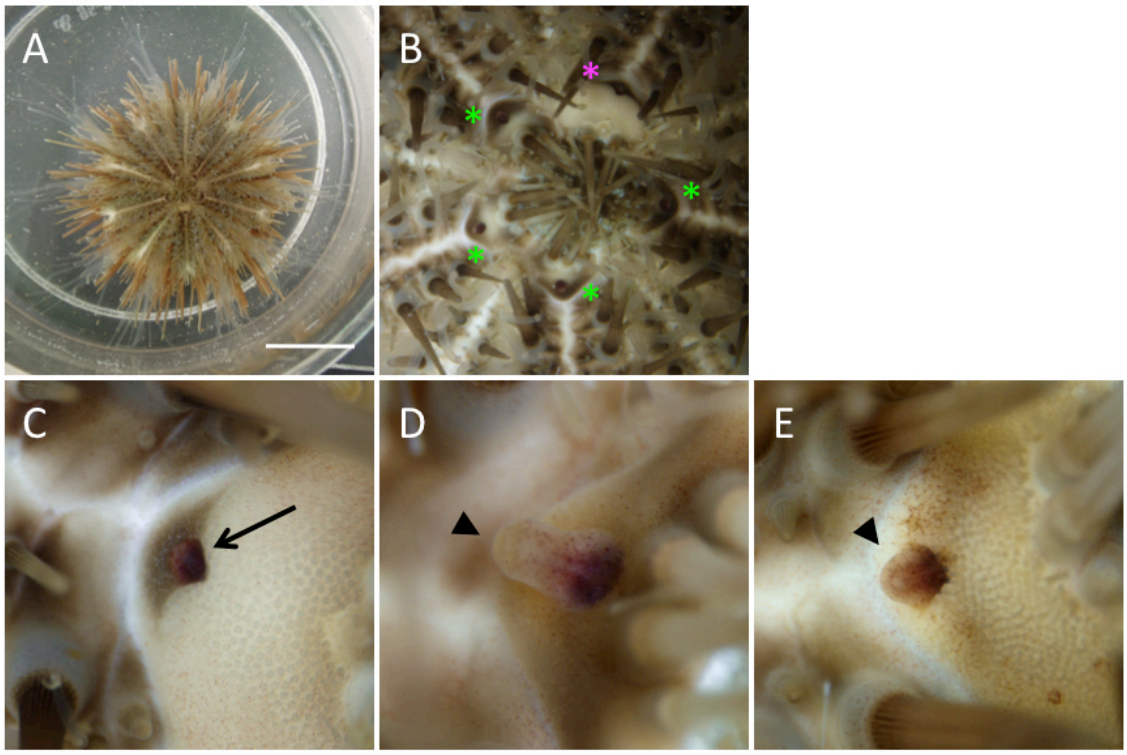

Figure 1. Males and females are easily distinguishable in *T. reevesii*. (A) Adult of *T. reevesii*. Bar is 20 mm. (B) Five gonopores are present around anus (asterisks). Magenta asterisk indicates the location of a gonopore on the madreporite. (C-E) Magnified images of gonopores of female (C) and males (D, E). Females have no protrusion (arrow in C), but males do (arrowheads in D and E).

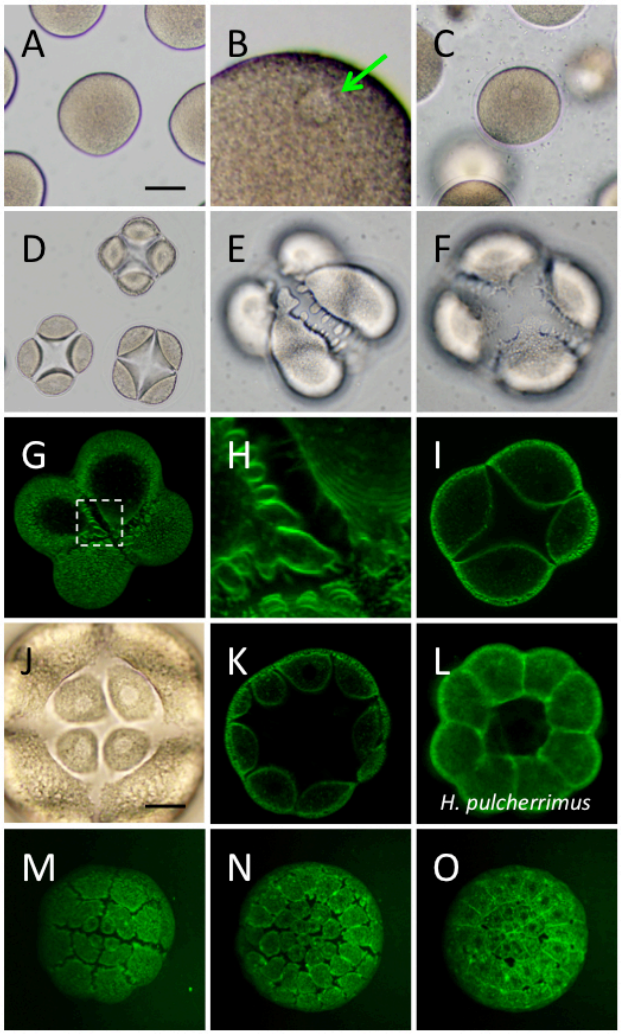

Figure 2. Eggs and early cleavage of *T. reevesii*. (A) Unfertilized egg. Bar is 40 μm. (B) Female pronucleus (arrow). (C) Fertilized egg with sperm. (D) Embryos at the 4-cell stage. (E, F) Magnified images of 4-cell stage embryo. (G) Stacked image of 4-cell embryo, whose membrane was stained with FM1-43. (H) Magnified image of dot-lined square region in (G). (I) Single optical section of 4-cell embryo. (J) Vegetal view of 16-cell stage embryo. Bar is 20 μm. (K) Single optical section of 32-cell stage embryo. (L) Single optical section of 32-cell stage of *Hemicentrotus pulcherrimus*. (M-O) Time-lapse images from 32-cell to 60-cell stage.

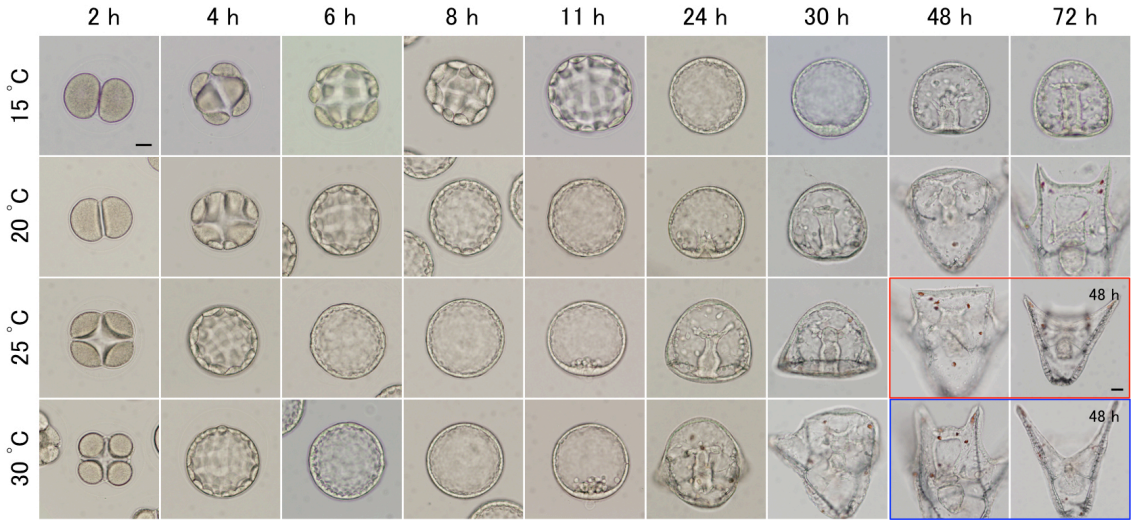

Figure 3. Early development of *T. reevesii* under 4 culture temperatures. Each image shows the developmental stage, with the culturing temperature (˚C) and time post-fertilization (h) indicated at the left and top of the panel, respectively. Embryos cultured for 72 hours at 25 and 30 ˚C without being fed were dead; thus, 48-hour embryos are shown. Bars are 20 μm.

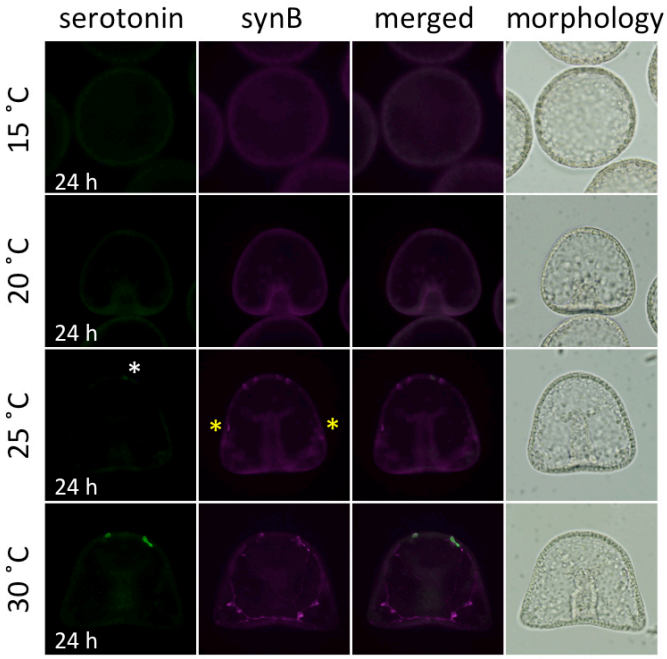

Figure 4. Neurogenesis at 24 hours under 4 culture temperatures. Epifluorescent images indicating that 15 and 20 ˚C cultured embryos have no neurons, but 25 and 30 ˚C ones have differentiated neurons. White asterisk indicates that the serotonergic neuron appears at the anterior tip of 25 ˚C-cultured embryo. Yellow asterisks show the

position of lateral ganglions at the same stage embryo.

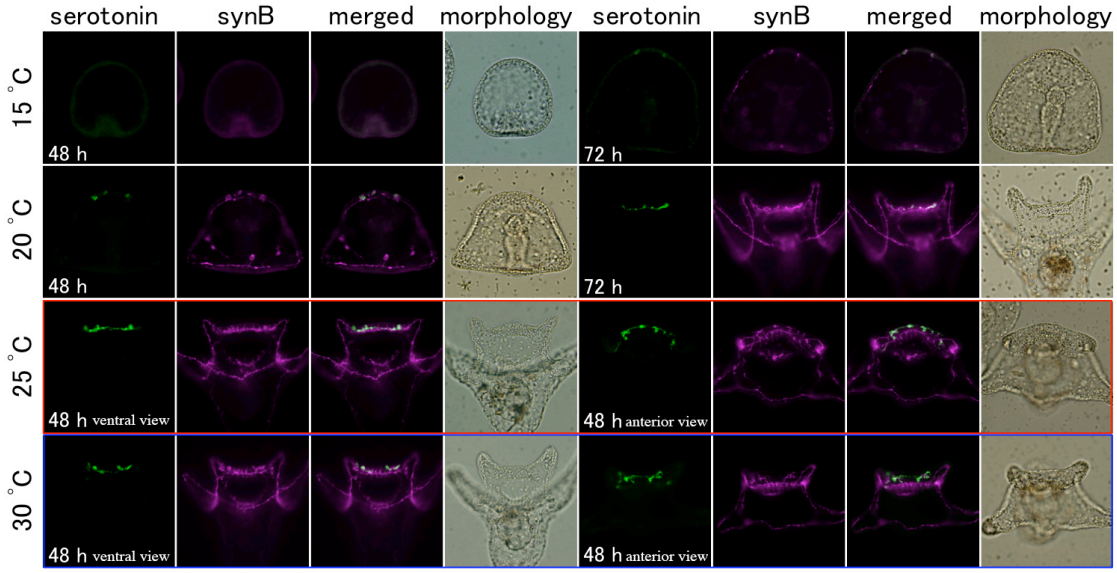

Figure 5. Neurogenesis at 48 and 72 hours. Epifluorescent images indicating that 15 ˚C embryos still have no neurons at 48 hours, but neurons appear at 72 hours. Although the axon elongation is immature in 20 ˚C-cultured embryos at 48 hours, the neural network is well developed at 72 hours. Embryos at 48 hours are only shown for 25 and 30 ˚C cultures and they have already had developed neural network at this time. Anterior views of 25 and 30 ˚C-cultured embryos strongly indicate that the serotonergic neurons in 4-arm pluteus stages are present at the dorsal side of anterior neurogenic ectoderm.

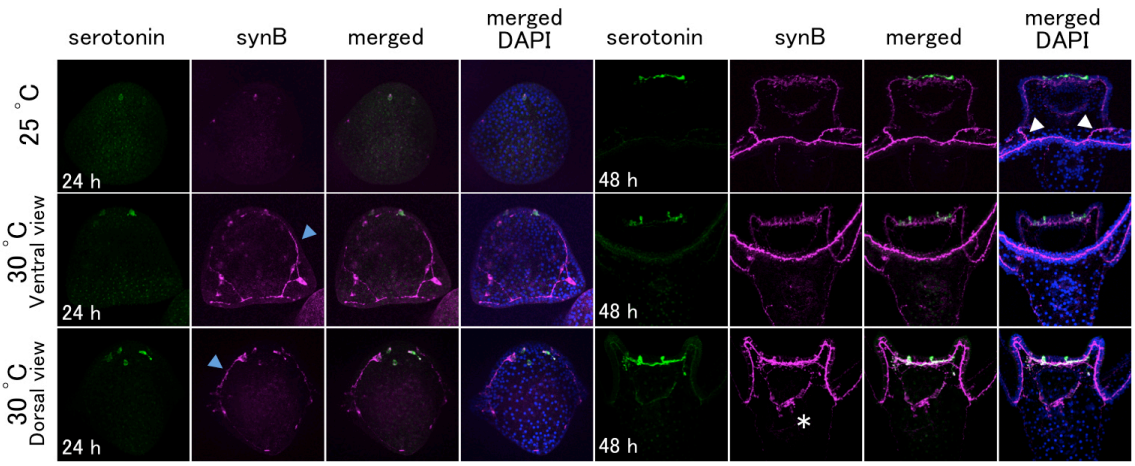

Figure 6. Detailed observation of neurogenesis using confocal microscopy. Embryos at 24 and 48 hours stained with anti-serotonin and anti-synaptotagmin B (synB) were

observed with a confocal microscope, and stacked optical sections are shown. Blue arrowheads indicate the extended axons. White arrowheads show the neurons present at the oral ectoderm. Asterisks indicate neurons at the lower lip ganglion.

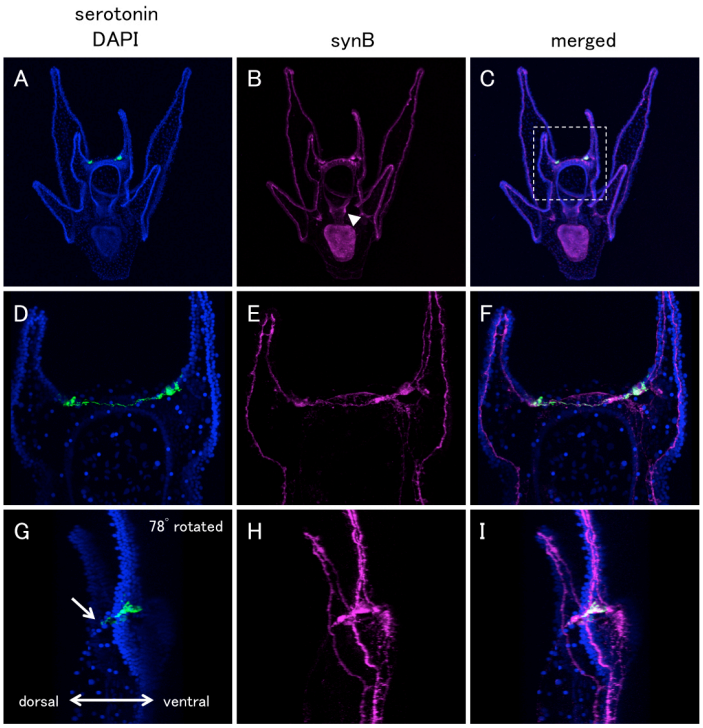

Figure 7. Embryos cultured for 7 days at 20 ˚C and stained with anti-serotonin and anti-synaptotagmin B (synB). (A-C) Overview of the stained embryos. Arrowhead in (B) indicates the neural plexus at the esophagus. (D-F) Magnified dorsal view of the oral lobe. (G-I) Rotated view (78º) of stacked images of 7-day embryos. The axons from serotonergic neurons are present on the dorsal side (arrows).